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Global Challenges for the 21st Century: the Role and Strategy of the Agri-Food Sector

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Abstract

Human activity is having an increasing global impact on the environment, geology and ecosystems. There is an 80% probability that world population will increase to between 9.6 billion and 12.3 billion by 2100 and could even reach 10 billion by 2056, 6 years earlier than projected. Historically, global production of food has outpaced consumption growth. This is evidenced by falling real prices of food, however this is now slowing, caused by constraints on supply and continued growth of demand. Paradoxically, as billions suffer food insecurity through lack of food, more than 2 billion people, approximately 30% of the world's population, are overweight or obese and this percentage continues to grow. It is also estimated that the world will need to close a significant food-gap by 2050, primarily because of continued population growth and changing diets. Increases in temperature of over two degrees Celsius are projected to have a negative impact on global yields of major crops. Agri-food production, including manufacture, food preparation and cooking, accounts for approximately 30% of all greenhouse-gas emissions and livestock production accounts for approximately 50% of this. The agricultural sector will increasingly be driven by these global changes, including a rising world population, rapid development of emerging economies, with western lifestyle aspirations, growing geopolitical instability around shortages of land, water and energy and 'one health' issues. However, a technological revolution is taking place, including breakthroughs in nutrition, genetics, informatics, satellite imaging, remote sensing, meteorology, precision farming and low impact agriculture. These changes will hopefully continue to drive major global investment in agricultural technologies. It is of vital importance that countries around the world recognize fully the opportunities and challenges and provide the appropriate framework support, investment and infrastructure. In this regard, increasing high quality livestock research will be essential to help address the looming food and environmental challenges and is a message that animal scientists and veterinarians around the world need to be making to governments and funding agencies. Farming practices globally will continue to change because of competing demands. Hence it is essential that the livestock sector benefits fully, both from continued improvements of current reproductive technologies and in the application of future reproductive technologies to meet these global challenges.

Keywords: food security, greenhouse gases, livestock, sustainability, technology.

Introduction

It has been proposed that the world is entering, or may have even entered, the anthropocene epoch from the holocene epoch, because human activity is having a significant global impact on the Earth's environment, geology and ecosystems (Zalasiewicz *et al.*, 2015). In a recent EU report (Expo Milano, 2015, EU Scientific Steering Committee Recommendations) there was a summary of some of the key global issues. For example, it stated that nearly 1 billion people are chronically hungry. In comparison, because of over consumption of food, coupled with reduced physical activity, approximately twice as many people are overweight. Furthermore in Paris in December 2015, 195 countries agreed to try and keep global temperature rise to well below the 2°C above pre-industrial levels, and to even pursue efforts towards 1.5°C, since increases in temperature of over two degrees Celsius are expected to have a negative impact on global yields of major crops (Intergovernmental Panel on Climate Change (IPCC), 2012; 2013; Climate and Global Production Shocks Report, 2015). Unfortunately however, the agricultural sector was largely absent from the talks in Paris (Benton and Bajželj, 2016). Hence the impact of enhanced human endeavor and activity, coupled with continued population growth, will result in increasing global challenges throughout the 21st century. This paper outlines a number of the global challenges, the impact of livestock production and possible mitigation strategies, including the development and benefit of new technologies. These are discussed together with possible strategies that countries, individually and in collaboration, could develop to meet the challenges, using examples of current programs in the UK and Brazil.

The Global Challenges

The focus of this review is on population growth, food security, climate change and livestock production. However the additional impact of other challenges, such as the links between animal and human health, zoonotic diseases, and the increase in antibiotic resistance, need to be recognized.

Population growth

World population at time of writing currently stands at around 7.5 billion, with a population growth of

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approximately 200,000 per day. At the dawn of agriculture, at approximately 8,000 B.C., the world population was estimated to be 5 million, with a growth rate of less than 0.05% to reach about 2 to 300 million by 1 A.D. A significant change occurred with the industrial revolution at approximately 1,800 A.D. with the second billion being achieved in only 130 years. Peak annual growth rate occurred in the 1960s when it was approximately 2.2%, but it is currently declining and is around 1.15% per year. The United Nations recently released population projections based on data until 2012. Analysis of these data reveals that, contrary to previous projections, and despite the decline in birth rate, the world population is unlikely to stop growing this century. Indeed there is an 80% probability that world population will increase to between 9.6 billion and 12.3 billion by 2100 and could even reach 10 billion by 2056, 6 years earlier than projected. Much of the increase is expected to happen in Africa, in part because of increased rates of fertility and a recent slowdown in the pace of fertility decline. Also, the ratio of working-age people to older people is likely to decline substantially in all countries, even those that currently have young populations (Gerland *et al.*, 2014). Current mean world figures from the World Bank indicate that the proportion of people over the age of 65 is currently greater than 8%, but this increases towards 20% for some countries in Western Europe.

Recent figures from the United Nations Department of Economic and Social Affairs, Population Division show more women than ever now use family planning, with some poorer regions recording the fastest pace of growth since 2000. In 2015 an estimated 64% of either married women or women living with a partner aged between 15 to 49 years used family planning, compared with 36% in 1970. However, despite the success of the past 40 years it has been concluded that investment in family planning is needed to keep up with demand and meet the need of women who are unable to access services (see Population Council). Furthermore in a recent comment in the Lancet (World Abortions 1990 to 2014, 2016) it was concluded that additional knowledge, regarding the incidence of induced abortion, is needed to motivate and inform efforts to help women avoid unintended pregnancies and to monitor progress toward that end. It was estimated that abortion rates have declined significantly since 1990 in the developed world, but not in the developing world. An important conclusion was that ensuring access to sexual and reproductive health care could help millions of women avoid unintended pregnancies and ensure access to safe abortion.

In summary, despite the success in contraceptive use, world population continues to grow, resulting in increased demand for food and resources. The Food and Agriculture Organisation (FAO) food demand projections and World Resources Institute (WRI; Ranganathan *et al.*, 2016*)*, estimate that the world will need to close at least a 60 percent "food gap" between the crop calories available in 2006 and expected caloric demand in 2050 (Foley, 2011). In conclusion, the food gap will stem primarily from continued population growth and changing diets.

Food security

Food security, as defined by the FAO, occurs when: all people, all of the time, have physical, social and economic access to sufficient, safe and nutritious food that meets their dietary needs and food preferences for an active and healthy life. Despite progress there are currently over 800 million people who are chronically hungry in the developing world, with more than 3 million children dying each year from the causes of under- and mal-nutrition and an estimated 160 million under 5-year-old children who are stunted and will carry the burden of this through their lives. Globally we are far from being food secure (World Hunger and Poverty Facts and Statistics, 2015). Indeed it is estimated that around 2 billion more people suffer from either iron deficiency (World Health Organization - WHO, 2012) or other micronutrient deficiencies caused primarily by the lack of access to food and this is in most cases due to relative or absolute poverty. For example, limited access to food and rapid food price inflation can be a cause of civil unrest and drive human migration. Paradoxically, as billions suffer food insecurity through lack of food, more than 2 billion people, approximately 30% of the world's population, are overweight or obese and this percentage continues to grow (WHO, 2015*)*. This is often associated with poverty and is a consequence of over-consumption of calories, the lack of access to appropriate nutrition, as well as a lack of physical activity. Furthermore, caloric over-consumption progressively increases personal, public-health and environmental costs and thereby increases the pressure not only on the global food supply, but also on national health systems. In summary, food and nutrition security is an issue for all societies. Importantly however, the FAO estimates that there is enough food in the world to provide every single person each day with approximately 2,770 kcal (FAO, 2012; World Hunger and Poverty Facts and Statistics, 2015).

Historically, global production of food has outpaced consumption growth as evidenced by falling real prices of food. However, this "outpacing" is now slowing because of constraints on supply, alongside continued growth of demand (European Commission, 2011). For example, more people are demanding more food that is more resource-intensive to produce such as meat. In addition, in most places there is considerable food wastage. On the supply-side, historic yield growth has slowed or even plateaued in recent years and this has been the case for the UK. The acceptability of technological solutions to increasing yields is sometimes resisted, as has occurred in the EU (European Commission, 2011). In addition, there is increased competition for land, water and other natural resources, which may impact on global food production and climate change and is also threatening production growth in many areas. A further constraint is that reducing the environmental impact of agriculture, aquaculture and fisheries, including greenhouse gas emissions, while maintaining production will probably

require changes in the way food is produced.

How can changing diets, including the type, combination, and quantity of foods people consume, contribute to a sustainable food future? As discussed, global population is projected to grow to nearly 10 billion people by 2050, with two-thirds of those people projected to live in cities. In addition, at least 3 billion people are expected to join the global middle class by 2030. As nations urbanize and citizens become wealthier diets change, with people generally increasing both their calorie intake and the share of resource-intensive foods, such as meat and dairy, in their diets. Over the last 40 years, the worldwide consumption per capita of milk has doubled, and meat consumption has more than tripled (Kearney, 2010).

At the same time globalization, technological advances, business and economic changes, and government policies are transforming entire food chains. Multinational businesses are increasingly influencing what is grown and what people eat. Together, these are driving trends toward Western-style diets, which are high in calories, protein and animal-based foods. Indeed in China meat consumption has increased nine fold (Kearney, 2010). Although some of this shift reflects health and welfare gains for many people, including increased longevity, the scale of this ongoing change in diet will make it harder for the world to achieve a number of the United Nations sustainable development goals, which include reducing hunger, improving healthier lives, improved water management, combating climate change, and protecting terrestrial ecosystems (Ranganathan *et al.*, 2016). In summary given recent trends, demand is likely to rise more quickly than supply towards the middle of the 21st century increasing the pressure to convert more land for farming. The combination of these drivers suggests that emissions from the agri-food sector will continue to grow. Changing some farming practices could offset some of this increase, but achieving such changes will be a challenge and require continued collaborative research and development across continents.

Climate change

The negative impact of climate change, including an increase in temperature of over two degrees Celsius (Intergovernmental Panel on Climate Change - IPCC, 2012, 2013; Climate and Global Production Shocks Report, 2015), on global yields of major crops will probably be spread unevenly over the globe. It is projected that crop production in low latitudes will experience negative effects, whereas in northern latitudes impacts may vary. The areas where climate change is expected to threaten crop productivity the most (Wheeler and von Braun, 2013) include countries in Africa and South Asia, that are home to many of the world's more than 800 million undernourished people (FAO, 2014). Food production of the globally most important commodity crops, maize, soybean, wheat and rice, comes from a relatively small number of major producing countries. The exposure of a large proportion of global production of the major crops is therefore concentrated in specific parts of the globe. This report stresses that extreme weather events, in these regions, will have the largest impact on global food production. Furthermore, simultaneous extreme weather events in two or more of these regions, creating a 'multiple bread basket' failure, would represent a serious production shock. However, understanding the underlying cause of extreme weather events in different production regions is currently under-researched.

There is an urgent need to understand the dynamics of meteorological events, such as the El Niño, which may become even more extreme, in order to quantify the likelihood of production shocks in major food-producing regions. Indeed it is projected that a catastrophe is developing currently in Africa because of crop failure caused by the impact of El Niño. It has been estimated that 50 million people, across a number of countries, could require food-aid during the next 12 months. Initial modeling by a US-UK Taskforce (Extreme Weather and Resilience of the Global Food System Report, 2015) suggests that what would be called an extreme food production shock in the late 20th century, will become more common in the future. The data suggest that a 1 in 200 year event for the climate in the late 20th century equates to a loss of approximately 8.5% production, and over the next decades (2011 to 2040), a 1 in 200 year event is about 15% larger in magnitude and equivalent to the loss of 9.8% of calorie production. Furthermore, according to the model an event that would have been called 1 in 100 years over the period from 1951 to 2010 may become as frequent as a 1 in 30 year event before the middle of the current century.

Agriculture and the production of food is a very significant emitter of greenhouse gases, causing emissions of CO_2 by agricultural machinery and the transportation of crops and animals, nitrous oxide from the use of fertilizers (synthetic and manure), and methane from livestock and flooded paddy fields for rice (Smith *et al.*, 2014). Furthermore, the demand for food has led to a global expansion of farmland at a rate of about 10 million hectares per year during the last decade. Some of this cleared land was tropical rainforest, adding more emissions and reducing the capacity of land to absorb and store carbon. There have been a number of estimates of greenhouse gas emissions from agriculture and food production that vary depending on methodology. For example, the estimate of 30% by Bajželj *et al.* (2013) includes the manufacture, preparation and the cooking of food. A more recent study calculated that agriculture, forestry and other parts of the land use sector is responsible for just under a quarter (approximately 10 to 12 $GtCO_2eq$ per year) of anthropogenic greenhouse gas emissions, mainly from deforestation and agricultural emissions from livestock, soil and nutrient management (Smith *et al.,* 2014). Furthermore, according to the Food and Agriculture Organization of the United Nations (FAO, 2014), agri-food production and forestry was estimated to account for approximately 20% of all greenhouse-gas emissions in the world (25% in America, 12% in Europe, 15% in Africa, 4% in Oceania and 44% in

Asia). Overall however, producing and cooking the food we eat results in approximately the same amount of emissions as those produced from personal travel, lighting, heating and air conditioning and domestic 'white goods' combined.

Livestock production

The livestock sector supports about 1.3 billion producers and retailers worldwide, and contributes 40 to 50% of agricultural gross domestic product (Herrero *et al.* 2016). These authors estimated that between 1995 and 2005, the livestock sector was responsible for greenhouse gas emissions of 5.6 to 7.5GtCO_2e per year which comprises approximately 50% of agricultural emissions. However, livestock accounts for up to half of the technical mitigation potential of the agriculture, forestry and land-use sectors. This will be through the application of management options that sustainably intensify livestock production, promote carbon sequestration in rangelands, reduce emissions from manures, and through reduction in the demand for livestock products. Although the mitigation potential of reductions in livestock product consumption is large, their economic potential is unknown at present (Herrero *et al.*, 2016).

Livestock, particularly ruminants, can eat a wider range of biomass than humans, although in the drive for greater efficiency, intensive systems of livestock production have evolved to compete with humans for high-energy crops such as cereals. In a study by Wilkinson (2011), feeds consumed by livestock were analyzed in terms of the quantities used and efficiency of conversion of grassland, human-edible crops and crop by-products into milk, meat and eggs, using the United Kingdom as an example of a developed livestock industry. It was concluded that by accounting for the proportions of human-edible and inedible feeds used in typical livestock production systems, a more realistic estimate of efficiency could be made by comparing systems. Therefore increasing efficiency in livestock production and reducing the share of animal products in human consumption are two possible strategies to curb the adverse environmental impacts of the livestock sector. In a recent study, Schrader *et al.* (2016) explored the opportunity for sustainable livestock production by modeling the impacts and constraints of a third strategy in which livestock feed components, that compete directly with human food crop production, are reduced. Thus animals are fed only from grassland and by-products from food production. They show that a strategy focusing on feed components, which do not compete directly with human food consumption, offers a viable complement to strategies focusing on increased efficiency in production and/or reducing the share of animal products in human consumption (Schrader *et al.*, 2016).

In conclusion, one of the key challenges in the livestock sector will be the development of technological advances in sustainable intensification. However, this approach will be only one mitigation route together with decreases in food waste and improvements in human diets (Bajželj *et al.*, 2014).

Agri-Food Technology Strategy

Agricultural science and technology is one of the world's fastest growing and exciting sectors within the global marketplace. As discussed, this market will be driven by global changes including a rising world population, rapid development of emerging economies with western lifestyle aspirations and growing geopolitical instability around shortages of land, water and energy. In parallel a technological revolution is also taking place, including breakthroughs in nutrition, genetics, informatics, satellite imaging, remote sensing, meteorology, precision farming and low impact agriculture. These changes will continue to drive major global investment in agricultural technologies. It is of vital importance that countries around the world recognize fully the opportunities and challenges and provide the appropriate framework support and investment.

Recognizing these challenges, including the continuing reduction in food self-sufficiency in the United Kingdom, the UK Government launched 'A UK Strategy for Agricultural Technologies' (UK Government, 2013). In 2016 the strategy was expanded to incorporate the food production and retail sector, as well as primary agricultural production, and is overseen by the AgriFood Technology Council. This strategy is aimed at improving the integration of UK's science and progressive food and farming businesses with the Government's support for trade, investment and international development. The aim is to help unlock a new phase of global leadership in agricultural innovation. The vision of the strategy is that the UK becomes a world leader in agricultural technology, innovation and sustainability; exploits opportunities to develop and adopt new and existing technologies, products and services to increase productivity; and thereby contributes to global food security and international development.

The mission of the strategy includes (1) additional investment from both government and industry in agricultural research and innovation, including the launch of four new research innovation centers (Agrimetrics, Crop Health and Protection, Livestock, Agricultural Engineering Precision) and investment in a significant number of applied research projects, supporting the speedier translation of research into practice i.e. from farm to fork through agri-food supply chains; (2) promoting the UK's expertise and capacity in the agri-tech sector to stimulate investment, creating a new generation of spin-outs and start-up ventures, increasing export opportunities and new collaborations with emerging and developing economies; (3) take a global lead in agricultural informatics and in establishing the metrics and techniques by which progress towards sustainable intensification can be assessed; (4) encourage investment in change, including enhanced skills and knowledge transfer, exploitation of shared data and widespread adoption of best practice. In addition, the

UK Government has just announced a £1.5 billion Global Challenges Research Fund, across the Research Councils, to support cutting-edge research that addresses the challenges faced by developing countries. There has also been a significant focus on skills given that it will be essential to have an increasingly skilled workforce to take full advantages of the rapid pace of technological progress, not only within the agricultural sector, but also across other sectors such as informatics and engineering.

Brazil has also promoted programs to mitigate the environmental threats associated with the global challenges, as discussed, and to reduce food shortage within its boundaries. The Brazilian Agricultural Research Corporation (Embrapa) - Ministry of Agriculture, Livestock and Food Supply launched a number of programs aiming to reduce poverty and hunger and also to stimulate agricultural technologies supporting environmentally safe food production. The "Brazil Without Extreme Poverty" program, which aims to increase familiar agricultural production and to enhance/create markets for these products, and the "Low Carbon Agriculture Plan" (ABC Plan) are perhaps the most relevant and emblematic actions from the Brazilian government within the context of the global challenges. A relevant program, with the ABC Plan directly linked to livestock production, is the National Integrated Crop-Livestock-Forestry (ICLF) Policy, which targets the rehabilitation of degraded pastures and forests by the development of integrated systems.

In conclusion, it is essential that the livestock sector can take full benefit of the funding initiatives that are being launched worldwide and continue to develop new reproductive technologies to be better placed to respond to the global challenges.

Reproductive Technologies in the 21st Century

In 2012 Murphy made a presentation to the Sociedade Brasileira de Transferência de Embriões (SBTE) on 'The future of animal reproduction'. The accompanying review paper discussed a range of current technologies, including artificial insemination, including classical genetic selection approaches (Murphy, 2012). The review paper also stressed the major limitations to the use of these technologies in extensive production systems, together with the use of additional technologies including prostaglandins to regress corpora lutea, progestagens to mimic luteal function, regulation of follicular development with estrogens or GnRH, and synchronization of ovulation with either GnRH and/or estrogens (Bó *et al.*, 2007; Lamb *et al.*, 2010; Baruselli *et al.*, 2011). Although these technologies have a number of benefits, there still remains substantial potential for improvement in the rate of successful pregnancy, despite these compounds being available for nearly 50 years.

Other current technologies include the use of sexed semen and embryo transfer which has become more widespread, particularly in South America who leads the world (over 70%) in the number of *in vitro* produced embryos (Blondin, 2015). However, the success of this technology is still limited by the efficacy of the superovulation protocols, including the variability of the follicular reserve (Mapletoft and Bó, 2011; Ireland *et al.*, 2011) and the variation between *Bos taurus* and *Bos indicus* breeds (Morotti *et al.*, 2015; Sartori *et al.*, 2016a), and *in vitro* embryogenesis protocols (Baruselli *et al.*, 2015). The review paper of Sartori *et al.* (2016b), published in these proceedings, describes the development and magnitude of the reproductive technologies in use in Brazil, and also discusses the technical bottlenecks that limit the impact on animal production efficiency. Among the current limitations, special focus has been given to sub-optimal protocols for oocyte maturation and embryo culture leading to low blastocyst development rates (averaging 25 to 50%), which compromise conception rates and successful pregnancy outcome. In addition, embryonic developmental competence is compromised further by inefficient freezing protocols. Once these bottlenecks are minimized, the impact of *in vitro* embryo production on livestock productivity is expected to increase significantly, particularly when used in combination with the development of DNA based strategies for donor selection as indicated below.

Continuing, and indeed even an increase, in livestock research and development will be essential to help address the looming food and environmental challenges and is a message that animal scientists around the world need to be making to their governments and funding agencies. However, the message should also be one of optimism, given the untapped potential of our farm species. The potential yields of plant and animal farm species, including constraints and opportunities in the 21st century, has been reviewed and considered extensively (Sylvester-Bradley and Wiseman, 2005). For livestock the opportunities include (i) increasing the proportion of twin births in cattle, (ii) more precise control of seasonality, litter size, lamb survival and puberty in sheep, (iii) increased fecundity in pigs, (iv) increased milk yield in dairy cows and sheep, (v) improving feed conversion efficiency, meat quality and growth in sheep and beef cattle. However, to obtain these benefits improved animal management, welfare and feeding strategies will be required (Sinclair and Webb, 2005; Webb *et al*., 2005; Garnsworthy and Thomas 2005). In all species the predicted trends for increased yields, accompanied by increases in efficiency, are projected to reduce environmental impact of animal agriculture, mainly through reductions in animal numbers required for a given output of meat and dairy (Garnsworthy, 2004). However it is important to realize that a more systems based approach to production will be required, together with the incorporation of new physiological and genetic technologies. Furthermore, despite large genetic improvements in the quantitative traits of growth, production, and efficiency of farmed livestock over recent decades, current evidence suggests that little variation has been lost and that improvements should indeed be sustainable in the future (Hill, 2016).

Traditional marker-assisted selection did not result in the widespread use of DNA information in

animal breeding (Meuwissen *et al.*, 2016). The main reason was that the traits of interest in livestock production are much more complex than expected and are determined by thousands of genes with small effects on phenotype (Haley *et al.*, 1993). However, advances in genotyping and sequencing a little over a decade ago have resulted in the development and application of genomic selection, which is arguably the most important breakthrough in animal breeding since the development of best linear unbiased prediction in the 1940s (see Hickey *et al.*, 2016).

Genomic selection assumes that all markers might be linked to a gene affecting the trait and concentrates on estimating their effect, rather than testing its significance. Three technological breakthroughs resulted in the current widespread use of DNA information in animal breeding: (i) the development of genomic selection technology, (ii) the discovery of massive numbers of genetic markers (single nucleotide polymorphisms, SNPs), and (iii) high-throughput technology to genotype animals for hundreds or even thousands of SNPs in a cost-effective manner. The use of whole genome sequence data is anticipated to have a significant impact on dairy and beef cattle, pigs, and poultry breeding (Meuwissen *et al.*, 2016). For example in sheep and goat breeding, rapidly reducing genotyping cost, coupled with a better understanding of how to maximize benefits of genomic selection should result in a dramatic rise in the adoption of these approaches (Rupp *et al.*, 2016). In beef cattle the development of accurate genomic evaluations in beef populations are more difficult than in dairy populations because of the presence of multiple breeds, the poor extent of phenotyping, lack of the use of artificial insemination (Berry *et al.*, 2016), and sheep and beef systems are generally lower-margin businesses and hence tend to be slower adopters of technology. However, the ongoing development of low cost sensors, such as ultra-wide band technology for estrous detection (Homer *et al.*, 2013), and associated information technology for the generation and capture of phenotypes should have a positive impact. These types of technologies, coupled with international initiatives, suggest that the necessary framework is in place for further development of genomic predictions certainly for beef, and possibly even for sheep (Berry *et al.*, 2016).

In addition and significantly, genome editing in livestock populations of cattle, sheep and pigs, has resulted already in viable zygotes and living animals. The idea of genome editing appears straight forward, in that base pairs at specific locations in the genome can be deleted, changed or added (Hickey *et al.* 2016). Importantly, these changes are permanent and if made in germ line cells, are heritable and advances in this technology are occurring regularly. From an animal breeding perspective, roles for genome editing include (i) fix favorable alleles for monogenic traits (e.g. disease resistance, myostatin, polledness), (ii) removal of recessive alleles that impact on fertility and (iii) increase the frequency of favorable alleles for polygenic traits (Hickey *et al.*, 2016). These advances, coupled with increased understanding of epigenetic effects (Sinclair *et al.*, 2007), will bring understanding, but both opportunities and challenges, that can potentially be included in livestock breeding programs. These include the need for a relatively inexpensive technology to sequence the epigenome on a large scale, and in large numbers of individuals, to accurately estimate epigenetic variance at a population level. Statistical methods also need to be developed to incorporate whole methylome information together with large environment and DNA sequence information. Practical implementation must be carefully evaluated to successfully incorporate epigenetic information in livestock breeding, including mate selection, in order to obtain genotypes that favor a certain epigenotype (González-Recio *et al.*, 2015). In summary, multidisciplinary genetic and management/nutrition practices could result in the incorporation of favourable epigenotypes into populations, in addition to technologies such as genome editing.

Conclusions

The food security, world population and climate change challenges are clear. Food security for the majority of the world's population is about nutrition for a healthy life, not calories to survive. Greater than 50% of the world's population are malnourished, either over- or under-weight, and the global economic burden of non-communicable diseases (Alleyne *et al.*, 2013) is growing rapidly. Indeed it is viewed as one of the major challenges for sustainable development in the 21st century. For example, in many countries, poverty and obesity are correlated, with inadequate diets in the urban poor being a significant issue, alongside other developing world micronutrient and calorie deficiencies.

As discussed, the IPCC challenge is to limit climate change to less than 2°C, which will require more sustainable consumption. As the emissions from agri-food are approximately 30% of global emissions, and are continuing to grow not shrink, the argument that demand is increasing and so we must grow more to meet this demand is increasingly recognized as being unsustainable. This will result in a stronger focus on the demand-side measures, such as dietary change and waste reduction.

There has been much debate on dietary change and on livestock production. For example, there is ongoing discussion that humans should eat less meat, particularly as the average person, in more than 90% of the world's countries and territories, consumed more protein than the estimated daily requirement (Ranganathan *et al.*, 2016). Alternatively humans should eat more grass fed beef and sheep, in addition to eating less meat, and/or eat more chicken versus eating less red meat. The recent WRI report indicates that there is no current "protein crisis" as on average every region of the world consumes more protein than is required, although it is expected that "demand driven desire" for protein consumption will grow. It is clear that a significantly more sustainable, or even a reduction, in ruminant meat and dairy consumption will be

indispensable for reaching the 2°C target, unless unprecedented advances in technology, such as the green revolution that occurred 50 years ago (Borlaug, 2000), are repeated (Hedenus *et al.*, 2014).

There are three possible ways we could respond to this sobering conclusion: (i) we carry on as we are and miss the IPCC Paris targets, and therefore perhaps lock us into 4 to 5°C of global warming by the end of the century. (ii) We rely on research and innovation to find ways to significantly increase yields to reduce the rate of land conversion and develop carbon capture and storage. (iii) We recognize that demand for food is driving emissions and work to make changes meet the supply-side improvements halfway (T. Benton, 2016; University of Leeds).

The first option is not a viable long-term strategy. The second option has possibilities, but there is little evidence of research budgets on the necessary scale that will be required being forthcoming, indeed globally research budgets have been decreasing in some countries. Furthermore there is a significant gap between mitigation potential and economic viability. Hence the third option seems, at least currently, the better way forward.

It is clear that our habits globally, with specific exceptions, have changed rapidly in recent decades. More food is consumed per capita, generally food is cheaper with respect to income, there is significantly more choice and availability, and importantly there is significant waste. However, there is no reason why habits could not change again to achieve a more sustainable lifestyle? A positive start would be to reduce food waste. For example, on a global basis, about a third of food is lost in either fields and/or storage, or wasted in the supply chain and in our homes. Wasting food is not just a waste of valuable resources it also causes additional emissions when ending up in landfill sites. For example, food waste costs the average UK family, with children, approximately US $1,000 per year.

One option is to reduce consumption of intensively produced meat and dairy. Raising livestock is a less efficient way of producing food than growing crops. Currently, a third of the crops we grow are fed to livestock to produce meat, and nearly half of the emissions from agri-food are related to meat production, more than the entire transport sector. If we used the land that is currently growing feed for animals to grow mainly food for humans, and consume only milk and meat from pasture/byproduct-fed animals, there is scope for very significant reduction in emissions. If we continue to consume increasing amounts of non-pasture fed animals, then the choice of meat is important because producing beef emits more than five times as much as chicken and pork, although as discussed previously, a robust assessment of feed efficiency is required (Wilkinson *et al.*, 2011) in the context of obtaining more realistic estimates of emissions.

Increasingly, people around the world eat more calories than are good for them and as stated previously, about two billion adults are either overweight or obese and this number continues to grow. In Europe, for example, the population consume around twice as much meat as is deemed healthy, whereas in the United States this number is three times. Hence a global switch towards more plant-based diets would reduce global mortality by up to 10% and food-related greenhouse gas emissions by as much as 70% by 2050 (Springman *et al.*, 2016). It means that adjusting diets and attitude to waste has the potential to make the Paris targets more achievable. In a very recent modeling study, Herrero *et al.* (2016) concluded that the mitigation potential of reductions in livestock product consumption is large, but their economic potential is unknown at present.

Finally, one further global challenge that will have increasing impact during the 21st century, and has not been discussed in detail in this review, is the link between animal health and human health, zoonotic diseases, and the increase in antibiotic resistance (see Wegener, 2012). The epidemiology of antimicrobial-resistant microorganisms at the human–animal interface is complex, although it is estimated that the volume of antimicrobials used in food animals exceeds their use in humans worldwide. The One Health agenda (Gibbs, 2014) is gaining significant traction worldwide when it comes to addressing zoonotic transmission of pathogens that are resistant to antimicrobials. There will be an increasing need to engage a wide range of stakeholders, not only researchers, but also farmers, veterinarians, food safety professionals, medical doctors, as well as environment and wildlife experts in monitoring and controlling activities and coordinating responses to this global challenge, that will definitely occur.

It is clear that more research and investment are needed to increase the affordability and uptake of mitigation practices, to moderate consumption of livestock products where appropriate, and to avoid negative impacts on livelihoods, economic activities and the environment. Importantly however, limited take-up of new farming methods, together with high costs, means that a relatively small proportion of what is technically possible is currently economically viable. Continued advances in the use of current reproductive technologies, together with the development of new reproductive technologies will be essential.

In conclusion, the future focus should be on the development of system-based approaches (Randolph *et al.*, 2007), together with the cost effective and welfare friendly use of new technologies and farming practices. This strategy should ensure that the application of new technologies, that will certainly become available during the 21st century, should assist in maximizing the efficiency and sustainability of livestock production and reduce environmental impact. In this regard, it is worth remembering the comment that “It is not the strongest of the species who survive, nor the most intelligent; rather it is those most responsive to change” (attributed to L. C. Megginson when interpreting Charles Darwin’s Origin of Species, 1963).

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Progesterone and conceptus-derived factors important for conceptus survival and growth

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Abstract

Progesterone (P4) from the corpus luteum (CL) is critical for the establishment and maintenance of pregnancy and plays a major role in regulating endometrial secretions essential for stimulating and mediating changes in conceptus growth and differentiation throughout early pregnancy in ruminants. Numerous studies have demonstrated an association between elevated P4 and acceleration in conceptus elongation. A combination of *in vivo* and *in vitro* experiments found that the effects of P4 on conceptus elongation are indirect and mediated through P4-induced effects in the endometrium. Despite effects on elongation, data on the impact of post-insemination supplementation of P4 on pregnancy rates are conflicting and typically only result in a modest improvement, if any, in fertility. Differences in conceptus length on the same day of gestation would suggest that factors intrinsic to the blastocysts transferred regulate development, at least in part, and would be consistent with the hypothesis that the quality of the oocyte regulates developmental competence. This paper will review recent knowledge on the effect of P4 on conceptus development in cattle and summarize strategies that have been undertaken to manipulate post fertilization P4 concentrations to increase fertility.

Keywords: cattle, conceptus, embryo, pregnancy, progesterone.

Introduction

Most embryonic loss in cattle occurs in the first few weeks after conception. Fertilisation success is typically high (~90%) but a significant proportion of the resulting embryos fail to develop to term. The majority of these embryos are lost between fertilisation and maternal recognition of pregnancy, which in cattle occurs around day 16 post-mating (Diskin and Morris, 2008; Wiltbank *et al*., 2016). Wiltbank *et al*. (2016) described four pivotal periods for pregnancy loss during the first trimester of gestation and discussed possible causes for pregnancy failure during these periods. Despite a relatively high fertilization rate (>85%), 20-50% of high-producing lactating dairy cows experience pregnancy loss during the first week of gestation. From days 8 to 27, concomitant with embryo elongation and maternal recognition of pregnancy, losses average approximately 30%. From days 28 to 60, losses of approximately 12% occur while in the fourth period, during the third month of pregnancy, pregnancy losses are reduced (approximately 2%), but may be elevated in some cows, particularly in those carrying twins in the same uterine horn (Wiltbank *et al*., 2016).

Optimal dialogue between the developing embryo and its mother is essential for successful pregnancy recognition and maintenance of pregnancy during the critical peri-implantation period of pregnancy when the stage is set for implantation and placentation that precedes fetal development (Guillomot, 1995; Hue *et al*., 2012; Spencer *et al*., 2015). However, this dialogue really only becomes absolutely essential as the time of pregnancy recognition approaches as evidenced by the fact that embryos are routinely transferred to a synchronous uterus up to about day 8 of development in commercial embryo transfer with good success. Indeed, pregnancies have been achieved following transfer of embryos as late as day 16 (Betteridge *et al*., 1980), although due to the filamentous nature by that time, it is impractical to do so.

Uterine epithelial cells secrete and/or transport a wide range of molecules, including nutrients, collectively referred to as histotroph that are transported into the fetal-placental vascular system to support growth and development of the conceptus (embryo/fetus and associated membranes). In turn, molecules secreted by conceptuses, in particular interferon tau (IFNT), the maternal recognition of pregnancy signal in ruminants, but also prostaglandins (PGs; Dorniak *et al*., 2011, 2012; Spencer *et al*., 2013), induce changes in the uterine endometrium which are essential if pregnancy is to be maintained.

There is a strong positive association between the post-ovulatory rise in concentrations of progesterone (P4) and embryonic development in sheep and cattle (Satterfield *et al*., 2006; Carter *et al*., 2008). Much has been written about the role of P4 in the establishment and maintenance of pregnancy. Many researchers have tried to manipulate P4 concentrations during the first two weeks after mating in an attempt to achieve higher pregnancy rates. Rather than repeat in detail what has already been written, the reader is directed to several other recent comprehensive reviews on the subject (Lonergan, 2011, 2015; Wiltbank *et al*., 2014; Spencer *et al*., 2015).

Establishment of pregnancy in cattle

Following fertilization in the oviduct, the early

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embryo undergoes the first mitotic cleavage divisions before entering the uterus at about the 16-cell stage on approximately day 4 after ovulation. It soon forms a morula and, by day 7, a blastocyst containing an inner cell mass and a single layer of trophectoderm surrounding a fluid-filled blastocoel cavity. After hatching from the zona pellucida on approximately days 8 to 9, the spherical blastocyst grows and changes in morphology from a spherical to ovoid shape during a transitory phase preceding the elongation of the trophectoderm to a filamentous form that usually begins between days 12 and 14. The conceptus continues to grow and secrete IFNT, which prevents prostaglandin-induced luteolysis and maintains the pregnancy. Unlike primate and rodent embryos which invade the endometrium soon after hatching, ruminant conceptuses do not invade the endometrium during implantation, which commences at approximately day 19 in cattle (Guillomot, 1995), but rather undergo an extended free-floating period of development during which they exibit rapid elongtion of the extra-embryonic tissues.

In vitro fertilization studies have demonstrated that contact with the female reproductive tract is not necessary in order for the embryo to reach the hatched blastocyst stage. However, the characteristic elongation of the conceptus prior to implantation is dependent on secretions from the uterus. This elongation does not occur *in vitro* (Flechon *et al*., 1986) and does not occur *in vivo* in the absence of uterine glands (Gray *et al*., 2002; Spencer and Gray, 2006). Several authors have attempted to induce elongation *in vitro* by growing blastocysts in confined spaces (Brandão *et al*., 2004; Alexopoulos *et al*., 2005; Zhao *et al*., 2015) but while lateral expansion occurs, events as they occur *in vivo* are not recapitulated.

Thus, exposure to the uterine environment is required for conceptus elongation. Uterine luminal fluid (ULF) contains embryotrophic substances, collectively termed histotroph, that drive elongation of the conceptus via effects on trophectoderm proliferation and migration as well as attachment and adhesion to the endometrial luminal epithelium (LE; Gray *et al*., 2001; Spencer *et al*., 2008; Bazer *et al*., 2010; Forde *et al*., 2014a). The ULF is derived primarily from transport and (or) synthesis and secretion of substances by the endometrial LE and glandular epithelium (GE), but also by the conceptus (Forde *et al*., 2015), and it is a complex and rather undefined mixture of proteins, lipids, amino acids, sugars (glucose, fructose), ions, and exosomes/microvesicles (Bazer, 1975; Gray *et al*., 2001; Bazer *et al*., 2012; Burns *et al*., 2014; Forde *et al*., 2014b). P4 induces the expression of a number of genes in the endometrial epithelium that are then further stimulated by factors from the conceptus (e.g., IFNT and PGs) and the endometrium itself (Dorniak *et al*., 2013; Brooks *et al*., 2014; Lonergan and Forde, 2014). In turn, the genes and functions regulated by these hormones and factors in the endometrial epithelia cause specific changes in the uterine histotroph that govern conceptus survival and elongation (Faulkner *et al*., 2013; Forde *et al*., 2014a, 2015).

Progesterone and the endometrium

A prerequisite for establishing uterine receptivity to implantation in all species studied thus far is loss of expression of P4 receptors (PGR) from uterine LE and then GE (Bazer *et al*., 2010). Paradoxically, it is sustained exposure of the endometrium to circulating concentrations of P4 that leads to this down-regulation of PGR as the luteal phase of the estrous cycle progresses. The concentrations of P4 in circulation modify the loss of expression of PGR in the endometrium such that, in animals in which P4 is high there is early loss of the PGR (Okumu *et al*., 2010) i.e. uterine receptivity to implantation is established earlier. Conversely, low or sub-optimal concentrations of P4 delay loss of the PGR and thus delay establishing uterine receptivity to implantation (Forde *et al*., 2011a). Thus, in simple terms, it would appear that elevating P4 immediately after estrus or mating simply advances the changes in endometrial gene expression which normally occur (Forde *et al*., 2009).

The transcriptome of the bovine endometrium has been described under a variety of physiological and experimental conditions (Forde *et al*., 2009, 2011a, b; Sandra *et al*., 2011; Bauersachs *et al*., 2012; Binelli *et al*., 2015). Temporal changes in gene expression in the uterus occur irrespective of whether the cow is pregnant or not and it is really only at the time of maternal recognition of pregnancy at around day 16 that major changes in gene expression between pregnant and cyclic endometrium are detectable (Forde *et al*., 2011b; Bauersachs *et al*., 2012). Forde *et al*. (2009) described the global transcriptome of the endometrium from day 5 to day 16 in pregnant and cyclic cattle under conditions of normal and elevated P4 and revealed how circulating concentrations of P4 regulate endometrial genes. This study found that P4 supplementation advanced the normal temporal changes in endometrial gene expression, particularly for genes associated with energy sources or contributors to histotroph, which may contribute to advanced conceptus development on day 13 and day 16.

Progesterone and conceptus elongation

Elongation of the ruminant conceptus is essential for normal pregnancy recognition and implantation. Mamo *et al*. (2011) described the global transcriptome profile of the bovine conceptus at five key stages of its pre- and peri-implantation growth (days 7, 10, 13, 16, and 19). Analysis identified differentially regulated genes organized in nine gene clusters forming a sequential transcript dynamics across these developmental stages. These data have been expanded upon by more recent studies (Valour *et al*., 2014; Barnwell *et al*., 2015, 2016; Ribeiro *et al*., 2016a, b).

Flechon *et al*. (1986) cut day 12 ovine blastocysts into pieces and cultured them *in vitro* for 24 h, to produce structures called trophoblastic vesicles (TV, blastocysts without the embryonic disc). Such TV survived *in vitro* for up to 10 days but failed to elongate. In contrast, TVs elongated *in vivo* after transfer to

recipients demonstrating that trophoblast elongation does not depend necessarily on the presence of the embryo proper, but can occur in TV composed only of the trophectoderm and the extraembryonic endoderm.

Earlier studies in ewes (Wilmut and Sales, 1981; Lawson and Cahill, 1983) and cows (Garrett *et al*., 1988b) suggested that maternal P4 regulates early conceptus growth and development. More recent studies have confirmed those findings and began to unravel the underlying biology. In particular, significant progress has been made in clarifying the role of P4 in the successful establishment of pregnancy in sheep and cattle, with particular emphasis on how P4 affects endometrial gene expression and conceptus elongation.

The stimulatory effect of P4 on trophoblast elongation is unequivocal. As mentioned earlier, however, this effect is likely a result of downstream effects of P4-induced changes in gene expression in cells of the endometrium (Satterfield *et al*., 2006; Forde *et al*., 2009, 2011a) resulting in changes in the composition of ULF or histotroph to which the developing embryo is exposed (Faulkner *et al*., 2013). Whether any of the effects of P4 are directly on the embryo has been assessed by experiments in which P4 was added to medium during the *in vitro* culture of embryos. Results of such studies have been varied and contradictory with some authors reporting positive effects of P4 (Ferguson *et al*., 2005, 2011; Merlo *et al*., 2007) while others have reported no effect (Reggio *et al*., 1997; Goff and Smith, 1998). Overall, however, despite the presence of PGR mRNA on embryos (Clemente *et al*., 2009), there is little convincing evidence that P4 has a direct effect on the early embryo. In our own laboratory, culture of embryos *in vitro* in the presence of P4 did not affect the proportion developing to the blastocyst stage in the presence or absence of oviductal epithelial cells (Clemente *et al*., 2009). This finding is consistent with the observations of Larson *et al*. (2011) who failed to observe a direct effect of P4 either from days 1 to 3 or 4 to 7 after fertilisation. Furthermore, addition of P4 to culture medium had no effect on conceptus elongation after transfer to synchronised recipients (Clemente *et al*., 2009). In two other *in vivo* studies, we failed to demonstrate an effect of elevated P4 on blastocyst development. In the study of Carter *et al*. (2008), no differences in embryonic development on day 5 or day 7 were observed when beef heifers were supplemented with exogenous P4 from day 3, despite dramatic effects on post-hatching elongation between days 13 and 16 of pregnancy. In a follow-up study, multiple *in vitro* produced embryos were transferred to the oviduct of beef heifers that did or did not receive a P4 insert on day 3 after onset of oestrus. There was no effect of P4 on the proportion of embryos that developed to the blastocyst stage by day 7 (Carter *et al*., 2010).

The effects of elevated P4 shortly after conception on the advancement of conceptus elongation have been convincingly demonstrated in cattle and sheep. Garrett *et al*. (1988b) administered 100 mg P4 on days 1, 2, 3 and 4 of pregnancy which increased concentrations of P4 in peripheral plasma on days 2 to 5 and significantly larger conceptuses on day 14. Using a P4 implant on day 3 of pregnancy, Carter *et al*. (2008) significantly elevated concentrations of P4 in plasma until day 8 and this was associated with larger conceptuses recovered at slaughter on day 16. Similarly, when ewes received daily injections of 25 mg P4 from 36 h post-mating, blastocyst diameter increased by 220% on day 9 and at the time of initiation of elongation of blastocysts to a filamentous conceptus on day 12 was advanced (Satterfield *et al*., 2006); these effects of P4 treatment on blastocyst development were blocked by administration of RU486, a PGR antagonist.

As mentioned above, using a combination of *in vitro* embryo production and *in vivo* embryo transfer techniques, we have shown that the effect of P4 on conceptus development is mediated exclusively via the endometrium (Clemente *et al*., 2009). Interestingly, the embryo does not need to be present in the uterus during the period of P4 elevation in order to benefit from it (Clemente *et al*., 2009), strongly suggesting that the effect of P4 is via advancement of the normal temporal changes that occur in the endometrial transcriptome (Forde *et al*., 2009) resulting in advanced conceptus elongation. In addition, reducing the output of P4 from the CL, for example, by treatment with prostaglandin F2α (Beltman *et al*., 2009b; Forde *et al*., 2011a, 2012) or by aspirating the contents of the preovulatory follicle just before the expected time of ovulation (O'Hara *et al*., 2012) results in a delay in the temporal changes in the endometrial transcriptome resulting in delayed conceptus elongation *in vivo*.

Barnwell *et al*. (2015) examined the effect of embryo source (*in vitro vs. in vivo* derived) and recipient P4 concentration at the time of embryo transfer on conceptus development on day 17. They reported no relationship between P4 concentration on day 7 at the time of embryo transfer and conceptus length on day 17. Strangely, when only longer conceptuses were considered, heifers with *in vitro* produced embryos had lower P4 than those with *in vivo* derived embryos. In contrast, Frade *et al*. (2014) reported that higher plasma P4 concentration at timed embryo transfer was associated with increased pregnancy rate in *in vitro*-produced embryo recipients.

Asynchronous embryo transfer

The regulatory effect of the uterus on bovine conceptus development, and the role played by P4, has been beautifully illustrated in studies comparing the outcome of synchronous and asynchronous embryo transfer. Such synchrony between the needs of the developing embryo and uterine secretions has long been recognized as being critical to the successful establishment of pregnancy (Pope, 1988). Indeed, embryo transfer studies in sheep and cattle have clearly demonstrated a need for close synchrony between embryo and the uterine environment of the recipient. Previous studies have established that pregnancy rates are reduced when embryos are greater than 48 h from synchrony with the recipient's uterine environment (Moore and Shelton, 1964; Rowson and Moor, 1966;

Rowson *et al*., 1972).

Asynchronous transfer of day 7 bovine blastocysts to the uteri of day 5 or day 9 recipients resulted in retarded (5.4 ± 0.4 mm) or advanced (50.4 ± 5.2 mm) conceptuses on day 14, respectively, compared to synchronous controls (day 7 to day 7: 15.7 ± 1.5 mm) or conceptuses derived from AI (12.0 ± 3.3 mm; Ledgard *et al*., 2012). Consistent with these observations, Geisert *et al*. (1991) reported that only 1 of 21 (4.8%) day 8 bovine blastocysts transferred to a day 5 uterus established pregnancy compared to 50% in synchronous controls.

Administration of P4 early in the estrous cycle of the recipient has been shown in some cases to effectively advance uterine receptivity for the transfer of older asynchronous embryos. In sheep, day 6 recipients after early exposure to exogenous P4, supported development of transferred day 10 blastocysts (Lawson and Cahill, 1983). In cattle, embryo transfer to P4-treated recipients (100 mg/day from day 1 to day 4) which showed estrus 72 h after the donor cows (i.e., day 8 blastocysts transferred into a day 5 uterus) resulted in pregnancy rates at day 35 similar to those of synchronous (±12 h) recipients (42.1 *vs*. 50%), while, as mentioned above, only approximately 5% of day 5 asynchronous recipients became pregnant (Geisert *et al*., 1991).

Similar data have been reported recently by Randi *et al*. (2015) who transferred multiple day 7 bovine blastocysts to synchronous (day 7) or asynchronous (day 5 or day 9) recipients (n = 10 per recipient). Transfer of day 7 blastocysts to a day 5 uterus resulted in fewer conceptuses surviving (20%) and delayed elongation in those that were recovered. In contrast, transfer to an advanced day 9 uterine environment resulted in the same level of survival as synchronous controls (~50%), but conceptus elongation was markedly advanced, in agreement with the observations of (Ledgard *et al*., 2012). Supplementation of day 5 recipients with P4 from day 3 increased circulating concentrations of P4 and increased conceptus length compared to day 5 controls; however, supplementation with P4 reduced the length of estrous cycles in approximately 50% of heifers.

Together, these studies indicate that P4 stimulates changes within the uterine environment which regulate receptivity and promote embryo survival and conceptus elongation. Manipulating P4 may be one way of strategically regulating the temporal changes that normally occur in the uterine environment in order to allow flexibility in the timing of embryo transfer. Given the above results indicating that transfer to an advanced uterus (i.e., uterus ahead of the embryo), which has had longer exposure to P4 results in an advancement in conceptus elongation and that such advanced conceptuses produce more IFNT (Kerbler *et al*., 1997; Rizos *et al*., 2012), one could reasonably hypothesize that transfer to an advanced uterus would result in improved pregnancy rates. However, interrogation of data from commercial embryo transfer operations does not support that hypothesis (Wright, 1981; Donaldson, 1985; Hasler *et al*., 1987; Heyman, 1988; Hasler, 2001; Rodrigues *et al*., 2003; Randi *et al*., 2015). For example, in the study of Randi *et al*. (2015), 4749 recipients received a single *in vitro* produced fresh blastocyst. Overall pregnancy rate was 43.5%, which is about the norm in such commercial IVF operations. Transfer of a day 7 blastocyst to a synchronous day 7 uterus resulted in a pregnancy rate of 47.3%. Transfer to a uterus one day behind (day 6: 46.6%) did not affect pregnancy rate. However, transfer to a day 5 (40.8%) or a day 8 (41.3%) uterus moderately impacted pregnancy rate while transfer to a uterus 2 days in advance (day 9: 24.4%) or 3 days behind (day 4: 27.0%) dramatically reduced pregnancy rates compared to results from synchronous transfer of blastocysts. Taking results of all of these studies together, it is clear that the accelerated conceptus elongation associated with transfer of a blastocyst to an advanced uterus does not necessarily translate into an improved pregnancy rate; rather, once synchrony is exceeded by approximately 48 h, pregnancy rates decline appreciably.

Supplementation of progesterone and pregnancy rate

Results of several retrospective studies have indicated a positive relationship between circulating concentrations of P4 in the week after breeding and subsequent pregnancy rate (Stronge *et al*., 2005; Diskin *et al*., 2006; Parr *et al*., 2012). Interestingly, there is both a linear and quadratic component to this relationship; that is, too much P4 may lead to a decline in pregnancy rate. Thus, both sub-and supra-optimal concentrations of P4 from days 4 to 7 after AI or a sub-optimal rate of increase in the concentration of P4 during this interval are negatively associated with embryonic survival. Cummins *et al*. (2012) reported that circulating concentrations of P4 were 34% greater in cows with similar genetic merit for milk production traits, but with extremes of good (Fert+) or poor (Fert-) genetic merit for fertility traits. In a follow-up study, Moore *et al*. (2014) investigated the factors affecting circulating concentrations of P4 in those cows. Concentrations of P4 were measured from days 1 to 13. CL volume was 41% greater and mean circulating concentrations of P4 were 79% greater in Fert+ cows compared with Fert- cows. The results indicate that greater circulating concentrations of P4 were primarily due to a greater capacity of CL to secrete P4 rather than differences in clearance rate of P4 in this lactating cow genetic model of fertility.

Ultimately, circulating concentrations of P4 are determined by the balance between the rate of P4 production by the CL and the rate of P4 metabolism, mainly by the liver. Production of P4 is mainly regulated by the number of large luteal cells (LLC) and constitutive production of P4 by these cells which in turn is dependent on the provision of sufficient cholesterol substrate, mainly in the form of high-density lipoprotein (HDL). Increasing the number of granulosa cells and thereby the number of LLC , by ovulation of larger or multiple follicles, results in increased P4 output by the CL. Circulating HDL may be manipulated

by diet and this has been used as a strategy to increase P4 (Cordeiro *et al*., 2015). Metabolism of P4 is primarily related to the rate of blood flow to the liver (Sangsritavong *et al*., 2002) and is affected by the physiological condition of the cow. Therefore, practical strategies aimed at changing inherent CL capacity through genetic selection (Cummins *et al*., 2012; Butler, 2013; Moore *et al*., 2014) or the manipulation of circulating concentrations of P4 will be most productive by focussing on increasing luteal tissue volume to increase P4 production and/or limiting P4 metabolism (Wiltbank *et al*., 2014).

In a study in which inseminated cows were blood sampled on week 5 of presumed pregnancy, 50% of cows with P4 < 2.8 ng/ml aborted before week 9 of gestation and 95% of cows with P4 of 6.0 ng/ml on week 5 maintained pregnancy (Starbuck *et al*., 2004). Kenyon *et al*. (2013) determined P4 concentration from days 4 to 28 relative to presumptive estrus necessary for maintenance of pregnancy in lactating Holstein cows. An early rise in P4 from day 0 to 14 was associated with establishment of pregnancy after embryo transfer. Cows with P4 concentration <5 ng/ml on day 14 were more likely to lose pregnancy from day 28 to 63. Faster rise in P4 concentration during the metestrus and early diestrus are associated with pregnancy establishment following embryo transfer, which suggests that early rise in P4 concentration has an indirect effect on embryo development through modulation of uterine environment and secretion of histotroph. Furthermore, the positive effects of early rise in P4 concentration appear to go beyond the phase of maternal recognition of pregnancy through adhesion and placentation stages.

Given the importance of P4 for pregnancy establishment and the known effects on uterine receptivity and conceptus development many researchers have attempted to manipulate P4 using a variety of strategies in the days immediately post-conception in order to improve conception rates. Clearly, increasing concentrations of P4 after ovulation stimulates conceptus elongation in beef heifers, dairy cows, and sheep. However, supplementation of cattle with P4 during early pregnancy has resulted in mixed outcomes in terms of embryonic survival (Beltman *et al*., 2009a; Parr *et al*., 2014).

Based on the demonstration that elevated P4 accelerates conceptus development and that larger conceptuses produce more IFNT, one could reasonably hypothesize that such advanced conceptuses would be more likely to establish pregnancy. However, data on the impact of post insemination supplementation of P4 on pregnancy rate are conflicting and, at best, indicate a modest positive response. For example, in one recent large study, Nascimento *et al*. (2013) reported the results of 2 separate analyses that evaluated the effect of hCG treatment post-AI on fertility in lactating dairy cows. The first study was a meta-analysis of 10 different published studies that used hCG treatment on days 4 to 9 post-AI in lactating dairy cows. Overall, hCG administration increased pregnancies per artificial insemination (P/AI) by 3 percentage points [34% (752/2,213) *vs*. 37% (808/2,184)]. In a subsequent field trial, lactating Holstein cows (n = 2,979) from 6 commercial dairy herds received hCG or not on day 5 after a timed AI Pregnancies per AI were greater in cows treated with hCG (40.8%) than control (37.3%) cows. Interestingly, the positive effect of hCG (overall approximately 3.5%) was restricted to first-lactation cows.

A variety of strategies can be used to increase peripheral P4, ranging from those that stimulate endogenous production such as: (i) manipulation of follicular development to increase the size of the preovulatory follicle and hence the CL (Baruselli *et al*., 2012; Mesquita *et al*., 2014; Ramos *et al*., 2015); (ii) direct stimulation of CL development with luteotrophic agents (Maillo *et al*., 2014); (iii) induction of accessory CL using appropriately timed administration of GnRH or hCG (Santos *et al*., 2001; Stevenson *et al*., 2007; De Rensis *et al*., 2010; Lonergan, 2011; Torres *et al*., 2013); or (iv) direct supplementation with exogenous P4 through injections (Garrett *et al*., 1988b; Geisert *et al*., 1991; Pugliesi *et al*., 2014) or P4-containing devices (Stevenson *et al*., 2007; Carter *et al*., 2008; O'Hara *et al*., 2014b, c).

Paradoxically, depending on the timing of administration, exogenous P4 can have a negative effect on CL lifespan resulting in short inter-oestrous periods due to premature CL regression (Ginther, 1970; Garrett *et al*., 1988a; Burke *et al*., 1994) while at the same time advancing conceptus development due to the changes induced in the endometrium (O'Hara *et al*., 2014a). This situation is clearly not compatible with successful maintenance of pregnancy. It is possible that a combination of exogenous P4, to induce the required stimulation of the endometrium and conceptus, and luteotrophic support, such as that provided by hCG, to avoid early CL regression, would provide a means of optimizing maternal recognition of pregnancy. Indeed, administration of hCG at the time of P4 injections on days 1 to 4 overcame the negative effect on CL lifespan (Ginther, 1970). In support of this notion, in a recent study (O'Hara *et al*., 2014b), administration of eCG, a glycoprotein secreted by the endometrial cups of pregnant mares with a relatively long half-life of about 2-3 days and with both LH- and FSH-like properties in cattle, to beef heifers on day 3 post oestrus in association with an intravaginal P4 insert reduced the number of short cycles and increased mean luteal tissue weight and circulating P4. However, the numbers of heifers involved was small.

We have recently shown that a single i.m. injection of hCG as early as day 2 or day 3 after oestrus resulted in a larger CL and increased circulating concentrations of P4 compared to controls (Maillo *et al*., 2014). However, the results of Souza *et al*. (2015) examining the effect of administration of long-acting injectable P4 (LAP4) and/or hCG on luteal function and conception rate of high producing dairy cows (n = 982) would suggest that this does not translate into improved pregnancy rates. Cows were assigned to one of four groups: (i) control; (ii) 900 mg LAP4; (iii) 2000 IU hCG; (iv) a combination of LAP4 and hCG. While treatments resulted in elevated P4, conception rate after

30 days was higher in the LAP4 group, but not in the hCG or LAP4 + hCG groups. Conception rates at 60 days, as well as pregnancy loss between 30 and 60 days after TAI were not affected by treatment.

Final remarks

One consistent observation from the multiple embryo transfer studies we have carried out, involving the transfer of 10-20 day 7 blastocysts to the uterus of synchronized recipients and recovery on day 14, is the variation in conceptus size on day 14, even amongst those recovered from the same uterus. Such differences in conceptus length on the same day of gestation may be related to an inherent lack of developmental competency or may simply be a consequence of asynchrony with the maternal environment. It would suggest that factors intrinsic to the blastocysts transferred regulate development, at least in part, and would be consistent with the hypothesis that the quality of the oocyte regulates developmental competence (Rizos *et al*., 2002).

Our current studies are aiming to understand the underlying factors that regulate conceptus elongation and to attempt to separate those intrinsic to the conceptus from those intrinsic to the uterus. In this regard, Barnwell *et al*. (2016) recently characterized differential patterns of mRNA expression between short and long bovine conceptuses recovered on day 15 of gestation which may be indicative of conceptus survival.

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Strategies to avoid drowning in the deep sequencing data flood

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Abstract

The enormous technological progress in the field of functional genomics during the last 15 years had a significant impact on animal sciences. With the development of Next Generation Sequencing it became feasible to analyze genomes and transcriptomes within short time frames and affordable costs. One major challenge of this rapid development is to manage the data flood and to perform data analysis and integration in an optimal manner. This review provides some information about a typical analysis pipeline for RNA-Sequencing (RNA-Seq) data and a strategy for the analysis of small RNA-Seq data derived from species with poor annotation for non-coding RNA genes. Furthermore, problems regarding gene annotation in livestock species and their possible implications for data analysis and interpretation are discussed. Despite of not yet solved problems and challenges with respect to data analysis and integration the approaches in the field of functional genome analysis opened up new ways to try to understand the complex trait fertility.

Keywords: animal breeding, bioinformatics tools, biology of reproduction, deep sequencing, Galaxy project.

The impact of functional genomics on life science research

The tremendous technological advances in the field of functional genomics during the last decades had a strong impact on research in animal sciences. This is reflected, e.g., by a dramatic increase of the number of publications containing respective keywords (Fig. 1). The first wave started with the broad application of DNA microarrays end of the nineties, and a similar rise is observed for studies using RNA sequencing (RNA-Seq). With respect to livestock the increase of the number of published transcriptome studies showed a shift of two to three years.

The development of Next Generation Sequencing (NGS) facilitated the analysis of genomes and transcriptomes in an extremely short time at affordable costs (Goodwin *et al*., 2016). With the newest instruments for the generation of so-called "short reads" (up to 2X 150 bp, Illumina HiSeq 4000) it is currently possible to obtain up to 1.5 Tera bases corresponding to 5 billion reads per run or 12 genomes or 100 transcriptomes or 180 exomes per instrument run which takes 3.5 days. Furthermore, Third Generation sequencers deliver extremely long reads and can be used to sequence full-length RNA molecules (messenger as well as long non-coding RNAs) or to bridge longer repetitive genomic sequences to fill the gaps of the current versions of genome sequence assemblies (Goodwin *et al*., 2016). But also in the field of proteome analysis, the techniques have advanced, particularly mass spectrometric methods. Improvement has been achieved mainly with respect to sensitivity and quantification (Zhang *et al*., 2014a, b). Furthermore, NGS techniques have been refined in order to analyze tiny amounts of RNA or DNA. Whereas early RNA-Seq library preparation protocols needed starting material (total RNA) in the microgram range, modern standard protocols start from 100 ng of total RNA. Special protocols were developed to perform RNA-Seq even for a few or single cells such as oocytes and early embryos but also with parts of neuronal cells (Liu *et al*., 2014; Hrdlickova *et al*., 2016; Marr *et al*., 2016).

With this rapid development, particularly for NGS, a big challenge came up with respect to data analysis, interpretation, and integration (Rajasundaram and Selbig, 2016; Sun and Hu, 2016; Suravajhala *et al*., 2016). More and more data sets are generated for the analysis of gene expression at the level of RNA and proteins as well as for the genome-wide identification of sequence variants correlating with the trait fertility (Bauersachs, 2014; Bauersachs and Wolf, 2015). The combination of data from genome-wide association studies (GWAS) or quantitative trait locus (QTL) studies with corresponding data derived from gene expression analyses has a great potential to improve the understanding of the trait fertility with respect to the effects of sequence variations on gene expression regulation. A number of attempts to integrate these data have been performed for cattle (Pimentel *et al*., 2011; Minten *et al*., 2013; Moore *et al*., 2016).

In addition to the classical gene products mRNA and protein also non-coding RNA molecules are investigated which mainly have a role in regulation of gene expression (Bidarimath *et al*., 2014; Kotaja, 2014). Particularly, microRNAs (miRNAs), short non-coding regulatory RNAs, play a major role in the regulation of gene expression mainly at the level of repression of translation of specific target mRNAs as well as mRNA degradation (Krol *et al*., 2010). The expression of miRNAs in endometrium and in the embryo/conceptus has already been investigated in a number of studies (Ponsuksili *et al*., 2014; Krawczynski *et al*., 2015a, b).

For various reasons, such as not well standardized data analysis pipelines, incomplete genome sequence assemblies for livestock species, and incomplete gene annotation the analysis and the comparability of different data sets is complicated. This

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is even more complicated if omics data has been generated in different labs using various technological platforms (Bauersachs, 2014). To solve these problems will be one of the main tasks for future research if the scientific community is interested in exploiting the potential of omics studies and in a real progress in the field, i.e., the understanding of fertility as a complex trait.

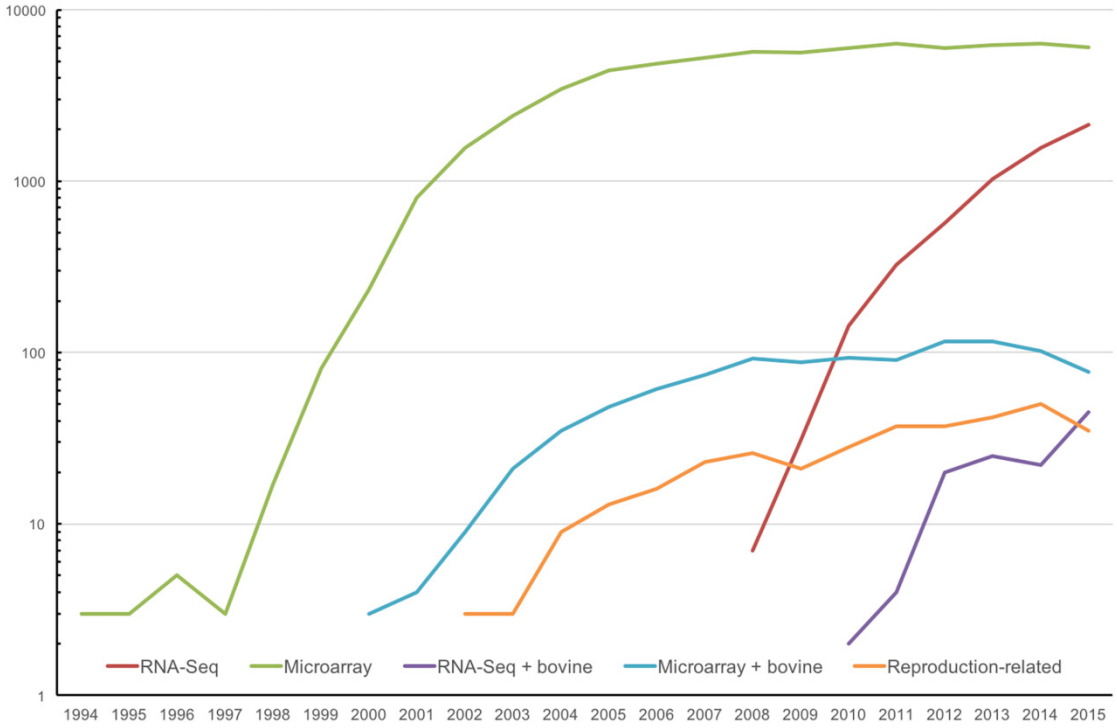

Figure 1. Pubmed search for abstracts containing keywords related to transcriptome analyses. RNA-Seq: keyword "RNA-Seq"; Microarray: keyword "microarray"; Microarray+bovine: keywords "microarray", "bovine", "*Bos taurus*", "cattle"; RNA-Seq+bovine: keywords "RNA-Seq", "bovine", "*Bos taurus*", "cattle"; Reproduction-related: using a combination of keywords for transcriptome analysis, livestock species, and reproductive organs.

Typical data analysis pipeline and statistical analysis

A typical data analysis pipeline for RNA-Seq data comprises several steps starting from the obtained sequence reads (Fastq files). Usually, the sequence reads are first trimmed based on quality scores (e.g. with Trimmomatic), i.e., bases with low quality at the ends (mainly found at the 3' end) are removed. Since RNA-Seq libraries often contain a certain percentage of cDNA inserts shorter than the read length, some reads run into the adapter sequence which has to be removed using a respective tool. To get information for the quality of the sequence data, Fastq files are checked before and after processing steps (e.g. FastQC) to ensure that all files have a comparable quality and to identify potential sequencing artifacts. After these data processing steps, the remaining reads are usually mapped to a reference genome or a transcriptome. The first is usually performed by the use of a spliced read mapper, e.g., Tophat2 (Kim *et al*., 2013) or HISAT (Kim *et al*., 2015). After assigning the sequence reads to a specific location in the genome the reads are counted for each exon, transcript or each gene. This can be performed on the basis of available gene annotation from NCBI or Ensembl. Alternatively, the data itself can be used to complement existing gene annotation using tools like Cufflinks or StringTie (Trapnell *et al*., 2012; Pertea *et al*., 2015). In the first years of RNA-Seq data analysis most of the tools were only available in command line mode running on Linux systems. With the integration into the Galaxy platform, a web browser-based genome analysis tool (Blankenberg *et al*., 2010), complex large-scale analyses can be performed without informatics or programming expertise (Giardine *et al*., 2005). Finally, these steps result in a read count table that is used for analysis of differential gene expression. Widely used tools for the analysis of read count data and the identification of differentially expressed genes (DEG) are the BioConductor R packages EdgeR (Robinson *et al*., 2010) and DESeq2 (Love *et al*., 2014). Since a local installation of Galaxy on a LINUX server is necessary to analyze bigger data sets such as RNA-Seq data an alternative way is to do the complete analysis of RNA-Seq data by the use of R and BioConductor on a desktop computer (Anders *et al*., 2013).

Analysis of small RNA-Seq data sets with special adaptation to poorly annotated species

For the analysis of small RNA-Seq data sets a modified analysis pipeline is needed compared to the basic analysis pipeline for RNA-Seq data since the resulting reads represent, at least in theory, the entire

sequence of a small ncRNA. The typical processing steps of the FastQ files starting with quality control up to adapter clipping are similar. However, the use of spliced mappers like TopHat2 (Kim *et al*., 2013) or HISAT (Kim *et al*., 2015) for the analysis of smallRNA-Seq data sets is not appropriate because small RNAs are usually neither spliced nor found in coding regions of annotated genes. This leads to the necessity of a different mapping and sequence annotation strategy. For example, mapping to a reference genome using the Burrows-Wheeler Alignment tool (BWA; Li and Durbin, 2009) to map against a reference genome or NCBI BLAST (Altschul *et al*., 1997) for short sequences which is also available in Galaxy (Blankenberg *et al*., 2010) are suitable options. The BWA aligner works best for well annotated genomes where almost all short ncRNAs are known. So, the obtained sequences are just mapped to the corresponding genes and miRNA sequences (canonical and isomiRs) can be easily analyzed with tools like miRDeep2 (Friedlander *et al*., 2012). Because the BLAST algorithm is too slow for the analysis of too high numbers of sequence comparisons the number of unique sequences found in smallRNA-Seq libraries have to be appropriately filtered, e.g., based on a counts per million (CPM) cut-off, to reduce the number of sequences from hundreds of thousands or even millions to several thousand. This filtering removes at the same time sequences without biological relevance or sequences which are very likely to be the result of sequencing artifacts. An example for this data analysis strategy is shown in Fig. 2.

A challenging problem for livestock species including pig and cattle is the rather low number of annotated small ncRNAs, which complicates the use of BWA for mapping and miRDeep2 for identification of miRNAs. Furthermore, small RNA libraries usually contain also many other small RNAs in addition to miRNAs, such as fragments of ribosomal RNAs (rRNA), transfer RNAs (tRNA), small nucleolar RNAs (snoRNA), small nuclear RNAs (snRNA), and Piwi-associated small RNAs (piRNAs) in case of germline cells (Cole *et al*., 2009). Although the prediction of novel miRNAs can be performed by the use of miRDeep2 (Friedlander *et al*., 2012), the annotation of sequence fragments derived from other RNA molecules is more difficult. In contrast in humans, a great variety of ncRNAs is known compared to other mammalian species. This information can be used to improve annotation of small RNA data from other species since many of these RNAs are highly conserved. The use of BLASTn-short (local installation in Galaxy) for sequence comparison to all available sequences for RNA molecules of the target species and the inclusion of ortholog information derived from well annotated species from different annotation sources significantly improves the annotation of identified sequences found in the small RNA-Seq results to 80-90%, depending on the species and the sample type (Jochen Bick, 2016; ETH Zurich; personal communication). The consideration of the frequent occurrence of sequences representing isoforms of miRNAs (isomiRs; Krawczynski *et al*., 2015a; Zhang *et al*., 2016) can further improve sequence annotation. IsomiRs result from imprecise and alternative cleavage during the pre-miRNA processing and post-transcriptional modifications. The isomiRs show different miRNA stability, sub-cellular localization, and target selection (Zhang *et al*., 2016). Since post-transcriptional modification during miRNA processing also leads to the addition of nucleotides not matching to the genome sequence those isomiRs cannot be easily mapped using BWA and/or miRDeep2. Using the annotation strategy based on BLASTn searches following statistical data analyses can be performed including various types of small ncRNAs or miRNAs only. Furthermore, based on the attempt to annotate as much as possible of the obtained sequences, percentages of read counts in relation to the total number of read counts can be calculated for individual types of ncRNAs. This can also help to identify technical or biological outliers in a data set.

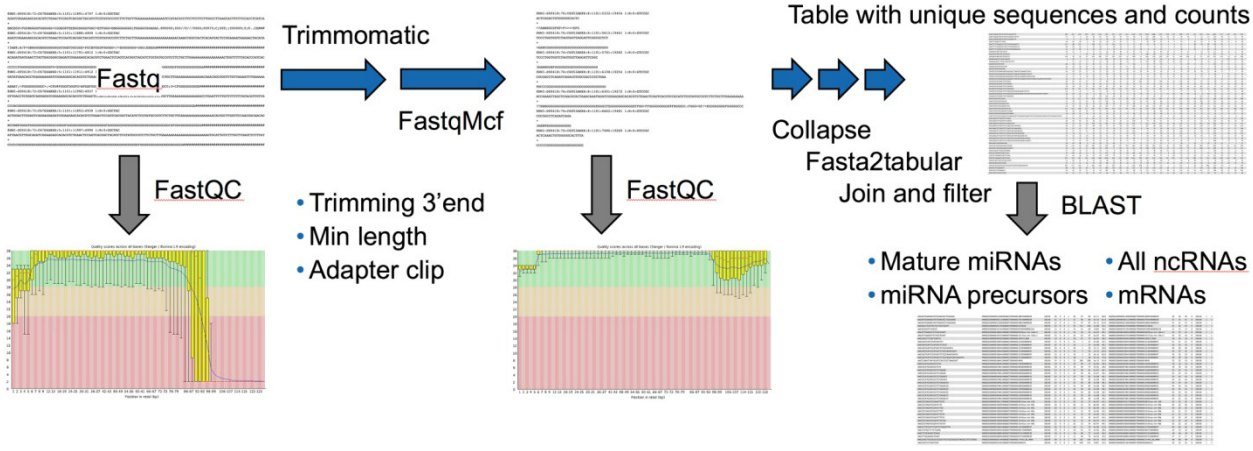

Figure 2. Workflow for data analysis of small RNA-Seq data performed by the use of Galaxy tools. The workflow goes from left to the right and includes processing of sequence files, generation of a read count table, and annotation of the obtained sequences. Fastq: sequence files derived from Illumina sequencer; FastQC: tool for quality control of fastq files; Trimmomatic and FastqMcf: tools for processing fastq files; BLAST: Basic Local Alignment Search Tool.

Gene annotation in livestock

The efforts to sequence the human genome (Lander *et al*., 2001) were extremely high and cost alone the US tax payer almost three billion dollars. In addition to the genome sequence itself large projects were performed to sequence full-length cDNAs from mRNA derived from almost all human tissues (Wiemann *et al*., 2001) to obtain information about transcribed regions in the genome, gene structures, and transcript isoforms. Meanwhile, genomes have been sequenced also for livestock species (Elsik *et al*., 2009; Wade *et al*., 2009; Groenen *et al*., 2012). However, gene annotation for these species is still based in large part on the comparison to human or mouse orthologous genes. In addition, different annotation pipelines, e.g., NCBI and Ensembl, provide gene models which show sometimes substantial differences making the correct assignment of genes annotated with different pipelines at the same genomic locus difficult. The corresponding information for the assignment of genes in Entrez Gene to genes in Ensembl is incomplete (NCBI->Ensembl) or incorrect (Ensembl->Entrez Gene). This is a serious problem if useful information such as ortholog annotation found in one database should be assigned to gene IDs of the other database.

For the functional annotation and downstream bioinformatics analysis the use of gene IDs of livestock species is not optimal and leads to information loss. The reason for that is incomplete annotation, i.e., many genes still do not have the official gene symbol and are not assigned to functional annotation databases such as Gene Ontologies (Ashburner *et al*., 2000) and KEGG pathway database (Kanehisa and Goto, 2000). To avoid this loss of information the putative human ortholog information can be used. One resource for ortholog information is for example EnsemblCompara (Vilella *et al*., 2009; Pignatelli *et al*., 2016). In order to combine information derived from different databases provided by the NCBI and Ensembl we are developing a Mammalian Ortholog and Annotation database (MOA-Db) integrated in the Galaxy platform in our group (Jochen Bick, 2016; ETH Zurich; unpublished results). A schematic overview of the MOA-Db is shown in Fig. 3.

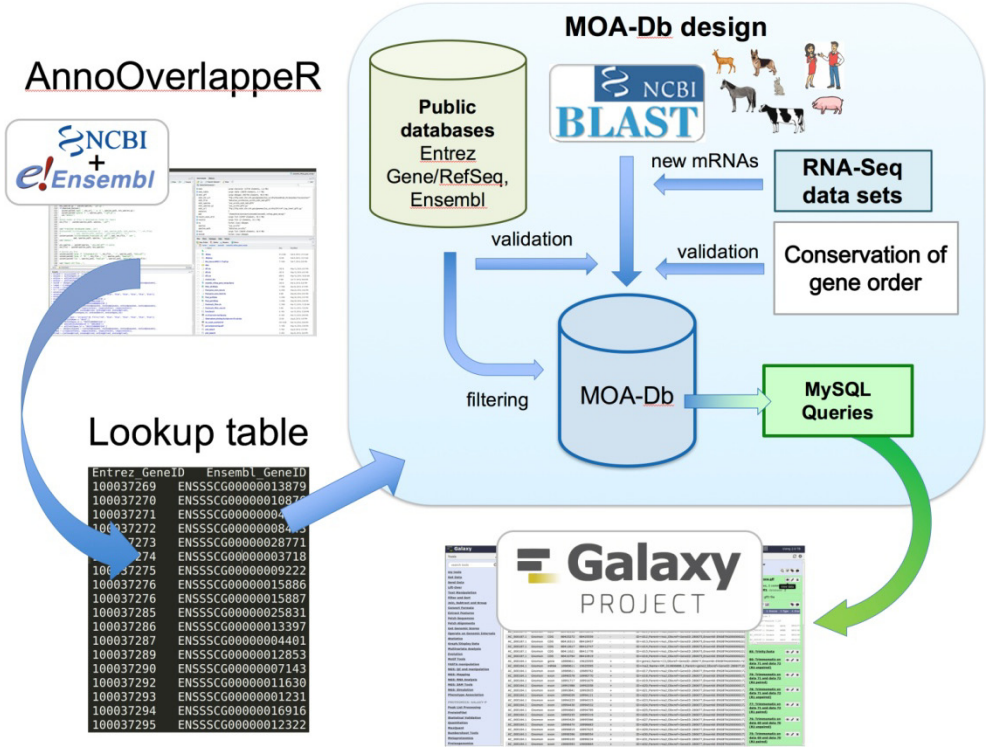

Figure 3. Development of a Mammalian Ortholog and Annotation database (MOA-Db). Based on information derived from public databases and available RNA-Seq data sets an annotation database is built for a number of mammalian species including gene annotation from NCBI and Ensembl as well as ortholog information. The ortholog relationships are based on information extracted from databases such as EnsemblCompara as well as on crosswise global BLAST comparisons of all transcripts annotated at NCBI for each species.

Conclusions

The development of functional genomics approaches opened new ways to improve our understanding of the complex trait fertility. After the first wave of enthusiasm it is becoming more and more evident that there is a number of big challenges in the context of data analysis and integration. The main problems are inherent in missing standards for data analysis pipelines, integration of different kinds of data sets, bias in data sets related to different laboratories, protocols, and the use of different platforms. Furthermore, a particular challenge is the integration of results from different omics approaches, such as genome, transcriptome, proteome, and metabolome analysis. A major obstacle for the integration of omics

data sets is the existence of a plethora of different databases and corresponding identifiers as well as incomplete, inconsistent, and not coordinated gene annotations, e.g., when comparing genome annotation at NCBI and Ensembl. In addition, an insufficient and/or erroneous gene or protein annotation leads to a significant loss of information and in the worst case to wrong data interpretation. Despite of all these problems and challenges, the development of the new sequencing technologies and the foreseeable even more exciting developments with respect to functional genomics technologies promise a new era of research in the animal sciences.

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Role of the oviduct and oviduct-derived products in ruminant embryo development

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Abstract

The fact that embryos can be obtained *in vitro* undermines the role of the oviduct. However, it has been demonstrated that when *in vitro* produced bovine zygotes are cultured in the oviduct of sheep, cattle or mice the embryo quality is improved compared to the embryos produced *in vitro*. Thus the oviduct is not simply a passive organ required only for transporting the embryo to the uterus but also provides a suitable microenvironment for the early embryo. The study of physiological mechanisms and interactions between the embryo and the oviductal environment is essential to understand the correct processes of early embryo developmental. This knowledge can be used to improve current *in vitro* procedures providing high quality embryos capable of continued development and implantation, and resulting in viable births.

Keywords: bovine, embryo development, *in vitro,* oviduct.

Introduction

In vivo, oocytes and embryos develop in a complex and dynamic environment. First, in the ovarian follicle, the oocyte grows and matures, achieving full developmental competence just prior to ovulation. Subsequently, in the oviduct, the oocyte undergoes fertilization and early embryonic development. Finally in the uterus, the blastocyst forms, hatches from the zona pellucida, elongates, and progressively attaches to the uterine wall (Spencer *et al*., 2007). Therefore, the environment where the early embryo develops has a significant impact on the subsequent embryonic development in the short and long term.

In vitro embryo production seeks to mimic the physiological conditions in which embryos normally develop to produce embryos at the appropriate stage and optimal quality. These characteristics are necessary to establish a pregnancy and to produce a healthy offspring after transfer.

In the last 20 years, researches on *in vitro* embryo production in ruminants have focused on two crucial questions: how to maximize embryo development and optimize quality of the blastocysts produced. Although a certain amount of progress has been made in both areas, the quality of *in vitro* produced blastocysts continues to lag behind those obtained *in vivo*. This inferiority of *in vitro* produced embryos is manifested in terms of morphology, cryotolerance, gene expression and pregnancy rate after embryo transfer (Lonergan and Fair, 2008).

It has been demonstrated that the oviductal environment supports embryonic growth up to the blastocyst stage after trans-species transfer across a wide range of species (Fair *et al*., 2001; Lazzari *et al*., 2002; Rizos *et al*., 2007). Using the sheep oviduct *in situ* for culturing *in vitro* produced zygotes, it was clearly shown that the key part of the process responsible for suboptimal embryo quality is the post-fertilization period (Galli and Lazzari 1996; Enright *et al*., 2000; Rizos *et al*., 2002a, b). Thus, studying the oviductal environment and the signals exchanged between the oviduct and/or the early embryo is crucial to improve our understanding of the underlying regulatory mechanisms controlling embryo development (Aviles *et al*., 2015). Furthermore, this knowledge would allow the development of *in vitro* models capable to produce embryos of better quality and also to study embryo-maternal interactions. In this review we will discuss the role of the oviductal environment on early embryo development and embryo quality based on evidence from both *in vivo* and *in vitro* studies in ruminants.

Role of the oviduct during early embryo development

The oviduct is a tubular structure, sustained by the mesosalpinx, that connects the ovary to the uterine horn. The oviduct is divided in five morphological and functional parts: (i) the infundibulum, (ii) the ampulla, (iii) the ampullary-isthmic junction, (iv) the isthmus and (v) the utero-tubal junction (Maillo *et al*., 2016b). The infundibulum is the most proximal structure to the ovary and is funnel-shaped, and its fimbriae receive the oocyte after ovulation. The ampulla is the wider part of the tubal structure. The ampullary-isthmic junction is the place where fertilization takes place (Hunter, 2012). The isthmus presents a narrow lumen and is the place where the sperm reservoir is established prior to fertilization; and also where the early stages of embryo development take place. The utero-tubal junction connects the isthmus to the uterus (Yániz *et al*., 2000).

The oviduct is an active organ that maintains and modulates the fluidic milieu for sperm capacitation, transport and fertilization of the mature oocyte and early embryonic development (Rodriguez-Martinez, 2007; Leese *et al*., 2008; Lloyd *et al*., 2009). After fertilization, the developing embryo passes through the isthmus, through ciliary movements and muscular contractions, until it reaches the uterus about 16-cell stage on day 4 (Ellington, 1991). Therefore, the first

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stages of bovine embryo development occur in the oviduct (Hackett *et al*., 1993). All these events generate an interest in better understanding the role of the oviduct as a multifunctional and specialized reproductive organ (Rodriguez-Martinez, 2007; Leese *et al*., 2008).

Oviductal epithelium

Oviductal epithelium is composed of two different cell types, ciliated and secretory. During gamete and embryo transport, the ciliary cells exhibit a synchronized movement leading to a directed flow of fluids (Abe and Hoshi, 1997). Secretory cells have microvilli on their apical side and secrete substances and growth factors, usually by exocytosis, associated with the first days of the oestrous cycle, which contribute to the development of the early embryo (Abe, 1996; Murray and Smith, 1997).

Sperms transiently adhere to the epithelial cells lining the caudal isthmus, constituting the sperm reservoir. This interaction is important because it lengthens the fertile lifespan of sperm, regulates capacitation and also controls the number of sperm present at the site of fertilization to limit the opportunity for polyspermy (for review, see Miller, 2015)

Populations of the different epithelial cells are dynamic during the phases of the oestrous cycle. The proportion of ciliated cells decreases in the infundibulum and the ampulla during the luteal phase compared with the follicular phase (Yániz *et al*., 2000). Moreover, cell morphology is modified as a function of embryo development and cycle stage (Suuroia *et al*., 2002). The height of ciliated cells decreases in the infundibulum and ampulla during the luteal phase and in the isthmus the height of secretory cells also diminishes (Abe *et al*., 1999). Furthermore, transcriptome approaches have identified different functional groups of genes involved in the regulation of the oviduct during the oestrous cycle (Bauersachs *et al*., 2004). Recently, Cerny *et al.* (2015) identified, in bovine oviductal epithelial cells (BOEC), a large number of differentially expressed genes (DEGs) between the follicular (1563 DEGs) and luteal (1758 DEGs) phases, with 616 DEGs exclusive to the ampulla and 811 DEGs exclusive to the isthmus. Similarly, we identified DEGs between the oviductal epithelial cells from the ampulla and isthmus of pregnant heifers collected on day 3 after oestrus. This may reflect morphological and functional differences for those regions (Maillo *et al*., 2016a).

Oviductal fluid

Oviductal environment are reflected in the composition of the oviductal fluid (OF). The OF is generated by (i) transudation from plasma into the oviductal lumen together with (ii) the secretion of substances synthesized by the secretory cells (Menezo and Guerin, 1997). OF composition is very complex, containing simple and complex carbohydrates, ions, lipids, phospholipids and proteins (Leese *et al*., 2001; Avilés *et al*., 2010). Some of these components are metabolic substrates, such as lactate, pyruvate, amino acids, and glucose, whose concentrations differ from those present in the uterine fluid and serum (Hugentobler *et al*., 2007, 2008).

Secretions present in the OF affect oocyte and sperm function (Killian, 2011; Mondejar *et al*., 2013) with proteins such as glycodelins, and lactoferrin involved on gamete interaction (Ghersevich *et al*., 2015) and oviductin, osteopontins and the complement protein C3 involved in early embryo development (Tse *et al*., 2008). In addition, these proteins together with others present in the OF have been previously reported to play direct roles in sperm motility, viability (Kouba *et al*., 2000), sperm-ZP binding (Banerjee and Chowdhury 1994), ZP hardening (Kratz *et al*., 2003), embryo-maternal interactions (Reed *et al*., 1998), oocyte (Hess *et al*., 1999), early embryo development (Lim and Hansel 1998), cell proliferation (Hulboy *et al*., 1997), differentiation and apoptosis, fertilization rates (Dinara *et al*., 2001), and pH (Ekstedt *et al*., 2004).

The oviduct-specific glycoprotein (OVGP1) is a component of the OF identified in many species in a highly conserved form. It is one of the most studied proteins in the OF. OVPG1 synthesis and secretion is dynamic and related to oestrogen (Buhi, 2002; Killian, 2004) and luteinizing hormone stimulation (Sun *et al*., 1997). OVGP1 binds to the zona pellucida (ZP) of the oocyte and early embryo suggesting a role in early embryo development (Buhi, 2002). It has been shown that embryo culture in the presence of oviductin increased embryo development *in vivo* in pigs (McCauley *et al*., 2003) and sheep (Pradeep *et al*., 2011). Coy *et al.* (2008, 2012) demonstrated that OVGP1 and heparin-like glycosaminoglycans from the oviductal fluid of sows and cows participate in the functional modification of the ZP, affecting the sperm-oocyte interaction and contributing to the control of polyspermy. Besides, OVGP1 and sperm interactions increased rates of fertilization and embryonic development (Killian, 2004). In addition, it is suggested that OVGP1 stabilizes the microenvironment surrounding by gametes and embryo, preventing dispersal of essential nutrients and ions, particularly during ciliary beating or muscular contraction, increasing the viscosity of luminal fluid (Hunter, 1994; Mondejar *et al*., 2012).

Proteomic studies of the OF have demonstrated that gametes modulate the oviductal environment in a favourable way to prepare the oviduct milieu for the arrival of the embryo (Georgiou *et al*., 2005). Sperm regulated twenty proteins, while the oocyte regulated only one protein (Ig kappa light chain). Three proteins were commonly regulated by both gametes (Complement Component C3, Ig kappa variable region, and haemoglobin beta chain), and one protein showed regulation by sperm and oocytes in opposing directions (Complement Component C3; Georgiou *et al*., 2007).

Embryo-maternal communication in the oviduct

As mentioned before, after fertilization the first few mitotic cleavage divisions take place in the isthmus

(Hunter, 1998). On day 3.5 to 4 after fertilization, the early embryo, at the 8- to 16-cell stage, moves from the oviduct to the uterus (Hackett *et al*., 1993) continuing the mitotic divisions forming first a compact agglomerated of cells called morula and, by day 7 to 8, a blastocyst.

For a successful pregnancy establishment, a complex signal exchange between the newly formed embryo and the mother is essential. In ruminants, the principal pregnancy-recognition signal produced by the embryo is interferon-tau, secreted by the trophoblast from day 10 up to day 21-25 (Spencer and Bazer, 2004). Alterations in the environment of the early embryo could have consequences in the subsequent development. Thus, a high proportion of embryonic losses occur between days 8 and 17 of pregnancy (Humblot, 2001; Thatcher *et al*., 2001).

The oviduct, as the first site of embryo development, is considered a starting point to examine putative signals between the embryo and the reproductive tract (Wolf *et al*., 2003). The embryo in the oviduct undergoes epigenetic changes responsible for further development, implantation and postnatal phenotype (Wrenzycki *et al*., 2005). However, the mechanisms involved in this embryo-maternal communication currently are mostly unknown (Fazeli, 2008).

Evidence *in vivo* in mice, by RT-qPCR showed changes in the oviductal gene expression depending on the presence or absence of embryos (Lee *et al*., 2002). Recently, new transcriptomic technologies (e.g., microarrays) have been used to elucidate the complex molecular dialogue between maternal tract and the embryo. Thus, in pigs Almiñana *et al.* (2012) showed that embryo-maternal communication exists at earliest stages of pregnancy, before the well-known embryonic signal of maternal recognition. In contrast, Maillo *et al*. (2015) did not find differences in the bovine oviduct transcriptome in the presence or absence of an 8- to 16-cell embryo *in vivo*. Obviously, multi-ovulatory species like mice and pigs cannot be directly compared with mono-ovulatory species such as cattle. Thus, the bovine model would provide new information on early embryo maternal communication that may be important for humans.

In this communication, the embryo might play a role as a modulator of the immune system in the maternal tract, inducing the down-regulation of immune related genes to allow the refractory uterus to tolerate the embryo and support its development (Almiñana *et al*., 2012). In a recent study from our group, it was necessary to transfer multiple embryos (up to 50) into the oviducts of heifers to detect differences in the transcriptome. When a single embryo was transferd into the oviduct (pregnant vs cyclic heifers) no differences were found, suggesting a local effect of the embryo (Maillo *et al*., 2015). More recently, Smits *et al*. (2016) reported a local influence of the embryo on the transcriptome of the equine oviduct epithelium.

Oviductal environment and *in vitro* models

In vitro systems are a valuable tool to study pathways and mechanisms, which are difficult to study *in vivo,* and cell cultures provide valuable aspects of physiologic or pathologic mechanisms. Studying the oviductal environment is crucial to understand the underlying regulatory mechanisms controlling embryo development (Aviles *et al*., 2015). The advantages of the oviductal environment have been demonstrated in different models; many physiological aspects have been clarified; however, many others still remain unknown (Hunter, 2012).

The culture of bovine oviductal epithelial cells (BOEC) as a monolayer may provide useful information on early embryo maternal interaction signals. Recently, Schmaltz-Panneau *et al*. (2014) described transcriptome changes in BOEC related to the presence of bovine embryos. BOEC are usually obtained from oviducts of slaughtered heifers or cows. When a BOEC line is established for *in vitro* embryo co-culture, it is essential to determine the stage of the oestrous cycle of the oviducts used. BOEC at oestrus have been successfully used as *in vitro* model simulating embryo maternal interactions (Rief *et al*., 2002). Recently, Cordova *et al*. (2014) used oestrus-metoestrus (day 0-3) BOEC for early (day 1-4) or late (day 4-7) embryo co-culture showing that the presence of the cells during the first four days of development, which correspond to the presence of embryos in the oviduct *in vivo*, accelerated the kinetics of blastocyst development and induced changes in genes involved in epigenetic control. The positive effects of these cells on the embryos are attributed to embryotrophic substances, such as growth factors secreted by the cells (Nancarrow and Hill, 1994; Vanroose *et al*., 2001). Besides, BOEC modulates the surrounding environmental conditions, decreasing the oxygen levels in the culture medium, preventing the formation of deleterious radicals as reactive oxygen species (ROS; Thompson *et al*., 2000; Vanroose *et al*., 2001), removing toxic substances from the medium (e.g., ammonia; Nancarrow and Hill, 1994) and decreasing the glucose and ion levels that could have detrimental effects on the embryos (Vanroose *et al*., 2001). The drawback of co-culture systems is that they have been associated with methodological complexity, lack of repeatability and biosanitary risk (Menezo and Guerin, 1997). To avoid the use of primary cultures that have a risk of contamination, the use of established cell lines allows standardized culture conditions and better control (Pegoraro *et al*., 2000). We recently reported that an established BOEC line can be used successfully after freezing and thawing as an *in vitro* embryo co-culture system, avoiding the lack of reproducibility between replicates, and did not differ from BOEC in suspension in terms of embryo development (Lopera-Vasquez *et al*., 2016a).

An alternative to co-culture, avoiding a direct contact between BOEC and embryos, is the use of conditioned media from BOEC which has a positive effect on embryo development and percentage live calves after transfer (Lim *et al*., 1997). The BOEC conditioned media is able to support embryo development to the blastocyst stage (Mermillod *et al*., 1993) through identified secreted embryotrophic

components such as OVGP1 (Briton-Jones *et al*., 2004), ET-1 (Reinhart *et al*., 2003), IGF (Xia *et al*., 1996; Winger *et al*., 1997), VEGF, EGF, IGF1, TGFβ2, and IL4 (Okada *et al*., 2005). However, many other secretions still remain unknown. Therefore, BOEC co-culture and/or their secretions must be a key for studying embryo maternal interactions and improve *in vitro* current systems.

As mentioned before, the OF is responsible for nurturing the embryo during the early stages of development. Therefore, using OF as a supplement during the *in vitro* embryo culture may affect embryo development and quality. Coy *et al*. (2008) evaluated the effect of oviductal fluid (30 min incubation) on the ZP of pig and cow oocytes and demonstrated an increase in the proteolytic resistance of the ZP reflected in a prolonged pronase digestion periods (3-8 h), and a modulation of sperm-ZP interaction through an increase in monospermy rate. Lloyd *et al*. (2009) exposed *in vitro* matured porcine oocytes to bovine OF for 30 min before fertilization, thereby increasing the blastocyst rate and quality in terms of morphology, cell number, as well as gene expression patterns of apoptotic and developmentally-related genes. Similarly, in cattle, Cebrian-Serrano *et al*. (2013) evaluated the effect of short-term incubation of matured oocytes with bovine OF; no effect on embryo development was observed but abundance of genes transcripts including *G6PD* and *SOD32* was reported (Cebrian-Serrano *et al*., 2013). In a recent study, we showed that only low concentrations of OF (<5%) in embryo culture media, in the absence of serum, had a positive effect on development and quality in terms of cryotolerance, cell number and expression of qualitatively related genes (Lopera-Vasquez *et al*., 2015).

The extracellular environment contains a large number of mobile membrane-limited vesicles called ‘‘extracellular vesicles’’ (EVs). EVs contain microvesicles (MVs), apoptotic bodies and exosomes. Originally, the EVs were associated with removal process of receptors and with cellular waste function (Thery, 2011). Subsequently, they were found to have immune effects (Raposo *et al*., 1996). These data opened the possibility that EVs could play a role in intercellular communication (Thery, 2011). EVs have been found in many biological fluids, including plasma (Caby *et al*., 2005), serum (Taylor and Gercel-Taylor, 2008), urine (Pisitkun *et al*., 2004) epididymal fluid (Gatti *et al*., 2005), amniotic fluid (Asea *et al*., 2008), follicular fluid (da Silveira *et al*., 2012), and milk (Admyre *et al*., 2007). A major discovery was that the content of EVs included both mRNA and miRNA and that EV-associated mRNAs could be translated into proteins by target cells (Valadi *et al*., 2007). EVs with features of exosomes released by immune cells have been demonstrated to selectively incorporate miRNA that can be functionally transferred as a consequence of fusion with recipient cells (Mittelbrunn *et al*., 2011).

The possible role of EVs in reproduction has been reported recently. Da Silveira *et al.* (2012) isolated MVs and exosomes of equine ovarian follicular fluid and, by proteomics and real-time PCR analysis, demonstrated the presence of proteins and miRNAs. The miRNAs were present in surrounding follicular cells, suggesting that MVs and exosomes play a role in mediating cell communication within the mammalian ovarian follicle (da Silveira *et al*., 2012). In addition, Sohel *et al*. (2013) demonstrated the exosome-mediated transport of miRNAs in the bovine follicular microenvironment. Similarly, Ng *et al*. (2013) identified and examined the presence and potential role of MVs and exosomes in the uterine cavity. MVs and exosomes miRNA has enabled bioinformatic identification of pathways that could be influenced if the exosomes are taken up by trophectoderm or epithelium at the time of implantation, or transferred to sperm as they transit the uterine cavity (Ng *et al*., 2013). The results from Burns *et al*. (2014) support the hypothesis that exosomes and MVs present in uterine luminal fluid of pregnant and cyclic ewes contain specific proteins, miRNAs, and mRNAs, that are capable of delivering their contents *in vitro*. Recently, the same group found EVs emanating from both the conceptus trophectoderm and uterine epithelia supporting the notion that MVs in uterine fluid have a biological role in conceptus-endometrial interactions which may be important for the establishment and maintenance of pregnancy (Burns *et al*., 2016). Al-Dossary *et al*. (2013) revealed the expression and secretion via oviductal exosomes of PMCA4a (Ca2+ homeostasis) in the female reproductive tissues and luminal fluids during oestrus, and their sperm-uptake, with possible roles in sperm viability during their storage in the oviduct and during capacitation and the acrosome reaction. Recently, the same group have identified oviductosomes (exosomes and microvesicles present in the oviductal fluid) in murine and bovine species although further studies are needed to determine their interaction with gametes/early embryo(s; Al-Dossary and Martin-Deleon, 2016). Furthermore, we provided evidence that extracellular vesicles derived from BOEC-conditioned media improved blastocyst quality and induced cryoprotection in *in vitro* culture to the same extent as classical co-culture with fresh BOEC monolayers (Lopera-Vasquez *et al*., 2016a). In addition, when extracellular vesicles were obtained from bovine isthmic oviductal fluid and added during the *in vitro* embryo culture, they had a positive effect on gene expression patterns of developmental-related genes compared with serum supplementation, suggesting an association between the oviductal environment and the developing embryo (Lopera-Vasquez *et al*., 2016b).

Concluding remarks

The content of the oviductal environment and its short and long term effects on early embryo development are extremly relevant and may provide new insights on embryo-maternal communication, improving assisted reproductive tecnologies. The challenge today is to develop *in vitro* culture conditions that will allow growth of the embryo based on the physiological components to which it is exposed *in vivo* to enhance the development of high/better quality embryos.

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Endocrine and metabolic differences between *Bos taurus* and *Bos indicus* cows and implications for reproductive management

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Abstract

Based on the considerable differences in ovarian morphology and function, as well as circulating hormones and metabolites between *Bos indicus* (*B. indicus*) and *Bos taurus* (*B. taurus*), researchers are using this acquired knowledge to optimize protocols for fixed-time artificial insemination (FTAI), and production of *in vivo* derived embryos by multiple ovulation or by *in vitro* embryo production (IVP). In *B. indicus*, at the time of follicle deviation, the dominant follicle is smaller and acquires ovulatory capacity at a smaller diameter than *B. taurus*. Moreover, despite ovulating smaller follicles and having smaller corpora lutea (CL), circulating concentrations of estradiol (E2) and progesterone (P4) are greater in *B. indicus* than *B. taurus*. These physiological differences may be related to greater circulating cholesterol, insulin and IGF1 in *B. indicus* than in *B. taurus*. For both genetic groups there is a negative relationship between circulating P4 and ovulatory response to the first GnRH treatment of a fixed-time AI (FTAI) protocol. Moreover, despite lower clearance rates of steroid hormones in *B. indicus* than *B. taurus*, the dose of 2 mg estradiol benzoate seems to be the most effective either for Nelore (*B. indicus* beef), Angus (*B. taurus* beef), or Holstein (*B. taurus* dairy) cows at the initiation of an E2/P4-based FTAI protocol to optimize synchronization and pregnancy per AI (P/AI). Several studies have shown that only one recommended dose of PGF2α at a FTAI protocol may be insufficient for adequate luteolysis in *B. indicus* and *B. taurus*. When submitted to multiple ovulation and embryo transfer, *B. indicus* cows and heifers need less FSH than *B. taurus* to achieve superovulation. Moreover, IVP has been more successful in *B. indicus* than *B. taurus* due to greater antral follicle count and anti-mullerian hormone, and better oocyte quality.

Keywords: artificial insemination, embryo transfer, Holstein, hormone, metabolism, Nelore.

Introduction

Recent studies have gathered a great deal of knowledge on the reproductive physiology of *Bos indicus* (*B. indicus*) heifers and cows, especially by performing direct comparisons with *Bos taurus* (*B. taurus*) under similar environmental, nutritional, and management conditions. Those studies have identified considerable differences in ovarian morphology and function, as well as circulating hormones and metabolites between these two genetic groups.

Currently, researchers are using this acquired knowledge to optimize protocols for fixed-time artificial insemination (FTAI), and production of *in vivo* derived (IVD) embryos by multiple ovulation or by *in vitro* embryo production (IVP). Accordingly, this manuscript is divided into sections that describe: 1) Differences in reproductive physiology between *B. indicus* and *B. taurus*, 2) Practical implications of the physiological differences between *B. indicus* and *B. taurus* for FTAI protocols, and 3) Practical implications of the physiological differences between *B. indicus* and *B. taurus* for embryo production.

Because Nelore (*B. indicus*) and Holstein (*B. taurus*) are among the principal beef and dairy cattle breeds used in Brazil, respectively, they are the most representative of the studies discussed in this article.

Differences in reproductive physiology between *B. indicus* and *B. taurus*

During the last decade, there was a substantial increase in studies that compared reproductive physiology variables between *B. indicus* and *B. taurus*. Although below we present a summary of those results, and a compilation of data represented in Fig. 1 and Table 1, the aim of this manuscript is not to extensively describe those data. Therefore, for detailed information, we suggest that the reader consult the publications cited in this review article.

The average ovarian antral follicle count (AFC) in *B. indicus* is twice the number observed for *B. taurus* (Fig. 1; Table 1). For example, at wave emergence, the number of 2 to 5 mm follicles present in the ovaries was 42.7 (ranging from 25 to 100) in Nelore and 19.7 (ranging from 5 to 40) in Holstein cows (Bastos *et al*., 2010). Greater AFC has been associated with greater circulating anti-mullerian hormone (AMH) in *B. indicus* as compared to *B. taurus* (Baldrighi *et al*., 2014; Batista *et al*., 2014). Another observed difference between *B. taurus* and *B. indius* was the size of largest follicle at deviation. In Holstein cattle, the diameter of the future ovulatory follicle at the time of deviation was between 8.3 and 9.8 mm (Ginther *et al*., 1996; Sartori *et al*., 2004; Bastos *et al*., 2010). In Nelore heifers, deviation happened when the largest follicle reached 5.4 to 6.2 mm (Sartorelli *et al*., 2005; Gimenes *et al*., 2008), and in nonlactating Nelore cows between 7.0 and 7.4 mm of

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diameter (Bastos *et al*., 2010; Sartori *et al*., 2016). Nevertheless, the time after ovulation or after wave emergence for follicle deviation was similar between *B. taurus* and *B. indicus* (Sartori *et al*., 2010a, 2016; Fig. 1) due to a slower growth rate of the follicle in Nelore (0.8 to 1.2 mm/d; Gimenes *et al*., 2008; Sartori and Barros, 2011) than Holstein cattle (1.2 to 1.6 mm/d; Sartori *et al*., 2001).

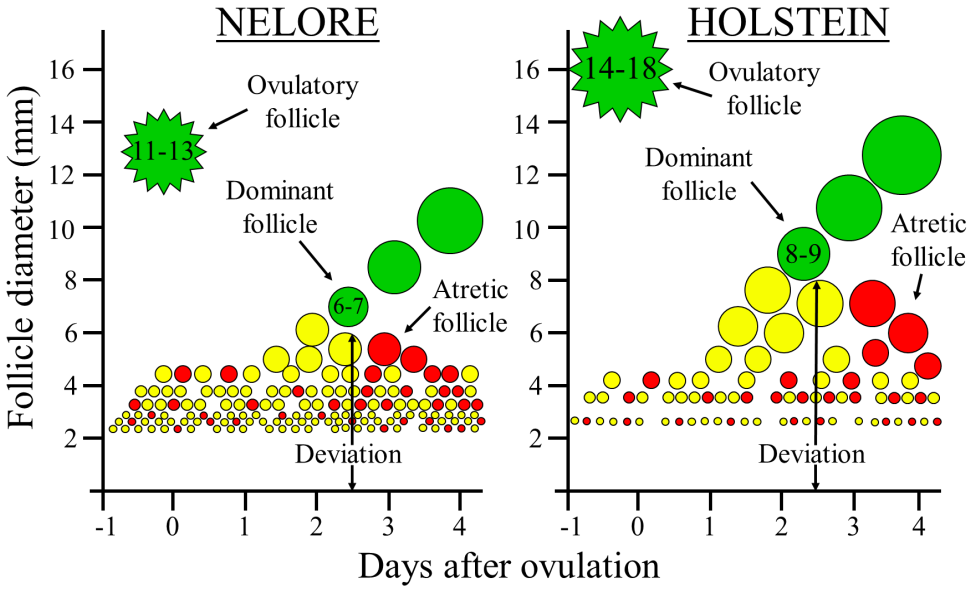

Figure 1. Schematic representation of follicle development in Nelore (*B. indicus*) and Holstein (*B. taurus*) cows based on data from the literature and personal data. Nelore have a greater population of small (2 to 5 mm) follicles in the ovaries throughout the entire estrous cycle than Holstein cattle. Moreover, although both breeds have follicle deviation between day 2 and 3 after ovulation, the diameter of the future dominant follicle at deviation is greater in Holstein cattle. The diameter of the ovulatory follicle is also greater in Holstein than Nelore cows (From Sartori *et al*., 2010a).

Although follicle deviation occurs in *B. indicus* when the dominant follicle reaches 5 to 7.5 mm in diameter compared to 8 to 10 mm in *B. taurus*, it is possible that additional growth is necessary for the dominant follicle to acquire ovulatory capacity in both breeds. Sartori *et al*. (2001) observed that Holstein cows with follicles 7 or 8.5 mm in diameter did not ovulate, even after administration of high doses of pLH (40 mg). However, 80% of the cows with follicles $\geq$10 mm ovulated after pLH administration. Conversely, Gimenes *et al*. (2008) reported that administration of 25 mg pLH in *B. indicus* heifers induced ovulation in 33.3, 80.0 and 90.0% of animals with follicles that were 7.0 to 8.4, 8.5 to 10 and >10 mm in diameter, respectively.

Diameter of the largest ovulatory follicle and of the corpus luteum (CL) also differs between *B. taurus* and *B. indicus*. *B. taurus* have larger diameter of ovulatory follicle (Fig. 1) and greater CL volume than *B. indicus*. However, circulating concentration of steroid hormones in *B. taurus* are lower than in *B. indicus* (Sartori and Barros, 2011; Sartori *et al*., 2016). In studies with Nelore cattle, the diameter of ovulatory follicle was between 11 and 14 mm (Figueiredo *et al*., 1997; Sartorelli *et al*., 2005; Sartori *et al*., 2016), and the maximum diameter of the ovulatory follicle in Holstein cattle was between 13 and 19 mm (Ginther *et al*., 1989; Sartori *et al*., 2002a, 2004). Similarly, the CL diameter of *B. indicus* ranged from 17 to 21 mm or ~2.5 to 5.0 cm^3 (Segerson *et al*., 1984; Rhodes *et al*., 1995; Figueiredo *et al*., 1997; Bó *et al*., 2003; Sartori *et al*., 2016), whereas the CL diameter of *B. taurus* ranged from 20 and 30 mm or ~4.0 to 14.0 cm^3 (Ginther *et al*., 1989; Bó *et al*., 2003; Sartori *et al*., 2004, 2016).

Despite having larger follicles and CL, *B. taurus* have lower circulating steroid hormone concentrations. For example, we have shown that non-lactating Holstein cows had larger ovulatory follicle diameter (14.2 *vs*. 12.9 mm) and CL volume (5.2 *vs*. 3.9 cm^3) than non-lactating Nelore cows (Sartori *et al*., 2016); however, Holstein cows had a lower preovulatory peak of circulating estradiol (E2; 12.5 *vs*. 16.2 pg/ml) and circulating progesterone (P4) concentrations (1.9 vs. 2.7 ng/ml) on day 7 of the estrous cycle compared with Nelore cows.

This inverse relationship between size of ovarian structures and circulating hormone steroids may be related to the reported differences in circulating insulin, IGF1, and cholesterol between breeds. Most of the studies have described greater circulating insulin and IGF1 in *B. indicus* than in *B. taurus* (Alvarez *et al*., 2000; Sartori *et al*., 2010b, 2013). Moreover, it has also been reported that, under similar nutrition management conditions, Nelore heifers have ~60% greater circulating cholesterol (precursor for the biosynthesis of steroid hormones) than Holstein heifers (196.8 *vs*. 123.5 mg/dl; Gandra *et al*., 2011). The potential effects of metabolic hormones or other substances on either different clearance rates of steroid hormones or E2 and P4 production by ovarian structures are discussed by Sartori *et al*. (2016).

Table 1. Comparison of reproductive variables between *B. indicus* and *B. taurus*.

	B. indicus vs. B. taurus
Estrous cycle length	=
Day of luteolysis	≤
Number of waves per cycle	>
Ovarian antral follicle count	>
Circulating AMH	>
Size of dominant follicle at deviation	<
Day of deviation after ovulation	=
Growth rate of dominant/ovulatory follicle	<
Maximum size of dominant/ovulatory follicle	<
Estradiol peak preceding ovulation	>
Duration of estrus	≤
Luteal tissue volume	<
Circulating progesterone	>
Steroid hormones clearance rate	<
Circulating insulin	>
Circulating IGF1	>
Circulating FSH	≤
Circulating cholesterol	>

*From Segerson *et al*. (1984); Alvarez *et al*. (2000); Bó *et al*. (2003); Carvalho *et al*. (2008); Gimenes *et al*. (2008); Sartori *et al*. (2010, 2013, 2016); Sartori and Barros (2011); Baldrighi *et al*. (2014); Batista *et al*. (2014).

Practical implications of the physiological differences between *Bos indicus* and *Bos taurus* for FTAI protocols

Artificial insemination is an important tool for genetic improvement. However, suboptimal estrus detection rates in cycling cows (Pinheiro *et al*., 1998; Lopez *et al*., 2004; Sartori and Barros, 2011; Fricke *et al*., 2014) and a substantial percentage of postpartum cows that are not cycling (Wiltbank *et al*., 2002; Meneghetti et al., 2009; Santos *et al*., 2009), produce the problem of low service rates (SR) in *B. indicus* and *B. taurus*. Thus, AI programs based on estrus detection may have lower reproductive efficiency and less cost-benefit than FTAI programs (Ribeiro *et al*., 2012).

The FTAI program allows for a large number of cows to be inseminated on the same day, with high reproductive efficiency and relatively low cost. Nevertheless, the success of the FTAI program depends on several factors, such as synchronization of follicular waves to optimize the period of follicular dominance in order to not ovulate too old (Cerri *et al*., 2009) or too young (Vasconcelos *et al*., 2001) of a follicle; synchronization of corpus luteum (CL) function and circulating P4; complete luteolysis at the end of the protocol; and synchronization of final ovulation with optimally scheduled FTAI.

Synchronization of emergence of follicle wave

There are two main methods for follicle wave emergence synchronization used in dairy and beef cattle: GnRH-based protocols, in which in the presence of a dominant follicle, GnRH induces ovulation and a new follicle wave starts (Pursley *et al*., 1995), or E2/P4-based protocols, that induces regression of all follicles present in the ovaries and a new wave starts within the following days, depending on the E2 ester used (Bó *et al*., 2003). In GnRH-based programs in dairy cattle, about 50 and 70% of the cows have a new wave properly synchronized by the Ovsynch and Double-Ovsynch programs, respectively (Giordano *et al*., 2012a). In E2/P4-based program in dairy cows about 70% of the cows have a new wave properly synchronized (Monteiro Jr *et al*., 2015a).

Effectiveness of GnRH-based protocols for follicle wave emergence in B. taurus *and* B. indicus

There are several factors that influence the proportion of cows ovulating to a GnRH/LH surge at the initiation of a FTAI protocol, such as day of the estrous cycle (Vasconcelos *et al*., 1999) or follicle diameter (Sartori *et al*., 2001; Gimenes *et al*., 2008). Although GnRH-based protocols have been successfully used in *B. taurus* cows (Pursley *et al*., 1995, 1997; Bridges *et al*., 2008), but not necessarily in *B. taurus* heifers (Pursley *et al*., 1995, 1997), there are conflicting data regarding their effectiveness in *B. indicus* cows (Fernandes *et al*., 2001; Bó *et al*., 2003; Baruselli *et al*., 2004; Ferraz Jr *et al*., 2016). Below, we describe results of experiments that may explain why GnRH-based protocols may have had suboptimal outcomes in the studies cited above.

Because it has been shown that *B. taurus* did not have consistent ovulation to a GnRH treatment even in the presence of a dominant follicle >10 mm in diameter (Martinez *et al*., 1999; Perry and Perry, 2009), factors other than follicle size, such as circulating concentration of P4, may affect ovulation to GnRH. For example, a study (Biehl *et al*., 2013) compared ovulatory response to the first GnRH (100 µg gonadorelin) treatment of the 5 days CO-Synch+CIDR program in Nelore heifers. The treatments were: HiP4 (n = 62; GnRH in heifers with a CL present at P4 device insertion); LoP4 (n = 35; GnRH in heifers with no CL present at P4 device insertion); and PGF-LoP4 (n = 65; GnRH in heifers that had a CL but were treated with 25 mg of dinoprost [PGF] 2 days before GnRH treatment and P4 device insertion). Ovulation to GnRH was

greater for LoP4 (85.7%) and PGF-LoP4 (95.4%) compared with the HiP4 (25.8%). Melo *et al*. (2016) have also shown a negative correlation between circulating P4 and ovulatory response to GnRH. Lack of ovulation to GnRH is likely related to the negative relationship between circulating concentration of P4 and magnitude of the GnRH-induced LH surge, which has been demonstrated in *B. taurus* heifers or cows (Colazo *et al*., 2008; Perry and Perry, 2009; Giordano *et al*., 2012a). For example, treatment with 100 µg of GnRH in the presence of high *vs*. low P4 resulted in a much lower peak of the LH surge (3.3 *vs*. 15.7 ng/ml). Nevertheless, greater doses of GnRH, even in the presence of elevated P4, can result in a greater magnitude of the LH surge and greater ovulation (Giordano *et al*., 2013). Moreover, it is possible that different analogs of GnRH may produce different outcomes, because it has been reported, for example, that gonadorelin induced a lower LH surge compared with other analogs of GnRH, such as lecirelin, fertirelin, or buserelin (Chenault *et al*., 1990; Picard-Hagen *et al*., 2015). Although the magnitude of a GnRH-induced LH surge in the presence of low *vs*. high P4 has not yet been tested in *B. indicus*, it seems likely that this problem may underlie some of the observed lack of ovulation to GnRH treatment in these cattle.

Thus, GnRH-based protocols are likely to have lower synchronization of the first follicular wave when they are initiated in the presence of a CL, primarily due to lack of ovulation of a dominant follicle at the initiation of the protocol. This is clearly related to the greatly reduced magnitude of the LH surge induced by GnRH in the presence of elevated P4. In addition, due to natural growth and regression of follicular waves during the luteal phase there are many times when an LH-responsive dominant follicle is not present, and therefore ovulation would not occur even with an optimized LH surge. Further, the presence of elevated P4 may reduce LH receptor expression in the dominant follicle and this could reduce responsiveness to an LH surge in dominant follicles (Dias *et al*., 2014). In spite of reduced ovulation to GnRH in the presence of elevated P4, and therefore reduced follicular wave synchronization, fertility is generally relatively high in cows that are given the first GnRH of an Ovsynch protocol in the presence of elevated P4.

In contrast, in a low P4 environment, GnRH induces ovulation in a high proportion of lactating dairy cows (Gumen *et al*., 2003). A low P4 environment would be expected in anovular cows or cows in the proestrous phase of the estrous cycle. In these cows, ovulation to the GnRH treatment is essential for optimal fertility, as cows with low P4 at the initiation of the protocol that did not have ovulation to GnRH had greatly reduced fertility (Giordano *et al*., 2012b). Comparisons of the use of EB (2 mg) *vs*. GnRH at the beginning of a FTAI program have reported variable results. For example, Pereira *et al*. (2013a) compared the 5-days Cosynch protocol, a GnRH-based protocol, to an E2/P4 protocol in Holstein cattle (n = 1,190) during the summer. They reported better synchronization rate for the GnRH compared to the E2 protocol (78.2 *vs*. 70.7%; P = 0.02). However, using only synchronized cows, percentage pregnant/AI (P/AI) was not different at the 32 days pregnancy diagnosis but was lower for GnRH than E2 at the 60 days pregnancy diagnosis (17.7 *vs*. 25.6%; P = 0.03) due to greater pregnancy loss for GnRH than E2-treated cows (21.7 *vs*. 6.7%; P = 0.01). In contrast, in high-producing dairy cows (n = 1,035), Melo *et al*. (2016) reported that, even with a low ovulation rate to GnRH (gonadorelin; ~ 35%) at the beginning of the protocol, cows that received GnRH tended to have greater P/AI that those receiving EB at the start of the protocol (38.2 *vs*. 33.7; P = 0.07).

Many GnRH-based protocols in *B. taurus* now use presynchronization strategies to assure that cows are at a stage of the estrous cycle that optimizes ovulation to GnRH. For example, Double Ovsynch uses an Ovsynch protocol to presynchronize the cows so that almost all cows (~94%; Herlihy *et al*., 2012) are cycling and at day 6 or 7 of the estrous cycle at the start of the breeding Ovsynch protocol. This produces a high ovulation rate to GnRH and high fertility during the protocol. These presynchronization strategies have not been adequately tested in *B. indicus* but are likely to be too complicated for most *B. indicus* management systems. Unfortunately, studies have not yet been performed that directly tested the ovarian responses and fertility of *B. taurus vs*. *B. indicus* in response to the same GnRH-based protocols.

Effectiveness of E2/P4-based protocols for follicle wave emergence in B. taurus *and* B. indicus

Early studies that established the physiological basis for these protocols utilized beef heifers and found that treatment with different esters of E2 simultaneously with P4 treatment led to suppression of the gonadotropins, regression of growing follicles, and emergence a new follicular wave about 4 days after E2 treatment (Bo *et al*., 1993, 1994, 1995). Although E2/P4-based FTAI protocols are widely used in *B. taurus* and *B. indicus* (Martinez *et al*., 2000; Ayres *et al*., 2008; Meneghetti *et al*., 2009; Souza *et al*., 2009; Sá Filho *et al*., 2011; Pereira *et al*., 2013b; Monteiro Jr *et al*., 2015b), the metabolism of E2 is different between *B. taurus* and *B. indicus* (Sartori *et al*., 2016). For example, in a 3 x 2 Latin Square design study (Bastos *et al*., 2011), the effect of dose of estradiol benzoate (EB; 1, 2 or 4 mg), given at the same time as a P4 intravaginal insert, on the synchronization of follicular wave emergence was evaluated in non-lactating Nelore (n = 13) and Holstein (n = 11) cows receiving a maintenance diet. The BCS and body weight were kept at 3.5 ± 0.1 and 3.0 ± 0.2 (scale of 1 to 5) and 535 ± 14 and 600 ± 23 kg for Nelore and Holstein cows, respectively. Two doses of prostaglandin F2α (PGF2α) were given 11 days apart, and simultaneously with the second PGF2α, cows were treated with EB and with an intravaginal P4 insert, which remained for 10 days. Ovarian dynamics were monitored daily by means of ultrasonography for 10 days after EB treatment. Only cows with follicular wave emergence synchronized by the protocol were included, i.e., when the emergence occurred between 1 and 6 days after treatment with EB + P4. In response to the treatments, 2.5% (1/39) of Nelore cows did not have a synchronized follicular wave emergence, with the only non-synchronized cow

being treated with 1 mg EB. As for Holstein cows, 15.1% (5/33) did not have a synchronized follicular wave emergence (three cows had received 1 mg and the other two cows, 2 or 4 mg EB). Regardless of breed, there was a dose-dependent effect of treatment with EB on the following variables: day of the follicular wave emergence, diameter of the dominant follicle 9 days after treatment with EB, circulating concentration of E2 24 h after treatment with EB, time that circulating FSH began to rise (beginning of FSH peak) after EB, and time of occurrence of the peak of circulating FSH (Table 2). The follicular wave emergence occurred 3.1 ± 0.3^{a}, 3.3 ± 0.1^{ab} and 3.9 ± 0.2^{b} days after treatment with 1, 2, or 4 mg of EB, respectively ($^{a,b}P \leq 0.05$), independent of breed, although circulating concentrations of E2 24 h after treatment with EB was much greater in Nelore than Holstein cows (Table 2). The diameter of the largest follicle 9 days after treatment was 12.2 ± 0.5^{a}, 11.5 ± 0.8^{ab} and 9.9 ± 0.7^{b} mm in cows that received 1, 2 or 4 mg of EB, respectively ($^{a,b}P \leq 0.05$), but there was no breed effect (Table 2). At follicle wave emergence, the number of 2 to 5 mm follicles present in the ovaries was greater in Nelore than in Holstein cows (30.8 ± 4.5 *vs*. 13.6 ± 1.1 AFC; Table 2). Furthermore, follicle deviation occurred, on average, 3.0 ± 0.2 days after wave emergence, independent of breed, when the diameter of the largest follicle reached 7.3 ± 0.4 and 9.0 ± 0.5 mm in Nelore and Holstein cows, respectively ($P < 0.05$). It was concluded that the timing of follicle wave emergence after treatment with EB + P4 was EB dose-dependent for both breeds.

The results presented above are, somewhat, contradictory, because despite a very different clearance rate of E2 (same doses, but very distinct concentrations of circulating E2 after EB treatment [>2-fold difference] in cows with little difference in body weight - Holsteins were only 12% heavier than Nelore cows), the behavior of wave emergence after EB + P4 treatment was not different between breeds (Table 2). Therefore, based on differences in circulating E2 after EB treatment between breeds, one might expect that lower EB doses should be used for Nelore and higher EB doses should be used for Holstein cattle at the initiation of an E2/P4-based FTAI protocol in order to optimize synchronization of follicle wave emergence. In contrast, based on the similar responses of both breeds to EB treatments in terms of follicle wave emergence, a similar E2 dose might be optimal for Nelore and Holstein cows.

Currently, both in *B. indicus* and *B. taurus*, the dose of EB recommended for initiation of a FTAI protocol is 2.0 mg given at the same time as treatment with P4/progestin. However, hypothesizing that the dose of 2.0 mg EB would be insufficient to effectively synchronize follicular wave emergence in lactating dairy cows, Monteiro Jr *et al*. (2015a) compared 2.0 *vs*. 3.0 mg EB at the beginning of a FTAI protocol. All cows were treated with EB (2.0 or 3.0 mg) at the time of introduction of a P4 insert (day 0). On day 7, cows were given 25 mg of PGF2α; on day 8, the insert was removed and cows were given 1 mg of estradiol cypionate (ECP). All cows received FTAI on day 10. Daily transrectal ultrasound evaluations of the ovaries were performed. There was no difference ($P > 0.10$) between treatments with 3.0 *vs*. 2.0 mg EB for proportion of cows with synchronized follicular wave emergence (71.4% [15/21] *vs*. 82.6% [19/23]), and time to wave emergence (3.6 ± 0.19 *vs*. 3.4 ± 0.17 d). However, treatment with 3.0 mg EB decreased the percentage of cows with a CL on day 7 of the FTAI protocol (19.8% [4/17] *vs*. 55.3% [13/21]; $P < 0.05$), indicating that the higher dose of EB caused increased CL regression during the protocol.

Using similar reasoning as in the study of Bastos *et al*. (2011), a study was done in *B. taurus* and *B. indicus* beef cattle comparing 1.0 *vs*. 2.0 mg EB + P4 at the initiation of a FTAI protocol. Pessoa *et al*. (2015) compared the treatments above during a resynchronization protocol starting 22 day (day 22) after the first FTAI and evaluated P/AI, pregnancy loss and induction of new follicular wave emergence in suckled beef cows. Thus, on day 22 after first FTAI all cows received an intravaginal P4 insert and, regardless of pregnancy status, 1426 cows (768 *B. taurus* and 728 *B. indicus*) were treated with either 1 or 2 mg EB. After 8 days (day 30), the P4 insert was removed and pregnancy diagnosis was accomplished by ultrasound. Non-pregnant cows were then treated with cloprostenol. On the same day, *B. taurus* cows received 10 mg FSH and 1 mg EB, whereas *B. indicus* cows were treated with 300 IU eCG and 1 mg ECP. The FTAI was performed 44 or 48 h after P4 removal on *B. indicus* and *B. taurus* cows, respectively. Pregnancy diagnosis was conducted again at 62 days after first FTAI. The P/AI after the first FTAI was similar (~44.0%; $P = 0.85$) between treatments regardless of breed. However, P/AI after resynchronization was lower ($P = 0.0001$) in cows treated with 1 compared to 2 mg EB (36.1 *vs*. 47.3%). Pregnancy loss at first FTAI was similar ($P = 0.37$) between treatments (3.8 *vs*. 5.5% for 1 and 2 mg EB), but the cumulative pregnancy was greater ($P = 0.01$) in cows treated with 2 mg EB (68.2%) than those treated with 1 mg EB (62.8%). Moreover, *B. indicus* cows had ovarian dynamics evaluated by ultrasound to assess induction of a new follicular wave emergence after treatment with 1 mg (n = 12) *vs*. 2 mg (n = 12) EB. Despite a similar interval from EB treatment to new follicular wave emergence ($P = 0.13$), the emergence of a new wave was more dispersed ($P = 0.03$) in cows treated with 1 mg EB (1.8 ± 1.3 d) compared with cows treated with 2 mg EB (2.3 ± 0.6 d). Therefore, a dose of 2 mg EB produced a more uniform emergence of the follicular wave and greater P/AI after resynchronization, without compromising the pregnancy established at the first FTAI in suckled *B. taurus* and *B indicus* beef cows. Preliminary results of a study (Prata and Sartori, 2016; ESALQ, USP, Piracicaba, SP, Brazil, unpublished) that is being performed with postpartum lactating Nelore cows followed a similar trend in P/AI for 1 *vs*. 2 mg EB treatment at the initiation of a FTAI protocol (40.9% [36/88] *vs*. 47.2% [42/89]; $P = 0.40$) but was not significantly different (day 0: EB + P4 insert; day 8: P4 insert removal, 0.6 mg ECP, 500 µg cloprostenol, and 300 IU eCG; day 10: FTAI).

Table 2. Results (least squares means ± SEM) of reproductive variables of non-lactating Nelore (*B. indicus*) and Holstein (*B. taurus*) cows treated with 1, 2, or 4 mg of estradiol benzoate (EB) and an intravaginal progesterone insert.

	Breed						P-value		
	Nelore			Holstein					
	1 mg EB (n = 11)	2 mg EB (n = 12)	4 mg EB (n = 12)	1 mg EB (n = 11)	2 mg EB (n = 11)	4 mg EB (n = 11)	Breed	Dose	BxD
Wave emergence after EB; day	3.1 ± 0.3[b]	3.4 ± 0.2[ab]	4.0 ± 0.2[a]	3.0 ± 0.3[b]	3.3 ± 0.3[b]	3.9 ± 0.3[a]	0.66	0.01	0.98
Antral follicle count at emergence; number	31.1 ± 5.8	32.4 ± 5.8	30.9 ± 5.8	11.8 ± 6.2	13.8 ± 6.1	14.1 ± 6.1	0.03	0.67	0.79
Diameter of largest follicle 9 days after EB; mm	11.5 ± 0.9[a]	11.5 ± 0.9[ab]	9.8 ± 0.8[b]	13.7 ± 1.2[a]	11.4 ± 1.1[ab]	10.4 ± 1.2[b]	0.34	0.05	0.47
Growth rate of dominant follicle; mm/day	1.3 ± 0.1	1.3 ± 0.1	1.1 ± 0.1	1.6 ± 0.1	1.6 ± 0.1	1.4 ± 0.2	0.03	0.22	0.95
Circulating estradiol 24 h after EB; pg/ml	57.0 ± 9.1[c]	85.4 ± 9.5[b]	148.1 ± 9.1[a]	25.8 ± 12.0[c]	42.3 ± 11.2[b]	71.0 ± 11.9[a]	< 0.01	<0.01	0.06
Maximum circulating FSH after EB, ng/ml	1.06 ± 0.06	1.07 ± 0.06	1.06 ± 0.06	1.30 ± 0.08	1.27 ± 0.08	1.10 ± 0.09	0.04	0.26	0.30
Onset of FSH surge after EB, h	57.8 ± 5.4[ab]	53.3 ± 3.5[b]	64.0 ± 3.2[a]	46.0 ± 4.7[b]	56.0 ± 4.4[ab]	60.9 ± 4.0[a]	0.26	0.04	0.30
Time of FSH surge after EB, h	78.0 ± 5.7[b]	82.7 ± 6.3[b]	110.0 ± 5.7[a]	91.6 ± 7.5[b]	90.1 ± 7.0[b]	101.9 ± 7.5[a]	0.46	< 0.01	0.25

[a,b,c]Effect of dose within breed (P < 0.05).

Corpus luteum function, luteolysis, and growth and ovulation of the preovulatory follicle

Despite having smaller CL, *B. indicus* have greater circulating P4, probably due to greater production, but also, a reduced metabolic clearance rate for P4 than in *B. taurus* (Sartori *et al*., 2016). Moreover, among *B. taurus* breeds, lactating dairy cows have even lower circulating steroid hormones (Sartori *et al*., 2002a, 2004) due to greater metabolic clearance associated with greater dry matter intake (Sangsritavong *et al*., 2002; Vasconcelos *et al*., 2003).

One of the first studies (Carvalho *et al*., 2008) to suggest lower P4 clearance rates in *B. indicus* than *B. taurus* was done using 70 nulliparous heifers of different genetic groups [*B. indicus* (Nelore and Gir), *B. indicus* x *B. taurus* (Nelore x Angus and Gir x Holstein), *B. taurus* (Angus and Holstein)]. Two PGF2α treatments were performed 24 and 12 days (day -24 and day -12) before a P4 implant insertion. On day 0, each heifer received the P4 implant, and 2 mg EB. In addition, half of the heifers from each genetic group received 25 mg of PGF2α on day 0. A second PGF2α treatment was performed on all heifers at the time of P4 implant withdrawal (day 8). On day 9, all heifers received 1 mg EB. There were no detectable differences in serum P4 concentrations among genetic groups on day 0, but P4 concentrations were greater on day 3 ($P < 0.05$) in *B. indicus* (6.8 ± 0.8 ng/ml) and *B. indicus* x *B. taurus* (5.7 ± 0.7 ng/ml) than in *B. taurus* (3.9 ± 0.4 ng/ml) heifers. Circulating P4 in *B. indicus* heifers on day 6 (5.7 ± 0.6^{a} *vs*. 4.0 ± 0.6^{b} *vs*. 3.2 ± 0.5^{b} ng/ml) and day 8 (5.3 ± 0.7^{a} *vs*. 3.3 ± 0.4^{b} *vs*. 3.0 ± 0.5^{b} ng/ml) exceeded ($^{a,b}P < 0.05$) those of *B. indicus* x *B. taurus* or *B. taurus* heifers, respectively. These results were corroborated by another study (Nascimento *et al*., 2012; Sartori *et al*., 2016) designed to compare circulating P4 profile in non-lactating Holstein (n = 20) and Nelore (n = 20) cows fed a maintenance diet after insertion of intravaginal P4 inserts. Cows did not have a functional CL at the time of implant insertion. There was an effect ($P < 0.05$) of breed (1.2 *vs*. 2.2 ng/ml, Holstein and Nelore, respectively), in which P4 concentrations were about 90% greater in Nelore than in Holstein cows, probably due the greater P4 metabolism in Holsteins (Fig. 2A). After removal of the P4 device, blood was sampled every 40 minutes (0.65 h) until 240 min, and a lower P4 concentration was observed in Holstein than in Nelore cows (Fig. 2B).

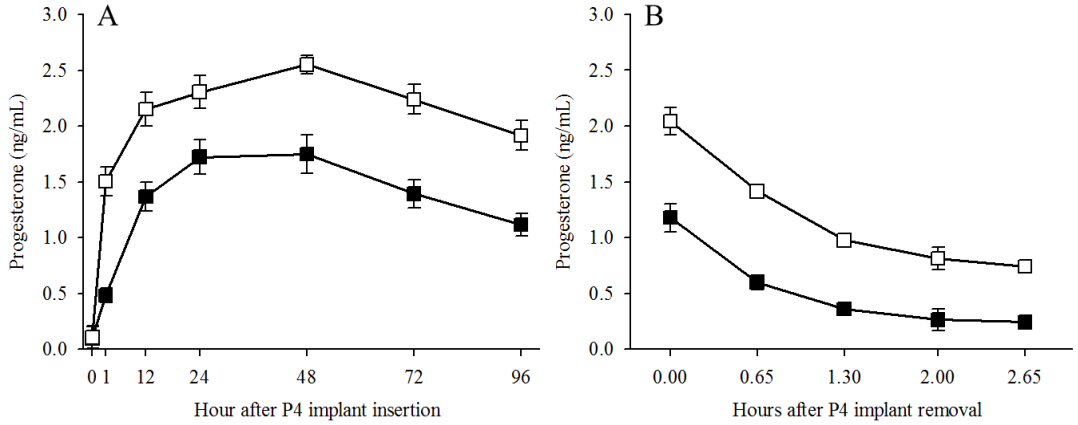

Figure 2. Circulating progesterone (P4) concentrations (least squares means ± standard error) of non-lactating Holstein (n = 20; ■) and Nelore (n = 20; □) cows fed with a maintenance diet. (A) Blood was sampled before (0 h), and 1, 6, 12, 24, and 48 h after P4 implant insertion. There were effects of breed and day ($P \leq 0.05$). (B) Blood was sampled immediately before (0 h) and 0.65, 1.30, 2.00, and 2.65 h after P4 implant removal. There were effects of breed and day ($P \leq 0.05$; From Sartori *et al*., 2016).

It has been shown that serum LH concentrations during the natural (Randel, 1976; Randel and Moseley, 1977), E2-induced (Rhodes *et al*., 1978), or GnRH-induced (Griffin and Randel, 1978) preovulatory LH-surge were lower in Brahman (*B. indicus*) than in British (*B. taurus*) breeds. Likewise, as discussed by Bó *et al*. (2003), gonadotrophin secretion patterns in the postpartum period also differ between *B. taurus* and *B. indicus*. Under the same conditions, *B. taurus* (Hereford × Shorthorn suckled cows) had higher plasma LH concentrations (0.7 ± 0.1 ng/ml) than suckled Brahman cows (0.6 ± 0.1 ng/ml) after 30 days postpartum and this difference appeared to increase over time (D'Occhio *et al*., 1990). In addition, a greater proportion of *B. taurus* cows had a higher pulsatile LH secretion than *B. indicus* cows (D'Occhio *et al*., 1990). In this study, LH concentrations were also affected by body condition and postpartum management (D'Occhio *et al*., 1990) suggesting that nutrition is one of the major concerns when evaluating postpartum activity in *B. indicus* and *B. taurus*. Moreover, independent of breed, or genetic group, suckling cows have lower LH-pulse frequency than lactating cows not carrying calf (discussed by Stevenson and Lamb, 2016). Despite that, non-pregnant, lactating Holstein cows had greater LH-pulse frequency than non-pregnant non-lactating Holstein cows (Vasconcelos *et al*., 2003). It has been shown that LH pulses are essential for growth of the dominant follicle (Ginther *et al*., 1996). Circulating P4 concentrations are expected to influence LH-pulse frequency because P4 inhibited the dominant follicle development in a dose-dependent manner (Adams *et al*., 1992; Santos *et al*., 2016). Therefore, cattle with too high of circulating P4, nursing cows, or cows under

nutritional restrictions may have reduced development of the dominant/preovulatory follicle potentially leading to reduced likelihood of ovulation and reduced size of the ovulatory follicle, which may compromise fertility under these conditions. These problems may be even more likely in *B. indicus* due to several factors, such as described above.

The importance of circulating concentrations of P4 during development of the preovulatory follicle on ovulation and fertility in cattle has been discussed by several authors (Wiltbank *et al*., 2006; Carvalho *et al*., 2008; Santos *et al*., 2016; Stevenson and Lamb, 2016). The general idea is that due to high LH-pulse frequency and physiologically low circulating P4 due to elevated steroid metabolism, lactating dairy cows ovulate larger follicles, and have high incidence of double ovulations, however oocyte and embryo quality may be hampered (Sartori *et al*., 2002b; Wiltbank *et al*., 2006, 2014; Santos *et al*., 2016). In *B. taurus* beef cattle, Stevenson and Lamb (2016) reported little effect of P4 environment during development of the preovulatory follicle on pregnancy risk. Moreover, Holstein heifers that received PGF2α at the initiation of a Cosynch + CIDR protocol, and therefore, had lower circulating P4 during the protocol, resulting in larger ovulatory follicles, had similar P/AI as heifers not receiving PGF2α at the initiation of the protocol (Stevenson *et al*., 2008). In contrast, studies in *B. indicus* heifers or non-lactating cows indicated that high circulating P4 concentrations during synchronization of ovulation protocols reduced the growth of the dominant follicle, which negatively affected ovulation rate and pregnancy success (Carvalho *et al*., 2008; Dias *et al*., 2009; Peres *et al*., 2009).

The results presented above may explain the contrasting differences regarding the effects of equine chorionic gonadotrophin (eCG) at the end of a FTAI protocol on final follicle development and ovulation between *B. indicus* and *B. taurus*. Whereas in *B. indicus*, the majority of studies has shown a positive effect of eCG on percentage of heifers or cows that ovulate at the end of the protocol, greater circulating P4 during the subsequent diestrus, and improved P/AI (Baruselli *et al*., 2004; Peres *et al*., 2009; Sá Filho *et al*., 2009, 2010a, b; Lemes *et al*., 2011; Sales *et al*., 2011), data in *B. taurus* are conflicting and, although some studies in beef cattle have shown improved ovulation rate and fertility by adding eCG to the FTAI protocol (Pessoa *et al*., 2016), several studies have shown no or little benefit of eCG, especially in lactating Holstein cows (Small *et al*., 2009; Souza *et al*., 2009; Ferreira *et al*., 2013; Pulley *et al*., 2013).

Regarding induced luteolysis by exogenous treatments, the responsiveness of CL to PGF2α seems to be very similar between *B. indicus* and *B. taurus*. To study the effect of the dose of a PGF2α analog and day of the estrous cycle at the time of treatment, Ferraz Jr *et al*. (2016) performed an experiment with non-lactating Nelore cows, in a 3 x 2 factorial design, in which three different doses of dinoprost tromethamine (12.5, 25.0, and 50.0 mg) were used on days 5 or 7 of the estrous cycle. In this study luteolysis was dose-dependent. Therefore, increasing the dose of PGF2α increased the proportion of cows detected in estrus and augmented the percentage of cows with circulating P4 concentration <1.0 ng/ml at 72 h after PGF2α treatment. Similar results were reported by Nascimento *et al*. (2014) who found that complete luteolysis did not occur in non-lactating Holstein cows on day 5 of the estrous cycle with either a full dose of PGF2α (25 mg of dinoprost tromethamine), two full doses 8 h apart, or double dose (50.0 mg). Giordano *et al*. (2013) evaluated whether increasing the dose of PGF2α (cloprostenol) during the Breeding-Ovsynch portion of the Double-Ovsynch protocol could improve the rates of ovulation and luteolysis and therefore increase P/AI. The authors observed a better luteolytic response to PGF2α (% of cows with complete CL regression) when the dose was increased from 500 µg to 750 µg in lactating Holstein cows (84.3 *vs*. 90.8%; P = 0.03). The greater cloprostenol dose increased luteal regression primarily in multiparous cows and increased fertility (P = 0.05) only at the pregnancy diagnosis 39 days after FTAI.

In addition to the dose of PGF2α, it is well known that the day of the estrous cycle impacts the proportion of cows with luteolysis after PGF2α (dinoprost tromethamine) treatment. Ferraz Jr *et al*. (2016) observed that Nelore cows that had CL on day 5 of the estrous cycle were more refractory to PGF2α than those on day 7 of the cycle. Moreover, in lactating Holstein cows, a second treatment of dinoprost tromethamine (+PGF2α; 24 h apart) increased the percentage of cows that had complete luteal regression (95.6%) compared with control cows (84.6%). Although one study found no detectable effect of the additional PGF2α on P/AI (control = 41.5% *vs*. +PGF2α = 44.7%; P = 0.34; Brusveen *et al*., 2009), recent studies have found significant improvements in P/AI when a second PGF2α treatment is given, particularly in multiparous cows (Carvalho *et al*., 2015; Wiltbank *et al*., 2015). Therefore, complete luteolysis is essential to optimize ovulation and fertility to a FTAI protocol. In fact, more lactating Holstein cows with P4 < 0.1 ng/ml, compared with P4 ≥ 0.1 and < 0.22 ng/mL at the time of AI, ovulated to an E2/P4-based FTAI protocol (81.2 *vs*. 58.0%) and had increased P/AI (47.4 *vs*. 21.4%; Monteiro Jr *et al*., 2015a). Similar results were observed by Pereira *et al*. (2013b) when lactating dairy cows were also synchronized with an E2/P4-based protocol and were either inseminated or served as embryo recipients. The P4 concentration at the time of AI or 7 days before embryo transfer (ET) altered fertility in FTAI cows, with higher P/AI in cows with P4 concentration <0.1 ng/ml compared with cows with P4 concentration ≥0.1 ng/ml. In ET cows, higher P/ET was found in cows with P4 concentration <0.22 ng/ml compared with cows with P4 concentration ≥0.22 ng/ml.

Practical implications of the physiological differences between *B. indicus* and *B. taurus* for embryo production

The physiological differences between *B. indicus* and *B. taurus* described above, need also to be

considered when managing females for *in vivo* derived (IVD) embryos by multiple ovulation and embryo transfer (MOET) or *in vitro* embryo production (IVP).

One of the protocols most used in *B. indicus* cows and heifers for MOET was developed by Barros and Nogueira (2005) and was called P-36. The protocol included insertion of an intravaginal P4 insert for 36 h after PGF2α administration and induction of ovulation with exogenous LH, administered 12 h after P4 insert removal (48 h after PGF2α administration). The FTAI was performed 12 and 24 h later since ovulation occurs between 24 and 36 h after LH administration. The effectiveness of the P-36 protocol has been confirmed (Baruselli *et al*., 2006; Nogueira *et al*., 2007). A variation of the P-36 protocol, in which the P4 device is removed 24 h after PGF2α (protocol P-24) and LH is administered 24 h later (48 h after PGF2α), has been utilized in Nelore cattle, apparently with comparable results to those obtained with P-36 protocol (Baruselli *et al*., 2006).

The use of the P-36 protocol in *B. taurus* breeds, however, has resulted in decreased number of viable embryos in comparison with conventional protocols with estrus detection. In Holstein (Baruselli *et al*., 2006) and Angus donors (Bó *et al*., 2006), viable embryo production was increased with the P-36 protocol when the ovulation induction treatment (LH or GnRH) was administered at 60 h (P-36/LH60), rather than 48 h (P-36/LH48) after PGF2α administration. On the other hand, even though delaying ovulation for 12 h in the P-36 protocol in *B. taurus* breeds (P-36/LH60 protocol) had positive effects on embryo production, the opposite occurred when used in *B. indicus* breeds. The P-36/LH60 protocol caused a decrease in embryo production when compared to P-36/LH48 protocol (Baruselli *et al*., 2006). Therefore, it can be inferred that ovulation in superstimulation protocols must be induced earlier in *B. indicus* donors; whereas, in *B. taurus* donors, it seems necessary to delay treatment with an ovulation inducer, thereby allowing an increase in follicle size, and acquisition of LH receptors.

Bos indicus breeds have a reduced capacity for LH secretion and a greater sensitivity to exogenous gonadotropins than *B. taurus* (Randel, 1984). Superovulatory response was evaluated in Nelore cows submitted to three different doses of Folltropin-V (100, 133 or 200 mg) in a crossover design. There were no significant differences in any of the variables evaluated, indicating that it was possible to reduce the dose of FSH to 100 mg in Nelore cows submitted to a FTAI superstimulatory protocol, without compromising superovulatory response and embryo production (Baruselli *et al*., 2006). Other studies in Nelore heifers successfully induced superovulation and embryo production using a smaller dose of FSH (70 mg; Guardieiro *et al*., 2014), which is very unlikely to be effective in *B. taurus*.

Especially due to the greater AFC and better oocyte quality in *B. indicus* in relation to *B. taurus*, IVP is much more successful in *B. indicus*. For example, Gir (*B. indicus*), Holstein and crossbreds (1/4 Holstein x 3/4 Gir or 1/2 Holstein-Gir) were compared for total and viable oocyte yield, and IVP embryos (Pontes *et al*., 2010). The number of total and viable oocytes, and embryos produced were greater in Gir than in Holstein cattle (17.1 *vs*. 11.4; 12.1 *vs*. 8.0; 3.2 *vs*. 2.2, respectively). Moreover, embryo production (5.5 blastocysts) was even greater in Holstein-Gir crossbreds compared to the other breeds. Another study was conducted to compare IVP between Nelore and Holstein heifers (Gimenes *et al*., 2015). More oocytes were recovered (37.1 *vs*. 15.4), more embryos were produced (7.3 *vs*. 1.1), and a greater blastocyst rate was obtained (28.3 *vs*. 14.1%) from Nelore than Holstein heifers. In another study (Sales *et al*., 2015), Gir cows had a greater number of oocytes recovered by ovum pickup (OPU; 23.4 *vs*. 14.9), better quality of oocytes demonstrated by greater cleavage rates (73.6 *vs*. 40.8%), greater number of blastocysts (3.8 *vs*. 0.7) and better blastocyst rates (36.7 *vs*. 12.1%) than Holstein donors.

Overall, the main findings of the studies described above indicate that *B. taurus* yielded less oocytes, as well as produced less blastocysts per OPU than *B. indicus* donors. This pattern seems to occur even when other *B. taurus* breeds are studied. Sudano *et al*. (2014) conducted an experiment in which non-lactating Simmental (*B. taurus*) and Nelore cows were compared for *in vivo* embryo production and IVP. Although the total number of recovered ova/embryos per cow (5.5 *vs*. 3.7) and transferable IVD embryos per cow (3.8 *vs*. 2.3) were not different between Nelore and Simmental, respectively, when IVP was performed, Nelore produced more oocytes per OPU (14.9 *vs*. 8.1) and had greater blastocyst rates per OPU (41.5 *vs*. 23.4%) than Simmental cows. The potential impact of metabolic or steroid hormones, associated or not with different feed intake regimens on embryo production in *B. indicus* and *B. taurus* cattle, has been discussed in more details by Sartori *et al*. (2016).

Final considerations

Bos indicus and B. taurus have been mainly used for milk and beef production around the world. Although, in general, *B. taurus* have been more intensely selected for production, a better adaptation to the tropical and sub-tropical environments makes *B. indicus* and crossbreds feasible options for production. Moreover, there are significant differences in the reproductive physiology between those genetic groups that affect the application of adequate tools for reproductive management. For example, *B. taurus* in general reach puberty sooner (Sartori *et al*., 2010a) and have a shorter gestation length as compared to *B. indicus* (Paschal *et al*, 1991). Therefore, in order to have a 12-months calving interval, *B. indicus* cows must conceive 10 days earlier than *B. taurus*. Differences in estrus behavior and ovarian function also make some adjustments of reproductive management necessary. The greater sensitivity to the negative feedback of steroid hormones on the hypothalamus-pituitary axis makes a dose reduction sometimes necessary during hormone treatments in *B. indicus*. On the other hand, due to greater steroid hormone clearance, especially in

lactating *B. taurus* dairy cows, higher doses of hormones may be necessary to optimize reproductive management strategies. Moreover, the greater AFC, greater oocyte quality, and greater sensitivity to gonadotropins in *B. indicus*, make embryo production much more affordable than in *B. taurus*.

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Using new analytical tools to produce better embryos *in vitro*

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Abstract

The purpose of this review is to summarize what we know about preimplantation embryo metabolism, focusing on ruminant species, and to discuss how this knowledge informs our approach to culturing embryos *in vitro*. The important relationship between embryo metabolism and viability will be emphasized, and theories of metabolic networks in embryos presented. Methods that have historically been used to study embryo metabolism will be compared and contrasted to a new method of evaluating embryo metabolism; metabolomics. Finally, the advantages and disadvantages of using metabolomics technologies to study embryo metabolism will be critically evaluated. The application of metabolomics to assisted reproductive technologies, and specifically to embryo culture, will be highlighted. We conclude that use of metabolomics to study embryo physiology will enlighten our understanding of embryo metabolic pathways in the context of a complete media that enables good blastocyst production. This way of thinking about embryo metabolism as dynamic, complex and interrelated biochemical pathways, informed by metabolomics, will allow us to develop the next generation of embryo culture medium to support and manipulate metabolism to promote embryo viability, as well as to identify the most viable embryos for transfer.

Keywords: embryo, *in vitro* culture, metabolism, viability.

Introduction

Embryos can develop successfully to the blastocyst stage in a wide variety of commercially available culture media. Although embryos produced *in vitro* exhibit only slightly lower pregnancy rates than those produced *in vivo*, they exhibit reduced survival following vitrification and have multiple associated problems during pregnancy and parturition, including heavier birth weight, extended gestation, and a higher incidence of fetal and neonatal loss, suggesting reduced embryo quality (Hasler, 2000; Rizos *et al*., 2002). Improvements have been made in the culture of embryos from domestic species in the last decade, but significant progress in optimizing *in vitro* embryo production remains elusive because we still do not fully understand embryonic metabolism. Preimplantation embryos exhibit an astonishing degree of metabolic plasticity, allowing them to use a variety of metabolic substrates via multiple pathways to support development in a variety of media that often bear little resemblance to the composition of oviductal or uterine fluid. This complicates the determination of optimal nutrient provisions to support development *in vitro*. Although embryos are capable of adapting their metabolic activity to utilize a variety of nutrients in their environment, the metabolic costs of adaptation to suboptimal culture conditions can compromise embryo viability, cryotolerance, maintenance of pregnancy, fetal growth, and offspring health. This relationship between metabolic activity and viability is central to the successful application of assisted reproductive technologies. Only by understanding the metabolic requirements of the embryo can we design culture systems that support the development of viable embryos with the best chance of resulting in healthy offspring.

The application of metabolomics to the analysis of embryo metabolism is helping to further this understanding. Metabolomics permits investigation of embryo physiology in a focused, in depth manner that has not been previously possible, allowing us to think about embryo metabolism as a complex interplay of multiple metabolic mechanisms. This technology has tremendous potential to expand our knowledge of embryo metabolism because it can be applied non-invasively to the study of embryo physiology via the simultaneous measurement of multiple substrates following culture in an optimized medium. A metabolomics approach not only provides information about suspected pathways of importance, but also about unknown regulatory mechanisms and metabolic intermediates. Information provided by metabolomics will inform the development of improved embryo culture media to reduce *in vitro* stress and adaptation, as well as methods to regulate metabolism *in vitro* to improve embryo quality. In addition, specific metabolic fingerprints characteristic of high quality embryos will be discovered.

Metabolic networks in mammalian embryos

Existing studies have provided a glimpse of the diverse metabolic mechanisms used by embryos, and hinted at the dynamic, tightly controlled biochemistry over the time course of preimplantation development. However, we have only begun to appreciate these mechanisms and how they are controlled. Interpretation of metabolic studies is complicated by *in vitro* conditions, and we still do not have a good understanding of how embryos are operating metabolically within the larger context of their environment, much less what pathways they should be

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utilizing to promote optimal quality. Several hypotheses, not necessarily mutually exclusive, have been proposed to understand embryo metabolism in a larger context. The Quiet Embryo Hypothesis proposes that viable embryos have lower overall metabolism because they are not responding to cellular stress (Leese, 2002; Baumann *et al*., 2007; Leese *et al*., 2007, 2008). Energy requirements are increased with stress, suggesting that elevated metabolism has a negative relationship with embryo viability. Embryo metabolism has also been hypothesized to mirror that of cancer cells, which use a metabolic strategy known as the Warburg Effect (Warburg, 1956; Krisher and Prather, 2012; Smith and Sturmey, 2013). Warburg metabolism is thought to support rapid cellular proliferation by providing precursors for macromolecular synthesis and oxidative stress management, and is known to be involved in the control of cellular differentiation (Vander Heiden *et al*., 2009, 2010; Panopoulos *et al*., 2012; Zhang *et al*., 2012). Although the tricarboxylic acid (TCA) cycle is a much more efficient means of producing ATP when compared to glycolysis, the embryo may have more important metabolic uses for glucose than simply ATP production. This may include redox control and macromolecular synthesis, including DNA, RNA, proteins and lipids to support rapid embryonic growth. It may be too simplistic to view embryo glucose metabolism as primarily a means to produce ATP, ignoring the embryo's biosynthetic requirements. In this case, fatty acids and/or amino acids likely support basal TCA activity to provide ATP.

Embryo metabolism

Although embryo metabolism has been well investigated, the basis of our current understanding primarily comes from studies of the murine embryo and its use of a limited number of substrates, primarily the carbohydrates glucose, lactate and pyruvate and occasionally the amino acid glutamine. The pioneering studies of Biggers and Brinster (Brinster, 1965a, b; Biggers *et al*., 1967) led to a model in which cleavage stage embryos primarily utilize pyruvate and lactate while a reliance on glucose metabolism via glycolysis characterizes the blastocyst prior to implantation, when higher glucose uptake is a signature of viability (Gardner and Leese, 1987; Gardner *et al*., 2001). Fifty years later, this model is still surprisingly accurate and widely cited as the generalized pattern of metabolic activity in the mammalian embryo. However, these studies were conducted in simple media (salts, carbohydrates, and protein) that do not support optimal development. For example, oviductal and uterine fluids contain all 20 of the basic amino acids (Harris *et al*., 2005; Hugentobler *et al*., 2007; Li *et al*., 2007), in contrast to early culture medium formulations in which glucose and lactate were present in supra-physiological concentrations and no amino acids were present (Brinster, 1965a; Whitten and Biggers, 1968).

In ruminants, pyruvate uptake exceeds that of glucose at the early cleavage stages (Rieger *et al*., 1992; Gardner *et al*., 1993; Thompson *et al*., 1996). Similarly, ~90% of ATP is derived from oxidative metabolism prior to compaction, with pyruvate and glutamine being the preferred substrates (Thompson *et al*., 1991, 1996; Rieger *et al*., 1992; Gardner *et al*., 1993). Even though glucose is not the "preferred substrate", early bovine embryos do utilize glucose, with increases in PPP and glycolysis during preimplantation development (Wales and Brinster, 1968; Leese and Barton, 1984; Pantaleon *et al*., 2001; Comizzoli *et al*., 2003). Glucose consumption, hexokinase activity, and lactate production increase from the zygote to morula stages (Wales and Brinster, 1968; Leese and Barton, 1984; Gardner and Leese, 1986, 1988; O'Fallon and Wright, 1986; Saito *et al*., 1994; Houghton *et al*., 1996). In post-compaction ruminant embryos there is a shift to glucose metabolism, with an increase in glucose uptake, lactate production, glycolytic activity, and the proportion of ATP produced via glycolysis (Thompson *et al*., 1991, 1996; Rieger *et al*., 1992; Gardner *et al*., 1993). Pyruvate uptake and oxidation also increase during blastocyst development (Rieger *et al*., 1992; Gardner *et al*., 1993; Thompson *et al*., 1993, 1996; Krisher *et al*., 1999; Khurana and Niemann, 2000), even though glucose is the primary substrate. Oxidation of lactate and pyruvate appear to be inversely related, with inclusion of one substrate in the medium inhibiting metabolism of the other during pre-compaction development (Khurana and Niemann, 2000). Isolated trophectoderm cells from bovine blastocysts consumed less glucose and more pyruvate, and produced more lactate than inner cell mass cells (Gopichandran and Leese, 2003).

Glucose is present in the bovine oviduct at a concentration of ~2.5 mM (Hugentobler *et al*., 2008, 2010). However, there are culture media that successfully support development of bovine preimplantation embryos with glucose (SOF; Tervit *et al*., 1972; Steeves and Gardner, 1999; Gandhi *et al*., 2000) and without glucose (CR1aa; Rosenkrans and First, 1994); (mSOF; Takahashi and First, 1992). Because the cow and pig embryo are able to develop *in vitro* from the 1-cell stage to blastocyst in the absence of exogenous glucose, without any known detrimental consequences, it may be possible that pyruvate is converted to phosphoenolpyruvate (PEP) by mitochondrial enzymes, which may participate in the reversible reactions of glycolysis to supply intermediates for the PPP.

With the exception of glutamine, the majority of metabolic studies have focused on carbohydrates. However, porcine, bovine, and ovine embryos will develop to the blastocyst stage with protein and/or amino acids (AA) as the only exogenous nutrient sources (Petters *et al*., 1990; Thompson *et al*., 1992; Sutton-McDowall *et al*., 2012). Numerous studies have shown that AA have beneficial effects on the development of embryos from multiple species when added to the culture medium (Liu and Foote, 1995; McKiernan *et al*., 1995; Lane and Gardner, 1997; Steeves and Gardner, 1999; Biggers *et al*., 2000; Lane *et al*., 2001; Suzuki and Yoshioka, 2006). Specific amino

acids are consumed (depleted from the medium) and produced (secreted into the medium) by embryos from mice, pigs, cattle, and humans (Houghton *et al*., 2002; Orsi and Leese, 2004; Humpherson *et al*., 2005; Wale and Gardner, 2012). Presumably some of the consumed amino acids are used for protein synthesis, but other possible fates for the amino acids are not clear. Amino acids can act as osmotic buffers, helping the embryo to maintain cellular homeostasis (Baltz and Zhou, 2012). Ammonium production by embryos cultured with amino acids also indicates that some amino acids are being converted to TCA cycle intermediates for generation of ATP (Gardner *et al*., 2001; Lane *et al*., 2001). Since ammonium can be inhibitory to development, embryos have mechanism to detoxify ammonium and prevent its build-up in the cytoplasm or the culture medium. Murine and bovine embryos are capable of producing glutamine from ammonium and glutamate and/or producing alanine from glutamate, pyruvate, and ammonium (Orsi and Leese, 2004; Wale and Gardner, 2013). The resulting alanine and glutamine are secreted into the medium, which has been observed in a number of studies (Houghton *et al*., 2002; Orsi and Leese, 2004; Humpherson *et al*., 2005; Wale and Gardner, 2013; Krisher *et al*., 2015).

The study of carbohydrate metabolism has overshadowed the contribution of fatty acid β-oxidation (FAO) until relatively recently. Cow, pig and cat oocytes have large stores of intracellular lipids while the mouse has fewer lipid stores, a fact reflected by the color of the cytoplasm (McEvoy *et al*., 2000; Leroy *et al*., 2005a). In humans and domestic ruminants, palmitic, stearic and oleic are the most abundant fatty acids in oocytes, while pig oocytes contain greater polyunsaturated fatty acids, particularly linoleic acid (Homa *et al*., 1986; Matorras *et al*., 1998; McEvoy *et al*., 2000; Kim *et al*., 2001). Even those species with a relatively low concentration of lipids, like mice, rabbits, and humans, have been shown to actively metabolize this nutrient source (Khandoker and Tsujii, 1998; Haggarty *et al*., 2006; Dunning *et al*., 2010; Paczkowski *et al*., 2014). Inhibition of fatty acid oxidation decreases embryonic development in both mice and cattle (Hewitson *et al*., 1996; Ferguson and Leese, 2006). The addition of fatty acids or carnitine to stimulate FAO to oocyte and embryo culture medium has primarily shown positive effects on development, although results are variable due in part to differences in type and concentration of fatty acid used (Spindler *et al*., 2000; Leroy *et al*., 2005b; Dunning *et al*., 2010; Marei *et al*., 2010; Somfai *et al*., 2011; Van Hoeck *et al*., 2011; Wu *et al*., 2011).

Historic approaches to measuring metabolism

To date, most of what we know about embryo metabolism has been determined using radiolabeled substrates or microfluorescence. Radiolabeled substrates provide information about specific pathways, depending on the location of the label on the original substrate and the end metabolite. Microfluorescence is based upon enzymatically coupled reactions associated with changing ratios of NAD(P)+:NAD(P)H. Both methods result in precise quantitation of substrate metabolism. Perhaps the most important consideration when interpreting these results is that embryo metabolism is not only affected by the conditions in which the embryo develops, but also the medium in which metabolism is assessed (Gardner and Leese, 1990; Lane and Gardner, 1998; Krisher *et al*., 1999; Gandhi *et al*., 2001). Another drawback to metabolic measurement is that we are unable to measure the metabolic pathways that are normally used by embryos *in vivo*, so we are never completely confident of what an embryo should be doing metabolically. Of course, we can compare the metabolism of *in vivo*-derived embryos to that of *in vitro* cultured embryos, but we must keep in mind that there will likely be some sort of adaptation to the *in vitro* environment (Lane and Gardner, 1998). Even given these caveats, metabolic studies have provided important information that has helped us understand metabolic mechanisms in mammalian embryos.

Metabolomics

It is only recently that technological advances in automation and information technology have allowed the basic techniques of metabolomics to be applied to the study of embryo metabolism (Hollywood *et al*., 2006; Brison *et al*., 2007; Seli *et al*., 2007; Krisher *et al*., 2015). Metabolomics offers multiple advantages over previous methods. This technology is able to measure uptake and production of multiple substrates by the embryo by non-invasively analyzing the medium following *in vitro* culture. This represents a significant advance in our ability to examine embryo metabolism in a complex environment during preimplantation development, compared to our current snapshots of isolated pathways measured in modified media not designed to support long term embryo culture. This approach also leaves the embryo viable for transfer, thus lending itself to the discovery of a metabolic signature characteristic of high quality embryos that could be used to select embryos for transfer (Singh and Sinclair, 2007).

Typically, medium is analyzed following *in vitro* embryo culture and compared to medium without an embryo to ascertain how the composition of the culture medium was altered, commonly referred to as the metabolic 'footprint' of the embryo. Although an indirect measurement, it provides specific information about what substrates the embryo is taking up and producing, providing clues as to the pathways in operation. Metabolomics provides information about how the embryos use substrates that we know are included in the culture medium using a targeted approach (measuring a predefined set of metabolites). In addition, a non-targeted approach can be used that will collect information about all detectable metabolites, known and unknown, to generate novel information about embryo biochemistry. While the non-targeted approach investigates a larger cohort of metabolites, the datasets are large and complex. Recent improvements in informatics workflow for metabolomics have helped

mitigate this issue, improving both metabolite annotation and interpretation on a large scale. Another key point to consider when using metabolomics to study embryo metabolism is that quantitation is usually relative. In this circumstance, the amount of a particular substrate taken up by the embryo is reported as a percentage of what was detected in medium without an embryo. This makes inclusion of appropriate media controls, collected from the same culture dish and treated identically to sample drops, paramount. Although this method of relative quantitation does not provide information about the concentration of any given substrate, it does provide data regarding those substrates embryos are consuming or producing in statistically significant quantities compared to the total amount available. Absolute quantitation is possible for some known metabolites by calibrating sample values to a standard curve, which permits better comparison between metabolomic studies as well as to metabolism studies carried out using other techniques. However, this increases cost and can be difficult to do for large numbers of metabolites. It is important to note that absolute quantitation is not necessary to make valid metabolomic comparisons. Often, both uni- and multivariate statistical analyses enable recognition of differences or changes in metabolite profiles that can be used as markers of disease or toxicity, even before specific metabolites are quantified, or even identified.

Several platforms can be adapted for metabolomics, although mass-spectrometry based approaches are ideally suited for the sensitivity, complex composition and low sample volume inherent in analyses of embryo culture media. Multiple platforms have been reported for analysis of embryo metabolism, including gas or liquid chromatography (GC and LC, respectively) and/or matrix-assisted laser desorption/ionization (MALDI) coupled to mass spectrometry (MS), as well as nuclear magnetic resonance (NMR), Raman, or near infra-red (NIR) spectroscopy. If MS is used, measurement of the molecules' mass, or the masses of distinctive fragments of that molecule following derivitization, results in a specific molecular fingerprint that then allows identification of the metabolite when compared to known databases. The sensitivity of these methods permits the analysis of individual embryos, negating the need for embryo pooling and providing the opportunity to associate specific metabolic profiles with embryo competence post transfer.

Along with the power inherent in the application of metabolomic technology to embryo metabolism, there are some limitations. It is only possible to detect net differences in culture medium with and without an embryo. If the same substrate is both consumed and produced by the embryo, resulting in a net change of zero, it will not be detected as metabolic activity. It is also not possible to differentiate between the same substrate originating from the culture medium or the embryo. For example, these techniques cannot distinguish between lactate from the culture medium and lactate produced by the embryo, only the total lactate value is obtained. Substrates labeled with stable isotopes (such as ^{13}C) can be used to overcome these problems, but not in embryos destined for transfer. Similarly, if the culture medium lacks a metabolite important for embryo metabolism and development, a metabolomics approach will not reveal its absence. An additional limitation of metabolomics is that to obtain information about many intermediates in metabolic pathways, which is critical to understanding pathway preference, the embryo must be analyzed directly and thus destroyed. Additionally, in most cases fewer metabolites will be analyzed than are actually detected in the complete sample spectrum. This may be because a targeted approach is used where only known metabolites are specifically examined, because some metabolites are unknown, or because some were not accurately detected. A final drawback to metabolomics technology is that current platforms are expensive and complex, requiring experts to both run the samples and analyze the data, resulting in relatively slow throughput and making them unrealistic for most assisted reproductive technology (ART) laboratories (Montag *et al*., 2013). However, blastocyst vitrification provides the time necessary to perform these complex analyses at a specialized core facility prior to embryo transfer.

Applications of metabolomic technology to assisted reproduction

Metabolomic profiling provides a large amount of information describing the metabolic activity of individual embryos. Now that we can successfully undertake such studies, we must consider the impact that this information might have in ART. Can the knowledge generated by this technology improve ART? Certainly, a primary outcome is that of basic knowledge leading to an improved understanding of embryo metabolism. Then we can expand our experiments to determine how embryo metabolism changes during preimplantation development, discover how embryo metabolism is altered by maternal disease, and in what manner embryo quality is reflected by metabolism. Finally, we can then address the overarching question of how these factors interact with the environment in which the embryo finds itself to influence competence. These studies should lead to the formulation of improved culture media that manage embryo metabolism to alter the activities of specific pathways critical to embryo quality that are not supported in conventional media.

Metabolomics has been used for research of embryo metabolism, providing novel basic information. Although analyzed in groups, the metabolome of mouse embryos has been defined using tandem mass spectrometry (LC-MS/MS) and capillary electrophoresis TOF-MS (Wale and Gardner, 2012; Yamada *et al*., 2012). Our laboratory has reported metabolomic analyses of mouse, bovine and human embryos using GC- and MALDI- MS relative to species, stage of development, embryo quality, maternal characteristics, and culture conditions (Krisher *et al*., 2015). Lipids are one class of metabolites that have begun to be studied in depth in oocytes and embryos using metabolomic

techniques. Using MALDI-MS, the lipid content of individual oocytes and embryos from several species was defined, and alterations in lipid profile of bovine embryos due to culture with serum were described (Ferreira *et al*., 2010). MALDI time of flight (TOF) MS was used to evaluate the lipid profile of human cumulus cells, demonstrating that phosphatidylcholine might be used as a marker of oocytes capable of producing an embryo that results in pregnancy (Montani *et al*., 2012). Desorption electrospray ionization mass spectrometry (DESI-MS) has been used to describe changes in lipid profile during preimplantation development in the mouse, and described differences between embryos produced *in vitro* and *in vivo* (Ferreira *et al*., 2012). In bovine embryos, MALDI-MS revealed differences in phosphatidylcholine and sphingomyelin due to *in vitro* culture as well as subspecies of origin (Sudano *et al*., 2012).

A relatively unheralded application of metabolomics in the ART laboratory is the identification of embryo-toxic contaminants in contact materials. Significant quality testing is currently performed, typically using the mouse embryo assay, to determine the suitability of specific lots of reagents and plastic ware for human embryo culture. Many products are tested by both the supplier and the end user, and products are detected that compromise embryo development. However, there is no understanding of the contaminating compounds present that render lots unsuitable. If these compounds could be identified, products could be prescreened to eliminate those with known contaminants causing negative effects on embryo growth. This would not only significantly reduce the chance that these products would reach the ART laboratory, but would also decrease resources used for testing.

Probably the most anticipated application of metabolomics to ART, however, is the development of a biomarker for embryo viability. Given that embryo metabolism is so closely linked to viability, a metabolic biomarker is of great interest (Nel-Themaat and Nagy, 2011; Gardner and Wale, 2013). Research has provided compelling evidence that metabolism, and amino acid turnover in particular, is related to embryo quality in humans and other mammalian species (Houghton *et al*., 2002; Brison *et al*., 2004; Sturmey *et al*., 2008, 2010; Hemmings *et al*., 2012; Gardner and Wale, 2013). To date, morphology is the most widely used method by which to identify viable embryos. However, it is widely accepted that this parameter provides only limited information about an embryos ability to implant and support a viable pregnancy (Botros *et al*., 2008). Initial reports suggested that metabolomic analyses may provide a better predictive tool for embryo selection, compared to morphology alone (Nagy *et al*., 2008; Marhuenda-Egea *et al*., 2010; Cortezzi *et al*., 2013). Retrospective studies using Raman and NIR spectroscopy, as well as electrospray ionization MS (ESI-MS), defined associations between spent media profiles and the potential for successful implantation in human ART (Nagy *et al*., 2008; Sakkas *et al*., 2008; Scott *et al*., 2008; Seli *et al*., 2010; Marhuenda-Egea *et al*., 2011; Pudakalakatti *et al*., 2013; Zivi *et al*., 2014). Randomized controlled trials based upon these retrospective results were undertaken but selection using the metabolomics based viability index did not increase pregnancy rate compared to selection based upon morphology alone (Hardarson *et al*., 2012; Vergouw *et al*., 2012; Uyar and Seli, 2014), possibly due to limitations in sensitivity of this platform (Gardner and Wale, 2013). Of interest, these studies did not identify specific metabolites, only calculated a viability index based upon the spectrum of unspent media. The goal here was biomarker-based prediction, not the generation of knowledge that would inform what we know of embryo metabolism.

Conclusions

It is clear that metabolic activity is a critical indicator of embryo viability. The success of assisted reproductive technologies involving even a small amount of time in culture is dependent on providing the embryo with an appropriate combination of substrates that will support normal metabolic activity and minimize cellular stress. Although our understanding of embryo metabolism has improved greatly since the early work of Biggers and Brinster (xxx), there is still much work to be done. To understand the relationship between metabolism and viability, we must examine the complex metabolic pathways in total and appreciate their interrelationships. We are just starting to realize the diversity of metabolic mechanisms present among embryos from different species. Previous studies have provided only snapshots of metabolic pathways in isolation. However, application of metabolomic technologies to the analysis of embryo metabolism permits visualization of metabolism in optimized culture conditions and in the context of a complete metabolic system. To date, metabolomic technology has been successfully applied to the study of embryo metabolism, although most studies have been descriptive in nature. These initial studies have provided important new information about the metabolic activity of embryos during development *in vitro*, and have begun to address the relationship between metabolism and quality. Now the field is poised to expand this work to address experimental hypotheses for basic research, and apply the knowledge gained. Ultimately metabolomic data will provide in depth detail of biochemical pathways used by embryos under various conditions, revolutionizing our understanding of embryo biochemistry and leading to the ability to manipulate metabolism *in vitro* to support improved embryo development, and allowing the identification of the most metabolically viable embryos.

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Functional aspects of seminal plasma and sperm proteins and their potential as molecular markers of fertility

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Abstract

Molecular components of sperm and in the media surrounding them influence bull fertility. Given this concept, proteins of the seminal plasma modulate crucial functions and events of reproduction, such as sperm motility and capacitation, cell protection, acrosome reaction, fertilization and embryonic development. Sperm proteins are also important for successful fertilization, egg activation and embryo development. Empirical associations between seminal and sperm proteins and fertility in the bovine indicate that these proteins are potential molecular markers of the male reproductive status.

Keywords: bull, fertility, proteins, seminal plasma, sperm.

Introduction

Pregnancy per artificial insemination (AI) is the best indicator of the reproductive potential of sires. However, this information becomes available only after animals are mature and have been selected for commercial use in the AI industry. Moreover, criteria such as sperm motility and morphology have limited associations with the actual fertility of sires, particularly in groups of animals that have been extensively selected by the AI industry (Killian *et al.*, 1993; Moura, 2005; Moura *et al*., 2006; De Oliveira *et al*., 2013; Dogan *et al*., 2015; Kaya and Memili, 2016). Significant differences in fertility exist among bulls with "normal" semen parameters and bulls with non-compensable sperm defects may never achieve adequate fecundity (De Oliveira *et al*., 2013; Dogan *et al*., 2015; Kaya and Memili, 2016). This information suggests that mechanisms by which sperm molecular profiles influence bull fertility are not fully understood. In this context, research effort has been made to identify molecular markers of gamete function and fertility in nearly all farm animals and humans. Candidates for such markers include sperm RNA, sperm proteins and molecules of the reproductive fluids, among others. All these studies are based on the hypothesis that molecular components of sperm and/or from the surrounding media influence the fertilizing capacity of sires. Considering this concept, analysis of both sperm and seminal plasma proteome will provide meaningful information about the mechanisms regulating sperm fertilizing potential and reproductive performance of sires. Thus, the present review discusses the roles of selected seminal plasma proteins and how their expression relates to fertility. Sperm proteins and their potential use as fertility markers are also discussed mainly focused on the bovine species.

Seminal plasma proteins

Proteins involved in sperm protection

Seminal plasma contains proteins that protect sperm in the epididymis (Kraus *et al*., 2005; Hinton *et al*., 1995), after ejaculation and in the female reproductive tract. Production of reactive oxygen species (ROS) is part of sperm physiology (MacLeod, 1943) but, when in excess, ROS disturb sperm homeostasis through formation of lipid peroxidation, and reduction of enzymes that regulate calcium influx and loss of ATP (Ohta *et al*., 1989; Aitken *et al*., 1993). To control the deleterious effects of ROS, epididymis secretes antioxidant enzymes (Hinton *et al*., 1996), such as glutathione S-transferase, tioredoxin peroxidase, superoxide dismutase, glutathione peroxidase (GSHPx) and catalase (Alvarez and Storey, 1983; Jeulin *et al*., 1989; Fouchécourt *et al*., 2000; Dacheux *et al*., 2006). GSHPx is one the main enzymes that protects sperm (Perry *et al*., 1992; Dacheux *et al*., 2005) and it catalyzes the reduction of hydrogen peroxide (Halliwell and Gutteridge, 1990). Increased GSPHx activity in ram semen is linked to antioxidant effects and maintenance of sperm viability (Casao *et al*., 2010). Another seminal plasma molecule known as acidic seminal fluid protein (aSFP) also has actions on the control of oxidative stress in the bovine reproductive tract (Einspanier *et al*., 1993; Schöneck *et al*., 1996). aSFP shares identity with molecules of spermadhesin family (Romão *et al*., 1997) and, in the bull, it is secreted by both the epididymides and accessory sex glands (Moura *et al*., 2007a, 2010). Binding of aSFP to ejaculated sperm occurs but it is lost after capacitation (Dòstolovà *et al*., 1994), suggesting that, unlike porcine spermadhesins (Caballero *et al*., 2004, 2005), bovine aSFP does not participate in sperm-oocyte interaction. However, aSFP has been associated with survival of bull sperm to cryopreservation (Jobim *et al*., 2004), suggesting that its anti-oxidant activities may be beneficial to reproductive technology.

Additionally, ion chelators of the seminal plasma, such as lactoferrin, protect sperm against effects of lipid peroxidation (Ochsendorf, 1999). Lactoferrin sequesters ionic iron (Nozaki *et al.*, 2003) and adsorbs sperm during epididymal transit (Jin *et al*., 1997) and ejaculation (Thaler *et al*., 1990), when it also has

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antimicrobial effect. Seminal albumin, in turn, binds to lipid peroxides, contributing to sperm protection (Alvarez and Storey, 1983), and presents a positive correlation with the percentage of normal sperm in bovine semen (Elzanaty *et al*., 2007). Clusterin is another seminal plasma molecule with protecting roles, acting as a chaperone (Humphreys *et al*., 1999) and inhibiting cell lysis by complement-mediated mechanisms present in female secretions (Ibrahim *et al*., 1999; Meri and Jarva, 2001). In the epididymis, some clusterin functions relate to maturation, lipid transport (Tenniswood *et al.*, 1992) and sperm membrane remodeling (Humphreys *et al.*, 1999). Clusterin chaperone activity is consistent with its ability to interact with different types of proteins *in vivo* (Carver *et al.*; 2003). *In silico* analysis of clusterin networking indicates its potential interactions with proteases and protease inhibitors, such as plasminogen, alpha-2-macroglobulin, TIMP-1, alpha-2-antiplasmin precursor and plasminogen activator inhibitor 1. Clusterin also has putative links to fibronectins, which participate in cell adhesion, healing of wounds and maintenance of cell structure; GTP protein-coupled receptors and modulators of cell growth, such as TGFB3. Seminal plasma clusterin is inversely related to the percentage of sperm with intact membrane in peccaries (*Peccari tajacu* L.; Santos *et al*., 2014) and with the percentage of normal sperm in semen samples of Brahman bulls (Boe-Hansen *et al*., 2015). In contrast, bull and ram sperm with morphologic defects exhibit extensive clusterin binding (Ibrahim *et al.,* 2001a, b). This association probably occurs as a result of clusterin´s ability to bind to the damaged portions of hydrophobic regions of sperm membrane (Bailey and Griswold, 1999).

Proteins associated with sperm motility

Seminal plasma contains proteins associated with processes of sperm motility (Baas *et al*., 1983), such as the kallikrein-cinins. In this case, kininogen present in seminal plasma acts as a specific substrate for kalikrein (Fink *et al.*, 1989), producing the main effectors for stimulation of sperm motility after ejaculation (Schill *et al.*, 1989). Studies have demonstrated positive correlation between seminal plasma kallikrein activity and sperm motility, and addition of exogenous kallikrein benefits bovine sperm motility as well (Somlev *et al*., 1996). Angiontensin converting enzyme (ACE) is another seminal plasma component related to the kalikrein system (Hohlbrugger *et al*., 1984). ACE catalyzes the formation of angiontensin II and binds to receptors on the sperm, intensifying events related to motility (Vinson *et al*., 1996). ACE activity in the ram seminal plasma positively correlates with sperm concentration and fertility (Métayer *et al*., 2001; Gatti *et al.*, 2004). Inhibition of ACE activity in the bovine seminal plasma decreases the number of sperm with progressive motility and inhibits acrosome reaction after *in vitro* capacitation (Costa and Thundathil, 2012).

Proteins involved in sperm capacitation

Phospholipid binding proteins belonging to the family of BSPs (Binder of Sperm Proteins) are present in the seminal plasma of several species, including bulls, bucks (male goats and rabbits), rams, men, male rodents, and stallions (Moura *et al*., 2007a; Manjunath *et al*., 2009; Souza *et al*., 2012; Plante *et al*., 2016). BSPs comprise approximately 60% of all proteins of the accessory sex gland fluid (Moura *et al*., 2007a) and seminal plasma (Manjunath and Sairam, 1987) from *Bos taurus* bulls and nearly the same amount in *Bos indicus* seminal plasma (Rego *et al*., 2014). In the bovine, BSP proteins are secreted as several isoforms with 14-15 kDa (BSP1 and BSP3) and 30 kDa (BSP5). Both BSP1 and BSP5 possess two fibronectin type II domains arranged in tandem and amino terminal extensions that are O-glycosylated at threonine residues. Such biochemical attributes allow BSP1 and BSP5 to interact with sperm and to modulate ligand-binding activities by similar mechanisms (Calvete *et al*., 1996; Manjunath *et al*., 2009), sharing functional similarities as well (Manjunath and Therien, 2002). Bovine BSPs are typical accessory sex gland proteins and there are no reports of their expression in the epididymis (Manjunath and Therien, 2002; Moura *et al*., 2007a, 2010). BSPs bind to bull sperm at ejaculation (Manjunath and Therien, 2002) and remain as such after sperm come in contact with oviductal secretions *in vitro* (Souza *et al*., 2008); after sperm become acrosome reacted and after sperm are subjected to freezing and thawing (Rodriguez-Villamil *et al*., 2016). BSPs also mediate the interaction between sperm and the oviduct epithelium (Gwathmey *et al*., 2006; Suarez, 2016). The most studied role of BSPs is their effects to bind and remove phospholipids and cholesterol from the sperm membrane, one of the initial events of capacitation (Thérien *et al*., 1999). Capacitating effects of BSPs seem to hold true in other species as well, such as mice (Plante and Manjunath, 2015) and humans (Plante *et al*., 2014). However, while ruminant BSPs originate mainly from the accessory sex glands (Manjunath *et al*., 2009; Souza *et al*., 2012; van Tilburg *et al*., 2014), its expression in mice and humans occurs in the epididimis.

Roles played by BSPs seem to go beyond sperm capacitation as experimental evidence indicates that BSP1 contributes to *in vitro* fertilization and embryonic development when added to the fertilization media. The study conducted by Rodriguez-Villamil *et al*. (2016) evaluated cumulus-oocyte complexes (COCs) incubated with frozen-thawed ejaculated sperm (18 h) in Fert-TALP medium containing: heparin and different concentrations of purified BSP1 (10, 20 or 40 µg/ml). With ejaculated sperm, cleavage rates were similar when Fert-TALP medium was incubated with heparin (74.1 ± 2.7%), 10 µg/ml BSP1 (77.8 ± 3.1%) or 20 µg/ml BSP1 (74 ± 2.0%). Day-7 blastocyst rates were equivalent after incubations with heparin (40.8 ± 5.0%) and 10 µg/ml BSP1 (34.1 ± 4.4%), but there were marked reductions in blastocyst formation after IVF

media were supplemented with 20 µg/ml BSP1 (22.4 ± 2.9%) and 40 µg/ml BPS1 (19.3 ± 4.1%; $P < 0.05$). Such results confirm that BSP1 is as efficient as heparin for induction of capacitation and the fertilizing capacity of frozen-thawed ejaculated sperm. However, the study also reports a damaging effect of BSP1 on *in vitro* embryo development. This outcome may be the result of BSP1-induced damage on sperm, given the exposure period during fertilization (18 h). In agreement with such results, studies show that excess of BSP proteins and time exposure are harmful to cryopreserved sperm because of membrane destabilization and excessive phospholipid and cholesterol efflux (Therien *et al*., 1995; 1998; Manjunath and Therien, 2002). Also, an earlier investigation claimed that the content of BSP5 in accessory sex gland fluid has a quadratic association with bull fertility (Moura *et al*., 2006), suggesting that too much BSP is detrimental to sperm physiology and/or embryo development. Finally, inclusion of BSP1 in the Percoll solution with frozen-thawed ejaculated sperm for a short period of time (30 min), prior to *in vitro* fertilization, was detrimental to embryo development (our unpublished results). In this case, 40 µg/ml BPS1 was as efficient as heparin to enhance the fertilizing capacity of sperm. Result obtained with the Percoll incubation substantiates the concept that damaging effect of BSP1 relates to exposure time of sperm to this molecule.

In other experiments, COCs were incubated with frozen-thawed cauda epididymal sperm (18 h) in Fert-TALP medium containing: no heparin, heparin, 10, 20 or 40 µg/ml BPS1. In this case, cleavage and blastocyst rates were similar after treatments with heparin (68.5 ± 1.3% and 24.7 ± 3.2%, respectively) or without heparin (65.5 ± 1.8% and 27.3 ± 1.6%, respectively). Cleavage was higher after treatment with any BSP1 concentrations (74.2 ± 2.7% to 79.0 ± 1.1%) than without heparin ($P < 0.05$). Also, cleavage was better with 40 µg/ml BSP1 (79.0 ± 1.1%) than with heparin (68.5 ± 1.3%; $P < 0.05$). Embryo development was higher ($P < 0.05$) after treatment with 20µg/ml BSP1 (35.6 ± 2.5%) and 40 µg/ml (41.1 ± 2%) than after incubations with heparin (24.7 ± 3.2%) or without heparin (27.3 ± 1.6%). It has been established that BSPs bind to membrane cholesterol and phospholipids but how this occurs is still a matter of debate. Interestingly, supplementing IVF media with BSP1 + heparin (for 18 h) did not improve the effects of BSP1 when fertilization occurred with frozen-thawed epididymal sperm (our unpublished results). Thus, we may conclude that: 1. heparin has limited effect on cauda epididymal sperm *in vitro*, as evaluated by fertilization and blastocyst formation; 2. BSP1 had better effect on embryo growth than heparin; 3. combination of BSP1 with heparin does not enhance cleavage rates and embryo development beyond those obtained with BSP1 only. When conducting studies with IVF systems, we also verified that BSP1 did not cause reductions in blastocyst rates after fertilization with epididymal sperm, in contract to what we had observed with ejaculated sperm. This obviously emphasizes that previous exposure or not of spermatozoa to seminal plasma determines how sperm will respond to BSP *in vitro*.

Despite all important roles played by BSPs, these molecules can also damage sperm during cryostorage as they extract phospholipids and cholesterol from the membrane in a concentration- and time-dependent manner (Manjunath *et al*., 2002; Plante *et al*., 2016). Such deleterious effects occur when sperm are exposed for a long time and/or to excess of BSP proteins, as it happens during cooling and freezing. In this regard, extenders used for sperm preservation, such as egg-yolk (EY) and milk, contain components that associate with BSPs (Manjunath *et al*., 2002). In the case of EY, its low-density lipoproteins interact with bovine BSPs (Bergeron and Manjunath, 2006) and milk proteins also interact with BSPs. This interaction between milk components and BSPs prevent excessive BSP binding to sperm and excessive phospholipid removal from the membrane, being thus the fundamental mechanism by which milk components protect sperm during cryopreservation (Plante *et al*., 2015). In fact, experiments conducted with goats (Menezes *et al*., 2016) show that not only did milk proteins (casein and β-lactoglobulin) bind to BSPs, but they also reduced BSP interactions with sperm membranes. Currently, BSP proteins are one of the most studied seminal plasma proteins in mammalian species. In addition to being abundant in ruminants, studies conducted in the bull confirm that BSPs have broad effects on ejaculated sperm, including capacitation, interaction with the oviduct epithelium and fertilization. The fact that BSPs interact with components of semen extenders suggest that these proteins are potential targets for the development of new fertility markers and biomolecules that can improve assisted reproductive technologies.

Proteins involved in acrosome reaction and fertilization

Seminal plasma phospholipase A2 (PLA2) participates in capacitation, acrosome reaction and sperm-oocyte membrane fusion (Soubeyrand *et al*., 1997; Pietrobon *et al*., 2005; Roldan and Shi, 2007). PLA2 also has antimicrobial effects and its expression in seminal plasma is associated with fertility in bulls (Moura *et al*., 2006). The analysis of seminal plasma proteome showed that osteopontin (OPN) was related to *in vivo* fertility of Holstein bulls (Killian *et al*., 1993; Moura *et al*., 2006) and to the fertilizing capacity of cauda epididymal sperm treated with accessory sex gland fluid during IVF trials (Henault *et al*., 1995; Moura *et al*., 2007b). In fact, the amount of OPN in the seminal plasma of high fertility bulls is 4-fold higher in comparison to that in low fertility sires (Moura *et al*., 2006). The importance of OPN in reproduction is also demonstrated by experiments using IVF. In this regard, the percentage of fertilized bovine oocytes was significantly reduced by addition of OPN antibodies to fertilization media and exposure of sperm or oocytes to antibodies against alphaV and alpha5 integrins before fertilization (Gonçalves *et al*., 2007). Also, pre-treatment of sperm and oocytes with OPN enhances

both *in vitro* fertilization and early embryo development in the bovine (Gonçalves *et al*., 2008a, b). The RGD amino acid sequence of osteopontin mediates its link with α5 and αv integrins (Denhardt, 2002; Wai and Kuo, 2004) and the ability of osteopontin to support cell adhesion is prevented when the RGD sequence is mutated (Liaw *et al*., 1995; Xuan *et al*., 1995). Treatment of sperm or oocytes with an RGD peptide, but not with an RGE sequence, reduced both the number of sperm bound to the zona pellucida and fertilization rates, similar to what was found using antibodies against osteopontin. Results involving RGD and anti-integrin antibodies suggest that OPN interacts with sperm through integrins (Gonçalves *et al*., 2007). Incubation of oocytes with osteopontin purified from bovine milk increased cleavage rates on day 4 (from 78.1 ± 1.3 to 85.8 ± 1.4 %), blastocyst development on day 8 (from 24.2 ± 1.2 to 33.8 ± 1.4 %) and hatched blastocysts on day 11 (from 10.6 ± 1.6 to 18.5 ± 1.4 %; Gonçalves *et al*., 2007). Following this concept, purified milk OPN improved sperm capacitation and, when added to the IVF media, OPN enhanced bovine blastocyst formation (Monaco *et al*., 2009). Moreover, freezing bull semen with different concentrations of OPN elicited better *in vitro* fertilization rates (85 to 78 ± 4 % *vs*. 75 to 69 ± 4 %) and blastocyst development on day 8 (45 ± 2.9 to 37 ± 1.6 % *vs*. 33 ± 2.3 to 29 ± 2.8 %) in comparison with untreated semen (Gonçalves *et al*., 2008a). In the swine, supplementation of fertilization media with recombinant rat OPN enhanced fertilization rates by 41% and reduced polyspermy (Hao *et al*., 2006). Exogenous OPN added to IVF media improved cleavage rates and swine embryo development, and inhibited apoptosis and DNA fragmentation (Hao *et al*., 2008). Moreover, a recent study described that anti-OPN antibodies efficiently decreased the rates of *in vitro* fertilization and blastocyst growth in mice (Liu *et al*., 2015). Such pieces of evidence gathered after experiments in bulls, porcine and mice support OPN's effects on fertilization and post-fertilization events.

Osteopontin is typically involved in cell adhesion, tissue and extracellular remodeling, inflammation and immune-mediated events (Denhardt, 2002; Wai and Kuo, 2004; Rittling and Singh, 2015; Bouleftour *et al*., 2016). Despite substantial information about the actions of osteopontin in several tissues, an understanding of its function in male reproduction is far from complete. Based on the current literature and general attributes of osteopontin, a plausible hypothesis has been suggested by several authors about OPN and fertilization. In this case, OPN secreted by the accessory sex glands binds to sperm during ejaculation through integrins and that the integrin-OPN complex interacts with the zona pellucida (D'Cruz, 1996). This model is supported by the fact that bovine oviductal fluid contains OPN (Gabler *et al*., 2003). Additionally, OPN binds to CD44 receptor, which usually participates in cell adhesion (Cichy and Puré, 2003), and has been expressed both in sperm (Bains *et al*., 2002) and oocyte membrane (Schoenfelder and Einspanier, 2003). In the bull, osteopontin binds to sperm acrosome cap at ejaculation (Cancel *et al*., 1999) and this sperm-OPN link still exists after sperm comes in contact with secretions of the oviductal fluid and undergoes acrosome reaction *in vitro* (Souza *et al*., 2008). In addition to sperm binding, OPN interacts with the zona pellucida and oolemma of bovine oocytes (Souza *et al*., 2008). In light of these findings, we propose that OPN adheres to sperm and this complex connects to the zona pellucida or to OPN-zona pellucida, given that osteopontin is capable of forming bonds with other OPN molecules with high affinity (Kaartinen *et al*., 1999; Goldsmith *et al*., 2002). When entering the periviteline space, OPN attached to the post-equatorial segment would mediate the interaction of sperm with the oolema, also through integrins and/or CD44. Integrins (αv and α5) have been identified in the bovine (Erikson *et al*., 2008) and human spermatozoa (Fusi *et al*., 1996; Reddy *et al*., 2003), as well as on human oolema (D'Cruz, 1996). The CD44 transmembrane glycoproteins are present in bovine sperm and oocyte. Interface of sperm osteopontin with oocyte integrins and CD44 receptors could trigger intracellular signaling, as reported for other cell types (Wai and Kuo, 2004; Rangaswami *et al*., 2006), and affect fertilization and early embryo development.

Sperm proteins and fertility

Molecular and cellular health of sperm are important for mammalian reproduction and proteins of spermatozoa are vital for fertility, ability of the sperm to fertilize and activate the egg and to support embryo development. Sperm proteins play important roles in sperm integrity, morphology and functions, including motility, capacitation, fertilization, egg activation and embryo development (Parisi *et al*., 2014). For example, Outer dense fiber protein 2 (ODF2) is a component of sperm tail structure and is involved in regulation of motility in mouse (Tarnasky *et al*., 2010). In addition, tubulins, such as tubulin beta-2C chain located in sperm tail, are down regulated in asthenozoospermia (Siva *et al*., 2010). Also, mutations in gene encoding for sperm cation channel protein (CatSper) in mouse result in less directed sperm movements in the mouse (Ren *et al*., 2001).

Sperm capacitation is important for sperm's ability to fertilize the egg and many proteins and pathways that have been shown to be involved with sperm capacitation. In this regard, extracellular signal-regulated kinase (ERK) pathway is involved in tyrosine phosphorylation and capacitation of boar sperm (Awda and Buhr, 2010), and casein kinase 2 (CKII) participates in EGF signaling in sperm from Holstein bulls with different fertility. Following fertilization, sperm proteins appear to play roles during egg activation and are also markers of fertility. For example, sperm phospholipase C Zeta 1 (PLCZ 1) is involved in intracellular Ca2+ oscillation (Cooney *et al*., 2010) and postacrosomal sheath WW domain-binding protein (PAWP) participates in porcine egg activation and pronuclear formation, with potential effects on embryo development as well (Wu *et al*., 2007). Moreover, using methods in proteomics, Peddinti *et al*. (2008) identified

125 differentially expressed proteins in sperm from high *vs.* low fertility bulls and Grant *et al*. (2015) demonstrated that superoxide dismutase (SOD) is expressed at higher levels in sperm from high fertility sires.

Sperm nuclear structure is also crucial important for fertility. Experiments have demonstrated that bull sperm contain a repertoire of histones fertility (De Oliveira *et al.,* 2013) and that amounts of Protamine 1 (PRM1) in sperm are directly correlated with bull reproductive performance (Dogan *et al.,* 2015). These studies indicate that different amounts of histones, as well as the ratios between protamines and histones, determine sperm viability and thus bull fertility.

Conclusions

In recent decades, new methods in proteomics have allowed the detection of unprecedented number of proteins in the seminal plasma and spermatozoa of humans, domestic and wild species. This obviously broadens our knowledge about the roles of molecules present in seminal fluid and sperm, and how they define male fertility. Empirical associations exist between some of those molecules proteins and fertility indexes and sperm parameters. Experiments also confirm cause and effect relations between male factors (such as osteopontin and BSPs), and *in vitro* fertilization and early embryo development. Therefore, seminal and sperm proteins are potential markers of fertility and they may be used in the animal biotechnology industry.

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Markers of pregnancy: how early can we detect pregnancies in cattle using pregnancy-associated glycoproteins (PAGs) and microRNAs?

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Abstract

Pregnancy detection has evolved over the last few decades and the importance of early pregnancy detection is critical to minimize the amount of time a cow spends not pregnant. Embryonic mortality (EM) is generally considered to be the primary factor limiting pregnancy rates in cattle and occurs early (<day 28) or late (≥ day 28) during gestation (day 0 = estrus). In cattle, the incidence of early EM is approximately 20 to 40% and the incidence of late EM is approximately 3.2 to 42.7%. Significant effort has been directed toward understanding the mechanisms resulting in early EM up to day 17; however, relatively little is known about the causes or mechanisms associated with EM after day 17. Based on work in these areas, numerous investigators are pursuing methods of early pregnancy or EM detection after day 17 of gestation. This review will highlight some of the technology and markers being used for early pregnancy detection and provide evidence for just how early pregnancy can be detected in the bovine. Advancements in early embryonic or pregnancy detection may lead to development of strategies to overcome early gestation losses.

Keywords: cattle, embryonic mortality, placenta, pregnancy.

Introduction

Successful pregnancy is the most important factor to ensure an efficient and economically sound beef or dairy operation. In order to reach that end point, reproductive loss must be avoided. Early identification of pregnancy failure is key to determining the most effective management strategies and the ability to predict this loss offers greater opportunity to minimize its impact. Loss of pregnancy may occur at any time between conception and calving; however, some time points are more critical than others. Pregnancy failure affects all cattle; however, high producing dairy cattle are more susceptible to decreased pregnancy rates than dairy heifers and beef cows (Diskin *et al.*, 2011; Pohler *et al.*, 2015a; Pohler *et al.*, 2016a, b). Although fertilization rate in cattle is often greater than 85% (Santos *et al.*, 2004, Diskin and Morris, 2008) there have been reports of differences in fertilization rates in beef and dairy cattle that result in a large amount of reproductive failure (Breuel *et al.*, 1993, Sartori *et al.*, 2002; Santos *et al.*, 2004, 2009); however, the focus of this review will be on post fertilization failure and detection. In addition, fetal losses (>day 45 of gestation) are low at approximately 3% (Inskeep and Dailey, 2005). Thus, reproductive loss during the embryo stage (day 0 to 44) of development is substantial. Early embryonic loss can be classified as loss that occurs before day 28 of gestation. Although the embryonic heartbeat can be detected by this time via real-time ultrasonography, the conceptus does not yet resemble a calf. Causes of early embryonic mortality include lethal genetic mutations, uterine asynchrony, and maternal recognition failure (Ayalon, 1978; Diskin and Morris, 2008). Early embryonic loss is generally accepted to account for 20 to 40% of pregnancy failure (Sreenan and Diskin, 1986; Inskeep and Dailey, 2005; Santos *et al.*, 2004, 2009). During the late embryonic period, through day 44, growth can be characterized by the development of limb buds, eye orbits and the formation of placentomes. Although late embryo mortality accounts for less than 10% of pregnancy loss, it has significant implications for the producer and has been suggested to cause greater financial burden then early EM (Diskin and Morris, 2008). By day 45, the conceptus takes the true form of a fetus with split hooves, ribs and displays limited movement (Curran *et al.*, 1986). After days 45 to 60 pregnancy loss decreases and is less than 5% through the second and third trimester of pregnancy.

Pregnancy failure is extremely costly to the producer. Lost revenue can be attributed to cost of feeding and managing nonpregnant cows, decreased weight of late born calves at sale time and a decreased calving percentage due to cows that lost pregnancy. In a study involving lactating dairy cows, pregnancy loss after 1 month of gestation cost producers an average of $555 (US) due to repeat breeding expenses, increased calving interval and increased probability of involuntary culling (De Vries, 2006). In beef cattle that have had pregnancy loss and manage to become pregnant to a subsequent breeding, there is still a significant amount of lost revenue from reduced weight of late born calves and decreased uniformity of the calf crop. Pregnancy diagnosis is a very important management tool that is underutilized in the United States. According to the USDA's 2008 National Animal Health Monitoring System (NAHMS) Beef survey, only 20% of operations

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utilize pregnancy diagnosis via palpation or ultrasound, a number that has remained virtually unchanged since the 1997 survey (United States Department of Agriculture - USDA, 2010). In comparison, the 2007 NAHMS Dairy survey reported 93% of operations perform pregnancy detection (USDA, 2009). Pregnancy diagnosis can identify open cows, help estimate calving dates, and help producers make culling decisions. This review will describe various methods of pregnancy diagnosis in cattle and research advancements that may allow for earlier detection of pregnancy.

Established methods of pregnancy diagnosis

Rectal palpation and ultrasonography

As the conceptus develops during gestation, fluid accumulates, and placentation advances, methods of pregnancy detection allow for manual transrectal palpation of the uterus per rectum and its contents. Transrectal palpation of the uterus, starting as early as day 35 of gestation allows for detection of a pregnancy by palpation of fluid and the amniotic vesicle within the uterus. Palpation of the uterus and its contents is traditionally practiced from 40 to 60 days after insemination with the earliest detection limit being approximately 30 to 35 days post insemination. Additional sensitivity can be achieved during this time point by using transrectal ultrasound for pregnancy detection (Lucy *et al.*, 2011). Ultrasound is the gold standard for determining pregnancy and confirming the presence of a viable embryo. Transrectal ultrasonography can be accurate as early as day 26 to 29 to diagnose pregnancy and visualize a discernable heartbeat (Pierson and Ginther, 1984; Kastelic *et al.*, 1988; Beal *et al.*, 1992). Doppler ultrasound may provide additional information based on visualization of blood flow to the placenta/conceptus; however, data supporting its use as a pregnancy diagnosis method has been mixed. Today, ultrasound is considered the only visual indicator of pregnancy in cattle and is used for comparison with all recent attempts at diagnosing earlier pregnancy in this review. With all palpation and ultrasound techniques, a highly experienced individual is required to complete these test.

Chemical based pregnancy tests

Earlier and more effective means of pregnancy diagnosis are constantly being sought and evaluated. Numerous chemical and biochemical based pregnancy tests have been developed and tested for use in cattle. Each has strengths and shortcomings that have led to their adoption or lack of use in various production schemes. One important consideration in evaluating pregnancy diagnosis tests is the difference between pregnancy specific and non-pregnancy specific methods. Pregnancy specific markers are physiologically present only in pregnant animals and produced specifically from the pregnancy; whereas non-pregnancy specific markers, while elevated during pregnancy, may be produced under other physiological conditions as well.

Progesterone is one example of a non-pregnancy specific diagnosis method. Progesterone is one of the more common chemical based pregnancy tests commercially available although overall a very small percentage of producers use it (USDA, 2009). Produced by the corpus luteum (CL), progesterone is a steroid hormone that is crucial for maintaining pregnancy; however, the cyclic profile of progesterone mandates that pregnancy detection must occur between luteolysis and the formation of a new CL. During this time period, non-pregnant cows should exhibit low progesterone levels, whereas progesterone concentrations in pregnant cows should remain elevated. Significant differences in progesterone concentration appear between pregnant and non-pregnant cows between day 20-24 post insemination in both serum and milk. Accurate positive pregnancy diagnosis varied between 60 and 100% for milk progesterone, however detection of non-pregnant animals varied between 81 to 100% (Nebel *et al.*, 1987; Sasser, 1987, Nebel, 1988). Longer luteal phases in some cows, cysts or persistent follicles may play a role in elevated progesterone concentrations during the pregnancy test period that yield false positive results (Pohler *et al.*, 2015a). Some discrepancies exist in evaluating the effectiveness of progesterone testing with regards to embryo loss. Research has shown that pregnant cows with progesterone concentration below 3.76 ng/ml at week 5 were more likely to experience embryonic mortality before week 9 than cows with a greater concentration of progesterone (Starbuck *et al.*, 2004). However, it should be noted that a majority of cows (77%) in the low concentration group maintained pregnancy through week 9. A later study by Pohler *et al.* (2013), demonstrated that serum concentration of progesterone between day 28 to 30 in pregnant cows was not predictive of pregnancy loss between a positive pregnancy diagnosis by progesterone and final pregnancy confirmation at day 70 of gestation.

Another steroid hormone, estrone sulfate is produced by the conceptus and can be detected at day 100 of pregnancy in cattle (Holdsworth *et al.*, 1982). Estrone sulfate is a pregnancy specific marker, though its late period of detection limits its use in domestic cattle. Although steroid hormone pregnancy tests have largely been replaced in cattle, sheep and swine, use of estrone sulfate has been used for pregnancy detection of non-domestic animals in the wild and zoos through noninvasive fecal and urine samples. Estrone sulfate has been successfully evaluated for use in pregnancy detection in hoofed stock, gorillas, orangutans, baboons and wild felids (Kumar *et al.*, 2013).

Early pregnancy factor (EPF), also known as early conception factor, appears in maternal circulation shortly after fertilization. In cattle, EPF is observed within 48 h after breeding and seems to contribute to maternal immune suppression and implantation preparation (Morton, 1998; Cordoba *et al.*, 2001). A study by Athanasas-Platsis, *et al.* (1989) demonstrated a critical role of EPF when mice that had been immunized against EPF had decreased embryo viability and an

increased incidence of pregnancy failure. Action of EPF is not confined to pregnancy as growth factor-like properties have been seen in tumors. A large percentage of embryo loss occurs after the recommended sampling time of commercial EPF assays (48 h to 7 days). Studies have indicated that commercial EPF tests have a sensitivity (or detection of pregnant animals) of 45 to 86% and a specificity (or detection of non-pregnant animals) of 4 to 28.8% (Cordoba *et al.*, 2001; Gandy *et al.*, 2001). In regards to EPF as a pregnancy detection method, commercial tests are unreliable at identifying non-pregnant animals that limits its use in this capacity.

The maternal recognition of pregnancy signal in cattle and other ruminants is interferon tau (IFNT). Interferon tau stimulates CL maintenance through endocrine-like actions, blocks estrogen receptors and paracrine mechanisms on the endometrium prompt IFNT stimulated gene production (Pohler *et al.*, 2015a, Spencer and Hansen, 2015). Due to the difficulty associated with detecting small concentrations of IFNT directly, research has focused on measuring IFNT-stimulated genes (ISGs) that have been upregulated in peripheral mononuclear blood cells (PBL). Conceptus IFNT mRNA concentration peaks in cattle at day 20 post-conception (Han *et al.*, 2006; Spencer and Hansen, 2015). Genes including ISG15, Mx1 and Mx2 are more highly expressed in peripheral blood leukocytes (PBL) of pregnant cows than non-pregnant cows (Han *et al.*, 2006; Gifford *et al.*, 2007). A study by Green, *et al.* (2010), showed that pregnant heifers had greater IFNT-stimulated gene expression than cows. They concluded that a IFNT-stimulated gene based pregnancy test would be possible for heifers at day 18 but not for cows whose response is more limited based on the current sensitivity of available assays (Green *et al.*, 2010). Unfortunately, ISGs are not unique to pregnancy which limits their use as a pregnancy detection tool to identifying non-pregnant animals (Han *et al.*, 2006; Gifford *et al.*, 2007; Pohler *et al.*, 2015a); however, a resynchronization protocol can be implemented in cows that are identified as non-pregnant which improves operation efficiency (Lucy *et al.*, 2011).

MiRNAs: potential biomarkers for pregnancy diagnosis

The search for easily accessible biomarkers of various diseases and physiological states has recently focused on circulating microRNAs (miRNA). Between 18 and 22 nucleotides in length, miRNAs play important roles in regulation of gene expression and have been found in biological fluids ranging from serum and amniotic fluid to urine and milk (Reid *et al.*, 2011; Pohler *et al.*, 2015a). MicroRNAs are released from cells of most tissue types in plasma membrane bound extracellular vesicles (EV), especially exosomes. The packaging of miRNA in EVs or exosomes is important from a detection standpoint as RNA-ases are unable to penetrate and breakdown the miRNA allowing them to be extracted from blood and serum (Reid *et al.*, 2011). Exosomes and EVs play a crucial role in intercellular communication, including promotion of sperm maturation, regulation of immune function, release of miRNA for a wide array of regulatory functions, as well as other roles currently being studied (Raposo and Stoorvogel, 2013). Serum and whole blood have proved an acceptable source of EV-derived miRNA profiles, thus providing a potential blood-borne biomarker candidate for various disease and physiological states (Häusler *et al.*, 2010; Reid *et al.*, 2011). Human based disease research has revealed significant differences in miRNA abundance for many cancers (Lawrie *et al.*, 2008; Häusler *et al.*, 2010), heart disease (Tijsen *et al.*, 2010) and sepsis (Wang *et al.*, 2010). In addition, circulating miRNAs in maternal serum have been observed as potential biomarkers of pregnancy status due to their significant impact on gene expression and regulation (Chim *et al.*, 2008). A study by Gilad *et al.* (2008) identified miRNAs that are increased in abundance in pregnant humans but not in non-pregnant females. This finding led to the rapid expansion of identifying miRNAs that were specific to pregnancy and across various species, although none have been thoroughly explained.

An initial study of pregnancy specific markers in mares identified 7 miRNAs that were only expressed in pregnant mares (Cameron *et al.*, 2011) compared with the non-pregnant controls. In addition, work in the sheep has confirmed the presence of miRNA in uterine lumen fluid in pregnant and cyclic sheep (Burns *et al.*, 2014). These data support the idea for a likely role of miRNA in conceptus-endometrial interactions during the establishment of pregnancy (Burns *et al.*, 2014). In addition, a follow up study to the one described above, provides evidence that EVs are produced from the trophectoderm and uterine epithelia in the pregnant ewe and are involved in intercellular communication (Burns *et al.*, 2016).

Many groups are now looking into miRNAs as biomarkers for pregnancy detection in the cow. There is increasing evidence that pregnancy specific miRNAs exist and may be potential markers for pregnancy diagnosis. In 2015, exosomal miRNAs were reported to be differentially expressed in pregnant versus non-pregnant cows and cows undergoing early embryonic mortality (Pohler *et al.*, 2015b). A recent study by Fiandanese *et al.* (2016) identified a potential miRNA, bta-mir 140, as an early biomarker for pregnancy detection. At day 19, bta-mir 140 was up regulated in all pregnant cows, and at day 13 onwards, it was upregulated in pregnant, non-lactating cows (Fiandanese *et al.*, 2016). Similarly, Ioannidis and Donadeu (2016) identified 6 miRNA (day 16: bta-miR-26a, bta-miR-29c, bta-miR-138, bta-miR-204. Day 24: bta-miR-1249, day 16 & 24: hsa-miR-4532) that were differentially expressed in pregnant heifers. Although refinement is necessary to pinpoint ideal miRNA for pregnancy diagnosis, results indicate that miRNAs have potential as an early pregnancy detection tool. Furthermore, miRNA may provide information to denote embryonic viability. Preliminary data from our laboratory indicate cows that experience embryo mortality compared to cows that have a successful pregnancy have a significantly increased abundance of specific miRNAs at days 17 and 24 of

gestation. Future studies are needed to assess the repeatability of these findings and to determine precise miRNA most applicable for embryo viability analysis.

Developing early pregnancy diagnosis method; pregnancy associated glycoproteins

The placenta

Proper placentation is crucial for pregnancy development and ultimately pregnancy success. Active placentation in the cow occurs between day 28 and 40 of gestation (Aires *et al.*, 2014). Bovine placentation involves adhesion between the maternal-caruncle structures and fetal cotyledonary tissues to form placentomes. Superficial interdigitation begins around day 20 in cattle between microvilli of the trophectoderm and uterine epithelium. True placentomes, marked by increased villi length and raised tissue in caruncular endometrium are distinct by days 31 to 33 of gestation. By day 39, placentomes are easily discernable and have long, occasionally branching villi (King *et al.*, 1979).

Binucleated trophoblast cells (BNCs) appear in the fetal chorion of ruminants at days 18 to 19 of gestation and comprise 15 to 20% of the trophectoderm throughout pregnancy. Binucleated trophoblast cells migrate to the maternal epithelium from the fetal chorion after maturation but do not penetrate past the basement membrane (King and Atkinson, 1987). Contact between the maternal and fetal interface at the microvilli junction allows migration of BNC's towards the basement membrane to begin (Wooding and Wathes, 1980, Wooding and Burton, 2008). Products of BNC's, including hormones, placental lactogen and pregnancy associated glycoproteins (PAGs), are packaged in secretory granules and enter maternal circulation across the basement membrane (Pohler *et al.*, 2015a).

PAG production

Pregnancy associated glycoproteins were identified in the 1980's during early attempts to develop pregnancy-specific markers that could be used for pregnancy diagnosis. Although PAGs are often thought of to be produced by BNCs, Green *et al.* (2000) reported that PAGs can be sorted into two separate families based on their expression in trophoblast cells. Some PAGs are expressed in both BNCs and mononucleated trophoblast cells while others are solely produced in BNCs (Green *et al.*, 2000). Although their physiological role is unknown, a large number of distinct PAGs and more than two dozen specific PAG genes have been described. Based on accumulation of PAGs at the junction between uterus and placenta and known proteolytic activity of certain PAGs, it has been hypothesized that PAGs may help process growth factors or may have adhesion actions (Wallace *et al.*, 2015). Based on evidence that PAGs may inhibit certain immune cells, they may also play a role in disguising antigens from the maternal immune system (Perry *et al.*, 2005). After appearance of BNCs and epithelial adhesion of trophoblast, the first sizable increase in PAG concentration occurs between days 22 to 24 of gestation. Concentrations of PAG continue to increase through day 36, followed by subsequent decrease in concentration until day 60 of pregnancy followed by a steady increase through the second and third trimesters of pregnancy. In the weeks preceding parturition, a substantial increase in circulating concentrations of PAG occurs that peaks at calving. This may be attributed to significant placental growth at the end of gestation or the release of stored PAG from other tissues (Green *et al.*, 2005; Pohler *et al.*, 2013). Eight weeks post parturition, PAGs are not detectable in maternal circulation (Green *et al.*, 2005).

PAGs and pregnancy diagnosis

Since their discovery, PAGs have been a target for pregnancy diagnosis. Pregnancy-specific protein B was the first identified PAG of interest by scientists looking for pregnancy specific markers that could be detected early in gestation in the 1980's (Butler *et al.*, 1982; Sasser *et al.*, 1986). Using early assays, PAGs were detectable at day 24 of gestation in cattle; however, the physiological function was unknown which is still the case today. Discovery of multiple PAG families and genes has contributed to understanding the increasing complexity of PAGs. The radioimmunoassay (RIA) first developed shortly after discovery of pregnancy specific protein B was the standard for PAG detection for many years (Zoli *et al.*, 1992). This assay was highly specific and the validating study concluded that PAGs were secreted into the maternal system and were unique to pregnant animals. A study by Green *et al*. (2005) validated an ELISA that specifically targeted PAGs secreted early in gestation that had a shorter half-life (4.3 days *vs*. 8.4 days) than the previous targets to reduce the potential for false positives in postpartum cows (Zoli *et al.*, 1992; Sousa *et al.*, 2003; Green *et al.*, 2005). The ELISA was demonstrated to accurately detect pregnant cows via serum concentrations of PAGs at day 28 post insemination. Studies comparing the efficacy of the PAG ELISA, PAG RIA and transrectal ultrasonography revealed comparable results for the diagnosis of pregnancy in cattle at day 28 of gestation although some differences were identified in the ability of certain assays to detect non-pregnant animals (Szenci *et al.*, 1998; Karen *et al.*, 2015).

Commercial PAG tests are currently available using both milk and blood samples, and include BioPRYN (BioTracking LLC. Moscow, ID USA), IDEXX Bovine pregnancy test (IDEXX Laboratories Inc. Westbrook, ME USA) and DG29 pregnancy test (Genex Cooperative Inc. Shawano, WI USA). BioPRYN accepts blood samples from heifers 25 days post breeding and cows 28 days post breeding, IDEXX recommends day 28 blood or milk samples and DG29 has been validated using day 29 blood samples. At the recommended sampling time, all commercial tests provide 98-99% true positive (pregnant) reading and false positive (reported as pregnant but open) rates range from 1-5% however, some variation may be due to late embryonic mortality.

Early pregnancy diagnosis

Current research is focused on finding markers and increasing sensitivity to identify pregnant cows before day 28 of gestation. Although diagnosis is limited to the time frame following the introduction of PAGs to maternal circulation at days 19 to 20, preliminary research indicates that PAGs may be effective at diagnosing pregnancy as early as day 24. Currently, research is focused on heifers, which exhibit greater PAG concentrations earlier in gestation compared with cows. A recent study in Brazil evaluated the accuracy of pregnancy diagnosis at day 24 in predominantly Holstein heifers following timed embryo transfer (Reese *et al.*, 2016). Serum PAG concentrations at day 24 differed between pregnant (2.98 ng/ml) and non-pregnant (0.69 ng/ml) heifers. Using receiver operating curve analysis, PAG concentrations greater than 1.39 ng/ml were 95% accurate in diagnosing a pregnant heifer at day 24 of gestation. Using a day 17 baseline sample, the difference between day 24 and day 17 samples predicted 79% of pregnancies. This, early pregnancy diagnosis using PAG is possible; however, more work is needed in this area. It is realistic to assume that day 24 PAG concentrations can diagnosis pregnancy, but high rates embryonic loss before the standard day 30 tests may decrease the efficiency and benefits of testing early. In a recent study, embryonic mortality between day 24 and 31 of gestation was 20.8% in lactating dairy cows, thus pregnancy loss following maternal recognition of pregnancy may be more prevalent than previously thought making early pregnancy diagnosis less useful (Pohler *et al.*, 2016a).

PAGs as indicators of embryo success

Recent studies have demonstrated a strong correlation between successful pregnancies and PAG concentrations during early gestation. Increased circulating PAG concentrations approximately day 28 of gestation are generally predictive of increased embryo survival, making PAG a likely marker for evaluating embryo viability and placental competence. In comparison to progesterone, which exhibit no difference between heifers or cows that undergo embryo mortality and those that maintain pregnancy, PAG concentrations are significantly different between heifers and cows (Fig. 1; Kill *et al.*, 2013). Serum PAG concentrations in cows that maintained pregnancy (4.53 ± 0.34 ng/ml) were significantly higher than cows that underwent pregnancy loss (3.14 ± 0.72 ng/ml) after fetal heartbeat detection at day 28 (Pohler *et al.*, 2013). All cows had a pregnancy with a fetal heartbeat at day 28, indicating a viable pregnancy at that time. Perhaps more importantly, PAGs seem to be particularly effective at identifying cows that will undergo late embryonic or early fetal mortality. As serum concentrations increased, the probability of embryo mortality significantly decreased. Late embryo mortality between days 31 and 59 was predicted with 95% accuracy if PAG concentrations were <1.4 ng/ml at day 31 after timed artificial insemination (Pohler *et al.*, 2016a). Pohler *et al.* (2016b) demonstrated that both *Bos indicus* and *Bos taurus* cows that experienced embryo mortality had similar and lower PAG concentration at day 28 despite the significant differences between PAG concentrations of successful pregnancy in B*os taurus* compared with B*os indicus* cows (Fig. 2).

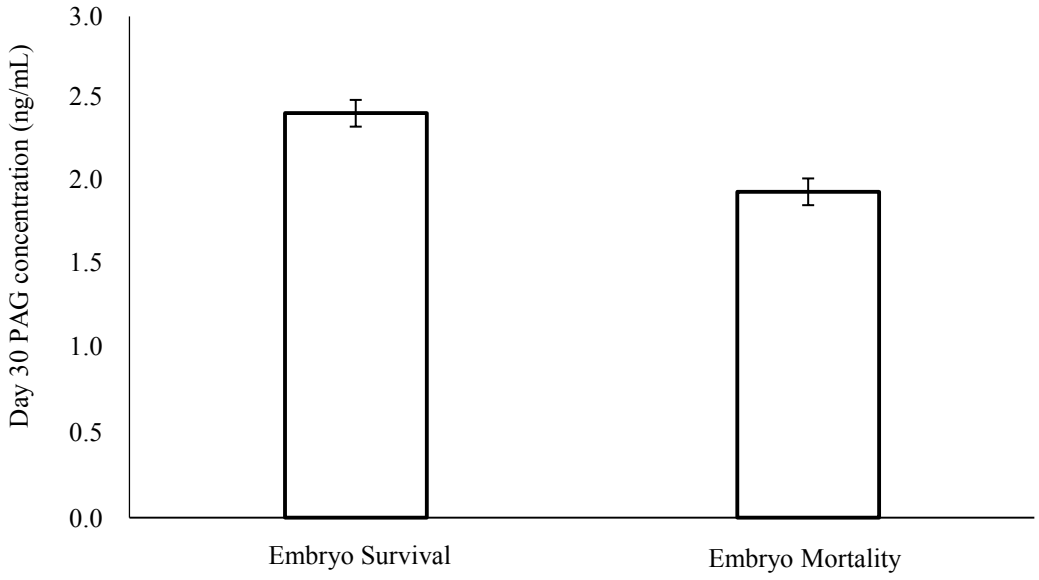

Figure 1. Serum concentrations of pregnancy-associated glycoproteins (PAGs) in heifers that had a embryonic heartbeat on day 30 and maintained pregnancy (Embryo survival; n = 406) or did not maintain pregnancy (Embryo mortality; n = 21). Heifers that had embryo mortality between gestation days 30 and 65 had significantly less PAG serum concentrations than heifers that maintained pregnancy.

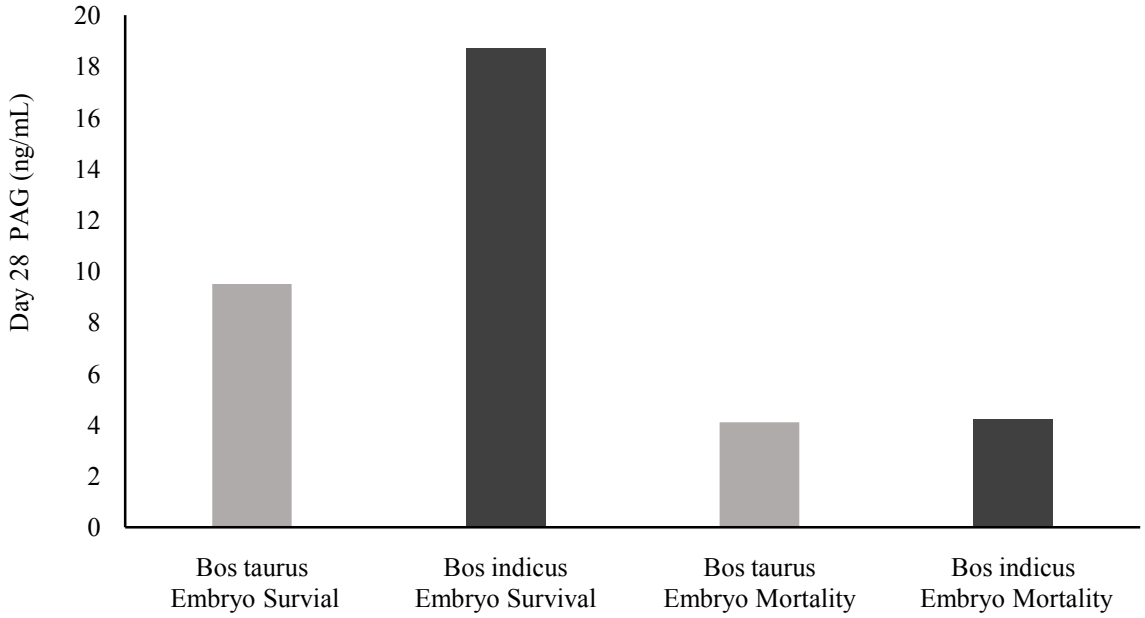

Figure 2. Serum concentrations of pregnancy-associated glycoproteins (PAGs) in samples collected on day 28 of gestation from pregnant *Bos taurus* cows and *Bos indicus* cows with a viable embryo based on fetal heartbeat. Cows were then categorized into whether they maintained pregnancy until day 72 (*Bos Taurus;* n = 1416) or day 100 (*Bos indicus;* n = 1365) of gestation (Embryonic survival) or embryonic mortality (between days 29 to 72 or 100; *Bos Taurus;* n = 171; *Bos indicus;* n = 213). Beef cows that experienced late embryonic mortality had decreased ($P < 0.05$) circulating concentrations of PAGs on day 28 compared to cows that maintained an embryo.

A multitude of factors have may affect circulating PAG concentrations including subspecies, parity and sire. Despite a correlation between embryonic size and placental size, results indicate no significant relationship between PAG concentration and embryo size during early gestation (Pohler *et al.*, 2014). Lack of correlation indicates that the decrease in PAG concentration in cows experiencing late embryonic mortality is indicative of impaired placental or endometrial function, not slow embryonic growth. In addition, *Bos indicus* cattle tend to have greater circulating PAG concentrations compared to *Bos taurus* cattle (Pohler *et al.*, 2016b). Any *Bos indicus* influence in the genetic base will increase PAG concentrations over a straightbred *Bos taurus* cow (Mercadante *et al.*, 2013). Others have reported that profiles of circulating PAG are similar between subspecies although *Bos indicus* cows may have a smaller relative increase in the weeks preceding parturition (Sousa *et al.*, 2003). High producing dairy cows exhibit a significant negative correlation between milk production and PAG concentration. In a study by Lopez-Gatius *et al.* (2007), each 1 kg increase in milk resulted in a decrease in PAG of 0.08 to 0.1 ng/ml. Perhaps even more interesting are the effects of parity and sire on PAG concentrations. Heifers have consistently been reported to have the greatest PAG concentration and as parity increases, mean PAG concentration subsequently decreased in a somewhat linear fashion until the 3 or 4th parity (Lobago *et al.*, 2009; Ricci *et al.*, 2015; Pohler *et al.*, 2016b). Recent work has examined sire differences on PAG concentration due to the paternal influence over trophoblast and placental development. Preliminary evidence suggests that pregnancies produced by bulls accounting for decreased rates of late EM may exhibit increased PAG concentrations compared to bulls that result in increased rates of late EM (Pohler *et al.*, 2016b).

Estrus expression at the time of insemination or prior to embryo transfer has been directly correlated with pregnancy success in both beef and dairy cattle (Perry *et al.*, 2005; Pereira *et al.*, 2016). In a recent study (Pereira *et al.*, 2016), lactating dairy cows undergoing TAI or embryo transfer had increased fertility and decreased embryonic morality if they exhibited estrus versus those that did not exhibit estrus. Furthermore, lactating dairy cows with pregnancy loss had decreased circulating concentrations of PAGs early in gestation (Pohler *et al.*, 2016a), similar to the current study. In a study with postpartum beef cows, there was an increase in PAG concentrations on day 28 of gestation when comparing estrotech patch scores at TAI (day 0). Surprisingly, previous work has not demonstrated an association with pre/postovulatory estradiol or progesterone production with PAG production early in gestation (Pohler *et al.*, 2013). Thus, these data indicate that cows which exhibit estrus and conceive have increased circulating concentrations of PAGs on d 28 and increased likelihood of pregnancy success compared with pregnant cows that did not express estrus at TAI. Future experiments are needed in this area to truly understand this relationship and potential mechanism that is underlying this increase in PAG production.

Conclusions

Although numerous detection methods can

accurately diagnosis pregnancy in cattle, PAGs and pregnancy specific miRNA are biomarkers that can be used before day 30 of gestation and may be useful in predicting embryonic mortality. Although pregnancy diagnosis may be possible earlier in gestation, benefits may be mitigated by high incidence of embryonic mortality after day 24.

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Effect of preovulatory follicle maturity on pregnancy establishment in cattle: the role of oocyte competence and the maternal environment

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Abstract

Reproductive technologies to synchronize estrus and ovulation in cattle have enhanced the ability to practically utilize artificial insemination to increase both genetic merit and reproductive management of beef and dairy herds. The ability to successfully synchronize a follicular wave and ovulation, in heifers and cows, has improved substantially in recent years. Consequently, pregnancy rates to a single fixed-time artificial insemination (FTAI) can approximate that of insemination following spontaneous estrus. Despite these advances, a subset of heifers and cows often has a physiologically immature dominant follicle at the time of GnRH-induced ovulation. These animals will exhibit reduced pregnancy rates and decreased embryonic survival if a pregnancy happens to become established. The physiological mechanisms underlying the preceding decreased fertility have been a focus of our laboratories and may include an effect of the follicular microenvironment on both oocyte competence and the maternal environment. Oocytes must have adequate opportunity to complete cytoplasmic and molecular maturation during the final stages of oocyte maturation that occur within the preovulatory follicle. Follicular status, during the proestrus period, must be such that adequate circulating concentrations of estradiol are present before FTAI to increase oviductal transport of gametes and enhance both the luteinizing capacity of granulosa cells and progesterone receptor population in the post-ovulatory uterus. Following ovulation, the follicle's transformation to a functional corpus luteum to secrete adequate amounts of progesterone is essential for the establishment of pregnancy. The physiological status of the preovulatory follicle, prior to FTAI, greatly affects the concepts discussed above and has an important impact on pregnancy establishment and maintenance in cattle.

Keywords: bovine, follicle, oocyte, pregnancy, synchronization of ovulation.

Introduction

Synchronization of estrus/ovulation and artificial insemination (AI) are powerful techniques for both genetic improvement and reproductive management in beef cattle (Seidel, 1995). However, the time and labor associated with the detection of estrus has been a deterrent to the adoption of AI in beef herds. Therefore, significant effort has been directed toward development of fixed-time AI (FTAI) protocols that allow heifers and cows to be inseminated at a predetermined time and achieve pregnancy rates that are similar to those following the detection of estrus and AI. Furthermore, FTAI protocols increase the proportion of heifers and cows that conceive early in the breeding season, which has important benefits for reproductive management and beef production. Significant progress has been made toward developing FTAI protocols that precisely control the time of ovulation. Consequently, increased effort has been directed toward understanding the ovarian, uterine, and embryonic mechanisms controlling the establishment and maintenance of pregnancy (see reviews by Pohler *et al.*, 2012; Bridges *et al.*, 2013; Geary *et al.*, 2013), with the purpose of developing strategies for increasing the pregnancy rate to a single insemination. The purpose of this paper is to review the effect of ovulatory follicle size, at the time of FTAI, on pregnancy rates and late embryonic/fetal survival, to discuss why physiologically immature follicles may be present at FTAI, and to discuss mechanisms by which the physiological maturity of a dominant follicle may affect the establishment and maintenance of pregnancy in beef cattle.

Overview of synchronization of ovulation

Ovarian mechanisms controlling the expression of estrus, ovulation of a competent oocyte, and establishment of an oviductal/uterine environment conducive to embryonic development is likely optimized when a female expresses estrus and ovulates spontaneously. However, when the preceding events are artificially manipulated with FTAI protocols, pregnancy rates can be reduced. Cattle have recurrent follicular waves, beginning prior to puberty and continuing until late gestation, and the development of FTAI protocols require both synchronization of follicular waves and the induction of luteolysis. Consequently, FTAI protocols for cattle frequently involve the following physiological sequence: 1) Turnover of a dominant follicle to initiate a new follicular wave. This is accomplished by administration of exogenous gonadotropin releasing hormone (GnRH; e.g. USA) or estradiol in the presence of progesterone (e.g. Brazil) to induce ovulation or dominant follicle turnover, respectively (see reviews by Bó *et al.*, 1995; Diskin *et al.*, 2002), 2) Induction of luteolysis, five to seven days later, by administration of prostaglandin F2α (PGF), and 3) Administration of

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estradiol or GnRH to induce ovulation following insemination. Essentially all FTAI protocols in the USA are variations of the preceding GnRH-PGF-GnRH injection sequence with some differences in timing of insemination and many protocols include a progestin between the first GnRH and PGF injections to better control estrus expression. For FTAI protocols, the timing of insemination is scheduled to result in an overlap between the period of oocyte viability following ovulation and availability of capacitated sperm in the ampulla of the oviduct. However, at the time of FTAI, there is a mixed population of heifers or cows that have or have not expressed estrus. Animals that have not expressed estrus by the time of FTAI require an injection of GnRH or estradiol to induce a preovulatory gonadotropin surge and ovulation so that all animals can be inseminated at the same time. Females that exhibit estrus prior to or at the time of FTAI normally have a spontaneous gonadotropin surge and experience higher pregnancy rates compared to those that fail to exhibit estrus (Perry *et al.*, 2005; Larson *et al.*, 2006). Therefore, a challenge with FTAI is to manipulate the estrous cycle or the induction of ovulation such that the follicular microenvironment is optimal for acquisition of oocyte competence and programming the maternal environment for the establishment and maintenance of pregnancy.

Effect of ovulatory follicle size on pregnancy in beef heifers and cows

In *Bos taurus* and *Bos indicus* cattle, antral follicles acquire the ability to ovulate in response to an endogenous or exogenous preovulatory gonadotropin surge at 7 or 10 mm in diameter, respectively, which is associated with the time of follicular divergence between the newly selected dominant follicle and subordinant follicles (Sartori *et al.*, 2001; Gimenes *et al.*, 2008). This time frame corresponds to acquisition of LH receptors in bovine granulosa cells by the selected follicle (see review by Lucy, 2007). However, a larger dose of LH was required to induce ovulation in a 10 mm follicle versus larger sized follicles (Sartori *et al.*, 2001), suggesting a difference in the physiological maturity of small versus large dominant follicles.

When ovulation is induced, the size or physiological maturity of the preovulatory follicle influenced pregnancy rate and late embryonic survival in beef and dairy cattle (Lamb *et al.*, 2001; Vasconcelos *et al.*, 2001; Perry *et al.*, 2005, 2007; Waldmann *et al.*, 2006; Dias *et al.*, 2009; Meneghetti *et al.*, 2009; Sá Filho *et al.*, 2009). In a study from our laboratory, postpartum beef cows induced to ovulate small dominant follicles (less than 11.3 mm in diameter) experienced lower pregnancy rates and higher incidences of late embryonic mortality than did those induced to ovulate large (greater than 11.3 mm in diameter) dominant follicles. Interestingly, ovulatory follicle size did not affect pregnancy establishment or maintenance when animals exhibited estrus and underwent spontaneous ovulation (Perry *et al.*, 2005). This led to the hypothesis that the physiological maturity, rather than the diameter, of a preovulatory follicle affects the establishment and maintenance of pregnancy (Perry *et al.*, 2005; Atkins *et al.*, 2013).

Why do heifers and cows have small dominant follicles at fixed-time insemination?

Our laboratories have utilized the CO-Synch FTAI protocol (GnRH-1 seven days before PGF, and GnRH-2 at FTAI 48 h after PGF; Geary *et al.*, 1998) to examine the effect of ovulatory follicle size on pregnancy establishment in beef heifers and postpartum cows (Perry *et al.*, 2005, 2007; Atkins *et al.*, 2013).Although this protocol has been modified for current use in the industry, we have continued to use it since it results in significant variation in dominant follicle size at GnRH-2. Approximately 40 to 50% of heifers (Atkins *et al.*, 2008) and 66% of postpartum beef cows (Geary *et al.*, 2000) have a dominant follicle capable of responding to GnRH-1. It is logical that small dominant follicles present at the time of GnRH-2 (FTAI) could result from failure to ovulate a dominant follicle and initiate a new follicular wave following GnRH-1 administration. Consequently, at GnRH-2 there will be heifers and cows that have and do not have a synchronized follicular wave. We hypothesized that cows that do not have a synchronized wave at GnRH-2 may have a small dominant follicle at GnRH-2. Alternatively, a slower growth rate of the dominant follicle could result in a small dominant follicle at GnRH-2. To test the preceding hypothesis we administered GnRH-1 to beef heifers, cycling postpartum cows, and anestrous postpartum cows at times when they would or would not have a follicle capable of ovulating to the induced gonadotropin surge (Atkins *et al.*, 2008, 2010a, b). Administration of GnRH-1 occurred on days 2, 5, 10, 15 and 18 or 2, 5, 9, 13, and 18 after estrus (day 0) in cycling heifers and postpartum cows, respectively. In beef heifers, day of the cycle at GnRH-1, but not ovulatory response to GnRH-1 had an effect on dominant follicle size at GnRH-2. Heifers receiving GnRH-1 in the latter part of the cycle (i.e. days 15 and 18) had a greater incidence of spontaneous luteolysis before PGF administration and earlier onset of estrus regardless of the presence of an accessory corpus luteum after GnRH-1, which resulted in smaller follicles at GnRH-2. Consequently, a strategy to reduce the presence of small, physiologically immature follicles at GnRH-2 in heifers may be to pre-synchronize their follicular development, such that follicles are in an earlier stage of the estrous cycle (≤day 10) at GnRH-1. In cycling cows, the day of the cycle at GnRH-1 did not affect dominant follicle size or the proportion of cows ovulating at GnRH-1. However, in both the cycling and anestrous groups, cows that ovulated in response to GnRH-1 had a larger follicle at GnRH-2 than cows that did not ovulate. In summary, induction of ovulation at GnRH-1 increased preovulatory follicle size at GnRH-2 in postpartum cows but not heifers.

Follicular determinants of pregnancy establishment in beef cattle

The decrease in pregnancy rate and late embryonic/fetal survival (days 28 to 70 post breeding) following GnRH-induced ovulation of physiologically immature follicles is likely due to a combination of decreased oocyte competence and (or) an inadequate preparation of the maternal environment for pregnancy establishment. Atkins *et al.* (2013) performed a reciprocal embryo transfer experiment to distinguish between effects of the follicular microenvironment on oocyte competence *vs*. the maternal environment. Single GnRH-induced ovulations were synchronized in recipient and donor postpartum beef cows. Animals were classified into large (≥12.5 mm) and small follicle (<12.5 mm) groups at GnRH-induced ovulation, and none of the animals were detected in estrus. Donor animals were inseminated, and embryos or unfertilized oocytes were recovered seven days later. Viable embryos from donors with small or large follicles were transferred into recipients with small or large follicles to differentiate between effects of the follicular microenvironment on oocyte competence and(or) the uterine environment. Evidence of inadequate oocyte competence and a compromised uterine environment in females induced to ovulate a small compared to a large ovulatory follicle was reported and is discussed in more detail below.

Oocyte determinants of fertility

Oocyte competence is defined as the oocyte's ability to resume meiosis, cleave after fertilization, develop to the blastocyst stage, and bring to term a successful pregnancy (Sirard *et al.*, 2006). Developmental competence is acquired throughout oocyte and follicular growth as the oocyte progresses through meiotic, cytoplasmic, and molecular maturation. During the period of oocyte growth, the bovine oocyte increases in size from an intra-zonal diameter of less than 30 µm in primordial follicles to greater than 120 µm in tertiary follicles (Hyttel *et al.*, 1997). Bovine oocyte competence has been examined by evaluating fertilization rate, cleavage rate, proportion of embryos that reach the blastocyst stage, as well as embryo quality (Otoi *et al.*, 1997; Hendricksen *et al.*, 2000; Atkins *et al.*, 2013) with increased oocyte competence observed in oocytes of larger size (Otoi *et al.*, 1997) and originating from larger follicles (Arlotto *et al.*, 1996; Hendricksen *et al.*, 2000; Atkins *et al.*, 2013).

Acquisition of oocyte competence can be divided into three major events: 1) Acquisition of the ability to undergo meiotic maturation, 2) Acquisition of cytoplasmic maturation, and 3) Accumulation and storage of mRNA transcripts and proteins (i.e. molecular maturation). In fetal life, DNA synthesis doubles the chromatin content in the oocyte. The chromatin enters the diplotene stage of meiosis I and is arrested in a state of intermediate chromatin condensation, which allows for transcription of mRNA that can be stored within the oocyte for weeks due to polyadenylation of the 3' untranslated region (Sirard, 2001). Oocytes remain in diplotene arrest until they are either removed from their surrounding follicular cells or exposed to the preovulatory gonadotropin surge. As the oocyte gains meiotic competence, it acquires the ability to be released from meiotic diplotene arrest, fully condense its chromatin, expel a polar body, and progress to metaphase II (MII). It is commonly accepted that actively growing oocytes are meiotically incompetent, and acquisition of meiotic competence is a progression that takes place as the oocyte grows (Sirard, 2001). At an intrazonal diameter of 100 µm, the bovine oocyte acquires the ability to resume meiosis, but full meiotic competence to reach MII is not acquired until the oocyte reaches a diameter of 110 µm, which is normally contained in a 3 mm bovine follicle (Hyttel *et al.*, 1997).

While oocytes from bovine follicles greater than 3 mm may be competent to resume meiosis, they must progress through cytoplasmic maturation or oocyte capacitation to attain full developmental competence. Early changes in the oocyte's ultrastructure occurred at the secondary stage of follicular development as the zona pellucida and cortical granules were synthesized (Sirard, 2001). However, few changes in oocyte ultrastructure were observed from this point until the follicle reached a size of 8 to 9 mm (Hendrickson *et al.*, 2000). As the follicle progressed to ovulatory size, morphological changes in the mitochondria, ribosomes, endoplasmic reticulum, Golgi complex, and cortical granules occurred as the oocyte transitioned from the germinal vesicle (GV) to MII stage (reviewed by Ferreira *et al.*, 2009). The preceding reorganization of organelles is presumably regulated by cytoskeletal microfilaments and microtubules and is essential to oocyte viability (e.g. providing ATP to the nucleus for meiotic maturation and fertilization, proper translation of proteins, and the production of a calcium gradient and cortical granule release to block polyspermy; reviewed by Ferreira *et al.*, 2009).

In cattle, transcripts produced and stored by the oocyte are essential for subsequent oocyte maturation and early embryonic development up to activation of the embryonic genome (reviewed by Sirard *et al.*, 2006). Molecular maturation refers to the transcription of the mRNA blueprint (i.e. transcriptome) as well as storage of transcripts through the incorporation and extension of a 3' poly(A) tail (Brevini-Gandolfi *et al.*, 1999). Maternal mRNAs are rapidly transcribed and stored beginning at the secondary follicle stage (Fair *et al.*, 1997) and throughout the period of rapid oocyte growth up to the 3 mm follicular size (Fair *et al.*, 1995). Past this point, transcriptional activity continued, at a lower rate, until condensation of the chromosomes following germinal vesicle breakdown (GVBD; Fair *et al.*, 1995; Mourot *et al.*, 2006; Mamo *et al.*, 2011).

Molecular maturation of the bovine oocyte is also influenced by the surrounding follicular cells where the innermost layer of cumulus cells, the corona radiata, possesses cellular projections (i.e. transzonal projections) that penetrate the zona pellucida and

directly contact the oolemma (Macaulay *et al.*, 2014). Although it is well known that small molecules (e.g. cAMP) can be delivered from cumulus cells to the oocyte, via transzonal processes, transport of mRNA to the oocyte has recently been reported and transported transcripts were observed to increase as the oocyte progressed from metaphase I (MI) to MII and to be associated with polyribosomes (Macaulay *et al.*, 2014, 2016). Transport of mRNAs is reportedly terminated upon exposure to the gonadotropin surge and subsequent breakdown of transzonal projections (Macaulay *et al.*, 2014).

Induced ovulation of small preovulatory follicles, in cows that have not expressed estrus, may negatively impact acquisition of oocyte competence. While meiotic competence is mostly complete by the time a bovine follicle reaches 3 mm, inadequate cytoplasmic and(or) molecular maturation could compromise oocyte competence in small preovulatory follicles at GnRH-induced ovulation. An inadequate transcriptome may be observed in oocytes from small preovulatory follicles, which are induced to ovulate prematurely, since transcription ends at GVBD and does not resume until activation of the embryonic genome. Analysis of the transcriptome of bovine oocytes from dominant follicles of postpartum beef cows that differed in size (smaller than 11.7 mm versus larger than 12.5 mm) or physiological status (estrous expression versus no estrous expression) revealed a list of differentially abundant transcripts that could regulate pathways associated with acquisition of oocyte competence (Dickinson, 2016).

Endocrine requirements for the establishment of pregnancy

Protocols for precisely synchronizing ovulation in beef and dairy cows have been developed and are widely employed by the industry (Binelli *et al.*, 2014; Bó and Baruselli, 2014; Colazo and Mapletoft, 2014). The next challenge in protocol development is to further increase the pregnancy rate following FTAI. Accomplishing this goal will require an increased understanding of the endocrine and physiological mechanisms controlling acquisition of oocyte competence, ovulation, fertilization, gamete transport, early embryonic development, maternal recognition of pregnancy, and placentation. Binelli *et al*. (2014) identified three biological principles of FTAI protocols that govern pregnancy success: 1) Regulation of circulating concentrations of progesterone to increase oocyte competence and efficacy of PGF-induced luteolysis prior to FTAI, 2) Adequate estradiol priming during proestrus, and 3) Adequate progesterone priming during the early luteal phase. In postpartum beef cows, GnRH-induced ovulation of small dominant follicles resulted in decreased circulating concentrations of estradiol at FTAI and decreased postovulatory concentrations of progesterone (Perry *et al.*, 2005; Busch *et al.*, 2008; Atkins *et al.*, 2010a, b, 2013). These concepts are discussed in more detail below.

Role of proestrus and preovulatory estradiol

Proestrus includes the period from luteolysis to the onset of estrus and is characterized by increased pulsatile secretion of LH, increased circulating concentrations of estradiol, estrogenic changes in the reproductive tract (e.g. cervix, uterus, and oviduct), and preovulatory follicular growth and maturation. Pregnancy rates following FTAI were positively associated with length of proestrus in beef (Mussard *et al.*, 2007; Bridges *et al.*, 2008, 2010; Geary *et al.*, 2013) and dairy (Santos *et al.*, 2010) cattle. Ovulation synchronization protocols that increase length of proestrus influence the follicular and uterine steroid environment by increasing serum concentrations of estradiol at estrus and progesterone during the subsequent luteal phase. Increased serum concentrations of estradiol at FTAI were associated with increased pregnancy rates (Jinks *et al.*, 2013). Therefore, the effects of increased proestrus on pregnancy rates were more likely an effect of increased estradiol rather than a function of follicular age (Bridges *et al.*, 2008).

Increased pregnancy rates associated with increased circulating estradiol at FTAI may be due to a direct effect of estradiol on the cumulus-oocyte complex, oviduct and uterine environment, and(or) an indirect effect on gamete transport. The bovine oocyte and surrounding cumulus cells contain estradiol receptor mRNA (Driancourt *et al.*, 1998; Beker-van Woudenberg *et al.*, 2004) and oocytes from preovulatory bovine follicles that had increased intrafollicular concentrations of estradiol were more likely to develop into blastocysts (Mermillod *et al.*, 1999). However, addition of estradiol to in vitro maturation media had either no effect or a negative effect on nuclear maturation of bovine oocytes (Beker-van Woudenburg *et al.*, 2004, 2006). Interestingly, treatment of beef cows with estradiol cypionate, during the preovulatory period, increased pregnancy rates in cows following GnRH-induced ovulation of small, but not large ovulatory follicles (Jinks *et al.*, 2013). Circulating concentrations of estradiol may affect the establishment and maintenance of pregnancy in a manner that is independent of oocyte competence. For example, increased follicular secretion of estradiol may increase pregnancy rates through modulating uterine pH (Perry and Perry, 2008a, b), by altering sperm transport and longevity (Allison and Robinson, 1972; Hawk, 1983), by inducing oviductal secretions (e.g. oviductal glycoprotein; reviewed by Buhi, 2002), by modulating progesterone action via induction of progesterone receptors in the uterus (Stone *et al.*, 1978; Zelinski *et al.*, 1982; Ing and Tornesi, 1997), and(or) by increasing luteal progesterone secretion. Madsen *et al*. (2015) demonstrated the necessity of preovulatory estradiol on embryo survival and placental attachment in beef cows using an ovariectomized cow model. In regards to the latter effect of estradiol, Atkins *et al*. (2013) reported that circulating concentration of estradiol at FTAI (day 0) was positively associated with serum concentrations of progesterone on day 7 and independent of ovulatory

follicle size. The ability of luteinized human granulosa cells to secrete progesterone increased when the cells were collected from follicles having increased follicular fluid concentrations of estradiol (McNatty, 1979). In addition, ewes treated with an aromatase inhibitor prior to induced ovulation had a delayed rise in serum progesterone (Benoit *et al.*, 1992). Consequently, estradiol may have a role in preparing follicular cells to luteinize.

Role of postovulatory progesterone

The preovulatory gonadotropin surge induces luteinization and transformation of the ovulatory follicle into a corpus luteum, which serves as the primary source of progesterone during the establishment and maintenance of pregnancy in cattle (Smith *et al.*, 1994). Luteal development is a continuation of follicular maturation; consequently, an inadequate follicular microenvironment (e.g. decreased gonadotropin stimulation and[or] estradiol production) may impair subsequent luteal function (Garverick and Smith, 1986). In beef heifers and postpartum beef cows, GnRH-induced ovulation of small dominant follicles was associated with decreased postovulatory concentrations of progesterone (Perry *et al.*, 2005; Atkins *et al.*, 2008, 2010a, b) and decreased pregnancy rates in postpartum beef cows (Atkins *et al.*, 2013). Potential mechanisms by which decreased circulating concentrations of progesterone, during the early luteal phase, might result in decreased pregnancy rates are discussed below.

In ruminants, the early conceptus relies on progesterone-stimulated production of growth factors and uterine secretions collectively known as histotroph for nourishment (Geisert *et al.*, 1992; Spencer and Bazer, 2002). Ovarian steroids can have an indirect effect on uterine function through estradiol induction of uterine progesterone receptors (Zelinski *et al.*, 1982; Ing and Tornesi, 1997) and progesterone effects on histotroph production (Garrett *et al.*, 1988). Alternatively, progesterone may also have a direct effect since the bovine embryo possesses progesterone receptor mRNA (Clemente *et al.*, 2009) and may respond directly to progesterone supplementation in culture (inconsistencies reviewed by Lonergan, 2009).

Beginning on day 9 after GnRH-induced ovulation and FTAI, circulating concentrations of progesterone were greater in pregnant versus nonpregnant postpartum beef cows (Perry *et al.*, 2005). A delayed rise in circulating progesterone may compromise pregnancy establishment due to decreased embryonic size and production of interferon-tau (IFN-tau). Production of IFN-τ from the trophoblast on approximately days 14 to 20 is an essential signaling mechanism for maternal recognition of pregnancy and IFN-tau has been shown to reduce pulsatile uterine PGF secretion by blocking expression of endometrial oxytocin receptors (reviewed by Spencer *et al.*, 2007). A delayed rise in progesterone, following ovulation, was associated with lower rates of bovine embryonic development and reduced IFN-tau production by day 16 embryos (Mann and Lamming, 2001). In summary, an adequate increase in the postovulation concentration of progesterone is necessary for pregnancy establishment and maintenance in cattle.

Conclusion

Ovulation of a competent oocyte, as well as adequate preovulatory secretion of estradiol and postovulatory secretion of progesterone are essential for the establishment and maintenance of pregnancy. When ovulation was induced with GnRH in postpartum cows not detected in estrus, positive associations among ovulatory follicle size, circulating concentrations of preovulatory estradiol, fertilization rates, embryo quality, circulating concentrations of progesterone during the postovulatory period, and pregnancy rate have been reported (Atkins *et al.*, 2013). In the preceding study, preovulatory estradiol at GnRH-induced ovulation and postovulatory progesterone seven days later were the two most important factors affecting pregnancy establishment. Continued research on FTAI protocols in modern beef and dairy production systems should focus on strategies to increase preovulatory estradiol, postovulatory progesterone, and oocyte competence to increase pregnancy rates to a single insemination.

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Maintenance or regression of the corpus luteum during multiple decisive periods of bovine pregnancy

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Abstract

In ruminants, there are specific times during the estrous cycle or pregnancy when the corpus luteum (CL) may undergo regression. This review has attempted to summarize the physiological and cellular mechanisms involved in CL regression or maintenance during four distinct periods. The first period is near day 7 when animals that are ovulating after a period of low circulating progesterone (P4), such as first pubertal ovulation or first postpartum ovulation, are at risk of having a premature increase in Prostaglandin F2α (PGF) secreted from the uterus resulting in early CL regression and a short estrous cycle. The second period is when normal luteolysis occurs at day 18-25 of the cycle or when the CL is rescued by interferon-tau secreted by the elongating embryo. The uterine mechanisms that determine the timing of this luteolysis or the prevention of luteolysis have been generally defined. Induction and activation of endometrial E2 receptors result in induction of endometrial oxytocin receptors that can now be activated by normal pulses of oxytocin. Of particular importance is the observation that the primary mechanisms are only activated through local (ipsilateral) and not a systemic route due to transfer of PGF from the uterine vein to the ovarian artery. In addition at the CL level, studies are providing definition to the cellular and molecular mechanisms that are activated in response to uterine PGF pulses or pregnancy. The third period that is discussed occurs in the second month of pregnancy (day 28-60) when undefined mechanisms result in CL maintenance of an ipsilateral CL but regression of a contralateral (opposite side from pregnancy) CL. The final period that is discussed is regression of the CL just prior to parturition. Although, cortisol from the fetus appears to be the primary initiator of luteolysis, PGF seems to be the final signal that causes regression of the CL. Thus, in all four periods, regression of the CL is likely to be caused by the direct actions of PGF that is secreted from the uterus. The uterine mechanisms that result in secretion of PGF seem to be normally inhibited during the early luteal phase, making short luteal phases not a normal event, and are altered during early pregnancy (day 18-25) resulting in prevention of luteolysis. During much of pregnancy, the mechanisms that cause PGF secretion from the uterus in response to oxytocin are intact but luteolysis does not normally occur, perhaps due to lack of efficient utero-ovarian transfer of PGF.

Keywords: interferon-tau, luteolysis, prostaglandin F2α.

Introduction

Alterations in the development, function, and regression of the corpus luteum (CL) is a primary feature of the reproductive cycle and pregnancy of mammals. The primary role of the CL in these processes is due to secretion of the hormone progesterone (P4). From a historical perspective, the first detailed description of CL was by Regnier deGraaf (1641-1673) when he described the "globules" and explained that in rabbits “the number of globules equals the number of offspring from a particular mating” (deGraaf, 1672 cited by Jocelyn and Setchell, 1972). Later, Gustav Born (1851-1900), an excellent histologist, forwarded the idea that it was a gland of internal secretion, based on the high vascularity and lack of ducts, and speculated that it could be involved in pregnancy (see excellent reviews by Simmer, 1971; Magnus and Simmer, 1972). Two of his students, Ludwig Fraenkel and Vilhelm Magnus, independently tested this hypothesis in their own laboratories using slightly different experimental methods. In Germany, Ludwig Fraenkel performed bilateral ovariectomy or electrocautery of all CL in mated rabbits and found that they did not maintain their pregnancies (Fraenkel and Cohn, 1901). In Norway, Vilhelm Magnus performed galvano-cautery of all CL as well as bilateral ovarian removal in mated rabbits and also reported that pregnancy was not maintained (Magnus, 1901). Both researchers reached the same conclusion that the CL was essential for maintenance of pregnancy. Magnus later called the pregnancy-maintaining endocrine secretion from the CL “differentieringsstofe” (differentiating stuff), the first name given to the hormone later known as progesterone (P4; Magnus, 1901). The initial reports were met with some skepticism. Fraenkel doggedly continued his research during the next decade on more than 160 rabbits eventually concluding that "Thus by the power of large numbers my thesis is proven: The ovary, in particular the CL, regulates the implantation and initial development of the embryo" (Fraenkel, 1910).

Possibly the most interesting biological properties of the CL are related to the incredible dynamics of this tissue. From the remains of the ovulated follicle, a new distinct structure is born (Smith *et al.*, 1994). After differentiation, the CL is composed of multiple cell types (Wiltbank, 1994). The thecal and

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granulosa cells of the follicle, differentiate into the small and large steroidogenic cells of the CL. This transformation involves a steroidogenic change as these cells develop the enzymatic machinery to produce tremendous quantities of P4. From a mass perspective, the granulosa cell is only 10 µm in diameter prior to the LH surge but increases to 38 µm when it develops into the fully-functional bovine large luteal cell. This calculates to an increase in volume from about 500 μm^3 in the granulosa cell to almost 30,000 μm^3 in the large luteal cell, over a 50-fold increase in cellular volume (Wiltbank *et al.*, 2012). In addition, there is rapid and extensive angiogenesis as the avascular follicular antrum remodels into a highly vascularized structure with every large luteal cell adjacent to multiple capillaries (Ellinwood *et al.*, 1978) and the highest blood flow per gram of tissue in the body (Wiltbank *et al.*, 1989). Then, abruptly, at specific, critical times during the cycle or pregnancy, the structure is put to death. For example, the bovine CL goes from a fully-functional structure of 4 to 6 g, to a structure with little production of P4, low blood flow (Zalman *et al.*, 2012), and structurally <20% of the original volume, a process known as luteolysis or CL regression.

There are four time periods that will be discussed in this review article when CL regression commonly occurs in dairy and beef cattle (Fig. 1). The first potential luteolysis period that will be discussed is at about day 7. In most beef cattle and many dairy cattle, the first post-partum estrous cycle and rarely, other later estrous cycles, will have a short duration of ~10 days, due to early regression of the CL at ~day 7. Luteolysis at this stage is not prevented by pregnancy but specific mechanisms prevent this early regression during most estrous cycles. The second period is the classical time for luteolysis in non-pregnant cows, near day 18-25. In pregnancy, maintenance of the CL during this period has been termed "Maternal Recognition of Pregnancy". There is a great deal of information available on the factors, events, and mechanisms related to luteolysis and prevention of luteolysis during this period. For example, there is a primary role for uterine prostaglandin F2α (PGF), acting in a local manner, as the initiator of luteolysis during this classical period of luteolysis, with many of the uterine and luteal mechanisms that determine this process being clearly defined. In addition, a primary role for embryonic interferon-tau (IFNT) in preventing luteolysis has been clearly demonstrated with the precise mechanisms still an active area of research. The third period is during the second month of pregnancy or day 30 to 60 when CL regression can occur but is generally prevented through mechanisms that remain undefined. The final period of luteolysis occurs near the end of pregnancy with an increase in circulating estradiol (E2) followed by a tremendous increase in circulating PGF that is associated with an abrupt decrease in circulating P4 due to CL regression. Regression of the CL at this time is critical for the timing and physiology of the normal parturition process. The CL is the primary source of P4 throughout the estrous cycle and pregnancy, although during mid to late pregnancy the placenta secretes a variable amount of P4 in individual pregnant cows (Fig. 1). This review will emphasize the similarities and differences during these times of luteolysis, in terms of origin of PGF and intrauterine and intraluteal mechanisms that underlie each occurrence of luteolysis.

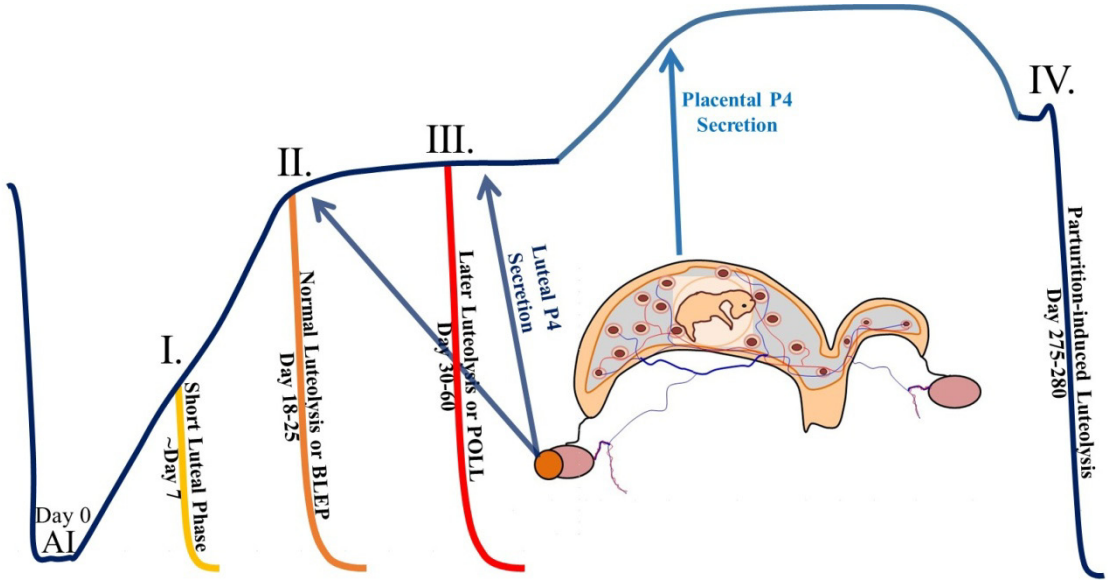

Figure 1. Theoretical diagram of circulating progesterone (P4) concentrations during pregnancy in cattle emphasizing the four key periods when luteolysis can occur. Period I - Luteolysis happens near day 7 and this leads to a short luteal phase. Pregnancy does not protect from this early luteolysis. Period II - Normal time of luteolysis at day 18-25 when prostaglandin F2α pulses from the uterus regress the corpus luteum. During pregnancy, secretion of interferon-tau from the uterus leads to Blockade of Luteolysis in Early Pregnancy (BLEP) and maintenance of CL structure and function. Period III - Later luteolysis that can occur during the second month of pregnancy leading to pregnancy loss. During these stages of pregnancy, there are still undefined mechanisms that produce Prevention of Later Luteolysis (POLL) extending the CL lifespan through later pregnancy. Period IV - Parturition-induced luteolysis occurs about 2 days before parturition and allows continuation of the cascade of events that produce parturition.

Period I: Short luteal phase and early CL regression - day 5-7

In ruminants, short cycles, due to an inadequate luteal phase, occur in some specific physiological situations. For example, short cycles have been documented in cattle and sheep following the first ovulation after puberty (Berardinelli and Butcher, 1979; Berardinelli *et al.*, 1979), following the first ovulation post-partum in dairy and beef cattle (Garverick *et al.*, 1992b), and after the first ovulation of the breeding season in ewes that were previously not cycling (Hunter, 1991). For example, in beef cattle that have been induced to ovulate by calf removal or treatment with an ovulation inducing agent, such as human chorionic gonadotropin (hCG) or gonadotropin-releasing hormone (GnRH), there is generally a very high incidence of shortened luteal phases (~80%). In dairy cattle, the first post-partum ovulation can be followed by a short cycle, but a much lower incidence has been reported (~27%; Table 1). It seems obvious that the presence of short cycles can reduce fertility since pregnancies are unlikely to occur because the embryo has generally not hatched from the zona pellucida and definitely not undergone embryonic elongation at the time of this early CL regression. Thus, fertility is absent in these cows, whether or not they have had fertilization and an appropriately-developing embryo.

Table 1. Incidence of short luteal phases or short cycles in dairy cattle.

Type of cattle	Short cycle (%)	Reference
Dairy cattle		
Lactating Holstein	76/281 (27.1%)	Fallon (1958)
Lactating Holstein	16/118 (13.6%)	Hinshelwood *et al*. (1982)
Lactating Holstein	9/27 (33.0%)	Stevenson and Britt (1979)
Lactating Friesian	5/18 (27.8)	Savio *et al*. (1990)
Lactating Holstein	21/39 (53.8%)	Staples *et al*. (1990)
Lactating Holstein	176/645 (27.3%)	Royal *et al*. (2000)
Total dairy	303/1,128 (26.9%)	

It is also clear that the short cycle is due to a shortened lifespan of the CL, for example in cattle, the CL regresses at 6-7 days after previous estrus. The cow then shows a subsequent estrus at 8-11 days after the previous estrus. As evidenced by the physiological conditions that result in short cycles, one of the primary risk factors for the shortened CL lifespan is an extended period without circulating P4 prior to the ovulation that produces the inadequate CL.

To understand the physiological mechanisms that produce the short estrous cycle, a number of different ruminant experimental models have been utilized. One model that has been utilized is the ewe in the non-breeding season that is induced to ovulate with GnRH treatment (Hunter, 1991; 0.25 μg every 2 h for 18-24 h, followed by an ovulatory dose of GnRH, 125 μg). Pretreatment for 36 h with P4 resulted in 100% of ewes with a normal CL lifespan, whereas, induction of ovulation with this protocol without pretreatment with P4 resulted in ~70% shortened luteal phases. In ewes, CL regression occurs between day 4 and 6 after the induced LH surge (Robinson *et al.*, 2008). In beef cattle, induction of the first post-partum ovulation by early weaning or temporary calf removal or by using hormonal treatments, such as GnRH or hCG, results in a high percentage of cows with short cycles, whereas pretreatment with P4 or progestogens before these induction procedures prevents these short cycles (Copelin *et al.*, 1988; Salfen *et al.*, 1995). Finally, in cycling dairy heifers or cows, a high percentage of short cycles is induced if cows are given a premature treatment with GnRH following induction of luteolysis with PGF (0 or 24 h after PGF given on day 6 or 7 of the estrous cycle; Peters and Pursley, 2003; Rantala *et al.*, 2009). Each of these models is characterized by inadequate circulating E2 during the proestrous period, generally ovulation of a smaller follicle, production of a CL with a shortened lifespan, and a short estrous cycle.

Using these animal models, the mechanisms that produce early regression of the CL have generally been divided into effects related to: 1) the preovulatory follicle, 2) the CL, and/or 3) the uterus. Related to the follicle, there are clear differences between the preovulatory follicle of ewes or cattle that have a shortened luteal phase including: decreased LH receptors in granulosa cells and decreased follicular E2 production, as evidenced by decreased circulating E2 or decreased E2 in follicular fluid (Hunter *et al.*, 1986; White *et al.*, 1987; Inskeep *et al.*, 1988; Braden *et al.*, 1989a, b). Thus, follicular function prior to short estrous cycles is clearly defective, and preovulatory circulating E2 concentrations are reduced in each of the physiological situations that result in a shortened luteal phase.

Related to the CL, initial development and function of the CL were similar until 4 days after the LH surge in ewes with normal or shortened luteal phases. After that time, the CL regressed rapidly in a manner that closely resembled the timing of CL regression at the end of the luteal phase (Hunter *et al.*, 1989). In beef cattle, the patterns of P4 were similar until day 5 after estrus and subsequently there was a rapid decrease in circulating P4 as CL regression ensued (Copelin *et al.*, 1987). One suggestion has been that short-lifespan CL lack gonadotropin support or gonadotropin responsiveness. Contrary to this idea, there were no differences in LH receptors or adenylate cyclase activity in early CL that were destined for short-lifespan vs. longer lifespan (Smith *et al.*, 1996). In addition, there were no detectable differences in LH secretion during the early luteal phase for these two types of animals (Garverick *et al.*, 1988). Further, treatments with luteotropins, such as treatment with

GnRH pulses or hCG, were unable to overcome the shortened luteal lifespan (Smith *et al.*, 1996). In addition, there are no differences in PGF responsiveness of CL destined to have normal or short lifespan (Copelin *et al.*, 1988). Thus, although the CL clearly regresses prematurely in cows or ewes with a short cycle, there is no evidence that the CL is functionally incompetent or that it regresses due to mechanisms that are internal to the CL.

Finally, most evidence is consistent with a primary role for the uterus in shortened luteal lifespan in ruminants. The most definitive experiments utilized hysterectomized cattle or ewes that were induced to ovulate. Intact cows had an estrous cycle of 8.8 days, whereas, hysterectomized cows that were treated similarly had a prolonged functional CL and did not return to estrus (Copelin *et al.*, 1987). Similarly, hysterectomy resulted in normal CL function in ewes destined to have short-lived CL (Southee *et al.*, 1988). Thus, it seems clear that the shortened luteal lifespan is due to a direct effect of the uterus. Active immunization against PGF (Copelin *et al.*, 1989) or inhibition of PGF secretion were both effective in extending the CL lifespan and increasing P4 secretion by the CL, indicating that uterine PGF is the primary cause of the shortened luteal phase. Consistent with this idea, cows destined to have short-lived CL have early secretion of PGF from the uterus (Zollers *et al.*, 1991) and an early increase in circulating metabolite of PGF that begins near the time of the first increases in circulating E2 during the first follicular wave (Southee *et al.*, 1988; Hunter *et al.*, 1989; Cooper *et al.*, 1991; Hunter, 1991; Garverick *et al.*, 1992b). In addition, intrauterine infusion of IFNT, the protein that normally causes maintenance of the CL during pregnancy in ruminants, can prevent short luteal phases (Garverick *et al.*, 1992a). At least part of the action of IFNT is a reduction in uterine secretion of PGF. Thus, all of these diverse types of evidence clearly point to premature secretion of PGF from the uterus as the direct initiator of premature luteolysis in cows with a short-lived CL.

There also seems to be a critical role for E2 from the follicle in development of the mechanisms associated with a shortened CL lifespan. Reduced preovulatory E2 can produce a short CL lifespan (Vasconcelos *et al.*, 2001; Peters and Pursley, 2003; Rantala *et al.*, 2009), even in animals with elevations in P4 prior to the preovulatory period. However, supplementation of early postpartum beef cattle with E2 alone was not sufficient to restore normal luteal phases but they required both elevated P4 prior to the preovulatory period and elevated E2 during the preovulatory period to have normal CL lifespan (Sa *et al.*, 2009). Thus, elevated circulating P4, followed by a decrease in P4 and an increase in circulating E2 are important components that regulate a lack of development of oxytocin responsiveness during the early luteal phase and prevent short-lived CL, in normal physiological situations (Hunter *et al.*, 1989; Hunter, 1991; Beard and Hunter, 1996). In addition, there is a critical role for E2 from the dominant follicle of the first follicular wave, after ovulation, in the mechanisms that underlie premature PGF secretion in short luteal phases (Beard and Hunter, 1994). This is evidenced by the fact that treatment with steroid-stripped follicular fluid inhibited growth of the dominant follicle and premature development of oxytocin-responsiveness in ewes destined to have a short-lived CL. Thus, it seems clear that elevated P4 followed by elevated E2 in the absence of P4 are critical for preventing premature E2-induction of oxytocin responsiveness during the early luteal phase and induction of early luteolysis.

Figure 2 shows a theoretical, simplified model comparing changes in the uterine endometrial cells during the early luteal phase in animals destined to have CL with normal or shortened CL lifespan. In animals destined to have a normal lifespan, at day 5-7 the endometrial cells do not have E2 responsiveness, due to action of P4 and E2 prior to ovulation. Therefore, even though there is an increase in E2 during the first follicular wave, this E2 does not have an effect on the uterine endometrial cells, preventing induction of oxytocin receptors, and preventing premature luteolysis. However, in animals that will have a short life-span CL, E2 responsiveness is present in the uterine endometrial cells and the increase in circulating E2 during the first follicular wave allows induction of oxytocin receptors, with subsequent oxytocin-induced PGF secretion, and premature luteolysis. These events resemble the events that occur at day 17-20 in the uterine endometrial cells of animals destined to have normal CL lifespan.

Period II: Normal luteolysis: role of uterus and PGF - days 18-25 of estrous cycle

Under normal conditions, if no fertilization occurs during an estrous cycle, the CL will regress at ~18-19 days after previous estrus. This process is referred to as normal luteolysis, and involves a decrease in P4 (functional luteolysis) as well as a decrease in CL volume and blood flow (structural luteolysis). Functional luteolysis occurs over a period of ~24 h in individual cows or sheep, if luteolysis is designated as circulating P4 < 1 ng/ml, or in ~32 h if <0.5 ng/ml is used as the designation for functional luteolysis (Ginther *et al.*, 2007).

The role of the uterus in determining the lifespan of the CL was first demonstrated in guinea pigs by showing that CL were maintained after hysterectomy (Loeb, 1927). In ruminants, a clear demonstration of the role of the uterus in luteolysis was published in 1956 by using hysterectomy of both sheep and cattle (Wiltbank and Casida, 1956). Sheep and cattle that had complete removal of the uterus did not return to estrus and CL lifespan was greatly prolonged. For example, sheep with CL marked with India ink were still present at 52, 53, 76, 98, and 107 days after hysterectomy. Similarly, cows that had complete hysterectomy had large, marked CL present at 27, 98, and 154 days after hysterectomy (Wiltbank and Casida, 1956). Thus, complete removal of uterus led to maintenance for a long but not yet clearly defined time. Interestingly, when part of the uterus (ipsilateral to the CL) remained after the hysterectomy surgery, CL regression occurred and

animals returned to estrus. Numerous hysterectomy and unilateral hysterectomy studies have been performed and the role of the uterus in luteolysis is clearly delineated (Collins *et al.*, 1966). In addition, ipsilateral (uterine horn on the same side as CL) hysterectomy invariably prolonged the lifespan of the CL, while contralateral hysterectomy consistently failed to affect the CL lifespan (Inskeep and Butcher, 1966).

It was soon realized that the uterine-derived luteolysin that regressed the CL was able to arrive at the ipsilateral CL through a local veno-arterial transfer pathway. The ovarian artery in ruminants is extremely convoluted and in close apposition to the uterine vein, thus allowing transfer of the uterine luteolysin, PGF, to the ovarian artery (Ginther and Del Campo, 1974; Mapletoft *et al.*, 1976a). In an experiment in which the convoluted ovarian artery was separated from the uterine vein, three out of four ewes maintained the CL until slaughter on day 25 (Barrett *et al.*, 1971). Experiments involving unilateral hysterectomy and subsequent cross-over anastomosis of the uterine veins provided strong evidence for the local delivery of a uterine luteolysin to the ovarian artery (Mapletoft *et al.*, 1976a). When the uterine vein from the intact horn was joined with the uterine vein on the hysterectomized side with the CL, the CL regressed at the normal time clearly demonstrating the involvement of the local utero-ovarian, veno-arterial pathway in luteolysis during this period (Mapletoft *et al.*, 1976a).

The possibility that PGF was the luteolytic agent was first suggested during a reproductive workshop in 1965 (Hansel, 1966). Evidence was subsequently provided in rats that treatment with PGF2α produced luteolysis (Gutknecht *et al.*, 1969; Pharriss and Wyngarde 1969). Thereafter, a series of experiments by McCracken using auto-transplanted ovaries demonstrated the luteolytic effect of PGF in sheep (Goding *et al.*, 1967; McCracken, 1971). There are now multiple types of evidence that convincingly demonstrate that PGF is the uterine luteolysin in ruminants (Knickerbocker *et al.*, 1988). First, PGF is abundantly produced in the uterus of non-pregnant ruminants (McCracken *et al.*, 1972) and there are increased concentrations of PGF in uterine venous drainage, uterine flushings, and uterine tissue near the expected time of luteolysis (Wilson *et al.*, 1972). Second, pulses of uterine vein PGF and the circulating concentrations of the PGF metabolite (PGFM; 15-Keto-13,14-dihydro-PGF2α) correspond to the time of decreasing P4 in non-pregnant heifers (Kindahl *et al.*, 1976; Ginther *et al.*, 2007). Third, inhibition of uterine PGF production prevents spontaneous luteolysis in both ewes and heifers (Lewis and Warren, 1977). Fourth, passive immunization with antibodies that are specific to PGF prolonged the lifespan of the CL (Fairclough *et al.*, 1981). Fifth, there is good evidence that PGF can be efficiently exchanged from the uterine vein to the ovarian artery in sufficient quantities to make the local mechanisms physiologically feasible (Lamond *et al.*, 1973; McCracken *et al.*, 1981). Finally, treatment with pulses of PGF that mimic the natural PGF pulses can induce complete luteolysis that resembles natural luteolysis (Schramm *et al.*, 1983; Ginther *et al.*, 2009; Atli *et al.*, 2012). Thus, evidence is compelling that luteolysis in ruminants is initiated by PGF coming from the non-pregnant uterus.

The pattern of PGF secretion has clearly been shown to be pulsatile. Figure 3 shows a typical pattern for PGFM, oxytocin, and P4 during luteolysis in an individual cow undergoing luteolysis between day 18 and 20. As shown, P4 concentrations decrease rapidly with most of the decrease occurring in a 24 h period. The decrease in P4 is associated with four distinct pulses of PGF, which are reflected in the circulating PGFM pulses shown in Fig. 3. Finally, pulses of circulating oxytocin occur routinely prior to luteolysis with little effect on circulating PGFM. However, during the time of luteolysis, each oxytocin pulse is associated with a pulse of PGFM. This pattern emphasizes the critical role for oxytocin responsiveness, i.e. oxytocin receptors, in production of PGFM pulses and ultimately luteolysis.

The cellular mechanisms within the uterine endometrial cells that produce the PGF pulses was shown in a simplified manner in Fig. 2 and is shown in greater detail in other reviews (McCracken *et al.*, 1999; Spencer and Hansen, 2015). Clearly the induction of E2 receptors has a central role in the mechanisms that initiate these pulses. During most of the normal luteal phase, expression of E2 receptors in endometrial cells is inhibited by elevated circulating P4 acting on P4 receptors in these cells. Near the end of the luteal phase, elevated P4 begins to downregulate P4 receptors and this allows E2 receptor expression near the time of luteolysis (Spencer and Bazer, 1995; Spencer *et al.*, 1995). Initiation of luteolysis is dependent upon activation of these E2 receptors by the increase in circulating E2 that accompanies development of a dominant follicle in the preovulatory follicular wave. This is illustrated by the delay in luteolysis that is caused by elimination of circulating E2 by either ultrasound-guided ablation of follicles (Araujo *et al.*, 2009) or inhibition of follicle growth by treatment with steroid-stripped follicular fluid (Salfen *et al.*, 1996). This delay in luteolysis is prevented by treatment with low doses of E2 (Salfen *et al.*, 1996; Araujo *et al.*, 2009). One of the major actions of E2 in the endometrial cell at this time is the induction of oxytocin receptors (Robinson *et al.*, 2001). After induction of oxytocin receptors, uterine endometrial cells produce pulses of PGF secretion in response to the natural pulses of oxytocin that occur during the day (Ginther *et al.*, 2012).

Thus, natural luteolysis is connected to distinct PGF pulses from the uterine endometrial cells that are induced by oxytocin pulses. Studies using [^{3}H]-PGF have indicated that during the peak of a PGF pulse, ~10% of secreted PGF will be transported from the uterine vein to the ovarian artery (Lamond *et al.*, 1973; McCracken *et al.*, 2010). How this PGF is transported between the vascular systems has been delineated in elegant recent studies that showed expression of a specific PG Transporter in the three layers, tunica intima, tunica media, and tunica adventitia, of both the utero-ovarian vein and the ovarian artery (Lee *et al.*,

2010; McCracken *et al.*, 2011). The primary PG Transporter is a member of the organic solute carrier family that has 12-transmembrane domains and is termed SLCO2A1, also known as OATP2A1 (Kanai *et al.*, 1995; Schuster, 1998, 2002). A specific inhibitor of the PG Transporter, DIDS, was able to block luteolysis but did not change expression of E2 receptor, oxytocin receptor, Cox-2, PG transporter, or uterine production of PGF (Lee *et al.*, 2013). Thus, all luteolytic mechanisms were intact but still efficient PGF transport, in this case out of the endometrial cells, is needed for the luteolytic process.

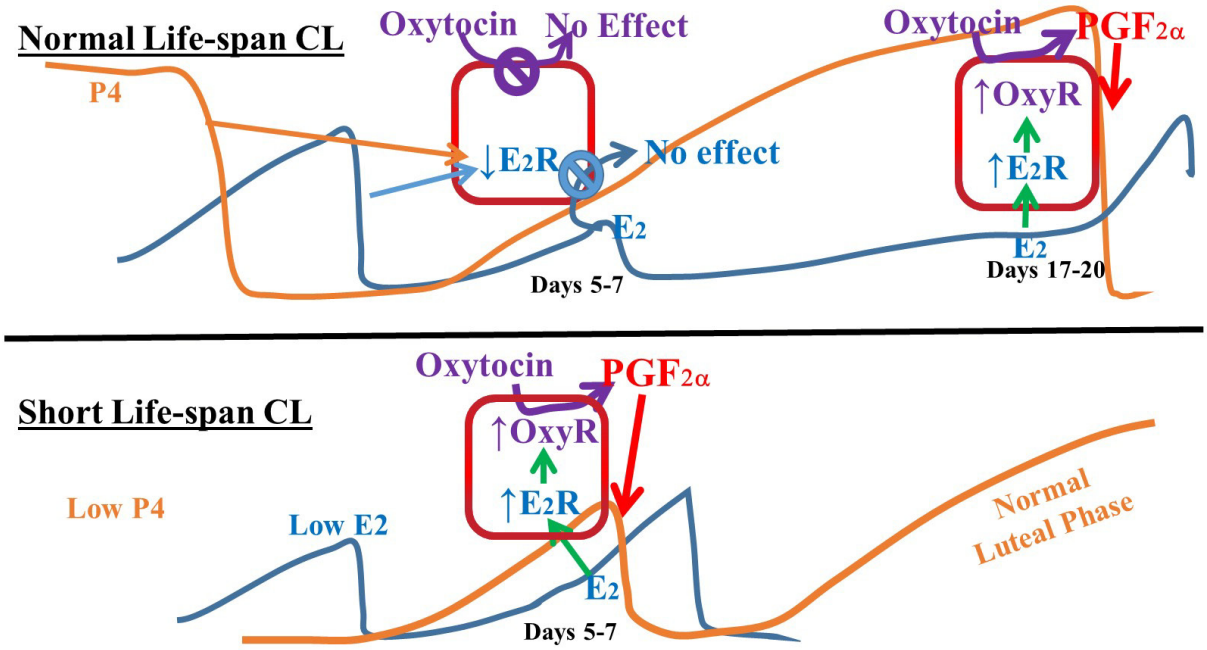

Figure 2. Proposed physiological model for the mechanisms that produce a CL with a short or normal life-span. The cell that is shown is the uterine endometrial cell at day 5-7 or at day 17-20 during the normal CL lifespan. Text discusses the mechanisms shown in the figure. E2R = estradiol receptor; OxyR = Oxytocin Receptor; P4 = progesterone; E2 = estradiol-17β; PGF2α = prostaglandin F2α.

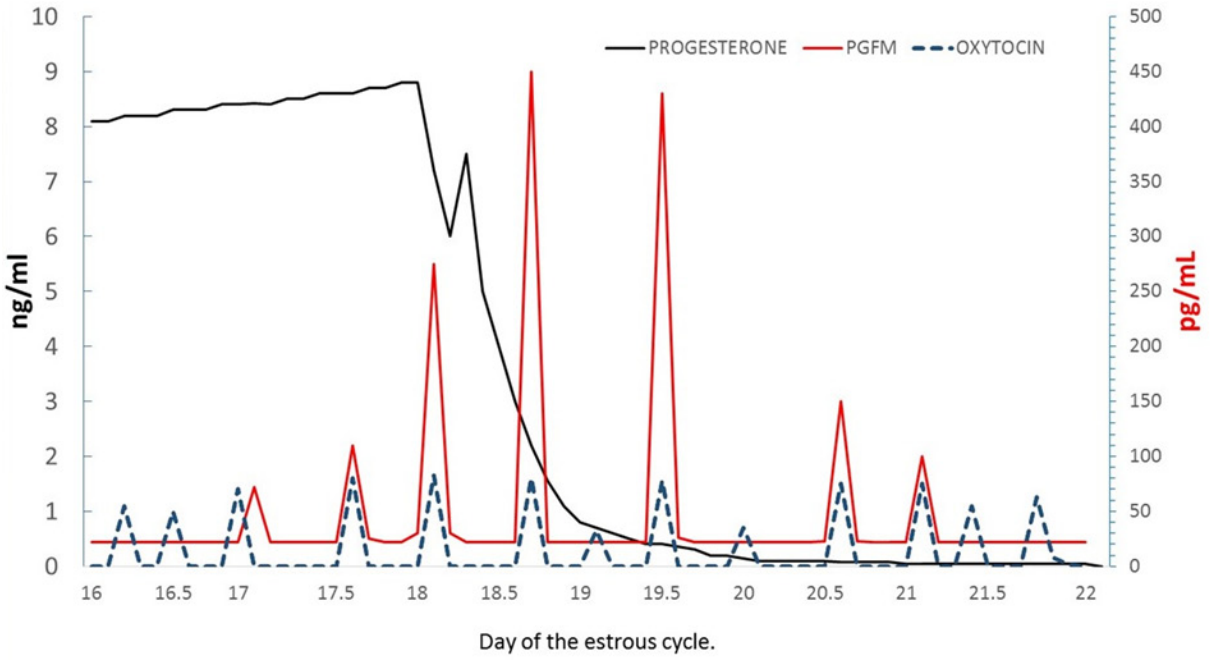

Figure 3. Diagram of the patterns of circulating progesterone, oxytocin, and PGFM concentrations during the time of luteolysis in cattle. As luteolysis approaches, pulses of oxytocin act on the uterine endometrial cells to cause production of pulses of PGF, detected as PGFM in the peripheral circulation, and these pulses cause the decrease in circulating P4 and luteolysis.

Once PGF reaches the CL, some of the most exciting cellular and molecular processes are activated, resulting in the complete luteolytic process. Although many studies have evaluated the processes that are involved in the biological action of PGF in the CL, most of these studies utilized supra-physiological doses of PGF. Recent studies have either monitored the mechanisms that follow natural PGF pulses (Ginther and Beg, 2009) or used more physiological pulses of PGF in an attempt to mimic and synchronize the mechanisms that occur during natural luteolysis (Ginther *et al.*, 2009; McCracken *et al.*, 2012). Our laboratory has given intrauterine injections of low doses of PGF in order to evaluate gene expression in response to synchronized PGF pulses (Atli *et al.*, 2012). Figure 4 shows a simplified view of some of the gene expression changes that are happening within the CL following each PGF pulse. Although only a single cell is shown, ostensibly a large luteal cell since they contain most of the PGF receptors, it should be understood that multiple cell types and complex processes are occurring during luteolysis.

Five key types of gene expression events are illustrated in response to each PGF pulse. The early response gene, Jun, is shown to increase in response to each PGF pulse, which is representative of many early response genes (Atli *et al.*, 2011). The PGF synthesis pathways are complex but two genes are shown, one involved in PGF production, prostaglandin G/H Synthase-2 (PTGS2), and one involved in PGF metabolism, prostaglandin Dehydrogenase (PGDH). After the first PGF pulse both genes are stimulated, in spite of the fact that PGDH will eventually be inhibited during the luteolysis process, after the third pulse. The continued induction of the PGF synthesis pathways and the eventual inhibition of the PGF metabolism pathways will allow intraluteal PGF production and an autoamplification pathway for PGF production (Tsai and Wiltbank, 1996, 1997). Production of P4 is eventually inhibited but, similar to PGDH, steroidogenic acute regulatory protein or StAR, is stimulated by the first pulse of PGF but then inhibited by pulse 2, 3, and 4. Eventually all of the steroidogenic pathway genes are inhibited but StAR is the most acutely regulated.

Many genes that are associated with immune function are stimulated during the luteolytic process. Some may be due to production of immunomodulatory molecules from luteal cells and eventually increased expression is due to influx of immune cells into the CL during the luteolytic process. Thus, immune-related genes begin to be stimulated mostly after the 2nd PGF pulse and continue to increase after the 3rd and 4th pulses. Alterations in angiogenic pathways are a key part of the luteolysis process. Expression of VEGFA is shown as an example of alterations in angiogenesis during pulses of PGF. After the first pulse there is a paradoxical increase in VEGF mRNA expression and then all subsequent pulses produce inhibition of genes that are involved in stimulating blood vessel formation and function.

Thus, this simple diagram illustrates some of the key pathways that are being activated during the luteolytic process. Transcriptional pathways, such as the ones regulated by some of the early response genes, are activated and are likely to mediate the subsequent changes in gene expression. Steroidogenesis is decreased as the CL proceeds through functional luteolysis. Structural luteolysis proceeds as blood vessels begin to breakdown and immune cells are involved in key structural and functional changes that occur during luteolysis. Finally, although PGF pulses from the uterus are the initiators of luteolysis, intraluteal production of PGF is probably important for expression of the complete luteolytic pathways.

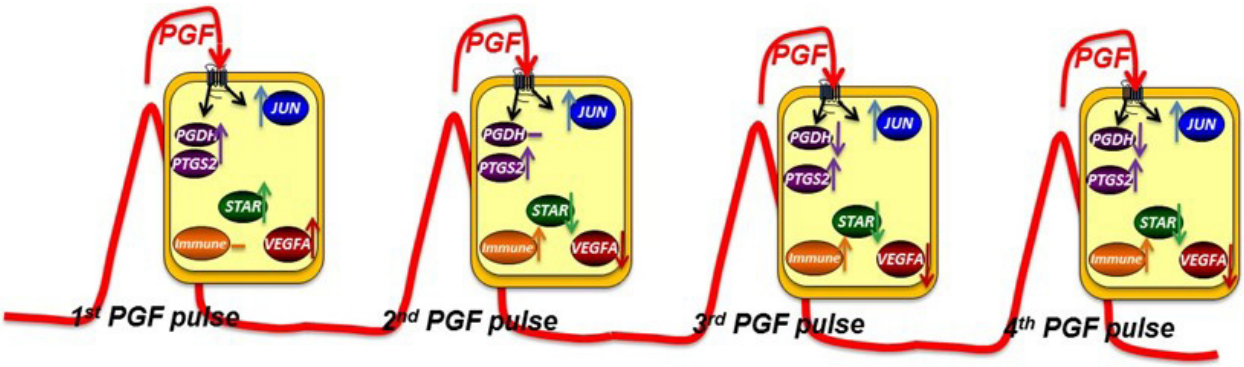

Figure 4. Physiological model of the changes in gene expression within the CL during intrauterine injection of low doses of PGF, designed to mimic physiological pulses of PGF occurring during luteolysis. See text for details.

Period II. Blockade of luteolysis in early pregnancy - day 18-25 of pregnancy

Pregnancy causes a blockade of luteolysis that has been sometimes termed "Maternal recognition of pregnancy". In ruminants, the pregnancy protects the CL from regression through local and not systemic pathways. This has been clearly demonstrated in a series of elegant experiments with isolated horns or vascular anastomoses. For example, transfer of embryos into a surgically-isolated uterine horn resulted in CL regression if the embryo was transferred contralateral to the CL but the CL was maintained if the embryo was transferred ipsilateral to the pregnancy in both cows (Del Campo *et al.*, 1977) and ewes (Moor, 1968). In surgically-isolated horns, surgical anastomosis of the main uterine vein from the gravid side to the uterine vein on the non-gravid side resulted in maintenance of

the CL on the non-gravid side in both ewes (Mapletoft *et al.* 1975) and cows (Del Campo *et al.*, 1980). This demonstrated that the pregnancy signal was local and not systemic and was carried in the local uterine vein. Similarly, anastomoses of the ovarian artery from the gravid side to the non-gravid side resulted in CL maintenance (Mapletoft and Ginther, 1975; Mapletoft *et al.*, 1976c) demonstrating that the signal passed from the uterine vein to the ovarian artery, only on the same side and not the opposite side from the pregnancy. Thus, it is clear that even though some pregnancy signals may escape the uterus, the critical pregnancy signal that protects the CL in ruminants during early pregnancy is acting locally by passing from the uterine vein to the ovarian artery and not through the systemic circulation. In addition, these studies demonstrated that maintenance of pregnancy is not just the absence of a signal, such as the absence of PGF pulses, but the active blockade of luteolysis. This is clear since uterine vein blood from the non-gravid horn is still present when the blood from uterine vein of the gravid horn is added and the CL is protected. The two ewes that had clots in the anastomosis had normal CL regression.

Early embryo transfer experiments showed that if the embryo was flushed from the uterus on day 13 or before in the ewe (Moor and Rowson, 1966; Moor *et al.*, 1969) or day 16 or before in the cow (Northey and French, 1980) there was normal timing of CL regression. In contrast if the embryo was flushed from the uterus after this "critical period", the CL lifespan was extended, demonstrating the time when the pregnancy signal began to be secreted by the embryo. In cyclic ewes, intrauterine infusion of homogenates or secreted proteins from day 14-15 embryos extended CL lifespan, whereas, homogenates of day 21-25 embryos did not alter CL lifespan (Rowson and Moor 1967; Godkin *et al.*, 1984b), demonstrating the limited interval during pregnancy when the signal is secreted by the conceptus. The active principal in the homogenates was heat and protease-labile, and had properties consistent with a low molecular weight protein (Rowson and Moor, 1967; Martal *et al.*, 1979; Godkin *et al.*, 1982). Later studies showed that a single protein, termed ovine or bovine trophoblast protein-1 at the time and later IFNT, was solely responsible for maintenance of the CL during pregnancy in ruminants (Godkin *et al.*, 1984a, 1997; Thatcher *et al.*, 1984).

Thus, during the critical period of day 17 to 25 in cattle, the embryo is dramatically elongating, nourished by histotroph in the uterine lumen. The ruminant trophectoderm secretes IFNT during elongation of the early embryo and IFNT has been shown to be the definitive signal for CL maintenance during early pregnancy (Roberts, 1996; Bazer *et al.*, 1997). In uterus, IFNT acts in a paracrine manner to prevent expression of estrogen receptor alpha and oxytocin receptor in endometrial luminal epithelium and superficial glandular epithelium, thereby altering release of luteolytic pulses of PGF (Spencer *et al.*, 2007b). Interferon-tau also stimulates expression of specific genes, termed interferon-stimulated genes (Antoniazzi *et al.*, 2013), in the uterus (Johnson *et al.*, 1999) and in peripheral tissues such as the CL (Oliveira *et al.*, 2008; Bott *et al.*, 2010) and peripheral blood cells (Gifford *et al.*, 2007; Shirasuna *et al.*, 2012). Thus, circulating IFNT that escapes the uterine lumen might prevent CL regression by acting directly on CL and this action could be independent or synergistic with the actions of IFNT on uterine PGF secretion.

Previous studies have also indicated that the CL of pregnancy has reduced sensitivity to PGF (Silvia and Niswender, 1984, 1986; Silvia *et al.*, 1984a). Much of this resistance to PGF action is ascribed to actions of prostaglandin E2 and E1 (termed PGE in this proposal) coming from the pregnant uterus. Indeed, PGE can block natural or PGF-induced luteolysis either *in vivo* or *in vitro* (Michael *et al.*, 1993; Miyamoto *et al.*, 1993; Fortier *et al.*, 2004; Weems *et al.*, 2011). In addition, recent studies showed that endocrine delivery of recombinant ovine IFNT, via uterine or jugular vein, protected the ovine CL from the luteolytic actions of PGF, maintaining intraluteal and circulating P4 and CL volume (Antoniazzi *et al.*, 2013).

It is clearly established that P4 from the CL is essential for maintenance of pregnancy and that IFNT from the elongating embryo is the definitive signal for CL maintenance during early pregnancy (Roberts, 1996; Bazer *et al.*, 1997; Spencer *et al.*, 2007b; Dorniak *et al.*, 2013). At this time, there is still substantial controversy regarding the precise endocrine pathways involved in maintenance of the CL by IFNT with three potential pathways being most supported but by different research groups. First, the classical mechanism is that IFNT changes uterine gene expression resulting in reduced pulses of PGF and thus lack of luteolysis (Thatcher *et al.*, 1984; Knickerbocker *et al.*, 1986; Danet-Desnoyers *et al.*, 1994; Spencer *et al.*, 2007a; Dorniak *et al.*, 2013). Second, IFNT increases uterine production of PGEs (PGE1 and PGE2) and PGE blocks the action of PGF at the CL, maintaining CL function (Ottobre *et al.*, 1984; Silvia *et al.*, 1984a, b; Wiltbank and Ottobre, 2003; Krishnaswamy *et al.*, 2009; Weems *et al.*, 2011, 2012; Lee *et al.*, 2012). Third, recent convincing evidence demonstrates that IFNT exits the uterine lumen and interacts directly with the CL and may directly block PGF action at the CL (Gifford *et al.*, 2007; Oliveira *et al.*, 2008; Bott *et al.*, 2010; Hansen *et al.*, 2010; Antoniazzi *et al.*, 2013).

Although it will not be possible in this manuscript to definitely select which of these endocrine mechanism(s) is most important in maintenance of the CL during early pregnancy, some perspective can be thought-provoking. Although the exit of IFNT from the uterus into the systemic circulation seems irrefutable, the systemic mechanism required for this pathway is not consistent with most of the older studies that definitely show a local and not a systemic mechanism involved in CL maintenance in ruminants. Thus, the third mechanism is unlikely to be the sole anti-luteolytic mechanism, although, recent studies showed that endocrine delivery of IFNT, via uterine or jugular vein, protected the ovine CL from the luteolytic actions of PGF, maintaining intraluteal and circulating P4 and CL volume (Antoniazzi *et al.*, 2013). Nevertheless, it seems

possible that endocrine delivery of IFNT could still be acting on the uterine endometrial cells. Related to the first mechanism, circulating PGF is generally found to be higher in the pregnant than the non-pregnant ruminant (Lewis *et al.*, 1977; Vincent and Inskeep 1986; Arosh *et al.*, 2004), although pulses of PGF may differ in pregnant and non-pregnant ruminants. Finally, previous studies have indicated that the CL of pregnancy has reduced sensitivity to PGF (Silvia and Niswender, 1984, 1986; Silvia *et al.*, 1984a). Much of this resistance to PGF action is ascribed to actions of PG E2 and E1 (PGE) coming from the pregnant uterus. In addition, PGE2, but not IFNT, is transported through the utero-ovarian plexus consistent with a local signal occurring during early pregnancy (Lee *et al.*, 2012). Further, the PGE2:PGF2α ratio in the utero ovarian vein was ~72-fold higher, and, in the ovarian artery, was ~115-fold higher on day 16 of pregnancy versus the estrous cycle of ewes, indicating the efficiency of utero-ovarian PGE2 transport. Based on all of these various findings, it seems likely that uterine PGE, secreted in response to IFNT from the elongating embryo, has a key role in protection of the CL from luteolysis during early pregnancy (day 18-25 in cattle).

Period III. Prevention of later luteolysis during pregnancy – day 30-60 of pregnancy

The second month of pregnancy is not a typical time to think of luteolysis. However, IFNT is likely to no longer be a major factor in maintaining the CL during the second month of pregnancy since IFNT secretion from the developing conceptus peaks by day 23 of pregnancy and then dramatically decreases during the next few weeks (Godkin *et al.*, 1988; Stojkovic *et al.*, 1995). This provokes the obvious question: What maintains the CL after day 30 of pregnancy when IFNT is no longer present? Related to this question is the observation of pregnancy loss during 30-60 days of pregnancy. For example, we recently summarized the results from 46 recent studies and ~25,000 pregnancies that were evaluated by ultrasound on ~day 32 and again at ~day 60 and found 11.95% pregnancy loss during this period (Wiltbank *et al.*, 2016). The pregnancy loss during this time period is even greater for clones. The primary cause of pregnancy loss during this period could be initial embryonic death and subsequent luteolysis or, alternatively, could be initiated by inappropriate luteolysis during this period followed by loss of the pregnancy (Giordano *et al.*, 2010). No studies have clearly differentiated these two distinct causes.

We became more interested in luteolysis during this time period, based on recent observations that we made on timing of CL regression in accessory CL that are contralateral to the pregnancy (Wiltbank *et al.*, 2016). In this experiment, we induced accessory CL in lactating cows by treatment with GnRH on day 5 after AI. Pregnant cows could therefore have an accessory CL present on either the same side as the pregnancy (ipsilateral) or on the opposite ovary (contralateral). Intriguingly, although ipsilateral CL rarely regressed, almost all contralateral CL regressed during the pregnancy. Of particularly interest, most accessory CL regressed during the second month of pregnancy. Thus, mechanisms are present on the same side as the pregnancy that allow maintenance of the ipsilateral CL, whereas, in the contralateral horn, mechanisms occur that result in regression of the CL. This result also demonstrates that local and not systemic mechanisms are responsible for maintenance or regression of the CL during this period.

What are the local mechanisms that result in maintenance of the CL during the second month of pregnancy, but that are not present, at least in sufficient quantities, to maintain the CL on the contralateral ovary? There are a few things to consider. First, it seems likely that the contralateral CL regresses due to PGF secretion from the contralateral uterine horn, since CL do not undergo spontaneous regression in ruminants with the uterus removed (Wiltbank and Casida, 1956; Mapletoft *et al.*, 1976b). Related to this idea, responsiveness to oxytocin and the ability of the uterus to synthesize and secrete PGF persists throughout pregnancy with increases in concentrations of PGFM of approximately 6-fold when oxytocin is administered to cows between day 50 and 280 of pregnancy (Schallenberger *et al.*, 1989; Fuchs *et al.*, 1996). Second, the uterine horns were not isolated in our experiment and therefore whatever local agent is involved in this process must have a difference in action on the ipsilateral than the contralateral ovary. Thus, there could be a protective substance that is present in larger concentrations on the ipsilateral than the contralateral side. Nevertheless, the most likely explanation, in our opinion, is that the ipsilateral uterine horn has greater blood flow than the contralateral horn, as previously reported (Ford *et al.*, 1979; Ford and Chenault, 1981; Panarace *et al.*, 2006). Thus in this scenario, high uterine blood flow would not allow efficient transfer of uterine-secreted PGF from the uterine vein to the ovarian artery on the ipsilateral side but efficient PGF transport would continue to occur on the contralateral side, potentially due to reduced uterine blood flow (Ford *et al.*, 1979). Thus, the blood flow explanation is the simplest explanation for the differential CL regression between ipsilateral and contralateral ovaries, although differential secretion of PGF or a luteal protective substance cannot be ruled out at this time.

One topic to consider is whether contralateral CL regression may represent a condition that is germane to the practical issue of pregnancy loss during the second month of pregnancy. Since pregnancy loss can be initiated by death of the embryo or alternatively by regression of the CL (Kastelic *et al.*, 1991; Giordano *et al.*, 2012), it seems likely that any losses due to CL regression utilize similar mechanisms as observed with contralateral CL regression during this time. Thus, a pregnancy that had not increased uterine blood flow sufficiently between 30 and 60 days of pregnancy, would be susceptible to loss by uterine-derived PGF. We speculate that PGF is secreted by the uterine horns throughout pregnancy, but the elevated uterine blood

flow does not allow this PGF to be transferred to the ovarian artery. Obviously a great deal of research remains to be done to fully explain this potentially critical period for CL regression or maintenance.

Period IV. Parturition-induced luteolysis– day 270-290 of pregnancy

In ruminants, the signal to end gestation arises from the fetus. It appears that the timing of parturition is encoded in the fetal genome and the mechanisms are activated by specific developmental events that occur in the developing fetus (Jenkin and Young, 2004). The signal from the fetus to initiate parturition in ruminants has been clearly shown to be the glucorticoid, cortisol, coming from the maturing hypothalamo-pituitary-adrenal axis. The circulating cortisol concentrations increase exponentially during the final weeks of gestation, producing what is termed the "cortisol surge" (Poore *et al.*, 1998). When the cortisol surge is mimicked by treatment of the lamb with betamethasone (glucocorticoid), a consistent change in gene expression happened at specific times after treatment: placental aromatase mRNA increased by 14 h, endometrial luminal epithelial cells dramatically increased E2 receptor expression by 28 h, and increased oxytocin mRNA and oxytocin binding at 28 h, with a consistent increase in electromyographic uterine contractile activity at 48-50 h, with definitive labor onset occurring at 56.6 ± 0.8 h after treatment (Wathes *et al.*, 1996). Thus, a similar sequence of events may occur in the uterus during parturition as occurs at the normal time of luteolysis at day 18-20, with first E2 receptor expression, then activation of the E2 receptors by circulating E2, and rapid induction of oxytocin receptors. Oxytocin binding to oxytocin receptors is likely to be driving myometrial contractions during labor, and induction of oxytocin receptors was observed during natural parturition (Wathes *et al.*, 1996) but not during betamethasone-induced parturition (Leung *et al.*, 1999). It seems likely that activation of oxytocin receptors by pulses of oxytocin underlies the large amount of PGF secretion and regression of the CL before parturition.

Figure 5 shows the changes in hormonal concentrations near the time of parturition in Holstein cattle (From adaptation of Rasmussen *et al.*, 1996; Mattos *et al.*, 2004). Concentrations of P4 remain elevated until about 36 h prior to calving and then promptly decrease to less than half the concentrations at 24 h before calving and reaching basal concentrations by 12 h before calving (Rasmussen *et al.*, 1996). The circulating concentrations of PGFM follow an opposite pattern with basal concentration up until about 48 h prior to calving when circulating PGFM starts to dramatically increase (Mattos *et al.*, 2004), most likely due to increased secretion by cells of the uterus. After parturition, concentrations of PGFM remain elevated, consistent with secretion from the uterus since the placenta has been lost by 24 h after parturition. The PGFM concentrations near this time are more than 5-fold greater (2000 pg/ml) than the peak concentrations of PGFM in a luteolytic pulse (300-400 pg/ml during peak luteolysis) during normal luteolysis on day 18-20 (Ginther *et al.*, 2010). Circulating E2 concentrations also increase dramatically and peak at more than 600 pg/ml or almost 100-fold greater than the peak circulating E2 concentrations near estrus (Sartori *et al.*, 2004). This E2 is coming from the placenta, as evidenced by the dramatic decrease in cows with normal placental loss after parturition and continued elevation in cows with retained placenta (Rasmussen *et al.*, 1996).

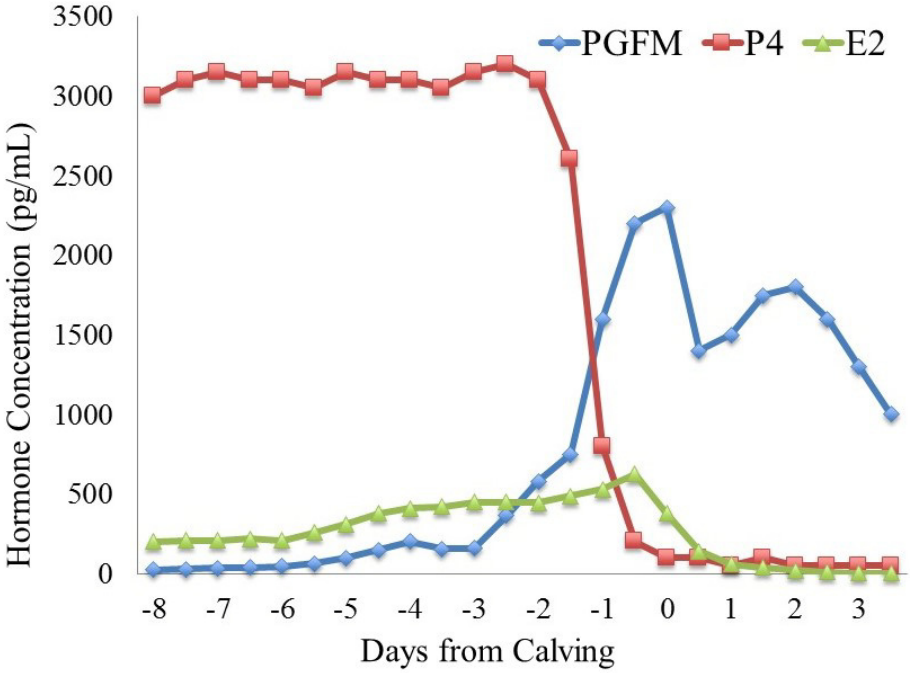

Figure 5. Circulating concentrations of progesterone (P4), estradiol (E2), and PGFM, normalized to the time of parturition in Holstein dairy cattle. From Rasmussen *et al*. (1996) and Mattos *et al*. (2004).

There are dramatic changes in PG production near parturition from different tissues and due to different regulatory mechanisms. During the last 15-20 days of gestation there is an increase in PGE2 in the fetal circulation, closely matching the slow increase in fetal cortisol during this time. There is also the dramatic increase in PGF secretion, reflected in the increase in PGFM, however this is a late event occurring within 2 days of parturition. The PGE2 originates from the placenta due to induction of PGH2 in placental cells by the low amounts of cortisol that begin to come from the developing fetus. This increase in PGE2 secretion is not dependent upon circulating E2, since treatment with an aromatase inhibitor does not alter the increase in PGE2 in fetal circulation (Whittle *et al.*, 2000). Interestingly, this PGE2 may be important for induction of aromatase near the time of parturition which drives the dramatic increase in E2 production by the placenta. Subsequently, rising cortisol, combined with the rising E2 concentrations, now induce PGH2 expression in maternal uterine cells and this causes the dramatic increase in PGF secretion observed just before parturition. That both E2 and cortisol are required for the increase in PGFM is demonstrated by the dramatic increase in PGFM (>500 pg/ml) in response to cortisol in the presence of physiological concentrations of E2 but no increase when cortisol is given in the absence of E2 due to simultaneous treatment with an aromatase inhibitor (Whittle *et al.*, 2000).

There is substantial evidence that PGF has an obligatory role in parturition-induced luteolysis, as seen by the patterns of PGFM discussed above. Treatments that initiate premature delivery, also induce the characteristic increase in PGF secretion from the uterus before luteolysis and parturition (Wu *et al.*, 2004). Inhibition of PGF production by treatment with nonsteroidal anti-inflammatory drugs (NSAIDs) will delay or prevent luteolysis near parturition (Jenkin, 1992; Sugimoto *et al.*, 1998; Jenkin and Young, 2004). Finally, mice that have a knockout of the PGF receptor do not undergo luteolysis and therefore do not go through normal parturition, unless the ovaries/CL are removed (Sugimoto *et al.*, 1997; Tsuboi *et al.*, 2003).

Conclusions

Three of the four time period of luteolysis have been well-characterized. In each case, there is a clear role for E2 receptors in the induction of oxytocin receptors. This happens prematurely in the short luteal phase, also during the normal luteolytic process at about day 18-20 in cattle, and during the luteolytic process that results in parturition. After oxytocin receptors are present, oxytocin then induces secretion of PGF from the uterine endometrial cells in each of these luteolytic events. Thus, uterine PGF secretion is an essential part of each time of luteolysis that has been well-studied. In the short luteal phase and normal luteolysis, uterine-produced PGF is transferred through a local veno-arterial pathways and sufficient PGF eventually reaches to CL to result in luteolysis, following multiple PGF pulses. During parturition, it seems likely that there are such high quantities of PGF being secreted by the uterus (10-fold higher PGFM) that PGF may reach the CL through the systemic circulation. After completing this review of the literature, it clearly does not seem typical and the authors could find no convincingly-researched physiological situation that demonstrates spontaneous regression of the ruminant CL in the absence of uterine-derived or exogenous PGF. Thus, the concept that PGF causes all types of luteolysis in ruminants is strongly supported by each type of well-investigated luteolytic event.

The key question that puzzles us after this review of the literature is how is the CL protected from luteolysis during pregnancy, after the interval when IFNT is secreted by the ruminant embryo. During most of pregnancy, the uterus will respond to oxytocin with PGF pulses (Schallenberger *et al.*, 1989; Fuchs *et al.*, 1996) but still luteolysis does not occur. For us, the two most logical responses to this physiological question are: 1) There is a substance protecting the CL from PGF action during much of pregnancy or 2) PGF does not reach the CL during most of pregnancy, perhaps due to lack of PGF transport via the normal local transport pathways. Future research will continue to unlock this and other mysteries about the endocrine and cellular/molecular mechanisms that produce luteolysis and protection from luteolysis in ruminants.

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Follicle development and selection: past, present and future

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Abstract

This paper reviews progress that has been made over the last 50 years and discusses how the development and application of new technologies have been utilized to increase our understanding of the development and selection of the dominant follicle. Our increased knowledge from research carried out worldwide, has demonstrated that the development of the dominant follicle, and importantly the production of a good quality oocyte, are controlled by a set of complex and interactive extra- and intra-ovarian control systems, impacted by underlying genetic and external environmental factors, such as nutrition. In totality this has resulted in improvements in fertility, as demonstrated by the impact of diet on oocyte quality and increased pregnancy rates. However, given the increasing global challenges of food security, coupled with climate change, more in-depth understanding of these complex multifactorial control systems will have even greater significance in overcoming today's livestock production challenges, including some that were present over 50 years ago. In conclusion, the continuing development of new technologies, coupled with new knowledge and understanding of these complex control systems, should ensure that ruminant fertility is maximized, while ensuring good animal welfare within sustainable production systems.

Keywords: cattle, follicle, ovary, reproduction.

Introduction

Understanding the mechanisms regulating ovarian follicle development is central to the diagnosis and treatment of infertility. Cyclical variation in the number and size of ovarian follicles in cattle was being studied in detail over 90 years ago (Hammond, 1927; Rajakoski, 1960; Bane and Rajakoski, 1961). Indeed these studies confirmed the presence of large follicles throughout the greater part of the estrous cycle. This hypothesis of phasic follicular development was also supported by the work of others around that time and more recently (Cupps *et al.*, 1959; Asdell, 1960, Ireland *et al.,* 1979). In a review published over 5 years ago (Scaramuzzi *et.al*., 2011), which provided an update on a previous review published nearly 20 years ago (Scaramuzzi *et al*., 1993) discussing factors that regulate folliculogenesis and ovulation rate in ruminants, there was a dedication to the work of Hannah Peters carried out in the 1960s and 1970s. This work was presented at an ovarian workshop in Glasgow, UK, over 40 years ago. Peters and her colleagues (Peters *et al*., 1975), in studies of ovarian function in rodents and humans, together with information from other scientists published during the previous 40 years, confirmed these earlier ruminant studies. Importantly Peters *et al.* went on to conclude that: (1) the initiation of follicular growth is continuous; (2) follicles grow sequentially and continue to grow until they die or ovulate; (3) the number of follicles that start to grow is dependent upon the size of the small follicle pool; (4) the initiation of follicular growth is independent of gonadotropins and involves intra-ovarian factors; (5) the continued growth of medium and large follicles become increasingly dependent upon gonadotropins and (6) exogenous gonadotropins reduce the incidence of atresia in large follicles.

Since this earlier work over 50 years ago, there have been major technological developments that have provided the tools to elucidate in more detail the patterns and control of follicular growth and confirm whether similar mechanisms are operating in ruminants, as well as in rodents and humans. For example, these technological developments have included more specific and sensitive hormone assays for gonadotropins (Niswender *et al*., 1969; Staigmiller *et al*., 1979), steroids (Webb *et al*., 1985) and metabolic hormones (Gutierrez *et al.,* 1997a); ovarian ultrasound scanning and ovum pick-up (Pierson and Ginther, 1984; Pieterse *et al.,* 1988); physiological models for investigating hormone feedback mechanisms (Hauger *et al.,* 1977; Martin *et al.,* 1988; Price and Webb, 1988; Campbell *et al.,* 1995; Gong *et al.,* 1995); gonadotropin receptor binding studies (Webb and England, 1982a, b; Ireland and Roche, 1983); more physiologically relevant i*n vitro* culture methods for follicular cells (theca (Campbell *et al.,* 1998), granulosa (Campbell *et al.,* 1996; Gutierrez *et al.,* 1997b), oocytes (Fouladi-Nashta *et al*., 2005)), preantral follicles (Gutierrez *et al.,* 2000); molecular biological techniques (Bao *et al.,* 1997; Armstrong *et al.,* 1998; Bao and Garverick, 1998; Garverick *et al.,* 2002) and ovarian tissue transplant studies (Gosden *et al.,* 1994; Baird *et al.,* 1999, Campbell *et al*., 2014).

The integration of this panoply of approaches

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has been crucial in increasing our understanding of the key mechanisms involved in the selection and development of the dominant follicle in ruminants, confirming some of the similar overarching mechanisms as concluded by Peters *et al.,* (1975) for rodents and humans. As will be discussed, studies carried out worldwide have demonstrated the complexity of the overall process, the redundancy of various control systems within the overall process and how these processes can be impacted by the interaction of extra- and intra-ovarian mechanisms, as well as environmental factors such as nutrition.

Follicle development

Folliculogenesis is a lengthy and intricately regulated process, including the dramatic proliferation and precisely controlled differentiation of both the somatic and germ cells (Webb and Campbell, 2007). Much of the earlier work focused on the mechanisms controlling antral follicle development because of the strategic and commercial importance of anovulatory infertility and controlled ovarian stimulation. However, more recently interest in early follicle development has increased because factors, such as nutrition, have been shown to have an impact throughout follicle development, including on oocyte quality. Furthermore, primordial and preantral follicles represent a significant store of oocytes, particularly from animals of high genetic merit, that could be developed to overcome the fact that 99.9% of these oocytes are lost to atresia.

Early follicle development

Primordial follicles represent the source from which follicles will be recruited for growth throughout reproductive life, with individuals containing around 100,000 to 250,000 at birth (Turnbull *et al.,* 1977). Twenty years of work mainly in sheep, but also cattle and pigs, initially transplanting ovarian cortex tissue (Gosden *et al*., 1994), and subsequently whole ovaries (Campbell *et al.,* 2014), to the ovarian pedicle, demonstrated that the tissue does develop a blood supply with subsequent follicle growth and pregnancies. These experiments confirmed the earlier histological experiments that primordial follicles take 3 to 5 months to reach the dominant follicle stage, with much of this time spent in the preantral stages of development.

These experiments provided some of the first evidence that gonadotropins can affect the rate of development of preantral follicles *in vivo*, but also suggested the existence of a gonadotropin-independent intraovarian feedback loop regulating both the rate of primordial follicle initiation and primary and secondary follicle development. Indeed, a large body of work from groups around the world demonstrated that preantral follicles, as well as acquiring steroid enzymes and gonadotropin receptors, acquire a panoply of local growth factors, including members of the insulin-like growth factor (IGF) family and transforming growth factor-β (TGF-β) super family (Campbell *et al*., 2003b, 2014; Webb *et al.,* 2003; McNatty *et al*., 2007; Webb and Campbell, 2007). Furthermore *in vitro* culture studies demonstrated that bovine preantral follicles are responsive to these factors (Gutierrez *et al.,* 2000).

Antral follicle development and maturation

Earlier work in sheep and cattle investigated the patterns of steroidogenesis during antral follicle growth. They demonstrated that testosterone and estradiol are elevated when peripheral preovulatory LH concentrations are rising (England *et al*., 1981). However, there was a significant increase in testosterone in both small and large antral follicles, whereas estradiol increased only in large follicles. Work in mono-ovulatory species, such as cattle, demonstrated that the largest (dominant) follicle is responsible for approximately 90% of the estradiol secreted at estrus (Staigmiller *et al*., 1982). It was also confirmed at this time that the number of thecal and granulosa cell LH receptors in ovulatory follicles was significantly correlated with both follicular fluid estradiol concentrations and with *in vitro* estradiol production. Importantly, the presence of LH receptors, particularly in granulosa cells, was demonstrated to be an excellent marker for the identification of the ovulatory follicle (Staigmiller *et al*., 1982; Webb and England, 1982a, b). Also that the increased number of thecal and granulosa cell LH receptors identified during the preovulatory period, is part of the final maturational process that ensures that ovulation will occur in response to the preovulatory LH surge. Subsequent work, including molecular biological studies up to the present day, have confirmed these findings and demonstrated the importance of local components of both the TGF-β and IGF systems that affect the maturation of the ovulatory follicle and functional competency of the subsequent corpus luteum (CL; Gregson *et al*., 2016).

The use of ovarian ultrasound scanning throughout the estrous cycle in cattle confirmed the wave-like pattern in ovarian follicle development, as small antral follicles progress from a gonadotropin-responsive to gonadotropin-dependent stages of development (Pierson and Ginther, 1984; Ginther *et al.,* 2003). The use of this technology also aided the further clarification of the different stages of follicular development: (i) early gonadotropin-independent (primordial to early preantral), (ii) gonadotropin-responsive (preantral to small antral) and (iii) gonadotropin-dependent (antral to large antral) stages of follicle development in monovular species. The progression of antral follicles through these stages of development requires FSH concentrations to reach a critical threshold level in the peripheral circulation for the synchronous recruitment of a cohort of gonadotropin-responsive small antral follicles (see Ginther *et al.,* 1996; Adams, 1999; Webb *et al*. 1999, 2003; Webb and Campbell, 2007). In one of the first comprehensive studies investigating the action of inhibin, it was concluded that there are basic differences in the way that ovarian feedback acts to control the secretion of LH and FSH in the ewe (Martin *et al.,* 1988). Follicle stimulating hormone secretion was

shown to be primarily controlled by the synergistic action of estradiol and inhibin on the anterior pituitary gland (Baird *et al.,* 1991), while the secretion of LH is inhibited during the follicular phase by the effect of estrogen at the level of the hypothalamus and pituitary gland and during the luteal phase by the synergistic action of estradiol and progesterone at the hypothalamic level.

Additional work in cattle, using laparoscopy, demonstrated that these large luteal-phase follicles, as well as follicles around the time of luteolysis, are also capable of ovulating in response to human chorionic gonadotropin (hCG; Price and Webb, 1989). While these less mature follicles can ovulate during the mid-luteal phase, progesterone production by these induced CL is attenuated (see Webb *et al.,* 1992; Gregson *et al.,* 2016). Gonadotropin-dependent follicles require a constant supply of FSH to continue their development, including the expression of P450 side chain cleavage (P450scc) and P450 aromatase (P450arom) in granulosa cells, together with steroid 17 alpha-hydroxylase (P450c17) in theca cells, which increases the production of estradiol within the granulosa cells of the follicle (Webb *et al*., 1999; Garverick *et al.*, 2002). When bovine follicles reach 7 to 9 mm in diameter, one or two of this group are selected to continue growing and become either the dominant or subordinate follicle (Webb *et al.,* 1999; Ginther *et al.,* 2003; Mihm and Evans, 2008). It has been demonstrated that the future dominant follicle acquires the ability to growth under low FSH conditions by transferring its gonadotropin dependency from FSH to LH (see Fig. 1; Campbell *et al*., 2003b). Furthermore, the decrease in FSH concentration suppresses the emergence of a new follicular wave (Campbell *et al.,* 1995). Numerous studies in both sheep and cattle have shown that a window, requiring appropriate LH concentrations, exists to enable normal pre-ovulatory follicle development (Beg *et al*., 2001; Garverick *et al*., 2002; Scaramuzzi *et al.,* 2011).

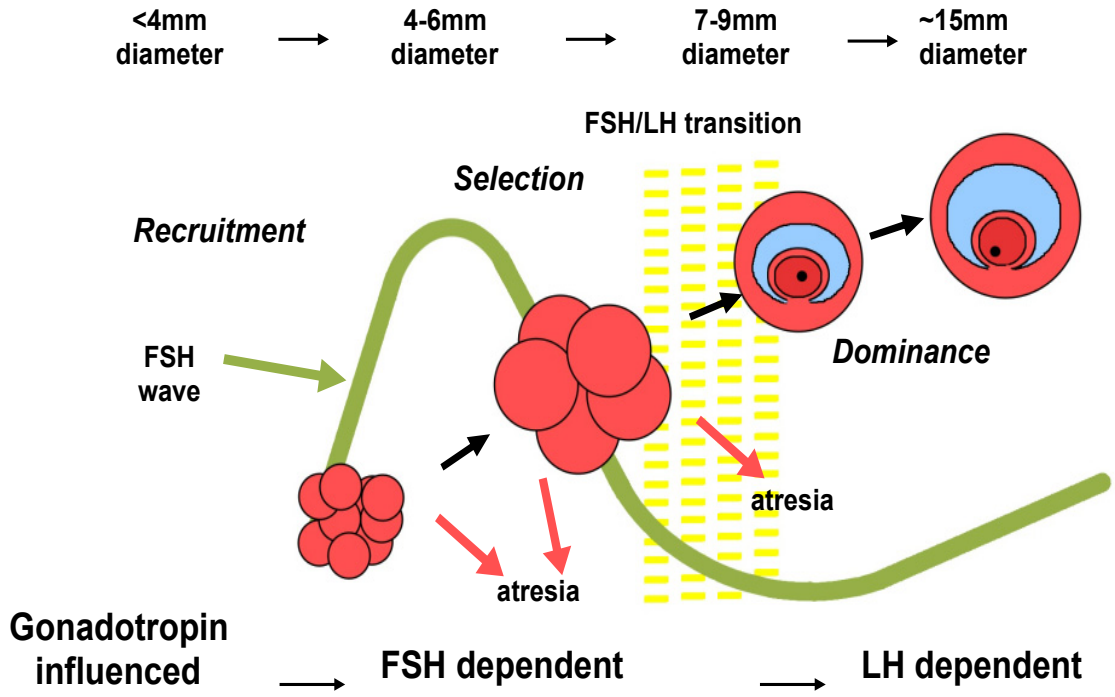

Figure 1. The role of gonadotropins in follicular selection and dominance in cattle. Note the transition period when follicles become LH dependent. Adapted from Webb and Campbell (2007).

Control and manipulation of ovulation rate

Initial work in sheep indicated that follicular dominance appears to be less pronounced in ewes, in comparison to mono-ovulatory species such as cattle (Driancourt *et al.,* 1991). Hence species such as sheep could be more amenable to manipulating ovulation rate for production purposes (see Webb *et al.*, 1999). Indeed treatment with either a variety of steroid antisera or a 3β-hydroxysteroid dehydrogenase inhibitor increased ovulation rate (Land *et al.,* 1983; Webb, 1987). These studies demonstrated that the mechanisms controlling ovulation rate, as distinct from those controlling the occurrence of ovulation, are operative in both the breeding season and seasonal anestrus. Similar experiments in cattle demonstrated that active immunization against a testosterone conjugate resulted in variable responses including anestrus and a high incidence of follicular cysts. However, those heifers that resumed spontaneous estrous activity did so with an increased incidence of multiple ovulations (Price *et al*., 1987).

Mechanisms controlling follicular recruitment into the population of gonadotropin-dependent follicles are different from the mechanisms controlling the selection of the follicles destined to ovulate (Webb *et al*., 1989). A significant amount of work worldwide, in a variety of breeds of sheep with differing ovulation

rates, showed no qualitative or quantitative differences in the pattern of secretion of either pituitary gonadotropins or ovarian hormones (Souza *et al*., 1997). It was postulated that in breeds that carried the major FecB gene, which confers a significant increase in ovulation rate, the major site of action is at the level of ovary. Indeed a number of other significant studies were published by several groups at the turn of the century confirming the single gene effect on ovulation rate was operating at the level of the follicle (see Hanrahan, 2003). This conclusion was confirmed in a subsequent physiological study (Campbell *et al.,* 2003a) where despite suppressing endogenous gonadotropins and subsequently stimulating ovulatory follicle development with exactly the same pattern and level of exogenous gonadotropins, FecB gene carriers continued to display the characteristic increase in the number of ovulatory follicles that mature and ovulate at smaller diameters, when compared with non-gene carriers. These results provided additional evidence that the FecB mutation acts at the level of the ovary by increasing the sensitivity of follicular cells to gonadotropic stimulation.

In a more recent study, a series of six experiments (Gong *et al*., 2016; Roslin Institute (Edinburgh), Midlothian, UK; University of Nottingham, UK unpublished observations) were carried out in cattle to define the physiological concentrations of FSH and LH required for the selection of the dominant follicle. The main conclusions from this study were (1) physiological concentrations of FSH given as a continuous infusion and for an adequate duration, in the presence of basal LH, are capable of inducing a multiple ovulatory response; (2) initial exposure to FSH followed by LH pulses alone can stimulate the development of multiple preovulatory follicles; (3) LH pulses appear not to have a major effect on the pattern of preovulatory follicle development, although adequate LH pulsatile support is required for full estradiol synthesis and the acquisition of ovulatory competence of preovulatory follicles and (4) that the duration of initial exposure to FSH and the ability to transfer the dependence from FSH to LH are critical for the selection of a single dominant follicle.

Overall these studies confirmed that it is more challenging to have controlled increases in ovulation rate in cattle, where there is a greater drive for the development of a single dominant ovulatory follicle, in comparison with sheep. The various patterns of follicular growth and maturation seen in the different breeds of sheep is due to the presence of a number of pathways through which higher ovulation rate can both be achieved, but also manipulated. Possible pathways of ovulation rate control are described in the review of Scaramuzzi *et al*. (2011).

These gonadotropin infusion studies in cattle also demonstrate that with a physiological pattern of gonadotropins, a single ovulation with a functional CL can be achieved. Taken together, the decades of previous work have resulted in a more detailed understanding of mechanisms controlling the development of the dominant follicle for improving MOET protocols. However current protocols still utilize supra-physiological concentrations of gonadotropins resulting in variable outcomes, both in the number of ovulations and the quality of the oocytes particularly in cattle. Recently however, a single injection of a preparation of long-acting recombinant FSH (rbFSH) was shown to produce similar superovulatory responses, resulting in the production of good quality embryos, when compared with a pituitary-derived FSH preparation administered twice daily for 4 days (Carvalho *et al.,* 2014). The authors concluded that more studies using different types of cattle and different doses of rbFSH are needed to confirm the findings reported in this preliminary study. In conclusion further work is still required.

Intra-ovarian control

Over the last two decades, much work has concentrated on the complex actions and interactions between locally produced hormones and growth factors (see Knight and Glister, 2003; Beg and Ginther, 2006; Webb and Campbell, 2007). Numerous interactions are involved in the control of follicle development during the early gonadotropin-independent stages of follicle development and are also involved in modulating the responsiveness of the follicle to gonadotropic signals during the later gonadotropin-responsive and gonadotropin-dependent stages of follicle development in monovular species. These systems include the insulin/IGF system (IGFs; Webb *et al*., 1999), the inhibin/activin system (Campbell and Baird, 2001; Campbell *et al.,* 2003b) and the TGF-β superfamily system (for example bone morphogenetic protein (BMPs), fibroblast growth factors (FGFs) and anti-Müllerian hormone (Souza *et al.,* 2002; Campbell *et al.,* 2006; Monniaux *et al*., 2010; Chaves *et al*., 2012; see Fig. 2).

Moreover, contrary to previous understanding, the oocyte is not a passenger within the follicle as increasing evidence has shown that it is able to modulate follicular development through the production of several oocyte specific secreted factors, among which growth differentiation factor-9 (GDF9; Juengel *et al*., 2006), BMP15 (Galloway *et al*., 2000) and BMP6 (Knight and Glister, 2003, 2006; Campbell *et al*., 2006) are implicated (McNatty *et al.*, 2007). Gap junction-mediated communication between the oocyte and the surrounding somatic cells is essential for the coordinated development of both cell types, including somatic cells providing the oocyte with metabolic substrates and meiosis-arresting signals (Themmen, 2005). The orderly, stage specific expression of this intrafollicular cascade, is essential for continuing and healthy ooctye and follicle development. One of the best described is the transition of the ovarian somatic cells, during the gonadotropin responsive phase, from a proliferative phenotype with a high mitotic index (MI) to a differentiative phenotype with low MI, increased levels of gonadotropin receptors, induction of steroidogenic enzymes and other endocrine and local factors (Webb and Campbell, 2007).

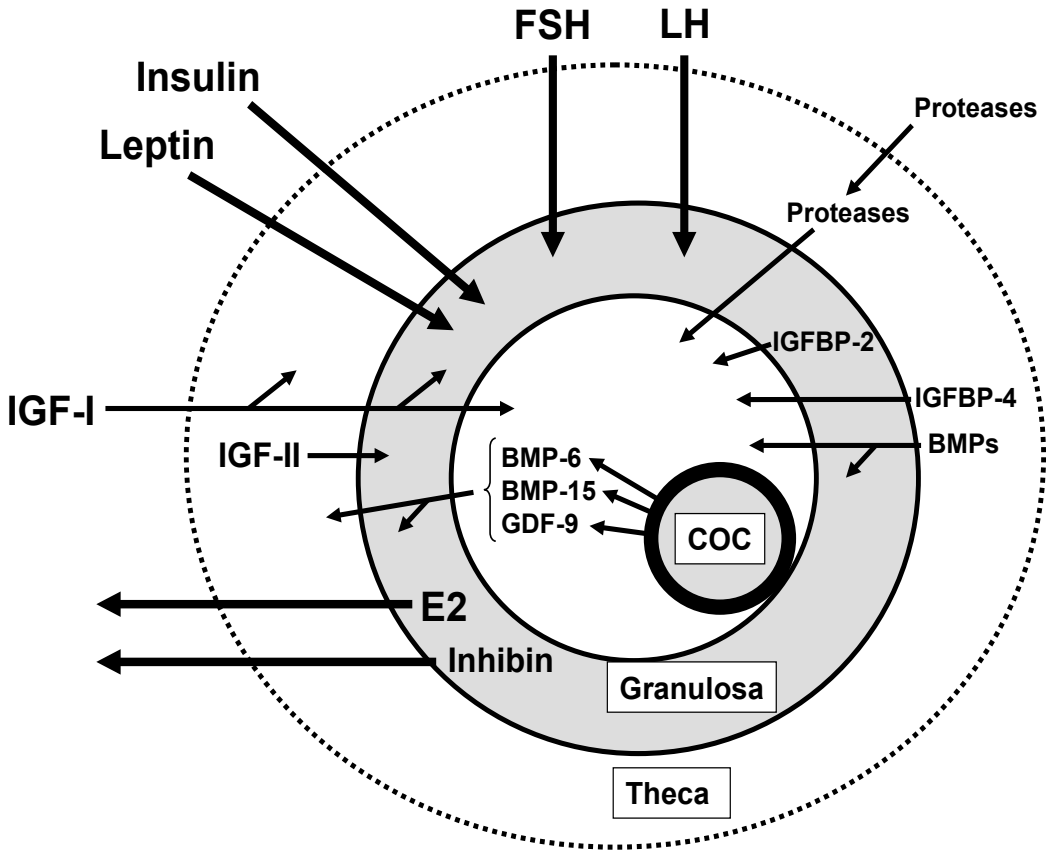

Figure 2. Peripheral gonadotropins and metabolic hormones work in conjunction with follicular growth factors (IGFs, IGFBPs, BMPs, GDF-9), released by granulosa, theca and the oocyte, to regulate development of the follicle and cumulus-oocyte complex (COC). Adapted from Garnsworthy *et al.* (2008).

Insulin-like growth factors (IGFs)

While carrying out studies to delineate the action of the classical gonadotropic hormones, there was an interesting result whereby dairy cows treated with recombinant growth hormone (bovine somatotropin; bST) to increase milk yield had significantly increased twinning rates (see Webb *et. al.,* 1994). Importantly heifers given a dose of bST similar to that given to lactating dairy cows to increase milk yield (Gong *et al.,* 1991) doubled the population of small antral follicles (2 to 5 mm in diameter), a finding that was subsequently confirmed in *Bos indicus* breeds (Buratini *et al*., 2000). Further studies (Gong *et al*., 1993a, 1997) demonstrated that (1) the number of small follicles were reduced as the dominant follicle grew and reached its maximum size; (2) bST could significantly enhance the recruitment of small follicles in heifers and that this increase was positively correlated with peripheral IGF-1 and insulin concentrations and (3) bST did not affect the turnover of follicular waves, nor the inhibitory action of the dominant follicle on its subordinate follicles. Hence bST can enhance the recruitment of small follicles through increases in peripheral concentrations of either IGF-1 and/or insulin concentrations. Indeed, pre-treatment of heifers with bST enhanced the superestimulatory response to pregnant mare serum gonadotropin or equine chorionic gonadotropin (PMSG-eCG)/FSH in terms of the number of ovulations, total number of ova/embryos recovered and number of transferable embryos. Importantly, the quality of embryos also appeared to be improved (Gong *et al.,* 1993b, 1996).

These previous studies demonstrated that growth hormone, IGF-1 and insulin have a significant role in the reproductive axis, as well as in the classical metabolic axis where they stimulate longitudinal growth and enhance muscle mass. *In vitro* studies have also demonstrated the action of IGF-1 on cell proliferation and steroidogenesis in ruminants, but questioned whether IGF-1 is produced in physiologically significant quantities by granulosa cells (see Lucy, 2000; Webb and Campbell, 2007). These previous results and other studies (Armstrong *et al*., 2000) supported the hypothesis that theca-derived IGF-2 is the major intraovarian IGF ligand produced by bovine antral follicles. The expression of mRNA encoding type 1 IGF receptor in granulosa cells from preantral follicles, but before the developmental stage when IGF-2 mRNA is expressed, suggested an endocrine role for IGFs in regulating preantral follicle growth.

As well as the IGF ligands and the receptors, binding proteins have also been shown to have a modulating role. The presence of at least 51 IGF binding protein (BP) isoforms corresponding to IGFBP-1 to -6, many of which were phosphorylated, were detected in bovine follicular fluid from subordinate follicles (Nicholas *et al.,* 2002). The results also confirmed that the total number of IGFBPs was reduced

in dominant follicles. This work demonstrated the high degree of conservation of IGFBP post-translational modifications between species. Furthermore, IGFBP-2 immunoactivity has been shown to be localized in bovine granulosa cells and the basement membrane of healthy preantral follicles, whereas IGFBP-4 immunoactivity was only localized in both theca and granulosa tissue (Armstrong *et al.,* 1998). Of particular interest was the lack of IGFBP-2 mRNA expression in large (>8 mm) gonadotropin-dependent follicles. Utilising serum-free cell culture systems, FSH was shown to inhibit the expression of IGFBP-2 mRNA in granulosa cells, whereas LH stimulated IGFBP-4 mRNA expression in theca cells. Furthermore a significant correlation was demonstrated between the presence of low molecular weight IGFBPs in bovine follicular fluid and caspase-3 activity of granulosa cells from individual follicles (Nicholas *et al*., 2005). In summary these results, along with others, demonstrate the central importance of the IGF system in ruminant follicular development and demonstrate avenues (see Fig. 2; Armstrong *et al*., 2003; Lucy, 2003) through which nutrition can impact on follicular development.

Bone morphogenetic/growth differentiation factors (BMPs/GDFs)

As discussed, these factors are mainly produced by the oocyte, pointing to the influence of these cells on follicular development (McNatty *et al.,* 2007, 2014). Naturally occurring mutations in sheep and humans (Otsuka *et al.,* 2011; Wang *et al*., 2013) of GDF9 (Hanrahan *et al.,* 2004; Juengel *et al.,* 2004; McNatty *et al*., 2007; Nicol *et al.,* 2009; Otsuka *et al.,* 2011; Wang *et al.,* 2013), BMP15 (Galloway *et al.,* 2000; McNatty *et al.,* 2001; Souza *et al*., 2001; Wilson *et al.,* 2001; Hanrahan *et al.,* 2004; Juengel *et al.,* 2004; Monteagudo *et al.,* 2009; Otsuka *et al.,* 2011) and their receptors (i.e. ALK6; Mulsant *et al*., 2001, Souza *et al.,* 2001; Wilson *et al.,* 2001), show increases in ovulation rates in these species. However, when mutations on GDF9 and/or BMP15 genes are homozygous, the ovaries do not show follicular development (i.e. streak ovaries; Hanrahan *et al.,* 2004; Juengel *et al.,* 2004; McNatty *et al.,* 2007; Nicol *et al.,* 2009). On histological examination, follicles in these animals are arrested at the primary stage, indicating that these factors are important signals for follicular development. Data from sheep shows clearly that the BMPs are ineffective in stimulating granulosa cell differentiation in the absence of FSH, but do reveal a clear interaction between the level of IGF and BMP exposure in terms of the induction of aromatase activity (Campbell *et al.,* 2006; see Fig. 2). Thus, in this species both BMP and IGF act to augment FSH-stimulated cellular differentiation.

In vitro and *in vivo* work, carried out in our laboratory, with BMP15 yielding equivocal results. Ovine granulosa cells treated with low doses of rhBMP15 alone showed mild increases in FSH-stimulated estradiol production while higher doses were markedly inhibitory. Bone morphogenetic protein 15 is, however, known to form heterodimers with GDF9 (Mottershead *et al.,* 2013) and exposure of granulosa cells to both rmGDF9 and rhBMP15 induced a 10-fold increase in estradiol production when compared to the same doses in isolation (A Marsh, J Hernandez-Medrano, BK Campbell, 2016; Dept Obstetrics and Gynaecology, University of Nottingham, UK, Faculty of Veterinary Medicine, UNAM, Mexico City, Mexico; University of Nottingham, Queen's Medical Centre, Nottingham, UK; unpublished results). Moreover, *in vivo* experiments in sheep using either *in situ* ovarian cannulation or an ovarian autotransplant model, showed that direct infusion of rhBMP15 resulted in small, but statistically significant increases in ovarian androstenedione and estradiol secretion. This was confirmed by direct ovarian infusion of antisera specific ovine BMP15 sequences (J Hernandez-Medrano, BK Campbell; 2016; Faculty of Veterinary Medicine, UNAM, Mexico City, Mexico; University of Nottingham, Queen's Medical Centre, Nottingham, UK; unpublished results). Therefore, the physiological importance of BMP15 in ruminant species appears more complex than indicated by the phenotypes observed in the sheep mutation carriers. However there is recent evidence (Behrouzi *et al*., 2016) that BMP15, GDF9, and TGF-β1 can be up regulated in bovine preovulatory follicles, providing a possible link with improved pregnancy rates.

Fibroblast growth factors (FGFs)

Follicular development is regulated by a combination of inhibitory as well as stimulatory mechanisms. Evidence from *in vivo* and *in vitro* studies in cattle point to an anti-steroidogenic inhibitory role for FGFs (Chaves *et al*., 2012). Interestingly, the capacity to prevent or attenuate FGF action appears to be included in the various mechanisms leading to selection of the dominant follicle and its continued growth towards ovulation. In the bovine follicle wall, expression of FGF17 and FGF18, members of the FGF8 subfamily, is highest in atretic granulosa and theca cells, respectively. This expression pattern, combined with the inhibitory effects of FGF17 and FGF18 proteins on estradiol and progesterone production from cultured granulosa cells, suggest their involvement in paracrine/autocrine loops leading to follicular atresia (Machado *et al.,* 2009; Portela *et al.,* 2010). Alternatively, FGF10, a member of the FGF7 subfamily, appears to exert its inhibitory role in a different context. Several lines of evidence point to the participation of FGF10 in the regulation of follicle selection. Expression of *FGF10* was detected in bovine oocytes and theca cells, where lower levels of the transcript were observed in healthy follicles. In contrast, FGF10 receptors (FGFR1B and FGFR2B) are expressed in both theca and granulosa cells at lower levels in healthy follicles. More importantly, FGF10 inhibited estradiol production from granulosa cells *in vitro* and when injected into the future dominant follicle, also blocked follicular growth (Buratini *et al.,* 2007; Gasperin *et al.,* 2012; Castilho *et al.*, 2015). More recently, mRNA abundance of FGF10 was reported to be lower in the future dominant follicle compared with

the largest subordinate follicle before morphological divergence, whilst levels of FGFR2B mRNA were higher in granulosa cells from the subordinate follicle (Castilho *et al.,* 2015). Data from this study also indicate that the mechanisms by which FGF10 supress follicular steroidogenesis involve a reduction in the sensitivity of the follicle to FSH and IGFs, as FGF10 decreased mRNA abundance of FSHR and IGFR-1 in granulosa cells. The interaction between the FGF and IGF systems in the control of follicular development is further substantiated by the inhibitory effect of IGF1 on FGFR2B expression in granulosa cells (Buratini *et al.,* 2007). Therefore, a feedback loop involving the FGF and IGF systems appears to be important for dominant follicle selection. The inhibition of the FGF10 system contributes to enhance IGF signalling, whilst IGF signalling attenuates the inhibitory influence of FGF10 in the future dominant follicle (see Fig. 3).

In addition to the regulation of steroidogenesis in the follicle wall, FGFs are expressed by the oocyte and appear to mediate oocyte-cumulus interactions in the antral follicle. When supplemented during *in vitro* maturation (IVM) of bovine cumulus-oocyte complexes, FGF10 enhanced cumulus expansion, while increasing PTGS2 expression and glucose uptake by cumulus cells. As glycolytic activity was not altered by FGF10, the increase in glucose uptake most likely provides more substrate for hyaluronic acid, thus leading to more exuberant expansion (Caixeta *et al*., 2013). Fibroblast growth factor 17 was also capable of enhancing cumulus expansion when added to the IVM medium, and interacted with BMP15 to increase embryo quality as measured by number of cells in the inner cell mass. In summary, the FGF system adds another level of complexity in the control of dominant follicle development.

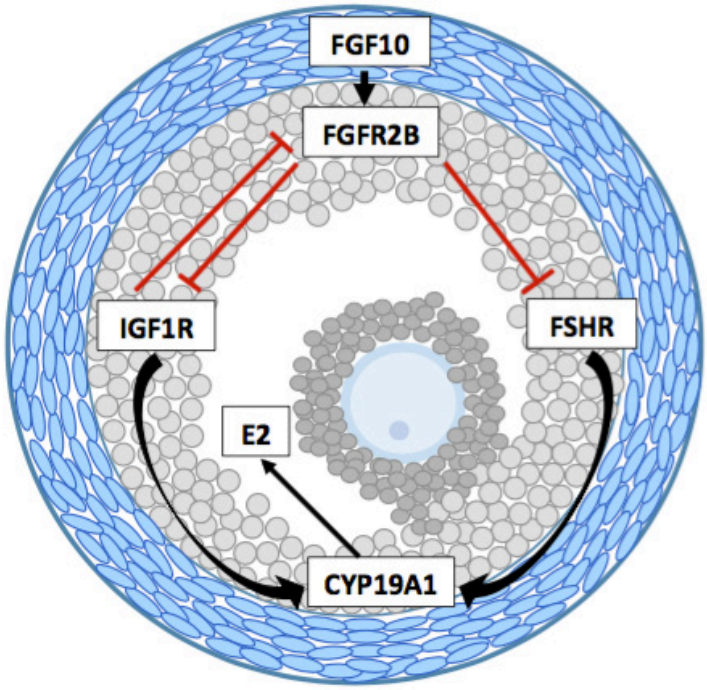

Figure 3. Proposed model for the participation of FGF10 in the control of estradiol production during follicle selection. FGF10/FGFR2B signaling is enhanced in the future subordinate follicle and decreases sensitivity to FSH and IGF, leading to a reduction in CYP19A1 expression and, consequently, estradiol production. The inhibition of IGF signaling by FGF10 would also contribute to enhance and prolong FGF10 signaling in the future subordinate follicle, as IGF signaling inhibits FGFR2B expression.

Vascular endothelial growth factor (VEGF)

Growth of the dominant follicle, and of the CL, also require the parallel growth of a capillary network to sustain them to ensure the supply of oxygen and trophic hormones. VEGF is a key signal that promotes angiogenesis and it is indispensable for the growth of antral follicles (Taylor *et al.,* 2007; Robinson *et al*., 2009) and CL (Guzmán *et al.,* 2014). However, as for the IGF-1 system, the VEGF system is complex and composed of two receptors and 6 ligand isoforms. It has been shown that VEGF mRNA, for isoforms 120, 164 and 205, are expressed in both granulsoa and theca cells. The inception of atresia was accompanied with disappearance of the expression of VEGF205 and a reduction in mRNA for VEGF164 and VEGF120 in granulosa cells. This was evident in theca cells in advanced stages of atresia (Rosales-Torres *et al.,* 2010). In addition, protein and mRNA expression of the extracellular domain of the VEGF receptors has been reported in the dominant follicle. These soluble proteins are released to act extracellularly, to bind and block the action of VEGF. The expression of soluble VEGF receptor 1 (VEGF-R1) declines as the follicle grows. In contrast, the sVEGF-R2, which has higher affinity to the ligand, increases as the dominant follicle grows (Macias *et al.,* 2012). This increase in an antagonistic soluble receptor could decrease follicle angiogenesis just prior

to ovulation to avoid excessive bleeding at the ovulation site.

Anti-Müllerian hormone (AMH)

Among the factors implicated in early follicular development is AMH (Durlinger *et al*., 2002; Ireland *et al*., 2011; Campbell *et al*., 2012; Monniaux *et al*., 2014; Estienne *et al*., 2015). AMH is a dimeric glycoprotein member of the TGF-β superfamily (Rey *et al.,* 2003; van Houten *et al*., 2010; Monniaux *et al*., 2014) and produced in granulosa cells from the primary follicle stage, increasing in preantral and small antral follicles, and gradually decreasing in mid- to large-antral follicles (Rico *et al.,* 2011; Campbell *et al*., 2012). Anti-Müllerian hormone knock-out studies in mice (Durlinger *et al*., 2002) and sheep (Campbell *et al*., 2012) have reported increases in the number of developing follicles suggesting that AMH is involved in both the regulation of transition from primordial into primary follicles (Gigli *et al.,* 2005) and the rate of progression of gonadotropin-responsive to the gonadotropin-dependent stage. Furthermore, recent studies of follicle number and AMH in cattle have concluded that the inherently high variation in the ovarian reserve (Mossa *et al.,* 2012), which is both heritable (Walsh *et al.,* 2014) and also affected by maternal undernutrition (Mossa *et al*., 2013), has a negative impact on ovarian function that may result in suboptimal fertility (Ireland *et al*., 2011). Furthermore it appears that AMH measurement can be used to assess fertility in cattle, as AMH is linked to follicle number (Monniaux *et al*., 2010; Ireland *et al*., 2011).

Regarding the mechanism of action, AMH has been shown to inhibit gonadotropin dependent growth of cultured mouse preantral follicles (Durlinger *et al*., 2002; Themmen, 2005; Bertold *et al.,* 2016) and in rat, pig and sheep granulosa cell cultures, AMH attenuates the FSH-dependent increase in aromatase activity (see Fig. 4), estrogen synthesis/release and LH receptor expression (di Clemente *et al*., 1994; Hernandez-Medrano *et al.,* 2012). Additionally, recent *in vitro* observations have demonstrated that AMH is a potent inhibitor of LH-stimulated androgen production in theca cells. This was supported by increasing concentrations of androstenedione in follicular fluid observed in small antral follicles following immunisation against AMH in sheep. Furthermore, AMH concentrations have been shown to increase *in vitro* (Elfituri, 2016) and *in vivo* (Narkwichean *et al*., 2014) following treatment of granulosa cells and ewes, respectively, with aromatisable and non-aromatisable androgens, thus providing further evidence that androgens may have a direct role in the regulation of AMH production. Further, the observation that readily diffusible VEGF variants associated with growing follicles (VEGF120 and VEGF164) act as negative regulators of granulosa cell AMH expression support a model whereby AMH may be central to the development of thecal cell function in gonadotropin responsive follicles, both in terms of steroidogenesis and angiogenesis. Figure 5 outlines a proposed model of the control of AMH expression in granulosa cells under the stimulatory influence of oocyte secreted factors (GDF9 and BMP15) and diffusion gradients originating from the oocyte and inhibitory influence of thecal cell derived androgen.

In conclusion there are a range of intra-ovarian factors that act at all stages of follicular development in concert with gonadotropin drive. These complex, and possibly, redundant systems ensure that the dominant follicle will produce a quality oocyte at ovulation. They also highlight the various sites whereby extra-ovarian and environmental factors can impact on ovarian function and follicular development.

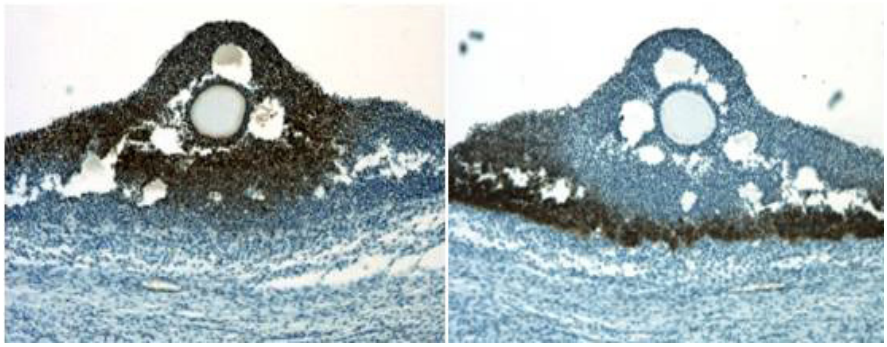

Figure 4. Expression of AMH (left) and Aromatase (right) protein in consecutive sections from the same gonadotropin-dependent large antral sheep follicle. Note AMH expression is absent from the differentiated granulosa cell layer but present in coronal/cumulus cells surrounding the oocyte and is directly inverse to aromatase expression. Adapted from Campbell *et al.* (2012).

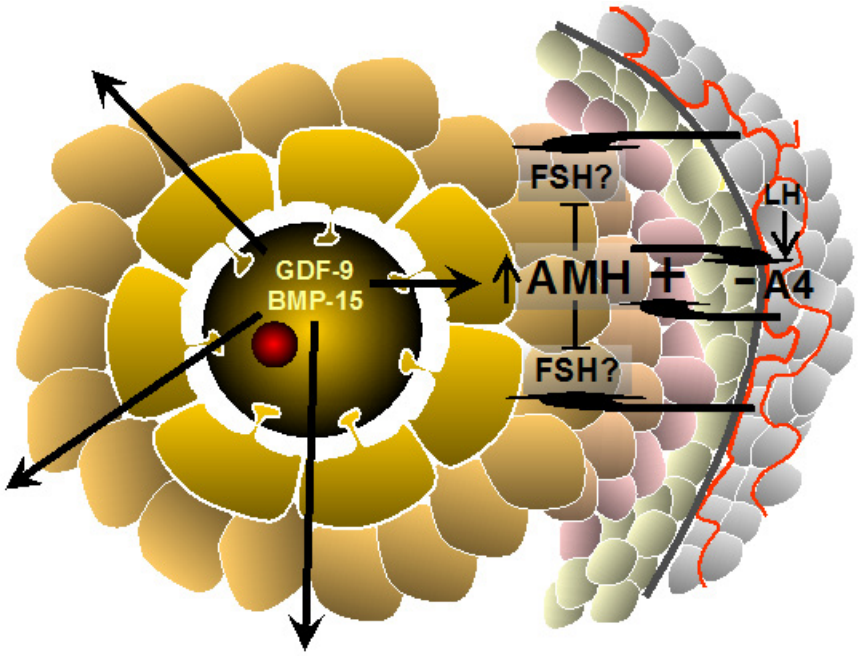

Figure 5. Proposed model of control of AMH expression in granulosa cells under the stimulatory influence of oocyte secreted factors and diffusion gradients. These originate from the oocyte. Also note the inhibitory influence of thecal cell derived androgen. Adapted from Campbell *et al.* (2014).

Nutritional influences

As discussed, even though follicular and oocyte development occurs as a sequence of well-synchronised events, they are susceptible to environmental interferences. This is clear from the effect that growth hormone, working via IGFs and insulin, can influence follicular development and oocyte quality (see Buratini *et al*., 2000; Webb and Campbell, 2007; Sartori *et al*., 2013). Indeed the response of domestic animals to their environment is an important determinant of reproductive efficiency. Environmental influences such as temperature, and the quantity and quality of food all impinge on various facets of reproductive function.

Nutritional supplementation has long been known to improve reproductive performance and to enhance the lambing rate of sheep and goat flocks (Scaramuzzi and Martin, 2008; Martin, 2009). As early as 1899 (Heape, 1899) it was recognized that fatter sheep, or sheep with higher nutritional intake produced a higher proportion of twin lambs. Subsequent studies have confirmed that as body weight of the ewe increases, there is also an increase in the ovulation and subsequent twinning rate (Coop, 1962; Morley *et al.,* 1978). Furthermore three decades ago in beef cattle, it was shown that calving cows in a higher body condition have improved reproductive performance (Wright *et al.,* 1987).

In comparison with cattle, follicular waves in small ruminants are not as clearly spaced. Furthermore, sheep and goats appear to have follicles capable of reaching maturity and ovulating within 60 to 80 h, when luteolysis is induced at any time of the estrous cycle. These observations led to the concept that the period required to achieve an increase in ovulation rate by flushing need not be longer than the length of the recruitment phase, if given at a time where follicles predestined for ovulation are selected (Gutierrez *et al.,* 2011). The precise requirement for this stimulus seems to be the period of increased LH pulse frequency, following decline in progesterone concentrations. Indeed, short term flushing prior to luteolysis increases ovulation rate when given either as an oral glycogenic substance (Letelier *et al.,* 2008; Gutierrez *et al*., 2011), or when lupin grains are added to the diet (Downing *et al*., 1995; Muñoz-Gutierrez *et al.,* 2004), or by intravenous infusion of either glucose (Muñoz-Gutierrez *et al.,* 2002; Gallet *et al.,* 2009) or glucosamine (Muñoz-Gutierrez *et al*., 2002). The increase in ovulation rate seems to be related to an increase in glucose, insulin and leptin concentrations, but without detectable changes in peripheral IGF-1 or FSH (Viñoles *et al*., 2005).

In further studies, the period of flushing was shortened to a single oral drench, using soluble substrates that acutely increase propionic acid in the rumen and glucose concentrations in blood (Ferraro *et al.,* 2009, 2016). In addition, the effect of these substances was short-lived, limiting the effect to a defined period of time. In two studies, glucogenic drenching, at the time of either luteolysis or progestin withdrawal, increased ovulation rate in sheep, with around 90% of ewes having multiple ovulations (see Table 1; Gutierrez *et al*., 2011). The increase in ovulation rate may be due to an increase in circulating concentrations of insulin, but not IGF-I, that lasted for 12 h after drenching (Martinez, 2004; Ferraro *et al.,* 2016). In addition, the rise in ovulation rate was not accompanied by an increase in the number of small (<4 mm) and large (>4 mm) follicles, nor was the diameter of the three largest follicles affected. However, there was a decrease in the number of mRNA transcripts for

P450 aromatase, with no changes in either the LH receptor or 3β-HSD (Ferraro, 2011). These studies confirm, in sheep, that the twelve hours following luteolysis are fundamental, not only for the selection of ovulatory follicles and the determination of ovulation rate, but that this period of development is very sensitive to the impact of environmental factors such as nutrition.

Table 1. Frequency of multiple ovulations in Pelibuey ewes treated with oral administration of a glycogenic solution (300ml of glycerol: water solution; 90:10 v/v) at the time of estrous synchronization with PGF2α or FGA+ PGF2α.

Treatment group	N	Ovulation type (%)				Ovulation rate
		Single	Double	Triple	Quadruple	
Synchronisation with PGF2α						
Control[a]	58	39.66	60.34	0	0	1.6 ± 0.06[a]
Glycerol[b]	74	10.81	71.62	16.22	1.35	2.08 ± 0.06[b]
Synchronisation with progestins						
Control[a]	55	39.62	56.60	3.77	0	1.64 ± 0.07[a]
Glycerol[b]	53	5.66	50.94	39.62	3.77	2.41 ± 0.09[b]

[a,b]Different superscripts within a column differ (P < 0.01). From Gutierrez *et al*., 2016; Faculty of Veterinary Medicine, UNAM, Mexico City, Mexico; unpublished observations.

Despite the recent improvement in reproductive performance of high-yielding dairy cows, promoted by both pharmacological control of follicular growth and ovulation for fixed time AI and genetic improvements, reduced fertility still has major implications for economic sustainability (Chagas *et al.,* 2007; Wiltbank *et al.,* 2011). In the case of high-yielding dairy cows reproductive performance had been declining for a number of decades due to the selection for increased milk yield (Royal *et al*., 2000), especially when animals are under negative energy balance (Butler, 2003). Energy balance is defined as energy intake minus energy output in milk. If energy intake is insufficient to meet the demands of milk secretion, as it usually is in early lactation, body reserves are mobilized. If energy intake exceeds requirements, excess energy is deposited as body fat, but over the long-term cows can adjust their energy intake in an attempt to keep levels of body energy reserves constant (Garnsworthy, 1988; Garnsworthy and Webb, 1999). In postpartum beef cows recent results demonstrated that an increase in lean tissue, in relation to body fat, can result in diminished serum concentrations of leptin and IGF-1 (Guzman *et al*., 2016). These hormonal changes compromise the response to estrus induction resulting in reduced estrus and estrous cycles after treatment, and pregnancies at the end of the breeding period, further demonstrating the interrelationship between the classical metabolic hormones and reproductive function.

In dairy cattle there has been significant research effort worldwide to both elucidate the underlying mechanisms and to devise nutritional strategies that can maintain milk production and quality, while stimulating dominant follicle development, maintaining oocyte quality and hence improving fertility. From the previous discussion it is clear that because of the interactions between extra- and intra-ovarian regulatory systems an integrated approach is required, because changes in one system can impact on other systems. There is also a requirement to consider general homeostatic and homeorhetic mechanisms simultaneously with the mechanisms involved in the initiation of ovarian cycles postpartum, development of the dominant follicle and the production of a good quality oocyte. For example, high insulin diets that result in higher peripheral insulin concentrations result in a significantly higher number of poor quality oocytes (Adamiak *et al.,* 2005), whereas the addition of fat to a high-starch diet improved blastocyst yield (Fouladi-Nashta *et al.,* 2007). In a large series of studies (see Garnsworthy *et al*., 2008), based on our increased understanding of the factors affecting follicular development and oocyte quality, it was demonstrated that the optimum strategy for improving fertility in high-yielding dairy cows was initially to feed a diet that stimulated follicular development and then to feed a diet that improved the developmental competence of oocytes (Garnsworthy *et al*., 2009). This information is now being applied within the UK dairy industry, in conjunction with genetic fertility index information, to reverse the previous long-term decline in fertility of high yielding dairy cows. However, as reviewed and concluded by Leroy *et al*., (2013), further work is required to elucidate both the direct and indirect effects of dietary lipid supplementation on oocyte quality and pregnancy rates.

Conclusions

Over the last half-century our understanding of the control of follicle development and selection in ruminants has increased immeasurably with the application of a range of new technologies. This has resulted in the realization that follicular development and selection of the dominant follicle and the production of a good quality oocyte are controlled by a set of complex and interactive systems, including (i) the requirement for continuous gonadotropic drive in the later stages of development, (ii) the interaction of a range of local intraovarian growth factor systems and (iii) genetic and environmental influences. A plethora of studies worldwide, have shown that this increased

understanding can result in improved fertility, as demonstrated by the impact of diet on oocyte quality and increased pregnancy rates. Importantly, continuing improvements in the reproductive performance of domestic ruminants will require further increased understanding of these multifactorial mechanisms that control ovarian follicle development. For example, there will be opportunities as new understanding and new technologies are developed, as demonstrated by the identification of alleles that could be beneficial for estrous behavior and hence provide an opportunity for selection of cows displaying stronger estrous activity (Homer *et al.,* 2013).

In conclusion, despite significant progress there is still a need for improved estrous detection and conception rates, reduced embryo loss and the ability to control superstimulatory responses more precisely. These are livestock production issues that were present over 50 years ago and which have become even more important to improve further given the increasing global challenges of food security and climate change.

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DNA methylation dynamics during oocyte and embryo development and its association with environmental induced alterations

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Abstract

Epigenetic mechanisms are fundamental to successful gametogenesis and development. The fertilized egg undergoes global DNA demethylation to facilitate remodelling from two differentiated gamete-specific states to a pluripotent embryonic state. Maintenance of appropriate levels of DNA methylation during preimplantation development is essential to embryo viability. Recent advances in epigenetic research have highlighted the susceptibility of foetal epigenetic programming to maternal health and nutritional status, particularly, at the time of conception. There is much evidence that maternal stress impacts on ovarian function, leading to compromised oocytes presented for fertilization in a suboptimal environment. Similarly, declining fertility has become a substantive issue in western countries, where it is primarily associated with high mean ages at childbearing. Thus the use of assisted reproduction technologies (ART) interventions to overcome low fertility is increasing steadily across the globe. In addition, the use of prolonged in vitro culture following the removal and storage of oocytes and/or ovarian tissue in advance of cancer treatment, or to circumvent ovarian aging, is increasing rapidly. ART is associated with compromised pre and post -natal outcomes, including premature birth, low birth weight, congenital abnormalities and elevated risk of epigenetic disorders. There is extensive evidence from studies in cattle that embryos produced by conventional ART protocols are susceptible to errors in epigenetic programming. The present review discusses the impact of intrinsic physiological status and external environments on oocyte and embryo DNA methylation with regard to data available from mouse, human and bovine models.

Keywords: epigenetic, oocyte, embryo, mammals.

Introduction

Genomic imprinting is an epigenetic process, which enables parent-of-origin expression of a cohort of mammalian genes (Fowden *et al*., 2006). The correct dosage of imprinted gene expression has been shown to be critically important to embryonic growth, development, placental function and postnatal behaviour and metabolism (Reik and Walter, 2001; Davies *et al*., 2005; Smith *et al*., 2006). Parent-of-origin expression of imprinted genes is facilitated through asymmetrical epigenetic marks on either the maternal or paternal allele. Generally, imprinted genes are arranged in clusters containing differentially marked, CpG rich domains, known as differentially methylated regions (DMRs); the most extensively studied of these marks is DNA methylation (Lucifero *et al*. 2004). Mammalian DNA methylation patterns required for genomic imprinting are subject to periods of dynamic reprogramming during development and are established at different developmental time points, depending on whether they are transmitted through the male or the female germline. Paternal DMRs acquire their methylation marks prior to birth in the prospermatagonia (Davis *et al*., 2000; Li *et al*., 2004); while DNA methylation marks at maternal DMRs are established postnatally in the growing oocyte (Hiura *et al*., 2006). Epigenetic reprogramming involves DNA methylation erasure and re-establishment at two points in the life cycle, firstly after fertilization in the zygote, and secondly in primordial germ cells (PGCs), which are the direct progenitors of sperm or oocyte. Following fertilization, global DNA demethylation occurs in the zygote to facilitate remodelling from two distinct differentiated gamete-specific states to a pluripotent embryonic state (Hales *et al*., 2011; Torres-Padilla and Ciosk, 2013). The epigenetic signature inherited from the gametes (excepting the parental imprints) is erased and developmental totipotency is restored. In the PGCs, parental imprints are erased and de novo establishment of new methylation landscapes that are different between male and female germlines restore germline developmental potential (Seisenberger *et al*., 2013). There is extensive evidence in cattle, human and laboratory model species that intrinsic physiological status and external environments, such as assisted reproductive technologies (ART), can dramatically alter the epigenetic landscape of gamete, embryonic, foetal and adult tissues, leading to impaired function/ adverse health outcomes in adult life. The mechanisms and implications with regard to data available from mouse, human and bovine models are reviewed.

DNA methylation dynamics during epigenetic reprogramming in the germline and preimplantation embryos

DNA methylation erasure

Recent studies have started to elucidate how global demethylation in the zygote and PGCs is orchestrated. It appears that active demethylation occurs on the paternal genome in one-cell embryos (Maher and Reik, 2000; Oswald *et al*., 2000) and passive demethylation occurs on the maternal genome from the two-cell until the blastocyst -stage (Reik *et al*., 2001). It would appear that the major wave of genome-wide demethylation occurs between fertilization and the two-cell stage in humans with only subtle changes in DNA methylation levels occurring thereafter (Guo *et al*., 2014). In mouse, the most important period of demethylation occurs at the zygote stage, followed by gradual demethylation until the blastocyst stage (Smith *et al*., 2012). In both human and mouse, demethylation of the paternal genome occurs much faster than that of the maternal genome (Guo *et al*., 2014; Smith *et al*., 2014). Several models for active DNA demethylation have been proposed (Morgan *et al*., 2005; Wu and Zhang, 2010): Active demethylation mediated by the ten-eleven-translocation (TET) family member, Tet3, appears to

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be important for both (Guo *et al*., 2014; Smith *et al*., 2014), while Base Excision Repair (BER) pathway may also be important (Xu and Walsh, 2014). Maintenance of appropriate levels of DNA methylation during preimplantation embryo development is essential for the viability of embryos (Barton *et al*., 2001). As well as the marked allele being resistant to demethylation, equally important appears to be the ability of the unmarked allele to resist de novo methylation following implantation (Proudhon *et al*., 2012; Rutledge *et al*., 2014).

PGCs arise in the epiblast of the developing embryo (Ginsburg *et al*., 1990), where they inherit the somatic epigenetic traits that are present in the cells of the epiblast at this time, including significant levels of global DNA methylation (Seki *et al*., 2005; Ohinata *et al*., 2009). Therefore, PGCs need to reprogramme from a somatic profile into that of germ cells that will give rise to the gametes, with the capacity to form the totipotent zygote in the next generation. DNA methylation is globally erased during migration of PGCs towards the genital ridge. Epigenetic reprogramming in PGCs requires remodelling of the chromatin structure, global changes in the transcriptional landscape and the resetting of imprint DNA methylation marks. It is essential for the development of the next generation that the parental imprints are erased in PGCs and that new imprints are established that reflect the gender of the embryo. These imprints are then maintained in the gametes derived from the PGCs and will contribute to the epigenome of the future zygote (Seisenberger *et al*., 2013).

The mechanisms involved in PGC demethylation have been difficult to elucidate, due to the inaccessible nature of PGCs. Up until recently, studies in mouse focused on the period between E11.5 and E13.5 (Reik *et al*., 2001; Hajkova *et al*., 2002). The short duration of this period, specifically the low number of cell cycles, lead to the concensus that DNA demethylation in PGCs was an active process. The protein activation-induced deaminase (*Aid*), has been conclusively shown to be involved in global DNA de-methylation in PGCs, whereas TDG, BER and the TET proteins have been proposed without definitive proof (Seisenberger *et al*., 2013). There is some evidence to support the possibility that DNA methylation erasure is initiated earlier, at the time of the down regulation of the DNA methylation machinery transcripts (Seki *et al*., 2005; Kurimoto *et al*., 2008; Guibert *et al*., 2012). If the period of global erasure is extended back to this stage, then passive demethylation also becomes a possibility.

Reprogramming of DNA Methylation

During spermatogenesis and oogenesis, epigenetic modifications are established which are required for normal embryonic progression (Bourc'his *et al*., 2001; Rideout *et al*., 2001; O'Doherty *et al*., 2012). Reprogramming the maternal genome, through appropriate DNA methylation of differentially methylated regions (DMRs), is central in regulating genomic imprinting (Li *et al*., 1993), a process in which a small cohort of genes are exclusively expressed from a single allele, according to parent-of-origin (Preece and Moore, 2000). Imprinted gene DMRs are actively established during mammalian gametogenesis (Reik *et al*., 2001), the vast majority of which acquire DNA methylation from the maternal germline during the oocyte growth phase (Ueda *et al*., 1992; Lucifero *et al*., 2002, 2004; Hiura *et al*., 2006). During postnatal mouse oogenesis, imprinting establishment occurs asynchronously at different imprinted genes during the transition from primordial to antral follicle stages (Lucifero *et al*., 2004; Hiura *et al*., 2006;). The DMRs tested in human and bovine oocytes have shown the expected pattern of methylation (Geuns *et al*., 2003, 2007; El Hajj *et al*., 2011; Heinzmann *et al*., 2011; O'Doherty *et al*., 2012; Urrego *et al*., 2014). However, there appear to be differences in the mechanisms and timings of imprint establishment among mammals, (see review by (Hanna and Kelsey, 2014)). For example, the oocyte growth phase in humans and cattle is quite protracted, lasting months (Fair, 2003; Fair *et al*., 1997). Acquisition of the maternal imprints commences in the final phase of oocyte growth and the individual imprints appear to be established in a size-specific manner (Geuns *et al*., 2003, 2007; Arima and Wake, 2006; Sato *et al*., 2007; O'Doherty *et al*., 2012). In bovine, for example, acquisition of the maternal imprints commences when oocytes reach a diameter of 110 to 120 µm; *SNRPN* and *MEST* are fully methylated by 120 µm, whereas *IGF2R*, *PEG10* and *PLAGL* were only partially methylated at this size (O'Doherty *et al*., 2012). Recent studies have established that gene transcription determines the characteristic DNA methylation landscape of the mature murine oocyte. Indeed it was determined that transcription events could account for 85-90% of DNA methylation established in the oocyte, including methylated CpG islands (CGIs) and imprinted DMRs (Veselovska *et al*., 2015). However, a small number of expressed genes escape DNA methylation, as well as a small number of CGIs within active transcription units. Indeed, it suggests that gene expression perturbations during oocyte follicular growth could result in alterations in DNA methylation in mature gametes, including at CGIs. Since a fraction of the oocyte DNA methylome is maintained to some extent in pre-implantation embryos just before the embryonic onset of de novo methylation, environmentally induced changes in gene expression in female germ cells could lead to alterations in the epigenome of the next generation, with possible transgenerational effects (Borgel *et al*., 2010; Smallwood *et al*., 2011). Thus, physiological or external factors that disrupt the transcription environment during oocyte follicular growth could result in alterations in DNA methylation in mature gametes.

Genome-wide studies in human gametes and embryos have revealed many similar mechanisms of global DNA methylation reprogramming between humans and mice (Guo *et al*., 2014; Smith *et al*., 2014). Imprinting marks must be maintained during the sensitive oocyte-to-embryo transition. As this stage of development is under maternal control, the role of maternal effect genes in genomic imprint regulation is of interest. Several key maternal effect proteins that protect imprinted methylation sites during preimplantation development have been identified and include: PGC7 (also known as STELLA or DPPA3): In mice, PGC7 was shown to maintain imprints at a subset of loci, including *Peg1, Peg3,* and *Peg10* domains and the paternally imprinted *H19-Igf2* (H19DMR) and *Rasgrf1* domains (Nakamura *et al*., 2007); zinc finger protein 57 (ZFP57), which is required for the methylation of the *Snrpn* domain in mouse oocytes and for the maintenance of DNA methylation after fertilisation at *Dlk1, Peg1, Peg3* and *Nnat* domains (Li *et al*., 2008); tripartite motif-containing 28 protein (TRIM28; also known as KAP1/TIF1b), TRIM28 may regulate epigenetic stability in mouse oocytes and embryos by protecting against passive demethylation (Messerschmidt *et al*., 2012) and finally, DNMT1 (Hirasawa *et al*., 2008). Two DNMT1 isoforms are present in mature oocytes and preimplantation embryos: oocyte-specific (DNMT1o) and somatic (DNMT1s) isoforms. DNMT1o accumulates during oocyte growth (Bao *et al*., 2000), and is the abundant form expressed in oocytes and preimplantation embryos (Mertineit *et al*., 1998). There are five known family members of the DNMTS (DNMT1, DNMT2, DNMT3A, DNMT3B AND DNMT3L*)*, which are believed to be responsible for establishing and maintaining methylation patterns (Ooi *et al*., 2009). Gene targeting studies in mice have demonstrated that the methyltransferases DNMT3A, DNMT3B and DNMT1 are indispensable for

embryonic survival (Li *et al*., 1992; Okano *et al*., 1999). These findings are likely to apply also in cattle, as we have demonstrated the presence of DNMT3A, DNMT3B and DNMT3L in bovine oocytes during the critical period of DNA methylation imprint acquisition (O'Doherty *et al*., 2012). Although not addressed here, it must be recognized that additional epigenetic modifications that are established in the oocyte, such as histone post-transcriptional modifications that signal active and silent chromatin and regulatory elements, also impact on decisions in the early embryo and any alterations or failures could lead to impaired epigenetic quality of the oocyte that might impact on early embryonic development and longer-term health.

Epigenetic effect of maternal physiological status on embryo and foetal-oocyte programming and adult offspring health

The protracted nature of oocyte growth in bovine and humans could result in repeated exposure of oocytes to environmental challenges, resulting in an accumulation of insults over the lifetime of the female. For example, it is likely that most females will experience alterations to their diet and nutritional status, development of metabolic and/ or infectious disease, exposure to environmental toxins and stress and in some cases administration of assisted reproductive therapies during their lives. Since an important fraction of the oocyte DNA methylome is maintained during preimplantation embryogenesis, environmentally-induced changes in gene expression in oocytes could lead to alterations in the epigenome of the next generation.

There is substantial evidence that the maternal environment pre-conception and during embryonic and foetal development, including inadequate nutrition and over-nutrition, influences the development and future health of the individual (Barker *et al*., 1989). The condition, health status and age of the oocyte donor (mother) can affect the quality of the oocyte and the health of the resulting offspring (Ashworth *et al*., 2009). This is especially relevant to the fertility of European women, where the average age at which women deliver their first child has increased to almost 30 years (http://www.unpopulation.org; 2014, United Nations Population Division Department of Economic and Social Affairs). The mechanisms behind these effects are unknown, but most likely alteration of gene expression in the oocyte and surrounding follicle cells, is instrumental. Studies in mice (Dahlhoff *et al*., 2014) and cattle (O'Doherty *et al*., 2014), have shown that DNA methylation in oocytes is modified by maternal diet or physiology. Postpartum lactating dairy cows are frequently pathogenically and metabolically challenged due to preferential partitioning of energy to milk production, leading to poor reproductive performance within their critical re-breeding window. The adverse metabolic environment induced by lactation alters the metabolomic, steroidogenic and transcriptomic profile of ovarian follicles during their development in postpartum lactating cows compared to non-lactating heifers (Bender *et al*., 2010; Walsh *et al*., 2012a, b). Metabolic differences include higher concentrations of saturated and lower concentrations of poly unsaturated -fatty acids and altered amino acid profiles in follicular fluid from lactating cows compared to those from dry heifers (Bender *et al*., 2010). Steroidogenesis is also compromised; dominant follicle estradiol and progesterone synthesis is reduced during differentiation and luteinization, respectively (Walsh *et al*., 2012b). At the level of the transcriptome the expression profiles of transcripts associated with steroid biosynthesis (Walsh *et al*., 2012b), immune cell function and chemotaxis (Walsh *et al*., 2012b) are also different. We have analysed the methylation status of a several candidate imprinted DMRs in fully-grown oocytes from postpartum cows. The resulting data revealed hypomethylation at the DMRs of a number of maternally methylated imprinted genes (*PEG3*, *PLAGL1* and *SNRPN*), in oocytes recovered from postpartum cows during the period of most acute negative energy balance (NEB) and greatest metabolic stress. The contribution of an inappropriate follicular fluid fatty acid profile to the aberrant methylation status was investigated in vitro. The findings confirmed the sensitivity of *PLAG1* to the follicular environment (O'Doherty *et al*., 2014). The most famous comparative situation in humans is the Dutch winter famine cohort; specifically, individuals conceived during famine presented hypomethylation of the *IGF2*-DMR compared with their non-exposed siblings, almost sixty years after exposure (Heijmans *et al*., 2008). Furthermore, genome-wide analysis of DMRs in the cohort detected a number of differentially methylated CpG regions, usually at regulatory regions (Ravelli *et al*., 1998; Oger *et al*., 2014). Moreover, the findings of an additional study indicated that the nutrition-dependent changes in methylation were gene- or tissue-restricted (Veenendaal *et al*., 2012). While NEB is a highly important issue for high yielding postpartum dairy cows, most Western societies do not expect to ever experience a scenario like the Dutch winter famine; obesity and high glycemic diets are possibly the most significant issues facing western women. Obesity induces multiple changes in the ovary, such as leptin resistance, lipotoxicity and local inflammation, all of which could have an impact on processes involved in establishment of the oocyte's normal epigenetic programme. There is much concern that maternal obesity and associated complications during early embryo life leave a "nutritional imprint" with long-term effects on the promotion of obesity and related conditions in adulthood (Ravelli *et al*., 1998). Data from animal models fed high fat diets clearly endorses the validity of these concerns. Consumption of a high fat (HF) diet during gestation (35 - 60% of calories from fat) is associated with offspring obesity, hypertension, abnormal cholesterol metabolism and cardiovascular disease, for review see Seki *et al*., 2012). Several rodent models have been employed to investigate the epigenetic changes induced by a maternal HF diet. Results from a selection of models demonstrate that a HF diet during pregnancy can induce epigenetic modification, including changes in DNMT expression (Liu *et al*., 2011; Zhou and Pan, 2011), altered DNA methylation of genes involved in obesity, and energy homeostasis (Martínez *et al*., 2014) hypomethylation of the *Mc4r* gene, which plays a role in body weight regulation in mice (Gong *et al*., 2010); and altered transcription and methylation status of *Igf-2* in fetal livers from dams fed HF diets (van Straten *et al*., 2010). Obviously studies on human tissues are more restrictive, however, recent studies employing genome-wide epigenetic analysis also revealed the potential for widespread DNA methylation variation in foetal tissues exposed to maternal gestational diabetes (Finer *et al*., 2015).

Extra-ovarian factors affecting oocyte development: The effect of Assisted Reproductive Technologies (ART) Research:

Assisted reproduction technologies are now responsible for the birth of tens of thousands of children yearly and up to 2% of all births in some countries (2004). A growing number of human and animal model studies have indicated the likely presence of postnatal consequences of ART and infertility, some associated with epigenetic alterations in conceptuses and placentae (Walter and Paulsen, 2003; Nelissen *et al*., 2014; Whitelaw *et al*., 2014). Ovarian

hyperstimulation has been associated with alterations in global DNA methylation levels in two-cell mouse embryos and reduced pre-implantation development in vitro (Shi and Haaf, 2002). The majority of mouse studies have found no effects on imprinting establishment, although several reports indicate that ovarian stimulation might interfere with the capacity to maintain imprinting during pre-implantation development, for review see Anckaert and Fair, 2015). In the human population, there have been reports of an increased risk for certain imprinting disorders following ART (Huntriss and Picton, 2008). These involved cases of Beckwith-Wiedemann Syndrome, Silver-Russell Syndrome and Angelman Syndrome. In addition to epigenetic defects localized to specific genes, alterations have also been observed at additional loci (Rossignol *et al*., 2006), suggesting a more global epigenetic disruption after assisted reproduction in humans. These concerns are reinforced by the detection of changes in both the epigenome and transcriptome in physiologically normal ART offspring (Batcheller *et al*., 2011).

There is much endeavor to expand the range of ARTs offered to patients to include ovarian cortical cryopreservation and subsequent retransplantation or in vitro culture to patients facing ovarian toxic chemotherapy. While there has been some progress (Laronda *et al*., 2014), currently, there is no data available to measure the epigenetic impact of such treatments on the cultured oocyte or resulting embryo. The findings from studies in mice suggest that the oocyte imprint establishment during in-vitro follicle culture conditions progresses normally (Anckaert *et al*., 2009, 2013; Hiura *et al*., 2006; Lucifero *et al*., 2002, 2004; Trapphoff *et al*., 2010) and candidate imprints were not perturbed in two-cell embryos obtained after IVF of oocytes derived from the same in-vitro follicle system (El Hajj *et al*., 2011), however, in vitro culture of the resulting embryos was associated with loss of imprinting for several genes in fetal and placental tissues (Mann *et al*., 2004; Rivera *et al*., 2008). This might indicate a different susceptibility of the in vitro grown and matured oocyte compared to the embryo, to culture-induced effects. Furthermore, care needs to be taken when extrapolating mouse data to support ART interventions in humans, as discussed above, the protracted natured of oocyte growth and the extended residency of the oocyte in the human ovary, may result in increased opportunities or sensitivity to aberrant epigenetic programming.

In cattle, research into developmental epigenetics has primarily focused on cloned bovine embryos, due to the high degree of pregnancy loss following transfer of embryos produced by somatic cell nuclear transfer (SCNT; Chavatte-Palmer *et al*., 2012). Nevertheless, evidence does exist to indicate that embryos produced by more conventional ART protocols are also susceptible to erroneous epigenetic programming (Urrego *et al*., 2014, for review). Global methylation profiling of bovine embryos revealed remarkable differences in the DNA methylation profile of blastocysts depending on developmental stages completed under in vitro culture condition. Furthermore, DNA methylation patterns of CpG islands and repetitive elements were also affected in the blastocysts depending on the stage of the embryo subjected to in vitro culture (Salilew-Wondim *et al*., 2015). Moreover, the data from cattle is supported by the extensive body of work describing differences at the gross morphological, ultrastructural, physiological, chromosomal, transcriptional and metabolic levels in in vitro-derived embryos compared with their *in vivo*-derived counterparts (reviewed in Lonergan and Fair 2008; Anckaert and Fair, 2015).

Conclusion

The regulation of oocyte and embryo development is a highly orchestrated, multi-regulatory process. DNA methylation is an important epigenetic regulatory mechanism. The prolonged growth phase and residency within the ovary endured by oocytes from larger mammals means that multiple opportunities for exposure to potential environmental hazard exists. Therefore it is important that in so far as possible, optimal physiological and external environments are achieved, through dietary management, health care and benign ex vivo assisted reproduction interventions.

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Dynamics and role of MicroRNAs during mammalian follicular development

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Abstract

Ovarian functions, which involve dynamically regulated processes of selection, recruitment and dominance, are known to be regulated by an array of genes, which are expressed in spatiotemporal manner in follicular somatic cells and gametes. This differential expression of genes in mammalian follicular cells is partly regulated by posttranscriptional gene regulators named microRNAs (miRNAs). In addition to the cellular miRNAs, growing number of evidences are available on the potential role of extracellular vesicles mediated transfer of miRNAs in follicular fluid. These extracellular vesicles are shown to be involved in cell-to-cell communication within the follicular environment. The molecular messages carried by the extracellular vesicles released into extracellular space are thought to be reflections of the physiological status of the cells from where they are released. Therefore due to their structural nature and potential to cargo several physiologically relevant molecules, exosomes have a great potential to be used as markers of oocyte developmental competence in follicular environment. Here, we review large sets of literatures to show the dynamic nature of miRNAs during various stages of mammalian follicular development, oocyte growth and the role of some of the miRNAs in ovarian cell functions. Moreover, the presence of microvesicle and exosome-coupled extracellular miRNAs in mammalian follicular fluid and their potential involvement in cell-to-cell communication are briefly discussed.

Keywords: exosomes, extracellular vesicles, folliculogenesis, microRNAs, ovary.

MicroRNAs in mammalian ovary

The mammalian ovary is an organ where series of dynamically regulated processes of follicular recruitment, selection, dominance, ovulation and atresia undergo. These processes are under tight coordination of paracrine and endocrine factors which in turn regulate the expression and interaction of multitude of genes in the ovary (Hunter *et al*., 2004). In line with an attempt to unravel the genetic regulation of ovarian functions, the first functional importance of microRNAs (miRNAs) in female reproduction was evidenced by tissue specific knocking out of the *Dicer1*; an important evolutionary conserved ribonuclease III enzyme involved in miRNAs biogenesis. The conditional inactivation of *Dicer1* in mouse ovarian granulosa, oviductal and uterine cells resulted in female infertility by decreasing the rate of ovulation, shortening the uterine horns and formation of oviductal cysts (Nagaraja *et al*., 2008). This has opened the door for further investigation of miRNAs and their role in mammalian ovaries. Subsequently, in the last decade intensive research on identification and functional analysis of miRNAs was performed in ovaries of various species. Several groups have reported that miRNAs are expressed in mammalian ovarian cells signifying their potential involvement in posttranscriptional regulation of important genes in the ovary. Construction of ovarian small RNAs complementary DNA (srcDNA) libraries revealed that miRNAs are the most abundantly expressed class of small RNAs in mouse ovary (Ro *et al*., 2007). A massive parallel sequencing of small RNA fraction extracted from newborn mouse ovary has identified 398 known and 4 novel microRNAs expressed in newborn mouse ovary (Ahn *et al*., 2010). Similarly, small RNA library constructed from cow ovary identified 50 known and 24 novel miRNAs, among which miRNAs; let-7a, let-7b, let-7c, miR-21, miR-23b, miR-24, miR-27a, miR-126 and miR-143 were found to be the most dominant ones (Hossain *et al*., 2009). The expression pattern of miRNAs in sheep ovaries during anestrus and the breeding season identified 97 known, 369 conserved and 17 predicted novel miRNAs (Di *et al*., 2014). Ovarian samples of uniparous and multiparous goats showed differential expression of miRNAs in which 8 miRNAs of the let-7 family; let-7b, let-7b-5p, let-7-5p, let-7c, let-7c-5p, let-7f-5p, let-7f, let-7 and miR-140, miR-320a were the top 10 abundantly expressed miRNAs in both the uniparous and multiparous goat ovaries (Ling *et al*., 2014). Deep sequencing of miRNAs in porcine ovary also identified the abundance of around 732 mature miRNAs (Li *et al*., 2011). Following the identification of miRNAs in ovaries of various species, studies in the last years have focused on the expression characterization and functional analysis of these regulatory molecules in follicular growth and ovarian function.

Expression dynamics of miRNAs during follicular development

During the course of mammalian folliculogenesis, different miRNAs are expressed in theca cells, granulosa cells (Salilew-Wondim *et al*., 2014; Gebremedhn *et al*., 2015), cumulus-oocyte-complex (Abd El Naby *et al*., 2013), follicular-fluid (Sohel *et al*., 2013) and the corpus luteum (McBride *et al*., 2012; Maalouf *et al*., 2016b). We have previously shown significant differences in the expression of

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miRNAs in bovine granulosa cells derived from subordinate and dominant follicles of early luteal phase (day 3 and 7 of the estrous cycle; Salilew-Wondim *et al*., 2014) and late follicular phase (day 19 of the estrous cycle; Gebremedhn *et al*., 2015). Interestingly, the extent of differences in the relative abundance of miRNAs in granulosa cells of the two follicular categories was minimal at day 3, whereas it was much pronounced at day 7 and 19. However, irrespective of the day of estrous cycle, miR-26a, miR-10b and the let-7 family members were the most abundantly expressed miRNAs in granulosa cells of both dominant and subordinate follicles (Salilew-Wondim *et al*., 2014; Gebremedhn *et al*., 2015) signifying their cellular housekeeping role in bovine follicular development. The dynamic nature of miRNA expression has been also evidenced in ovine species during follicular-luteal transition in which miR-21, miR-125b, let-7a and let-7b were the most abundantly expressed miRNAs during comparative analysis of ovine growing follicles, preovulatory follicles, early and late corpus luteum (McBride *et al*., 2012). Similarly, microarray technology was used to investigate the association of miRNA expression with bovine follicular growth and atresia. For this, genome-wide miRNA expression analysis was performed on tissues samples derived from small growing (4-8 mm), large healthy (12-17 mm) and atretic follicles (Sontakke *et al*., 2014). Among the 17 differentially expressed miRNAs, miR-144, miR-202, miR-451, miR-652, and miR-873 were enriched in larger healthy follicles compared to the small growing follicles. Moreover, 57 miRNAs were differentially expressed between large healthy and large atretic follicles. To determine the involvement of selected miRNAs obtained from in vitro studies; miR-21, miR-23a, miR-145, miR-503, miR-224, miR-383, miR-378, miR-132, and miR-212 in regulating equine follicular development, Donadeu and Schauer (2013) aspirated follicular fluid from dominant follicles during ovulatory and anovulatory seasons. It was shown that the relative levels of miR-21, miR-23b, miR-378 and miR-202 were higher in ovulatory follicles, whereas miR-145 tends to have higher level in anovulatory follicles. The level of miR-21, miR-132, miR-212, and miR-224 was increased in leading follicles, whereas the expression of their predicted target genes; *PTEN*, *RASA1* and *SMAD4* was reduced, indicating their potential involvement in regulating cell survival, differentiation and steroidogenesis during follicular selection in equine ovary (Schauer *et al*., 2013).

Depending on their genomic localization, miRNAs are grouped and termed as clusters (Mathelier and Carbone, 2013). MiRNA expression data analysis revealed an interesting pattern of co-enrichment or degradation of miRNA clusters in specific stages of bovine follicular development. Accordingly, the miR-183-96-182 cluster, miR-132-212 cluster and miR-424-450-542 cluster miRNAs were significantly enriched in granulosa cells of preovulatory dominant follicles while the miR-17-92 cluster miRNAs were enriched in granulosa cells of the subordinate follicles obtained at day 19 of the estrous cycle (Gebremedhn *et al*., 2015).

Potential role of miRNAs in controlling ovarian functions

During the course of follicular development, proliferation and differentiation of granulosa cells are essential for proper follicular recruitment, dominance, maturation, ovulation and atresia. Several studies have indicated the involvement of miRNAs in regulating the proliferation, differentiation, survival, cell cycle transition and/or apoptosis of granulosa cells (Carletti *et al*., 2010; Sirotkin *et al*., 2010; Yin *et al*., 2014; Andreas *et al*., 2016; Gebremedhn *et al*., 2016). Interestingly, in vitro granulosa cell culture models have become most popular and widely used to understand the potential role of miRNAs in ovarian functions. For this reason, majority of the functional studies of miRNAs in granulosa cells are performed through in vitro loss-and-gain of function by modulating the levels of selected miRNAs (Maalouf *et al*., 2016a).

In an attempt to determine the role of human miR-15a in controlling basic granulosa cell functions, Sirotkin *et al.* (2014) transfected primary human granulosa cells with anti-miR-15a and miR-15a precursor followed by evaluation of the expression of marker genes for cell proliferation (*MAPK/ERK1,2* and *PCNA*), apoptosis (*Caspase3* and *Bax*) and hormonal levels (Progesterone, Estradiol and Testosterone) in spent culture media. It was shown that inhibition of the miR-15a increased the protein accumulation of proliferation and apoptosis marker genes, reduction in the release of progesterone and testosterone and promotes the release of estradiol. Contrary to this, overexpression of miR-15a resulted in opposite effect. Evidences showed that multiple extra- and intraovarian factors such as the members of the TGF-β superfamily members (van den Hurk and Zhao, 2005; Knight and Glister, 2006), activin receptor-like kinases (ALK) and the Smads (Miyazawa *et al*., 2002; Florio *et al*., 2010) are implicated in regulation of follicular development. Members of the TGF-β superfamily, which are known to be involved in granulosa cell proliferation and differentiation (Hsueh *et al*., 1984; Hirshfield, 1991), were reported to be posttranscriptionally regulated by miRNAs in granulosa cells (Yao *et al*., 2014). In that study, over expression of miR-224 enhanced TGF-β1 induced proliferation of granulosa cells by targeting *Smad4* gene. Conversely, inhibition of miR-224 suppressed the proliferation of granulosa cells induced by TGF-ß1 treatment. Similarly, miR-181a expression decreased in mouse granulosa cells treated with activin A in dose dependent manner and overexpression of miR-181a inhibited cell proliferation by targeting acvr2a, which in turn resulted in suppression of *CCND2* and *PCNA* (Zhang *et al*., 2013). Recently, we showed that the miR-183-96-182 cluster coordinately target the expression of *FOXO1* and promote proliferation and cell cycle transition of bovine granulosa cells. Overexpression of the miR-183-96-182 cluster miRNAs reduced the proportion of cells under G0/G1 arrest and increased the proportion of cells entering the S-phase of the cell cycle. Interestingly, selective degradation of *FOXO1* using siRNAs also promoted the cell

proliferation and cell cycle transition. This evidence supports the idea of a coordinated role of this miRNA cluster in repressing anti-proliferation genes and their downstream transcripts (Gebremedhn *et al*., 2016). During various stages of ovarian follicular development, majority of the follicles undergo atresia being triggered by granulosa cells apoptosis (Portela *et al*., 2015). Accumulated evidences are available regarding the regulation of miRNAs in granulosa cells apoptosis and determine the follicular cells fate to either ovulation or atresia. In one of the early miRNA functional study, miR-21 as one of the three highly induced miRNAs in murine granulosa cells in response to ovulatory dose of LH administration, induces granulosa cells apoptosis upon its inhibition. In the same study, *in vivo* anti-miR-21 treated ovary resulted in reduced rate of ovulation compared to the untreated contralateral ovary (Carletti *et al*., 2010). The let-7g induces apoptosis in pig granulosa cells by targeting the *TGFBR1* gene (Zhou *et al*., 2015), while porcine atretic follicles are highly enriched with let-7g and its overexpression promoted apoptosis of granulosa cells (Cao *et al*., 2015). MiR-26b promotes porcine granulosa cells apoptosis and induces follicular atresia by targeting *SMAD4* (Liu *et al*., 2014) and *ATM* (Lin *et al*., 2012). MiR-34a promotes granulosa cell apoptosis by targeting *INHBB* in porcine ovary (Tu *et al*., 2014). Similarly, functional analysis of the miR-17-92 cluster, which was found to be enriched in bovine granulosa cells derived from the subordinate follicles of day 19 of the estrous cycle, revealed the regulatory role of this cluster in granulosa cells proliferation, differentiation and steroidogenesis by targeting *PTEN* and *BMPR2* genes. Overexpression of the miRNA cluster promoted cell proliferation, and decreased proportion of differentiating cells (Andreas *et al*., 2016).

MiRNAs in steroidogenesis and their hormonal regulation

According to the two-cell-two-gonadotropin theory, luteinizing hormone stimulates thecal cells to produce androgens, and follicle-stimulating hormone stimulates granulosa cells to produce estrogens from androgens (Barnes *et al*., 2000). Growing evidences indicate that miRNAs act as regulators of steroidogenesis (Schauer *et al*., 2013; Toms *et al*., 2015; Donadeu *et al*., 2016). The effect of miRNAs on the release of major ovarian steroid hormones was determined by transfecting human primary granulosa cells with 80 different human pre-miRNAs constructs, and the release of progesterone was inhibited by 36 miRNAs. Whereas, 10 miRNAs promoted progesterone release (Sirotkin *et al*., 2009). MiR-378 decreases ovarian estradiol production by targeting aromatase. However, overexpression of the aromatase 3'-UTR in granulosa cells enhanced aromatase expression at protein level, possibly mediated by the binding of miR-378 within this region, thereby reducing the binding of this miRNA to the endogenous aromatase 3'-UTR (Xu *et al*., 2011). In a separate study, the progesterone receptor (PGR) was targeted by miR-378-3p which leads to decrease in the protein and mRNA levels and its downstream target genes; *ADAMTS1*, *CTSL1*, and *PPARG*, which are involved in follicular maturation and remodeling (Toms *et al*., 2015). A recent study showed that level of miR-378 in the follicular wall was correlated with estradiol, the estradiol: progesterone ratio and *CYP19A1* (Donadeu *et al*., 2016). MiR-34a and miR-320 inhibit estradiol release from human granulosa cells and murine ovaries, respectively (Sirotkin *et al*., 2009; Yao *et al*., 2014). However, miR-320 could stimulate testosterone and progesterone in murine ovaries. Interestingly, miR-383 has been shown to act as positive regulator of estradiol production in mouse granulosa cells by inhibiting *RBMS1*, which leads to c-Myc inactivation and steroidogenesis (Yin *et al*., 2014). Moreover, miR-132 is involved in the cAMP signaling pathway and promotes estradiol synthesis via the translational repression of *Nurr1* in ovarian granulosa cells and significantly induce expression of *Cyp19a1* through *Nurr1*; a direct target that suppresses *Cyp19a1* expression (Wu *et al*., 2015). Similarly, miR-133b is involved in follicle-stimulating hormone (FSH)-induced estrogen production by down-regulating *Foxl2* and inhibiting the Foxl2-mediated transcriptional repression of *StAR* and *CYP19A1* to promote estradiol production (Dai *et al*., 2013). The overexpression of miR-20a, which is validated to target PTEN and BMPR2 genes, induced the release of progesterone in in vitro bovine granulosa cell culture (Andreas *et al*., 2016)

Recently, we investigated the effect of supra-physiological level of gonadotropins introduction during the process of controlled ovarian hyperstimulation on the level of extracellular miRNAs in bovine follicular fluid and blood plasma (Noferesti *et al*., 2015). It was shown that, a total of 57 and 21 miRNAs were differentially expressed in follicular fluid and blood plasma, respectively, derived from hyper-stimulated heifers compared to the unstimulated control counterpart. Bioinformatics analysis revealed that pathways including the TGF-ß signaling, MAPK signaling and oocyte meiosis are significantly enriched by target genes of the miRNAs selectively upregulated in follicular fluid and blood plasma of hyper-stimulated heifers (Noferesti *et al*., 2015). Despite the fact that the choice of the mechanism of release of miRNAs into extracellular space is not understood well, we have detected circulatory miRNAs in exosomes and Ago2 fraction of follicular fluid and blood plasma of hyperstimulated and unstimulated control heifers. This may evidence the releasing mechanism of miRNAs into extracellular space in both follicular fluid and blood plasma was not affected by super-stimulation treatment.

Extracellular miRNAs in follicular fluid

While the majority of miRNAs are found intracellularly, tremendous numbers of miRNAs have been found to circulate in the extracellular space and biological fluids, including follicular fluid (Donadeu and Schauer, 2013; Sohel *et al*., 2013; Santonocito *et al*., 2014). Mammalian follicular fluid consists of a

complex mixture of nucleic acids, proteins, metabolites and ions, which are known to be secreted by the oocyte, granulosa cells, theca cells and blood plasma components that come to the follicular fluid via theca capillaries (Revelli *et al*., 2009; Rodgers and Irving-Rodgers, 2010). The follicular fluid creates very suitable microenvironment for the growth and development of oocytes and its biochemical composition varies depending on the physiological status of the follicle. As any alteration in the follicular fluid composition can be associated with the oocyte quality, the follicular fluid components may provide useful diagnostic information on oocyte developmental competence and ovarian functions (Revelli *et al*., 2009). Several biochemical components including hormones, growth factors, cytokines and chemokines, which are secreted by follicular cells into follicular fluid, are known to promote oocyte maturation. Evaluation of any of specific components within the follicular fluid at any stage of follicular development revealed the physiological status of the animal in general and the oocyte health or growth status in particular (Sohel *et al*., 2013). In addition to autocrine and paracrine mode of communication inside the ovarian follicle, recently alternative mechanisms come into play. This mechanism in which the oocytes and follicular somatic cells exchange signals is mediated by the so called microvesicles and exosomes carrying RNAs (mRNA, miRNA, lncRNA and other types of RNAs), proteins and DNA fragments.

Extracellular vesicles can be broadly classified into 3 main classes: Microvesicles/ microparticles/ ectosomes, which are produced by outward budding and fusion of the plasma membrane, whereas exosomes are formed within the endosomal network and released upon fusion of multivesicular bodies with the plasma membrane. The release of extracellular vesicles (EVs), including exosomes and microvesicles, is a phenomenon shared by many cell types as a means of communicating with other cells. EVs released into the extracellular space can potentially reach distant tissues via circulation and once up taken by neighboring and/or distant cells, EVs can transfer functional molecules that may alter the status of recipient cells, thereby contributing to both physiological and pathological processes.

Extracellular microvesicles and exosomes in follicular fluid carrying miRNAs as cargo molecules have been detected in bovine (Sohel *et al*., 2013; Noferesti *et al*., 2015; Navakanitworakul *et al*., 2016), equine (da Silveira *et al*., 2012, 2014, 2015) and human (Xiao *et al*., 2016). The presence of EVs (microvesicles or exosomes) in follicular fluid may support the notion that they play a significant role in ovarian functions. The size and concentration of EVs in the follicular fluid between different sized follicles has been investigated (Navakanitworakul *et al*., 2016). In the same study while no significant changes were observed in the size of the extracellular vesicles between small, medium and large follicles, the concentration of extracellular vesicles decreased progressively as the follicle size increases. A microarray or sequencing based expression profiling of exosomes from bovine follicular fluid identified miRNAs enriched in follicular size dependent manner (Navakanitworakul *et al*., 2016) and oocyte developmental competence (Sohel *et al*., 2013). Interestingly, in the former study those miRNAs enriched in exosomes isolated from small follicle were found to be associated with cell proliferation, while miRNAs abundant in large follicle were found to be related to inflammatory response pathways. Similarly, significant number of miRNAs have been identified to be differentially carried by exosomes or Ago2 protein complexes isolated from follicular fluid harboring oocytes with different growth status (Sohel *et al*., 2013). Those differentially expressed miRNAs from both exosomal and non-exosomal fraction of the follicular fluid were found to be involved in molecular pathways (MAPK signaling pathway, focal adhesion and regulation of actin cytoskeleton), which are known to be main regulators of follicular development and oocyte growth.

During the course of bovine follicular development, expansion of cumulus cells is essential for oocyte maturation and release through ovulation for a successful fertilization and early embryo development. LH induced increase in the expression of genes such as prostaglandin-endoperoxide synthase 2 (*Ptgs2*), pentraxin-related protein 3 (*Ptx3*), and tumor necrosis factor alpha-induced protein 6 (*Tnfaip6*) are essential for the expansion of cumulus cells. Recently, co-incubation of bovine COC with extracellular vesicles isolated from follicular fluid aspirated from small and large follicles under in vitro condition resulted in cumulus expansion and increased expression of the aforementioned genes (Hung *et al*., 2015). Moreover, age related differences in exosome mediated miRNA abundance in follicular fluid of mare has been evidenced (da Silveira *et al*., 2015). This differential expression of exosomal miRNAs has been associated with the expression of granulosa cells genes associated with TGF-β signaling pathway, which is the most dominant pathway modulating follicular development and oocyte growth.

Potential role of extracellular miRNAs in cell-to-cell communication

Ovarian follicular development and oocyte maturation are well coordinated processes and mediated by a constant exchange of signals between ovarian somatic cells and the oocytes (Zuccotti *et al*., 2011). These exchanges of signals are made possible through the establishment of gap junctions between the surrounding somatic cells and the oocytes (Cecconi *et al*., 2004; Gilchrist *et al*., 2008). In the follicular environment the physical contact between the surrounding cells especially granulosa and oocytes is mediated by the expression of key molecules mainly connexines (*CX37* and *CX43*) which are also expressed in early stages of mammalian embryo development (Gittens and Kidder 2005; Gittens *et al*., 2005). Mutual communication between the oocyte and cumulus cells is achieved through secretion of both oocyte or cumulus

cells secreted factors and are shown to affect each other in relative abundance of transcripts at mRNA (Regassa *et al*., 2011) and miRNA level (Abd El Naby *et al*., 2013). The oocyte secreted factors which pass through the gap junctions include ions, metabolites and amino acids that are necessary for oocyte growth, as well as small regulatory molecules that control oocyte development (Gilchrist *et al*., 2008). In addition to the direct communication, the transfer of signals between the different follicular cell types could be facilitated by extracellular vesicles circulating in the follicular fluid.

The release of EVs, including exosomes and microvesicles, is a cellular characteristics shared by many cell types as a means of communication with other cells and also potentially removing cell contents. This phenomenon is more evident in follicular environment where cell-to-cell communication is vital for the growth of the oocytes. MiRNAs carried by microvesicles and exosomes in follicular fluid are also found to be present in the surrounding follicular cells including theca cells, granulosa cells and cumulus-oocyte-complex (Sohel *et al*., 2013). In addition to the identification of miRNAs carried by exosomes in mammalian follicular fluid, several in vitro experiments have been conducted to simulate the cell-to-cell communication mediated by exosomes (da Silveira *et al*., 2012, 2014; Sohel *et al*., 2013; Di Pietro, 2016). For this, we have previously shown that membrane labeled exosomes isolated from bovine follicular fluid harboring either growing or fully grown oocytes, were co-cultured with granulosa cells in vitro (Sohel *et al*., 2013). The uptake of exosomes by cultured granulosa cells and resulted in subsequent increase in endogenous cellular miRNAs and altered gene expression. Similar experiment in equine has also evidenced the uptake of microvesicles by cultured granulosa cells in vitro and in vivo experiments (da Silveira *et al*., 2012). Exosomes isolated from mid-estrous and preovulatory follicles and known to cargo *ACVR1* and miR-27b, miR-372, and miR-382 were found to alter the ID2 (an *ACVR1* target gene) in preovulatory mare follicle (da Silveira *et al*., 2014).

All in all, the follicular fluid will provide a potential environment for identification of marker molecules associated with follicular development and oocyte competence. Moreover, with the identification of extracellular vesicles as cargo for molecular signals, tremendous opportunities will be opened for therapeutics in the field of fertility treatments.

Conclusion

Mammalian folliculogenesis or ovarian functionality is well coordinated process involving a molecular cross-talk between the various somatic cell types and the developing gamete in the follicular microenvironment to attain successful ovulation, fertilization and development of embryo and to give birth to healthy offspring. Understanding the genetic regulation of these processes paves the way to identify molecular profiles and their regulatory mechanisms associated with ovarian function and facilitates the development of diagnostic markers for oocyte developmental competence. Especially, exploring follicular fluid for the presence of extracellular miRNAs, which could be indicators of ovarian functions and subsequently competence of oocytes, facilitates the development of non-invasive diagnostic tools to address female infertility. Moreover, future researches need to focus in deciphering the exact sources and role extracellular miRNAs in follicular fluid towards the development of therapeutic tools for fertility treatments associated with ovarian disorders.

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Factors that interfere with oocyte quality for *in vitro* production of cattle embryos: effects of different developmental & reproductive stages

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Abstract

The success of IVP is ultimately dependent on the number and quality of the cumulus-oocyte complexes (COC) harvested during the OPU procedure. Several factors appear to be critical to oocyte quality including follicle size, environment factors such as heat-stress, genetic background, age and lactation status of donor animals, all having a remarkable influence on the results of IVP. The aim of this review is to highlight some critical areas that can help veterinary practitioners to enhance OPU efficiency and successfully implement IVP into their routine practice. Focus will be given to recent findings in the literature and underlying physiological aspects that may be interfering with the quality of oocytes addressed to IVP in cattle at younger ages (calves and prepubertal heifers), pregnant vs non-pregnant status, and possible interactions with lactation and days postpartum during OPU.

Keywords: bovine, embryo, IVF, oocyte, pregnancy.

Introduction

Oocyte collection by ovum pick-up (OPU) associated with *in vitro* production of embryos (IVP) are important technologies that can improve efficiency of both dairy and beef herds (Merton *et al*., 2003; Pontes *et al*., 2010). The IVP industry is evolving fast in the last decade, and some parallel technologies such as genomics and the discovery of embryo production markers (i.e. AMH assay) are driving a more targeted and efficient genetic progress through IVP. For example, the use of genomic technology allows the identification of genetic superior animals at much earlier ages. As a result, producers are pushing both the industry and the scientific community to develop techniques that are more suited to younger animals sent to OPU-IVP routines, accelerating the genetic gain by decreasing generation intervals.

Despite of animal age in which OPU is performed, the overall success of IVP is ultimately dependent on the number and quality of the cumulus-oocyte complexes (COC) harvested during the OPU procedure. Evidently, several factors appear to be critical to oocyte quality including follicle size (Lonergan *et al*., 1994; Seneda *et al*., 2001), environment factors such as heat-stress (Torres-Júnior *et al*., 2008; Ferreira *et al*., 2011; Ferreira *et al*., 2016), genetic background (Gimenes *et al*., 2015; Sales *et al*., 2015), age (Batista *et al*., 2016a) and lactation status of donor animals (Baruselli *et al*., 2016), all having a remarkable influence on the results of IVP. Additionally, there is a growing body of scientific literature demonstrating the direct role of hormonal milieu during follicle development on oocyte quality. Focus has been given to the possible positive effects of circulating levels of P4 in determining oocyte quality in IVP (Lonergan, 2011). However, a recent study from our research group has demonstrated that neither exposure to lower levels of LH, nor cycles of P4, are limiting to oocyte viability and development to blastocyst stage (Batista *et al*., 2016b). Further research is needed to elucidate some of these rather complex interactions between reproductive hormones and oocyte quality.

Cattle breed is also one of the key factors that influence the efficiency of OPU-IVP. Interestingly, *Bos indicus* (also known as zebu breeds) and *Bos taurus* donors seem to have significant differences regarding the results of IVP (Pontes *et al*., 2010; Guerreiro *et al*., 2014a; Gimenes *et al*., 2015). It appears quite noticeable in most of the published literature that *Bos indicus* females have much greater numbers of oocytes retrieved during OPU; presumably due to greater numbers of ovarian follicle population and plasma anti-Müllerian concentration (Batista *et al*., 2014). Furthermore, zebu donors also have been shown to yield greater amounts of viable oocytes compared to *Bos taurus* donors (Guerreiro *et al*., 2014a; Gimenes *et al*., 2015).

Some management issues such as the type of diet and level of feed intake, as well as heat stress are widely known to have a great impact on oocyte quality and IVP. However, the interaction of these key factors may differ in *Bos taurus* and *Bos indicus* cattle. The level of energy intake for example, can affect circulating levels of insulin and IGF-1 (reviewed by *Sartori et al*., 2016). As a result, excessive increase in insulin concentration in blood, mainly in dairy cows later in lactation, are negatively associated with oocyte quality (reviewed by Baruselli *et al*., 2016). Exposure to heat stress is widely known to be deleterious to oocyte competence in *Bos taurus* cattle (Ferreira *et al*., 2011; Ferreira *et al*., 2016). Surprisingly, *Bos indicus* donors although more resistant to environmental heat stress conditions, are also affected by heat stress and may require nearly four months to recover oocyte quality to pre-heat stress levels, even after a short period of heat exposure (Torres-Júnior *et al*., 2008).

Altogether, several factors can be detrimental to

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oocyte quality and must be taken into account during IVP in cattle. Thus, the aim of this review is to highlight some critical areas that can help veterinary practitioners to enhance OPU efficiency and successfully implement IVP into their routine practice. Focus will be given to recent findings in the literature and underlying physiological aspects that may be interfering with the quality of oocytes addressed to IVP in cattle at younger ages (calves and prepubertal heifers), pregnant vs non-pregnant status, and possible interactions with lactation and days postpartum during OPU.

Effect of different developmental & reproductive stages on oocyte quality and *in vitro* embryo production

Oocyte quality and IVP in donor calves

In cattle the population of ovarian follicles at birth is estimated at 235,000 (Ericksson, 1966; Betteridge *et al*., 1989). However, as in all mammals, this follicle population quickly decreases with aging (Ericksson, 1966). Therefore, young heifers have more antral follicles in their ovaries (Desjardins and Hafs, 1969), which could be associated with more efficient IVP. Previous studies have shown that calves require gonadotropin stimulation before oocyte collection and IVP to achieve acceptable results (Presicce *et al*., 1997; Maclellan *et al*., 1998; Taneja *et al*., 2000). Among the hormonal protocols employed in calves selected for oocyte recovery, most use follicle-stimulating hormone (FSH) treatment before using laparoscopic OPU (LOPU) and IVP technologies (Armstrong *et al*., 1992; Fry *et al*., 1998).

Recently, our research group conducted a series of studies to evaluate different hormonal protocols for collection of oocytes followed by IVP in *Bos indicus* (Nelore) and *Bos taurus* (Holstein) calves (Batista *et al*., 2016a). An experiment was conducted at a commercial beef farm near Paulínia city (São Paulo, Brazil). A total of 45 Nelore donors were used: 30 calves (3 to 4 month) and 15 cyclic heifers (18 to 24 month). In a second experiment conducted at the São Paulo University Campus (USP, Pirassununga Campus, SP, Brazil), a total of 34 Holstein donors were used, including 24 calves (3 to 4 month) and 10 cyclic heifers aged 14 to 16 month. All calves were randomly assigned to receive a superstimulatory treatment with pFSH (calves with pFSH, n = 15) or not (calves without FSH, n = 15). Cycling heifers were subjected to a transvaginal ultrasound - guided OPU followed by IVP procedure at random stages of the estrous cycle.

All the calves underwent to LOPU. Calves without FSH were also subjected to LOPU at random stages of the estrous cycle. Superistimulated calves were treated before LOPU with an intravaginal progesterone device (day 0, Eazi-Breed CIDR, 0.33 g; Zoetis, São Paulo, SP, Brazil). After 5 days, calves received 4 treatments of porcine FSH (pFSH; 140 mg of pFSH; Folltropin, Agener, SP, Brazil) administered twice daily in decreasing doses (40 mg [day 5, AM], 40 mg [day 5, PM], 30 mg [day 6, AM]) over a 2-day period. The LOPU was performed 12 h after the last treatment with pFSH (day 7).

In *Bos indicus* donors, the number of retrieved COCs was greater in calves with FSH and in cycling heifers, compared to calves without FSH ($P = 0.04$). Furthermore, COC culture rate was greater in calves treated with FSH and in cycling heifers, compared to calves without FSH ($P = 0.01$). However, cleavage rate was similar for all 3 groups (calves without FSH, calves with FSH, and cycling heifers; $P = 0.41$; data shown in Table 1). Despite these positive effects of FSH treatment, the number of blastocysts produced was similar in calves with and without FSH, and this number was lower than in cycling heifers ($P < 0.0001$; Table 1).

In *Bos taurus* donors, the number of visualized follicles ($P = 0.01$) and recovered oocytes ($P < 0.0001$) was greater in calves with FSH compared to calves without FSH and to cycling heifers. The number of cultured COCs was similar in calves without FSH and in cycling heifers, and both groups had fewer cultured COCs than did calves with FSH ($P < 0.0001$). Despite these positive effects of FSH treatment, the number of blastocysts produced was similar in calves with and without FSH, and this number was lower than in cycling heifers (data summarized in Table 2).

The results presented herein demonstrate that it is possible to produce embryos for calves using LOPU/IVP. The FSH treatment could be used as a superstimulation treatment to improve the LOPU/IVP efficiency in young calves. However, further studies are needed to improve embryo production in calves compared to mature animals.

Table 1. Number of visualized follicles, COCs and blastocysts (mean ± SEM) after LOPU-IVP in *Bos indicus* (Nelore) donor calves and after OPU - IVP in *Bos indicus* (Nelore) cycling heifers.

Item	*Bos indicus*			
	Calves without FSH	Calves with FSH	Cycling heifers	P value[d]
Total follicles visualized	19.7 ± 4^{z}	32.3 ± 5.9^{y}	47.1 ± 6.3^{x}	0.003
Total COCs retrieved	13.5 ± 3.6^{b}	20.9 ± 5.1^{ab}	29.9 ± 5.3^{a}	0.04
Recovery rate (%)[e]	68.5^{a}	64.7^{b}	63.6^{b}	0.02
COCs cultured	4.7 ± 1.4^{c}	11.3 ± 4.0^{b}	18.1 ± 4.0^{a}	<0.0001
COCs cultured rate (%)[f]	35.1^{b}	54.3^{a}	60.6^{a}	0.01
Cleavage rate (%)[g]	47.0	52.2	50.3	0.41
Blastocysts produced	1.7 ± 0.7^{b}	2.3 ± 0.8^{b}	9.3 ± 2.0^{a}	<0.0001
Blastocyst rate (%)[h]	12.9^{b}	11.3^{b}	30.9^{a}	<0.0001

[d]Data with different superscripts in the same line differ with $P \leq 0.05$ ($a \neq b \neq c$) or $P \leq 0.06$ ($x \neq y \neq z$). [e]Total number of COCs/number of follicles aspirated. [f]Number of COCs cultured/number of follicles aspirated. [g]Number of cleaved zygotes/ number of COCs. [h]Number of blastocyst/number of COCs.

Table 2. Number of visualized follicles, COCs and blastocysts (mean ± SEM) after LOPU-IVP in *Bos taurus* (Holstein) donor calves and after OPU - IVP in *Bos taurus* (Holstein) cycling heifers.

	Bos taurus			
	Calves without FSH	Calves with FSH	Cycling heifers	P-values
Total follicles visualized	22.7 ± 4.2[b]	54.3 ± 9.5[a]	24.9 ± 3.6[b]	0.01
Total COCs retrieved	11.7 ± 2.4[bx]	22.4 ± 5.4[a]	9.2 ± 1.7[cy]	<0.0001
Recovery rate (%)[1]	51.3[a]	41.3[a]	36.9[b]	0.01
COCs cultured	3.6 ± 1.0[b]	12.3 ± 3.5[a]	4.7 ± 1.3[b]	<0.0001
COCs cultured rate (%)[2]	30.7[b]	37.7[a]	51.1[a]	0.02
Cleavage rate (%)[3]	17.8	30.5	26.1	0.47
Blastocyst produced	0.4 ± 0.2	0.7 ± 0.4	0.5 ± 0.3	0.78
Blastocyst rate (%)[4]	2.9	2.0	4.3	0.60

[d]Data with different superscripts in the same line differ with P ≤ 0.05 (a ≠ b ≠ c) or P ≤ 0.06 (x ≠ y ≠ z). [e]Total number of COCs/number of follicles aspirated. [f]Number of COCs cultured/number of follicles aspirated. [g]Number of cleaved zygotes/ number of COCs. [h]Number of blastocyst/number of COCs.

Oocyte quality and IVP in prepubertal and pubertal heifers

Several research labs have successfully produced viable embryos from prepubertal heifers (Armstrong *et al*., 1992; Fry *et al*., 1998; Taneja *et al*., 2000). However, there are some concerns that oocytes from young females have a lower developmental capacity than those from adult donors (Khatir *et al*., 1996; Presicce *et al*., 1997; Majerus *et al*., 1999)

Recently, our research group performed a study at Santa Rita farm located near Descalvado city in São Paulo state (Guerreiro *et al*., 2014b), where a total of 120 donors of four animal categories were used, as follows: prepubertal heifers (n = 30), pubertal heifers (n = 30), lactating cows (n = 30) and non-lactating cows (n = 30). Donors were submitted to OPU without previous synchronization of the follicular wave. Six OPU sessions were performed with five animals of each category, 20 donors *per* session.

Immediately before the OPU, all follicles were quantified and all visible follicles (≥2 mm) were punctured and total recovered structures, quantity and quality of viable oocytes were registered. All viable oocytes were submitted to IVP and their development (cleavage and blastocyst rate) was evaluated. Sex-sorted sperm from the same bull and semen batch were used to fertilize oocytes from all donor categories in all OPU sessions. Produced embryos (n = 206) were transferred into crossbred recipients (*Bos taurus* x *Bos indicus*).

No difference was observed between experimental groups, regarding total number of aspirated follicles (P = 0.08). Despite similar number of total recovered oocytes (P = 0.12), prepubertal heifers had an intermediate quantity of viable oocytes, and non-lactating cows produced more viable oocytes (P = 0.03), when compared to lactating cows. Still, prepubertal donors had lower cleavage rate (P < 0.0001) and lower blastocyst rate (P < 0.0001) compared to other categories (Table 3).

Thus, it is concluded that prepubertal Holstein donors have low competence for *in vitro* embryo production, being non-lactating cows the most efficient category for IVP. Embryos originated from prepuberal animals resulted in inferior conception rate in comparison to embryos produced from lactating cows and non-lactating cows [prepubertal: 0.0% (0/15)[b]; pubertal: 9.7% (3/28)[b]; lactating cows: 28.6% (10/25)[a]; non lactating cows: 32.7% (36/74)[a]; P < 0.05; Fig. 1]. However, similar conception rate was verified for embryos produced from pre-pubertal and pubertal donors.

Table 3. Number of aspirated follicles, oocytes and embryos produced after OPU-IVP in prepubertal and pubertal heifers, and in lactating and non-lactating cows from the Holstein breed. Data is presented as mean ± standard error mean.

Item	Heifer		Cows		
	Prepubertal	Pubertal	Lactating	Non-Lactating	P value
N	30	30	30	30	
Total follicles aspirated	18.3 ± 2.1	17.3 ± 1.2	14.0 ± 1.0	17.7 ± 1.7	0.08
Total COC retrieved[1]	14.2 ± 2.2	13.1 ± 1.1	9.8 ± 1.1	14.6 ± 1.7	0.12
COCs cultured	10.5 ± 1.8[ab]	8.3 ± 0.8[ab]	6.5 ± 0.9[b]	11.5 ± 1.4[a]	0.03
Cleavage rate (%)[2]	68.6[b]	98.8[a]	87.6[a]	10.1[a]	<0.0001
Blastocysts produced	0.5 ± 0.2[b]	1.1 ± 0.2[b]	1.2 ± 0.4[b]	4.2 ± 0.6[a]	<0.0001
Blastocysts rate (%)[3]	4.8[c]	12.7[b]	18.0[b]	36.5[a]	<0.0001

[1]COC - cumulus oocyte complex. [2]Number of cleaved embryos/viable COCs. [3]Number of blastocysts/viable COCs. [a,b,c]Different letters within rows indicate statistical difference (P < 0.05).

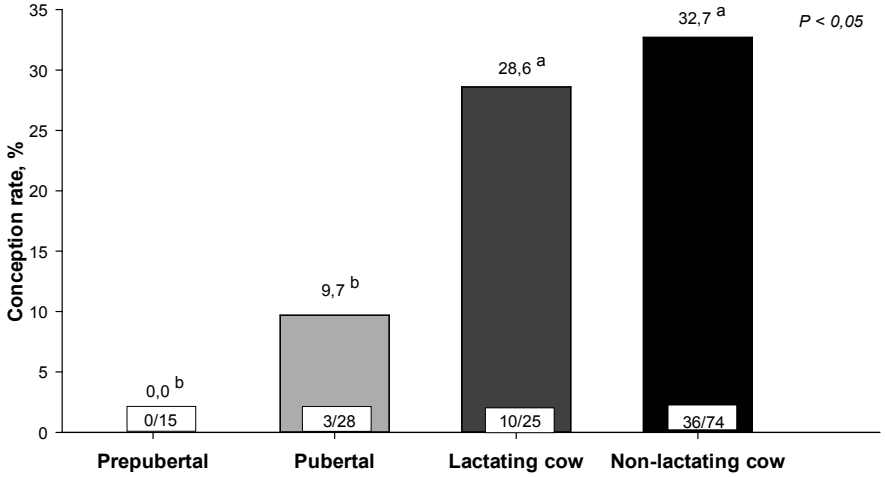

Figure 1. Conception rate after embryo transfer. Donors were prepubertal and pubertal heifers, and lactating and non-lactating cows from the Holstein breed.

We performed another trial with prepubertal Nelore heifer (*Bos indicus*) housed at Instituto de Zootecnia in Sertãozinho, São Paulo state (Batista *et al*., 2016; FMVZ/USP, São Paulo, Brazil; unpublished data). In this experiment we evaluated OPU-IVP production at different ages and cyclicity status. The experimental design included: prepubertal heifers aged 8 to 12 month (n = 24), prepubertal heifers aged 18 to 22 month (n = 20) and cycling heifers aged 22 to 26 month (n = 25). Data is summarized in Table 4.

Briefly, prepubertal heifers aged 8 to 12 month had lower numbers of visualized follicles, lower numbers of recovered oocytes than older heifers, despite cyclicity status. There were no differences across experimental groups in terms of the rate of COCs cultured or their cleavage rate. However, number of blastocysts produced, as well as blastocyst rate increased both with increasing age and after animals became cyclic (Table 4).

The results of OPU/IVP in prepubertal heifers demonstrate reduced efficiency compared to cycling heifers and adult animals. Further studies should be conducted to try to improve the efficiency of production in this age category.

Table 4. Number of visualized follicles, COCs and blastocysts (mean ± SEM) after OPU - IVP in *Bos indicus* (Nelore) prepubertal and pubertal and heifers.

Item	Prepubertal heifers (8 - 12 month) (n = 24)	Prepubertal heifers (18 - 22 month) (n = 20)	Pubertal heifers (22 - 26 month) (n = 25)	P value[a]	
				Age[b]	Ciclicality[c]
Total follicles visualized	19.7 ± 2.1	41.3 ± 5.28	34.0 ± 3.3	<0.0001	0.0002
Total COCs retrieved	13.4 ± 1.7	30.8 ± 5.8	22.6 ± 3.2	<0.0001	<0.0001
Total COCs cleaved	5.6 ± 0.8	14.8 ± 2.5	13.3 ± 1.9	<0.0001	<0.0001
COCs cultured	7.6 ± 1.0	16.8 ± 2.7	15.1 ± 2.2	<0.0001	<0.0001
COCs cultured rate (%)[d]	57.0	54.0	60.0	0.13	0.45
Cleavage rate (%)[e]	73.0	88.0	84.0	<0.0001	0.25
Blastocysts produced	1.5 ± 0.3	4.7 ± 0.9	7.2 ± 1.2	<0.0001	<0.0001
Blastocyst rate (%)[f]	20.2	28.1	47.0	0.05	<0.0001

[a]Effect of evaluated group. [b]Effect of age in the prepubertal group (8-12 month *vs*. 18-24 month). [c]Effect of cyclicity (cyclic *vs*. non cyclic). [d]Number of viable oocytes/number of total oocytes. [e]Number of cleaved oocytes/number of cultured oocytes. [f]Number of blastocysts/number of cultured oocytes.

Oocyte quality and IVP in pregnant donors

Recently, our research group studied the effect of pregnancy on oocyte quality and IVP of Holstein heifers (Bayeux *et al*., 2016). We evaluated 179 Holstein donors (*Bos taurus*) of 3 categories: prepubertal heifers (8 to 10 month; n = 60); pubertal heifers (10 to 12 month; n = 60) and pregnant heifers (14 to 18 month; n = 59). All animals underwent ovum pickup (OPU) at random stages of the estrous cycle. Sex-sorted sperm from the same bull and semen batch were used to fertilize oocytes from all donor categories in all OPU sessions. Pubertal heifers had a greater number of recovered oocytes as well as COCs cultured compared to other categories. In contrast, cleavage rate was similar between pubertal and pregnant heifers. Interestingly, pregnant heifers had a greater number of embryos produced per OPU and ultimately greater blastocyst rate when compared to other heifer-categories (Table 5). These results indicate that pregnant heifers were more efficient in terms of IVP compared to prepubertal and pubertal Holstein (*Bos taurus*) heifers.

Table 5. Number of recovered oocytes, COCs cultured, blastocysts, cleavage and blastocyst rate after OPU - IVP in *Bos taurus* (Holstein) donors in different categories.

Item	Heifers			P value
	Prepubertal (n = 60)	Pubertal (n = 60)	Pregnant (n = 59)	
Number of COCs retrieved	9.8 ± 1.3^{b}	15.6 ± 1.4^{a}	9.8 ± 1.6^{b}	0.001
COCs cultured	4.6 ± 0.6^{b}	9.1 ± 0.9^{a}	5.6 ± 1.1^{b}	0.001
Cleavage rate (%)[d]	31^{b}	56^{a}	78^{a}	0.001
Blastocysts produced	0.13 ± 0.1^{c}	0.9 ± 0.2^{b}	1.8 ± 0.3^{a}	<0.001
Blastocyst rate (%)[e]	2^{c}	14^{b}	37^{a}	<0.001

[d]Number of cleaved oocytes/number of cultured oocytes. [e]Number of blastocysts/number of cultured oocytes. Abbreviations: COC, cumulus - oocyte complex; IVP, *in vitro* embryo production; OPU, ovum pickup. Data with different superscripts in the same row differ at $P < 0.001$.

Oocyte quality and IVP during the early postpartum in donors

To assess the impact of early postpartum period on IVP in beef and dairy cattle, we have recently performed a study utilizing *Bos indicus* (Nelore; Pierucci *et al*., 2015) and *Bos taurus* (Holstein; Sala, 2013) cows. Ultrasound-guided follicular aspirations were performed every 14 days, from 30 to 86 days postpartum. Then, within breed, cows were blocked when 50% of the animals were cycling, which occurred at 30 days postpartum for *Bos taurus* and at 44 days postpartum in *Bos indicus* cows. Thus, we ended up with 2 experiments: experiment 1, *Bos taurus* cows with CL present (n = 14) and without CL present (n = 11) at 30 days with postpartum, and experiment 2, *Bos indicus* cows without CL present (n = 7) and without CL present (n = 8) at 44 days postpartum.

There was a significant effect of postpartum period, only for Nelore cows, in the number of aspirated follicles, recovered oocytes, and number of viable oocytes. In contrast, number of blastocysts (Fig. 2) as well as blastocyst rate did not differ with increasing days postpartum and cyclicity, both in *Bos indicus* and *Bos taurus* cows (Fig. 3).

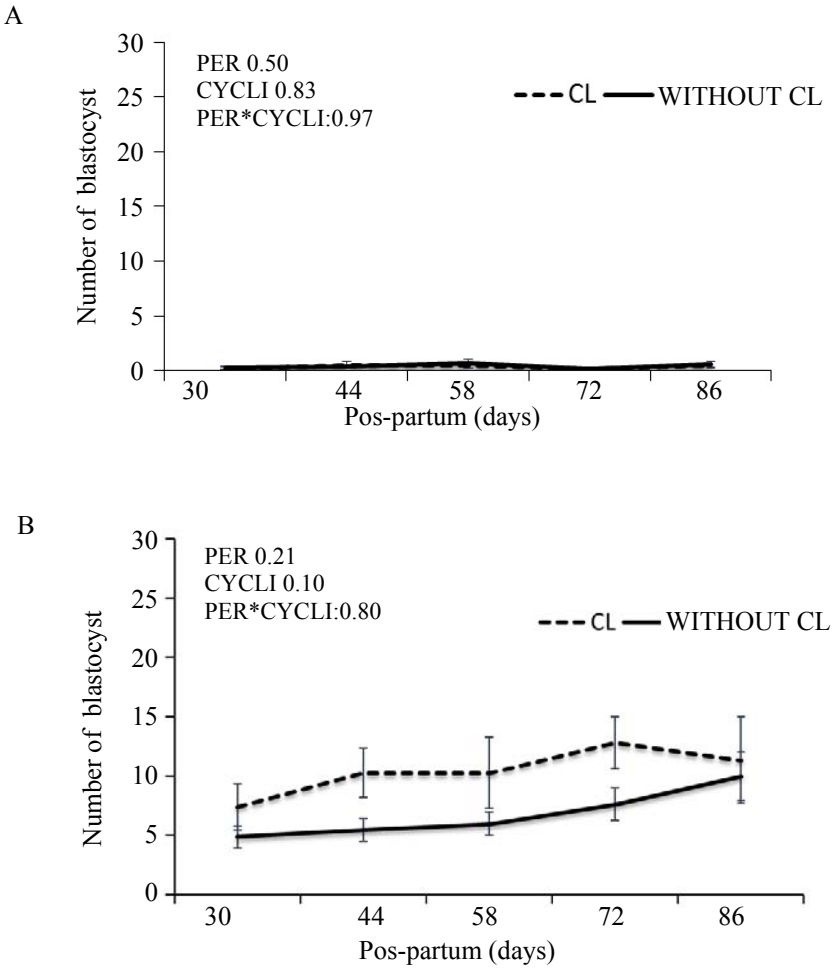

Figure 2. Number of blastocysts according to days postpartum in Holstein *Bos taurus* (A) and Nelore *Bos indicus* cows (B).

As observed in a previous study (Matoba *et al*., 2012), these results do not provide evidence of an effect of lactation-induced metabolic stress on oocyte developmental competence, in the early postpartum in dairy and beef cows, in terms of morphological ability to develop following *in vitro* fertilization (IVF). Previous research has shown impairment in fertility during early post partum due to metabolic disorder, mainly related to negative energy balance (Leroy *et al*., 2012). In these studies, fertility was evaluated after artificial insemination, with significant endocrine and metabolic alterations in the microenvironments of the dominant follicle. In studies with OPU/IVP, follicles are aspirated with approximately 2 to 3 mm, before the growth phase of the dominant follicle. This may partly explain the difference in results between experiments.

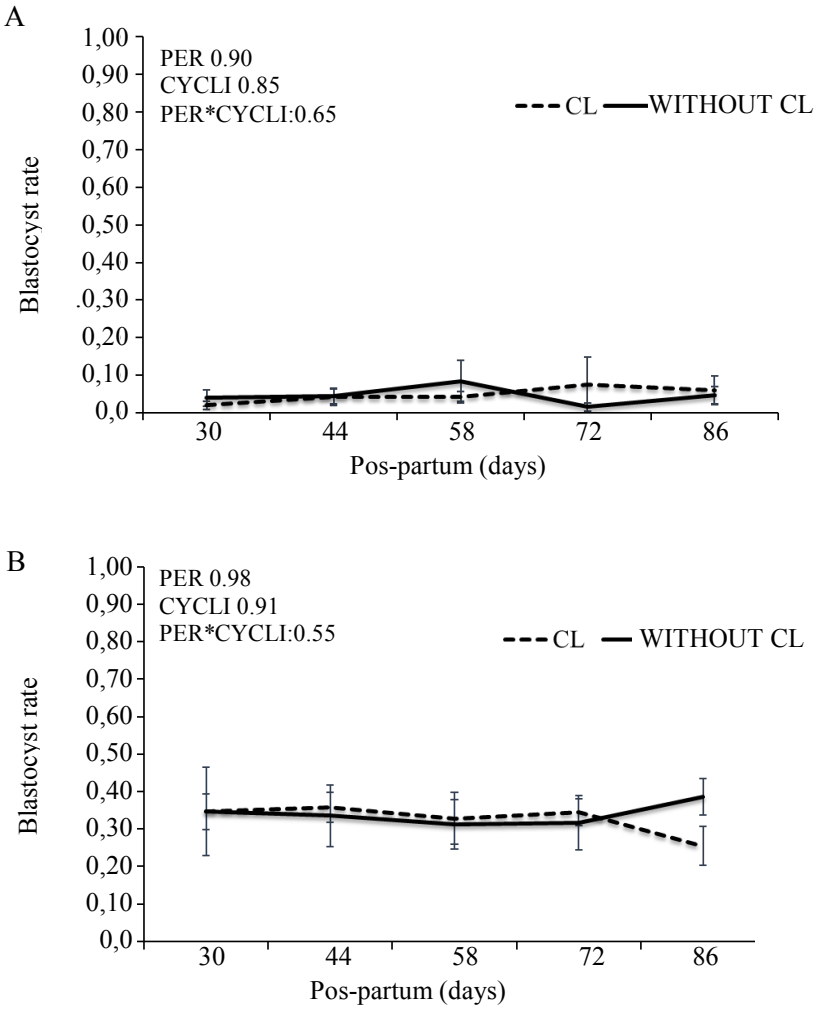

Figure 3. Blastocyst rate according to days postpartum in Holstein *Bos taurus* (A) and Nelore *Bos indicus* cows (B).

Oocyte quality and IVP during the during early or late lactation period

Clearly evaluating the effects of lactation on oocyte quality can be rather complex. For example, testing the isolated effects of lactation status per se on oocyte quality might be confounded by interactions with heat-stress for example, since lactating cows are generally more susceptible to heat stress. Also, lactating cows may suffer from different metabolic problems as lactation progresses until the dry-period. In addition, the type of diet and level of dry matter intake between lactating and non-lactating cows may also influence results of OPU-IVP. Despite of that, the negative effects of excessive energy intake was shown to compromise *in vitro* oocyte developmental competence, especially in over-conditioned (high body condition score) females (Adamiak, 2005). The mechanisms mediating these negative effects on oocyte competence may be related to endocrine alterations, such as hyperinsulinemia, peripheral insulin resistance, and increased glucose, NEFA and IGF-I, which may interfere with glucose transport in embryo cells and induce increased rates of apoptosis.

Lactating cows have been selected for milk production, making the modern dairy cow prone to peripheral insulin resistance to maintain milk production. Several factors have been involved to induce insulin resistance in lactating cows including excessive negative energy balance in early postpartum. Intriguingly, lactating dairy cows are again prone to insulin resistance that appears to happen more evidently in animals with greater body condition scores and having an excessive intake of diets with a high energy content. This is a rather common issue particularly in commercial dairies utilizing a single diet throughout the entire lactation. To

test whether stage of lactation and possible interactions with insulin resistance might influence oocyte quality, our research group (Baruselli *et al*. 2016), utilized Holstein cows that were either at early or late days in milk production at the moment of OPU-IVP. Results of this study clearly showed that insulin resistance associated with late lactation period (later lactation cows had greater circulating insulin levels) can disrupt oocyte quality and lower the efficiency of IVP. For example, we observed that cows at later lactation had a greater number of recovered oocytes per OPU session. In contrast, number of blastocysts, as well as blastocyst rates, were greatly reduced in cows at later lactation. In addition, a number of apoptotic genes were upregulated in cows with greater days in milk. These findings corroborate previous studies showing that lactating cows at later lactation are prone to insulin resistance, which clearly seem to lower oocyte viability during IVP procedures.

Table 6. Ovum pick-up and *in vitro* embryo production results from high producing Holstein cows during early or late lactation periods.

Item	Stage of lactation		P value
	Early	Late	
No. of animals	70	67	
DIM, days	110.5 ± 20.8	425.6 ± 21.0	N/A
Milk production, kg/day	34.3 ± 1.2	23.4 ± 1.2	<0.0001
No. of insemination	0.7 ± 0.2	7.0 ± 0.2	<0.0001
No. of lactation	2.4 ± 0.1	1.9 ± 0.2	0.05
BCS (1-5 scale)	2.79 ± 0.06	3.15 ± 0.07	<0.0001
No. of follicles	14.8 ± 2.4	22.7 ± 2.4	0.0016
Recovery rate, %	46.4 ± 4.4	53.8 ± 4.5	0.10
No. of oocytes	7.3 ± 2.0	14.3 ± 2.0	0.0004
No. of viable oocytes	4.6 ± 1.6	9.7 ± 1.6	0.0010
No. of cleaved oocytes (day 3)	4.7 ± 0.6	3.9 ± 0.6	0.10
Cleavage rate, (%)	48.0 ± 0.1	41.4 ± 0.1	0.08
No. of blastocyst (day 7)	2.2 ± 0.4	1.4 ± 0.3	0.06
Blastocyst rate (%)	23.0 ± 0.1	13.3 ± 0.1	0.0005

Adapted from Baruselli *et al*., 2016.

Oocyte quality and IVP in non-lactating donors

Oocyte quality has been considered an important factor contributing to the low fertility reported for high producing lactating dairy cattle (Walsh *et al*., 2011). Thus, we hypothesized that OPU-IVP procedures would result in a higher number of blastocysts per OPU session in non-lactating than in lactating donors. Our data showed higher number of blastocysts per OPU session in non-lactating than in lactating donors (Vieira *et al*., 2014). Non-lactating cows produced a higher blastocyst rate (41.9 *vs*. 13.4%; P = 0.001) and a higher number of transferable embryos per OPU (3.5 ± 0.5 *vs*. 1.3 ± 0.3; P = 0.003) than lactating Holsteins cows. Similar results were observed in a previously mentioned trial performed by our group (Table 3).

Dairy cows have a peculiar metabolic system, linked to nutrition and disruption of endocrine profiles. The metabolic profile of lactating dairy cows is commonly characterized by lower concentrations of progesterone and estradiol (Wiltbank *et al*., 2006) and increased concentrations of NEFA (nonesterified fatty acids) and BHBA (b-hydroxybutyrate; Leroy *et al*., 2005); and this peculiar metabolism has been associated with a suboptimal follicle microenvironment, compromising oocyte quality and resulting in a failure to conceive (Sartori *et al*., 2002, 2004; Wiltbank *et al*., 2006; Leroy *et al*., 2008a, b; Walsh *et al*., 2011). Therefore, the greater challenge of lactating cows to maintain an optimal reproductive efficiency may help explain the lower results observed in IVP. Thus, non-lactating donors may be considered the preferred donor in OPU-IVP programs, due to the higher yield of embryos per OPU session.

In another experiment (Sales *et al*., 2015) we studied the effects of different dietary energy levels [100 and 170% for maintenance (M) and high energy (1.7M), respectively] on metabolic, endocrine, and reproductive parameters in non-lactating *Bos indicus* (Gir; n = 14) and *Bos taurus* (Holstein; n = 14) cows submitted to OPU and IVP each 14 days. We measured glucose and insulin concentrations and performed glucose tolerance tests and the relative quantification of transcripts (PRDX1, HSP70.1, GLUT1, GLUT5, IGF1R, and IGF2R) from oocytes recovered at the end of the experimental period. No interactions were observed between the effects of breed and dietary energy level on the qualitative (viable oocytes, quality grade, and oocyte quality index) and quantitative (oocytes recovered) variables. There were no effects of dietary energy level on the qualitative and quantitative oocyte variables. *In vitro* embryo production (cleavage and blastocyst rates and number of embryos) was similar between diets, but the 1.7M diet reduced *in vitro* embryo production in *Bos indicus* cows after 60 days of treatment. Moreover, *Bos indicus* cows on the 1.7M diet showed lower transcript abundance for the HSP70.1, GLUT1, IGF1R, and IGF2R genes. All cows fed 1.7M diets had greater glucose and insulin concentrations and greater insulin resistance according to the glucose tolerance test. These results suggest that intake of a high energy diet for a long period reduces *in vitro* embryo production in non-lactating *Bos indicus* cows by causing

a hyperinsulinemic state, and promoting down-regulation of genes involved in cellular metabolism.

Conclusions and future directions

In conclusion, the IVP from younger beef or dairy cattle seem quite possible, although improvements are still needed to further improve this technology that came as a complement for genomic testing. More importantly, veterinarians working with OPU-IVP need to account for varying physiological aspects when working with specific cattle breeds (*Bos indicus vs*. *Bos taurus*). For example, avoiding working with cows submitted to a high energy diet for a long period and/or under heat stress, both factors that may induce poor oocyte quality, is highly advisable.

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Advances and limitations of *in vitro* embryo production in sheep and goats

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Abstract

This review summarizes the latest advances and main limitations for the implementation of *in vitro* embryo production programs in sheep and goats. We describe the laparoscopic assisted technique for oocyte retrieval and propose new insights for follicular manipulation to improve oocyte quality. Further description of the routine conducted in our laboratory for the *in vitro* process of oocyte maturation, fertilization and embryo culture is presented, with emphasis in the main issues for the success of the technique. Protocols for fixed time embryo transfer (FTET) are proposed and the optimal number of *in vitro* produced (IVP) embryos to be transferred *per* female is discussed. In addition, we present pregnancy outcomes and birth rates recently obtained with FTET with IVP embryos cryopreserved by vitrification with new minimum volume methods. In summary, due to important refinements for *in vitro* embryo production in sheep and goats achieved in the recent years, this technology is now available for its implementation in commercial programs for genetic improvement, for the production of genetically engineered sheep and goats, and for basic research in reproduction.

Keywords: cryopreservation; ewe, goat, LOPU, superovulation.

Follicular aspiration by laparoscopy

The success of an *in vitro* embryo production (IVEP) program for either research purposes or industry application depends largely on the availability of a continuous number of good quality oocytes. Although slaughterhouse ovaries represent a very useful cheap and rich oocyte source for research projects, the application of this technology in the industry and livestock requires oocyte retrieval from live animals. Follicular aspiration by laparoscopy (laparoscopic ovum pick-up; LOPU) is the best available technique to obtain oocytes from sheep and goats, firstly described by Robin Tervit in New Zealand and Hernan Baldassarre in Argentina in the early 1990s (Tervit *et al.*, 1992; Baldassarre *et al.*, 1994). This approach has the advantage of eliminating the poor results observed with the traditional laparotomy technique, besides the possibility of repeating the procedure several times in the same female, and allowing the production of offspring from prepubertal animals (Baldassarre and Karatzas, 2004). LOPU is used to recover oocytes for conventional IVP, but also for the production of zygotes for the generation of transgenic founders (Baldassarre *et al.*, 2003b) or for propagating transgenic animals by somatic cell nuclear transfer (Baldassarre *et al.*, 2003a). Recently, this technique has also been proposed as a useful tool to assist the generation of genome edited sheep and goats (e.g. CRISPR system; Menchaca *et al.*, 2016).

During the LOPU procedure, the animal is restrained on a standard laparoscopy table under general anesthesia. In our laboratory, general anesthesia is induced with ketamine and diazepam by i.v. route and is maintained with isofluorane inhalatory anesthesia. LOPU technique is further described by H. Baldassarre (Menchaca *et al.*, 2016), and briefly, consist of a laparoscopy equipment with a 5 mm diameter and 0° angle telescope, two 5.5 mm trocar/cannula sets (one for the laparoscope and one for the forceps), one 3.5-5 mm trocar/cannula set for the aspiration pipette, a 5 mm atraumatic grasping forceps, a fiber optic cable and a light source. The oocyte aspiration set consists of a collection tube with an inlet connected through tubing to the aspiration pipette and an outlet connected through tubing to a vacuum pump. The aspiration pipette consists of a 20G short bevel needle glued to the tip of a 30 cm-long acrylic tubing, with a 3 mm external diameter and 1 mm internal diameter. The ovarian surface is exposed by pulling from the fimbria in different directions using the forceps, and the follicles are punctured one by one using the aspiration pipette. For a trained team, in average it takes about 15-20 min per donor (including preparation) and recovery rate of cumulus-oocyte complex (COC) ranges from 50 to 90%.

The possibility to use prepubertal females of 1-3 month old as oocyte source is another advantage of the LOPU technique, reducing the generational interval and accelerating the process of genetic improvement (Paramio, 2010). In addition, LOPU can be performed repeatedly in the same female with an interval between aspiration sessions of 4 days without affecting oocyte retrieval and quality (Gibbons *et al.*, 2007). These two possibilities remarkably increases the capacity of multiplication of a female. The incidence of injuries caused to the ovary after successive LOPU sessions has been under study. In general, most of the reports did not found severe ovarian lesions that interfere with follicular aspiration, oocyte retrieval or embryo yield after 3 to 7 repeated hormonal treatments and LOPU sessions (Pierson *et al.*, 2004; Gibbons *et al.*, 2007; Teixeira *et al.*, 2011; Sanchez *et al.*, 2014). Other authors reported the presence of small adhesions in around 30% of sheep after repeated LOPU over a 10-week period (McEvoy *et al.*, 2006). In our experience, although some adhesion or fibrosis may be eventually

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found in few animals after several follicular aspiration sessions, it does not represent a relevant limitation.

Manipulation of follicular growth

In order to recover a high number of good quality COCs per LOPU session, the ovarian status must be synchronized and stimulated using gonadotropins. For synchronization, different protocols have been used in both sheep and goats and most of them are based in estrus synchronization treatments, mainly with progesterone or progestogen-containing intravaginal devices (e.g. CIDR; sponges). Usually, the devices are applied during 9-11 days together with a luteolytic dose of prostaglandin (PG) F2alpha or an analog at the time of gonadotropin treatment start. After synchronization, several treatments have been proposed for follicular stimulation prior to LOPU, being the most popular the multiple FSH injection regime and the so-called oneshot regime, consisting of one dose of FSH plus equine chorionic gonadotropin (eCG) described by Baldassarre and colleagues (Baldassarre *et al.*, 2002).

Different studies in sheep and goats have shown that *in vitro* matured (IVM) oocytes obtained by follicular aspiration have lower oocyte competence when compared to those maturated *in vivo* (Cognie *et al.*, 2003), suggesting unsuitable IVM conditions, but also low quality of the oocyte population obtained by aspiration. Oocyte competence is affected by the follicular size from which the COC is collected (Crozet *et al.*, 1995) and also by follicular wave dynamics, growth status and dominance further studied in cattle (Adams *et al.*, 2008). The ideal method should ensure a homogeneous pool of medium (~4 mm) and young follicles in growing or static phase containing healthy oocytes, and never in late atresia. With this in mind, recently we have tested new approaches for follicular control before LOPU in sheep. Previously, we had described the Day 0 protocol for stimulation of the first follicular wave, for superovulation in conventional embryo production or MOET programs (Menchaca *et al.*, 2002, 2007, 2009, 2010). This protocol synchronizes the ovulation (defined as Day 0 of the cycle) to induce the emergence of the first follicular wave 72 to 84 h after progesterone device removal and eCG administration. In Day 0 Protocol for MOET programs, FSH is administered in several doses (e.g. 6 or 8 doses) twice a day with the first dose given 84 h after device removal (i.e. soon after ovulation), coinciding with follicular recruitment of wave 1. Recently, we have validated this protocol associated to LOPU. In this case, a single dose of 80-100 mg of pFSH reconstituted in sodium hyaluronic acid (MAP-5, Vetoquinol) for slow releasing was given on Day 0 (i.e. 84 h after device removal). LOPU was performed 72 h after FSH dose (i.e. on Day 3). Preliminary data show higher number of aspirated follicles and collected oocytes using the Day 0 protocol with pFSH in MAP-5 when compared with those obtained from no FSH treated ewes (Cuadro *et al*.; IRAUy, Montevideo, Uruguay; unpublished results). Interestingly, the effect of pFSH in MAP-5 used in Day 0 protocol was potentiated when an intravaginal progesterone releasing device (DICO 0.3 g) was inserted from Day 0 (i.e. at FSH injection) until the day of LOPU. Follicular development in the presence of high progesterone levels significantly enhanced COCs quality, oocyte fertilization rate, and embryo production (Cuadro *et al*.; IRAUy, Montevideo, Uruguay; unpublished results). The addition of an intravaginal progesterone releasing device during the follicular development seems to be recommended during the stimulation of wave 1.

***In vitro* maturation**

The procedure for *in vitro* embryo production in sheep and goats has been extensively studied and is further described in previous reviews (Cognie *et al.*, 2004; Paramio, 2010; Souza-Fabjan *et al.*, 2014; Paramio and Izquierdo, 2014, 2016). For that reason, this document describes only the main features and weaknesses of this process.

Regarding IVM, probably most of the effort has been focused on improving the conditions of *in vitro* culture media. In that sense, a variety of media system have been studied, proposed and adopted. In general, most laboratories use TCM199 supplemented with different components for this stage. This medium can be supplemented with gonadotropins as LH, FSH or a combination of both since it increases the number of oocytes reaching MII and improves the rate of viable embryos (Moor and Trounson, 1977). In addition, cysteamine supplementation acts as a glutathione precursor, improving oocyte maturation clearly demonstrated in several species included sheep and goats (de Matos *et al.*, 2002; Cognie *et al.*, 2003). Estradiol is another proposed component to be included into maturation medium since it is present in the preovulatory follicular fluid. However, its effect is controversial (Guler *et al.*, 2000) and seems to be depending on the presence/absence of other components. Although in lower concentration than follicular fluid, estradiol is present in another component normally added to the IVM medium, the estrus sheep/goat serum. Estrus sheep/goat serum is routinely used in our laboratory, although its variability is well known, containing each batch different levels of metabolites, growth factors, hormones, proteins, lipids, etc., making it difficult to standardize culture conditions. In addition, as a biological component, serum represents a sanitary risk that may be relevant for commercial application of IVEP. On the other hand, chemically defined IVM media have greater repeatability and are safer from a sanitary point of view, deserving future investigation, validation and adoption. A deeper knowledge about the role of follicular environment in oocyte maturation, the function of its components and the interaction between the oocyte and cumulus cells, will help to improve IVEP in the future.

Probably, there are as many variations in embryo culture media as IVEP labs in the world. In our laboratory, maturation medium is composed of TCM-199 supplemented with estrus sheep serum, FSH, LH, cysteamine and antibiotics. Excellent and good quality

oocytes are incubated in groups of 25-30 per 100 µl drops of IVM medium under mineral oil for 24 h at 39°C in a humidified atmosphere containing 5% CO_2. Normally, more than 90% of metaphase II oocytes is expected to obtain under these conditions.

In vitro fertilization (IVF)

There are different strategies to prepare ovine/caprine semen for IVF, similar to those used in cattle. Motile spermatozoa are obtained by Percoll gradient or swim-up method and added to COCs in fertilization medium after IVM. Sperm capacitation is normally achieved with the use of heparin added to the fertilization medium and during semen preparation before IVF (e.g. during swim-up). In addition, ionomycin has been tested to improve fertilization rates in goats with good results (Urdaneta *et al.*, 2004).

Some laboratories remove cumulus cells at the end of IVM just before IVF (Cognie *et al.*, 2004). The effect of cumulus cells during IVF has been studied in sheep (Menchaca *et al.*, 2012) and goats (Souza *et al.*, 2013). We found a positive effect of cumulus cells during IVF, with greater cleavage rate (85 *vs*. 77%; $P < 0.05$) and developmental rates (37 *vs*. 17%; $P < 0.05$) when cumulus cells were maintained during IVF than when removed prior to IVF, respectively ($P < 0.05$). Similar improvement has been reported in goats with greater blastocysts yield (48 *vs*. 37%; $P < 0.05$) when cumulus cells were maintained during IVF (Souza *et al.*, 2013). After testing several protocols and components, in our laboratory we use synthetic oviduct fluid (SOF) supplemented with heparin, hypotaurine and estrus sheep serum, both for IVF medium and sperm preparation medium. Spermatozoa are capacitated by swim-up with an incubation time of 15 min, and an insemination dose of $1x10^6$ total sperm per drop of 100 µl SOF under mineral oil containing 20-30 oocytes. Oocytes and sperm are co-incubated for 18 to 22 h at 39°C in a humidified atmosphere containing 5% CO_2. Working under these conditions with frozen semen, processing more than 6,000 COCs during the last year, the cleavage rate at 48 h from insemination ranged from 80 to 90% in more than 80% of the IVP sessions.

In vitro culture (IVC)

Four events are crucial during IVEP: cleavage, embryonic genome activation (8-16 cells), morula compaction, and blastocyst formation accompanied by the formation of the inner cell mass and trophectoderm (Lonergan *et al.*, 2003). These events are negatively affected by inadequate culture conditions and several strategies have been designed to mimic the female tract in the lab, with approaches that include *in vivo* culture of zygotes on the oviduct of temporary recipients, *in vitro* co-culture with somatic cell support, and semi-defined media to suit embryo requirements (Paramio and Izquierdo, 2016).

Different culture media have been tested in small ruminant's embryos; however, SOF medium first described in the 1970s (Tervit *et al.*, 1972) is the most used among laboratories to culture sheep embryos and has been adapted to numerous species including cattle, pigs and goats. In our experience, changing or refreshing IVC medium on Day 3 after insemination improved blastocyst yield on Day 6 from 33.6% (78/232) to 42.0% (102/243; $P < 0.05$), probably by providing fresh nutrients and removing toxic metabolites (Vilariño *et al.*, 2012). Usually embryos are maintained in groups during IVC since this improves blastocysts yields than those single cultured (Gardner and Lane, 1993). In our laboratory, IVC is performed in groups of 20-30 embryos in 100 µl drops using SOF under mineral oil and supplemented with BSA, essential and nonessential amino acids and antibiotics, at 39°C in 5% O_2, 5% CO_2 and 90% N_2. Expected blastocyst rate under these conditions is around 30 to 40% (number of blastocysts on Day 6 from COCs in IVF).

Transfer of IVP embryos

Embryo transfer outcomes could be affected by the embryo quality and by intrinsic and extrinsic factors from the recipient (breed, age, reproductive status, nutrition and health). Regarding embryo quality, survival rate progressively increases with the stage of embryo development for *in vivo* derived embryos (Bari *et al.*, 2003). Although information for IVP embryos in small ruminants is scarce, the convenience of embryo transfer in blastocyst stages instead of morulae is largely accepted. Regarding the maternal component, the recipient female should provide a competent luteal function with sufficient progesterone production as well as a suitable uterine environment for embryo development and placentation. The luteal activity is determined, at least in part, by the treatment applied to synchronize the ovulation. In this sense, traditional hormonal protocols consist of progesterone or progestogen-based treatment usually administrated with intravaginal devices for 10 to 14 days (goats and sheep, respectively) and associated to eCG administration i.m. at device removal, with a luteolytic dose of PGF2alpha in goats. Alternatively, short-term protocols with progestogen treatment during 6-7 days have been used in sheep with similar outcomes than traditional treatments (Menchaca *et al.*; IRAUy, Montevideo, Uruguay; unpublished data). Using this protocol for estrus synchronization, around 90% of estrus and ovulation occurs in average 30 and 60 h from device removal, respectively (Menchaca and Rubianes, 2004). In our routine in sheep, the intravaginal device is inserted on Day 0 and removed in the evening of Day 6 plus PGF2alpha and 300 IU eCG, and marker vasectomized males are introduced into the flock from 24 to 48 h after device removal. Fixed time embryo transfer (FTET) is performed on Day 15 (i.e. 8.5 days after device removal and around 7 days after the onset of estrus) to transfer day 6 blastocysts (i.e. 7 days after follicular aspiration). FTET is carried out only in painted females (i.e. those that were mounted by the males) and the embryos are transferred into the uterine horn ipsilateral to the corpus luteum, assessed by laparoscopy. Expected pregnancy rate is approximately

50-60% when FTET is conducted with fresh embryos. Alternatively, FTET may be performed into the oviduct on Day 10, i.e. 3.5 days after device removal and one day after *in vitro* insemination and ovulation.

The recommended number of IVP embryos to be transferred per recipient is 1 or 2 embryos when FTET is performed with blastocysts, depending on the species, breed and productive system. In our experience, when transferring two IVP embryos around 30% of pregnant ewes get pregnant with twins. In a recent trial in which FTET was conducted with 240 IVP embryos in Merino recipients (a breed having around 1.1 of prolificity), pregnancy rate was about 10% greater when two embryos were transferred ($P < 0.05$). However, birth weight was 20% lower in twins ($P < 0.05$) and lamb survival rate after birth was much greater with single pregnancies (about 30%; $P < 0.05$) (Menchaca *et al*.; IRAUy, Montevideo, Uruguay; unpublished results). For this reason, under typical rangeland conditions as in Uruguay and Argentina, the objective should be to achieve one born lamb per ewe, which ensure a suitable birth weight and lamb survival rate.

Are we ready to skip the fresh embryo transfer?

One of the greatest difficulties of *in vitro* embryo technology in livestock is the low cryotolerance of the produced embryos in comparison to *in vivo* derived (IVD) embryos. For this reason, usually IVEP programs are conducted with fresh embryos, which in large-scale programs with embryos produced every week during long periods of time, require a large and continuous number of ready-to-use recipients. In this context, embryo cryopreservation deserve to be considered.

Conventional slow freezing methods were developed for IVD embryos, and in fact, they are widely applied in sheep and goats in commercial MOET programs. In general, this conventional method results in very low survival rates when applied to IVP embryos (Massip, 2001). *In vitro* produced embryos have some intrinsic differences with IVD embryos that negatively affect cryotolerance, associated with excessive accumulation of lipids, altered metabolism, changes in structural and physic characteristic, among others (Seidel, 2006). For this reason, vitrification methods have been further studied in parallel with the development of IVEP technology in different species, applied in livestock and endangered species, but also demanded in human assisted reproduction for oocyte and embryo cryopreservation. Several studies have been conducted to test different vitrification methods for small ruminants embryos (Traldi *et al.*, 1999; Dattena *et al.*, 2000; Baril *et al.*, 2001; Martínez *et al.*, 2006; Gibbons *et al.*, 2011; Morato *et al.*, 2011). In general, the effectiveness of vitrification technique depends on several factors as the stage of embryo development, embryo origin (*in vivo* or *in vitro*), volume and cooling rate, cryoprotectant media, and the species, among others (Arav, 2014).

During the last years, novel concepts related to vitrification have supported the development of minimum volume methods, reducing the volume of cryoprotectants and increasing the cooling and warming rates (Yavin and Arav, 2007). We have conducted a series of experiments with ovine embryos to improve survival rate after cryopreservation by using vitrification with minimum volume methods (Cryotop and Spatula MVD). These two vitrification methods have been previously reported for human (Kuwayama, 2007) and mice embryos (Tsang and Chow, 2009), respectively. In a recent study, we found that ovine IVP embryos vitrified with Cryotop and Spatula MVD showed acceptable *in vitro* survival and development rate (dos Santos Neto *et al.*, 2015). In a more recent study (dos Santos Neto *et al*.; IRAUy, Montevideo, Uruguay; unpublished results), we transferred 437 embryos to compare *in vivo vs. in vitro* sheep embryos subjected to vitrification by Cryotop or Spatula MVD methods, or to conventional freezing. As expected, regardless the cryopreservation method, pregnancy establishment was greater with IVD than with IVP embryos (pregnancy rate 68.8 *vs*. 22.3%, $P < 0.05$). Interestingly, recipient females receiving IVD or IVP embryos resulted in greater pregnancy rate ($P < 0.05$) when vitrification was performed by Cryotop method (77.8 and 55.1%) than Spatula MVD (59.3 and 18.4%) or conventional freezing methods (64.9 and 11.1%, respectively). Thus, in these conditions, the Cryotop method reached >50% of pregnant females transferred with IVP embryos. Pregnancy losses from 30 days of gestation to delivery tended to be greater for IVP than IVD embryos (14.9 *vs*. 5.8%; $P = 0.08$), with no effect of the cryopreservation method (dos Santos Neto *et al.*; IRAUy, Montevideo, Uruguay; unpublished data). Therefore, minimum volume vitrification methods seems to be interesting for future implementation in IVEP programs in sheep. However, some refinements are still necessary in order to have an easy and robust method to be applied on the field, and thus, finally take advantage of the benefits of cryopreservation.

Concluding remarks

The cumulative work achieved during several years by the contribution of many researchers, briefly summarized in this review, have transformed the IVEP in a supporting tool for livestock, genetic engineering and research. However, some limitations needs to be solved in order to improve the outcomes and simplify the technique. Oocyte competence is variable and should be improved, mainly by controlling follicular wave dynamics to offer more healthy oocytes for LOPU. *In vitro* COCs maturation, fertilization and embryo culture is a well-standardized procedure, but still requires some fine-tuning since most of the embryos die during the process before hatching. Embryo transfer and recipients management may be improved by FTET and standardized protocols for ovarian synchronization, as proposed in this review. In addition, fresh embryo transfer is a limiting factor for international exchange of genetic material and requires continuous availability of recipients. In this sense, new minimum volume methods for vitrification seems to be

attractive allowing acceptable pregnancy rates in our system. In summary, although some challenges are still present in IVEP in sheep and goats, substantial improvements have been achieved during the last years and this technology is available to be applied on the field.

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In vitro culture systems: how far are we from optimal conditions?

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Abstract

Over the past decades *in vitro* production (IVP) of bovine embryos has been significantly improved. Nevertheless, embryos generated *in vitro* still differ from their *in vivo* produced counterparts. Embryos must adjust to multiple microenvironments at preimplantation stages. Consequently, maintaining or mimicking the *in vivo* situation *in vitro* will aid to improve the quality and developmental competence of the resulting embryo.

Keywords: cattle, embryo, *in vitro* production.

Introduction

The birth of the first IVF calf derived from an *in vivo* matured oocyte in 1981 (Brackett *et al*., 1982) and the discovery of heparin as capacitating agent for bull sperm in 1986 (Parrish *et al*., 1986) were the two key events starting an era of intense research resulting in efficient *in vitro* production (IVP) systems for preimplantation embryos including *in vitro* maturation (IVM) of the oocyte to the metaphase II, *in vitro* fertilization (IVF), and subsequent *in vitro* culture (IVC) of embryos to the blastocyst stage. The first calves produced entirely from IVM-IVF-IVC were born in 1987 (Fukuda *et al*., 1990).

Another milestone was the development of ultrasound-guided transvaginal oocyte aspiration in humans and the adoption in the bovine in 1988 (Pieterse *et al*., 1988). The current technology of OPU/IVP harvesting immature oocytes form living cows can routinely be performed twice a week for an extended period of time without any long-term detrimental effects on the donor's cow fertility (Chastant-Maillard *et al*., 2003). Attempts were undertaken to combine OPU with colour Doppler ultrasonography which is a useful, noninvasive technique for evaluating ovarian vascular function, allowing a visual observation of the blood flow in a delimited area in the wall of preovulatory follicles (Brännström *et al*., 1998). Blood flow determinations of individual preovulatory follicles prior to follicular aspiration for IVF therapy provide an important insight on the intrafollicular environment and may predict the developmental competence of the corresponding oocyte (Coulam *et al*., 1999; Huey *et al*., 1999). In cattle, it has been shown that the time interval between the individual OPU sessions had an effect on the quality of oocyte and embryos at the molecular level, whereas differences in the perifollicular blood flow did not (Hanstedt *et al*., 2010). An increase in the blood supply to individual follicles appears to be associated with follicular growth rates, while a reduction seems to be closely related to follicular atresia (Acosta *et al*., 2003; Acosta 2007). Taken together, OPU can be considered a mature technique and no major improvements should be expected in the technology and its results in the near future. At present the application of IVP combined with ovum pick up (OPU) from valuable donors is increasing (again) due to developing breeding strategies based on genomic selection using SNP (single nucleotide polymorphism) chips. Depending on the chip used, thousands of these SNPs can be analyzed even in a biopsy taken from an embryo. This technology is now reaching routine usage for genomic selection (GS) in cattle (Ponsart *et al*., 2013).

With regard to IVP efficiency, approximately 80-90% of immature bovine oocytes undergo nuclear maturation *in vitro*, about 80% undergo fertilization, 30-40% develop to the blastocyst stage, and around 50% of the transferred embryos establish and maintain a pregnancy (Wrenzycki *et al*., 2007; Galli *et al*., 2014; Lonergan *et al*., 2016).

IVM

Cumulus-oocyte-complexes (COC) collected from ovaries of slaughtered or euthanized animals or from living animals via ovum pick-up (OPU) require *in vitro* maturation (IVM) as they are arrested at the germinal vesicle (GV) stage. Maturation involves a series of events that begin in fetal life with the initiation of meiosis. At birth, the oocytes are arrested at the diplotene stage (germinal vesicle stage, GV). After puberty when they are exposed to preovulatory surges of LH and FSH they proceed with meiosis and are arrested again at the metaphase II, the stage at which they are ovulated (Monniaux *et al*., 2014). In addition, optimal conditions for cumulus cells surrounding the oocyte need to be considered as there is a complex bi-directional communication between these two cell types (Gilchrist, 2011; Monniaux, 2016).

Proper maturation of the oocyte to metaphase II is a prerequisite for fertilization and pre-implantation development. It is possible to achieve blastocyst rates of up to 70% if *in vivo* matured oocytes are used. In contrast, if oocytes are matured *in vitro*, blastocyst rates are only half that of those matured *in vivo*. This rather limited success may be attributed to the heterogeneous population of oocytes which are normally retrieved from follicles of 3-8 mm rather than from preovulatory follicles. In contrast to the *in vivo* ovulated oocyte, these oocytes lack development up to the preovulatory stage

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and are matured *in vitro*. Therefore, much effort has been devoted to the establishment of noninvasive and non-perturbing means for selecting the most competent oocytes (Fair, 2010; Krisher, 2013; Wrenzycki and Stinshoff, 2013).

IVM of immature oocytes occurs by a different mechanism from that of *in vivo* matured oocytes. IVM is initiated immediately following the removal of the immature oocyte from small antral follicles, and such oocytes may have neither the time nor the correct environment to complete the necessary changes required for subsequent successful development (Krisher, 2013; Wrenzycki and Stinshoff, 2013; Lonergan and Fair, 2016).

Recently, the so-called simulated physiological oocyte maturation (SPOM) system has been introduced (Albuz *et al*., 2010). It prevented spontaneous resumption of meiosis after mechanical oocyte retrieval and thereby improved *in vitro* embryo development. However, due to the fact that similar outcomes were not easily to achieve, a revised version has been reported (Gilchrist *et al*., 2015). At the moment, most laboratories practicing IVM of cattle oocytes use a relatively simple oocyte maturation system.

IVF

IVF is a complex procedure whose success requires appropriate oocyte maturation, sperm selection, sperm capacitation and IVF media.

Semen samples contain a heterogeneous population of sperm cells. *In vivo*, the sperm cells are thought to be selected by various mechanisms within the female reproductive tract, with the result that the small number of spermatozoa found near to the oocyte are typically those best able to penetrate the zona pellucida and fertilize the oocyte. When using IVF, however, these natural selection mechanisms are circumvented. The most common method for preparing spermatozoa for IVF is by centrifugation them through a concentration gradient, such as a 45% Percoll mixture layered on a 90% solution.

A variant of colloid centrifugation using only one layer of colloid (in which case there is no gradient) has been developed. Single-layer centrifugation (SLC) through a species-specific colloid has also been shown to be effective in selecting spermatozoa with good motility, normal morphology and intact chromatin (Thys *et al*., 2009; Goodla *et al*., 2014; Morrell *et al*., 2014; Gloria *et al*., 2016). An alternative method is the swim-up procedure. The disadvantages of swim-up are that it takes approximately 45-60 min to do and only 10-20% of the spermatozoa in the sample are recovered. For colloid centrifugation, only 25 min preparation time is needed (including the centrifugation) and a recovery rate of >50% is commonly achieved (Thys *et al*., 2009), although this does depend on the sperm quality of the original sample.

Once IVM is complete, oocytes are ready to be fertilized. This involves the coincubation of oocytes with sperm cells. Most laboratories allow for 18-19 h of coincubation. The changes a sperm cell has to go through before it can fertilize an oocyte are summarized under the term capacitation. Media have been developed to support this process, e.g. TALP medium. As mentioned earlier, the primary capacitation agent is heparin. The majority of semen used for IVF is frozen-thawed. The most common final sperm concentration used in the IVF drop is 1×10^6 sperm/ml.

IVC

IVC of bovine embryos is the last step in the IVP procedure and involves approximately 6 days of culture from the presumptive zygote onwards. The most common media for culturing bovine embryos are variations of the original synthetic oviduct fluid (SOF) medium (Tervit *et al*., 1972). SOF is now part of most routine bovine IVP systems with/without serum. Embryos are cultured in only one medium throughout the entire time or a sequential system in which the medium formulation changes at certain time points in the culture period. These sequential media try to mimic the physiological changes that embryos encounter *in vivo* when they move down the oviducts and into the uterus. Parameters which vary from lab to lab are diverse, e.g. the volume of medium and the atmosphere in the incubator.

In vitro culture (IVC) conditions have been enhanced in the last years, mainly by adjustment of media formulations. However, while over 30% blastocyst formation could be achieved in most culture systems, it soon became obvious that quantity did not always match quality (Wrenzycki *et al*., 2005; 2007 Lonergan *et al*., 2006;) and that serum supplementation was detrimental to embryo/fetal development as one main causal factor of the so-called large offspring syndrome (LOS), characterized by abnormally advanced embryonic and fetal growth, altered gene expression patterns, and high perinatal losses (Young *et al*., 1998; Lazzari *et al*., 2002). A large field study demonstrated that the incidence of LOS was greatly reduced by *in vitro* culture in cell-free and serum-free SOF media (van Wagtendonk-de Leeuw *et al*., 2000). Such observations highlight the importance of the post-fertilization culture environment for the quality of the resulting blastocyst. However, the existence of diverse embryo culture media and methods makes it very challenging to define the optimal components of embryo culture media.

The success of an IVP laboratory may stem not only from improvements of the IVC per se, but from the entire IVP system (Gardner, 2008; Baltz, 2012; Leese, 2012). The latter includes: incubation conditions, gas phase, culture media, oil overlay, plastic ware, and embryo density and the volume of the medium. In addition, the skills of the staff involved in the entire process have to be considered as part of the system.

In general, IVP can be considered to be at an advanced stage of progress. However, an aspect that may change in the future is automation and miniaturization of the IVP process by better mimicking the *in vivo* environment, e.g. using microfluidics (Wheeler *et al*., 2007) or an encapsulation technology (Blockeel *et al*., 2009) to obtain IVP embryos of similar

quality as the *in vivo* ones.

Quality assessment of preimplantation embryos

The ultimate test of the quality of an embryo is its ability to produce live and healthy offspring after transfer to a recipient. Morphology and the proportion developing to the blastocyst stage are used as criteria to assess developmental competence. Evaluation of embryo morphology remains the method of choice for selection of viable embryos prior to transfer. It is the most practical and clinically useful approach to assess of embryo viability (Van Soom *et al*., 2003). A bovine embryo grading system developed previously (Lindner and Wright, 1983) is, with minor modifications, still widely applied in this field (Hasler, 2001), listed in the IETS Manual. However, sometimes embryo morphology alone is not accurate enough to act as the sole criterion for the prediction of embryo developmental potential *in vivo*. Better non-invasive markers and improved techniques are required. These techniques can provide more valuable information on embryo viability. For examples, measurement of oxygen consumption using the nanorespirometer (Lopes *et al*., 2007) as well as amino acid profiling (Sturmey *et al*., 2010) can be employed to predict developmental competence and embryo viability. Although non-invasive approaches are improving, invasive ones have been extremely helpful in finding candidate genes to determine embryo quality (Wrenzycki *et al*., 2007; Rizos *et al*., 2008; Graf *et al*., 2014).

Conclusion

In vitro production (IVP) of bovine embryos follows a well-developed procedure that is commercially available for this species. However, despite all the improvements in oocyte and embryo culture, at best only 30-35% of immature bovine COC develop to the blastocyst stage which might represent a reasonable efficiency. But the *in vivo* situation still cannot be mimicked sufficiently well. The quality of the embryos produced is still impaired in comparison with their *in vivo* counterparts. This suggests that there are still improvements to be made in increasing oocyte and embryo developmental competence. More basic research is needed on molecular mechanisms e.g. epigenetic reprogramming during early embryonic development as well as detailed studies on the composition and interactions of culture media. By altering the conditions of oocyte maturation and embryo culture respectively to mirror more closely that which occurs *in vivo*, it may be possible to produce not only more blastocyst stage embryos, but more importantly, blastocysts of better quality.

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Fertility programs for lactating dairy cows, their physiological basis, and the factors that are critical for their success

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Abstract

Lactating dairy cows have unique reproductive responses compared to when they were heifers that result in distinctly different reproductive measurements and pregnancy outcomes that can be partially overcome with pharmacological strategies. These parameters include circulating progesterone and estradiol concentrations, ovulatory follicle and corpus luteum diameter, incidence of anovulation and double ovulations, time in estrus, pregnancies per artificial insemination and pregnancy losses. Circulating concentrations of progesterone during diestrus are approximately half that in cows compared with heifers. This marked difference in progesterone is likely the explanation for an increased size in diameter of the ovulatory follicle and incidence of double ovulations in cows compared with heifers. Differences in diameter of the ovulatory follicle may explain why cows have greater corpora lutea diameters compared to heifers. The increase in double ovulations appears to be a key driver in the increase in twinning and pregnancy loss as dairy heifers transition to primi- and multiparous cows. Reduced estradiol concentrations in cows at time of estrus helps to explain the decreased duration of estrus in cows compared with heifers. Concentrations of progesterone during growth of the ovulatory follicle may be a key driver in differences in pregnancies per AI in cows compared with heifers. The difference in circulating progesterone may be related to LH overstimulation of the oocyte/cumulus complex in cows compared to when they were heifers. Pharmacological strategies have been developed in lactating dairy cows to manipulate ovarian development to create a hormonal environment similar to that of heifers. Three primary strategies, Presynch-11, G6G and Double Ovsynch appear to enhance fertility of dairy cows. This review discusses how these three strategies manipulate ovarian development similar to that of heifers and why compelling data indicate these programs should be referred to as "fertility programs."

Keywords: corpus luteum, dairy, follicle, ovsynch, progesterone.

Introduction

The evolutionary role of the ovary is to produce oocytes capable of fertilization. The supporting cast of ovarian structures, follicles and corpora lutea, are responsible for the hormonal environment, production of oocytes and maintenance of pregnancy. The physiological outcomes of these structures in lactating dairy cows when compared with nulliparous heifers is associated with negative reproductive outcomes (Wiltbank *et al.*, 2006). Pharmacologic manipulation of ovarian structures can reverse these effects of lactation and enhance fertility (Wiltbank *et al.*, 2011). The physiological basis for development of pharmacological programs to improve fertility of lactating dairy cows comes from studies that characterized key differences in reproductive indices in primiparous and multiparous cows (referred heretofore as "cows") *vs*. nulliparous heifers (referred heretofore as "heifers"; Sartori *et al.*, 2002, 2004; Wolfenson *et al.*, 2004). Differences in circulating concentrations of progesterone (P4) appears to be the key driver of many of these indices. Lactating cows have reduced circulating concentrations of P4 during the estrous cycle compared to heifers (Sartori *et al.*, 2002, 2004; Wolfenson *et al.*, 2004). This likely results in greater number of pulses of LH during the luteal phase of the cycle and in turn drives the growth of larger dominant and ovulatory follicle diameters in cows compared with heifers (Bergfeld *et al.*, 1996). These differences in P4 and LH pulsatility also create differences in length of follicular waves (Wolfenson *et al.*, 2004). In this case, cows with lower concentrations of P4 have longer inter-wave intervals compared to heifers (Wolfenson *et al.*, 2004) due in part to increased numbers of LH pulses that drive the growth of a dominant follicle for longer periods. This, in turn, leads to more cows with an ovulatory follicle with a greater antral age (measured from onset of wave to ovulation) that developed under greater numbers of LH pulses compared to heifers. Heifers have greater chances to have three waves of follicle growth during a slightly shorter estrous cycle if the second wave dominant follicle becomes atretic prior to endogenous luteolysis (Savio *et al.*, 1990). An increase in double ovulations as dairy heifers transition to primi- and multiparous cow appears to be a key driver in the increase in twinning (Wiltbank *et al.*, 2000, 2006; Sartori *et al.*, 2004; Lopez *et al.*, 2005). It appears the increase in twinning has a significant impact on pregnancy loss (López-Gatius *et al.*, 2002; López-Gatius and Hunter, 2005). Metabolic differences such as milk production and increased dry matter intake may explain these differences in reproductive function (Wiltbank *et al.*, 2006).

Reproductive inefficiency is an obstacle to dairy farm profitability and sustainability. During the past 50 years, reproductive efficiency of lactating dairy cows progressively decreased due primarily to two key reproductive parameters, low estrus detection and pregnancies per AI (Lucy, 2001; Washburn *et al.*, 2002). Current reports indicate that estrus detection rate

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is ~36% in lactating cows and 70% in heifers. The dramatic change in estrus detection rate from the transition of heifer to cow may be attributed to changes in circulating concentrations of estrogen (Sartori *et al.*, 2004; Wolfenson *et al.*, 2004; Wiltbank *et al.*, 2006) as well as differences in environment, with cows spending more time on concrete (Platz *et al.*, 2008; Martin *et al.*, 2015) and being more susceptible to heat stress (Her *et al.*, 1988; Orihuela, 2000). Fortunately, estrus detection rate (or service rate when referring to cows timed-inseminated) is significantly enhanced when cows are timed-inseminated with Ovsynch (Pursley *et al.*, 1997a). This is because Ovsynch can be utilized as a tool to control time to first AI and subsequent inseminations following a negative pregnancy diagnosis. Unfortunately, the second greatest obstacle, pregnancies per AI, is not enhanced with Ovsynch (Pursley *et al.*, 1997a, b). In the past 40 years, pregnancies per AI in lactating dairy cows decreased from around 65% in the 1950's (Spalding *et al.*, 1975; Butler and Smith, 1989) to approximately 35% (Strickland *et al.*, 2010; Fricke *et al.*, 2014) while pregnancies per AI in heifers remained steady at about 70% (Pursley *et al.*, 1997b; Escalante *et al.*, 2013). Ovsynch was developed to synchronize the time of ovulation to allow for timed-AI. Now, studies have focused on ways to improve follicular and luteal dynamics during Ovsynch to improve pregnancies per AI of lactating dairy cows (Vasconcelos *et al.*, 1999; Bello *et al.*, 2006; Wiltbank and Pursley, 2014).

Three key physiological events transpire in fertility programs in a greater percentage of cows than in conventional synchronization programs: 1) A new estrous cycle and 2) subsequent ovulation of a first wave dominant follicle are induced within an 8-h period. And, 3) complete luteolysis is controlled prior to endogenously induced luteolysis. These three events must occur to make these programs different than other synchronization programs.

This review focuses on specific pharmacological interventions utilizing only gonadotropin-releasing hormone (GnRH) and prostaglandin F2α (PGF2α) to manipulate ovarian development in lactating cows to generate physiological outcomes similar to nulliparous heifers and enhance fertility. These interventions manipulate antral age of the ovulatory follicle and the hormonal environment during its growth necessary to induce ovulation of a single competent oocyte. The outcomes improve the chances of a pregnancy to a single AI compared to AI following estrus and are now referred to as Ovsynch-based "fertility programs for dairy cows." (Wiltbank and Pursley, 2014) They are distinguishable from other Ovsynch-based synchronization programs in the way follicles and corpora lutea are manipulated.

Limitations of solely using Ovsynch

Ovsynch is based on three pharmacological treatments (Pursley *et al.*, 1995). The first treatment, GnRH, may induce an LH surge and may cause a mature functional dominant follicle(s; DF) to ovulate (Sartori *et al.*, 2001). In turn, ovulation of the DF induces subsequent emergence of a new follicular wave ~1.5 days later (Pursley *et al.*, 1995) followed by development of a new dominant follicle during the next 7 days. If a follicle does not respond to the GnRH it is likely the cow is in the first 3 to 4 days of a follicular wave *(*Bello *et al.*, 2006). During this early stage of a wave, the largest growing follicle may be too immature (e.g., no LH receptors (Xu *et al.*, 1995) to respond to the GnRH-induced LH surge caused by the first GnRH treatment (Sartori *et al.*, 2001). If the DF is not responsive to a GnRH induced LH surge, it may develop into an ovulatory follicle during the remainder of the Ovsynch treatments or possibly become atretic prior to luteolysis (Vasconcelos *et al.*, 2003). If the follicle becomes atretic prior to PGF2α, a new follicular wave will emerge. The new DF from that wave will most likely not be mature enough 2 days later to respond to the LH surge induced by the final GnRH of Ovsynch. If the PGF2α induces complete luteolysis, cows will likely display signs of estrus 3 to 4 days following AI following maturation of the newest pre-ovulatory follicle. This artifact is a common asynchrony of Ovsynch.

The second treatment, PGF2α, is administered to induce luteolysis, thus enabling the DF of the *new* follicular wave to develop into a pre-ovulatory follicle. Luteolysis following a single dose of PGF2α may not be effective, particularly in multiparous cows. This will be discussed in more detail later in this paper. The third treatment, GnRH, is administered 56 to 60 h after PGF2α (Brusveen *et al.*, 2008) to induce a pre-ovulatory LH surge that triggers ovulation of the DF 24 to 32 h later (Pursley *et al.*, 1995). This is highly effective in cows with functional pre-ovulatory follicles.

Cows treated with Ovsynch yield overall pregnancies per AI similar to those obtained after breeding to detected estrus (37 *vs.* 39%, respectively; $P > 0.10$; Pursley *et al.*, 1997a). Up to 40% of cows may not have an ovulation synchronized with Ovsynch programs (Pursley *et al.*, 1995; Vasconcelos *et al.*, 1999; Bello *et al.*, 2006). Non-synchronized cows will not be inseminated at an appropriate time relative to ovulation, or may not have luteolysis, thereby decreasing their chances of becoming pregnant (Vasconcelos *et al.*, 1999; Bello *et al.*, 2006). Improving synchronization rates alone of Ovsynch could have a dynamic impact on reproductive performance. Vasconcelos *et al.* (1999) attributed most of the variability in synchronization rate in cows to the stage of the estrous cycle in which Ovsynch was initiated. Cows started on Ovsynch between d 5-9 of the estrous cycle had a greater probability of synchronizing and therefore had a greater chance of pregnancy (Vasconcelos *et al.*, 1999; Bello *et al.*, 2006).

Difference between fertility and synchronization programs

Studies indicate that synchronization rates can be significantly improved when lactating dairy cows are treated with a pre-synchronization program utilizing PGF2α and GnRH compared to Ovsynch alone or

Presynch-12 or -14/Ovsynch (Bello *et al.*, 2006; Souza *et al.*, 2008; Herlihy *et al.*, 2012). The key reason for greater synchrony was the synchronous initiation of a new estrous cycle, which allowed the first GnRH of Ovsynch to be administered near day 6 or 7 of the new cycle (Vasconcelos *et al.*, 1999; Bello *et al.*, 2006). The data presented next will demonstrate that initiating the first GnRH of Ovsynch near day 6 or 7 of the estrous cycle enhances function of the ovulatory follicle and increases the percentage of cows that have ovulation to the final GnRH of Ovsynch (Vasconcelos *et al.*, 1999; Bello *et al.*, 2006). This, in turn, allowed more cows the opportunity for pregnancy, and translated into increased pregnancies per AI (Bello *et al.*, 2006).

Control of the emergence of the ovulatory follicle is critical for optimal synchronization

Attaining consistent ovulation in response to first GnRH of Ovsynch constitutes the first key step to optimizing synchronization of ovulation to Ovsynch in lactating dairy cows (Vasconcelos *et al.*, 1999; Bello *et al.*, 2006). Ovulation to first GnRH of Ovsynch is followed by emergence of a new follicular wave, from which the ovulatory follicle of Ovsynch develops (Pursley *et al.*, 1995). Thus, variation in response to first GnRH leads to extreme variation in the timing of emergence of the ovulatory follicle of Ovsynch. This, in turn, results in substantial variation in size of ovulatory follicles at the time of the final GnRH of Ovsynch (Vasconcelos *et al.*, 1999; Bello *et al.*, 2006). This variation leads to a reduced chance of pregnancy (Vasconcelos *et al.*, 1999; Bello *et al.*, 2006).

The G6G program, for example, decreased variability in size of the ovulatory follicle and increased synchronization rate to Ovsynch (Bello *et al.*, 2006). In these experiments, cows were treated with 25 mg PGF2α then 2 days later 100 μg GnRH. Then, cows received the first GnRH of Ovsynch either 4, 5, or 6 days later in experiment 1 (Bello *et al.*, 2006), and either 6, 7 or 8 days later in experiment 2 (Bello and Pursley, 2007). Controls in both experiments received only Ovsynch. Compared to Ovsynch alone, 6 days from the presynchronization treatment of GnRH until the first GnRH of Ovsynch significantly improved percentage of cows ovulating to first GnRH, percentage of cows responding to PGF2α by luteolysis, and percentage of cows with both a luteolytic response to PGF2α and ovulation to the final GnRH of Ovsynch. These improvements were repeated in experiment 2 for day 6 compared to controls. In the two studies combined, ovulation rate to the first GnRH of Ovsynch averaged 90% in the day 6 groups (n = 76). In addition, it appears that 7 days from presynchronization GnRH to the first GnRH of Ovsynch also improved these responses, particularly when cows initiated a new estrous cycle by responding to both presynchronization treatments. Thus, day 6 or 7 of the estrous cycle appear to be the ideal d of the estrous cycle to initiate Ovsynch to maximize ovulatory response to the first GnRH and luteolysis following PGF2α (Bello *et al.*, 2006; Bello and Pursley, 2007).

Additional data reveal that cows ovulating in response to the first GnRH of Ovsynch yielded significantly less variability in pre-ovulatory follicle size at the final treatment of GnRH, a greater chance of luteolysis in response to the PGF2α of Ovsynch, and a greater chance of ovulating to the final GnRH (Vasconcelos *et al.*, 1999; Bello *et al.*, 2006). Also from this study (Bello *et al.*, 2006), a positive linear relationship was detected between concentrations of estradiol at the final GnRH of Ovsynch and the probability of pregnancy. In addition, a quadratic relationship was also detected between ovulatory follicle size at final GnRH and the probability of a pregnancy. Cows with follicle sizes associated with a greater chance of pregnancy also had greater serum concentrations of estradiol. Thus, it is of critical importance to optimize the size of the ovulatory follicle to allow these follicles to secrete as much estradiol as possible at the time of the final treatment of GnRH of Ovsynch.

These two experiments were designed to only test the impact of this presynchronization scheme on follicle and CL development in response to the Ovsynch treatments (Bello *et al.*, 2006; Bello and Pursley, 2007). In these preliminary data, we show nearly a doubling of percent cows pregnant in the 6 or 7 days groups compared to Ovsynch alone. Figure 1 describes some of the potential differences between two presynchronization schemes. Presynch utilizes two injections of PGF2α 14 days apart and 11 to 14 days prior to the start of Ovsynch. Since PGF2α only directly controls luteolysis, time to estrus and ovulation can be quite variable, as a result d of the cycle at the start of Ovsynch can be variable too. The likelihood of initiating ovulation to the first GnRH of Ovsynch is approximately 61% in the 11 days interval, and approximately 45% in the 14 days interval, between second PGF2α and first GnRH of Ovsynch (Galvão *et al.*, 2007). In addition, cows treated with Ovsynch that are anovular will not respond to the PGF2α injections of Presynch, but will likely respond to the GnRH of the new proposed program, thus allowing the initiation of Ovsynch at the optimal time of a subsequent follicular wave.

If cows were on day 6 of the cycle at time of first GnRH of Ovsynch, 97% of cows had accessory CL induced from the GnRH-induced LH surge, had significantly greater P4 concentrations, and had a greater probability of a pregnancy (Bello *et al.*, 2006; Bello and Pursley, 2007). Cows with both a day 7 and 13 corpora lutea at time of PGF2α of Ovsynch have approximately 50% greater P4 concentrations compared to cows with only a day 13 corpus luteum (Pursley and Martins, 2011). Fertility programs enhance the percentage of cows that respond to the first GnRH of Ovsynch, and in turn, allows for more cows with accessory CL, greater concentrations of P4 at time of induced luteolysis, and a greater chance for pregnancy.

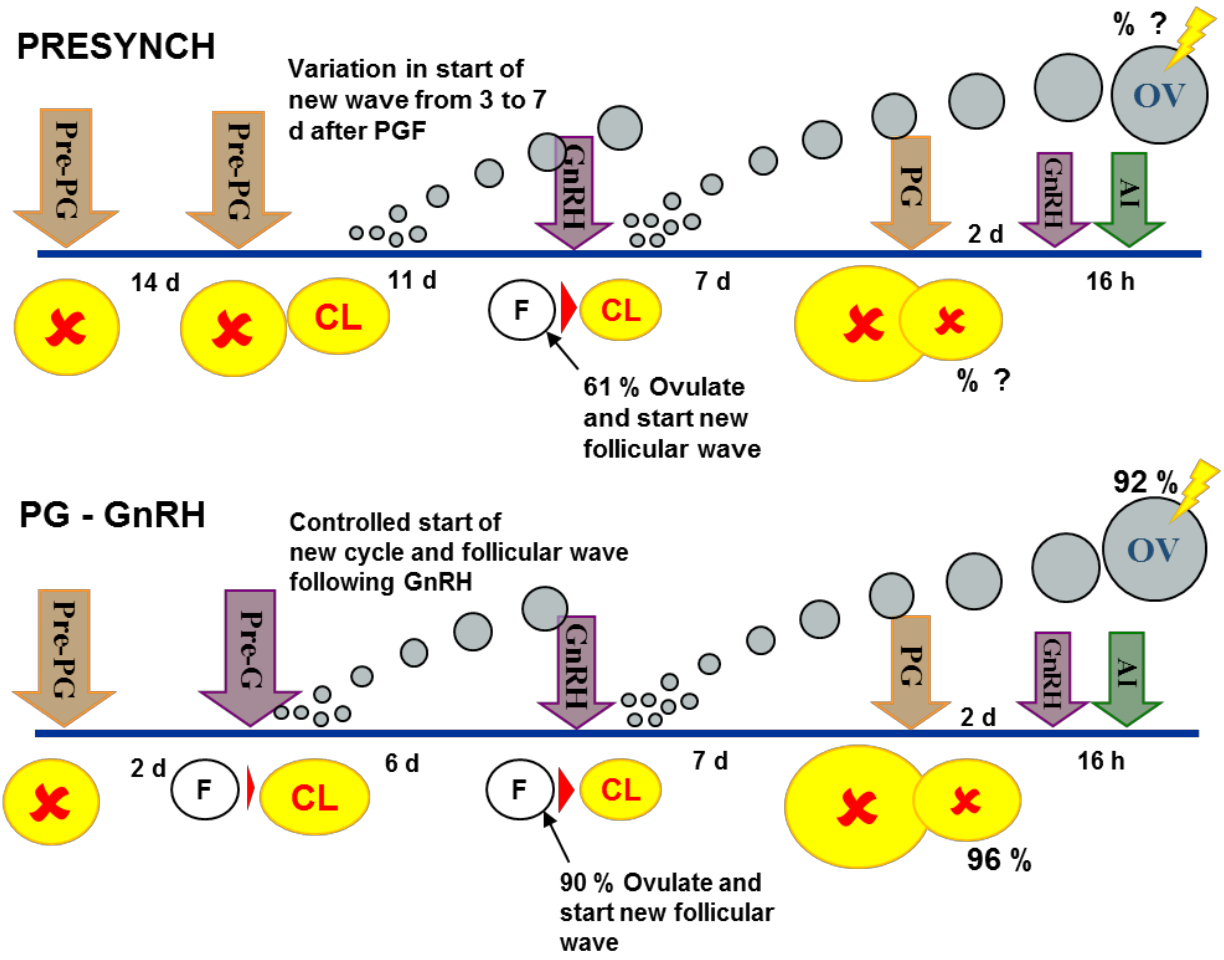

Figure 1. Description of potential difference in control of follicle development in Presynch versus the PGF2α – GnRH presynchronization scheme proposed. Control of a new estrous cycle in more cows may allow the PGF2α – GnRH scheme greater control of the ovulatory follicle following the first GnRH of Ovsynch. PG: PGF2α; F: Dominant follicle; AI: artificial insemination.

Enhancing CL regression

Circulating concentration of P4 during fertility treatments appears to be one of the most important markers of subsequent pregnancy success following timed-AI. In addition to the effect of levels of P4 prior to PGF2α on fertility of lactating dairy cows previously discussed; serum concentrations of P4 following PGF2α of Ovsynch has also been associated with P/AI of lactating dairy cows after timed-AI (Souza *et al.*, 2007; Brusveen *et al.*, 2009; Martins *et al.*, 2011a). However, in this case, a slight increase on serum concentration of P4 appears to be detrimental for the success of timed-AI outcomes. Studies using synchronization programs reported that cows with functional CL that do not decrease circulating P4 to basal levels have small to no chances of conceiving following timed-AI. In previous studies, probability of pregnancy decreased as circulating concentrations of P4 increased (Souza *et al.*, 2007; Brusveen *et al.*, 2009; Martins *et al.*, 2011a). Time to reach complete luteolysis after PGF2α of Ovsynch appears to also influence fertility since cows with a delay on P4 clearance had impaired fertility following timed-AI (Martins *et al.*, 2011a, b). Therefore, it is essential that cows with functional CL have complete luteal regression following PGF2α of Ovsynch, which is characterized as a drop of circulating P4 to basal levels prior to timed-AI. Reports of percentage of lactating dairy cows without complete luteolysis following PGF2α of Ovsynch are between 5 and 30% (Souza *et al.*, 2007; Brusveen *et al.*, 2009; Bisinotto *et al.*, 2010; Martins *et al.*, 2011a; Giordano *et al.*, 2012). In addition, similar results were obtained using either of the two PGF2α products available in the U.S.: dinoprost tromethamine (Lutalyse and ProstaMate) and cloprostenol sodium (Estrumate and estroPLAN). Although the mechanisms involved on the resistance of a mature day 7 or older CL to undergo complete luteolysis are not well characterized, some factors appear to influence the proportion of cows with complete luteolysis following PGF2α of Ovsynch. A previous study from our laboratory identified that parity and service could affect percentage of cows with complete luteolysis (Martins *et al.*, 2011a). A greater percentage of cows in first service underwent luteolysis compared to second and greater services (79 *vs.* 71%, respectively). Primiparous cows were also more likely to have complete luteolysis compared to multiparous cows (94 *vs.* 81%; Martins *et al.*, 2011a). This same study also reported that cows with greater circulating of

P4 at time of PGF2α of Ovsynch had a greater probability of complete luteal regression (Fig. 2; Martins *et al.*, 2011a). This result was unexpected since cows with two CL, a mature and accessory CL, at time of PGF2α of Ovsynch have higher serum P4 at time of PGF2α of Ovsynch and were believed to have problems with luteolysis due the number of CL and the young age of the accessory CL (day 7).

In order to enhance percentage of cows with complete luteolysis following PGF2α of Ovsynch, two different approaches have been tested: repeated administrations with PGF2α commercial label dose (Brusveen *et al.*, 2009) and increased label dose of PGF2α administered once (Giordano *et al.*, 2013). Percentage of cows with complete CL regression was increased when an additional PGF2α treatment was administered 24 h after the PGF2α of Ovsynch (95.6%) compared to cows with only the PGF2α of Ovsynch (84.6%; Brusveen *et al.*, 2009). There was an increase in percentage of cows with luteolysis. P/AI in cows with two *vs*. one PGF2α treatment was 52.7 *vs*. 47.0% (Brusveen *et al.*, 2009). Based on data from Martins *et al.* (2011a), cows that do not have complete luteolysis have nearly a 0% chance of becoming pregnant. The 11% of cows that did not have complete luteolysis (95.6 - 84.6%) likely did not become pregnant and mathematically this calculates to nearly a 6% difference.

Giordano *et al.* (2013) tested if a 50% increase in the label dose (0.5 mg *vs*. 0.75 mg) of a PGF2α analogue (cloprostenol) would increase the percentage of cows with complete luteolysis following PGF2α of Ovsynch (Giordano *et al.*, 2013). The greater dose of PGF2α increased the percentage of cows with complete luteolysis (87.7 *vs*. 79.2%) and tended to increase P/AI 39 d after AI in multiparous cows (45.4 *vs*. 40.9%; Giordano *et al.*, 2013). However, it did not influence primiparous cows (92.8 *vs*. 89.7%; Giordano *et al.*, 2013). These studies indicated that some cows and/or their CL are more resistant to achieve complete luteolysis with the regular label dose of PGF2α. Taken together, there is compelling evidence indicating that insufficient luteolysis after PGF2α of Ovsynch has a direct impact on reproductive performance of lactating dairy cow and that two administrations of PGF2α 24 h apart can overcome this problem. Therefore, fertility treatments incorporated the use of second PGF2α treatment 8 to 24 h after the PGF2α of Ovsynch to enhance complete luteolysis rate and the chances of pregnancy following timed-AI.

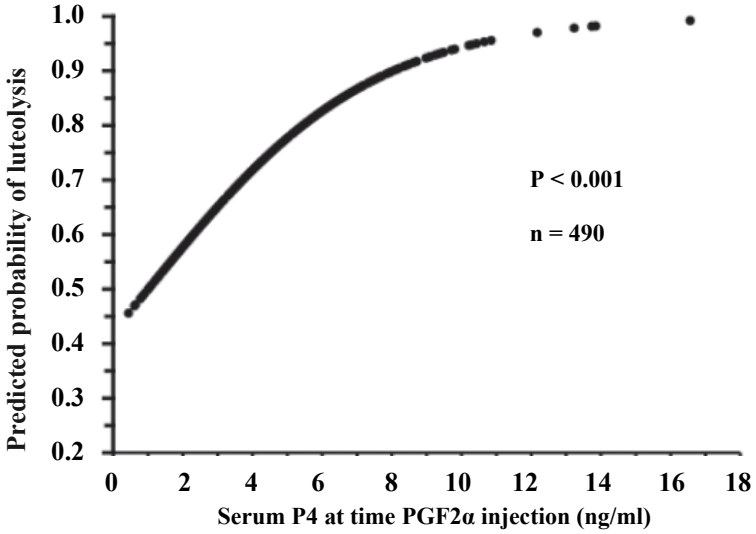

Figure 2. Predicted probability of complete luteolysis based on concentrations of progesterone (P4 < 0.5 ng/ml 56, 72, and 96 h after PGF2α injection) at time of PGF2α injection of Ovsynch in lactating dairy cows with functional corpus luteum (CL; P4 concentrations ≥0.24 ng/ml 24 h and ≥0.09 ng/ml 56 h after treatment; n = 490) at time of treatment. Published in Journal of Dairy Science (Martins *et al.*, 2011a).

Summary

It is critical to induce ovulation either during the estrous cycle or in an anovular condition to generate a new follicular wave and an accessory corpus luteum. Ovulation rate following a GnRH-induced LH surge is greatest on day 6 or 7 of the estrous cycle during the latter stages of the first follicular wave. It is also critical to initiate the induction of luteolysis of the spontaneous-formed CL as well as the accessory CL when the dominant follicle from the new follicular wave is at an ideal stage of maturity. Induction of complete luteolysis of these corpora lutea is critical and cannot be jeopardized; therefore, it is imperative to utilize two doses of PGF2α 8 to 24 h apart. In essence, inducing the initiation of a new wave and causing ovulation of the DF from that new wave manipulates the age of the ovulatory follicle similar to that of heifers during an estrous cycle (Sartori *et al.*, 2002, 2004; Wolfenson *et al.*, 2004). Presynch-10 or 11, G6G, and Double Ovsynch create these physiological differences at a greater rate compared to Ovsynch alone and Presynch 12 or 14 and may increase pregnancies per AI outcomes 30 to 60% (Bello *et al.*, 2006; Galvão *et al.*, 2007;

Souza *et al.*, 2008; Ribeiro *et al.*, 2012; Astiz and Fargas, 2013; Dirandeh *et al.*, 2015).

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Aspects and mechanisms of low fertility in anovulatory dairy cows

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Abstract

Postpartum anovulation is a natural process that is observed in most mammals, including women. In lactating dairy cows, the interval from calving to first ovulation typically averages 4 to 5 weeks, but a substantial proportion of cows have not resumed estrous cyclicity by 60 days postpartum. Extended delay in resumption of first postpartum ovulation is known to exert long-lasting detrimental effects on fertility in dairy cows including the lack of spontaneous estrus and subsequent timely insemination postpartum, but when anovular cows have the estrous cycle synchronized for artificial insemination (AI), still pregnancy per AI is reduced and the risk of pregnancy loss increased. Many risk factors exist for extended postpartum anovulatory periods such as negative nutrient balance and diseases, and these risk factors are also known to depress fertility by themselves. A key feature in anovular cows when inseminated is that they develop the ovulatory follicle under subluteal or low concentrations of progesterone. Progesterone from the corpus luteum is pivotal for follicle development, oocyte competence, embryo growth, and endometrial function; however, many of these effects exerted by progesterone are mediated either by secretion of gonadotropins influencing follicular function and oocyte competence or by endometrial histotroph secretion influencing embryo/conceptus growth and developmental biology. Sub-optimal concentrations of progesterone during follicle growth in anovular cows affect morula and early blastocyst quality, alter conceptus gene expression and affect endometrial function increasing the synthesis of prostaglandin F2α. When anovular cows receive sufficient supplemental progesterone during the antral stages of development of the ovulatory follicle, then pregnancy per AI is reestablished and resembled that of estrous cyclic cows that developed the follicle during diestrus. Preliminary data suggest that a minimum concentration of progesterone during follicle growth is needed to optimize fertility in anovular cows, at least 2.0 ng/ml.

Keyword: anovular, dairy cow, fertility, progesterone.

Introduction

Lactating dairy cattle are notorious for having less than adequate fertility as measured by suboptimal pregnancy per artificial insemination (P/AI) or by the increased pregnancy loss during the first months of gestation (Santos *et al.*, 2004). Recent data from the United States' dairy herd indicate that P/AI has remained somewhat stable in the last 10 years, at around 33%, whereas pregnancy rate, which is the rate at which cows become pregnant measured at 21-days intervals, increased and calving interval declined substantially (Bisinotto *et al.*, 2014). It is thought that the improvements observed in reproductive performance in dairy herds in the United States are, in part, the result of better implemented reproductive programs. However, expressive gains in genetics for daughter fertility have also been achieved and likely P/AI will increase in the near future.

One of the impediments for proper fertility in dairy cows is delayed resumption of estrous cyclicity during the first 2 months postpartum (Santos *et al.*, 2009). The lack of estrous cyclicity, a phenomenon also denoted as anovulation (Wiltbank *et al.*, 2002), usually affects 25% of dairy cows by approximately 65 days in milk (Walsh *et al.*, 2007; Santos *et al.*, 2009). Nevertheless, some herds might have up to 40% prevalence of anovular cows by the end of the voluntary waiting period when poor peripartum health and/or poor nutritional management are in place (Walsh *et al.*, 2007; Santos *et al.*, 2009). Anovular cows subjected to synchronization programs for AI on estrus or timed AI usually have reduced P/AI (Santos *et al.*, 2009; Bisinotto *et al.*, 2010) and increased pregnancy loss compared with estrous cyclic herdmates (Santos *et al.*, 2004; Bisinotto *et al.*, 2010). Similar to anovular cows, those cows that develop the ovulatory follicle during the last week of growth under low concentrations of progesterone have P/AI as low as that of anovular cows (Bisinotto *et al.*, 2010). Such response suggests that one of the culprits for low fertility in anovular cows is likely mediated by the lack of adequate concentrations of progesterone controlling the final stages of follicle growth and/or affecting the endocrine milieu during proestrus and the uterine function during the post-ovulatory period (Cerri *et al.*, 2011a, b; Shaham-Albalancy *et al.*, 1997, 2001).

A strategy to counteract the negative effects of low endogenous concentrations of progesterone on fertility has been the supplementation with exogenous sources at strategic times in an attempt to improve fertility in dairy cows. Nevertheless, responses to progesterone supplementation are variable (Bisinotto and Santos, 2012; Wiltbank *et al.*, 2012b) and oftentimes reproductive physiologists and veterinary practitioners have taken the simplistic tactic of "one approach fits

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all", which does not seem to have been very effective (Bisinotto and Santos, 2012; Wiltbank *et al.* 2012b). The questions that need to be asked and eventually answered are what cows require progesterone supplementation, when should progesterone be supplemented, and how much progesterone needs to be delivered to optimize establishment and maintenance of pregnancy in lactating dairy cows. Another and more long-term approach is to genetically select cows based on genomic markers linked to improved resumption of ovulation postpartum or shorter anovulatory intervals, so the prevalence of anovular cows by the end of the voluntary waiting period is reduced.

The role of progesterone in controlling reproduction

The steroid hormone progesterone is synthesized by follicles and the corpus luteum (**CL**; Mason and Savard, 1964), placenta (Conley and Ford, 1987), suprarenal glands (Wagner *et al.*, 1972), and the central and peripheral nervous systems (Schumacher *et al.*, 2004), with that of luteal origin having the greatest importance for regulation of reproductive events in cattle. Therefore, progesterone is considered one of the key ovarian steroids regulating the estrous cycle in females. Furthermore, inadequate concentrations during distinct phases of the cycle and during pregnancy are known to impair fertility in dairy cattle.

Luteal progesterone was determined to be a key regulator of reproduction in females after the observation that the presence of CL was required for the establishment and maintenance of pregnancy (Fraenkel and Cohn, 1901; Magnus, 1901; Corner and Allen, 1929). Subsequent studies described the complex mechanisms through which progesterone stimulates the secretion of nutrients and growth factors by the endometrial glands, collectively referred to as histotroph (Bazer *et al.*, 2008). Recent studies have documented and emphasized the role of progesterone in priming the uterus for proper bovine conceptus elongation after hatching from the zona pellucida (Brandão *et al.*, 2004; Alexopoulos *et al.*, 2005), underlying the importance of progesterone stimulation of uterine gland secretion for successful establishment and maintenance of pregnancy in cattle.

Nevertheless, the importance of progesterone regulating reproduction in dairy cattle goes beyond its role as coordinating uterine functions during peri-fertilization and early embryonic periods. Progesterone plays important roles in coordinating antral follicle grew and oocyte quality through its effects on luteinizing hormone (LH) pulsatility. Lactating cows classified as having low progesterone concentrations in the week preceding spontaneous estrus had less P/AI than herdmates with high concentrations of progesterone during the same period (Folman *et al.*, 1973). Similarly, cows in which the ovulatory follicle developed in the absence of a mature CL had reduced fertility compared with those in which follicles grow during diestrus (Bisinotto *et al.*, 2010). These differences in fertility in cows with low compared with moderate to high concentrations of progesterone during the development of the ovulatory follicle are, in part, attributed to the permissive role of progesterone regulating follicle growth and oocyte maturation.

Progesterone exerts a regulatory effect on the hypothalamus-pituitary-gonadal axis (Clarke and Pompolo, 2005) and differences in progesterone concentrations in plasma affect follicular fluid composition (Cerri *et al.*, 2011a), cumulus expansion and oocyte competence (Fair and Lonergan, 2012), embryo quality (Rivera *et al.*, 2011), and uterine function during the subsequent estrous cycle (Shaham-Albalancy *et al.*, 1997, 2001; Cerri *et al.*, 2011a). It has been estimated that the CL of dairy cows produce large quantities of progesterone (Wiltbank *et al.*, 2012a), but extensive catabolism by splanchnic tissues in high-producing cows results in concentrations in blood that are often considered inadequate for optimum reproduction (Wiltbank *et al.*, 2006).

Little is known about the optimum concentrations of progesterone pre- and post-insemination that maximizes fertility in dairy cows. Numerous examples exist of failures in response to supplemental progesterone to improve fertility in cattle (Monteiro Jr *et al.*, 2014; Wiltbank *et al.*, 2012b; 2014). In some cases, failures occurred because the delivery systems used were either inappropriate or less than ideal from the biological point of view. In other cases, even a depression in fertility was observed with supplemental progesterone after breeding in cows receiving embryo transfer (Monteiro Jr *et al.*, 2015).

Low progesterone affects follicle and embryo quality and influences endometrial function

Progesterone, although critical for establishment and maintenance of pregnancy, is known to exert little if any direct effect on oocytes and early embryo development (Lonergan *et al.*, 2016). The impacts of progesterone mediating embryo quality and conceptus elongation are indirect and likely caused by a combination of effects on LH pulse frequency during follicle development and on endometrial function during the post-ovulatory period (Lonergan *et al.*, 2016; Santos *et al.*, 2016).

Bovine follicular cells such as those of the cumulus-oocyte complexes are responsive to progesterone and differences exist in steroidogenic activity between dominant follicles of the first compared with those of the second follicular wave (Badinga *et al.*, 1992). First wave dominant follicles, which develop under low concentrations of systemic progesterone have high aromatase activity and concentrations of progesterone within the follicular fluid is rather high compared with those in blood plasma (Badinga *et al.*, 1992; Cerri *et al.*, 2001a). In fact, manipulating concentrations of progesterone in plasma did not alter the concentrations of progesterone within the follicular fluid in dairy cows (Cerri *et al.*, 2001a). Therefore, the effects of low concentrations of progesterone in plasma on follicular function very likely are mediated by changes in LH pulsatility, and not by a direct effect of progesterone on follicular cells.

It is well described that progesterone receptors are present in the arcuate nucleus of the hypothalamus in kiss-releasing neurons and activation of those receptors attenuates kisspeptin release which depresses gonadotropin-releasing hormone (GnRH**)** pulsatility (Clarke and Pompolo, 2005). Progesterone influences the release of LH, which can be noted by the reduction in LH pulse frequency during the luteal phase compared with the follicular phase of the estrous cycle in the sheep (Clarke, 1995) and during metestrus compared with diestrus in dairy cows (Endo *et al.*, 2012). In fact, treatment with exogenous progesterone decreased the frequency of LH pulses in ovariectomized ewes (Goodman and Karsch, 1980; Goodman *et al.*, 1982) and postpartum dairy cows (Nation *et al.*, 2000). Nevertheless, data suggest that progesterone does not affect the amount of GnRH receptors in the pituitary gland (Schoenemann *et al.*, 1985) and the negative feedback of progesterone on LH secretion is observed primarily at the level of the hypothalamus (Clarke and Pompolo, 2005), not the pituitary (Lane *et al.*, 2009). Because GnRH neurons do not express progesterone receptors (Herbison *et al.*, 1996; Skinner *et al.*, 2001), the action of progesterone on GnRH secretion is mediated by changes in upstream release of kisspeptin. When cows are exposed to low concentrations of progesterone, LH concentrations increase, follicle growth is accelerated, and follicular composition is altered (Cerri *et al.*, 2001a, b). Although these changes are unlikely to resemble those depicted in models for prolonged follicular dominance, the alterations in LH pulsatility elicited by low concentrations of progesterone likely influence oocyte quality by effects on gap junctions, protein phosphorylation and reactivation of meiosis from the diplotene stage of prophase I (Santos *et al.*, 2016). The advancement in oocyte maturation induced by increased LH pulsatility when concentrations of progesterone are low compromises early embryonic development and conceptus elongation (Wiltbank *et al.*, 2011).

Wiltbank *et al.* (2011) assigned cows to start the Ovsynch timed AI protocol on day 7 of the estrous cycle, but manipulated concentrations of progesterone such that in one treatment, cows developed the pre-ovulatory follicle under low concentrations of progesterone and in another treatment under high concentrations of progesterone. Treatments were achieved by administration of prostaglandin (PG) F2α concurrent with the initiation of the Ovsynch protocol to regress the pre-existing CL on day 7 of the estrous cycle. By doing that, the authors created two treatments, one in which the pre-ovulatory follicle developed only with the newly formed CL (low progesterone) and another treatment in which the pre-ovulatory follicle developed with both, the pre-existing CL and the newly formed CL (high progesterone). Oocytes-embryos were collected by flushing the uteri of cows on day 7 after AI. A total of 81 oocytes-embryos were collected from 168 cows flushed. Fertilization was similar between treatments, but embryo quality was markedly depressed in cows in the low progesterone treatment (Table 1). Similar depression in embryo quality has been observed when lactating dairy cows subjected to superstimulatory treatments initiated the follicle stimulating hormone (FSH) treatment under low concentration of progesterone in early metestrus (Rivera *et al.*, 2011). Interestingly, in the embryo donor experiment, supplementing progesterone during early metestrus with two intravaginal inserts reestablished concentrations of progesterone similar to those observed in cows in which the FSH started in early diestrus and rescued embryo quality (Rivera *et al.*, 2011).

Table 1. Effect of concentration of progesterone during follicle development on embryo quality in lactating dairy cows[1].

	Treatment[2]		
Item	Low progesterone	High progesterone	P-value
Fertilization, % (n/n)	78.8 (26/33)	77.1 (37/48)	0.43
Mean grade quality, 1 to 4	2.4	1.5	0.01
Grade 1, % (n/n)	34.6 (9/26)	67.6 (25/37)	0.01
Grade 2, % (n/n)	26.9 (7/26)	18.9 (7/37)	0.29
Grade 1 and 2, % (n/n)	61.5 (16/26)	86.5 (32/37)	0.02
Grade 4, % (n/n)	34.6 (9/26)	8.1 (3/37)	0.01

[1]Data from Wiltbank *et al.* (2011). [2]Cows were synchronized to start the Ovsynch timed AI protocol (GnRH, 7 days PGF2α, 56 h GnRH, 16 h AI) starting on day 7 of the estrous cycle. Low progesterone cows received an injection of PGF2α 1 day after initiating the Ovsynch timed AI protocol to regress the pre-existing CL, whereas high progesterone cows received no further hormonal treatment.

It is interesting that the negative effects of developing the ovulatory follicle under low concentrations of progesterone are not limited to reduced embryo quality. Anovular cows and those estrous cyclic initiating the timed AI protocol under low concentrations of progesterone have increased risk of pregnancy loss (Bisinotto *et al.*, 2010). It is well established that progesterone in the preceding estrous cycle influences endometrial function in the subsequent cycle (Shaham-Albalancy *et al.*, 1997, 2001). Cows that

develop the ovulatory follicle under low concentrations of progesterone have an earlier upregulation of endometrial expression of estrogen receptor-α protein in the postovulatory period and produce more PGF2α following an oxytocin challenge than cows that develop the ovulatory follicle under high concentrations of progesterone (Cerri *et al.*, 2011a). In fact, these cows are more likely to suffer from short luteal phases (Cerri *et al.*, 2011a), which is devastating to maintenance of pregnancy in cattle.

Although early embryo quality is compromised in anovular cows, many still present an elongated conceptus by day 15 of gestation. Nevertheless, conceptuses from anovular cows have marked changes in transcriptome indicating molecular changes that can compromise subsequent survival and explain the increased pregnancy loss later in gestation. Ribeiro *et al.* (2016c) identified 500 transcripts differently expressed between anovular and estrous cyclic lactating Holstein cows. Of those, 262 were upregulated and 238 were downregulated in anovular cows. Many of those genes were related to the process of transitioning from a tubular to a filamentous conceptus in dairy cattle (Ribeiro *et al.*, 2016a). Functional analysis of transcriptome data evaluated by Ribeiro *et al.* (2016c) observed that apoptosis, 14-3-3 signaling pathway, and autophagy were predicted to be increased in conceptus from anovular compared with those from estrous cyclic cows. These data suggest that conceptuses that survive to day 15 of gestation have altered molecular signatures that might favor mechanisms of cell death.

It is important to emphasize that one cannot ignore that anovulation is linked to numerous events that take place in early lactation such as excessive negative nutrient balance and diseases. Catabolism induced by negative energy balance and associated peripartum diseases are known to affect reproduction in dairy cows and to predispose them to have extended periods of anovulation (Ribeiro *et al.*, 2016a, b; Santos *et al.*, 2009). Diseases of inflammatory nature such as those that affect the reproductive tract and the mammary gland have catastrophic links with fertility in dairy cows. Cows that develop diseases are more likely to remain anovular and both influence fertility in dairy cattle. In fact, disease in early lactation leave long-lasting imprinting marks in the molecular features of conceptus (Ribeiro *et al.*, 2016b), which likely respond for some of the reduced P/AI observed in anovular cows.

Identification of anovular cows and those that respond to progesterone supplementation

As in most biological systems, there is likely an ideal concentration of each hormone that would likely maximize pregnancy in dairy cattle. For progesterone, this concentration or range of concentration remains unknown and likely varies with the stage of the reproductive cycle. Nevertheless, it is clear that anovular cows and those that develop the ovulatory follicle with low concentrations of progesterone have marked reductions in P/AI (Bisinotto *et al.*, 2010; Wiltbank *et al.*, 2012b, 2014).

One of the challenges with anovular cows is that absence of a CL following an ultrasonographic examination of the ovaries or a single measurement of progesterone concentration in blood plasma or serum are likely to inflate the prevalence of the problem. Cows in proestrus, estrus and metestrus usually will have either a small CL or no visible CL at all, and concentrations of progesterone will be low, typically below 1.0 ng/ml. This is why most studies characterizing the prevalence of anovular cows use two sequential ultrasound examinations of the ovaries 7 to 14 days apart or two sequential measurements of progesterone concentrations in blood in the same interval (Santos *et al.*, 2009). In most cases, this approach to diagnosing anovular cows is not practical under field conditions because of the labor involved, particularly in large farms.

One approach that has been used effectively is a single ultrasonographic examination of the ovaries at a strategic time, when low progesterone is known to depress fertility (Silva *et al.*, 2007). In fact, a single ultrasound examination at the beginning of the synchronization protocol is capable of identifying not only most of the anovular cows, but also cohort of cows known to have low P/AI (Bisinotto *et al.*, 2010, 2013). A similar method has been the norm of identification of low fertility cows in grazing farms in New Zealand and elsewhere (Rhodes *et al.*, 2003). In fact, estrous cyclic cows that received the timed AI program initiated during proestrus, estrus, or metestrus supposedly ovulated the first wave follicle when inseminated and had P/AI that was similar to that observed in anovular cows (Bisinotto *et al.*, 2010). The observation that the presence of CL and not estrous cyclic status has the greatest impact on P/AI is critical considering that approximately 25% of the cows receiving the first AI postpartum (Santos *et al.*, 2009) and 22 to 46% of those receiving resynchronized AI (Fricke *et al.*, 2003; Silva *et al.*, 2009) lack a CL when the synchronization protocol is initiated. Therefore, the use of a single ultrasonographic examination of the ovaries when the synchronization protocol is initiated is suggested as the method of choice to select cows for therapy with supplemental progesterone.

Supplementation with progesterone

The concentration of progesterone during diestrus in dairy cows is determined by the rates of luteal steroidogenesis and clearance from the circulation (Wiltbank *et al.*, 2012a). Therefore, more extensive catabolism usually results in reduced concentrations of progesterone in blood. The same concept would apply when cows receive exogenous progesterone. Treatment of growing heifers with progesterone inserts usually result in changes in plasma concentrations much greater than those typically observed when the same insert is used in high-producing dairy cows (Macmillan *et al.*, 1991; Cerri *et al.*, 2009), which is typically attributed to the more extensive catabolism of steroids by the splanchnic tissues in cows of increased nutrient intake and hepatic metabolism. Thus, when the same delivery

method is used in cows with distinct metabolic rates, it is not a surprise that concentrations of progesterone in plasma will greatly differ.

The fact that concentrations of progesterone vary with the type of cattle receiving a given intravaginal insert has major implications to designing supplementation systems because practitioners usually have available methods to deliver progesterone that are designed for a given type of cattle, but eventually use in all categories of cows and heifers in the farm. An example is the controlled internal drug-release system designed by Welch *et al.* (1984) as a vaginal device for use in sheep that became an alternative to the nylon sponges that were formerly used to deliver progesterone (Welch, 1984). This system later adapted to be used in cattle (Macmillan *et al.*, 1990), but it was designed to be used in heifers to facilitate adoption of AI (Macmillan *et al.*, 1991). Later, the same technology was adopted for use in lactating cows of moderate frame size and low genetic potential for production (Macmillan and Peterson, 1993). Use of the same delivery system in high-producing lactating dairy cows typically result in concentrations of progesterone in plasma that are only 20 to 30% of those observed in growing heifers or small-frame low producing cows (Cerri *et al.*, 2009).

Plasma concentrations during use of intravaginal inserts

For the subsequent discussion, the example of the controlled internal drug-release (**CIDR**) insert will be used because of familiarity with the literature, although the information herein would likely apply to any intravaginal insert that releases 80 to 90 mg of progesterone daily.

The original CIDR insert developed for cattle that still is marketed in many countries contains 1.9 g of progesterone. When used in ovariectomized heifers, it resulted in progesterone concentrations of 5.6 ng/ml for a period of 12 days, and the concentrations ranged from 8.7 ng/ml in the first hours after placement of the device in the vagina to 2.5 ng/ml at device removal on day 12 (Macmillan *et al.*, 1991). These are typical concentrations of heifers in early to mid diestrus and more than enough to block estrus, LH surge and ovulation. In fact, when applied to intact heifers in diestrus, the concentrations of progesterone increased 5 to 6 ng/ml within the first 24 h of treatment (Macmillan *et al.*, 1991). On the other hand, the same insert used in dry grazing New Zealand cows that had the CL regressed by PGF2α resulted in a mean concentration of progesterone of 2.8 ng/ml for a period of 10 days (McMillan *et al.*, 1991). Concentration was highest on the day of device insertion (4.1 ng/ml) and slowly declined to 1.9 ng/ml after 10 days of use. It is known from *in vitro* and *in vivo* drug release assessments that the CIDR insert releases on average approximately 89 mg of progesterone daily (Rathbone *et al.*, 2001, 2002) and the release is dependent primarily on the surface area of the device in contact with the mucosa of the vagina. However, it is unlikely that the delivery system is stable and not variable throughout the treatment period. In fact, although concentrations of progesterone in high-producing lactating dairy cows average 1 ng/ml when 89 mg of progesterone is delivered daily by the intravaginal insert, considerable cow to cow variability exist (Cerri *et al.*, 2009), either because of the pharmacokinetics vary among cows or because the delivery is not constant in all inserts. Likely both occur and explain the variability in blood progesterone responses when lactating dairy cows are treated with intravaginal inserts.

The re-engineered CIDR that is marketed in the United States and other countries contains 1.38 g of progesterone, but it is supposed to release the same amount daily as the original device containing 1.9 g (Rathbone *et al.*, 2001; 2002). Cerri *et al.* (2009) evaluated the concentrations of progesterone when estrous cyclic high-producing Holstein lactating cows received a new (1.38 g of progesterone) or a 7-day used CIDR insert after regressing the CL. The authors showed that a device releasing 89 mg of progesterone daily (Rathbone *et al.*, 2001, 2002) increased concentrations in plasma by approximately 0.8 to 1.0 ng/ml (Cerri *et al.*, 2009). Concentrations increased in the first 15 min and reached a plateau by 90 min after insertion of the device. Similar to the findings of Macmillan *et al.* (1991), concentrations declined over the course of use of the device, but in the lactating Holstein cow, they dropped to 0.5 to 0.7 ng/ml after 7 days of use. These concentrations of progesterone in dairy cows are sufficient to block estrus and the LH surge and ovulation, but not ideal to improve fertility when the goal is to supplement progesterone (Bisinotto and Santos, 2012; Bisinotto *et al.*, 2015a). This probably explains why previous studies in which a single CIDR was incorporated into timed AI programs demonstrated inconsistent responses in anovular cows (Bisinotto and Santos, 2012). The incremental progesterone from a single insert is likely insufficient to optimize follicle or oocyte maturation during the final stages of development before AI, or even to prime the endometrium for proper post-insemination function during conceptus development and maintenance of pregnancy.

Because release of progesterone from intravaginal devices is dependent primarily on the surface area in contact with the vaginal mucosa (Rathbone *et al.*, 2001, 2002), it is not a surprise that addition of multiple devices increases progesterone in plasma in a parallel manner to the number of inserts used (Macmillan *et al.*, 1991; Lima *et al.*, 2009). This is important because in many countries approval of new devices is costly, but opportunities exist for extra-label use of current devices to target individuals that might require daily doses of progesterone of at least 180 to 200 mg such as high-producing anovular dairy cows.

Incorporation of supplemental progesterone in synchronization programs

Programs for synchronization of ovulation and timed AI have been implemented worldwide as a management tool for the systematic control of reproduction in dairy herds. Timed AI programs allow for submission of all eligible cows to insemination with

satisfactory P/AI, which typically improves pregnancy rates especially when detection of estrus is inefficient (Tenhagen *et al.*, 2004) or when replacing breeding by natural service (Lima *et al.*, 2012). Fertility of estrous cyclic and anovular lactating dairy cows induced to ovulate the first-wave dominant follicle is usually compromised (Bisinotto *et al.*, 2010). Our work has clearly demonstrated that first-wave follicles that develop concurrently with the CL and, therefore, under low concentrations of progesterone result in alterations in the follicular fluid composition (Cerri *et al.*, 2011a), alterations in endometrial function (Cerri *et al.*, 2011a), reduced embryo quality (Rivera *et al.*, 2011; Wiltbank *et al.*, 2011), and compromised P/AI (Bisinotto *et al.*, 2010). More importantly, our work has shown that progesteron is likely to mediated these changes in reproductive responses of cows ovulating the dominant follicle of the first follicular wave (Bisinotto *et al.*, 2013). In fact, reduction in progesterone concentraiton during development of the second wave follicle markedly reduced embryo quality in single ovulating dairy cows (Table 1; Wiltbank et al., 2011).

Timed AI programs provide a unique platform for the manipulation of the ovulatory follicle in order to improve P/AI in dairy cows. One of these opportunities is the supplementation of progesterone to cows that are identified as being anovular or those in which the stage of the cycle results in low concentration of progesterone during the final phase of follicle development. Because lack of a CL when the timed AI protocol is initiated is predictive of low fertility, it then becomes logical that identification of cows without CL would be one of the targeted populations to receive supplemental progesterone (Bisinotto *et al.*, 2013, 2015a).

It is important to emphasize that synchronization programs based on the use of estrogens such as those with estradiol benzoate do require a source of exogenous progesterone (Baruselli *et al.*, 2004), otherwise cows without a CL when estrogens are administered or those that regress the CL after the treatment with estrogens will not have a properly synchronized estrus or ovulation.

Supplemental progesterone during the timed AI protocol according to presence of CL

Numerous studies have evaluated the impact of supplementing exogenous progesterone during timed AI protocols on fertility of dairy cows. Bisinotto *et al.* (2013, 2015a) evaluated the effect of supplementing progesterone in GnRH-PGF2α based synchronization protocols. The authors showed that increasing progesterone in blood above 2.0 ng/ml with use of two intravaginal inserts restored fertility in cows without CL similar to that of cows in diestrus. The data of Bisinotto *et al.* (2015a) suggested that a minimum of approximately 2.0 ng/ml was needed during the development of the ovulatory follicle to optimize fertility in high-producing dairy cows (Fig. 1). Such response likely explains the lack of benefit of a single intravaginal insert that results in 0.8 to 1.0 ng/ml in cows without a CL (Bisinotto and Santos, 2012). In fact, when progesterone was supplemented to cows without a CL with the use of 2 intravaginal inserts (Bisinotto *et al.*, 2013, 2015a), to supply approximately 180 mg of progesterone released per day (Rathbone *et al.*, 2001, 2002), then the P/AI was similar to that of cows in diestrus when the timed AI protocol was initiated (Table 2). The increments observed were of approximately 10 percentage units in P/AI (Bisinotto *et al.*, 2013, 2015a; Lima *et al.*, 2009).

Most experiments evaluating the use of supplemental progesterone were not necessarily designed considering that the intravaginal device might not supply the amounts needed to improve fertility in dairy cow. Because of that, it is then not surprising that the responses to supplemental progesterone during synchronization programs have been equivocal (Bisinotto and Santos, 2012). The inconsistency in results led us to conduct a systematic review of the literature with the objective to evaluate whether progesterone supplementation using a single intravaginal insert during timed AI programs benefit fertility in lactating dairy cows (Bisinotto *et al.*, 2015b). A total of 25 randomized controlled experiments including 16,683 dairy cows, half supplemented and half untreated controls were included in the meta-analysis. A portion of the studies, 21 experiments including 13,762 cows (82.5% of the all cows) had information whether they were in diestrus or did not have a CL when the timed AI protocol was initiated. Additional information collected included detection or no detection of the estrus during the timed AI protocol and if cows had or did not have the estrous cycle pre-synchronized before enrollment in the timed AI program. The meta-analysis revealed that progesterone supplementation increased P/AI on day 32 and 60 after insemination by 8 (relative risk [RR] = 1.08; 95% confidence interval [CI] = 1.03 to 1.17) and 10% (RR = 1.10; 95% CI = 1.03 to 1.17), respectively. Interestingly, the benefit of treating cows with progesterone during the timed AI protocols was greater in cows without CL (P/AI on day 60: RR = 1.18; 95% CI = 1.07 to 1.30) than those in diestrus (RR = 1.06; 95% CI = 0.99 to 1.12). Also, progesterone supplementation benefited P/AI only when all cows were subjected to timed AI. When detection of estrus was performed throughout the synchronization protocol, and cows could be inseminated if observed in estrus, then progesterone did not increase P/AI. Collectively, these results clearly demonstrate that cows without CL benefit from progesterone supplementation, but delivering 80 to 90 mg of progesterone/day to high-producing Holstein cows is not ideal. For instance, the 16% increase in RR of P/AI on day 60 after insemination when anovular cows received supplemental progesterone with a single insert to deliver 90 mg/day translated into an increment of 6.0 percentage units (from 27.3 to 33.3%) in P/AI (Bisinotto et al., 2015b), still less than the value typically observed when anovular cows received two inserts to deliver twice the progesterone (Table 2). The results of the meta-analysis also demonstrate that one of the benefits of progesterone supplementation is to better synchronize estrus/ovulation in these programs because detection of estrus and insemination during the protocol abolished the positive effects of supplementation on fertility.

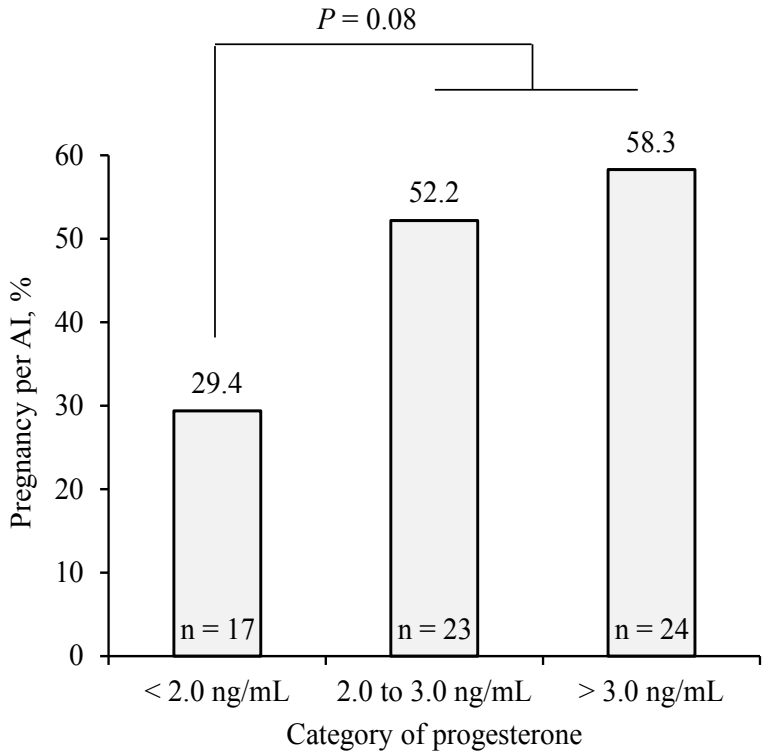

Figure 1. Pregnancy per AI on day 32 after insemination based on the concentrations of progesterone during follicle growth in cows receiving 2 intravaginal inserts containing progesterone during follicle growth in the Ovsynch protocol. Cows were categorized as having progesterone < 2.0 ng/mL (1.66 ± 0.32 ng/ml), from 2.0 to 3.0 ng/ml (2.53 ± 0.26 ng/ml), or > 3.0 ng/ml (3.97 ± 0.89 ng/ml) between the day of the first GnRH and that of the PGF2α. Data from Bisinotto *et al.* (2015a).

Table 2. Effect of presence of corpus luteum (CL) and progesterone supplementation for cows without CL at the initiation of the timed AI protocol on fertility responses.

	Treatment[1]		
	No CL control	No CL and progesterone	Diestrus
		% (n.)	
Estrus at AI			
Bisinotto *et al.* (2013)	34.2 (234)	36.2 (218)	35.0 (946)
Bisinotto *et al.* (2015a)	35.8 (652)	39.6 (635)	30.6 (640)
Mean estrus at AI	35.4	38.7	33.2
Pregnant day 60			
Bisinotto *et al.* (2013)	28.6 (234)	43.7 (215)	47.3 (941)
Bisinotto *et al.* (2015a)	28.9 (642)	37.2 (630)	33.9 (633)
Lima *et al.* (2009)	23.0 (87)	32.9 (85)	35,9 (334)
Mean pregnancy day 60	28.3	38.4	40.9
Pregnancy loss			
Bisinotto *et al.* (2013)	6.9 (72)	5.1 (99)	4.7 (467)
Bisinotto *et al.* (2015a)	8.5 (208)	11.4 (260)	8.8 (231)
Lima *et al.* (2009)	16.7 (24)	9.7 (31)	8.4 (131)
Mean pregnancy loss	8.9	9.7	6.4

[1]All cows were subjected to the 5-days timed AI protocol (Bisinotto *et al.*, 2013), Ovsynch-56 protocol (Bisinotto *et al.*, 2015a) or Heatsynch protocol (Lima *et al.*, 2009). No CL control = cows without a CL on the day of the first GnRH that received no supplemental progesterone; No CL progesterone = cows without a CL on the day of the first GnRH that received two intravaginal inserts containing each 1.38 g of progesterone; Diestrus = cows with CL on the day of the first GnRH of the timed AI protocol.

Conclusion

Delayed resumption of ovulation beyond 60 days postpartum affects a large proportion of dairy cows. Development of the ovulatory follicle under low concentrations of progesterone is the hallmark in anovular cows when subjected to synchronized inseminations. Suboptimal concentration of progesterone during follicle growth is one of the impediments for adequate fertility and markedly decreases P/AI in cows subjected to synchronization of estrus and ovulation. The compromised fertility observed in anovular cows is attributed to changes in the follicle/oocyte which carryover to the developing embryo, but also legacy effects on the conceptus and uterus that influence receptivity to pregnancy and maintenance of the CL. Supplementing progesterone to high-producing dairy cows has not always improved fertility in a consistent manner. In many cases, the inability of progesterone to improve P/AI is attributed to the delivery method that has not always been ideal for the type of cow under question. When sufficient progesterone is supplied to anovular cows and those without a CL at the initiation of the timed AI protocol, then fertility is restored similar to that of cows in diestrus. Based on the limited data available, it is suggested that a minimum of 2.0 ng/ml is needed during follicle development to improve fertility in dairy cows. Reaching such concentrations with supplemental progesterone increased P/AI by approximately 10 percentage units, equalizing that of cows in diestrus. On the other hand, when supplemental progesterone increases plasma concentrations by only 0.8 to 1.0 ng/ml then, although benefits were also observed, they usually ranged from 3 to 5 percentage units increment in P/AI, which is not sufficient to reach the values of P/AI observed in cows in diestrus.

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Update and overview on assisted reproductive technologies (ARTs) in Brazil

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Abstract

The impressive increase in the use of assisted reproductive technologies (ARTs), especially in cattle, during the last few years in Brazil is well known worldwide. In 2015, there were over 13.7 million artificial inseminations (AI), of which, about 77% were carried out using fixed-time AI (FTAI). This technology has helped to substantially improve reproductive efficiency in beef and dairy cattle. In relation to embryo transfer, production of *in vivo* derived (IVD) embryos remained relatively stable, with average production of 30-40,000 embryos per year, whereas *in vitro* production (IVP) of embryos had a substantial increase, from about 12,500 IVP embryos in 2000 to more than 300,000 IVP embryos after 2010. The increasing availability and use of sex-sorted sperm was one of the factors responsible for a recent shift from the predominance of IVP embryos from beef breeds to dairy breeds in Brazil. Moreover, there was also an increase from 13% in 2014 to 29% in 2015 in the percentage of vitrified/frozen embryos. Moreover, the successful use of protocols for fixed-time ET (FTET) due to their high efficiency and ease of implementation, has facilitated the dissemination of ET programs all over Brazil. However, there is room for improvement, since there are several reports of high pregnancy loss and high peripartum loss, when IVP embryos are used. The production of healthy cattle by somatic cell nuclear transfer has also increased in the last few years in Brazil, but despite substantial progress in reducing postnatal losses, no drastic increase in cloning efficiency up to parturition has occurred.

Keywords: artificial insemination, bovine, embryo, *in vitro* production, superovulation.

Introduction

Currently, Brazilian cattle industry has one of the largest commercial herds in the world, about 208.3 million head (Associação Brasileira das Indústrias Exportadoras de Carne - ABIEC, 2014). Brazil produced 10.7 million tons of beef in 2014 (ABIEC, 2014), being second place in the world ranking of meat production. Moreover, the dairy herd in Brazil ranks in the fifth position worldwide (Food Agriculture Organization of United Nations - FAO, 2012). Despite the magnitude of the herd, the annual Brazilian production of milk in 2014 was 24.741 billion liters, with a productivity of only 1,380 L of milk/cow/year (Instituto Brasileiro de Geografia e Estatística - IBGE, 2014). This is obviously very low production if compared, for example, with data from the USA herd (10,096 L of milk/cow/year), currently the largest producer of milk in the world (United States Department of Agriculture - USDA, 2014). However, both Brazilian beef and dairy productivity is increasing, which is directly related to technological advances in animal breeding, such as greater use of artificial insemination (AI) and embryo transfer (ET).

To have an idea on the evolution of these biotechnologies, in 2002 only 5-6% of heifers and cows were artificially inseminated in Brazil, about 7 million AIs, with only 1% of inseminations being through fixed-time artificial insemination (FTAI). In contrast, in 2015, about 13 million AIs were performed corresponding to 10-12% of females of reproductive age and 77% of these inseminations were performed by FTAI (Pietro Baruselli, 2016; School of Veterinary Medicine and Animal Science, USP, São Paulo, SP, Brazil; unpublished).

In relation to embryo production in cattle, there are two different scenarios. While production of *in vivo* derived (IVD) embryos remained relatively stable over the last 15 year, with average production of 30-40,000 embryos per year, the *in vitro* production (IVP) of embryos had a substantial increase from about 12,500 IVP embryos in 2000, to over 348,000 IVP embryos in 2014, representing almost 60% of the world embryo production.

Sex-sorted sperm has been widely and increasingly used in Brazil, especially for AI or IVP. Unfortunately, epidemiological data on the use of sex-sorted sperm in Brazil are not available. Regarding IVP, data from the last 3 years from one of the main labs in Brazil confirm other data from the literature that there is a reduction in embryo production per cultured oocyte if sex-sorted sperm is used for *in vitro* fertilization when compared with conventional unsorted sperm (23.6% [311,788/1,323,541] *vs*. 28.5% [242,259/848,939]; $P < 0.01$).

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For cloning, data from the Brazilian Association of Zebu Breeders (ABCZ) show a gradual increase in registered *Bos indicus* calves (predominantly of Nelore and Gir breeds) produced by somatic cell nuclear transfer (SCNT) during the years 2010 (n = 5), 2011 (n = 23), 2012 (n = 22), and 2013 (n = 41). Unofficial data indicate a continuous increase in number of healthy calves produced by SCNT from 2014 to 2016.

This manuscript aims to present an update and overview of the assisted reproduction technologies (ARTs) in Brazil focused on AI and ET in cattle and to describe reports on how these technologies have positively influenced the reproductive efficiency of dairy and beef herds.

Artificial insemination

As mentioned above, Brazil has one of the largest cattle herds in the world; however the use of AI is still low. In 2015, there were over 13.7 million inseminations, which correspond to 10-12% of cows and heifers of reproductive age (Fig. 1). Out of this total AIs, about 4.7 million were performed in dairy cows, with a decrease of 12.4% compared with the previous year. In beef cattle, 9 million inseminations were performed, with an increase of 16.2% in relation to 2014. In 2015, more than 10.5 million FTAIs were performed, with an increase of 11.2% compared to 2014, and FTAI now represents ~77% of all AIs carried out in Brazil (Fig. 1). These data demonstrate that FTAI is increasing the use of AI across Brazil with a doubling in the overall use of AI during the last decade, but over a 10-fold increase in the use of FTAI from ~1 million protocols in 2005 (11% of all AIs) to 10.5 million protocols in 2015 (77% of all AIs).

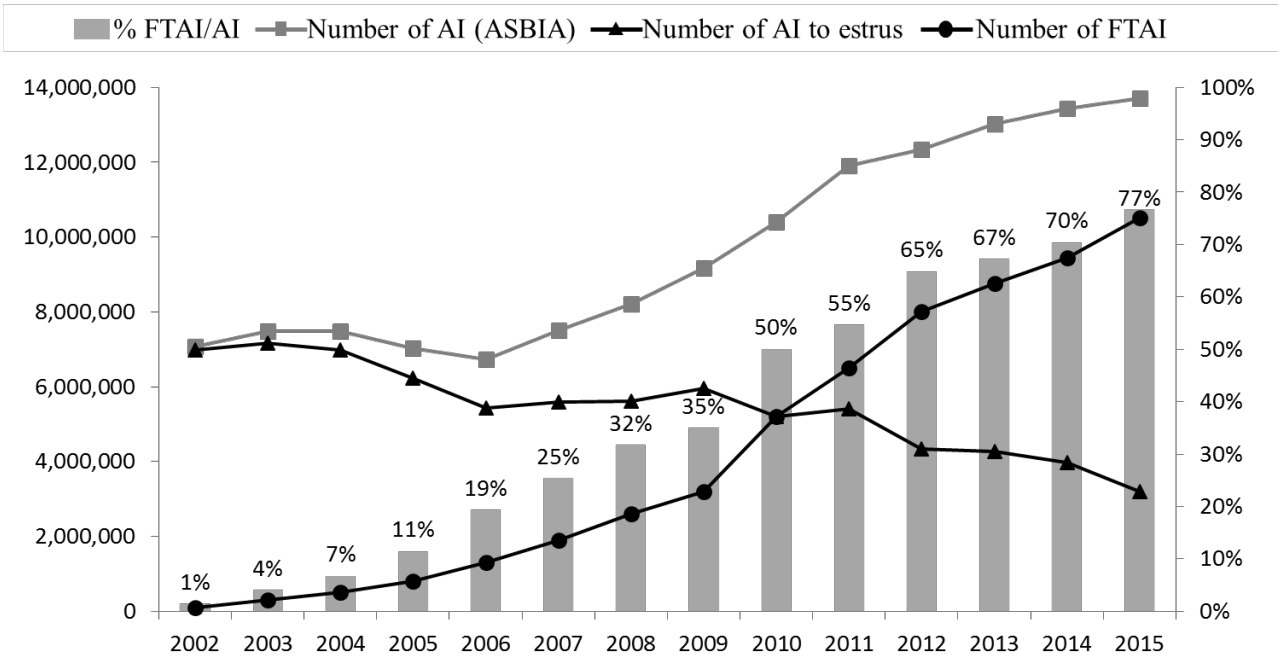

Figure 1. Data of artificial insemination (AI) based on cows and heifers bred to estrus or to fixed-time AI (FTAI) systems in Brazil during the period of 2002 to 2015. Numbers of FTAI were estimated based on hormones/products sold for each FTAI protocol.

Use of FTAI in dairy cattle

Although most dairy cows and heifers are bred by bulls in Brazil, AI is the preferred ART for most progressive dairy farms. When AI is employed, the question practitioners and producers ask is whether they should breed cows to estrus or FTAI. In fact, this doubt is understandable because studies that properly compared insemination to estrus *vs*. insemination to a FTAI protocol have described lower (Strickland *et al*., 2010; Carvalho and Fricke, 2016; University of Wisconsin-Madison; unpublished), similar (Rabiee *et al*., 2005; Nascimento *et al*., 2013b), or greater (Nascimento *et al*., 2013a) pregnancies per AI (P/AI) when cows are bred to estrus. However, suboptimal estrus detection rates in cycling cows (Lopez *et al*., 2004; Fricke *et al*., 2014) and a substantial percentage (~24%) of cows that are not cycling (Wiltbank *et al*., 2002; Santos *et al*., 2009), produces the problem of low service rates (SR) and, in general, lower 21-days pregnancy rates (21-day PR = P/AI x SR, every 21 days after the voluntary waiting period; VWP) for cows bred to estrus than cows bred to FTAI (Nascimento *et al*., 2013a; Wiltbank and Pursley, 2014).

In order to evaluate the impact of intensifying the use of FTAI on reproductive efficiency in a dairy herd in Brazil, an analysis of 4,512 AIs (1,688 in primiparous and 2,824 in multiparous cows) was performed between 2009 and 2014. These data were from a dairy farm, managed in a free stall system with a yearly rolling herd average milk yield of 10,700 kg during the period. Based on changes in the reproductive management strategy, data were compared between the times before (year 2009-2011) and after (year 2012-2013) intensifying the use of FTAI. Before the more intensive reproductive management program, cows

received two treatments with prostaglandin F2α (PGF2α) at ~40 and ~54 days in milk (DIM) and were bred if detected in estrus from 40 to 72 DIM. During this time cows were visually checked for standing estrus twice a day combined with use of pedometers as an estrus detection aid. Cows not bred by ~73 DIM were then enrolled in a FTAI protocol. Pregnancy diagnosis was conducted every 14 days. In 2012 and 2013, cows received one PGF2α treatment at ~40 DIM and were bred to any detected estrus until ~54 DIM, when cows that were not inseminated were then enrolled in a FTAI protocol. Pregnancy diagnosis was conducted every 7 days. In both situations, even after AI to estrus or to FTAI, cows observed in estrus were inseminated. The main FTAI protocol used during the period of the study was the following. Day-10: Progesterone insert + 2 mg estradiol benzoate (EB) or 100 µg GnRH, D-3: 500 µg cloprostenol sodium; Day-2: P4 insert removal + 500 µg cloprostenol sodium + 1.0 mg estradiol cypionate (ECP), D0: FTAI (Melo *et al*., 2016).

When reproductive management was intensified, the proportion of cows inseminated by FTAI increased ($P < 0.01$) from 29.1% (559/1920) to 56.9% (1474/2592), and cows were inseminated earlier (Fig. 2).

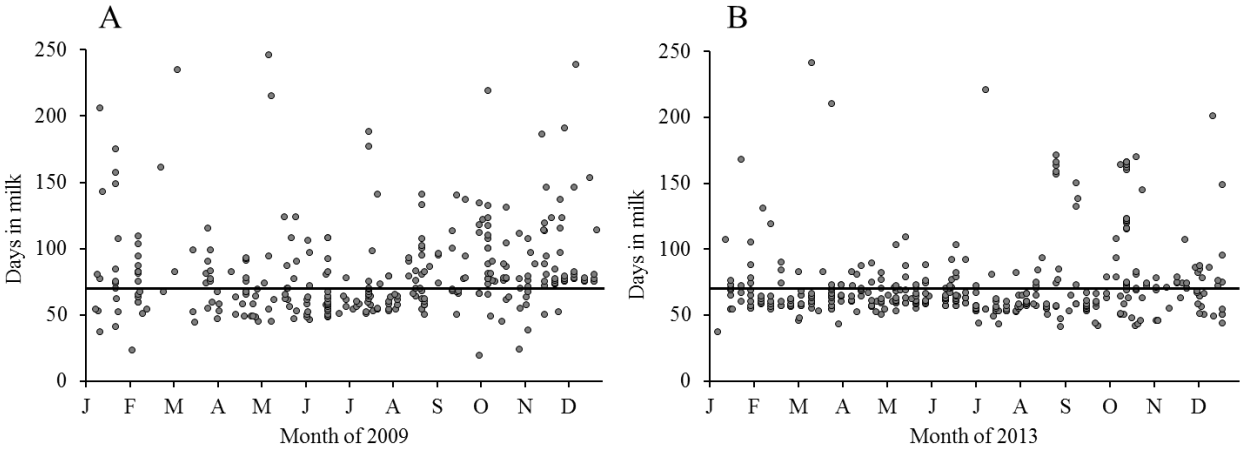

Figure 2. Distribution of first postpartum AI according to days in milk (DIM) in lactating dairy cows receiving reproductive management strategies before (year 2009); A), or after (year 2013); B) intensifying the use of FTAI. Horizontal lines represent 70 DIM.

Data from a survival analysis show that after intensifying the use of FTAI, cows were inseminated for the first time earlier ($P < 0.01$; Fig. 3A) and became pregnant sooner ($P < 0.01$; Fig. 3B).

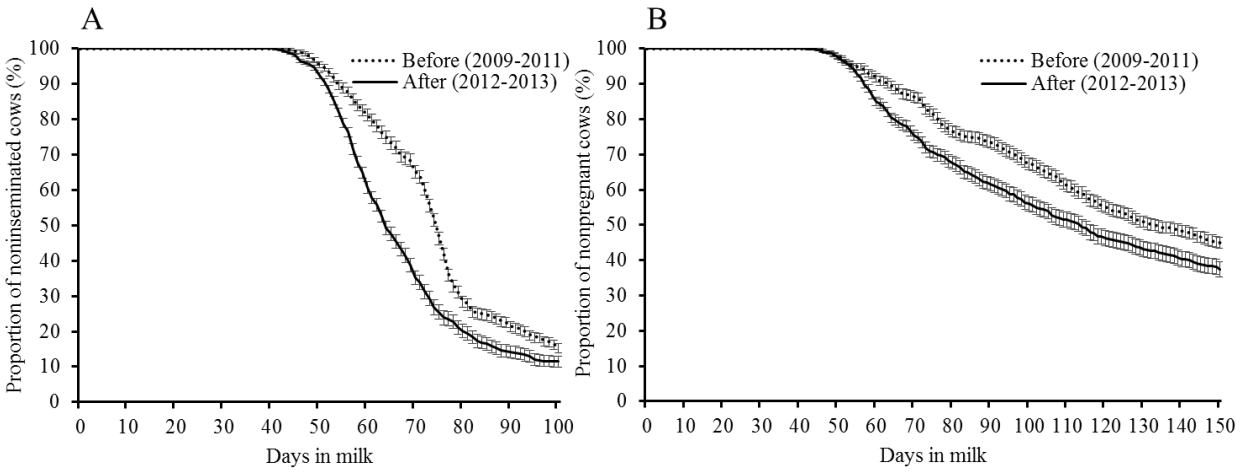

Figure 3. Survival curve by days in milk for proportion of noninseminated (A; $P < 0.01$) and nonpregnant (B; $P < 0.01$) dairy cows receiving reproductive management strategies before (year 2009-2011), or after (year 2012-2013) intensifying the use of FTAI.

The results related to the reproductive performance of cows are shown in Table 1. There was a significant decrease in the proportion of cows not inseminated by 70 DIM after the intensification of FTAI, resulting in more cows pregnant by 103 DIM. Moreover, with the more intensive use of FTAI during 2012 and 2013, overall fertility also increased, as seen by greater P/AI at 30 and 60 days, with no change in pregnancy loss (Table 1). This improved P/AI may be resulting from several factors, such as better cow comfort, health and nutrition, but especially due to improvements in the FTAI protocol (Binelli *et al*., 2014).

Table 1. Proportion of noninseminated cows at 70 days in milk (DIM) and proportion of pregnant cows at 103 DIM, pregnancy/AI, and pregnancy loss in dairy cows receiving reproductive management strategies before (year 2009-2011), or after (year 2012-2013) intensifying the use of FTAI.

	Before (2009-2011)	After (2012-2013)	P value
Noninseminated cows at 70 DIM, % (n/n)	64.8% (374/577)	35.0% (314/898)	<0.01
Cows pregnant at 103 DIM[a], % (n/n)	34.2% (184/538)	45.4% (408/899)	<0.01
Pregnancy/AI, % (n/n)			
31 days	27.9% (539/1,920)	37.1% (903/2,592)	<0.01
59 days	23.8% (463/1,920)	32.4% (777/2,592)	<0.01
Pregnancy loss between 31 and 59 days, % (n/n)	14.1% (76/539)	14.0% (126/903)	0.99

[a]Equivalent to three estrous cycles after the voluntary waiting period.

There was a major effect of intensification of FTAI on 21-days PR (Fig. 4), which increased linearly throughout the evaluated years, resulting in a decrease of approximately 35 days (from 180 days in 2009 to 145 days in 2013) on days open, or time from calving to conception. This improved 21-days PR was a result of greater SR associated with increased P/AI as reproductive management was progressively intensified (Table 1).

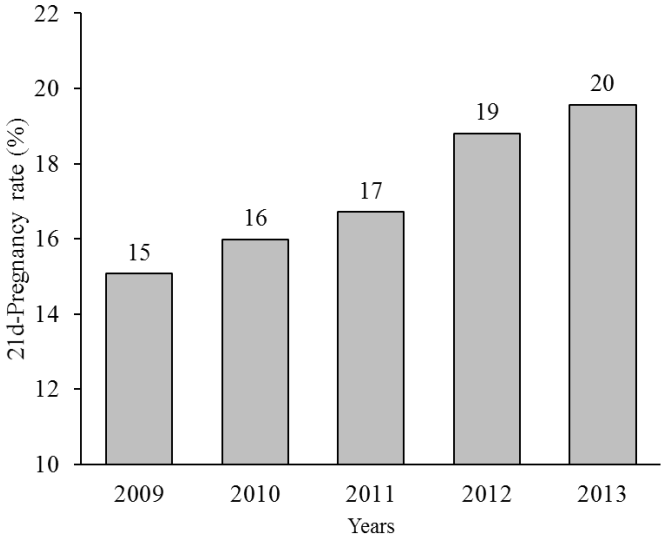

Figure 4. Results of 21-days pregnancy rate throughout the years in dairy cows receiving reproductive management strategies before (year 2009-2011), or after (year 2012-2013) intensifying the use of FTAI.

Thus, the intensive use of FTAI improved reproductive efficiency on this farm and this appears to be the best current alternative for other dairy farms in Brazil. We have also analyzed a large database of AI and FTAI from eight Brazilian dairy farms that were using a typical reproductive management strategy for Brazilian dairy herds and the results were very similar to those observed on the example farm above, prior to intensification of the reproductive management strategy [i.e., P/AI at 30 days = 29.0% (10029/34472), P/AI at 60 days = 24.9% (4076/16315), and pregnancy loss between 30 and 60 days = 14.7% (706/4782)]. Moreover, high pregnancy loss between 30 days and calving [28.2% (2832/10029)] and low birth rates [20.8% (7197/34472)] are of major concern, which may justify, even more, the intensification of reproductive management.

Use of FTAI in beef cattle

Most of beef cattle herds in Brazil are composed of *Bos indicus* and it is noteworthy that zebu cattle have longer postpartum anestrus and low body condition score (BCS) when kept on pasture (Bó *et al*., 2003), resulting in economic losses because of the increased interval from calving to conception and reduced P/AI (Bó *et al*., 2007). In a pasture-based cow-calf production system, the use of reproductive programs, such as synchronization of ovulation for FTAI (synchronization protocols based on P4 and E2), is essential to produce high pregnancy rates (PR) in the breeding season and it has been increasingly incorporated in cow-calf operations (Pessoa *et al*., 2016).

Data generated by the GERAR group (Specialized Group in Applied Reproduction to the Herd; created by a partnership between the School of Veterinary Medicine and Animal Science, São Paulo State University in Botucatu, and Zoetis, São Paulo) that is composed of more than 250 Brazilian technicians which discuss innovations and results for FTAI, show the evolution of P/AI from 2007 to 2015 in millions of heifers and cows submitted to FTAI (Fig. 5; Table 2). The main FTAI protocol used during the period of the study was the following. Day-11: Progesterone insert + 2 mg EB, D-4: 12.5 mg dinoprost tromethamine, Day-2: P4 insert removal + 0.6 mg ECP + 300 IU equine chorionic gonadotropin (eCG) or calf removal for 48 h, Day-0: FTAI (Meneghetti *et al*., 2009; Peres *et al*., 2009; Sá Filho *et al*., 2009).

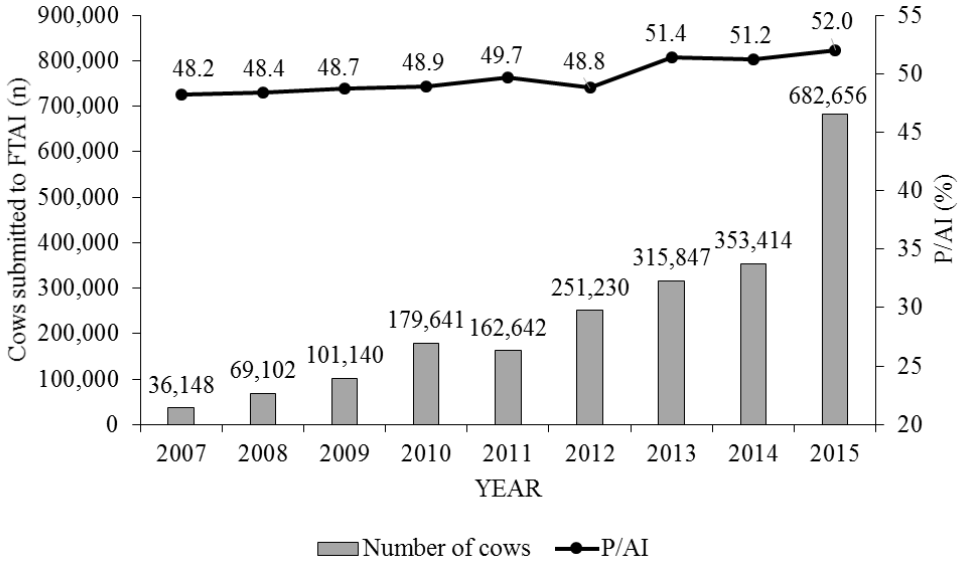

Figure 5. Number of beef heifers and cows submitted to FTAI and P/AI between 2007 and 2015.

Table 2. Pregnancy per AI of heifers, primiparous, multiparous and non-lactating beef cows submitted to FTAI between 2007 and 2015.

Year	Heifers %, (n)	Primiparous%, (n)	Multiparous %, (n)	Non-lactating, % (n)
2007	39.6% (3,037)	44.5% (5,249)	49.7% (22,519)	45.1% (1,510)
2008	44.8% (4,944)	42.6% (9,763)	50.9% (44,628)	45.6% (5,354)
2009	50.5% (8,347)	43.4% (15,476)	49.9% (70,308)	46.5% (5,526)
2010	39.7% (24,372)	48.5% (18,819)	50.7% (123,380)	49.4% (9,566)
2011	49.3% (21,810)	41.6% (22,453)	51.2% (105,440)	52.0% (11,076)
2012	47.1% (42,030)	44.1% (32,345)	50.2% (130,236)	52.1% (10,252)
2013	49.0% (58,032)	47.8% (42,467)	53.1% (189,726)	50.1% (24,432)
2014	46.8% (56,026)	48.0% (47,882)	53.0% (200,082)	50.8% (26,091)
2015	48.5% (124,687)	47.1% (80,690)	54.1% (392,511)	51.5% (69,734)

As shown in Table 2, the fertility in all types of beef cattle has been relatively constant (~50%) during the last 3 years. Nevertheless, there is likely to be room for improvement in many of the herds since some herds (~24%) had average P/AI greater or equal to 60% (Fig. 6). These herds are likely to have more intensive reproductive programs, better nutrition with fewer cows with low BCS (Fig. 7), and may use cattle with better fertility traits, such as Nelore X Angus crossbreds (Fig. 8).

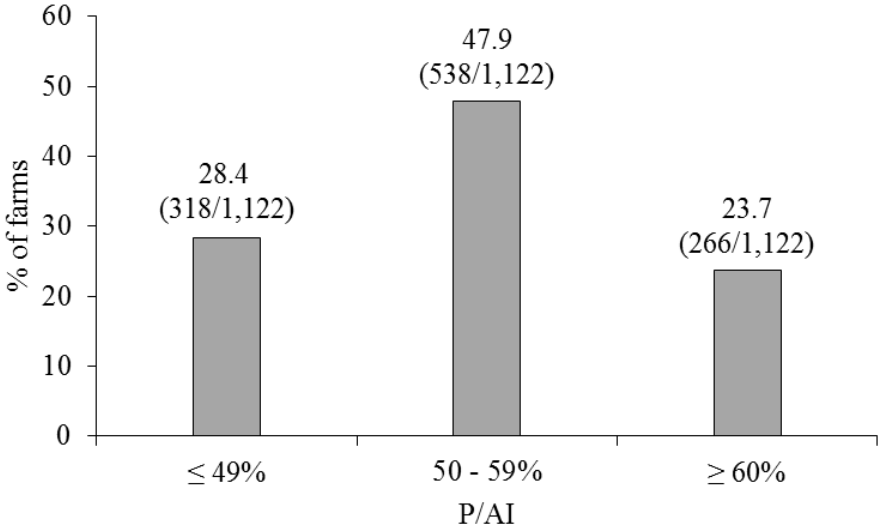

Figure 6. Distribution of farms according to P/AI of beef cows submitted to FTAI in 2015.

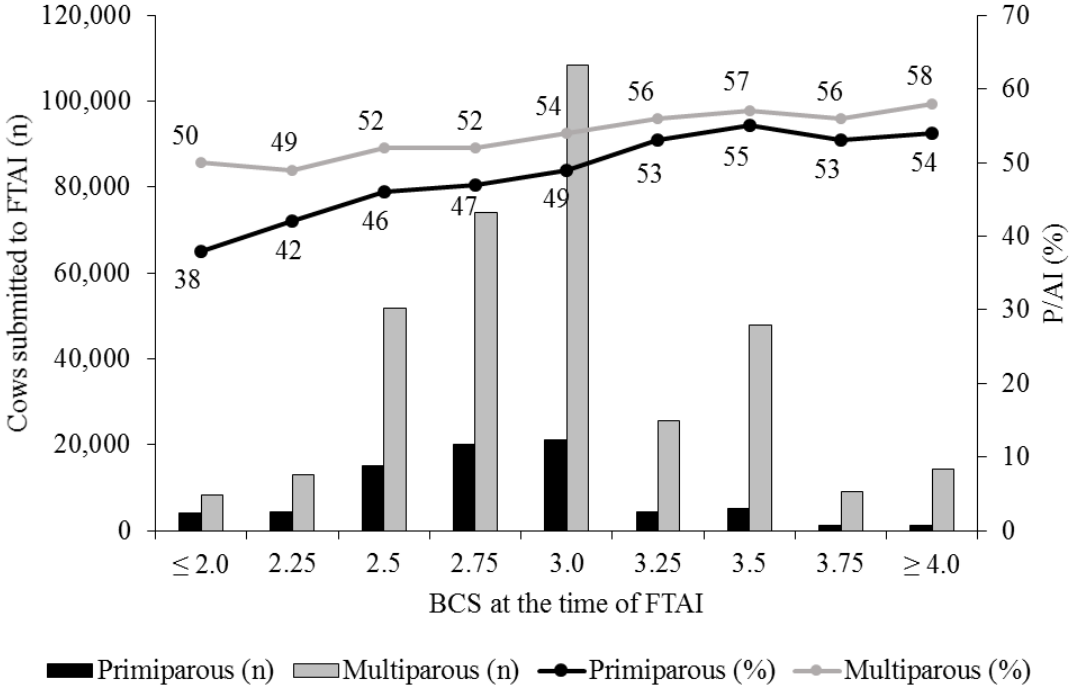

Figure 7. Number and P/AI of primiparous and multiparous beef cows submitted to FTAI according to BCS.

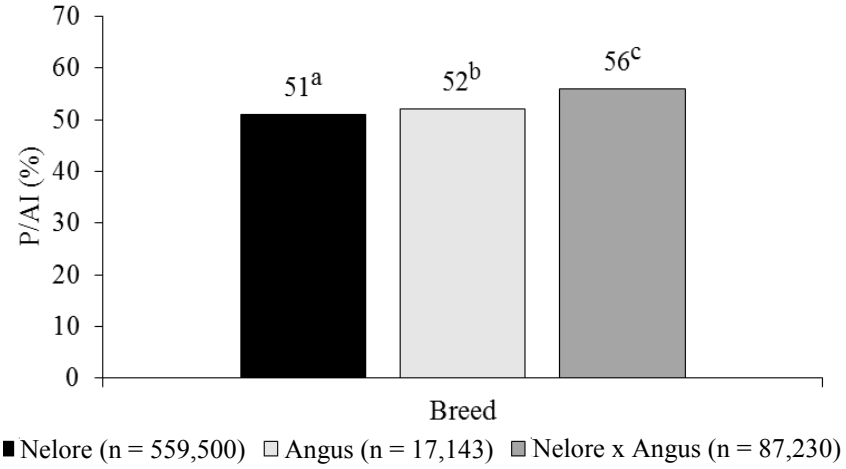

Figure 8. Breed effect on P/AI of cows submitted to FTAI. $^{a,b,c}P < 0.01$.

Embryo transfer

Evolution of the embryo industry in Brazil from 1995 to 2014

One of the most remarkable aspects of the use of ARTs in Brazil was the evolution of the cattle embryo industry during the last 15 years, particularly the emergence and later widespread use of IVP. In the early 90's, the Brazilian embryo industry was already substantial, and the country was the largest embryo producer, outside Europe and North America. However, the adoption of IVP after the year 2000 boosted the embryo industry, and since 2005, Brazil accounts for more than 20% of the world embryo production. In 2014, Brazil produced 348,468 embryos *in vitro*, which corresponds to 59.0% of the total world IVP (Perry, 2015).

The success of IVP in Brazil was due to a complex interplay of technical and economic factors that likely explain why it initially diverged from the trends elsewhere (Faber *et al*., 2003). Initially, in the period from the emergence of the first commercial IVP companies in 1999 to 2003, there was a relatively high cost and low efficiency of IVP (Hasler, 2000), but this was balanced by the high commercial value of the donors used. Thus, during this initial growth phase (first phase) IVP expanded mainly within the market of high genetic merit cows and the number of both IVD and IVP embryos increased similarly (Fig. 9).

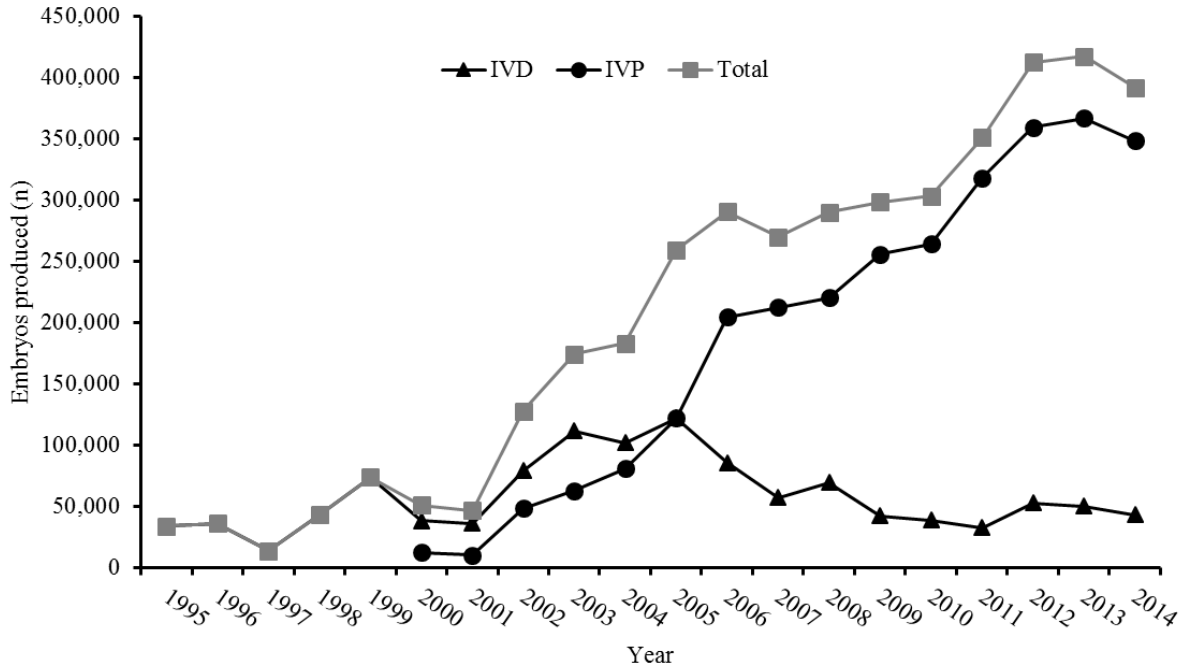

Figure 9. Production of bovine embryos in Brazil, according to the technique employed, during the period of 1995 to 2014. IVD: embryos produced by superovulation (*in vivo*); IVP: embryos produced *in vitro*.

A second phase of growth in the use of IVP embryos occurred between 2003 and 2010, driven largely by a shift from the production of embryos from high genetic merit animals to the production of replacement bulls. Prior to this second phase, the large size of beef cattle population in Brazil and the relatively low use of AI at that time (~6%; Baruselli *et al*., 2012) resulted in a repressed demand for such animals (bulls), especially in the Nelore breed. However at the peak of this second growth phase, in 2005, embryo production in Nelore (214,500) accounted for 82.7% of all embryos produced in the country, and for 90.0% of the embryos from beef breeds (Viana *et al*., 2012). Meanwhile, embryo prices began to decrease in Brazil due to many factors including: increasing efficiency of embryo production protocols, increased recovery of cumulus-oocyte complexes (COC) and greater blastocyst rates obtained in *Bos indicus* breeds (Pontes *et al*., 2009; Viana *et al*., 2012), and an increase in the scale of embryo production in commercial embryo production companies in Brazil. The IVP industry became more competitive, and eventually replaced multiple ovulation and embryo transfer (MOET) as the technique of choice for embryo production. Total embryo production increased rapidly, reaching numbers over 250,000 for the years after 2005.

We are currently in the midst of the third growth phase with increasing use of sex-sorted sperm in IVP, which occurred mainly in dairy breeds. In dairy breeds, production of a high percentage of female calves has many economic advantages and use of in IVP allows the production of approximately 90% of the embryos with the desired sex (Morotti *et al*., 2014). Thus, this third growth phase of the Brazilian embryo industry after 2010 has been marked by a clear shift from the predominance of beef breeds to dairy breeds. For example, in 2014, embryo production in dairy breeds increased by 46.5% and the total numbers of embryos produced from dairy breeds exceeded, for the first time, the number of embryos from beef breeds, (270,367 of 391,805, or 69.0% of total embryos). The expansion in the dairy sector also highlighted a new trend in the Brazilian embryo industry, the use of large-scale IVP to produce crossbred calves (Pontes *et al*., 2010). Producers and veterinarians explored the possibilities of obtaining the gains due to heterosis while maintaining herds with specific crossbred values (F1, ¾, etc.). For example, 79.3% of embryos produced in dairy breeds in 2014 were from Gir x Holstein crosses.

The inherent characteristics of dairy production, such as smaller herds and lack of a set breeding season, limits the availability of recipients, and thus required the development and use of cryopreservation alternatives. In 2015, the three main commercial laboratories in Brazil produced more than 276,000 embryos from ~50,000 donors with a blastocyst rate ~30% (more than 1 million oocytes used for IVF). Of those embryos, 111,000 were conventional embryos and 165,000 were produced using sex-sorted sperm and 29% (80,000) of these embryos were vitrified or frozen. In addition, these laboratories reported an increase in embryo production of more than 30% compared to 2014 (211,000 IVP embryos) and the percentage of vitrified/frozen embryos in these laboratories increased from 13% in 2014 to 29% in 2015. The continuing development and use of the direct transfer technique (over 9,000 embryos in 2015) is likely to lead to further increases in the use of cryopreserved IVP embryos. Moreover, the successful use of protocols for fixed-time ET (FTET), due to their high efficiency and ease of implementation, has facilitated the dissemination of ET

programs across Brazil.

Use of IVP embryos for reproductive management in dairy cattle

As seen above, the use of IVP embryos in dairy herds has increased in recent years. In 2015, the two largest laboratories that produce embryos from dairy breeds transferred more than 27,000 embryos, obtaining reasonable pregnancies per ET (P/ET), and acceptable pregnancy losses (Table 3), especially when beef cows, crossbreds, or heifers are used as embryo recipients. Moreover, the best IVP embryos are usually selected for vitrification, which may explain the observation of similar pregnancy losses for fresh and vitrified embryos, as presented in Table 3.

Table 3. Pregnancy per ET (P/ET) at 30 and 60 days and pregnancy loss between 30 and 60 days for fresh and vitrified IVP embryos from different dairy breeds in Brazil.

	30 days P/ET %, (n/n)	60 days P/ET %, (n/n)	Pregnancy loss % (n/n)
Gir			
Fresh	46.5 (4322/9294)	42.3 (3933/9294)	9.0 (389/4322)
Vitrified	34.1 (726/2128)	31.3 (667/2128)	8.1 (59/726)
Girolando (5/8 Holstein x 3/8 Gir)			
Fresh	45.1 (2214/4909)	43.1 (2116/4909)	4.4 (98/2214)
Vitrified	32.0 (340/1063)	30.7 (326/1063)	4.1 (14/340)
Holstein			
Fresh	38.2 (2409/6302)	34.4 (2170/6302)	13.3 (320/2409)
Vitrified	37.8 (1033/2735)	34.6 (947/2735)	8.3 (86/1033)
Jersey			
Fresh	35.4 (118/333)	33.6 (112/333)	5.1 (6/118)
Vitrified	40.3 (133/330)	37.8 (125/330)	6.0 (8/133)

However, results can vary from farm to farm, and rigorous evaluation and monitoring are necessary for this technology to be used on a large scale as a substitute for AI or FTAI. The following data describe two cases in which the use of IVP embryos enhanced reproductive efficiency and/or profitability.

The first dairy farm has 1,500 crossbred lactating cows (Girolando [5/8 Holstein x 3/8 Gir] breed) producing more than 25,000 kg of milk per day. The farm uses an intensive ET program, in which all cows receive IVP embryos using sex-sorted sperm in order to increase numbers of genetically-superior calves to be used as replacement heifers or for sale. Fig. 10 shows the number of embryos transferred from 2004 to 2015 in this farm. Between 2004 and 2010, there was a minor increase in embryos transferred, however, after that, there was a continuous increase in the use of ET. Over the past 3 years, more than 85% of calves that were born on this farm were females. Currently, only high-genetic merit cows (top 10%) are used as donors, providing embryos for the entire herd.

In 2015, more than 6,500 embryos were transferred, with acceptable P/ET at 30 days (43%) and 21-days PR (~20%). However, high incidence of pregnancy loss between 30 and 65 days (15%) and between 30 days and birth (30%) is an important issue. In addition, other factors such as low BCS, absence of CL at the beginning of the protocols for fixed-time ET (FTET), and subclinical mastitis affected ($P < 0.05$) P/ET and 21-days PR (Pereira and Coelho, 2016).

In addition, this farm also uses fresh, vitrified, and frozen embryos, and a study was done to compare P/ET among these treatments (Fleury *et al*., 2015). Grade I blastocysts or expanded blastocysts (Stringfellow and Seidel, 1998) were transferred to previously synchronized recipients. The P/ET were 51.4% (133/259) for embryos transferred fresh, 35.9% (84/234) for vitrified, and 42.1% (96/228) for direct transfer embryos. The P/ET obtained from IVP embryos vitrified or frozen were not different between each other, but they were lower than the P/ET obtained when IVP embryos were transferred fresh ($P < 0.05$). Therefore, these results highlighted the aspects of cryopreservation of IVP embryos with the convenience of direct transfer as compared with vitrification.

The second farm has 1,100 lactating cows (Holstein and Girolando breeds) with average milk production of 30 kg/day. The reproductive management consists of use of AI or transfer of IVP embryos. As shown in Table 4, despite having greater pregnancy losses, the IVP technique was chosen as a better reproductive management strategy for this dairy farm, as compared to AI, due to greater P/ET *vs*. P/AI, and greater birth rates for ET *vs*. AI. In addition, the use of sex-sorted sperm for IVF allowed an increased number of heifers born with IVP and greater genetic improvement.

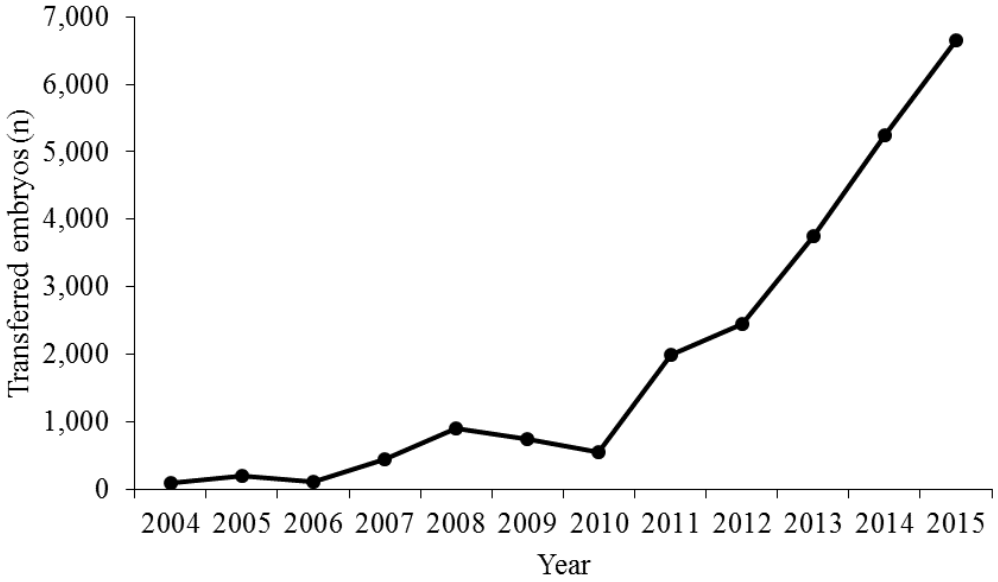

Figure 10. Number of embryos transferred from 2004 to 2015 in one dairy farm. From Pereira and Coelho (2016).

Table 4. Pregnancy per AI or P/ET at 30 days, birth rate, and pregnancy loss between 30 days and calving for Holstein and Girolando (5/8 Holstein x 3/8 Gir) lactating cows submitted to AI or ET on the same dairy farm.

	P/AI or P/ET at 30 days, % (n/n)	Pregnancy loss, % (n/n)	Birth rate, % (n/n)
Holstein			
AI	23.0[a] (895/3899)	39.3[A] (352/895)	13.9[a] (543/3899)
ET	43.1[b] (1026/2382)	43.6[B] (447/1026)	24.3[b] (579/2382)
Girolando			
AI	30.9[a] (1053/3413)	26.1[a] (275/1053)	22.8[a] (778/3413)
ET	45.4[b] (926/2038)	33.6[b] (311/926)	30.2[b] (615/2038)

[a,b]P < 0.01 within column and within breed. [A,B]P < 0.10 within column and within breed.

Pregnancy losses for the farms described above are much greater than those shown in Table 3, probably due to the use of different embryo recipients, as well as quality of IVP embryos selected for transfer. For the farms described above (Table 4), lactating cows were primarily used as recipients, whereas data presented in Table 3 are mainly from non-lactating embryo recipients. In fact, data of other dairy farms (n = 7) in which IVP embryos were transferred to lactating cows show acceptable P/ET at 30 days [42.9% (7204/16771)], however, pregnancy loss between 30 and 60 days [15.9% (820/5147)], and pregnancy loss between 30 days and calving [33.4% (2323/6956)] are high, resulting in low birth rates [28.8% (4663/16170)]. Greater pregnancy loss in lactating dairy cows as compared to heifers or non-lactating cows has been well-described elsewhere (Santos *et al*., 2004; Sartori, 2004).

Use of IVP embryos in beef cattle

Similar to what was reported for dairy cattle, data for beef cattle from the same IVF labs in Brazil, demonstrate acceptable P/ET at 30 days, especially when fresh embryos were transferred (Table 5). Pregnancy losses between 30 and 60 days may also be considered acceptable, and are similar for fresh or vitrified embryos (Table 5). However, as discussed in the next section of this manuscript, results are still not ideal, if compared with other ARTs.

Table 5. Pregnancy per ET (P/ET) at 30 and 60 days and pregnancy loss between 30 and 60 days for IVP embryos from beef breeds in Brazil.

	P/ET at 30 days, % (n/n)	P/ET at 60 days, % (n/n)	Pregnancy loss, % (n/n)
Nelore			
Fresh	44.4 (5,311/11,964)	40.4 (4,838/11,964)	9.1 (483/5,311)
Vitrified	34.8 (3,181/9,143)	31.8 (2,905/9,143)	8.6 (276/3,181)
Senepol			
Fresh	43.3 (3,408/7,874)	38.0 (2,996/7,874)	12.3 (421/3,408)
Vitrified	37.7 (2,967/7,873)	34.2 (2,694/7,873)	9.2 (273/2,967)

Reproductive efficiency of FTAI vs. FTET in beef cattle

Despite the many advances in the use of ARTs in Brazil, there is still substantial room for improvement, especially regarding cryopreservation/vitrification of IVP embryos. Below, we describe results of a study that evaluated reproductive efficiency in beef cows submitted to FTAI, or receiving the transfer of vitrified IVD or IVP embryos by FTET (Sartori *et al*., 2013).

Nelore (*Bos indicus*) cows (with a calf or not) were synchronized with the same protocol within a 3-months period (Fig. 11). For FTAI, 346 cows were bred on day 0 using frozen/thawed semen of five bulls. For ET, cattle received IVD (n = 274) or IVP (n = 573) vitrified embryos (produced with semen from seven bulls, of which, three were the same bulls used for FTAI) on days 6, 7, or 8 of the protocol after confirming the presence of a CL. The same groups of cows were used for all treatments. Transfers of IVD and IVP embryos, but not FTAI were concurrent, and there were two time-periods for AI or ET for each treatment group. Pregnancy was diagnosed by transrectal ultrasonography on day 30 after ovulation. Presence of an amniotic vesicle with an embryo was used as indicator of pregnancy. Pregnant cows were re-examined 30 days later, on day 60 of expected gestation.

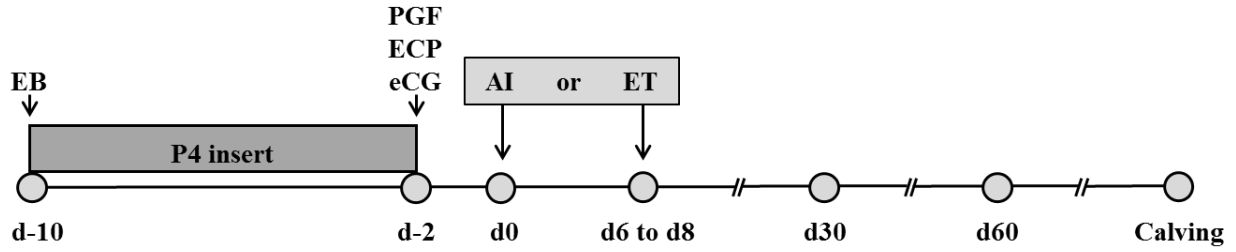

Figure 11. Schematic illustration of the protocol for FTAI or FTET in embryo recipient cows. Day-10: placement of an intravaginal insert of progesterone and 2 mg of estradiol benzoate (EB) i.m. Day-2: insert was removed and cows received i.m. treatments of 0.150 mg sodium cloprostenol (PGF2α), 300 IU equine chorionic gonadotropin (eCG) and 0.6 mg estradiol cypionate (ECP). Day 0: FTAI. Day 6 to Day 8: Embryo transfer. Day 30: Ultrasonography for pregnancy diagnosis. Day 60: Ultrasonography to confirm pregnancy.

All data regarding pregnancy diagnosis, pregnancy losses, and reproductive responses are shown in Table 6. The FTAI group had better results for almost all variables that were analyzed. Cows that received FTAI had greater P/AI at 30 and 60 days than cows receiving IVD or IVP embryos. However, when comparing cows that received ET, there was no detectable difference for P/ET at 30 days. Nevertheless, at 60 days, cows receiving IVP embryos had lower P/ET than cows receiving IVD embryos. Pregnancy loss between 30 and 60 days was lower for cows receiving FTAI, intermediate and not different from the other groups for cows receiving IVD embryos, and greater for cows receiving IVP embryos. For unknown reasons, FTAI cows had relatively high and similar rates of later pregnancy loss as IVP cows. Fewer cows receiving IVD embryos had later pregnancy losses, as compared with cows from the two other groups (Table 6). Moreover, gestation length was shorter for FTAI cows than for cows receiving IVD or IVP embryos (293.4 ± 5.3[a] [275 to 303], 296.7 ± 6.3[b] [270 to 315], and 296.8 ± 7.1[b] [277 to 319] days, respectively; mean ± SD [range]; $P < 0.001$). Another important aspect to be considered was that for all calculations mentioned above, for the FTAI group, 100% of cows submitted to the protocol were considered in the analyses, however for the ET groups, only data from cows that had a CL at the time of transfer (~80%) were analyzed. When this variable was used for analysis, more healthy calves were born per cow submitted to a synchronization protocol for the FTAI group and less for the IVP group (Table 6).

Table 6. Pregnancy per AI or P/ET, pregnancy loss, abortion, and peripartum loss in Nelore cows that received fixed-time AI (FTAI) or vitrified *in vitro* produced (IVP) or *in vivo* derived (IVD) embryos.

	FTAI	IVD	IVP
30 days pregnancy, % (n/n)	50.3[a] (174/346)	39.4[b] (108/274)	34.0[b] (195/573)
60 days pregnancy, % (n/n)	47.7[a] (165/346)	35.4[b] (97/274)	28.6[c] (164/573)
Embryo/fetal loss (30 to 60 days), % (n/n)	5.2[b] (9/174)	10.2[ab] (11/108)	15.9[a] (31/195)
Later pregnancy loss (60 days to calving), % (n/n)	15.2[a] (25/165)	6.3[b] (6/96)	16.5[a] (27/164)
Peripartum loss, % (n/n)	2.1[b] (3/140)	4.4[ab] (4/90)	9.5[a] (13/137)
Total loss, % (n/n)	21.3[b] (37/174)	19.4[ab] (21/108)	36.4[a] (71/195)
Healthy calf born per synchronization protocol, % (n/n)	39.6[a] (137/346)	25.4[b] (87/342)	17.3[c] (124/716)

[a,b,c]$P < 0.05$.

Cloning

The birth of Vitória in 2001, a Simmental calf clone produced from embryonic cells, marked the beginning of the cloning era in Brazil. Subsequently, the production of cloned calves from fetal fibroblasts and from adult cell lines in 2002 was reported by different research groups. This was followed by production of many other cloned calves, demonstrating the potential of using SCNT commercially, in cattle and possibly other species. Private companies and producers were interested in applying this technology in animal production, especially for high genetic value animals. A technical committee was subsequently formed by researchers from several universities and research centers in 2007 to set the criteria for creating the Genealogical Register of Zebu breeds for the Ministry of Agriculture. However, the registration of cloned animals was released by the Ministry only after May 2009. By that time, about 70 cloned cattle had already been born and commercialized in Brazil. The registration of these cloned cattle by the breed associations, although not representing a complete dataset, at least provides information about how SCNT is being used in Brazil. Therefore, since 2005, cloning services have been provided by commercial laboratories in Brazil for propagation of valuable genetics, either for animal production purposes or for preservation of rare genotypes. With respect to endangered livestock, not much has been done in Brazil, other than the production of two cloned heifers of the Junqueira breed in 2005. Nevertheless, in 2012, the Brazilian Agricultural Research Corporation and the Brasilia Zoological Garden began collecting and freezing blood and umbilical cord cells from wild animals that had died (Scientific American, March 11, 2013. http://www.scientificamerican.com/article/cloning-endangered-animals), mostly in the Cerrado savanna; however, no cloned animal has been produced from these samples.

In contrast, for animal production the situation is quite different. Data from the Brazilian Association of Zebu Breeders (ABCZ) show a gradual increase in registered *Bos indicus* calves (predominantly of Nelore and Gir breeds) produced by SCNT during the years 2010 (n = 5), 2011 (n = 23), 2012 (n = 22), and 2013 (n = 41). Unofficial data indicate a continuous increase in number of healthy calves produced by SCNT from 2014 to 2016.

It is important to point out, however, that somatic bovine cloning is still besieged by low efficiency (number of live calves as a proportion of embryos transferred). The epigenetic modifications that are established during cellular differentiation are likely to be a major factor producing this low efficiency since they may act as barriers to the proper reprogramming of somatic nuclei. The 30 days P/ET is similar for cloned embryos and IVP embryos, however the overall efficiency is low due to the large proportion of pregnancies that are lost during gestation (Gerger *et al*., 2016) and in neonatal and postnatal periods (Chavatte-Palmer *et al*., 2004; Panarace *et al*., 2007).

After many years of research, no dramatic increase in cloning efficiency has been observed, with the rate of survival of cloned embryos still varying from 0 to 12% (De Bem *et al*., 2011; Sangalli *et al*., 2014; Gerger *et al*., 2016). Some improvements in survival rate can be expected by using specific and intensive management and clinical procedures during the perinatal and postnatal periods (Meirelles *et al*., 2010).

In the last 3 years the results described in Brazil (Table 7) are very similar to those reported in the literature. The 30-days P/ET is similar to results with IVP embryos (~40%), however the pregnancy loss is still very high, as shown in Table 7, and is similar to the losses described by Panarace *et al*. (2007). Nevertheless, post-partum death appears to be decreasing (78% survival in 2016) due to a better understanding on how to care for newborn calves. This gives some hope that this technology may be of practical use in the future, although the problems of nuclear reprogramming and exceedingly high pregnancy losses still need to be unraveled.

Table 7. Pregnancy per ET (P/ET) at 30, 60 and 90 days, birth rate, pregnancy loss and postpartum loss of bovine embryos produced by somatic cell nuclear transfer.

	P/ET at 30 days, % (n/n)	P/ET at 60 days % (n/n)	P/ET at 90 days % (n/n)	Birth rate %, (n/n)	Pregnancy loss 30 days to birth % (n/n)	Postpartum survival %, (n/n)
2014	42.0 (126/300)	26.5 (80/300)	25.0 (75/300)	12.0 (36/300)	71.4 (90/126)	58.3 (21/36)
2015	34.6 (128/370)	15.7 (58/370)	12.4 (46/370)	10.0 (37/370)	71.1 (91/128)	59.4 (22/37)
2016	44.7 (83/186)	27.4 (51/186)	26.0 (48/186)	12.4 (23/186)	72.3 (60/83)	78.2 (18/23)

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The timing of puberty (oocyte quality and management)

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Abstract

This review aims at giving an overview on the physiological events leading to puberty onset in mammals and more specifically in cattle. Puberty is an important developmental milestone in mammals involving numerous changes in various physiological regulations and behaviors. It is a physiological unique event integrating several important central regulations at the crossroad of adaptation to environment: reproductive axis, feeding behavior and nutritional controls, growth, seasonal rhythm and stress. Puberty onset is also an important economic parameter in replacement heifer program and in genomic selection (genomic bulls). The quest for advanced puberty onset should be carefully balanced by its impact on physiological parameters of the animal and its offspring. Thus one has to carefully consider each step leading to puberty onset and set up a strategy that will lead to early puberty without being detrimental in the long term. In this review, major contributions in the understanding of puberty process obtained in rodents, primates and farm animals such as sheep and cattle are discussed. In the first part we will detail the endocrine events leading to puberty onset with a special focus on the regulation of GnRH secretion. In the second part we will describe the neural mechanisms involved in silencing and reactivating the GnRH neuronal network. These central mechanisms are at the crossroad of the integration of environmental factors such as the nutritional status, the stress and the photoperiod that will be discussed in the third part. In the fourth part, we will discuss the genetic determinants of puberty onset and more particularly in humans, where several pathologies are associated with puberty delay or advance and in cattle where several groups have now identified genomic regions or gene networks associated with puberty traits. Last but not least, in the last part we will focus on the embryologist point of view, how to get good oocytes for in vitro fertilization and embryo development from younger animals.

Keywords: Glial-neuronal communication, GnRH, hypothalamus, neuroendocrinology, timing of puberty, transcriptional regulation

Introduction

Puberty is an important developmental milestone in mammals involving numerous changes in various physiological regulations and behaviors. It is a physiological unique event integrating several important central regulations at the crossroad of adaptation to environment: reproductive axis, feeding behavior and nutritional controls, growth, seasonal rhythm and stress.

Puberty, puberty onset, peri-pubertal, reproductive maturity what's the difference

Puberty onset results from a complex and integrated sequence of biological events leading to progressive maturation of sexual characteristics that ultimately lead to attainment of full reproductive capacity. This sequence is referred as the timing of puberty. Puberty timing in mammals is the result of evolution allowing females to attain ideal pelvic anatomy and size, complete growth and maximize skeletal mineralization, prior to the demands of pregnancy, lactation and offspring rearing.

Puberty is defined as the moment of the first emission of gametes, *ie* the first ovulation in females and the first spermatozoa entering the epididymis in males. Therefore puberty is expressed as a date or as an age. From this definition, it is obvious that puberty can be easily detected in females by detecting the first ovulation. However in males there is no non-invasive method to assess the presence of epididymal spermatozoa and it is usually defined according various physical and behavioral changes. Therefore puberty is very often studied through the modifications observed before and immediately following the first emission of gametes. In that case it is better to speak of peri-pubertal period. For example in females, breeders usually monitor the exterior signs of receptivity (age at first estrus). However one has to keep in mind that estrus behavior can exist without a proper ovulation and the reciprocal is also true: ovulation can occur without any sign of estrus behavior. For males, breeders look at the sexual behavior too: mounting behavior and erection. Here again, this behavior does not mean that is there any spermatozoa in the ejaculate.

The strict definition of puberty onset as the first emission of gametes does not mean that the animals are able to breed yet. They can produce and release gametes but reproduction is more than that. Females usually need a period of time after puberty onset to have regular ovarian cycles and to get their uterus capable of supporting a pregnancy. For males, the concentration of spermatozoa in the ejaculate should reach a certain threshold to give an adequate fertility; here again this can take some time after the puberty onset. Reproductive maturity is another phenomenon and the mechanisms leading to puberty onset are different from those leading to reproductive maturity.

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Endocrine basis of puberty

Brief overview of the endocrine events across the estrus cycle

Post-pubertal females present estrus cycles, which is the reflection of the ovarian cyclicity. During the late follicular phase, the preovulatory follicles release high estradiol levels in the blood stream. The starting point of all endocrine events leading to ovarian cyclicity is the secretion of a neurohormone: the gonadotropin releasing hormone (GnRH). GnRH acts on the gonadotrope cells located in the anterior pituitary and promote the synthesis and release of both gonadotropins luteinizing hormone (LH) and follicle stimulating hormone (FSH). LH and FSH will act at the ovary level to promote the release of gonadal steroid estrogens and progestagens and to promote follicular growth. Granulosa cells and thecal cells collaborate to synthesize and release estrogens, among which 17-β-estradiol (E2) is the most prevalent estrogen in most species. The amount of estrogen released is dependent on the number of granulosa and thecal cells. Considering the growth of a sphere, the amount of estrogen that can be synthetized is proportional to the cubic of the follicle radius. Therefore the final growth of the dominant follicle is accompanied by a huge increase in E2 production. High E2 levels are responsible for the expression of estrus behavior. In most species studied, high E2 levels will also exert a positive feedback on GnRH secretion leading a large amount of GnRH release that causes a large amount of LH release: the pre-ovulatory GnRH and LH surges (see Fig. 1). LH surge occurs just before the ovulation and last several hours after, contributing to the luteinization of granulosa and thecal cells of the ovulated follicle. *De facto*, E2 levels drop and the positive feedback disappears, stopping its repressive action on GnRH secretion. In parallel progesterone (P4) secretion increases and exerts a negative feedback on GnRH secretion. High P4 levels have a positive action on E2 receptors (ERs) expression. Without P4-priming during the previous cycle, ERs expression is low and despite high E2 levels during the preovulatory phase, the estrus behavior, which is strongly dependent on ERα signaling, is poorly expressed. Once luteolysis occurs, P4 levels drop, the negative feedback is suppressed and GnRH secretion increases again leading to LH and FSH release and a new follicular phase starting.

Evolution of gonadotropin secretion in the pre-pubertal period

The key decisive event required for puberty to occur is an increase in pulsatile gonadotropin releasing hormone (GnRH) release from GnRH neurons leading to gonadotropins LH and FSH secretion. In mature adult, the GnRH is released in the portal veins in a pulsatile manner. GnRH secretion is difficult to assess. As a matter of fact, GnRH is released in capillaries within the ME that form portal veins along the pituitary stalk. From these portal veins, pituitary capillaries emerge and the GnRH is released in the intercellular space and reaches anterior pituitary cells. GnRH concentration in the portal veins varies between 4-100pg/ml (Caraty *et al*., 1982; Clarke and Cummins, 1982; Levine *et al*., 1982; Irvine and Alexander, 1987; Gazal *et al*., 1998), which gives a very small amount of GnRH for the small blood volume considered. Thus, the amount of GnRH that passes in the general circulation is very small; the concentration is well below the detection threshold of known hormonal assays. Moreover GnRH half-life is very short, a few minutes. Due to its small peptidic structure, circulating endopeptidases degrades rapidly the GnRH. Therefore to assess the GnRH secretion, blood should be punctured from pituitary portal vessels or from *canulae* inserted in the third ventricle (Gazal *et al*., 1998) and this can only be performed in large animals and requires invasive surgical procedures (Clarke and Cummins, 1982; Levine *et al*., 1982). An alternative is to follow LH secretion since it has been clearly demonstrated that a GnRH pulse precedes every LH pulse (Clarke and Cummins, 1982; Caraty *et al*., 1989).

In the female Rhesus monkey the early prepubertal period is characterized by an increase in pulsatile release with a concomitant increase in pulse frequency and pulse amplitude. In the midpubertal phase, only an increase in GnRH pulse amplitude is noticed and the global GnRH secretion is increased during the night (Watanabe and Terasawa, 1989). This is in contrast to ewes and heifers where the midpubertal period is characterized by an increase in LH pulse frequency associated with a decrease in pulse amplitude (Day *et al*., 1987). In heifers, the frequency of LH pulses is usually in a range of 2 to 4 pulses/24 h 100 to 50 days before puberty onset. The amplitude of LH pulses is high, reaching 6-8 ng/ml. From 50 days before to puberty onset, the frequency of LH pulses increased to reach 15-20 pulses/24 h and the mean amplitude of LH pulses decreased to values (<2 ng/ml; Day *et al*., 1987). Such increase in LH pulse frequency was also reported in female lambs (Claypool and Foster, 1990). In humans, this increase in pulsatile LH secretion is also observed but occurs during the night phase (Wu *et al*., 1996).

In spite of numerous physiological studies in model animals, little is known about the key events leading to GnRH neurons progressive activation at puberty onset. The scientific community admits that puberty onset is preceded by gradual changes in trans-synaptic and glial inputs to the GnRH neuronal network. The trans-synaptic changes consist of a coordinated increase in excitatory inputs and/or a reduction in inhibitory influences. Glial cells could also participate in regulating extracellular glutamate concentration, and in releasing growth factors and small diffusible molecules that directly or indirectly stimulate GnRH secretion. In addition to the classical excitatory glutamatergic neurons, kisspeptin signaling through GPR54 was discovered in 2005 as a powerful stimulator of GnRH release (Messager *et al*., 2005). Nevertheless, how these key events are triggered through environmental and nutritional factors is far from being understood.

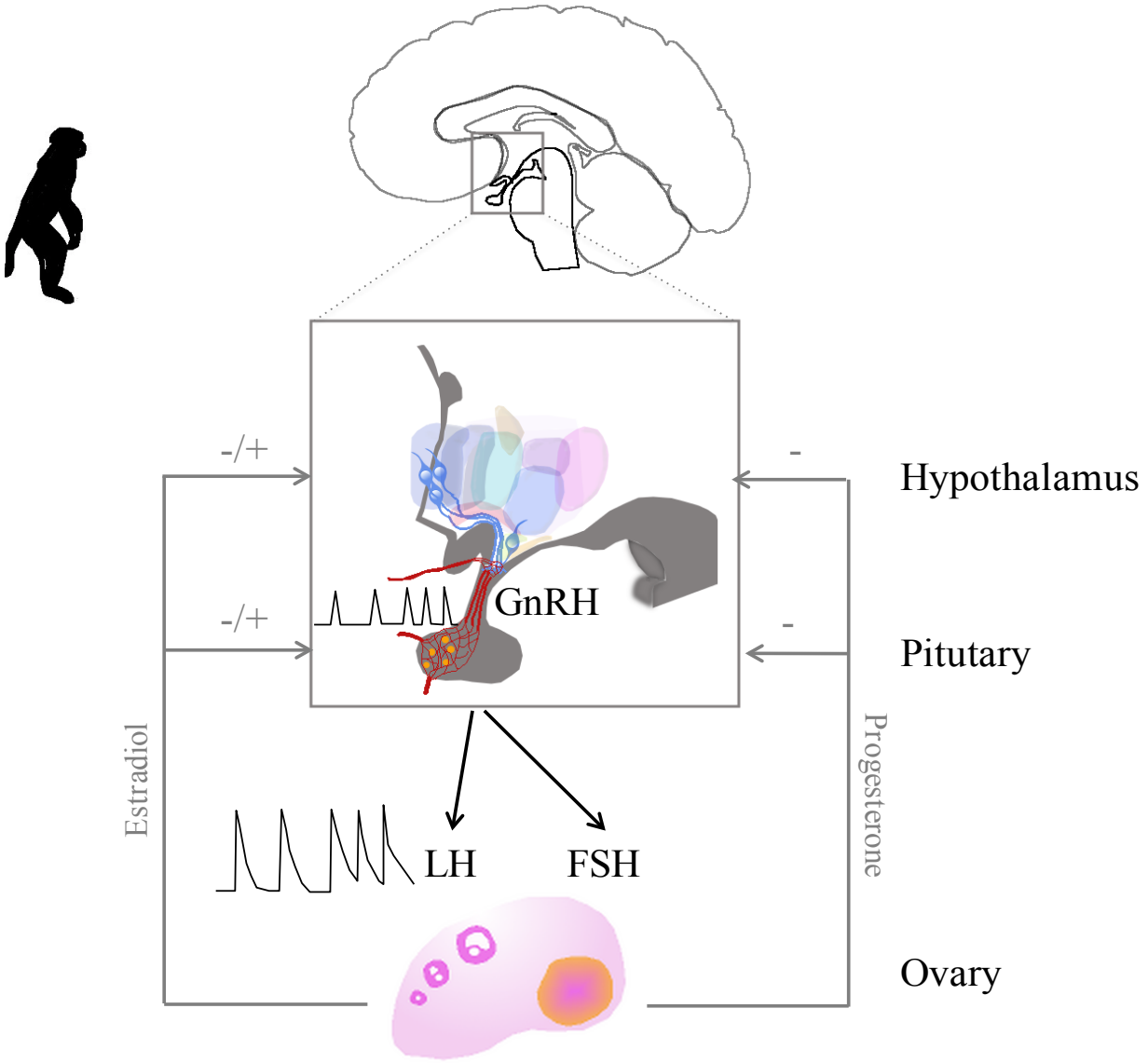

Figure 1. Schematic representation of the hypoyhalamic-hypophysis-ovary axis. GnRH neurons somas are mostly located in the preoptic area and send their axons towards the median eminence where GnRH is released in a pulsatile manner into capillaries. Median eminence capillaries merge to form the portal vessels on the ventral part of the anterior pituitary and give rise to pituitary capillaries. GnRH then can diffuse within the anterior pituitary and reach gonadotropic cells that release the gonadotropins: LH and FSH. LH and FSH will reach the general blood circulation and act on the ovaries to stimulate both oocyte and follicle growth and gonadal steroids secretion. Progesterone exerts an negative feedback at the pituitary and hypothalamic levels, estradiol at low concentration exerts a negative feedback at both pituitary and hypothalamic levels, but at high concentration (during estrus) it will have a positive feedback at both pituitary and hypothalamic levels.

GnRH control

The two modes of secretion

GnRH secretion is characterized by two modes of secretion: pulsatile and continuous (the surge). These two modes have been described in the pioneering work of Ernst Knobil in the rhesus monkey where he described a tonic and a phasic mode of LH secretion controlled by two different areas within the hypothalamus Preoptic area (POA) and mediobasal hypothalamus (MBH), respectively (Nakai *et al*., 1978). The pulsatile pattern of GnRH secretion was confirmed in the 80's when a trans-nasal surgical approach allowed the collection of blood from the portal vessels between the hypothalamus and the pituitary (Clarke and Cummins, 1982; Levine *et al*., 1982).

GnRH/LH secretion is pulsatile during the follicular and the luteal phases, the surge mode occurs during the pre-ovulatory period. In most species where GnRH and LH secretions have been monitored simultaneously, the LH secretion profile is a good estimate of the GnRH pulsatile secretion: a GnRH pulse always precedes one LH pulse. The frequency of pulsatile secretion varies across the estrus cycle. For example in the ewe, the follicular phase is characterized

by a high frequency ie 1 pulse *per* hour, and low amplitude of LH pulses, whereas the luteal phase is characterized by a low frequency ie 1 pulse *per* 6 h but high amplitude of LH pulses (Moenter *et al*., 1991). The GnRH pulse frequency is decoded by the GnRH receptor (GnRH-R) expressed by gonadotropic cells: high frequency favors the expression of the β-LH subunit whereas low frequency favors the expression of the β-FSH subunit (Bédécarrats and Kaiser, 2003; Thompson and Kaiser, 2014).

Anatomy of the GnRH neuronal network

The GnRH is a small peptide (10 amino-acids) issued from the processing of pre-pro-GnRH encoded by the *Gnrh1* gene. The pre-pro-GnRH is processed in GnRH neurons to give the GnRH and the GnRH-associated peptide (GAP), Both are packed in large dense core vesicles (LDCV) for further release (Clarke *et al*., 1987). The GnRH is synthetized and secreted by a specialized population of neurons: the GnRH neurons. In most mammals the GnRH neurons' somas are located in the POA with a few cell bodies located in the MBH and the axons project towards the median eminence at the bottom of the MBH. However in primates, the repartition is different with the majority of GnRH neurons' somas located in the MBH and just a few in the POA. Axonal projections are projected to the median eminence where GnRH is released in blood capillaries and transported in portal vessels to the capillaries network of the anterior pituitary where it will stimulate the expression and release of the gonadotropins FSH and LH.

Extracerebral embryonic origin of GnRH neurons

During embryogenesis, the GnRH neurons originate from the medial part of the nasal embryonic placode at early embryonic age 30 (E30) in sheep (Caldani *et al*., 1987; Caldani *et al*., 1995), E11.5 in mouse (Schwanzel-Fukuda and Pfaff, 1989; Wray *et al*., 1989), 6-7weeks of pregnancy in humans (Schwanzel-Fukuda *et al*., 1996). They then migrate along olfactory-, vomero-nasal- and terminal nerves to finally enter the forebrain through the cribiform plate. In the sheep, this phase of nasal migration is completed at E45, in the mouse at E13.5. Once in the brain they turn ventro-caudally to reach their final location in the POA/MBH. The phase of intra-cerebral migration is completed at E60 in the sheep and E16.5 in the mouse. Once settled, they grow their axonal projections toward the ME. This phase of axonal growth is terminated at E70 in sheep and E18.5 in the mouse. Once connected to the ME, it is believed that GnRH secretion occurs since it is correlated with the first observation of β-LH expression in the pituitary cells (Messaoud-Toumi *et al*., 1993). Primary cultures derived from embryonic nasal explants from E26 sheep embryos, E35 rhesus monkey embryos or E11.5 mouse embryos allow the development of functional secreting GnRH neurons that form a network in vitro. The GnRH secretion is pulsatile and the frequency is correlated to what it is observed in vivo according to each species considered (Duittoz and Batailler, 2000; Constantin *et al*., 2009). These *in vitro* approaches suggest that the pulsatility of secretion is an endogenous property of the GnRH network and that this property develops during the fetal life.

Functionality of the fetal GnRH neuronal network

Whether the pulsatile secretion develops in utero and plays a role in development has been studied particularly in the sheep species. Several groups have carried on a series of experiments on sheep fetuses. In chronically catheterized ovine fetuses, both LH and FSH exhibit a similar trend of peak values in midgestation (70-100 days) with a progressive decrease in plasma concentrations towards term (145 days; Sklar *et al*., 1981). Measurements from 55-60 days of gestation embryos gave low values of plasma LH and FSH concentrations. This pattern is similar to the one described in human fetuses with high concentration values of LH between 15-29 weeks of gestation (Kaplan and Grumbach, 1976; Clements *et al*., 2009). The ovine fetal pituitary gland has the capacity to respond to exogenous GnRH as early as 60 days of gestation with a maximal amplitude occurring during mid-gestation (Mueller *et al*., 1981). The pulsatile nature of LH fetal secretion was clearly assessed by serial blood sampling during a 4 h period in ovine fetuses at mid-gestation (Clark *et al*., 1984; Fig. 2). If we put in parallel the physiological maturation profiles of LH secretion and the development of the GnRH neuronal network we can clearly see the correlation between those events. Thus, once GnRH neuronal migration and axonal growth toward the median eminence is completed, the GnRH secretion can take place and induce the expression of gonadotropin subunits (Messaoud-Toumi *et al*., 1993). LH and FSH will act on the fetal gonad to stimulate gonadal steroid synthesis, and this is particular evident in male ovine fetuses where a spurt in testosterone secretion is detected at mid-gestation. This increase in testosterone in male fetuses or new born has been demonstrated in numerous mammals (Foster and Hileman, 2015; Plant *et al*., 2015; Prevot, 2015). In precocious mammals such as ovine and bovine species, the spurt occurs during the last third of gestation and is terminated at birth, whereas in altricial species such as rodents, the spurt occurs during the last days of pregnancy and during the first week post-natal (mice; Sisk and Foster, 2004). After this "mini puberty", the frequency of GnRH/LH secretion dramatically decreases and steroid levels drop; the infancy period is starting.

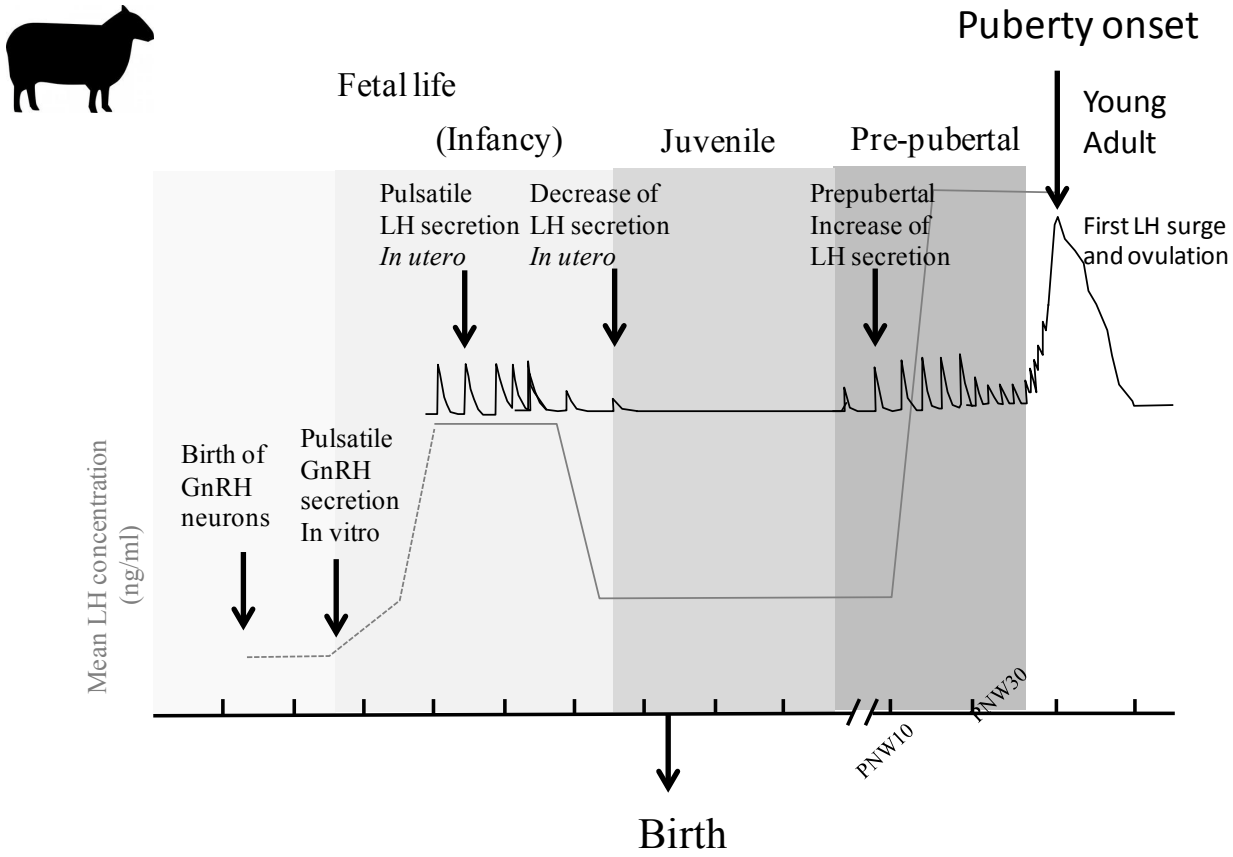

Figure 2. Schematic representation of the evolution of LH secretion from fetal life to adulthood in the ovine species. At 26 days of gestational age (G26), the first GnRH neurons are detected in the medial part of the nasal placode. From G26 to G35, GnRH neurons are born in the nasal placode and migrate along the nasal septum to reach the cribriform plate (intra nasal migration). From G35 to G45, GnRH neurons migrate into the brain and reach their final location in the preoptic area. From G45 to G60, GnRH neurons send axonal projections towards the external part of the median eminence (Caldani et al., 1995). At G60, the first expression of *LHB* is detected, suggesting that GnRH secretion is functional. From G80 to G120, LH is released in a pulsatile manner and contributes to the secretion of testosterone in male fetus and the sexualization of external genitalia and brain structures. From G120 to postnatal day 60 (PN60) there is virtually no LH secretion. From postnatal week 10 (PNW10) to PNW20, LH pulsatile secretion reappears with low frequency and high amplitude. From PNW20 to PNW30, the frequency of LH pulses increases and the amplitude decreases. The first preovulatory LH surge signs the onset of puberty.

Puberty: endocrine or brain revolution?

The pubertal transition involves both gonadal and behavioral maturation. The increase in the frequency of GnRH release and gonadotropins secretion progressively leads to the onset of gonadal functions: gametogenesis and steroid production. These steroids act in turn onto the brain to remodel neural circuits particularly those involved in sexual behaviors, but not only (Forger *et al*., 2015). In humans, several neurological or psychiatric diseases appear or are exacerbated at puberty (autism, schizophrenia, epilepsy, anorexia nervosa …).

Several decades of research have tempted to answer the question of the timing of the reactivation of GnRH secretion and the onset of puberty. As mentioned earlier, the hypothalamic-pituitary gonadal axis is functional during fetal/perinatal period, leading to the sexualization of external genitalia and specific regions of the nervous system. This activation is limited in time but offers a window of sensitivity to external factors such as endocrine disruptors (Parent *et al*., 2015; Hines *et al*., 2016).

Inhibitory mechanisms

Steroid-dependent mechanism

Early studies highlighted the role of the steroid negative feedback, the so-called "gonadostat" hypothesis (Frisch and Revelle, 1970). The "gonadostat" theory implies a higher sensitivity of GnRH neuronal network to the negative feedback of steroids: a steroid-dependent mechanism. In the prepubertal period, GnRH secretion is less sensitive to the negative feedback of gonadal steroids, the GnRH pulse frequency increases leading to gonadotropin secretion and gonadal activation. In the sheep species, early post-natal gonadectomy leads to immediately increased levels of gonadotropins as in the postpubertal period. Replacing

steroid gonadal hormones causes gonadotropins levels to go back to initial prepubertal values (Foster and Hileman, 2015). Similar findings were found in other mammals: hamster, ferret (Sisk and Foster, 2004). In heifers the negative feedback of estradiol declined as puberty approached (Day *et al*., 1987). However, in rat and rhesus monkey, the gonadostat theory is not sufficient to account for the low gonadotropins levels during infancy (Sisk and Foster, 2004). Interestingly, a steroid dependent mechanism exists at the end of the juvenile period of female rhesus monkey (Rapisarda *et al*., 1983).

Steroid independent mechanism

In rat and monkeys, after neonatal castration, gonadotropins levels remain low during the infantile period and increase progressively in the juvenile period to reach high levels as those expected at puberty. Such findings have been also reported in humans suffering from gonadal dysgenesis (Winter and Faiman, 2009). Although the precise neuronal target of gonadal steroid feedback is not clearly known, POA and the ArcN are involved in sensing estradiol negative feedback in gonadectomized prepubertal rats (Uenoyama *et al*., 2015). GnRH neurons, although located in the POA, are not considered as the primary target since they do not express the estrogen receptor α (ERα) albeit they do express ERβ (Hrabovszky *et al*., 2000; Herbison and Pape, 2001) but this later isoform does not seem to be involved in puberty onset. To account for this steroid-independent system, one assumption is that during infancy, inhibitory brain circuits block GnRH secretion; this break is released at puberty concomitantly with the onset of stimulatory brain circuits. GABA (γ-aminobutyiric acid) neurons are involved in the inhibition of GnRH neurons during juvenile period in several species. In the rhesus monkey, Terasawa's group showed the existence of a GABAergic break on gonadotropin secretion during the juvenile period. GABA level in the pituitary stalk (PS) and ME of juvenile monkeys is high but decreases during the peripubertal period (Mitsushima *et al*., 1994; Terasawa, 2005). The local infusion of GABA-A receptor antagonist bicuculline in the PS-ME of juvenile female rhesus monkey induces a rise in gonadotropins levels and the onset of ovarian cyclicity (Keen *et al*., 2011). The infusion of anti-sens mRNA encoding GAD67 (glutamic acid decarboxylase 67), a key enzyme for the synthesis of GABA, in juvenile rhesus monkey females triggered puberty onset with estrus cyclicity and ovulation (Kasuya *et al*., 1999; Fig. 3).

Both mechanisms co-exist to a different degree according to the species considered and also to the sex. One theoretical hypothesis would be that the steroid-independent mechanism provides a coarse regulation and will program the year (month) of puberty onset and the steroid-dependent mechanism will program the week/day when the first ovulation occurs.

Excitatory mechanisms

The pubertal reduction in GABAergic inhibition is accompanied by an increase in glutamate levels in the PS-ME, as well as an increase in the levels of the stimulatory neurotransmitters such as noradrenaline and Neuropeptide Y (NPY; Gore and Terasawa, 1991).

Neuropeptide Y

NPY is an appetite-stimulating neuropeptide and a neuromodulator of neuroendocrine functions. The interactions between NPY and neuroendocrine networks are complex and depend upon the sex and steroid environments. For example NPY is a potent stimulator of LH secretion in sex-steroid primed rats (Allen *et al*., 1985), whereas its intra-cerebroventricular (ICV) administration in gonadectomized rats inhibits LH release (McDonald *et al*., 1989). In the male Rhesus monkey, NPY exerts a negative effect on the GnRH pulse generator in prepubertal animals (Majdoubi *et al*., 2000). However in the female Rhesus monkey NPY release in the ME increases and is responsible for the observed increase in LH secretion at puberty onset (Gore *et al*., 1993). Two populations of NPY containing neurons have been described in the ArcN and the authors suggest that these two populations have distinct roles during the prepubertal period and at puberty onset (Majdoubi *et al*., 2000; Fig. 3). In the prepubertal ewe, NPY stimulates the expression of *Lhb* (β-LH subunit) in gonadotrope cells (Wańkowska and Polkowska, 2009). Neuroanatomical studies in prepubertal ewes demonstrate the presence of NPY inputs on Kp neurons in the ArcN (Polkowska *et al*., 2014) and on GnRH neurons in the POA (Norgren and Lehman, 1989; Tillet *et al*., 1989), thus suggesting two distinct pathways that can be involved in the stimulatory effect of NPY. The existence of 5 NPY receptors subtypes coupled to various signaling pathways and the existence of different hypothalamic and pituitary targets, can account for such opposite effects observed according to the sex, the steroid environment and the physiological state (Pralong, 2010).

Glutamate/NMDA

The excitatory amino acid glutamate and especially its NMDA subtype receptor are important components of the neural system that regulates sexual maturation. Multiple daily injections of NMDA agonists to immature rats (Smyth and Wilkinson, 1994) and monkeys (Urbanski and Ojeda, 1990) induce precocious puberty. On the contrary, administration of the non-competitive NMDA antagonist, MK801, delays puberty onset (Veneroni *et al*., 1990). GnRH neurons receive direct glutamatergic inputs and express NMDA and kainite receptors (Fig. 3). Hypothalamic glutamate contents increase during the prepubertal period and reach maximal values at puberty onset. Glutamate

receptors are ubiquitous in the CNS and they play important roles in many processes involving excitatory mechanisms, whether this increase in glutamatergic signaling is specific to puberty onset or whether it's a more general developmental process is not known (Parent *et al*., 2005).

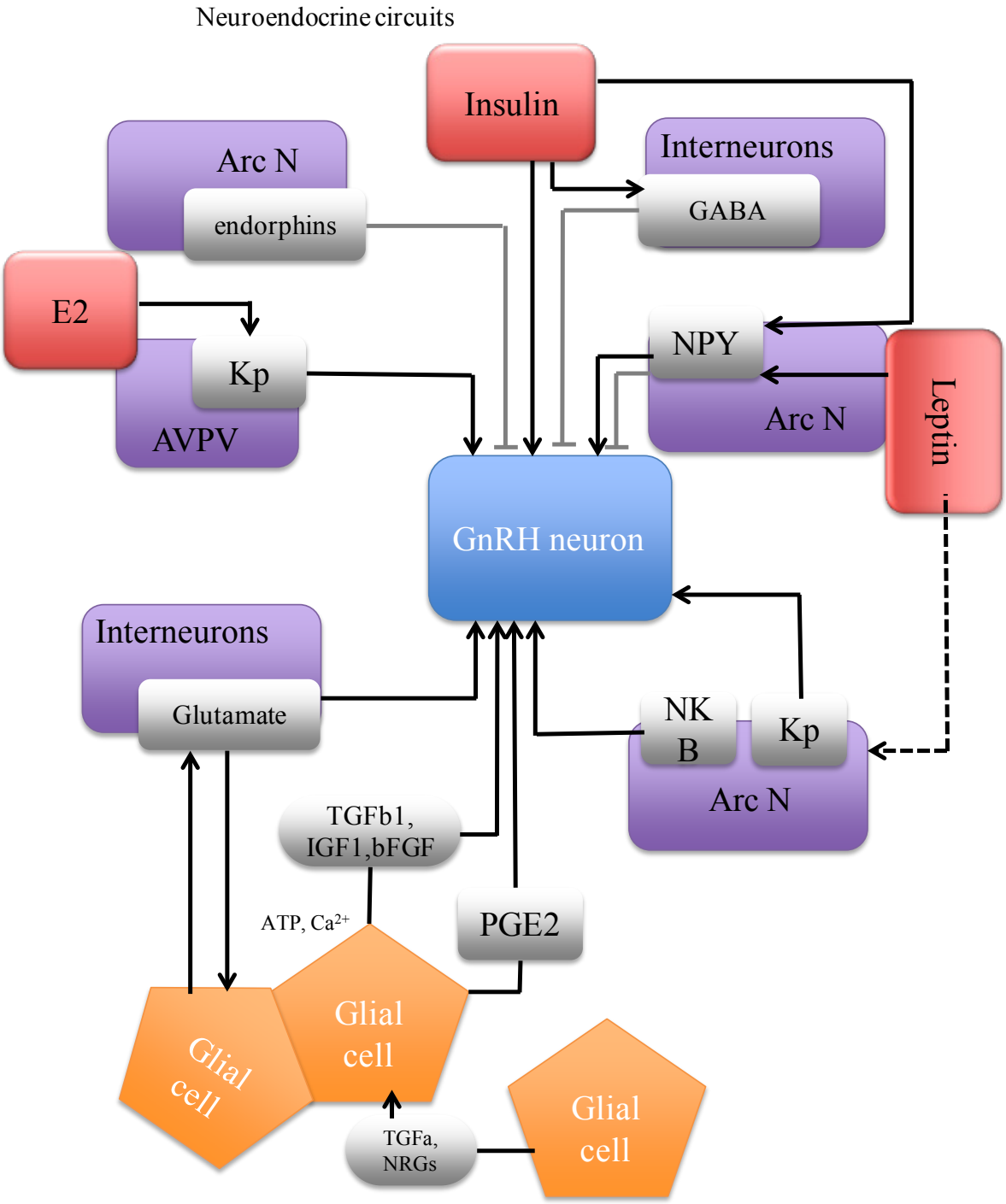

Figure 3. Schematic representation of neuroendocrine circuits. GnRH neurons receive inputs from Kp neurons located in the AVPV and ArcN, NKB neurons located in the ArcN , endorphin neurons located in the ArcN , GABA and glutamate from interneurons (grey boxes = neurotransmitters, purple boxes = neuroanatomical structure). Kp neurons, NPY neurons and GABA neurons are sensitive to E2, leptin and insulin respectively (hormones = red boxes). Glial cells (astrocytes and tanycytes, orange pentagons) in the microenvironment of GnRH neurons can release glutamate, growth factors such as TGFβ1, bFGF that stimulates the activity of GnRH neurons. Glial cells can also uptake glutamate from extracellular space. Glial cells also release neuregulins (NRGs) and TGFα, stimulating the release of PGE2 by neighboring glial cells, which stimulates GnRH neurons. Glial cells also release Ca^{2+}, ATP that regulate GnRH neurons' activity.

Kisspetin/GPR54

In 2004, a new key component was discovered, originally named metastin due to its anti-mitotic properties and now named Kisspeptin (Kp; Matsui *et al*., 2004; Seminara, 2005). *Kiss1* encodes a 54 amino-acids peptide Kp-54 (Kisspeptin-54) that cleaves into several shorter forms (Kp14, Kp13 and Kp10) forming the Kp family. Kp neurons strongly regulate the activity of GnRH neurons. Kp acts through a G-protein coupled receptor (GPCR): GPR54. Kp neurons are found in two distinct populations: ArcN and anteroventral periventricular nucleus (AVPV; Fig. 3). GnRH neurons express GPR54 and Kp fibers contact GnRH terminals in the ME. In humans, mutations in the GPR54 gene lead to an hyponadotropic hypogonadism (HH) characterized by a deficiency in pituitary secretion of gonadotropins which results in the impairment of pubertal maturation and of reproductive function (de Roux *et al*., 2003). Genetic models in rodents highlighted the central role of Kp/GPR54 system in the onset of puberty (Colledge and de Tassigny, 2009; Fig. 3). The Kp/GPR54 system is strongly regulated by metabolic factors and environmental factors, and could represent the central hub for decoding metabolic and environmental cues.

Glial regulation

When speaking of neuroendocrine regulations, most scientists focus on the roles played by neuronal circuits, neurotransmitters and neuromodulators and their cognate receptors. During the prepubertal period, although neuronal networks synaptically-connected to GnRH neurons govern the increase in GnRH secretion; glial cells contribute to the processes engaged through several mechanisms. Glial is a generic adjective to characterize several cell populations that are associated with GnRH neurons: astrocytes, tanycytes and olfactory unsheathing cells. For the sake of simplicity, we use the generic term, bearing in mind that different phenotypic cell types support it. One mechanism involves the production of growth factors acting on serine/threonine kinase receptors. Growth factors such as Transforming Growth Factor α (TGFα) and neuregulins acting on erbB receptors play a major role in glia-GnRH neurons communication. Activation of erbB receptors in glial cells associated with GnRH neurons, leads to the release of prostaglandin E2 (PGE2), which stimulates the electrical activity of GnRH neurons and the GnRH release (Prevot *et al*., 2003a, b, 2005; Ojeda *et al*., 2008). Other growth factors such as TGFβ, IGF1, bFGF are secreted by glial cells and regulate directly the activity of GnRH neurons (Ojeda *et al*., 2010; Fig. 3). Besides the secretion of growth factors, glial cells release small molecules such as calcium, glutamate and ATP that affect the GnRH neuronal activity. Glial cells can also uptake K^+ ions and glutamate that accumulate in the extracellular space during neuronal activity through glial specific dedicated transporters. These mechanisms are of major importance in regulating neuronal electrical activity and excitability. These mechanisms of regulation are tightly dependent upon the distance between the membrane of the glial and the synaptic cleft (Giaume *et al*., 2010).

Another mechanism that can affect glia-GnRH neurons interactions is the modulation of adhesiveness of glial cells onto GnRH neurons. Glial cells interact with GnRH neurons via homophilic interactions involving Neural Cell Adhesion Molecule (NCAM) and synaptic cell adhesion molecule (SynCAM1). In contrast, the poly-syalilated form of NCAM, PSA-NCAM, prevents hemophilic interactions between adjacent glial and GnRH neuronal cells. Heterophilic interactions also exist via the neuronal membrane protein contactin and the glial receptor like protein tyrosine phosphatase-b (Parent *et al*., 2007). These cell-to-cell interactions can trigger intracellular signaling cascades that can affect both glial and neuronal activities (Viguie *et al*., 2001; Parkash and Kaur, 2007; Sharif *et al*., 2013). Altering cell-to-cell communication through glial gap junctions or hemichannels decreases dramatically GnRH neuronal activity and GnRH secretion *in vitro* (Pinet-Charvet *et al*., 2015). Gap-junctions have previously been reported in the hypothalamus, particularly in the ArcN of female rats, where they are regulated by estrogen (Perez *et al*., 1990). Hypothalamic tanycytes, particularly the β-type which is closely associated with GnRH nerve terminals in the ME, express functional connexin-43 (Cx-43) hemichannels encoded by *Gja1*, which play a role in a glucose-sensing mechanism by releasing ATP (Orellana *et al*., 2012). The *Gja1* (Cx-43) promoting region contains AP1 and AP2 sites and a series of half palindromic estrogen response elements suggesting that Cx-43 (*Gja1*) expression can be directly regulated by estrogen levels (Yu *et al*., 1994). Taken altogether, these studies suggest that glial cells might exert a control of GnRH neuronal network as important as the classical transynaptic model.

Therefore, several layers of neuronal and glial components are involved in controlling the onset of puberty, increasing the complexity of the system. The most important question remains: what determines the timing of the inhibitory break removal and/or the timing on excitatory inputs onset?

Puberty: environmental cues

The timing of puberty is maybe the best example of the interaction between genotype and environment. Puberty is a physiological event integrating several important central regulations at the crossroad of adaptation to environment: reproductive axis, feeding behaviour and nutritional controls, growth, seasonal rhythm, corticotropic axis and stress.

Nutrition and metabolism

Nutritional factors have been considered for a long time as the key factor in puberty onset. In humans, until the mid-20th century, a gradual decline in age at menarche (first menstruation) has been reported in most industrialized populations. It is generally admitted that

this trend was due to gradual improvements in nutrition and healthcare (Sørensen *et al*., 2012) giving birth in the 70ies to the critical fat mass hypothesis according which, for a given species a critical fat mass is necessary for puberty onset. The link between nutrition and puberty onset was confirmed in numerous studies on laboratory animals, and also in farm animals. Adequate growth and adiposity are critical for the onset of puberty in mammals. Food restriction (Foster and Olster, 1984; Suttie *et al*., 1991) and excessive exercise during the juvenile period delay the onset of puberty (Manning and Bronson, 1989, 1991). The mechanism involved is the maintenance of the juvenile high sensitivity to the negative feedback sensitivity to gonadal steroids. In contrast, increased adiposity advance the onset of puberty (Kaplowitz *et al*., 2001; Rosales Nieto *et al*., 2014). This occurrence is associated with attenuation of estradiol negative feedback and increased pulsatile release of LH (Gasser, 2006). Therefore, nutritional cues interact with gonadal steroid feedback to time the onset of puberty in females. These findings led to the concept of nutritional programming of puberty in cattle. Age at puberty in cattle is indeed influenced by food intake, food composition and body weight (BW). It is usually admit that puberty occurs at 55-65% of adult BW, depending on the breed considered (Freetly *et al*., 2011). However, the cost of supplemental feeding to reach this target BW earlier is not always compensated by a sufficient improve in reproduction and calf production (Davis Rincker *et al*., 2011). The permissive nutritional signals for puberty onset are metabolic cues such as glucose, insulin and leptin for the most studied factors. These metabolic markers signal the brain that the somatic growth and energy stores are sufficient to sustain pregnancy and lactation without threating the mother and foetus' health. Interestingly, these factors are also important for males, although the mechanisms involved may differ. Since the discovery of the fat-signalling hormone leptin (Zhang *et al*., 1994), whose blood level is proportional to the amount of adipose tissue (Frederich *et al*., 1995), a great amount of research work has tried to demonstrate that leptin is a hormonal messenger signalling the metabolic state for initiating puberty and also for fertility. Studies performed in rodents suggested that leptin administration could advance the onset of female puberty (Ahima *et al*., 1997). Humans with leptin deficiency due to mutations in the leptin gene or in the leptin receptor, and mouse models with inactivated leptin gene or leptin receptor gene, are obese and do not undergo puberty (Chehab *et al*., 1996). However leptin administration in healthy juveniles does not advance puberty onset. In ewes (Henry *et al*., 2011) and cows (Amstalden *et al*., 2002) leptin administration does not affect the secretion of LH but leptin prevents fasting-induced reduction in LH pulsatility in prepuberal heifers (Maciel, 2004). In addition to leptin, other hormones such as insulin, or nutrients such as glucose, fatty acids and amino-acids have been shown to regulate GnRH neuronal activity in a direct manner or via a complex glial/neuronal network. Among the critical neuronal pathways, hypothalamic NPY/agouti-related protein (AgRP) and proopiomelanocortin (POMC) neurons located in the ArcN are considered as the two major pathways mediating nutritional cues. Theses neurons express the leptin receptor and target GnRH neurons, setting the physical pathway for the control of puberty onset. A small subpopulation of Kp neurons in the ArcN express LepR (Louis *et al*., 2011) and may constitute another target for nutritional regulation see Sánchez-Garrido and Tena-Sempere (2013) for a review. However selective ablation of LepR in Kiss1 expressing neurons does not alter puberty onset and fertility (Donato *et al*., 2011).

Taken altogether, these studies support a permissive role of leptin in the metabolic gating of pubertal maturation (Barash *et al*., 1996; Cheung *et al*., 1997).

Photoperiod

In photoperiodic species, puberty onset will depend on the timing of the birth. For example in the ovine species lambs born at the end of the winter or during spring time reach puberty at the next breeding season in autumn, a younger age that those born during autumn, reaching puberty at the following breeding season 10-12 months later. This delay in puberty in autumn-born ewe lambs is due to a prolonged hypersensitivity to the negative steroid feedback (Foster and Hileman, 2015). Similar findings were observed for photoperiodic short-lived animals such as Siberian hamsters where spring born individuals mature rapidly and breed during the summer whereas young born in lid to late summer have a delayed puberty the next spring (Butler *et al*., 2007). Exposing Holstein heifers to long day photoperiod enhance BW gain and hasten the onset of puberty (Rius *et al*., 2005), a result that has been observed also for the seasonal Murrah buffalo species (Roy *et al*., 2016). In photoperiodic species, the variation in food intake and metabolism is an adaptive physiological mechanism allowing the storage of energy resources in anticipation of the harsh days of winter. The immune response is also sensitive to photoperiod, short days photoperiod enhance immunological defenses. This seasonal plasticity of the immune system is highly conserved and is in opposite phase with the breeding season, one explanation would be that the energy cost of both activating reproduction and maintaining the immune function at its higher level is too high (Walton *et al*., 2011). In dairy cows, short days photoperiod improve mammary gland capacity, prolactin secretion and immune function (Dahl, 2008).

Stress and corticotropic axis

Prolonged or chronic stress results in the suppression of gonadotropin secretion and the inhibition of reproduction. Acute stress has variable effects (Tilbrook *et al*., 2000). Studies on adaptive response processes highlighted a positive link between childhood adversities with accelerated female reproductive development. Longer-term health costs are traded off for increased probability of reproducing before dying

via a process of accelerated reproductive maturation. Early adversity, early sexual maturation form the core component linking stress physiology with poor health later in life (Hochberg and Belsky, 2013).

Puberty: genetic determinants

While the timing of pubertal onset varies within and between different populations, it is a highly heritable trait, suggesting strong genetic determinants. Previous epidemiological studies estimate that 60-80% of the variation in pubertal onset is under genetic regulation (Parent *et al*., 2003; Gajdos *et al*., 2010). Abnormal pubertal timing affects up to 5% of adolescents and is associated with adverse health and psychosocial outcomes.

Genetic factors associated with delay of puberty in Humans

Idiopathic hypogonadotropic hypogonadism (IHH) is defined by absent or delayed sexual development, with puberty being either absent or incomplete by the age of 18 years. Deleterious mutations in genes coding for factors necessary for the migration of GnRH neurons lead to hypogonadotropic hypogonadism (IHH), which is the absence of puberty associated with low levels of gonadotropins and gonadal steroids. IHH is frequently accompanied by non-reproductive abnormalities such as anosmia (Kallmann's syndrome). In the Kallmann's syndrome, which associates IHH and anosmia, mutated genes encode for proteins involved in the development of GnRH neurons (Franco *et al*., 1991; Hardelin *et al*., 1992, 1991). The disruption of the migration of GnRH neurons causes them to stay into the nasal region or at the level of the cribriform plate, and they do not reach their final location in the hypothalamus. The Kallmann's syndrome is associated to mutations in *KAL1 , FGFR1* (Dodé *et al*., 2003)*, NELF* (Miura *et al*., 2004; Xu *et al*., 2011)*, PROKR2* (Dodé *et al*., 2006)*, FGF8* (Hardelin and Dodé, 2008)*, CHD7* (Kim *et al*., 2008)*,* and *WDR11* (Kim and Layman, 2011) genes encoding for anosmin, FGF receptor 1 (FGF-R1), NMDA receptor synaptonuclear signaling and neuronal migration factor (alias Nasal Embryonic Factor), prokinecitin receptor 2, FGF-8, chromodomain helicase binding protein 7, WD repeat domain 11, respectively (Fig. 4). In normosmic IHH (nIHH), the development of GnRH neurons is not affected but the functionality of the GnRH secretion is altered. n-IHH cases are associated with mutations in *GNRH* (Chevrier *et al*., 2011), *KISS1* (de Roux *et al*., 2003; Bianco *et al*., 2011), *DAX1* (Habiby *et al*., 1996; Merke *et al*., 1999), *GNRH1* (Bouligand *et al*., 2009; Chan *et al*., 2011), *LEPR*/*LEP* (Clement *et al*., 1998), PCSK1 (Jackson *et al*., 2003), *PROKR2/ PROK2* (Dodé *et al*., 2006), *SEMA3A/ SEMA7A* (Hanchate *et al*., 2012; Young *et al*., 2012), *TACR3/ TAC3* (Topaloglu *et al*., 2009; Topaloglu, 2010), *DMLX2* (Tata *et al*., 2014) genes encoding GnRH-R, GPR54, nuclear receptor 0B1, GnRH, Leptin-R, leptin, protein convertase subtilisin/kexin type 1, prokinecitin receptor 2, prokinecitin, neurokinin-B receptor, semaphorins-3a and -7a, neurokinin-B and Rab-connectin-3, respectively (Fig. 4). Most cases of IHH are sporadic, consistent with the affected individuals being infertile, but familial transmission has also been well described. Kindred analysis suggests that IHH is a wider spectrum of disease with individuals and relatives sharing an apparent common genotype but displaying a variety of reproductive or non-reproductive phenotypes. Oligogenicity could be one explanation for this phenotypic variation (Mitchell *et al*., 2011).

Oligogenic and complex genetic environmental interactions have now been identified, with physiological and environmental factors interacting in genetically susceptible individuals to alter their reproductive capacities.

Genetic factors associated with precocious puberty in Humans

Human precocious puberty is defined as the development of secondary sexual characteristics and elevated sexual hormones before 8 years of age in girls and 9 years of age in boys. There are two major forms of premature sexual maturation: inappropriate early activation of HPG axis that induces central precocious puberty (CPP) and peripheral precocious puberty (PPP) due to the increase of sex steroids with no activation of the HPG axis. Precocious puberty is highly deleterious since it will cause short stature, psychosocial problems and increase the risk of adulthood diseases.

Mutations in the *LHCGR* gene coding the LH receptor (LH-R) and leading to constitutive activation of the LH-R without ligand were the first mutations characterized in various family cases of peripheral precocious puberty limited to the male (Layman, 1999). These mutations affected only the male offspring and were without effect on the females. Recently cases of central precocious puberty have been associated with genetic variants affecting Kp signalling: mutation in the *KISS1* gene encoding Kp (Silveira *et al*., 2010; Mazaheri *et al*., 2015) or activating mutation of the *KISS1R* gene encoding GPR54 the Kp receptor (Teles *et al*., 2008; Silveira *et al*., 2010; Fig. 4). One of these mutations was present at heterozygous state in patient's mother and grandmother suggesting incomplete sex-dependent penetrance. Another possibility is that other genes could be involved in this phenotype evoking the oligogenicity concept in central precocious puberty as was well described for IHH (Mitchell *et al*., 2011).

Other cases of central precocious puberty are associated with mutations in the imprinted *MKRN3* gene encoding the makorin ring finger protein 3, a gene located in the imprinted Prader Willi syndrome region (Settas *et al*., 2014; Simon *et al*., 2015; Fig. 4). Data from Human cases and animal models suggests that *MKRN3* plays an inhibitory role in the reproductive axis and may represent a new pathway in pubertal regulation (Ong *et al*., 2009; Simon *et al*., 2015). MKNR3 is expressed ubiquitously.

Before 2000, clinical studies were individual case studies but now with the improvement of the

methods of sequencing of the genome, the increase of the capacities of calculation and the improvement of the algorithms, the studies of association of genomic data allow to find genetic variants associated to the age in the puberty. With this process, more than 100 loci involved in the susceptibility to precocious puberty have been discovered. Among them the *LIN28B* locus is one of the most significant (Ong *et al*., 2009; Elks *et al*., 2010; Fig. 4). *LIN28B* is a human homolog of *lin28* of *Caenorhabtidis elegans*, which was originally identified as a heterochronic regulator of developmental timing (Ambros and Horvitz, 1984) Deleterious mutations in *lin28* resulted in precocious larval to adult development and a partial transformation in sexual phenotype (Ambros, 2011). The Lin28 proteins are potent and specific post-transcriptional repressors of the biogenesis of let-7 miRNAs, which are time-specific expressed miRNAs that control developmental timing (Zhu *et al*., 2010).

A recent meta-analysis suggests that the variant allele carriers, especially people with heterozygote genotype for *ESR1* XbaI polymorphism and the wild allele for *ESR1 PvuII* polymorphism, are associated with precocious puberty susceptibility (Luo *et al*., 2015; Fig. 4).

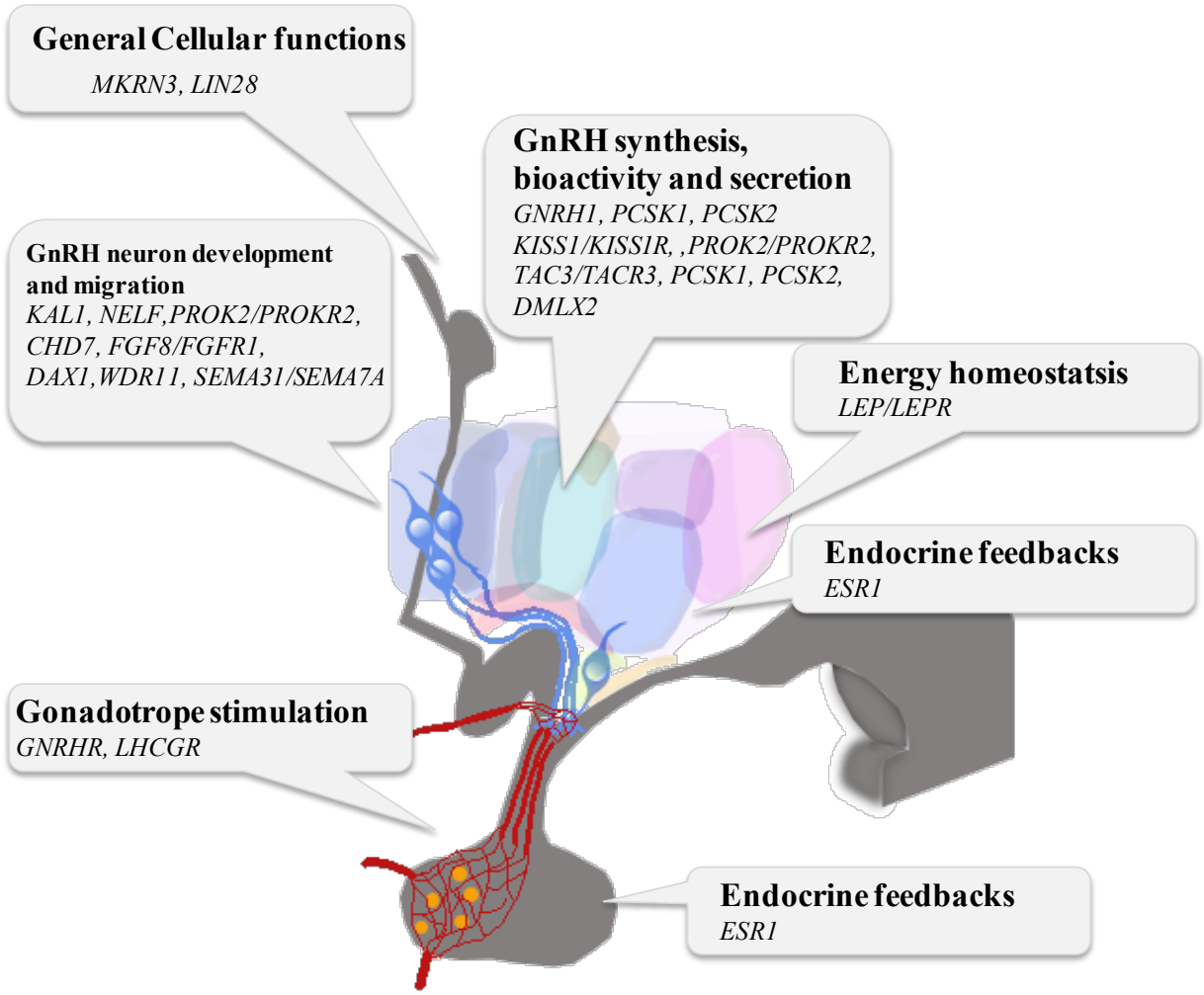

Figure 4. Genetic factors associated with pathological puberty delay or advance in humans. This figure summarizes how genetic factors associated with pathological conditions in humans are affecting cellular processes at hypothalamic and pituitary levels. From general cellular function GnRH neuron development and migration, GnRH synthesis, bioactivity and secretion, energy homeostasis, gonadotrope stimulation and endocrine feedbacks.

Genetic factors associated with age at puberty in cattle

Age at first calving usually varied between 24 and 36 months, according to cattle breeds and is considered a key factor in terms of profitability and efficiency in both dairy and beef cattle. Likewise, bull puberty also shows significant differences within and among breeds. In dairy cattle, age at first has continually decreased during the last decades. Improvement in nutrition and health have certainly contributed to an improve BW gain, but genetic selection for improved breeding and economic efficiency may also have indirectly impacted the onset of puberty (precocity; Mourits *et al*., 2000). Indeed, comparison of performances of 1970s and 1990s heifers from the same breed in New Zealand showed that modern heifers reached puberty at an earlier age than their predecessors, with a higher body weight than 20

years ago, meaning that mature size is different (Macdonald *et al*., 2007). As first calving at 24 months of age is becoming a common and general goal, one can safely assume that first-calving age will continue to decrease in the short term (Le Cozler *et al*., 2008).

Despite its economic importance, only a few studies have been conducted to identify genes and mutations associated with onset of puberty in either bulls or heifers. Most of these studies were done in beef cattle (mainly Angus), tropical breeds such as Brahman and Nelore cattle (*Bos indicus* cattle) and crosses which are reportedly older at puberty when compared with most *Bos taurus* breeds (Lunstra and Cundiff, 2003). Several parameters have been measured as a phenotype to study heifer puberty, from simple traits such as age at first service, age at first calving and age at first oestrus to more expensive and difficult to measure ones such as age at first *corpus luteum* (ultrasonography) or plasma progesterone concentration. For males, scrotal circumference, sperm quality (concentration, motility and morphology) as well as LH or IGF-1 circulating blood concentration have been monitored. One has to be aware that the nature of the quantitative puberty traits thus differs between studies. Moreover, their physiological meaning might be different than the strictly defined puberty onset. For example age at first oestrus does not mean age at puberty onset since oestrus behaviour is usually not present before the third oestrus cycle. Age at first calving is not age at first oestrus since the genital tract need several oestrus cycles to be fully developed in order to insure a full-length pregnancy.

Moderate to high heritability has been computed for heifer's age at puberty (0.2 to 0.48) and scrotal circumference (0.22 to 0.42; Vargas *et al*., 1998), suggesting that timing of puberty is likely to be a multigenic trait. Genetic correlations have also been observed between scrotal circumference or male IGF-1 blood concentration and heifer's age at puberty, suggesting that some common pathways may be involved in the two genders (Martinez-Velazquez and Gregory, 2003; Morris *et al*., 2010; Johnston *et al*., 2013).

Despite the multigenic nature of puberty onset, some major key player genes have been identified in humans, stimulating association studies in cattle, focused on some candidate genes. Polymorphisms in *GNRHR*, *LHR* and *IGF* were search for association with age of puberty in Angus male cattle (Lirón *et al*., 2012), showing significant association with one SNP located in IGF1. Likewise, polymorphisms in the *LHR*, *FSHR* and *GNRHR* were analysed in the Nellore breed, showing only association between *FSHR* and early puberty phenotype (Milazzotto *et al*., 2008). Furthermore, seven genes from the IGF1 pathway (*IGF1R, IGFBP2, IGFBP4, EIF2AK3, PIK3R1, GSK3B* and *IRS1*) were shown to be associated with heifer puberty in both Tropical Composite or Brahman breeds (Fortes *et al*., 2013). These findings support the hypothesis that IGF1 regulates arrival to puberty in male calves and also impact heifer puberty. In contrast to human and mouse, there are no evidences that genetic variation within *GNRH, LH* and its receptors could impact the regulation of pubertal timing in cattle. Based on their known effect on sexual precocity in mammals, 57 candidate genes related to lipid metabolism were also studied on a large panel of 1689 precocious and non-precocious Nellore heifers. Statistical analysis revealed that SNPs located within the *FABP4* and *PPP3CA* gene had a significant effect on sexual precocity (Dias *et al*., 2015).

Genome-wide association studies (GWAS) using microsatellites or SNPs have also been set up to identify QTL regions and highlight to new candidate genes. A search for markers associated with heifer's age at puberty and age at first calving in the Animal QTLdb (Hu *et al*., 2016) retrieves about 350 markers located within roughly 200 QTL regions, irrespective to breeds. Likewise, 10650 makers within 60 regions have been associated with male puberty, mainly on the X chromosome. Several candidate genes have been proposed starting from these regions and regulatory networks have been constructed (Fortes *et al*., 2010a, b, 2011, 2016). These findings suggest an enrichment of genes involved in axon guidance, cell adhesion, ErbB signaling, and glutamate activity, pathways that are known to affect pulsatile release of GnRH, which is necessary for the onset of puberty. In addition several TF were proposed as regulator of heifer's puberty, including *ESRRG, PPARG, HIVEP3, TOX, EYA1, NCOA2*, and *ZFHX4*. Combining GWAS and expression analysis in a multi-tissue omics also identified several key transcriptional regulators such as *PITX2, FOXA1, DACH2, PROP1, SIX6* … (Canovas *et al*., 2014). U6 spliceosomal RNA was also proposed as a positional candidate gene associated with age at first calving (Nascimento *et al*., 2016).

Interestingly, only a few common genes can be identified between genes located within QTL associated with either heifer's or bull puberty and genes already known in human to be involved in puberty onset: *HDAC8* and *NR0B1* may play a role in male puberty, whereas *CHST8, GABRA1, LEP* and *PROP1* may influence female puberty (Fig. 5). This finding suggests that cattle could provide new insight into the genetic basis of puberty in mammals. Consistent with the hypothesis of common pathways between genders, 16 common genes can be identified within heifer and bull QTL regions: *ARL2, CAPN1, CDC42EP2, DPF2, FRMD8, MRPL49, PARPBP, POLA2, SAC3D1, Slc22a20, SNX15, SPDYC, TIGD3 , TM7SF2 , VPS51, ZFPL1*.

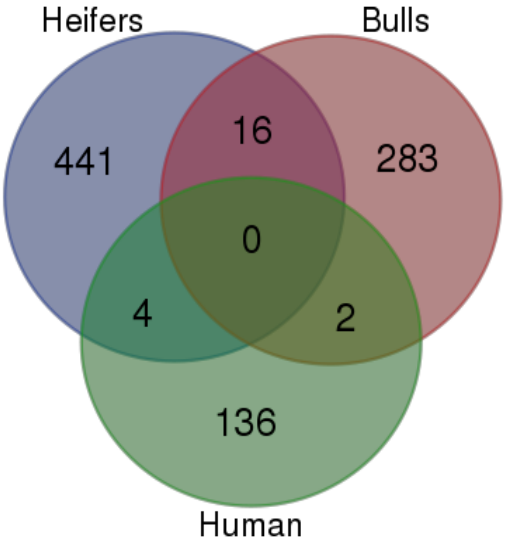

Figure 5. Only a few genes known to be associated with puberty onset in human are also located within cattle QTL regions. Cattle QTL regions were identified using the Animal QTLdb, taking into account "Age at first calving" for females and "Scrotal circumference" for males. Regions associated with "Age at puberty" were spread over female or male according to the experiment. QTL regions were defined as the critical mapping interval for linkage studies or a 500kb interval centered on the most significant marker for GWAS studies. Ensembl database was used to list genes located within these intervals and OMIM was used to establish a list of genes associated with puberty in Human. The Venn diagram presents the number of common genes between these lists, showing a limited number of common QTL and genes involved in bull's and heifer's puberty and only a few human candidate genes located within cattle QTL regions.

How to get good oocytes at younger age?

The overall goal of a replacement heifer program is to rear heifers to reach a desired age and body weight early so that they initiate puberty, establish pregnancy, and calve easily at a minimal cost. In addition to the investment needed to raise heifers from birth to calving, heifers that calve earlier spend a greater proportion of their life producing milk, and therefore returning profit to a dairy, whereas heifers that calve later spend more time in a non-productive period before initiation of lactation. The development of replacement heifers is a major economic investment for all beef and dairy operations. The costs associated with heifer development cannot be recovered if heifers do not conceive and remain productive in the herd; therefore, heifers need to conceive early in the breeding season or risk being culled. Breeders can use various levers to meet these objectives.

Advancing puberty

Feeding and photoperiod (ovine species) were the two main levers used by farmers to advance puberty. Young juvenile heifers fed with high-concentrate diet have a better weight gain and an advanced puberty onset compared to control heifers. The timing of this nutritional support is important, there is a developmental window during the early juvenile period (between 4-6.5 months) during which, high-concentrate diet will be effective on the timing of puberty onset. Feed restriction after this point will have little effect on the timing of puberty (Cardoso *et al*., 2015). One could imagine that the qualitative nutritional value and the timing of nutritional programming are of importance and should benefit from a research effort in this field.

Although this is not recommended by Europe, hormonal treatment can be used to advance puberty onset. Hormonal treatments are efficient to advance the first ovulation when administrated during the late juvenile period (8-10 months) in pre-pubertal heifers. They involve the administration of GnRH agonists or hCG (human chorionic gonatodotropin). The GnRH agonist Buserelin acetate is commonly used for oestrus synchronization or for treating post-partum anoestrus in adult females. Continuous infusion of GnRH or GnRH agonist (Deslorelin) using sub-cutaneaous implants or minipumps to 8-10 months' old heifers stimulate LH secretion and induce ovulation 30-48 h after the placement of the implant (Dodson *et al*., 1990; Grasselli *et al*., 1993). However luteinisation and the production of progesterone are not consistently observed and this may cause short luteal phases. The continuous exposure to GnRH or GnRH agonists induces the desensitization of the GnRH-R signalling. After GnRH agonist implants removal, the animals do not respond to exogenous GnRH treatment for 12 days (Bergfeld *et al*., 1996). For these reasons, hCG is usually preferred. hCG will mimic the effect of endogenous LH surge and stimulate the ovulation of the dominant follicle. The luteotropic effect of hCG guarantees the formation of a functional *corpus luteum* and will have a beneficial effect on the initiation of pregnancy. Its major side effect is that hCG is a human hormone and as such its repeated administration causes the development of an acquired immunity that impedes future treatments to be efficient (De Rensis *et al*., 2010; Dahlen *et al*., 2011). Both GnRH agonists- and hCG-based treatments rely on peptidic or proteic

substances that are not an environmental issue. In contrast to oestradiol- and progesterone- based hormonal treatments that have been used in the past in Europe or are still in use on the American, Asian and Australian continents. It would be interesting to test for Kp long life agonists that have been developed for the ovine species to see whether they could offer a more physiological activation of the central GnRH controlling system and thus avoiding the desensitization of GnRH-R signalling (Beltramo *et al*., 2015).

Collecting prepubertal oocytes

Another strategy is to overcome these problems by using *in vitro* production techniques and oocytes collection by Ovum Pick-Up (OPU) techniques. Despite the fact that large follicles are present before puberty, that good quality oocytes evaluated by the presence of compact cumulus can be collected by OPU, that the proportion of cleavages up to 8 cells after *in vitro* fertilization is correct, the rate of blastocysts obtained is low and their ability to produce successful pregnancy after embryo transfer is poor in comparison to data obtained from adult oocytes (Armstrong *et al*., 1992; Levesque and Sirard, 1994; Majerus *et al*., 1999; Landry *et al*., 2016). Ovarian stimulation using FSH can improve the rate of blastocyst formation, underlining the importance of hormonal environment to insure the oocyte competency to sustain development (Khatir *et al*., 1996). Different factors have been studied and sustain the cytoplasmic immaturity of prepubertal oocytes (Gandolfi *et al*., 1998; Oropeza *et al*., 2004; Bernal-Ulloa *et al*., 2016). Gene expression in blastocyst embryos relies mostly on post-transcriptional control of maternal transcripts accumulated during oocyte maturation. In calf oocytes, the expression of maternal transcripts differs from that of adult oocytes. Transcripts of PRDX2 and PRDX1 genes are in less quantities in oocytes collected from prepubertal animals in comparison to adult animals (Romar *et al*., 2011).

Conclusions

With the introduction of genomic selection 15 years ago, international agricultural politics have started to modify selection strategies, which now include puberty traits in order to advance puberty onset with the objective of reducing generation intervals. The selective pressure on onset of puberty will undoubtedly increase in a near future. Indeed, advances in molecular genetics have now made it possible to predict the total genetic value of animals by using genome-wide dense marker maps leading to the forthcoming of Genomic Selection (GS; Humblot *et al*., 2010). GS is of particular interest in cattle since the generation interval is long, artificial insemination bulls should be tested on their progeny before dissemination and some important traits such as fertility have a low heritability, due probably to a great sensitivity to environmental factors. Yearling bulls that have genomic breeding values information but lack phenotypic data on their daughters are often referred to as “genomic bulls”. There has been an immense shift among the AI companies toward the use of genomic bulls in the past 3 years. Some AI companies use almost all genomic bulls as sires of sons, whereas other companies use a combination of genomic bulls and progeny-tested bulls (Schefers and Weigel, 2012). Instead of waiting a minimum of 4.5 years to use progeny-tested bulls as sires of sons, AI companies could now use the best DNA-tested young bulls by roughly 1 year of age. Due to economical constrains, AI companies are now looking for animals having an advancement of their puberty.

It’s therefore of major importance to understand the link between these phenotypic changes, genetic determinants and environment. Indeed, GS de facto reduces the interval of generation and will speed up the selection process. This could be a great opportunity but may also increase the risk of disseminating unsuitable traits by lack of knowledge of their related pathways. Therefore, before implementing GS for QTL associated with puberty traits, it’s crucial to evaluate whether or not this selection process may affect other reproductive characteristics or reduce the robustness and increase vulnerability to environmental changes. There is clearly a need for basic research on factors that control puberty in order to improve heifer development and fertility (Perry, 2016) and address the question of robustness.

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Circles around the farm animal embryo – a Danish perspective

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Abstract

With focus on the farm animal embryo, a short overview is given about my research activities over the last 35 years. These activities have been described in five circles, covering different key aspects of my work. The first circle included studies on the basic biology related to oocyte maturation and follicular endocrinology in superovulated dairy cows. Methods were developed to characterize the donor cows with respect to their production of transferable embryos, and some were implemented into a Danish MOET breeding plan. The second circle dealt with in-vitro embryo production in cattle with development of a protocol to produce such embryos at high and consistent levels. Several comparisons were made to reveal consequences of the artificial in-vitro methods on oocytes and embryos, but also through studies of the newborn calves. The third circle was related to development and implementation of a number of technologies within this broad field; examples are mentioned for both oocyte recovery from donor cows, different steps in the in-vitro embryo production system, new ways to perform vitrification and nuclear transfer, and finally a new system to determine oxygen consumption in single embryos. In the fourth circle is described activities from the last years, where work was done with focus on the pregnancy rates after insemination of the dairy cow in their post-partum period, and where somatic cell nuclear transfer was developed both as a technology in itself as well as a helping technique to produce transgenic pigs as models for important human diseases. The fifth and final circle is addressing and thanking the many colleagues and collaboration partners that I have been involved with during all the years to do this work. Nothing could or would have been the same without them and their participation.

keywords: cattle, embryo technology, pig, reproductive biology, overview.

Introduction

My first AETE meeting was in 1987 in Lyon where the 3rd meeting was held. Since I have been at all but three of these annual highlights, and at every meeting I have been listening with interest to the distinguished scientists receiving the AETE award. Today, it is my privilege and honor to be that person, and since I was told this great news a year ago, I have gradually learned to use this opportunity and reflect over my scientific career in ways that I usually do not.

Having worked with farm animal embryos for more than 30 years gives many possibilities to do different things. My choice - to some extent also influenced by coincidences - has been to focus on the farm animal embryo in kind of circles around the days before, during and after fertilization, and I have always had Denmark as the center for my activities. Within those frames, most of my research interests have been divided between biology and technologies, from a basic to an applied aspect, and then using collaborations very much.

So, it has been fairly limited circles, made in a rather small country, and always with the embryo in focus. In the following, I will give a short overview of my scientific activities using some kind of a chronological approach. It will not be a real literature review, but a self-centered presentation. This is not often allowed, but on this special occasion I think it is. To prepare this has been interesting for me, but of course I hope that it will also be that for at least some of you.

The superovulated donor cow

From the beginning of the 1980ies, I started as Torben Greve's first PhD-student at the Royal Veterinary and Agricultural University in Copenhagen. He had been very much involved in establishing the basics on superovulation and embryo transfer in cattle, also in practice, but time had come to search for a better understanding of some of the reasons for the often varying and rather unpredictable results. Together with another PhD-student, Poul Hyttel, my first years in research were therefore focused on morphological and endocrine aspects of the preovulatory period in superovulated dairy cattle. What were the actions and consequences of the exogenous FSH treatment on the developing follicles and oocytes? Poul's focus was ultrastructure, while mine was the hormones and the more clinical and applied aspects.

Over several years we used more than 130 cows and heifers to study preovulatory oocyte morphology and follicular endocrinology and to describe their overall reaction patterns, and to relate these to the resulting oocyte and later embryo quality. Some of the main results were presented at my first IETS conference held in Colorado Springs in 1986 (Callesen *et al*., 1986), where characteristics of donors with good versus bad oocytes were presented. This was followed by several also more practice-related studies (e.g. Callesen *et al*., 1995) also to identify the good or bad donors. For this, donors were characterized through their patterns of progesterone or estradiol concentrations in plasma and milk (Callesen *et al*., 1988, 1990) together with detailed estrus observations (Callesen *et al*., 1993c), or by use of ultrasound examinations of follicular

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development and ovulation (Purwantara *et al*., 1993, 1994). Also in these years, problems associated with the FSH preparations used for superovulation (e.g. causing premature ovulations, Callesen *et al*., 1987b) were addressed by attempts to control the superovulatory reaction by use of anti-PMSG (Callesen *et al*., 1992) or by controlling the LH-contents (Schmidt *et al*., 1988).

On the practical side, larger projects based on MOET breeding plans were started in Europe, also in Denmark, where I got involved in such a project after having left Copenhagen and moved to Foulum in the western part of Denmark. This resulted in work on e.g. donor selection (Greve and Callesen, 1989), but we also made several studies on the embryo recipients; some of this work was presented at several AETE-meetings (Callesen *et al*., 1993a, b; 1994a, b). The contact to practice also gave some challenges when the more theoretical and experimental work met the real practical world (Liboriussen *et al*., 1995; Callesen *et al*., 1996), for example with the breeding potentials following the realized outcome of superovulation (Callesen *et al*., 1994c).

In vitro production of bovine embryos

During the 1980ies, the procedure for *in vitro* production of bovine embryos was being established based on work around the world with the first IVF calf reported from USA in 1983. In Copenhagen, Kang Pu Xu - another of Torben Greve's PhD-students - worked hard on this technology (e.g. Xu *et al*., 1987), and a prominent result was achieved when the first IVP calf in Europe was born here in 1987 (Greve *et al*., 1989).

In my Foulum-group we worked several years to optimize the bovine IVP-procedure. Peter Holm went through the tedious work of testing close-to-everything in relation to media and conditions for *in vitro* production (e.g. Holm *et al*., 1994, 1995), before this resulted in a quite useful modified SOF medium (Holm *et al*., 1999). Parallel to this work, we were still interested in morphological differences between vivo versus vitro oocytes (e.g. Hyttel *et al*., 1989b), also illustrating some of the implications for vivo and vitro fertilization (e.g. Hyttel *et al*., 1989a, 1991). At the AETE meeting in Venice, some of this work was summarized (Holm and Callesen, 1998).

With the gradual improvement of the IVP systems, we got a stronger interest in some of the consequences of such artificial *in vitro* conditions, stimulated by the disturbing reports about the so-called Large or Abnormal Offspring Syndrome. Thus, we worked on differences between vivo versus vitro embryos, both in cattle and pig (e.g. Hyttel *et al*., 2000), on chromosomal problems after *in vitro* production (Viuff *et al*., 1999), and finally also on the resulting calves (Jacobsen *et al*., 2000). As part of these studies, I spent some months in New Zealand and Australia in 2001 to study pregnancies and calves after both IVP and cloning. In Europe, these concerns were also subject for many discussions in the public with focus on animal ethics (e.g. Callesen *et al*., 1999), and also at the AETE meetings, where questionnaires were the basis for workshop discussions in e.g. Santander (den Daas and Callesen, 2000).

The tools for working with reproductive technologies

To work in such a biological area, technologies are required, and over the years I have been involved in several such technical developments in quite different fields.

Ovum pick-up

Oocyte collection through the abdominal wall was developed in the human field in the first part of the 1980ies, and together with a medical doctor we made some of the first attempts in cattle. It was a para-lumbar approach (presented at the 1987 IETS meeting; Callesen *et al*., 1987a), and several lessons were made - one was that it can result in recovery of oocytes, but also that a cow patient will kick you when you prick her with a needle; a big surprise for the medical doctor! Soon after, especially the group from Utrecht led the way into the much more convenient vaginal approach that also became routinely used in human.

In vitro *embryo culture*

We continued to work on *in vitro* culture, driven by Gabor Vajta's urge for simplicity and reliability. One area was an incubator system based on having the culture dish in a foil bag that was submerged into a water bath (Vajta *et al*., 1997b). This provided very stable temperature conditions, required minimal use of gases, and each culture dish had its own chamber. However, only few would - and will - accept a water bath in their culture lab. Another area was related to the anticipated need for an embryo to establish a local *in vitro* environment during its development. This was obtained by hand-making small holes or impressions in the bottom of standard plastic dishes (Vajta *et al*., 2000). The method was subject for a course also given at the AETE meeting in Lyon in 2004. A third interest was to see how far the embryo actually could be cultured *in vitro* (Brandão *et al*., 2004; Vajta *et al*., 2004). We learned that the trophoblastic cells grew quite well to form up to an almost 2 cm long structure … but the very early embryo proper did not.

Time-lapse systems

To study embryo morphology frequently during *in vitro* culture, Peter Holm built his own time-lapse system in the mid-1990ies (Holm *et al*., 1998). This was a fairly simple and cheap system, but it provided what was needed from the oocyte's and embryo's point of view, allowing complete, high and stable *in vitro* development for up to 9 days together with taking pictures every 20-30 min. The resulting films were rather boring during IVM, but there was much more to see when monitoring the pre-implantation period in both cattle (e.g. Holm *et al*., 2002) and pig (e.g. Callesen and Holm 2016), also with cloned and parthenogenetic embryos (Holm *et al*., 2003). Through

this work I established an interesting collaboration with the Danish company Unisense A/S that later developed the EmbryoScope®, an instrument that today is used in several human fertility clinics around the world.

Vitrification

A challenge in cryopreservation was the more fragile types of eggs, such as oocytes in general and embryos from certain species such as the pig; none of these structures really tolerated traditional slow freezing. Parallel to a visit from Masashige Kuwayama (Kuwayama *et al*., 1997), the vitrification technology was taken further by Gabor Vajta, resulting in a thin-straw system (Vajta *et al*., 1998). Now also early-stage embryos from both cattle and pig became possible to cryopreserve (Vajta *et al*., 1997a, c). An impressing illustration of the potentials of this technology was three calves born after having been vitrified/warmed two times before transfer: first as *in vitro* matured oocytes, second as blastocysts after *in vitro* fertilization and *in vitro* culture (Vajta *et al*., 1998).

Oxygen consumption

Working with embryos, it has for a long time been a wish to complement the morphological evaluations of embryo development with functional measures. Together with the company Unisense A/S, experts in microsensor technology, we established a system for measuring oxygen consumption from single embryos, using the bovine as a model. Together with a Portugese PhD-student, Ana Lopes, we used it for single-day measurements first on *in vitro* produced embryos (Lopes *et al*., 2005), but later also on flushed vivo embryos, that were afterwards transferred, illustrating the relation between embryo "respiration" and viability (Lopes *et al*., 2007b). In another approach, we installed the oxygen consumption system inside the previously mentioned time-lapse system, so repeated oxygen measurements could be made on the same embryos during seven days of *in vitro* culture, resulting in very detailed oxygen consumption curves (Gundersen *et al*., 2006, reviewed by Lopes *et al*., 2007a). The technology thus works, but its technical complexity has so far not made it useful for other than special research purposes.

Using the basic circles in a wider context

My focus on the embryo for several years, having interest in both biological and technological issues, has taken me into a number of broader applications.

One was to question our traditional approach where we attempt to make the conditions for the embryos as pleasant as possible during their stressful *in vitro* period (Callesen *et al*., 2012) and instead combine the various methods into a pro-active and challenging testing system to select the most robust embryos. This so far theoretical idea was subject for an IETS presentation in Argentina (Callesen *et al*., 2010), but it still remains theoretical.

A second area was in the post-partum cow, in which period oocyte and embryo qualities are key issues when it comes to establishment of a new pregnancy. The start of estrus cyclicity requires the endocrine systems to be in positive balance with the follicular development in the ovaries, and a successful outcome after insemination requires the whole reproductive system to be ready-for-use. In three different studies, indirect measures for these internal events were studied with particular reference to use in practice. In one, the vaginal discharge was characterized during the first period after calving and related to the cow's progesterone profiles (Gorzecka *et al*., 2011a, b, c). In another, focus was on metritis in the same period, working on the bacterial population and its effect on reproduction, as well as establishing a uterine scoring system (Elkjær *et al*., 2013a, b) as basis for deciding when to perform the first insemination (Elkjær *et al*., 2013c). In the third study, estrus cyclicity and reproductive performances were followed for a longer period after calving, namely in a system with extended lactation (Gaillard *et al*., 2016).

The third area was somatic cell nuclear transfer of pigs ("cloning"). We have been working on this complex technology over more than 20 years, first in cattle with birth of calves as a result (Smith *et al*., 1994), since in cattle and pigs with a zona-free approach (Booth *et al*., 2001a, b), and then with Gabor Vajta's handmade-cloning system (HMC; Vajta *et al*., 2003; Kragh *et al*., 2004) that resulted in the first piglets born in 2006 (review by Vajta and Callesen, 2012). The HMC system was going through a number of optimizations for example with different cytoplasmic volumes (Li *et al*., 2015) and with gilt versus sow oocytes (Li *et al*., 2014; Pedersen *et al*., 2015). Further, different pre-treatments were tested with cells and oocytes being exposed to a frog extract (Liu *et al*., 2014) or embryos to a high pressure treatment (Lin *et al*., 2014). Another very important side of our cloning work was related to the recipient animal, both in their selection and pre-treatment, but also with the transfer method used (Schmidt *et al*., 2010). Finally, the outcome was also being analyzed thoroughly (Liu *et al*., 2015), both related to the period around birth and to the piglets born with their reasons for not surviving this challenging procedure (Schmidt *et al*., 2011, 2015). Combining all these aspects, we built up a system that over four years produced very satisfying results (Callesen *et al*., 2014), and today we have a number of cloned piglets that are transgenic for different serious human diseases (e.g. Luo *et al*., 2011; Staunstrup *et al*., 2012; Al-Mashhadi *et al*., 2013; Jakobsen *et al*., 2014 - and more are coming). Over the next years, the medical doctors will reveal if these transgenic piglets can serve as useful animal models for the different diseases.

To all my collaborators

The type of research that I have described does not work well if you are sitting alone on a desert island with some paper and a pencil … no, we need each other.

In the different activities, we can have different roles, influenced by background education, experience, time etc. For all of these activities described above, I believe to have had a significant role in making them happen, but all have only been possible because we have been working as a group. We have never been a large group and Denmark is not a large country. However, Torben Greve learned me the importance of travelling around, meeting colleagues, presenting at scientific meetings. It may mean some fairly big travel expenses, but it is worth it. I first saw that as a young PhD-student at my first international meeting in 1983 in Helsinki, and since I have been at many such meetings at IETS, AETE, SBTE, ICAR and several others to meet you, discuss with you and visit you. From such an approach, even the small Danish groups have been around for some years now, and surprisingly, one of the Danes now stands here on this occasion. So, size does not always matter, if I may say so.

Final remarks

Through the years I have been working around the farm animal embryo in the days before, during and after fertilization, and this has been done in different species, in different contexts, with different technologies, in different collaborations. From such a view, I may have become a generalist in this field, but I still do consider the superovulated cow to have a special place in my scientific heart.

Speaking about my heart: My almost 35 years in research - so far - have given me so many contacts to colleagues, and today I am lucky and proud to consider quite many of you to have become friends. Thanks to all I have met during this travel around the embryo, nothing like that could or would have been done without you.

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Practical applications of sperm selection techniques for improving reproductive efficiency

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Abstract

Several selection techniques are available for processing spermatozoa. Apart from sperm washing to remove seminal plasma, only "swim-up" and colloid centrifugation have been used to any extent to prepare spermatozoa for *in vitro* fertilization, and only colloid centrifugation has been used to prepare sperm samples for artificial insemination. Single-layer centrifugation (SLC) through a species-specific colloid has been shown to be effective in selecting spermatozoa with good motility, normal morphology and intact chromatin in a range of species. This method is less time-consuming than swim-up, and has been scaled-up to allow whole ejaculates to be processed in a practical manner. The applications of SLC are as follows: to improve sperm quality in insemination doses or in samples for *in vitro* fertilization, to increase the shelf life of normal sperm doses, to remove pathogens (viruses, bacteria), to improve cryosurvival by removing dead and dying spermatozoa before freezing or after thawing, to select spermatozoa for intracytoplasmic sperm injection, and to aid conservation breeding.

Keyword: artificial insemination, chromatin integrity, filtration, *in vitro* fertilization, migration.

Introduction

The purpose of this review is to compare and contrast the sperm selection techniques that are available for selecting spermatozoa for use in various Assisted Reproduction Technologies (ART), with particular reference to those techniques used in preparing sperm samples for artificial insemination (AI) and *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI). The reasons for needing to select sperm will be described first, followed by a summary of the techniques available. The main part of the review will focus on colloid centrifugation, particularly Single Layer Centrifugation as an emerging (and promising) technology for sperm selection.

Why are sperm selection techniques needed?

It is difficult to produce good quality blastocysts from poor quality gametes. Semen samples contain a heterogeneous population of spermatozoa, some of which may possess the attributes necessary for successful fertilization. The "desirable" spermatozoa are thought to be selected by various mechanisms within the female reproductive tract, with the result that the small number of spermatozoa found in the vicinity of the oocyte are typically those best able to penetrate the zona pellucida and fertilize the oocyte. When using ART, however, these natural selection mechanisms are circumvented, allowing most spermatozoa to be found near the oocyte. The main reason for using a sperm selection technique in assisted reproduction is to select good quality (hopefully, functional) spermatozoa and to separate them from the rest of the sample, including any seminal plasma or extender that may be present (Morrell and Rodriguez-Martinez, 2009). Since seminal plasma contains decapacitation factors, spermatozoa must be removed from seminal plasma before they can be used in IVF. In the female reproductive tract, separation of spermatozoa from seminal plasma and selection of normal spermatozoa occurs at various sites, ranging from mechanical separation in the cervix (in animals that have vaginal deposition of semen during mating) to selective binding to oviductal epithelial cells (Suarez, 2007). The separation techniques that are performed in the laboratory mimic these selection mechanisms and are therefore biomimetic techniques.

Spermatozoa face many challenges in the female reproductive tract before they can reach the site of fertilization and penetrate the oocyte. There are fewer challenges to overcome in the *in vitro* situation, for example, spermatozoa do not have to navigate their way through the reproductive tract to reach the oocyte, and therefore the parameters of sperm quality linked to fertilizing ability, and the timing of events such as capacitation, may be different *in vitro* and *in vivo*. It is not only penetration and activation of the oocyte that is important but also the ability of the zygote to continue to develop; sperm quality, particularly chromatin integrity, appears to be important in this process.

Which sperm selection techniques are available and which are used in practice?

In previous reviews, sperm preparation techniques have been divided into those that separate spermatozoa from seminal plasma e.g. sperm washing and simple filtration, and those that also select a sub-population of spermatozoa, e.g. migration and colloid centrifugation. These techniques have been described in detail previously (Morrell and Rodriguez-Martinez, 2009, 2010; Morrell, 2012) and are summarized in Table 1. The methods that have been used consistently in practice are sperm migration (in the form of "swim-up") and colloid centrifugation. Both of these techniques separate the spermatozoa from seminal plasma and extender but whereas "swim-up" separates motile spermatozoa from immotile ones, colloid centrifugation allows the separation of morphologically normal, motile spermatozoa with intact chromatin from the rest of the sample (Morrell *et al*., 2009), and also

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removes seminal plasma proteins that are coating the surface of the spermatozoa (Kruse *et al*., 2011). Swim-up has been used to prepare spermatozoa for IVF, whereas colloid centrifugation has been used for both IVF and AI. The disadvantages of swim-up are that it takes approximately 45-60 min to do and only 10-20% of the spermatozoa in the sample are recovered. For colloid centrifugation, only 25 min preparation time is needed (including the centrifugation) and a recovery rate of >50% is commonly achieved (Thys *et al*., 2009), although this does depend on the sperm quality of the original sample.

The remainder of this review will focus on colloid centrifugation for preparation of sperm samples for ART.

Table 1. Sperm separation and selection methods.

Method	Seminal plasma removed	Sperm quality enhanced	Removal of potential pathogens	Fertility improved
Fractionation during collection	Mostly	Survival during storage increased	May be decreased Bacterial contamination may be reduced	Possibly
Sperm washing; simple filtration	yes	Kinematics may change; survival during storage increased	No	No
Sperm migration	Yes	Selection for motility	Viruses removed, bacteria may be present	Possibly
Filtration eg through glass wool, Sephadex	Partial removal	Selection for morphological normality, acrosome integrity	No	Possibly
Colloid centrifugation	Yes	Selection for motile, morphologically normal, intact membrane and acrosome, intact, chromatin.	Mostly	Yes
Hyaluronic acid binding	(spermatozoa are washed first)	Selection for binding to HA droplets or hyaluronan	Not tested	Yes
Sexing by flow cytometry	Yes	No, selection is for X or Y chromosome	Not reported	No

Modified from Morrell and Rodriguez-Martinez (2009*)*.

Background to colloid centrifugation

Heterogeneous cell populations can be separated into sub-populations according to density by centrifugation through colloids, a technique commonly known as density gradient centrifugation. During centrifugation on a gradient, cells move to a point corresponding to their own density, known as the isopycnic point (Pertoft, 2000). The apparent densities of spermatozoa from several species were identified by Oshio (1988). However, when colloids are used to prepare spermatozoa for ART, the density of the bottom layer of colloid is chosen to be less than that of mature spermatozoa, with the result that the spermatozoa pass through the colloid and collect in a pellet at the bottom of the tube. Immotile, morphologically abnormal, acrosome reacted spermatozoa or those with damaged chromatin are mostly retained at the interface between the semen and the colloid (Morrell *et al*., 2009).

The first colloid to be used for sperm preparation consisted of polyvinylpyrrolidone (PVP)-coated silica particles (Percoll, GE, Uppsala, Sweden) e.g. Serafini *et al*. (1990). Different densities of Percoll were produced by mixing the Percoll with various salt solutions, for example, TALP (Parrish *et al*., 1995) concentrated Tyrodes salts (Matás *et al*., 2010). However, approximately 20 years ago there was some controversy about whether the PVP-coating could be toxic to spermatozoa (Avery and Greve, 1995). Although Motoshi *et al*. (1996) observed no detrimental effects to bull spermatozoa after their exposure to PVP when capturing them for ICSI, there were reports of toxicity to mouse spermatozoa similarly exposed (Mizuno *et al*., 2002). This issue has not been satisfactorily resolved, partly because of species differences but also differences in PVP from various sources (Balaban *et al*., 2003). Subsequently it became apparent that some batches of Percoll had high endotoxin levels that were detrimental to sperm survival, which necessitated testing each batch of Percoll for sperm toxicity prior to use (Mortimer *et al*., 2000). The subsequent availability of silane-coated silica colloids provided an alternative to Percoll and resolved both the toxicity and the endotoxin issues.

Other issues with Percoll were related to loss of the acrosome during passage through the colloid. Cesari *et al*. (2006) reported a significant proportion of lost acrosomes from bull spermatozoa after preparation by Percoll density centrifugation compared to swim-up. This observation was in contrast to Somfai *et al*. (2002) who observed that preparation by Percoll density gradient resulted in a higher proportion of spermatozoa with intact acrosomes than swim-up. However, the centrifugation force used was greater in the study by Cesari *et al*. (700 g compared to 300 g) which may have contributed to the lost acrosomes.

A variant of colloid centrifugation using only one layer of colloid (in which case there is no gradient) has been developed. This method, known as Single Layer Centrifugation (SLC), has gained popularity when preparing spermatozoa for AI since larger volumes of semen can be processed in this manner than by density gradient. It is too time-consuming and impractical to attempt to layer several colloids of different densities in large tubes to create density gradients for large volumes of semen. Initially the SLC

technique was developed in 15 ml centrifuge tubes using 4 ml colloid but is was subsequently scaled-up to use 15 ml colloid in a 50 ml tube (Morrell *et al*., 2009), then to 20 ml colloid in a 100 ml tube and eventually to 150 ml colloid in a 500 ml tube (Morrell *et al*., 2011). Subsequently a small volume of colloid (1 ml) was used to prepare thawed red deer semen in an eppendorf tube (Anel-López *et al*., 2015). Recently a comparison was made between the original 4 ml colloid (Small SLC) and 1 ml of colloid, either in a 15 ml centrifuge tube (Mini-SLC) or in an eppendorf tube (Mini-EP) when preparing bull spermatozoa for IVF; although the sperm quality was good in all three variants, the sperm yield was highest in the Mini-SLC. Fortyfour blastocysts were produced from the Mini-SLC sperm preparation compared to 36 from the control (swim-up) preparation (Abraham *et al*., 2016). Although the difference was not statistically significant for this particular bull, the spermatozoa from this bull are known to work well in IVF (Y Sjunnesson; 2016; Division of Reproduction, Clinical Sciences, Swedish University of Agricultural Sciences, Uppsala, Sweden; personal communication). It would be interesting to try the Mini-SLC preparation technique for semen that does not normally work well in IVF.

Properties of SLC-selected sperm samples

The SLC technique has been used in a variety of species to prepare sperm samples for assisted reproduction. Most of the work on SLC has been carried out with stallion semen, mainly to prepare spermatozoa for AI but also for cryopreservation and for ICSI (Colleoni *et al*., 2011). The SLC-selected sperm samples tend to have better sperm quality than unselected controls, in terms of sperm motility (kinematics measured by computer assisted sperm analysis), membrane integrity, chromatin integrity, and normal morphology. Selected stallion sperm samples survive longer than unselected samples (Johannisson *et al*., 2009; Morrell *et al*., 2010) and retain their fertilizing capacity during cold storage for at least 96 h (Lindahl *et al*., 2012). They show improved pregnancy rates compared to controls, even for stallions of "normal" fertility (Morrell *et al*., 2014b). Spermatozoa can be separated from most of the bacteria contaminating a stallion ejaculate and also from equine arteritis virus in the semen of "shedding" stallions (Morrell *et al*., 2103). Selected spermatozoa show improved cryosurvival compared to unselected controls (Hoogewijs *et al*., 2011), and survive longer post-thawing (Hoogewijs *et al*., 2012). An additional interesting observation is that the yield of stallion spermatozoa after SLC was highly correlated with the fertility of the male after insemination of unselected samples (Morrell *et al*., 2014d), implying that SLC could be used as a diagnostic tool to indicate the potential fertility of a breeding stallion.

Boar sperm samples may have very good quality initially, as assessed by commonly used laboratory assays, which makes it difficult to see an improvement after selection. However, some improvement in sperm quality after SLC has been reported (Morrell *et al*., 2009). The SLC-selected samples showed enhanced ability to fertilize oocytes in IVF, necessitating a reduction in sperm dose to avoid polyspermy (Sjunnesson *et al*., 2013). The selected samples survive cryopreservation better than unselected samples (Martinez-Alborcia *et al*., 2012). Boar spermatozoa can be separated from bacteria contaminating the ejaculate during semen collection (Morrell and Wallgren 2011) and from porcine circovirus added to the ejaculate (Blomquist *et al*., 2011).

Most of the studies with bull semen have used SLC to prepare spermatozoa for IVF e.g. Thys *et al*. (2009), but there are some reports of its use with fresh semen e.g. Goodla *et al*. (2014). One report indicated that preparing fresh bull semen by SLC resulted in improved sperm quality in the thawed samples in terms of the proportion of sperm with high mitochondrial membrane potential and with high superoxide production, indicating high metabolic activity (Johannisson *et al*., 2016). Recent studies with bull spermatozoa in our laboratory have been to develop the reduced volume SLC mentioned earlier (MC Abraham *et al*.; 2016; Division of Reproduction, Clinical Sciences, Swedish University of Agricultural Sciences, Uppsala, Sweden; unpublished data), and also to use a low density colloid formulation to compare actual fertility of sperm samples in IVF (M Sabés Alsina, JM Morrell, Y Sjunnesson; 2016; Division of Reproduction, Clinical Sciences, Swedish University of Agricultural Sciences, Uppsala, Sweden; unpublished data). One of the problems with using IVF to detect differences between sperm treatments is that the method of preparing the sperm sample can influence the result. Using either swim-up or the normal colloid centrifugation will select motile or good quality spermatozoa, respectively, thus negating the effect of the quality of the original sample. In order to evaluate differences in fertility between treatments a low density colloid can be used just to separate the spermatozoa from the seminal plasma.

Conservation breeding

Isolated studies of the use of colloids to prepare spermatozoa have been reported in rare or endangered breeds of sheep (Jiménez-Rabadán *et al*., 2012), donkeys (Ortiz *et al*., 2015), and bears (Alvarez-Rodriguez *et al*., 2016), as well as in cats (Chatdarong *et al*., 2010), dogs (Dorado *et al*., 2103), red deer (Anel-Lopez *et al*., 2015), llamas (Trassoras *et al*., 2012) and camels (Malo *et al*., 2016). In these cases, either the sperm samples were prepared by SLC prior to freezing, to improve cryosurvival, or the most motile spermatozoa were selected from thawed sperm samples to improve their usability. The latter method is very useful when preparing spermatozoa for IVF or ICSI, but may not be so useful for AI particularly if large numbers of .spermatozoa are needed for an insemination dose.

Another article described using a low density colloid to remove somatic cells from an epididymal

suspension of spermatozoa of the gray wolf, to enable DNA to be extracted (Muñoz-Fuentes *et al*., 2014). In this case it is important to recover all the sperm cells while separating them from non-sperm cells in the sample. In other cases, epididymal sperm samples may be needed for gamete cryopreservation, in which case it is important to select mature spermatozoa and separate them from cellular debris in the sample. This aspect is particularly relevant for conservation breeding purposes, when it is important to rescue genetic material from rare individuals, which may mean extracting epididymal spermatozoa after the death of the animal. Testicular fragments may also be a source of germplasm for conservation purposes; recent studies with cat testicular tissue showed that SLC could be used to enrich testicular sperm cells from cell suspensions (Chatdarong *et al*., 2016). The latter authors conclude that SLC could be a useful selection tool for recovering testicular sperm cells from wild cats *post mortem* for conservation purposes.

One of the challenges with creating gene banks for species conservation is to cryopreserve material in such a manner that it will be usable at a future time. Few checks are carried out to see if frozen spermatozoa retain fertilizing capacity when thawed, partly because of the lack of suitable females or the opportunity to carry out a controlled fertility trial. Many sperm samples are frozen in media containing egg yolk or similar material of animal origin, with the result that it may not be appropriate to use it in the future from the point of view of disease transmission. Although breeding males of domestic species are tested for various virus diseases before freezing their semen, such a luxury is not practical for wild animals. In addition, it is only possible to test for known viruses; there is no means of knowing whether "emerging" diseases have been present in the wild population for some time, with the result that it may be possible to infect or re-infect animal populations by using untested frozen sperm samples.

Removal of pathogens

As mentioned previously, SLC has been used to separate spermatozoa from bacteria contaminating the ejaculate during semen collection (reviewed by Morrell and Wallgren, 2014). Almost all semen samples are contaminated by bacteria during semen collection and these bacteria tend to multiply due to the ready availability of nutrients supplied by the semen extender. Apart from competing with spermatozoa for nutrients in semen extenders, bacteria may produce metabolic byproducts that are detrimental to spermatozoa, and Gram-negative bacteria produce lipopolysaccharide from their cell walls that is toxic to spermatozoa. High bacterial loads in semen doses can cause a decrease in sperm motility and viablity, and also an increase in agglutination and in the acrosome reaction. Females inseminated with such contaminated semen may return to oestrus after insemination, or there may be high embryonic mortality, endometritis, systemic infection and/or disease, or a reduced litter size in polytocous species.

Therefore, antibiotics are added to semen extenders to control bacterial growth in semen doses for international trade. The antibiotics to be used and the doses are specified in various regulations e.g. European Council Directive 90/429/EEC, Annex C2 (European Union, 2012). However, such a non-therapeutic use of antibiotics is problematic in view of current attempts to reduce antibiotic use. It is now known that antibiotic resistance can develop very quickly and spread to other bacterial species within the same host, or even in different hosts. Therefore, SLC offers an alternative to the use of antibiotics since it can separate spermatozoa from a large proportion of the contaminating bacteria in a sperm sample. Some bacteria are more difficult to remove than others, e.g. those that tend to aggregate or form biofilms, presumably because the density of the "unit" formed is then similar to that of the spermatozoa, or because some bacteria can hook on to spermatozoa and are thus carried through the colloid.

Economics of Single Layer Centrifugation

The disadvantage of using colloid centrifugation to prepare sperm samples for IVF or ICSI is the extra cost involved. It is very difficult to determine the "value" of embryos produced from particular sires, which makes it impossible to generalise about the economics of using SLC to prepare sperm samples. However, since the sperm samples have to be separated from seminal plasma and/or cryoextender, SLC takes less time than swim-up and has the advantage of selecting spermatozoa with good chromatin integrity. In addition, semen from a male of superior genetic merit is usually used, resulting in embryos of considerable "value". The added advantage of being able to process the semen on only 1 ml of colloid instead of the 4 ml used previously, adds considerably to the merits of SLC as a selection technique. Thus the extra cost involved in purchasing colloid will be more than compensated by the production of more embryos or their enhanced survival and implantation rate. Other factors such as the biosecurity of the semen, reduced antibiotic usage, and the requirements of embryo production for export, must also be considered. Thus, any processing steps that can improve the quality of the semen, and potentially the number of good quality embryos produced, will add value to embryo production.

Concluding remarks

Sperm selection techniques are needed to prepare spermatozoa for assisted reproduction. Colloid centrifugation, especially Single Layer Centrifugation, can be particularly beneficial since it not only separates the best quality spermatozoa but also separates them from bacteria and viruses that may be present in seminal plasma. The fertility trials that have been carried out to date in a limited number of species indicate that the selected spermatozoa may have enhanced fertilizing capacity compared to unselected controls. If these

observations also hold for other species, especially rare breeds and endangered species, the technique will be particularly relevant for conservation breeding. Recent developments in reducing the volume of colloid needed to prepare bull spermatozoa for IVF may be particularly advantageous, especially when deciding which sperm preparation technique to adopt.

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Embryo maternal immune interactions in cattle

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Abstract

Mammalian embryo implantation requires the priming of the maternal immune system, but, not the provocation. There are many examples of conditions where a disturbed or aberrant immune profile during embryo implantation leads to pregnancy loss. However, these studies are primarily associated with human and mouse species; data is generally limited for cattle and livestock. Most available information centres on the endometrial response to interferon tau (IFNT), a type I antiviral cytokine, which is the maternal recognition factor for cattle and sheep. Interferon tau secretion by the embryo and detection by the dam is critical to corpus luteum (CL) maintenance and pregnancy retention. However, the large volume of bovine endometrial and conceptual transcriptomic data highlights a broader more integral role of the maternal immune system in the establishment of pregnancy in cattle. When taken together with available immunohistochemistry and flow cytometry data from livestock, mouse, and human, a profile of immune cell involvement from ovulation to conception and placentation emerges. The key events of pregnancy establishment in cattle and the involvement of the maternal immune system will be discussed.

Keywords: Cow implantation pregnancy immune-system embryo.

Introduction

The maternal immune system plays a critical role in mammalian embryo implantation. Successful establishment of pregnancy requires the activation of a controlled immune response that is simultaneously responsive and tolerant towards paternal antigens and the semi-allogenic embryo. The discipline of Reproductive Immunology has received considerable attention from a human clinical point of view and much data has been gathered from patients and generated from various mouse and *in vitro* model systems. In contrast, information from cattle mostly revolves around the endometrial response to the maternal recognition factor for cattle and sheep, the type I antiviral cytokine, interferon tau (IFNT), detection of which by the dam is critical to corpus luteum (CL) maintenance and the establishment of pregnancy. The greatest source of information has come from the large volume of bovine endometrial and conceptal transcriptomic data that has been generated in the past decade. The emerging knowledge clearly indicates that regardless of specificities in placentation physiology, an appropriate maternal immune response is just as critical to the establishment of pregnancy in cattle as it is in human and rodents.

In cattle, the first three to four cell cycle divisions post fertilization occur in the oviduct, such that the embryo enters the uterus on approximately day 4 post fertilization. There it undergoes a number of cell divisions to form the morula which, after differentiation, forms a blastocyst consisting of the inner cells mass (which will eventually give rise to the embryo/foetus) and an outer cell mass consisting of trophectoderm cells which ultimately give rise to the placenta. Up to this stage, the embryo is encased in the glycoprotein shell, the zona pellucida. Therefore the endometrial lining is not exposed to paternal antigens again until hatching, which occurs from day 8 to 9 post fertilization. Transcriptomic analysis of the bovine endometrium during this early stage of pregnancy indicates little or no change in gene expression in response to the zona-enclosed blastocyst stage embryo (Forde *et al*., 2011; Forde and Lonergan, 2012). Once hatched, the blastocyst forms an ovoid-shaped conceptus between days 12-14 and the elongation process begins. Elongation entails rapid proliferation of the conceptus trophectoderm cells, reaching 3-4 mm or more in length by day 14 (Randi *et al*., 2015), and 25 cm or more in length by day 17. As the embryo elongates, the trophectoderm and endometrial luminal epithelium (LE) become closely apposed, see Spencer *et al*. (2007), for review. During this period the conceptus relies on maternal secretions, collectively termed histotroph, for survival (Bazer, 1975). In contrast to mouse and human species, implantation in cattle is non-invasive. It is characterized by a superficial attachment and adhesion of the trophectoderm to caruncular and intercaruncular areas, commencing about day 19 (see Brooks *et al*., 2014), for review. During implantation, bovine trophectoderm cells form binucleate cells (BNCs) as well as trinucleate cells (TNCs), TNCs are products of fusion between binucleate cells and uterine epithelial cells (Wooding and Beckers, 1987) and are only located in the endometrium (Wooding, 1992). These multinuclear cells may play a role in implantation, contributing to the adhesion between conceptus and uterine endometrium at the placentomes. In cattle, several integrin family members (ITGs) have been characterized at the uteroplacental interface during trophectoderm attachment (MacIntyre *et al*., 2002; Pfarrer *et al*., 2003) and placentation (Pfarrer, 2006) periods and are believed to play constitutive roles. Similarly, the transmembrane glycoprotein, vascular cell adhesion -molecule (Osborn *et al*., 1989), is also regarded as a cell adhesion mediator during the processes of lymphocyte homing (May *et al*., 1993), angiogenesis

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(Ding *et al*., 2003) and allantoic membrane fusion to the chorion (Gurtner *et al*., 1995). The key events and interactions between the embryo and the dam are presented and reviewed.

The role of the embryo in the maternal immune response

Response to insemination

During transmission of seminal plasma (SP) at coitus, cells of the maternal immune system recognize various signaling constituents of semen, including interleukin (IL) -8, transforming growth factor beta (TGFB) and IFNG. In addition, sperm antigens are recognized as foreign (Robertson, 2005). The recognition of non-self initiates activation of a maternal immune response, which may ultimately confer immunological tolerance to paternal antigens that will be expressed by the embryo that develops after fertilization (Moldenhauer *et al*., 2009). The first stage of the maternal immune response is characterized by an influx of eosinophils and neutrophils to the uterine lumen. Data from mice show that chemoattractants released by these cells, such as granulocyte macrophage colony-stimulating factor (GM-CSF) and (IL) -6, attract both monocytes and dendritic cells, potentially creating an environment that regulates the inflammatory status of responding macrophages and increases expression of factors which promote early embryo development (Robertson *et al*., 1996, 2000; Robertson, 2007; Bromfield *et al*., 2014). Data from mice indicate that the absence of changes in the reproductive tract caused by SP can alter the developmental program of the developing conceptus (Bromfield, 2014). This cell-free, fluid fraction of the ejaculate is significantly diluted during semen preparation for use in AI programs, thus cows bred in this manner are only exposed to trace amount of SP. However, the relatively high pregnancy rates achieved in cattle following artificial insemination (AI) or embryo transfer (ET) suggest that maternal exposure to SP is not a critical component of the maternal immune response in cattle (Lima *et al*., 2009; Odhiambo *et al*., 2009).

Molecular response of maternal endometrium to the embryo

The presence of a rapidly elongating embryo is certainly registered by the maternal endometrium, as there is a dramatic change in global gene expression at this time (Forde *et al*., 2011). The type 1 interferon, interferon tau (IFNT), is the main signaling factor in maternal detection/recognition of pregnancy in cattle and sheep (Hansen *et al*., 1999; Choi *et al*., 2003). IFNT is secreted by the elongating conceptus, specifically the trophectoderm (Robinson *et al*., 2006). It is believed that the luminal epithelium of the uterine endometrium is the primary target for IFNT (Roberts *et al*., 1992; Imakawa *et al*., 2002); IFNT binds to a common receptor complex with two polypeptide subunits (IFNAR1 and IFNAR2; Rosenfeld *et al*., 2002). There is evidence to suggest that IFNT can reach the stroma, the uterine myometrium (Ott *et al*., 1998, Johnson *et al*., 1999, Hicks *et al*., 2003) and most likely, the circulating immune cells and the ovaries as well (Shirasuna *et al*., 2012). IFNT acts on the endometrium to stimulate the expression of genes that promote conceptus growth and development and induce uterine receptivity (Hansen *et al*., 1997, Johnson *et al*., 2000, Song *et al*., 2007; Mansouri-Attia *et al*., 2012). Candidate and global gene expression analysis revealed that a classical Type I IFN response during the peri-implantation period is induced by the conceptus/IFNT (Li and Roberts, 1994; Spencer *et al*., 2008; Mansouri-Attia *et al*., 2012;). Induced endometrial classical Type I IFN stimulated genes (ISGs) include, 2',5'-oligoadenylate synthetase 1, *OAS1* or *ISG15, MCP1* Chemokine (C-X-C motif) ligand 5; *CXCL5*, (for review, see Forde and Lonergan, (2012). The expression of several chemokines is induced in endometrial tissues including chemokine ligands 10 (*CXCL10*) and 9 (*CXCL9)*; (Nagaoka *et al*., 2003b, Imakawa *et al*., 2006). Endometrial CXCL10 attracts immune cells to the caruncular regions of the endometrium (Nagaoka *et al*., 2003a), and by acting through the CXCL10 receptor, CXCR3, this chemokine regulates TE cell migration and integrin expression (Imakawa *et al*., 2006).

Conceptus-maternal communication is vital for the successful establishment and maintenance of pregnancy, Sub-optimal communication, resulting from impairment of conceptus development and/or from abnormal uterine receptivity, contributes to a high incidence of embryonic mortality (see Lonergan and Forde, 2014, for review). Using RNA sequencing, Mamo *et al*. (2011) described the temporal changes in transcriptional profiles of the bovine conceptus from a spherical blastocyst on day 7 through days 10, 13, 16 and 19, corresponding to the formation of an ovoid conceptus, initiation of elongation, maternal recognition of pregnancy to a filamentous conceptus at the initiation of implantation on day 19. Generally, genes encoding trophoblast kunitz domain proteins, pregnancy-associated glycoproteins, cytoskeletal transcripts, heat shock proteins and calcium-binding proteins had highest expression levels at each of these stages of development (Lonergan and Forde, 2014; Mamo *et al*., 2011). Bauersachs *et al*. (2012) carried out a gene set enrichment analysis of several global transcriptomic datasets from days 15, 16, 17, 18 and 20 of the oestrus cycle or pregnancy and identified a conceptus-induced signature in the endometrium during the process of pregnancy recognition. Together with progesterone (P4), IFNT regulates endometrial gene expression necessary for the establishment of the proper uterine environment during the implantation period (Klein *et al*., 2006). A panel of approximately 30 genes was identified as expressed on day 16 as part of the early endometrial response to the conceptus and may represent early endometrial markers of a viable pre-implantation conceptus (Bauersachs *et al*., 2006, Mansouri-Attia *et al*., 2009a), reviewed by Lonergan and Forde (Forde and Lonergan, 2012). Although most data demonstrates that the main molecule affecting the

endometrium is IFNT, additional conceptus secreted molecules, including prostaglandins (PG; Dorniak *et al*., 2012, Spencer *et al*., 2013) and cortisol (Dorniak *et al*., 2013b), also act on the endometrium. An additional, but critically important action of IFNT, is the attenuation of endometrial PGF2a secretion, to maintain luteal production of P4. IFNT binds to IFNARs on the endometrial luminal epithelium and superficial glandular epithelium to inhibit transcription of the *ESR1* gene through a signalling pathway involving IFN regulatory factor (IRF) 2. These antiluteolytic actions of IFNT on the *ESR1* gene prevent *ESR1* expression and, therefore, the ability of oestrogen to induce expression of OXTR required for pulsatile release of luteolytic PGF (Spencer *et al*., 2007).

There has been much interest in determining the differences in global transcriptome profiles in embryos derived following natural mating or artificial insemination compared to those produced using assisted reproductive technologies (ART), such as *in vitro* embryo production or cloning. It is now widely accepted that ART derived embryos have significantly altered gene expression patterns compared to their *in vivo* derived counterparts (Clemente *et al*., 2011; Gad *et al*., 2012) What is most striking, is that these embryos elicit diverging responses from their recipient maternal endometrium, even though IFNT production levels was found to be similar in these pregnancies (reviewed by Sandra *et al*., 2015), suggesting that other pathways than IFNT-mediated, are involved in recognition of pregnancy. Comparing endometrial transcriptomes of cows that were recipients of *in vivo*, IVF-derived or SCNT -embryos revealed distinct patterns of gene expression among the three groups (Bauersachs *et al*., 2009; Mansouri-Attia *et al*., 2009b). Moreover, studies show that the supply of numerous amino acids and derivatives was significantly lower in the endometrium of SCNT conceptus-carrying females (Groebner *et al*., 2011b; Dorniak *et al*., 2013a).

It is likely that the class I major histocompatibility complex (*MHC-I*) also plays a role in embryo maternal interaction and modulation of the maternal immune response. The *MHC*, termed the bovine leukocyte antigen (*BoLA*) in cattle and the human leukocyte antigen (Davies *et al*., 2006) in humans, encodes a collection of immune and non-immune related molecules (see Kelley *et al*., 2005, for review). The class I region of the *MHC* includes the classical, or class *Ia* genes, the non-classical (*NC*), class *Ib*, genes and a number of pseudogenes. Although not directly homologous, classical class I genes have common characteristics across all species, such as high levels of polymorphism and high expression levels; their main function is to discriminate between self and non-self by presenting antigenic peptides to cytotoxic T lymphocytes, thus eliciting an immune response. Non-classical class I genes are generally non-polymorphic, have lower expression levels and varied functions (Hughes *et al*.,1999; Ellis, 2004). Currently there are circa 90 full length class I cDNA sequences validated and listed in the bovine *MHC* database (http://www.ebi.ac.uk/ipd/ mhc/bola). There are six or more classical *BoLA* class I genes, expressed in a number of different combinations, such that no more than three are expressed on a haplotype (Ellis *et al*., 1999; Birch *et al*., 2008). The existence and genomic location on chromosome 23, of five bovine *MHC-Ib* genes (named *NC1-NC5*), is recorded on the database. Their expression has been demonstrated in early cleavage stage bovine embryos (Doyle *et al*., 2009) binucleate cells (Bainbridge *et al*., 2001) and in first and second trimester and term trophoblast tissues (Davies *et al*., 2006). In general, the classical class I genes are found to be down-regulated or modified in the trophoblast cell populations in many mammalian species (for review, see Ellis *et al*., 2004). *MHC-I* mRNA expression by bovine embryos is both transcript and embryo stage-specific (Doyle *et al*., 2009) and can be regulated by a number of cytokines including IFNG, IL-4, and LIF (O'Gorman *et al*., 2010; Al Naib *et al*., 2011). Humans express three classical class I genes (HLA-A, -B and -C), and a number of non-classical genes, including HLA-G. HLA-G is expressed by human trophoblast (Ellis *et al*., 1999), which exists in both membrane-bound and soluble (secreted) alternatively spliced forms. A literature survey on the role of soluble HLA-G (sHLA-G) reported that sHLA-G secreted by early embryos may be necessary for implantation and could represent a good non-invasive marker for pregnancy rate following IVF (Fuzzi *et al*., 2002; Rizzo *et al*., 2007). However, the association between sHLA-G concentration and implantation success is not robust (Tabiasco *et al*., 2009).

We have investigated the mRNA expression profiles of bovine embryos as different stages of pre and peri -implantation development. Embryos were recovered from slaughtered pregnant beef-cross heifers at days 16, 17, 20, 24 and 34 post AI. The relative abundance of trophectodermal *NC-MHC-I (BoLA- NC1, NC2, NC3 & NC4)* mRNA expression was analysed using quantitative real time PCR. mRNA expression of *NC BoLA* sequences was detected at all stages investigated, with expression increasing linearly with embryo development (Reddy *et al*., 2011). In human, successful trophoblast invasion appears to depend upon the appropriate combination of fetal HLA-C expression by trophoblast and killer cell immunoglobulin-like receptors (Rouas-Freiss *et al*., 1997) by maternal uterine natural killer (NK) cells, moreover, inappropriate combinations could lead to poor placentation as seen in pre-eclampsia (Hiby *et al*., 2004). It appears that extra-villous human fetal trophoblast cell HLA-G expression may also potentiate maternal immuno-tolerance through modulation of CD4+ T, CD8+ T and NK -cell activity (Rouas-Freiss *et al*., 1997; Bainbridge *et al*., 2000; Fournel *et al*., 2000; Mansouri-Attia *et al*., 2012; Tilburgs *et al*., 2015). In general, MHC class I or class I-like ligands bind to KIR and Ly-49 multigene family members. The KIRs are expressed by NK cells and subsets of T cells (Vilches and Parham, 2002); whereas leukocyte immunoglobulin-like receptors (LILR) are expressed by several types of leukocytes (Long, 1999). Binding of MHC-I ligands either inhibits or activates their effector functions. In cattle, inhibitory (KIR2DL or

KIR3DL) and activating (KIR2DS and KIR3DS) members have been identified (Storset *et al*., 2003). Non-classical BoLA are produced in both nonsoluble and soluble forms, so it has been speculated that the soluble BoLA also bind LILR receptors on leukocytes in cows, which could inhibit the leukocyte (Rapacz-Leonard *et al*., 2014). However, to date, their interaction with trophoblast MHC-I ligands has not been detailed.

Maternal immune cell response to pregnancy

Although studies on the involvement of the maternal immune system in the establishment of pregnancy in cattle are few in number, particularly, for early pregnancy, monocyte (Mo), macrophages MØ and dendritic cells (DCs) appear to be the key actors during the implantation period (Fair, 2015). Macrophage recruitment to the pregnant endometrium has been described for a wide range of mammalian species, including the mouse (Fest *et al*., 2007), human (Mincheva-Nilsson *et al*., 1994, McIntire *et al*., 2008), sheep and cattle (Tekin and Hansen, 2004; Oliveira and Hansen, 2009; Oliveira *et al*., 2010; Mansouri-Attia *et al*., 2012). Some of the roles associated with macrophages at this time include clearing cellular debris and regulating apoptosis (Straszewski-Chavez *et al*., 2005), and regulation of placental lactogen concentrations at the fetal–maternal interface (Kzhyshkowska *et al*., 2008). However, these roles may be more important for mouse and human, where implantation is quite invasive. An additional role, which may be more relevant to ruminant species, is regulating the activation of anti-conceptus immune responses (Oliveira *et al*., 2010) in response to IFNT stimulation and antigenicity of the conceptus due to paternal antigen and classical MHC protein expression (Doyle *et al*., 2009). In cattle, using immunofluorescent labeling of immune cell markers, we observed an initial expansion of Mo, MØs (CD14+-cells), and DC (CD172a–CD11c+) populations in the endometrial stroma on day 13 of pregnancy (Mansouri-Attia *et al*., 2012). At the same time there was a decline in the number of CD11b positive cells; the loss of CD11b expression is characteristic of monocytes acquiring a stationary phenotype (Mansouri-Attia *et al*., 2012). Which supports their accumulation in the endometrial stroma in response to the embryo. Similarly, a human and mouse -specific role of Dendritic cells is involvement in decidua formation (Blois *et al*., 2007; Plaks *et al*., 2008). Immunofluorescent labeling of CD172a and CD11c in bovine endometrium sections, revealed a large population of immature cells within the endometrial DC population during early pregnancy (Mansouri-Attia *et al*., 2012). Immature DC's have been associated with the initiation and maintenance of peripheral tolerance (Dietl *et al*., 2006) and their presence has been positively associated with the establishment of healthy pregnancies in women (Tirado-Gonzalez *et al*., 2010). The expansion of these populations in the maternal endometrium is likely to be induced by IFNT.

The maternal immune response to pregnancy in humans, has long been described as a Th1/Th2 dichotomy with an imbalance toward a Th2 type immune response (Wegmann, 1988; Raghupathy, 1997). However, with more in depth transcriptomic and proteomic profiling, this view has been expanded, to take in to account the reported endometrial expression of Th1-type cytokines during implantation and proposed associated requirements for inflammatory signaling during the establishment of pregnancy (Lin *et al*., 1993; Chaouat, 2007). Using fluorescent labeling of lymphocyte subset markers on endometrial sections, we identified CD4+, CD8+, gamma delta T and FoxP3+ lymphocyte populations in both pregnant and cyclic cattle from day 5 to 16 of pregnancy/oestrous cycle. The population sizes did not appear to be temporally regulated during the oestrous cycle, or by the presence of an embryo (Oliveira *et al*., 2013). Although the population size did not alter, the gene expression profile of these cells was temporally modified; inflammatory/Th1 immune factors *IFNA, LIF, IL1B, IL8,* and *IL12A* were down regulated during the luteal phase of the oestrus cycle, while Th2 factors *LIF* and *IL10* were upregulated. Our findings suggested that the inflammatory status of T-lymphocytes is modulated during the oestrous cycle, taken together with the similar transcriptome profiles of cyclic and pregnant endometrial tissue, it would appear that the default mechanism in the uterus is to prepare for, and expect, pregnancy (Forde *et al*., 2011). In contrast to our findings, Correia-Álvarez *et al*. (2015) reported reduced numbers of CD45-positive leukocytes in the endometrium three days after transfer of *in vitro* produced bovine day 8 blastocysts to the uterus of heifers, compared to those with sham transfers. Similarly, Groebner *et al*., 2011a reported fewer CD45-positive leukocytes in the zona basalis of pregnant animals on day 18 of pregnancy, simultaneous with an increase in transcripts and elevated enzymatic activity of the tryptophan (Trp) -degrading enzyme indoleamine 2, 3 dioxygenase 1 (IDO). The Authors proposed that the elevated enzyme activity resulted in local Trp ablation, which lead to the reduced the number of leucocytes in the zona basalis of pregnant animals on day 18. However, neither group identified the specific leukocyte subset that was regulated in their study. Endometrial TGFb2 expression is also down regulated during the ovine and bovine implantation period, but appears to increase specifically in the placentome at this time (Mansouri-Attia *et al*., 2012). Several roles have been proposed for TGFb2 during placentation: 1) chemoattractant for Mo recruitment to the placentation foci; 2) regulator of trophoblast invasion and 3) regulation of Mo inflammatory status (Wahl *et al*., 1987; Graham and Lala, 1991).

The final lymphocyte to address is the NK cell, which is an essential player in the establishment of pregnancy in mouse and human. Using immunofluorsecent labeling of CD335+ cells, we found these cells to be surprisingly elusive in bovine endometrial tissue, in cyclic animals and particularly during the early stages of pregnancy (Oliveira *et al*., 2013). There is evidence from an *vitro* study suggesting that uterine NK cell expansion could be restricted by

IFNT (Skopets *et al*., 1992). Given that in mouse and human, uterine NK cells are critically involved in local vascular remodeling and regulation of trophoblast invasion during implantation (Mor *et al*., 2011), the restricted NK expansion might be a contributory mechanism by which non-invasive implantation develops in cattle, see review by Bazer *et al*. (2009).

Peripheral response of the maternal immune system to early pregnancy

In addition to the local uterine immune response, extra-uterine tissues, including peripheral blood cells (PBL) and the corpus luteum, respond to conceptus secretions (Sandra *et al*., 2015). The systemic effect of the conceptus has also been investigated with regard to IFNT and the expression of ISG in peripheral blood leucocytes (PBL; Oliveira and Hansen, 2008) and (Ott and Gifford, 2010). As observed in the endometrium, gene expression of ISGs (*MX1, MX2, OAS1, ISG15*) is induced in bovine PBLs (Green *et al*., 2010; Pugliesi *et al*., 2014) by day 18. These suggest that PBL ISG expression could be evaluated to determine cow pregnancy status (Forde and Lonergan, 2012), or to characterize the post insemination PBL profile of cows that maintain their pregnancies or those that ultimately re-cycle.

Summary

The role of embryo derived IFNT and the importance of maternal macrophage and dendritic cells in the establishment of pregnancy in cattle is widely understood. Further support for basic research in the area of bovine reproductive immunology is essential to generate new knowledge of the mechanisms involved in maternal – embryo immunological cross-talk. This information will lead to a better understanding of the optimal maternal immunophenotype to support early embryo development and implantation and facilitate the optimization of transition and post-partum -cow management to ensure this phenotype is achieved prior to breeding.

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