



## 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 *in 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- $\beta$  (TGF- $\beta$ ) 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- $\beta$  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 grow 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).

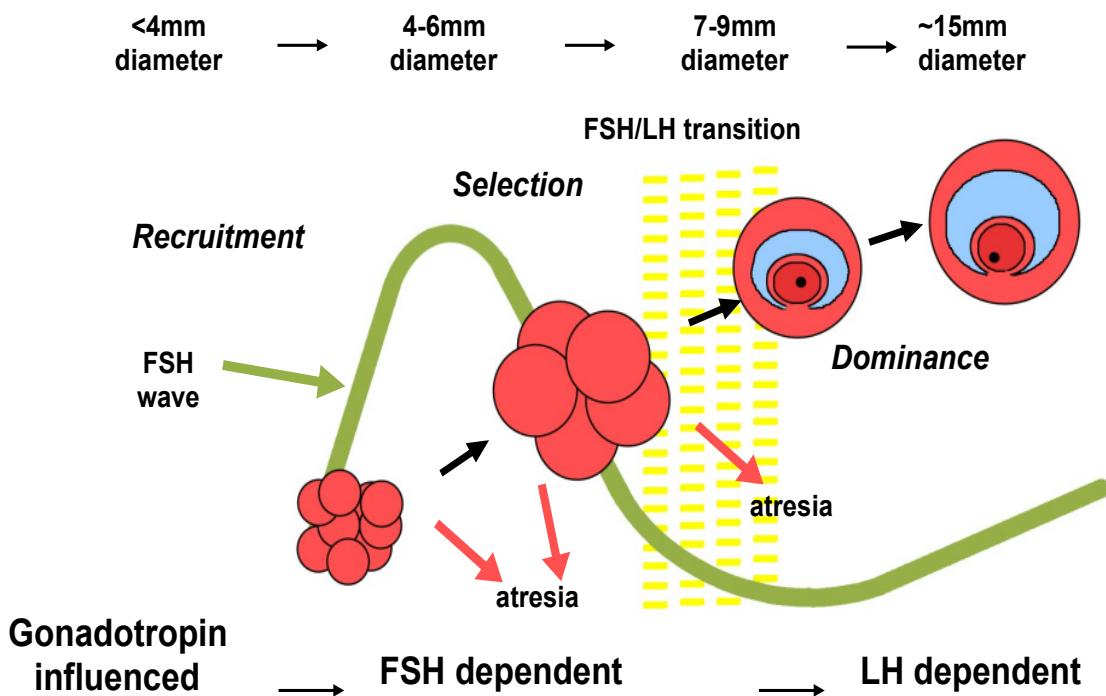


Figure 1. The role of gonadotropins in follicular selection and dominance in cattle. Note the transition period when follicles become LH dependent. Adapted from Webb and Campbell (2007).

#### *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 3 $\beta$ -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- $\beta$  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 oocyte 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).

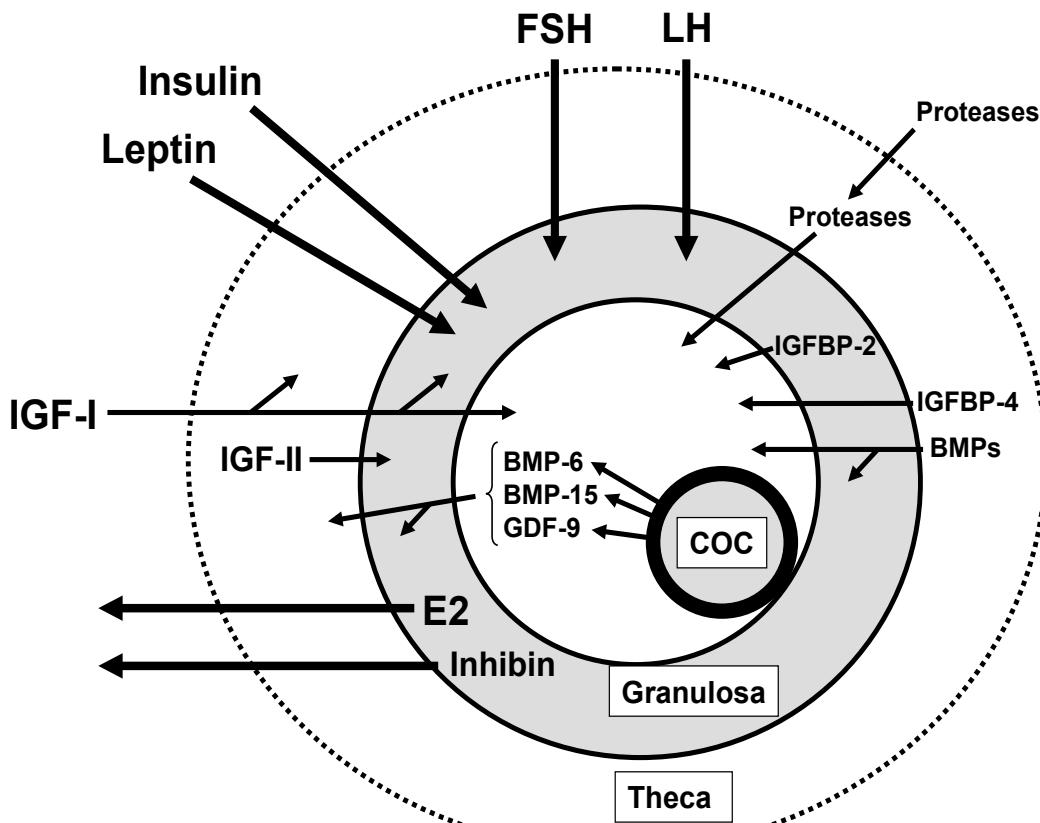


Figure 2. Peripheral gonadotropins and metabolic hormones work in conjunction with follicular growth factors (IGFs, IGFBPs, BMPs, GDF-9), released by granulosa, theca and the oocyte, to regulate development of the follicle and cumulus-oocyte complex (COC). Adapted from Garnsworthy *et al.* (2008).

#### Insulin-like growth factors (IGFs)

While carrying out studies to delineate the action of the classical gonadotrophic 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 superstimulatory 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- $\beta$ 1 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 suppress 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.

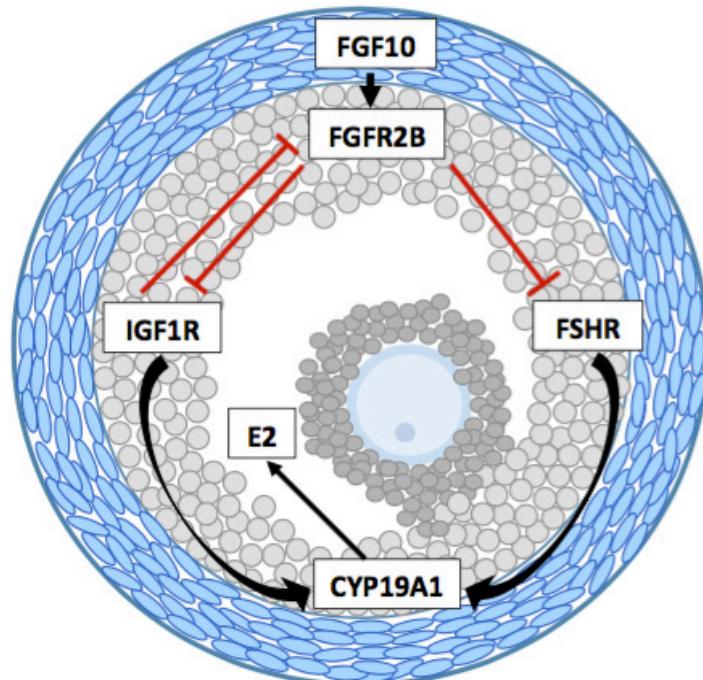


Figure 3. Proposed model for the participation of FGF10 in the control of estradiol production during follicle selection. FGF10/FGFR2B signaling is enhanced in the future subordinate follicle and decreases sensitivity to FSH and IGF, leading to a reduction in CYP19A1 expression and, consequently, estradiol 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 granulosa 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 (sVEGF-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- $\beta$  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.

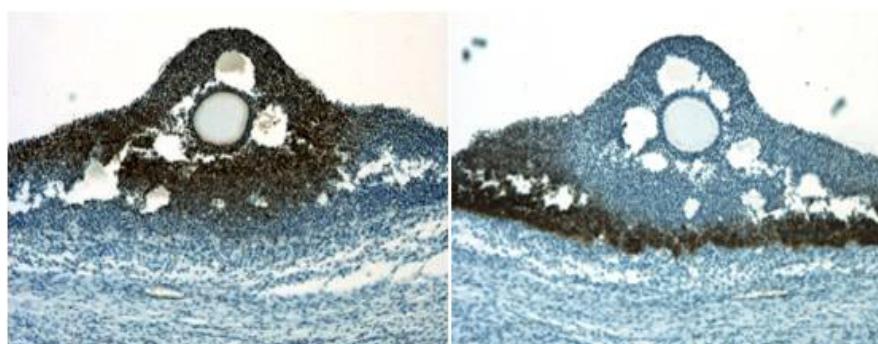


Figure 4. Expression of AMH (left) and Aromatase (right) protein in consecutive sections from the same gonadotropin-dependent large antral sheep follicle. Note AMH expression is absent from the differentiated granulosa cell layer but present in coronal/cumulus cells surrounding the oocyte and is directly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

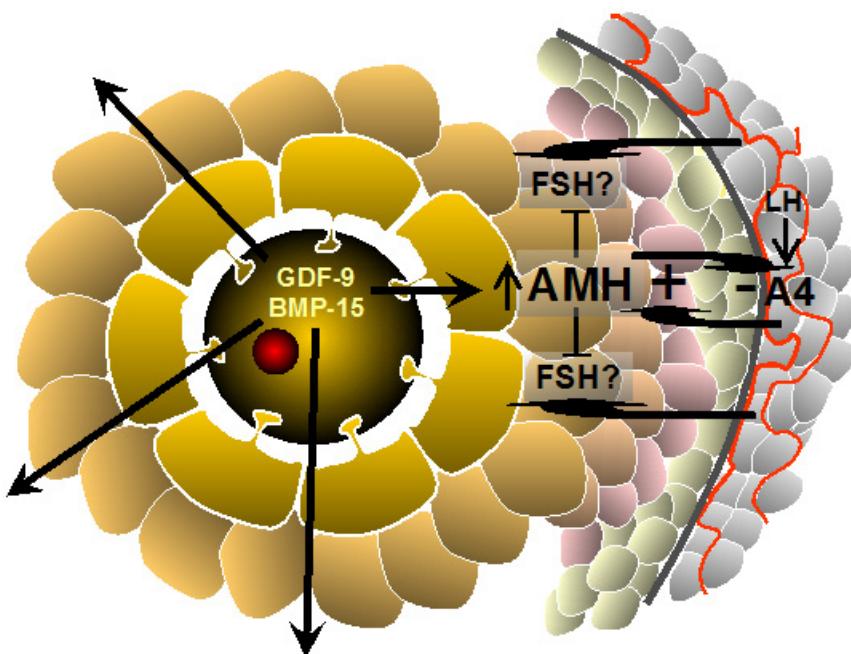


Figure 5. Proposed model of control of AMH expression in granulosa cells under the stimulatory influence of oocyte secreted factors and diffusion gradients. These originate from the oocyte. Also note the inhibitory influence of thecal cell derived androgen. Adapted from Campbell *et al.* (2014).

### 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 3 $\beta$ -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 PGF2 $\alpha$  or FGA+PGF2 $\alpha$ .

Treatment group	N	Ovulation type (%)				Ovulation rate
		Single	Double	Triple	Quadruple	
Synchronisation with PGF2 $\alpha$						
Control <sup>a</sup>	58	39.66	60.34	0	0	1.6 ± 0.06 <sup>a</sup>
Glycerol <sup>b</sup>	74	10.81	71.62	16.22	1.35	2.08 ± 0.06 <sup>b</sup>
Synchronisation with progestins						
Control <sup>a</sup>	55	39.62	56.60	3.77	0	1.64 ± 0.07 <sup>a</sup>
Glycerol <sup>b</sup>	53	5.66	50.94	39.62	3.77	2.41 ± 0.09 <sup>b</sup>

<sup>a,b</sup>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 intraovarian 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 gonadotrophic 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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