Impact of early life nutrition on the molecular and physiological regulation of puberty onset in the bull

D.A Kenny*,†, K. Keogh*, C.J. Byrne‡

*Animal and Bioscience Department, Teagasc, Dunsany, Co. Meath, C15 PW93, Ireland
†School of Agriculture and Food Science, University College Dublin, Belfield, Dublin, D04 F6X4, Ireland
‡Laboratory of Animal Reproduction, Department of Biological Sciences, University of Limerick, Limerick, V94 T9PX, Ireland

Corresponding author: David A. Kenny
Address: Animal and Bioscience Department, Teagasc, Dunsany, Co. Meath, C15 PW93, Ireland
Email: david.kenny@teagasc.ie

Abstract

The advent of genomic selection has accentuated interest in procuring saleable semen from young genetically elite bulls as early in life as possible. However, the timing of availability of semen, for commercial use will be determined by the age at which these young animals reach puberty and subsequent sexual maturity. Enhancing early life nutrition stimulates the functionality of the hypothalamic-pituitary-testicular (HPT) axis, mediated through complex biochemical interplay between metabolic and neuroendocrine signals and culminating in enhanced testicular growth, steroidogenesis, spermatogenesis and ultimately earlier onset of sexual maturation. Indeed, recent evidence indicates that the timing of sexual precocity, which is dictated by an early gonadotropin rise (8-20 weeks of age) in the bull, is determined by prevailing metabolic status during calfhood and is not compensable even where prior undernutrition is followed by dietary augmentation. However, despite this, the precise neuronal mechanisms regulating these developmental processes remain to be elucidated for the bull. Although early life nutrition clearly affects the sexual maturation process, there is little evidence for latent effects on post-pubertal semen characteristics. Equally, post-pubertal fertility,

measured as *in vitro* fertilisation and early embryogenesis, is not influenced by nutritional status during early life. Current efforts employing high throughput nucleic acid and proteomic sequencing and targeted immunofluorescence coupled with systems biology based gene network analyses, can provide further insight on how nutrition may mediate the biochemical interaction between neuroendocrine and testicular cellular processes. Such information can be harnessed to identify potential genomic targets as part of genomically assisted breeding programmes as well as facilitating development of strategic nutritional regimens to optimize sexual maturation and subsequent semen availability from genetically elite young bulls.

Keywords: nutrition, metabolic status, RNAseq,

Background

Although the advent of genomic selection in cattle breeding has undoubtedly increased the rate of genetic gain, the realisation of the full economic potential of this technology is undoubtedly hampered by the length of the generation interval (Konig et al., 2009). Indeed immense pressure is now been placed on the exploitation of male germplasm; elite sires can now be selected weeks after their birth, meaning that semen from these animals will be in demand as soon as they are capable of producing it. As a young bull will only produce 30-50% of the quantity of semen of a mature bull in his first year at stud (Amann and DeJarnette, 2012), the imbalance between supply and demand occurs immediately. Thus, there is major interest, worldwide in strategies to advance puberty and sexual maturation in the bull (Kenny and Byrne, 2018). Puberty in bulls is defined as the ability to produce an ejaculate containing >50 x 10^6 sperm cells with >10% progressive linear motility (Wolf et al., 1965). As semen quantity and quality can be a difficult phenotype to define, due to logistics of frequent semen collection and

analysis, more easily measurable proxy traits such as reaching a scrotal circumference ≥28 cm have also been used (Lunstra et al., 1978). However, scrotal circumference is influenced by both nutrition and breed; therefore, it will not be as sensitive or accurate for predicting age at puberty as conventional semen based analyses.

An acknowledged increase in secretion of the gonadotropins luteinizing hormone (LH) and follicle stimulating hormone (FSH) occurs in bulls between 8 and 20 weeks of age in response to increased gonadotropin releasing hormone (GnRH) pulsatility (Rawlings et al., 2008). The timing of this gonadotropin rise apparently dictates age at attainment of puberty (Evans et al., 1995). Luteinizing hormone stimulates differentiation of Leydig cells in the testes that secrete testosterone, whereas FSH stimulates the differentiation and proliferation of Sertoli cells (SC) (Berndtson et al., 1987, Rawlings et al., 2008). The secretion of these gonadotropins begins to decline in response to androgen negative feedback in the form of increased testosterone secretion in the testes (Rawlings and Evans, 1995). In recent years, research has focused on modulation of this early life gonadotropin secretion, with the aim of altering its timing or the magnitude of secretion; however, to date there have been no studies that examine whether it is the timing or magnitude of this rise that has a greater influence on age at attainment of puberty in bulls. Many studies including our own have clearly shown that enhancing the metabolic status of bull calves in early life hastens the onset of puberty (Brito et al., 2007a, Dance et al., 2015, Byrne et al., 2018a). Coincident with this, advancements in biotechnology have also facilitated more in depth investigations into biochemical interactions between metabolism and its regulatory role on sexual maturation (Liron et al., 2017, English et al., 2018a).

Physiological regulators of puberty

The timing and systemic concentrations of reproductive hormones secreted early in life have a major impact on testicular development and thus on the age at which an individual bull reaches puberty (Aravindakshan et al., 2000, Brito et al., 2007c). Based on datasets from our own laboratory, we have summarised the typical profiles of FSH, LH and testosterone (TT) in adequately fed young pre-pubertal bulls, depicted in Figure 1.

Released from the hypothalamus in pulses (Levine et al., 1982), GnRH is responsible for the secretion of LH and FSH from the anterior pituitary (Burgus et al., 1971, Matsuo et al., 1971). As GnRH plays such a pivotal role in the hypothalamic-pituitary-testicular (HPT) axis, manipulation of its secretion can lead to positive physiological changes in the reproductive development of the male.

The secretion of GnRH in bull calves is thought to be minimal during the infantile period (birth to 8 weeks). Based on analysis of blood from the hypophyseal portal system, pulsatile secretion of GnRH begins at approximately 2 weeks of age (Rodrigeuz and Wise, 1989). However, these latter authors reported that LH could not be detected in jugular blood until 8 weeks of age. In contrast, in work conducted by our own group and others LH has been detected in the systemic circulation of bull calves as early as 2 weeks of age (Figure 1, Byrne et al., 2018b; English et al., 2018; Evans et al., 1996). Nevertheless, based on these results, any attempt to manipulate neuroendocrinology with the objective of altering sexual development in the bull should begin as early as possible, postnatally.

Pharmacological interventions during early life development of bulls can also be used to advance sexual development. Many studies focus on manipulation of FSH, given its positive influence on SC proliferation (Orth 1984; Bagu et al. 2004). For example, bull calves treated every second day from 4 to 8 weeks of age with exogenous FSH exhibited an increase in

systemic FSH concentrations (Bagu et al. 2004), which led in turn to a hastening of puberty. Histological evaluation of the testes at 56 weeks of age in that study revealed that FSH-treated bulls had a greater number of SC, elongated spermatids and spermatocytes (Bagu et al. 2004). More recently, although intramuscular administration of FSH every 3.5 days from day 97- 171 of age resulted in a greater number of SC per seminiferous tubule and earlier onset of puberty, post-pubertal daily sperm production was unaffected (Harstine et al., 2018a, Harstine et al., 2018b).

It is widely accepted that adequate exposure to LH is vital for normal testicular function and the initiation of Leydig cell differentiation in the testes (Chandolia et al., 1997). Recent work from our own group (Byrne et al., 2018b) and others (Brito et al., 2007b, Dance et al., 2015) has shown that enhanced early life nutrition, during the aforementioned window of increased GnRH pulsatility, can positively affect LH secretion and testosterone synthesis and release. Indeed, the effects of enhanced early life nutrition and coincident increased systemic concentrations of LH and testosterone on testicular growth and morphological development were already evident at 18 weeks of age when comparing Holstein-Friesian calves offered a high versus a moderate plane of nutrition. This was manifested as greater paired testicular weights, increased seminiferous tubule diameter with a greater number of spermatogenic cells at a more advanced stage of maturity (higher percentage of spermatogonia) (English et al., 2018a). Consistent with this precocious testicular development, calves reared on the high plane of nutrition reached puberty approximately four weeks earlier than their contemporaries on the more moderate dietary allowance. Moreover, it has been clearly shown that delayed puberty as a result of suboptimal performance during the first six months of life cannot be readily mitigated through improving nutritional status in the ensuing pre-pubertal period (Brito et al., 2007b, Byrne et al., 2018a).

Endocrine regulation of hypothalamic-pituitary-testicular function

The studies cited above highlight the importance of an improved metabolic status for hastening the onset of puberty in bulls. Metabolic hormones (i.e. insulin like growth factor-1 (IGF-1), insulin, ghrelin, leptin, adiponectin) are secreted in response to enhanced nutrition, signalling the prevailing metabolic status to the hypothalamic-pituitary-gonadal axis, thus regulating the ontogeny of sexual development (Brito et al., 2007d).

IGF-1 and insulin

The essential role of IGF1/IGF1R in testicular development, SC and germ cell proliferation and differentiation in humans and various animal models has been reviewed by Cannarella et al. (2018). These authors highlight that FSH and IGF1 pathways are intimately connected, and IGF1R appears essential for FSH action in animals. In bulls the importance of the mitogenic influence of IGF-1 is further highlighted by the associated temporal secretion patterns of LH and testosterone (Dance et al., 2015, Byrne et al., 2018b). In the hypothalamus, IGF-1 receptors are present on GnRH neurons with the number of these receptors increasing with age in mice (Daftary and Gore, 2003). *In-vitro*, treatment of GT1-7 cells (model for GnRH secretion) with IGF-1 increased GnRH secretion (Anderson et al., 1999), likewise *in vivo*, treatment of castrated rams with exogenous IGF-1 increased LH secretion (Adam et al., 1998). The autocrine and paracrine actions of IGF-I have also been demonstrated in somatic cells in the testis (Cailleau et al., 1990, Wang and Hardy, 2004) with the presence of IGF-1R demonstrated in SC, Leydig cells, and spermatocytes of rodents (Wang and Hardy, 2004, Villalpando et al., 2008). Furthermore, FSH and IGF1 pathways are intimately connected, and IGF1R appears essential for

FSH action in animals (Cannarella et al., 2018). Indeed in bulls, *in-vitro* culture of SC from testes of 8 week old calves demonstrated that a combination of IGF-1 and FSH led to greater proliferation of SC, compared to treatment with either IGF-1 or FSH alone, further highlighting the important interactive role of IGF-1 and FSH in SC differentiation and proliferation (Dance et al., 2017). As IGF-1 is secreted in response to improved metabolic status, these results indicate that improving early life nutritive status nutrition may lead to earlier and greater SC proliferation, thus facilitating earlier onset of spermatogenesis and potentially improved long-term spermatogenic potential.

The contribution of insulin to the functionality of the HPG axis has been demonstrated by studies in which mice undergoing a specific excision of the insulin receptor in the hypothalamus (Bruning et al., 2000) displayed hypogonadism as a result of impaired release of GnRH. In addition, the identification of insulin receptors on the testes of rats (Abele et al., 1986) highlight that this hormone also has a direct effect on reproductive function in the male. In mice, when insulin receptor, IGF1 receptor or both receptors were inactivated in germ cell lineage or in SC, the inhibition of insulin/IGF signalling via receptor removal appears to result in minimal effects on germ cells and spermatogenesis (Pitetti et al., 2013). However, a 75% reduction was recorded in the size of testes and daily sperm production of adult mice lacking both insulin and IGF1 receptors in SC, as a result of a reduced proliferation rate of immature SC during the late fetal and early neonatal testicular period. In addition, the authors reported that FSH requires the insulin/IGF signalling pathway to mediate its proliferative effects on immature SCs (Pitetti et al., 2013). Collectively, these results emphasize the essential role of the insulin/IGF signalling pathway in FSH-mediated SC proliferation (Pitetti et al., 2013). In bulls, the importance of IGF-1 to Sertoli cell proliferation has been reported (Dance et al., 2017); however, the precise role of

insulin has yet to be elucidated. Nonetheless, an enhanced plane of nutrition resulted in elevated systemic concentrations of insulin as well as other metabolic hormones in early life, which was consistent with advanced testicular development and greater SC abundance during calfhood as well as earlier onset of puberty in Holstein-Friesian bulls (Byrne et al., 2018a, Byrne et al., 2018b, English et al., 2018a).

Adipokines

White adipose tissue (WAT) has traditionally been known for its role in energy storage and release when energy expenditure is greater than energy intake; however, WAT is metabolically active and thus can influence biochemical activities across many bodily systems. The function of WAT in metabolic and reproductive processes is complex, but it has been postulated that there is cross-talk between adipokines and the HPT axis. Adipose tissue produces a host of adipokines including leptin, adiponectin, resistin and apelin that impact many physiological systems including metabolism, reproduction and immunity function (Henry and Clarke, 2008). For additional insight into the impact of these adipokines on reproductive function during the pre-pubertal period in bulls the reader is referred to two recent reviews by Kenny et al. (2018) and Kenny and Byrne (2018). A brief synopsis is outlined below.

Leptin has been implicated as a metabolic signal involved in regulation of GnRH pulsatility (Amstalden, 2003). However, the stimulatory properties of leptin on LH secretion are normally only appreciable if the animals are undergoing severe nutrient restriction (Amstalden et al., 2000). Additionally, it is now evident that there are no leptin receptors on hypothalamic GnRH releasing neurons (Quennell et al., 2009), and *in situ* hybridization and immunohistochemistry approaches provide evidence that leptin signalling to the hypothalamus in cattle is mediated through the actions of the neuropeptide kisspeptin (Cardoso et al., 2015). Additionally,

proopiomelanocortin neurons in the arcuate nucleus (ARC) comprise a critical metabolic-sensing pathway thought to regulate the reproductive neuroendocrine axis (Cardoso et al., 2015), with kisspeptin neurons also acting as a relay for steroid feedback on GnRH secretion.

RNASeq data generated in our own laboratory from calves offered a high compared to moderate plane of nutrition highlight greater mRNA expression of genes involved in cellular energy production and branched chain amino acid degradation in subcutaneous adipose tissue in these calves (English et al., 2018b). This was consistent with histological evidence for greater adiposity (number and size of adipocytes) and a lower number of pre-adipocytes in subcutaneous adipose tissue of calves offered the high compared with the moderate plane of nutrition. Systemic concentrations of branched chain amino acids regulate metabolic processes in the liver via signalling to the ARC, which in turn leads to a down regulation of gluconeogenesis (Arrieta-Cruz and Gutiérrez-Juárez, 2016).

Although no differences in systemic concentrations of protein were detectable, calves offered a high plane of nutrition had a greater abundance of transcripts for adipokines such as leptin (LEP) and adiponectin (ADIPOQ) in subcutaneous adipose tissue (English et al., 2018b), concomitant with characteristics of precocious sexual development at 18 week of age such as greater paired testes weight, seminiferous tubule development and Sertoli cell abundance.

Effect of metabolic status on testicular Sertoli cell proliferation

The central role of the SC in influencing spematogenic potential together with its morphology and molecular physiology has been reviewed in detail by Franca et al. (2016). In order to assess the influence of metabolic status on testicular SC abundance in the bull, *in vivo*, we employed a multiple regression analysis using the data of English et al., (2018a) on body growth rate,

systemic metabolites, metabolic and reproductive hormones at various timepoints in bull calves between 2 and 18 weeks of age. The analysis showed that liveweight at 15 weeks of age accounted for 72% of the variation in SC number ($y = 12.53651 + 0.10854x$; adj $r2 = 0.72$; $P < 0.001$), highlighting the importance of ensuring optimum growth rates to ensure high weight for age in early calfhood, a period of high plasticity for SC proliferation and testicular development. Given the putative role of IGF-1 and FSH in SC proliferation (Dance et al., 2017), discussed earlier, unexpectedly concentrations of neither hormone contributed to the observed variation in SC abundance, in our analysis. Nevertheless, as expected there was a strong positive correlation between liveweight and systemic concentrations of IGF-1 at 15 weeks of age ($r = 0.73$; $P < 0.01$) thus supporting a likely mediating role of the latter in modulating SC differentiation.

Notwithstanding our observations on the concurrent positive effect of early life nutrition on proliferation of testicular SC, in a companion study (Byrne et al., 2018a) we failed to observe evidence for latent effects on SC abundance when bulls were slaughtered at 18 months of age. The resistance of SC to the prevailing influence of nutrition in adult animals is clearly evident from the data of Guan et al. (2014), who report no difference in Sertoli cell number between 24 month old rams offered either a high compared to a moderate plane of nutrition, despite the latter losing 10% of their initial bodyweight.

It has been reported (Sinowatz and Amselgruber, 1986) that Sertoli cell differentiation ceases at approximately 40 weeks of age in the bull (i.e. around the time of onset of puberty), whereas other authors have postulated that this cessation occurs at 25 weeks of age, coincident with an observed decrease in systemic concentrations of FSH (Bagu et al., 2004, Rawlings et al., 2008).

Our data highlights that the advantages of enhanced early life nutrition on Sertoli cell number may be compensated for beyond the early pre-pubertal period (8-20 weeks of age). This

argument is further strengthened when considering that although advancements in puberty are observed following enhanced early life nutrition, there is no evidence of latent effects on semen production potential in the same bulls (Harstine et al., 2015, Byrne et al., 2018a). In contrast, Holstein-Friesian bulls offered 130% versus 70% of energy and protein requirements had an increased number of harvestable sperm post-puberty supporting the role of enhanced early-life nutrition to optimise lifetime reproductive potential (Dance et al., 2016). In addition to this there were no reported negative effects of a moderate to high plane of nutrition on semen quality when offered post six months of age in the same Holstein-Friesian bulls (Dance et al., 2016, Byrne et al., 2018a). It is worth noting that in these studies, the frequency of semen collection was at monthly and fortnightly intervals, respectively, and this may not be reflective of exhaustion of sperm reserves experienced by these bulls in an AI centre, for example. In this regard, it would be worth examining whether there are latent effects of early life nutrition on the regeneration potential of bulls were they subjected to a more aggressive semen collection schedule. Although offering a high plane of nutrition, post six months of age, is reported to have no negative effects in the Holstein-Friesian bulls discussed above; there are reported deleterious effects when beef bred bulls are offered high plane of nutrition from six months of age (Coulter et al., 1987). Angus and Angus x Simmental bulls offered a high energy diet after weaning had greater scrotal temperature gradient; indicating that this group was not able to reduce testicular temperature at the same rate as their contemporaries offered a moderate plane of nutrition (Coulter et al., 1997). Analysis of semen from these bulls indicated that this reduced thermoregulation ability led to a reduction in the proportion of morphologically normal and motile sperm. In summary, there appears to be higher potency in disturbance of thermoregulation of the testes via nutrition when

offered to beef versus dairy bulls. This is a result of greater fat deposition and alterations in testicular vascular cone development (Kastelic et al., 2018).

Molecular physiology of sexual maturation in the bull calf

Neuroendocrine

The hypothalamus plays a major role as the homeostatic regulator of the body, receiving signals from metabolic hormones including leptin, ghrelin, insulin and IGF-I in the ARC region (Amstalden et al., 2011). It is now widely accepted that the brain undergoes periods of high plasticity during fetal and early post-natal life with the greatest response observed when metabolic perturbations are experienced during these periods (Schwartz et al., 2000, Calikoglu et al., 2001). The HPT axis controls the secretion of male hormones and thus spermatogenesis (Ramaswamy and Weinbauer, 2014).

A recent targeted PCR study by our research group has demonstrated that ghrelin receptor (*GHSR)* was down regulated in the ARC and anterior pituitary when Holstein-Friesian bulls were offered a high compared with a moderate plane of nutrition (English et al., 2018a), consistent with the inhibitory effect of this hormone on GnRH pulsatility (Chouzouris et al., 2016). The information on neuroendocrine regulation of puberty in bulls in currently very limited; however, neuroendocrinological mechanisms have been studied more extensively in heifers. The mRNA abundance of agouti related protein (AGRP) has been reported to be lower in heifers undergoing accelerated versus restricted growth (Allen et al., 2012), whereas pro-opiomelanocortin (POMC) mRNA abundance was increased in heifers achieving higher ADG.

Molecular control of onset of puberty in the bull

Given the central role of nutritional status in governing the functionality of the hypothalamic-pituitary-testicular axis which in turn regulates sexual development and puberty onset there is substantial interest in uncovering the underlying molecular mechanisms controlling this relationship. Additionally, unravelling the underlying biology contributing to precocious puberty may lead to the identification of the key 'gatekeeper' genes controlling puberty onset that, following appropriate validation, could be used in genomic selection breeding programs. A recent global transcriptomics (RNAseq) study by our research group showed that improved early life nutrition increased the expression of genes involved in both cholesterol and androgen biosynthesis within the testes (English *et al.*, unpubl.). However, in contrast, whereas thousands of genes were expressed in both the ARC and anterior pituitary tissues of the same calves, no genes affiliated with reproductive function or development were differentially expressed in either tissue (English *et al.*, unpubl.). This apparent discrepancy between hypothalamic-anterior pituitary gene expression patterns with that of the testes may be due to the transitory nature of gene transcripts within the brain (Bondy and Lee, 1993) and/or the fact the impact of prevailing nutrition may have been manifested much earlier in the brain tissues with latent effects on testicular obvious at the time of tissue harvest. Indeed the sizeable effect of plane of nutrition on testicular transcriptional activity (>1300 differentially expressed genes (DEG) detected) was consistent with a two fold increase in testicular weight and advanced stage of development for both seminiferous tubule morphology and spermatogenesis in bull calves offered a higher plane of nutrition (English *et al.*, unpubl.).

Although molecular based evaluations of the effect of early life-nutrition on DEG profiles can yield interesting preliminary information, individual genes obviously do not work alone, instead interacting across tissues to elicit a subsequent physiological phenotypic outcome. Thus study of

gene networks can provide the potential to uncover gene to gene interactions governing a particular phenotype of interest, with this information important for the identification of key or 'hub' genes that play important central roles in complex phenotypes (Wang et al., 2014). Consequently, using existing RNAseq datasets we recently performed gene co-expression analyses to reveal further insights into the genes that are affected by dietary manipulation and may be contributing to the advancement in sexual development typically observed in young bull calves offered nutritionally enhanced diet. Networks of co-expressed genes were derived from the RNAseq datasets (arcuate nucleus, anterior pituitary, testes and adipose tissue) of the aforementioned studies of English et al. (unpubl.). We employed the weighted gene co-expression network analysis (WGCNA) systems biology approach (Langfelder and Horvath, 2008) to examine the association between gene networks and testicular abundance of SC, a key trait positively associated with post-pubertal spermatogenic potential and fertility in the bull. The main outcomes of this analysis are summarised in Figure 3.

Genetic variants putatively associated with sexual maturation in the bull calf

Adipose

The function of adipose tissue in metabolic and reproductive processes is complex but it has been postulated that there is cross-talk between adipose tissue and the hypothalamic-pituitary-testicular axis (English et al., 2018). This relationship is established within our own results through the negative association between a network of co-expressed genes within the adipose dataset and genes of the GnRH signalling pathway. This negative correlation is paralleled with the up-regulation of *LEP* in bull calves offered a high compared to a moderate plane of nutrition (English et al., 2018). Greater leptin secretion from adipose tissue can augment gonadotrophin

synthesis and secretion ultimately contributing to Sertoli cell development in well-nourished calves.

In addition to the above, our gene co-expression analysis also revealed that animals offered a high plane of nutrition and having greater testicular Sertoli cell abundance displayed gene expression profiles in adipose tissue that were positively associated with oxidative phosphorylation as well as fatty acid elongation. This is in agreement with the documented earlier attainment of puberty in animals displaying greater adiposity, as evidenced by the immunohistological analyses of English et al. (2018). Additionally, our gene co-expression analyses revealed genes involved in PI3K-Akt signalling as positively correlated with Sertoli cell number. Indeed, within the published literature, Roa and Tena-Sempere (2014), Jin and Yang (2014) and Acosta-Martinez (2011) have all proposed a role for the PI3K-Akt signalling pathway for the integration of metabolism with reproductive processes, with our results indicating a contribution to puberty attainment dependent on prevailing dietary management. However, we did not establish evidence for a role for this biochemical signalling pathway within the hypothalamic-pituitary-testicular axis.

Arcuate Nucleus and Anterior Pituitary

As outlined above, our molecular evaluations through differential gene expression analysis yielded limited results for both the arcuate nucleus and anterior pituitary tissues of Holstein-Friesian bulls offered varying planes of nutrition, when tissues were analysed on their own. However, subjecting the same datasets to WGCNA revealed genes contributing to sexual development and specifically SC abundance that weren't apparent from differential expression analyses alone. For example, within the arcuate nucleus we identified two separate networks of

co-expressed genes that were positively correlated with Sertoli cell number. Of these two networks one included genes primarily involved in processes including ubiquitin mediated proteolysis, whilst the other comprised of genes coding for proteins of the inner mitochondrial membrane. These results indicate that increased cellular metabolic activity within the arcuate nucleus of animals on the high plane of nutrition is contributing to the greater testicular abundance of SC evident in these animals.

Currently, information on the molecular regulation of puberty in bulls is quite limited, particularly so in relation to genetic variations associated with puberty. Dias et al. (2017) and Fortes et al. (2013) have each identified SNPs associated with puberty in cattle using SNP-detection from RNAseq data and GWAS, respectively. One gene in particular, *PENK*, identified by Dias et al. (2017) as associated with puberty, was also included within each of the two arcuate nucleus co-expression networks positively associated with SC number. *PENK* was also previously identified as a gene harbouring a SNP associated with fertility in Brangus heifers (Peters et al., 2013). This gene codes for pro-enkephalin, forming a part of the opioid system, influencing the hypothalamus-pituitary-gonadal axis (Subrian et al., 2011). The opioid system produces neuroendocrine products that are involved in the release of GnRH and consequently FSH and LH from the anterior pituitary (Subrian et al., 2011). Thus the positive correlation observed in our analysis between *PENK* and SC number indicates that expression of this gene within the arcuate nucleus may be influencing SC development, more than likely through interaction with the FSH receptor that is expressed exclusively in SC (McLachlan et al., 1995).

Similar to the arcuate nucleus region, within the anterior pituitary, two separate networks of co-expressed genes involved in cellular proteolysis and the mitochondrion as well as an additional network involved in kinesin complex and chromatin remodelling were all positively associated

with SC number. The kinesin complex and chromatin remodelling network complements the differential expression of genes involved in cellular division within this tissue type as reported by English et al. (unpubl.). A number of genes of these co-expressed networks have previously been implicated in pubertal status in cattle or have defined functions in reproductive processes. These included the secretogranin genes, *SCG2* and *SCG3* were both positively associated with SC number and were also identified to be harbouring SNPs associated with puberty at the transcript level in the data of Dias et al. (2017). Secretogranins are neuroendocrine secretory proteins, involved in the packaging of peptide hormones and neuropeptides into secretory vesicles for uptake in target cells. Additionally, *PROP1* encodes a protein involved in the development of the pituitary gland as well as the production of hormones including LH, FSH and GH (D'Elia et al., 2001; Raetzman et al., 2002; Scully and Rosenfeld, 2002) and was also associated with SC number. Indeed from their work on pubertal status in heifers, Canovas et al. (2014) concluded that *PROP1* was a key transcription factor involved in the regulation of puberty.

A network of genes, including genes involved in the RNA-induced silencing complex, within the anterior pituitary was negatively associated with SC number was of particular interest. This multi-protein complex elicits a gene-silencing effect leading to transcriptional repression. The negative correlation between these genes and SC number highlights a continuation of cellular transcription and prevention of transcriptional repression within the anterior pituitary that may be contributing to the greater SC number evident in the animals on the high plane of nutrition.

Testes

Although the hypothalamus and anterior pituitary demonstrated a limited number of DEG associated with reproductive function in our differential expression study, an up regulation of

genes associated with cholesterol and androgen biosynthesis was evident within testicular tissue of bull calves offered a high plane of nutrition, consistent with higher systemic concentrations of testosterone (English et al., 2018a). Bull calves offered a high plane of nutrition in that study displayed histological characteristics of mature testes including greater SC abundance, more advanced lumen development and stage of spermatogenesis as discussed above and had reduced testicular expression of genes involved in SC maturation such as *CLAUDIN11* and *AMH* at 18 weeks of age. *CLAUDIN11* and *AMH* have been previously associated with disruption in SC tight junctions (Guan et al., 2014) and a greater number of SC still at the differentiation stage (Vigier et al., 1984), respectively. Despite CLAUDIN11 and AMH displaying differences when analysed by qPCR, these genes were not differentially expressed between planes of nutrition in RNAseq data. Our WGCNA highlighted a network of co-expressed genes within the testes dataset that was positively correlated with SC number including genes with a primary function involved in cholesterol biosynthetic process and cholesterol and lipid homeostasis. Cholesterol is essential for the production of all steroid hormones, and consequently its availability is vital for their optimal production (Hu et al., 2010). One gene in particular within this network, *CBLN2*, believed to be involved in synaptogenesis induction, was previously harbouring SNPs associated with puberty in cattle (Dias et al., 2017).

Conclusions

Systemic concentrations of metabolic hormones, IGF-1, insulin and to a lesser extent leptin, typically increase in response to an enhanced plane of nutrition in calves that is associated in turn with results elevations in LH secretion. This leads to advanced testicular development during early life with concomitant increases in testosterone production. The potential of early life nutrition to dictate a bull's lifetime performance should not be underestimated. Indeed, there is

now substantial evidence that dietary restriction in early life cannot be mitigated by enhancing nutritional input thereafter nor does a reduction in plane of nutrition after an animal reaches six months of age impinge on the advantages obtained from prior preferential dietary management. Molecular based evaluation of tissues of the HPT-axis as well as endocrinologically important tissue such as adipose provides evidence for the interaction between metabolic tissues and those of the HPT in regulating sexual development in the bull. In particular biological processes including oxidative phosphorylation, proteolysis, RISC and cholesterol biosynthesis contribute to the development of SC within the testes. Further analysis and validation of key 'hub' genes and proteins with the HPT and associated metabolic tissues may contribute towards marker assisted genomic selection programs for the identification and selection of bulls with greater propensity towards sexual precocity.

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Figure legends

Figure 1. Mean serum luteinizing Hormone (LH), follicle stimulating hormone (FSH) and testosterone concentrations in pre-pubertal Holstein-Friesian bulls (Adapted from Byrne *et al.*, 2018b; English *et al.*, unpubl.)

Figure 2. Mean (± SEM) systemic concentrations of adiponectin, IGF-1, insulin and leptin in pre-pubertal Holstein-Friesian bull calves (Adapted from Byrne et al., 2018a).

Figure 3. Biological processes contributing to Sertoli cell development. Transcriptional profiling of tissues of the HPT-axis as well as adipose tissue revealed biological processes that contribute to Sertoli cell development within the testes. All processes presented were positively correlated with Sertoli cell abundance, with the exception of GnRH signaling within the adipose tissue and RISC within the anterior pituitary, that were negatively associated with Sertoli cell development.