LATE GESTATION MATERNAL OVERNUTRITION ALTERS GENE EXPRESSION AND HISTOMORPHOLOGY IN NEONATAL FOAL TESTES

by

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A thesis submitted in partial fulfillment
of the requirements for the degree

of

Master of Science

in

Animal and Range Sciences

MONTANA STATE UNIVERSITY
Bozeman, Montana

May 2023

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DEDICATION

I would like to dedicate this thesis to every supporter that has enabled me to pursue my dreams and goals over the course of my life.

Specifically, the completion of this thesis would not have been possible without the support and guidance from my mentor and advisor, Dr. Amanda Bradbery. Thank you for believing in my abilities (more than I sometimes do) and pushing me to be better every day. The teaching, research, and learning opportunities you have provided me are invaluable. Thank you for opening doors for the advancement of my career and allowing me the opportunity to stick around at MSU a bit longer. I am looking forward to becoming a better scientist and teacher under your guidance in these next few years.

To my fellow graduate students, thank you for your friendship and support throughout the course of this degree. Having a sounding board for ideas, rants, questions, and life planning has been invaluable to my thought process and response to life in general. Thank you for supporting my ideas and efforts in the Graduate Student Organization, dealing with far too many emails from me, and indulging my requests for socialization. I hope that the bonds we have formed now continue to grow throughout the course of our lives. To my colleagues sticking around for another degree, I look forward to many more family dinners, office chats, writing groups, and brain breaks in our future.

Thank you to my committee members, Dr. Sarah McCoski, Dr. Rodrigo Marques, and Dr. Christian Posbergh for your guidance and assistance in the completion of this thesis and support of my graduate studies over the past two years. Your support and mentoring have shaped me into a better student and scientist.

To my wonderful boyfriend, Teddy – thank you for your unwavering support of my educational, career, and life choices. Over the past four years you have shown an uncanny ability to keep me focused and grounded while also bringing joy and laughter to every day, and I appreciate that more than you know.

To my family members who are no longer with us – Dad, Aunt Irene, Tia Linda, Grandpa, Grandma Ann, and Nana – thank you for nurturing me, supporting my growth, and always showing interest in my passions.

Lastly, I could not be where I am today without the support of my amazing mom, Debbie Trauner – my day one, ride or die, and best friend. (Derek, you're pretty cool too, I guess). Mom, thank you for allowing me to pursue my passions no matter the circumstances and never letting me believe I couldn't do something if I put my mind to it. Sorry I picked an expensive hobby but thank you for always supporting that hobby and allowing me to pursue it as far as I wanted to (which is multiple graduate degrees, apparently). Life hasn't always been kind to us, but I am glad to have had you by my side through all of it. These few sentences do not even begin to encompass how grateful I am for your unending support and friendship. Thanks, mom!

ACKNOWLEDGEMENTS

This project would not be possible without the knowledge, contributions, and collaboration from Dr. Amanda Bradbery, Dr. Sarah McCoski, and Dr. Carey Satterfield. Thank you to Texas A&M University for providing the animals and facilities necessary for completion of this project. Funding for this project was provided by Montana State University College of Agriculture.

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ABSTRACT

While obesity has deleterious effects on metabolism, performance, lameness, and health in mature horses, little is known about the impacts to reproductive health in foals from overfed dams. The objective of this study was to determine the effects of maternal overnutrition on neonatal foal testicular development. To test this, 16 Quarter horse mares were used in a completely randomized design and stratified by expected foaling date, body weight, and body condition score into one of two dietary treatments offered twice daily beginning on gestational d 235: control (CON; n = 8) fed to meet nutrient requirements and overfed (HIGH; n = 8) fed 40% above CON. At 5 h postpartum, foals were humanely euthanized, and testes were harvested, weighed, and stored for analyses. RNA was extracted and submitted for RNA sequencing. Histomorphometric evaluations were performed using a hematoxylin & eosin stain. ImageJ software was used to evaluate individual seminiferous tubule (ST) area, ST number, and % area of STs relative to interstitial tissue, as well as Sertoli cell numbers per ST. Data were analyzed using the MIXED procedure in SAS. Testes from HIGH male foals (n = 4) were 61% larger than testes from CON male foals (n = 4; P < 0.01). RNA sequencing revealed 56 differentially expressed genes between HIGH and CON groups (P-adjusted < 0.05). These genes represent biochemical pathways involved in oxidative phosphorylation, fatty acid metabolism, and thermogenesis, which are integral for testicular development and function. Gene ontology analyses revealed differential gene expression was associated with biological processes relevant to reproductive function and spermatogenesis, including cellular respiration and mitochondrial function. Histological analyses revealed no difference in individual ST area (P = 0.76) or Sertoli cell number within STs (P = 0.57); however, testes from CON foals had 2.3x more STs per 10X field than HIGH foals (P = 0.01) along with STs representing a greater percentage of the image area relative to interstitial tissue (P = 0.05). These data from the neonatal foal indicate that late-gestation maternal overnutrition alters fetal testicular development with potential long-term implications.

INTRODUCTION

Equine breeders and producers are greatly impacted by the reproductive success of their herds, yet little attention is given to fertility and reproductive efficiency when selecting equine breeding stock. Compared to livestock industries that incorporate factors such as age at puberty, first service conception rates, dystocia rates, and sperm motility and morphology into their breeding stock selection, equine producers tend to use performance success, temperament, lifetime earnings, conformation, offspring earnings, and coat color to select breeding stock. In horses, the average conception rate is 62-67% per cycle or up to 85% at end-of-season following standard industry breeding practices such as live cover or artificial insemination (AI) (Sullivan et al., 1975; Nath et al., 2010; Lane et al., 2016; Lebedeva et al., 2021). These rates are dwarfed by end-of-season conception rates in beef cattle of up to 97.5% (Lardner et al., 2015; Moorey and Biase, 2020). Great effort and capital compensate for these low pregnancy rates and suboptimal fertility in horses. Assisted reproductive technologies (ARTs) such as AI, embryo transfer, and intra-cytoplasmic sperm injection (ICSI) allow poor quality sperm to be viable for breeding. While ARTs are a valuable tool for horse producers, they may contribute to the propagation of mediocre reproductive traits by compensating for low fertility.

These reproductive complications are further exacerbated by suboptimal management leading to stress, health complications, and malnutrition. While obesity has deleterious effects on metabolism, performance, lameness, and overall health in mature horses, little is known about how the reproductive health of foals may be impacted when their dams are overfed. To date, a single study in the horse has investigated the effects of gestational plane of nutrition on testicular development, however, maternal diets represented a control/traditionally fed or a forage-

only/underfed group. Maternal undernutrition resulted in delayed testicular maturation in horses at 12 months of age, marked by low lumen scores and lower expression of genes associated with testicular maturity (Robles et al., 2017). Work in other species suggests maternal overnutrition can lead to impaired testicular development and premature aging (Rodriguez-Gonzalez et al., 2015; Weller et al., 2016).

This study explores the implications of late-term maternal overnutrition on morphology and gene expression in the testes of neonatal foals. The findings of this study will be critical to advancing management strategies to improve reproductive efficiency, performance, and health of equine breeding stock and their offspring.

LITERATURE REVIEW

Obesity and Overfeeding in the Equine Industry

Obesity and overfeeding are an ever-growing problem in the equine industry. While there are many scales for assessing body condition score (BCS), horses with a BCS of ≥ 7 on the Henneke 9-point scale are generally accepted as obese, while horses with a BCS of > 5 are considered overweight (Henneke et al., 1983). The Henneke body condition scale provides guidelines for evaluating adiposity in horses based on six major anatomical areas and overall appearance and was originally developed to assess nutritional status in gestating mares. An ideal and healthy BCS is a 5 out of 9 on the Henneke scale, with some horses fluctuating depending on the season, available feed supply, and use of the horse. Broodmares are typically considered ideal at a BCS of 6 out of 9 to ensure adequate nutritional status for conception and pregnancy maintenance. Despite clear expectations and descriptions of each score, BCS scales are subjective and frequently misunderstood, leading to improper evaluation of adiposity and subsequent lack of management to reduce weight in overweight horses.

Particularly in pleasure and moderate-level performance horses in developed countries, up to 51% of the population is considered overweight or obese (Thatcher et al., 2012). Thatcher's study, completed in Virginia, is supported by several studies completed in other regions worldwide (Wyse et al., 2008; Stephenson et al., 2011; Giles et al., 2014). In the United Kingdom, obesity prevalence in leisure horses was 54% after correction for underestimation of BCS by owners (Stephenson et al., 2011), while 45% of horses were considered overweight or obese in Scotland (Wyse et al., 2008). A study completed in England determining only obesity (BCS ≥ 7) found that 27.08% of horses were obese at the end of winter, 35.41% at the end of

summer, and that 41.76% of horses had at least one instance of obesity over the course of the study (Giles et al., 2014).

To exacerbate this issue, many horse owners tend to underestimate BCS. Stephenson et al. (2011) found that more than 50% of the owners surveyed rated their horse's BCS at least one grade lower (on a 5-point scale) than the researcher's rating, indicating a drastic difference in how owners perceive their horse's weight and health. Similarly, in Australia, 40% of owners significantly underestimated their horse's BCS (Potter et al., 2016). The shift from horses being viewed as pets rather than livestock and working animals contributes to this tendency to overfeed and underestimate weight and body condition. Owners may underestimate their horse's BCS due to lack of education on proper parameters of the scoring system or bias preferring the aesthetics of an overweight horse. In show animals, this is compounded by the tendency of judges to prefer overweight ponies (Pratt-Phillips et al., 2022). Judges across discipline and breed associations were found to incorrectly identify BCS in horses and ponies – identifying overweight horses as average and average horses as thin. The same judges were more likely to penalize a "thin" horse or pony over an overweight horse or pony (Munjizun and Pratt-Phillips, 2021). This prevalence of obesity, combined with ignorance or lack of understanding from owners, creates a cycle that perpetuates weight gain and unhealthy BCS in developed equine populations worldwide. Obesity and overfeeding in broodmares have the potential to impact the health, performance, and reproductive success of equine offspring through fetal programming.

Developmental Programming from Maternal Malnutrition

Fetal programming is defined as how environmental factors during pregnancy and fetal development play a role in offspring health and production outcomes later in life. Maternal

nutrition plays a key role in fetal programming. Dr. David Barker first proposed this hypothesis of fetal origins of adult disease – also known as developmental origins of health and disease – suggesting that adverse nutrition during gestation can impact health outcomes of the offspring in adult life (Barker, 1998). One of the most known examples of maternal nutritional programming is the Dutch famine or Dutch Hunger Winter during World War II. Children with mothers who were pregnant during this famine later experienced a variety of health issues including obesity, high blood pressure, cardiovascular issues, renal dysfunction, memory loss, and diabetes (De Rooij et al., 2022). Maternal overnutrition can cause obesity, disrupted reproductive cyclicity and hormonal regulation, increased follicular atresia and lower follicle numbers, and altered onset of puberty in offspring (Connor et al., 2012; Léveillé et al., 2014; Weller et al., 2016). Addiction and hedonistic behavior can also be caused by maternal overnutrition, along with altered learning capability and other behavioral or neurologic impairments in mice and rats (Montalvo-Martínez et al., 2018; Sarker et al., 2018; Hasebe et al., 2021).

Both under and overnutrition have known impacts, however overnutrition has been studied less in livestock species. In some cases, the application of undernutrition or overnutrition can result in similar changes in offspring phenotype and development. This is particularly apparent in ovine models, which experience intrauterine growth restriction (IUGR) following both gestational overnutrition and undernutrition. Offspring from both underfed and overfed groups had similar changes in pancreatic β-cell number and cardiac ventricular weight:fetal weight prenatally, and similar changes in plasma insulin, plasma glucose, plasma leptin, growth rate, adiposity, and appetite postnatally compared to control groups (Ford and Long, 2011). Studies of maternal undernutrition are suitable models for most livestock species encountering

drought or other diet-limiting conditions, while overnutrition is more realistic for horses, pets, and humans. Discoveries on the effects of maternal overnutrition in animals may serve as an appropriate and useful model for humans.

Transgenerational impacts of maternal nutritional programming have been demonstrated across species. Rats from protein-restricted mothers were born with lower birth weights and brain weights, and impaired cerebral function, which persisted to the F2 generation (Zamenhof et al., 1971). F2 children of fathers (F1) whose mothers (F0) were exposed to famine during the Dutch Hunger Winter were heavier and had higher body mass index (BMI) compared to fathers who were not prenatally exposed to famine (Veenendaal et al., 2013). Additionally, sex-specific transgenerational responses have been recorded. Lower food supply available to paternal grandfathers was linked to increased mortality in grandsons but not granddaughters, while lower food supply available to maternal grandfathers was linked to increased mortality in granddaughters but not grandsons (Pembrey et al., 2006). Sex-specific responses to maternal nutritional programming in both F1 and F2 generations provide evidence of a complex network of factors that may affect offspring for generations to come. To understand the impacts of fetal programming on reproductive health in male offspring, the development and function of the male reproductive system must first be described.

Male Reproductive Development

Prenatal

Information specific to the horse regarding the timeline of organogenesis and gonadal development is limited. Genital ridge formation occurs by day 30, with phenotypic sexual differentiation beginning around day 40 (Figure 1; Gier and Marion, 1969). Sex differentiation is

complete prior to day 45 of gestation in horses, and fetal sex can be determined by day 60 of gestation by transrectal ultrasound (Figure 1; Aurich and Schneider, 2014; Scarlet et al., 2021). The presence of sex-determining region Y (SRY) protein, also known as testes determining factor (TDF), initiates differentiation of pre-Sertoli cells, allowing the progression of male sexual differentiation (McLaren, 1991; Albrecht and Eicher, 2001; Skinner and Anway, 2005; Kashimada and Koopman, 2010). Anti-Müllerian hormone (AMH) secreted by early Sertoli cells initiates sexual differentiation by stimulating regression of the Müllerian ducts, followed by organization of cells into seminiferous cords within the testes. Early Sertoli cells organize themselves around primordial germ cells, with mesenchymal cells migrating shortly thereafter to surround the Sertoli-germ cell complex (Jost et al., 1981; McLaren, 1991). Seminiferous tubules are well developed and evenly distributed throughout the testes by gestational day 60 in male equine fetal gonads (Figure 1; Scarlet et al., 2021).

Equine fetal gonads undergo a period of hyperplasia during development unique from other mammals, beginning as early as day 40 of gestation (Figure 1; Barreto et al., 2018). The hyperplasia of interstitial cells in male and female gonads contributes to rapid growth throughout mid-gestation, where fetal gonads can become larger than the dam's mature ovaries (Cole et al., 1933; Wesson and Ginther, 1980; Barreto et al., 2018). This hyperplasia peaks around day 250 of gestation, after which interstitial cells begin to degenerate and undergo hypotrophy, contributing to the regression of gonadal size until parturition (Figure 1; Cole et al., 1933; Wesson and Ginther, 1980).

Most equine fetal growth occurs during the third trimester of gestation. This increase in growth is accompanied by an increase in energy demands of the mare (Meyer and Ahlswede,

1978; Platt, 1984; NRC, 2007). Due to increased energy demands, industry standard practice includes supplementing mares starting in the third trimester of gestation to meet additional nutrient requirements, provided in the form of ration balancers, protein tubs, or mare and foal concentrates. During the third trimester, the testes complete their final stages of development and descend into the scrotum. Movement through the inguinal canal begins between day 270 and 300 of gestation, with full descent of both testes occurring after day 315 of gestation (Figure 1; Bergin et al., 1970). Movement through the inguinal canal can only begin after the aforementioned decrease in size of the testes after day 250 of gestation.

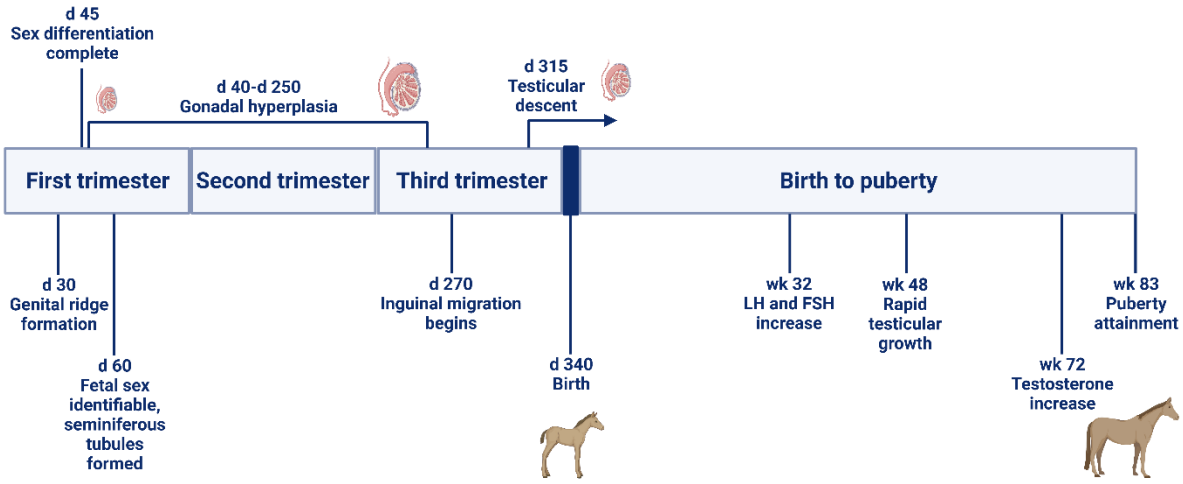


Figure 1. Timeline of reproductive development in male equine offspring from conception through puberty. d = day. wk = week. LH = luteinizing hormone. FSH = follicle stimulating hormone. Adapted from Cole et al., 1933; Gier and Marion, 1969; Wesson and Ginther, 1980; Naden et al., 1990; Clay and Clay, 1992; Aurich and Schneider, 2014; Barreto et al., 2018; Scarlet et al., 2021.

Postnatal

By 2 weeks of age, testes should now be present in the scrotum (Bergin et al., 1970). Stud colts maintain an infantile phenotype until week 32 postpartum (~7 months), marked by low

gonadotropin concentrations and minimal gonadal changes. Increased levels of LH and FSH at this point, along with rapid testicular growth beginning at 48 weeks, indicate the beginning of the pre-pubertal period (Figure 1; Naden et al., 1990; Clay and Clay, 1992).

Puberty (defined as the first ejaculate containing $\geq 50 \times 10^6$ total sperm at 10% motility) occurs around 83 weeks (~19 months) (Figure 1; Naden et al., 1990; Clay and Clay, 1992). Considering that spermatogenesis takes 57 days in horses, it is estimated that spermatogenesis begins at or before 75 weeks (~17 months) of age. This coincides with a rapid increase in testosterone concentrations beginning at week 72 (Figure 1; Naden et al., 1990). Following attainment of puberty, testicular growth and spermatogenesis continue to increase until 2 years of age and are estimated to continue developing until 5 years of age (Johnson and Thompson, 1983; Naden et al., 1990). Completion of puberty leads to functionality of the testes, including full spermatogenic potential.

Function of Mature Male Reproductive Organs

Mature testes produce sperm and play a fundamental role in hormonal signaling and feedback in the male reproductive system. Seminiferous tubules serve as the sole site of spermatogenesis, which is regulated by endocrine signaling within the hypothalamic-pituitary-gonadal (HPG) axis. Gonadotropin releasing hormone (GnRH) from the hypothalamus stimulates release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the pituitary (Figure 2). In the interstitial tissue of the testes, between seminiferous tubules, Leydig cells produce testosterone in response to LH, stimulating sperm production within the tubules. Testosterone binds to androgen binding protein (ABP) produced by the Sertoli cells in response to FSH (Figure 2). Testosterone from the Leydig cells and inhibin from Sertoli cells elicit negative

feedback to the hypothalamus and pituitary, regulating the release of GnRH and overall endocrine signaling for the male reproductive system (Figure 2). Testosterone is also responsible for secondary sex characteristics, including increased muscle development and libido. Maternal malnutrition is linked to altered sex hormone concentrations and associated changes in sexual behavior (Rhees and Fleming, 1981; Jacobs et al., 2014). Male offspring from overfed dams had decreased plasma testosterone and FSH, along with reduced displays of mating behavior compared to offspring of control fed dams (Jacobs et al., 2014). Similar effects were seen regardless of the timing of overnutrition – for groups overfed solely before pregnancy, during pregnancy and lactation, or before, during, and after pregnancy (Jacobs et al., 2014).

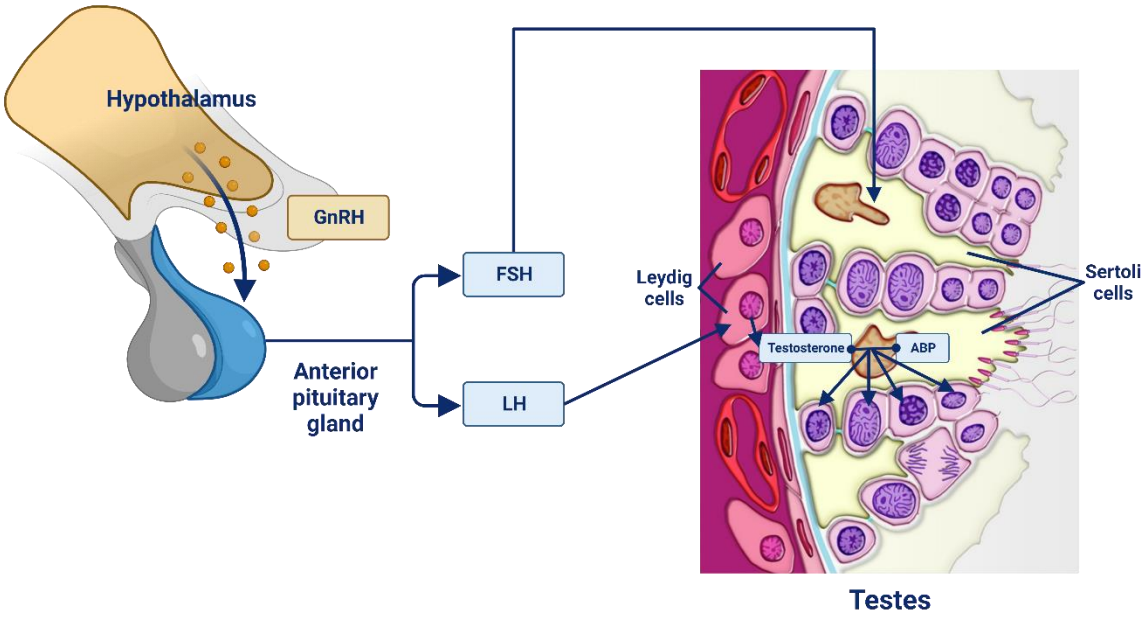


Figure 2. Hypothalamic-pituitary-gonadal (HPG) axis regulating spermatogenesis. Adapted from Jennings and Premanandan, 2017; Shah et al., 2021.

Spermatogenesis occurs through constant proliferation and maturation of spermatogonia. Beginning in the basement membrane as primordial germ cells/spermatogenic stem cells, sperm undergo a succession of mitotic and meiotic divisions. As they mature, these cells move away from the basement membrane into the adluminal compartment containing meiotic and post-meiotic germ cells, ultimately moving towards the lumen in the center of the seminiferous tubule. Sertoli cells within the tubules act as "nurse cells", supporting the development of spermatogonia into mature sperm cells. Sertoli cells also form tight junctions at the basement membrane of the seminiferous tubules, helping form the blood-testis-barrier. This barrier regulates substances in circulation and prevents immune cells from entering the tubules, protecting maturing spermatocytes.

Neonatal seminiferous tubule area and Sertoli cell number are correlated with future sperm production potential. Decreased seminiferous tubule area and lower Sertoli cell number are associated with decreased sperm counts and fertility at sexual maturity (Orth et al., 1988; Rodriguez-Gonzalez et al., 2015). The Sertoli cell number is established pre- and perinatally in mammals; therefore, maternal insults to Sertoli cell development can persist without opportunity for re-alimentation later in life.

Developmental Programming of Reproductive Health

Maternal plane of nutrition is one such maternal insult implicated in impaired fetal development, with impacts to morphology, hormone signaling, neuroendocrine regulation, fertility, and gene expression. Maternal overnutrition adversely affects normal morphological development of testicular tissue. Previous work in cattle overfed from day 60 of gestation found smaller diameter, length, and volumetric proportion of seminiferous tubules in calves from

overfed dams (Weller et al., 2016). Similarly, male rats from dams overfed from weaning through pregnancy and lactation had smaller seminiferous tubule area as well as fewer spermatogonia and spermatocyte numbers (Rodriguez-Gonzalez et al., 2015). Interestingly, the same differences are not observed in sheep. Lambs from dams overfed throughout pregnancy did not show different seminiferous cord or Sertoli cell numbers at the start of the third trimester compared to control lambs (Da Silva et al., 2003). Similarly, no differences in Sertoli cell number were found after maternal undernutrition throughout pregnancy in sheep (Andrade et al., 2013).

For these sheep models, researchers hypothesize that maternal nutrition primarily influences the male reproductive system through the hypothalamic-pituitary-adrenal (HPA) and HPG axes during development and puberty rather than through gonadal histology or gene expression differences *in utero*. While no differences in testicular morphology or gene expression were discovered at the beginning of third trimester from underfed or overfed ewes (Da Silva et al., 2003; Andrade et al., 2013), a similar experimental model resulted in impaired reproductive development in male offspring from birth through week 35 of age (Da Silva et al., 2001). Male offspring from adolescent ewes overfed throughout gestation had lower birth weights and testicular volume compared to offspring from control fed dams. In these growth-restricted offspring, puberty was delayed by 5 weeks and peak testosterone levels remained lower throughout development and puberty (Da Silva et al., 2001).

In rats, maternal overnutrition decreases fertility, increases sperm abnormalities, and decreases key reproductive hormones such as LH and testosterone (Rodriguez-Gonzalez et al., 2015; Meneghini et al., 2022). Rats from overfed dams presented with a higher percentage of

sperm that failed to complete capacitation, reduced mitochondrial activity in sperm, and increased tail abnormalities (Meneghini et al., 2022). These alterations could affect sperm motility and ultimately the ability of sperm to successfully fertilize an oocyte. Similarly, decreased sperm viability, motility, and concentration were associated with decreased fertility after maternal overnutrition (Rodriguez-Gonzalez et al., 2015). Also in rats, adult offspring from overfed dams had decreased testicular size, decreased testosterone, and increased serum AMH levels (Kalem et al., 2018). The diets in these studies, however, provided overnutrition before, during, and after pregnancy.

Maternal overnutrition and obesity affect the antioxidant defense systems in the testes of male offspring. These defense systems are key for maintaining reproductive health and preventing premature aging by combatting reactive oxygen species (ROS). Testes are particularly vulnerable to ROS because of their high levels of unsaturated fatty acids and potential for lipid peroxidation (Aitken and Roman, 2008). Altered oxidative stress biomarkers such as superoxidase dismutase (SOD), glutathione peroxidase (GPx), catalase, malondialdehyde (MDA), nitrotyrosine reactivity, and dichlorofluorescein (DFC) were found in the testes and sperm of rats with mothers who were overfed throughout pregnancy (Rodriguez-Gonzalez et al., 2015; Bautista et al., 2017). These changes in oxidative stress were associated with decreased fertility and sperm quality, as previously described by Rodriguez-Gonzalez et al. (2015)

It is widely accepted that an organism's phenotype is a result of its genotype combined with environmental influences. While an organism's DNA is consistent across its cells, levels of transcription of certain genes into RNA and ultimately proteins can be impacted by environmental cues. Alterations in gene expression impact cell function, in turn causing

morphological changes in body tissues and impacting the animal. Techniques such as RNA sequencing and qPCR can be used to determine changes in these gene expression levels, beginning to elucidate how various conditions impact tissue or cell types. Work in cattle found lower expression of steroidogenic acute regulatory protein (*StAR),* insulin-like growth factor 1 *(IGF1),* insulin-like growth factor 2 *(IGF2),* and insulin-like growth factor 1 receptor (*IGF1R)* genes in fetal testes of calves with overfed dams (Weller et al., 2016). Investigation of gene expression levels in conjunction with morphological evaluation can begin to build a story of biological changes induced by maternal overnutrition and how they may affect function of specific organs and organ systems.

Developmental Programming in the Horse

Research on fetal programming effects from maternal malnutrition in the horse is newly emerging. Recent studies using an equine model indicate that gestational undernutrition can negatively impact testicular development, bone development, and insulin regulation/glucose tolerance in horses (Robles et al., 2017). Offspring from dams fed a forage-only diet throughout gestation had narrower cannon bones at 19 and 24 months of age (Robles et al., 2017). Reduced testicular development at 12 months of age was marked by lower lumen scores associated with lower expression of genes associated with testicular maturity such as cytochrome P450 family 19 (*Cyp19*), gap junction alpha-1 protein or connexin 43 *(Cx 43),* androgen receptor *(AR),* and *STAR* as well as higher expression of *AMH,* associated with testicular immaturity. The same offspring tended towards insulin dysregulation at 24 months of age after an offspring overnutrition challenge (Robles et al., 2017). Maternal obesity at the time of insemination produced offspring

who had higher inflammatory markers, greater insulin resistance, and greater risk of developing osteochondrosis (Robles et al., 2018).

Maternal overnutrition in horses changes muscle fiber size and type as well as gene expression and insulin signaling in skeletal muscle of neonatal foals (Bradbery et al., 2021a). Foals from dams overfed during late gestation had larger muscle fibers and a shift towards type I fibers, indicating potential for decreased athletic performance later in life. These foals also displayed muscle-specific changes in gene expression and protein signaling related to insulin regulation, indicating the potential for future metabolic dysregulation (Bradbery et al., 2021a). Maternal overnutrition has also been shown to alter pancreatic islet development which can contribute to development of metabolic disease later in life. Foals from overfed dams had increased islet number and size – a marker of β-cell compensation and potential precursor for insulin resistance (Bradbery et al., 2021b).

Implications in the Horse

While obesity is not typically considered within breeding soundness exams, maternal obesity and overnutrition may contribute to dysregulated reproductive function in male equine offspring. Despite the commonality of overfeeding in the equine industry, minimal research has described the impacts of maternal plane of nutrition on foal reproductive health. Male offspring that are maintained as stallions and advance to breeding may have decreased fertility based solely on how their mothers were fed during gestation. Environmental factors including management and nutrition as these offspring mature can further exacerbate delayed development and decrease reproductive function. It is essential that horse industry professionals account for the effects of maternal nutrition on reproductive development and success of equine offspring.

Determining the impacts of maternal overnutrition on offspring reproductive health will allow for adjustment of management and feeding practices to optimize equine health and performance for future generations of equine athletes. This study was designed to accomplish the objectives of (1) determining histoarchitectural differences in prepubertal testes of neonatal foals in response to maternal overnutrition and (2) evaluating the effects of third trimester maternal overnutrition on the expression of genes in testes tissue associated with testicular development, puberty, and reproductive efficiency. We hypothesized that despite their larger size, testes in colts from overfed mares would have delayed development and changes in gene expression that could detrimentally impact future reproductive performance.

MATERIALS AND METHODS

Animals and Feeding

All animal work was performed at Texas A&M University and approved by the Texas A&M University Institutional Animal Care and Use Committee (AUP #2016-0072). Animal work was performed as previously described (Bradbery et al., 2021a; Bradbery et al., 2021b). Briefly, sixteen Quarter horse mares (initial body weight (BW) 541 ± 17.3 kg, initial BCS 5.96 ± 0.91; LSMeans ± SEM) were used in a completely randomized design. Mares were bred within a 30-day period to a single stallion to reduce genetic variability of the foals.

Mares were maintained on the same pasture at a BCS of 6 until the third trimester of gestation. Prior to third trimester, mares were stratified by BW, BCS, and expected foaling date into one of two dietary treatments beginning on gestational day 235. Both groups received *ad libitum* access to coastal bermudagrass hay (*Cynodon dactylon*) and fresh water for the duration of the study. Treatments consisted of a control (CON; $n = 8$) where all nutrients were fed to meet dietary requirements during late gestation, and an overfed group (HIGH; $n = 8$) where mares received an additional 40% above CON (NRC, 2007). From gestational day 235, CON mares received 0.1% BW as-fed commercial ration balancer (Empower Topline Balance, Cargill Animal Nutrition, Elk River, MN), while HIGH mares received the same as CON plus an additional 0.7% BW as-fed commercial mare and foal concentrate (SafeChoice Mare and Foal, Cargill Animal Nutrition, Elk River, MN; Table 1). Mares were fed twice daily, individually, and concentrate amounts were adjusted every 14 days in accordance with changes in BW to follow NRC guidelines (NRC, 2007). Intake was monitored for refusals, and all horses consumed the entirety of their diets.

Table 1. Nutrient analysis of maternal dietary treatments beginning on gestation day 235.

	Coastal bermudagrass hay	Forage balancer[a]	Mare and foal concentrate[b]
DM, %	90.06	89.14	89.94
CP, %	6.50	34.16	18.26
ADF, %	36.46	7.63	16.84
NDF, %	61.58	16.88	32.17
DE, Mcal/lb	1.07	1.56	1.45
Ca, %	0.29	3.46	1.45
P, %	0.16	1.81	1.07
K, %	1.09	1.82	1.48
Na, %	0.02	0.50	0.49
Cl, %	0.30	0.98	0.98

[a]Nutrena Empower Topline Balance (Cargill Animal Nutrition, Elk River, MN) fed at 0.1% BW daily (as-fed).
[b]Nutrena SafeChoice Mare and Foal (Cargill Animal Nutrition, Elk River, MN) fed at 0.7% BW daily (as-fed).

Tissue Collection and Preservation

At 5 h postpartum, foals were humanely euthanized using pentobarbital (Beuthanasia-D, Merck Animal Health, Madison, NJ), and tissue was harvested. All organs were harvested and weighed with a digital scale. Testes collected from each resulting male foal (CON, $n = 4$; HIGH, $n = 4$) were weighed as a pair including the tunica dartos with the epididymis removed. One testicle from each animal intended for histological analyses was preserved in 4% paraformaldehyde. Testicular samples intended for mRNA analysis were snap frozen in liquid nitrogen and stored at -80°C.

Morphological Analysis

A standard hematoxylin and eosin (H&E) staining procedure was used to determine the effects of maternal overnutrition on foal testicular histoarchitecture. Briefly, sections of paraffin-embedded foal testes 5 µm thick were cut and mounted on Superfrost Excell microscope slides

(Thermo Fisher Scientific, Pittsburgh, PA). Sections were deparaffinized with solvent (CitriSolv, Decon Labs, Inc., King of Prussia, PA) and rehydrated in a series of decreasing concentrations of ethanol followed by double distilled water. Following deparaffinization, Modified Mayer's hematoxylin (Modified Mayer's Solution, Abcam Inc., Cambridge, MA) was used to stain nuclear components of cells, followed by bluing reagent (Abcam Inc., Cambridge, MA) for bluing of the hematoxylin stain. Eosin Y (Abcam Inc., Cambridge, MA) was used to counterstain non-nuclear cell components. Following staining, sections were dehydrated in a series of increasing concentrations of ethanol, then rinsed in CitriSolv (Decon Labs, Inc., King of Prussia, PA), and Permount Mounting Medium (Thermo Fisher Scientific, Pittsburgh, PA) was used for mounting glass cover slips.

A minimum of 10 images per animal at 10× magnification were used to determine seminiferous tubule number, area, and seminiferous tubule:interstitial tissue ratio using ImageJ software version 1.53t (National Institutes of Health, Bethesda, MD). Images were selected randomly from 2-3 sections per animal using methodical scanning on an Evos M5000 Imaging System (Life Technologies Corporation, Bothwell, WA). Area was determined using the Freehand Selection tool on Image J, where scale was set using the Set Scale tool to calibrate ImageJ measurements to the scale bar provided on the image from EVOS M5000 imaging. Area was measured for all complete tubules fully within an image field and averaged across all images to obtain mean seminiferous tubule area for each animal. A minimum of 20 tubules at 40× magnification were selected from each animal to determine Sertoli cell number per seminiferous tubule. Sertoli cell number was determined using the Cell Counter plugin in ImageJ.

RNA Extraction and Sequencing

From snap-frozen samples, 15-20 mg of testes tissue was used for total RNA extraction using the PureLink RNA Mini Kit (Life Technologies, Austin, TX). RNA was extracted according to manufacturer instructions for frozen animal tissue and suspended in RNase-free water. Briefly, testes tissue was homogenized in lysis buffer using a rotor-stator homogenizer. After homogenization, 70% ethanol was added, and the sample was processed through the spin cartridge to bind RNA to the cartridge membrane. Following this process, impurities were removed with a series of washes and RNA was eluted into RNase-free water. RNA concentration and quality were assessed using a NanoDrop One spectrophotometer (ThermoFisher Scientific, Pittsburgh, PA). RNA quality data are presented below (Table 2).

Table 2. RNA concentration and quality at time of isolation, determined by NanoDrop One. (R = CON, Y = HIGH).

Sample ID	Concentration (ng/µL)	A260/280	A260/230
R2	417.1	2.09	2.23
R4	278.7	2.09	1.75
R5	525.3	2.08	2.19
R11	459.3	2.09	2.24
Y3	595.7	2.10	2.29
Y5	547.5	2.10	2.07
Y8	435.0	2.11	2.25
Y13	450.2	2.09	2.18

RNA sequencing was completed by NovoGene (Sacramento, CA) using an Illumina-based sequencing platform. Reads were filtered to remove reads with adapter contamination, reads with more than 10% of uncertain nucleotides, and reads with over 50% of low-quality nucleotides. Clean reads were aligned to the reference genome using HISAT2 v2.0.5. Data were aligned to the EquCab3.0 reference genome.

To quantify the number of reads mapped to each gene, featureCounts v1.5.0-p3 was used. The number of fragments per kilobase of transcript sequence per millions of base pairs sequenced (FPKM) of each gene were calculated based on the length of the gene and the count of reads mapped to this gene. Gene expression levels were quantified by the abundance of transcripts mapped to the genome. Expression level was depicted in FPKM, which considers sequencing depth and gene length. Gene expression levels were compared across all samples in the CON and HIGH groups.

Before differential gene expression analysis, read counts were adjusted by edgeR software. Error rate (Q score) was determined using Qphred = -10log10(e), with e representing the sequencing error rate per sample. Q scores were assigned to each sample, Phred10 (Q10) relates to a 90% correct base calling rate, Phred20 (Q20) indicates a 99% correct base calling rate, Phred30 (Q30) indicates a 99.9% correct base calling rate, and Phred40 (Q40) indicates a 99.99% correct base calling rate. Low Q scores are correlated with a higher percentage of error. GC content distribution of each sample was analyzed to assess sample stability and quality for gene expression quantification.

Read alignment to the reference genome was performed using the HISAT2 alignment program. Results of the read alignment provided mapping results for each sample. Mapping quantified the distribution, number, and percentage of reads mapped to the genome, as well as position (exon, intron, intergenic) strand (+/-), and the number of spliced reads.

Mapping information was placed into the Cufflinks assembler, where transcript fragments were compared to reference transcripts. Sufficiently different transcript fragments were considered novel and defined as novel genes, novel exons of known genes, or modified

versions of known transcripts. Novel gene annotation was determined through bioinformatic pathway analysis. A literature search was completed to determine differentially expressed genes related to the terms “testes” or “reproduction”.

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of differentially expressed genes (DEGs) was completed using clusterProfiler v3.8.1 software. Stringency was relaxed to include DEGs with an unadjusted *P*-value < 0.05 for enrichment analysis.

Statistical Analysis

Samples were numerically coded in a blinded fashion and processed randomly to eliminate bias. Means are reported as LSMeans ± SEM. All data were tested for normality and analyzed using the MIXED procedure in SAS v9.4 (SAS Inst. Inc., Cary, NC). Horse within treatment serves as a random effect, with main effect of treatment. Significance was determined when $P \leq 0.05$ and a trend towards significance when $0.05 < P \leq 0.10$.

For RNA sequencing data, differential expression analysis was performed using the edgeR R package (3.22.5). The *P*-values were adjusted using the Benjamini-Hochberg method (Benjamini and Hochberg, 1995). The threshold for significant differential expression was set at an adjusted *P*-value of less than 0.05. GO terms and KEGG pathways with an adjusted *P*-value of less than 0.05 were considered significantly enriched by DEGs.

RESULTS

Foal Parameters and Testicular Histoarchitecture

Foal birth weight did not differ between HIGH and CON groups (Bradbery et al., 2021b). Testes from HIGH male foals (32.93 ± 1.93 g) were 61% larger than testes from CON male foals (20.50 ± 1.93 g; $P < 0.01$; Bradbery et al., 2021b). Histological analyses revealed no difference in individual seminiferous tubule area ($P = 0.76$) or Sertoli cell number within seminiferous tubules ($P = 0.57$; Table 3, Figure 3). However, testes from HIGH foals (9.35 ± 2.48) had 2.3× less seminiferous tubules per 10× field than CON foals (21.68 ± 2.48; $P = 0.01$), along with seminiferous tubules representing a lesser percentage of the image area relative to interstitial tissue (HIGH 1.60 ± 0.61%; CON 3.66 ± 0.61%; $P = 0.05$; Table 3, Figure 3).

Table 3. Effect of maternal overnutrition on foal testicular histoarchitecture (represented as LSMeans).

	Treatment[1]			*P*-Values
Item	CON	HIGH	SEM	Trt[2]
Mean individual tubule area (μm^2)	1999.30	2070.86	153.81	0.76
Tubule number per field	21.68[a]	9.35[b]	2.48	0.01
Tubule:interstitial (% 10× field)	3.66[a]	1.60[b]	0.61	0.05
Sertoli cell number per tubule	36.63	34.31	2.69	0.57

[1]Treatments consisted of CON fed to meet nutritional requirements and HIGH fed 40% above CON beginning gestation day 235 to term.
[2]Trt = main effect of treatment.
[a,b]Superscripts indicate main effect of treatment ($P \leq 0.05$).

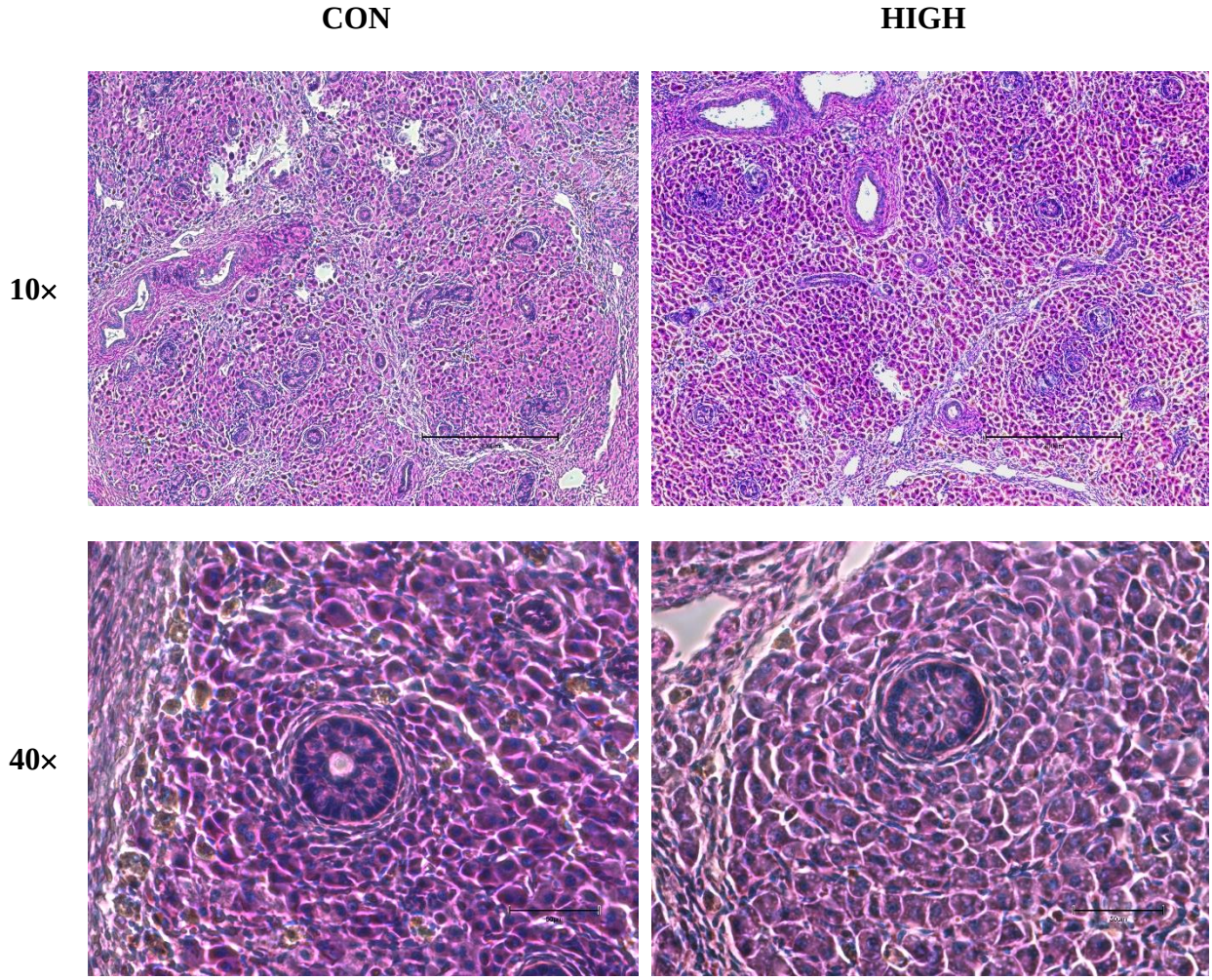


Figure 3. Testicular histoarchitecture at 10× (scale bar = 300 µm) and 40× (scale bar = 50 µm) in foals from control-fed mares (CON) or overfed mares (HIGH).

Foal Testicular Gene Expression

RNA sequencing averaged 49,439,422.75 reads per sample, with 97.28% reads mapped to the equine genome. Fifty-six DEGs were revealed between HIGH and CON groups (*P*-adjusted < 0.05; Table 4). Of these, 48 were down-regulated in the HIGH group while 8 were up-regulated. Six transcripts were found to be related to “testes” or “reproduction”.

Table 4. List of differentially expressed genes (DEGs) for HIGH vs. CON (*P*-adj < 0.05).

Gene Name	Gene ID	log_2FoldChange	*P* - adjusted
FAM227A	ENSECAG00000006137	3.111698	6.55E-06
-	novel.643	1.625173	0.000142
CCDC57	ENSECAG00000015187	-1.728	0.000831
-	novel.2696	-3.50133	0.001332
ENTPD2	ENSECAG00000016865	-1.46128	0.005701
-	ENSECAG00000033570	-3.29605	0.005701
EMILIN3	ENSECAG00000010749	-2.25832	0.011947
KRT86	ENSECAG00000009201	6.886329	0.011947
TOP1MT	ENSECAG00000013129	-1.36591	0.012093
CUX2	ENSECAG00000007841	-2.52509	0.012093
COL16A1	ENSECAG00000024119	-1.31597	0.012093
DEFB4B	ENSECAG00000007755	-1.85609	0.012093
-	ENSECAG00000035459	-3.4971	0.012093
PREX1	ENSECAG00000014676	-1.55027	0.013861
-	ENSECAG00000039441	6.047039	0.013861
RNF207	ENSECAG00000024940	-1.81101	0.013861
-	novel.2526	-6.56345	0.01389
HDAC11	ENSECAG00000002905	-1.4251	0.013981
RASAL3	ENSECAG00000009236	-1.66675	0.014833
EOMES	ENSECAG00000021052	-2.88338	0.016549
SEMA4F	ENSECAG00000010510	-1.80722	0.017377
IGSF10	ENSECAG00000023161	6.721094	0.017451
CYP26B1	ENSECAG00000022724	-2.11908	0.018831
PTPRU	ENSECAG00000012344	-1.29575	0.020248
-	novel.1102	-3.01504	0.020854
CD4	ENSECAG00000009049	-1.77742	0.026633
ADAMTSL4	ENSECAG00000019154	-1.6218	0.026633
-	ENSECAG00000020551	1.199878	0.028302
-	ENSECAG00000035886	-1.8019	0.028302
-	novel.283	-6.2214	0.028302
-	novel.2671	-1.32584	0.029365
GDPD5	ENSECAG00000010098	-1.58953	0.030367
LHB	ENSECAG00000007548	-2.38917	0.033633
APOD	ENSECAG00000022047	-1.18336	0.033763
CDH3	ENSECAG00000009528	-2.1743	0.033763
CHDH	ENSECAG00000012652	-2.09148	0.034963
KIF19	ENSECAG00000019912	-1.72165	0.037325
RUNX3	ENSECAG00000014360	-2.34433	0.038975
-	ENSECAG00000037646	2.132845	0.038975
SPOCD1	ENSECAG00000009300	-3.09537	0.038975
ANHX	ENSECAG00000013669	-2.27051	0.03946

Table 4 Continued.

OTC	ENSECAG00000022838	1.426556	0.03946
KIF21B	ENSECAG00000024729	-2.93244	0.040874
NAV2	ENSECAG00000017807	-1.5076	0.043404
APOBR	ENSECAG00000016962	-2.26781	0.044233
-	ENSECAG00000037978	-6.07389	0.047106
PLCH1	ENSECAG00000016623	-1.51269	0.048473
TMPRSS3	ENSECAG00000000233	-5.90598	0.048473
RF02204	ENSECAG00000040419	-5.83981	0.048473
LAMB3	ENSECAG00000008204	-2.12511	0.048473
KLF15	ENSECAG00000021092	-1.18323	0.048473
OBSCN	ENSECAG00000016720	-2.2133	0.048473
SHC2	ENSECAG00000009683	-2.07471	0.048473
TKTL1	ENSECAG00000021992	-2.16475	0.048473
PVRIG	ENSECAG00000020068	-1.79971	0.048473
AMH	ENSECAG00000036148	-1.47837	0.048473

Eleven KEGG pathways were represented by DEGS in the HIGH and CON groups (*P*-adjusted < 0.05, Table 5). Most notably, pathways representing biological processes such as oxidative phosphorylation, fatty acid metabolism, and thermogenesis were primarily upregulated, while natural killer cell mediated cytotoxicity was primarily downregulated (Figure 4).

Table 5. List of all differentiated KEGG pathways for HIGH vs. CON (*P*-adj < 0.05).

Description	KEGG ID	# DEGs Up	# DEGs Down	*P* - adjusted
Parkinson disease	ecb05012	55	12	6.93E-05
Oxidative phosphorylation	ecb00190	36	2	0.0031
Fatty acid metabolism	ecb01212	17	4	0.0031
Huntington disease	ecb05016	53	16	0.0031
Protein export	ecb03060	11	0	0.008827
Proteasome	ecb03050	16	0	0.008827
Alzheimer disease	ecb05010	57	20	0.008827
Thermogenesis	ecb04714	42	12	0.008827
Fatty acid elongation	ecb00062	10	2	0.008827
Endocytosis	ecb04144	27	32	0.010671
Natural killer cell mediated cytotoxicity	ecb04650	8	21	0.026002

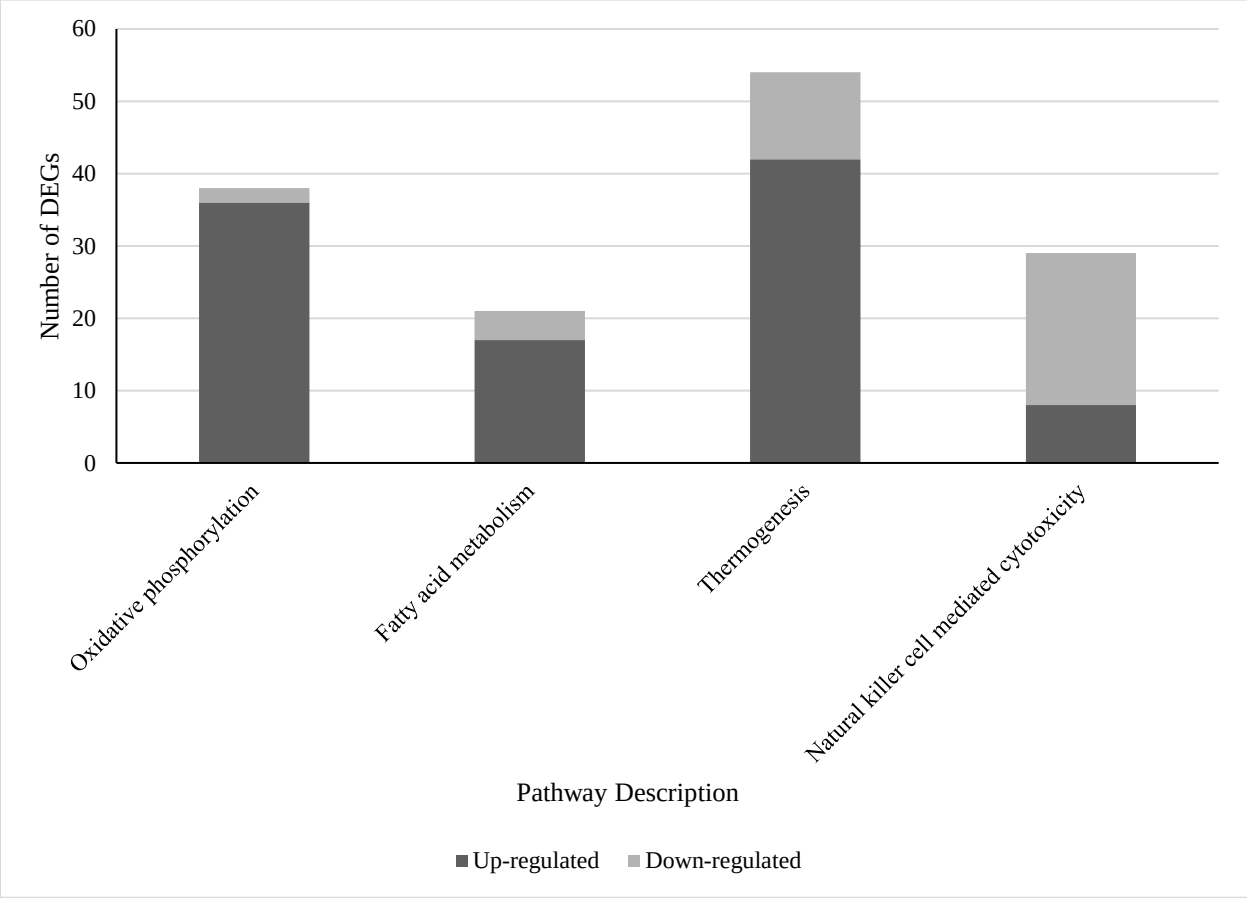


Figure 4. Differentiated KEGG pathways of interest for HIGH vs. CON (*P*-adj < 0.05).

Forty-eight GO terms were represented by DEGs in the HIGH and CON groups (*P*-adjusted < 0.05, Table 6). The top 5 differential GO terms by number of DEGs per term were endoplasmic reticulum part, drug metabolic process, cofactor binding, endoplasmic reticulum subcompartment, and nuclear outer membrane-endoplasmic reticulum membrane network (98, 94, 92, 86, and 86 DEGs, respectively). Two biological processes were found to be different, including drug metabolic process (*P*-adj = 0.008) and cellular respiration (*P*-adj = 0.007, Figure 5). DEGs in the cellular respiration term were found to be primarily upregulated (35 up, 3 down).

Table 6. List of all differentiated GO terms for HIGH vs. CON with (*P*-adj < 0.05).

GO Term Description	Category[1]	GO ID	# DEGs Up	# DEGs Down	*P* - adjusted
cellular respiration	BP	GO:0045333	35	3	0.006775
drug metabolic process	BP	GO:0017144	67	27	0.007818
mitochondrial envelope	CC	GO:0005740	72	10	0.00424
endoplasmic reticulum exit	CC	GO:0070971	7	2	0.00424
endoplasmic reticulum part	CC	GO:0044432	79	19	0.00424
myelin sheath	CC	GO:0043209	28	10	0.00424
endoplasmic reticulum	CC	GO:0005789	70	15	0.005946
endoplasmic reticulum subcompartment	CC	GO:0098827	71	15	0.00698
mitochondrial membrane part	CC	GO:0044455	44	1	0.007347
nuclear outer membrane-endoplasmic reticulum membrane network	CC	GO:0042175	71	15	0.007347
mitochondrial proton-transporting ATP synthase	CC	GO:0005753	9	0	0.00744
proton-transporting ATP synthase complex	CC	GO:0045259	9	0	0.00744
receptor complex	CC	GO:0043235	6	60	0.010145
mitochondrial inner	CC	GO:0005743	49	2	0.010759
mitochondrial membrane	CC	GO:0031966	64	7	0.014416
integral component of endoplasmic reticulum	CC	GO:0030176	23	5	0.015916
peptidase complex	CC	GO:1905368	21	1	0.016158
inner mitochondrial membrane protein complex	CC	GO:0098800	30	0	0.016158
proteasome complex	CC	GO:0000502	17	0	0.016158
myosin complex	CC	GO:0016459	3	14	0.016158
endopeptidase complex	CC	GO:1905369	17	0	0.018018
rough endoplasmic reticulum membrane	CC	GO:0030867	7	0	0.018018
T cell receptor complex	CC	GO:0042101	0	7	0.018018
nucleoid	CC	GO:0009295	11	2	0.018018
mitochondrial nucleoid	CC	GO:0042645	11	2	0.018018
organelle inner membrane	CC	GO:0019866	50	4	0.024947
extracellular matrix	CC	GO:0031012	8	38	0.025097
intrinsic component of endoplasmic reticulum	CC	GO:0031227	23	5	0.025097
mitochondrial protein	CC	GO:0098798	47	0	0.026714

Table 6 Continued.

proton-transporting two-sector ATPase complex	CC	GO:0016469	14	0	0.028597
P-P-bond-hydrolysis-driven transmembrane transporter	MF	GO:0015405	20	12	0.004023
cofactor binding	MF	GO:0048037	63	29	0.004023
primary active transmembrane transporter	MF	GO:0015399	20	12	0.004023
lyase activity	MF	GO:0016829	23	16	0.004023
ATPase activity, coupled to movement of substances	MF	GO:0043492	17	16	0.006794
ATPase activity, coupled to transmembrane movement of substances	MF	GO:0042626	17	12	0.007306
cation-transporting ATPase	MF	GO:0019829	14	4	0.007306
active ion transmembrane transporter activity	MF	GO:0022853	14	4	0.007306
ATPase coupled ion transmembrane transporter	MF	GO:0042625	14	4	0.007306
electron transfer activity	MF	GO:0009055	18	4	0.009204
ATPase activity, coupled to transmembrane movement of ions, rotational mechanism	MF	GO:0044769	12	0	0.009204
glutathione transferase	MF	GO:0004364	9	2	0.012264
proton-transporting ATP synthase activity, rotational	MF	GO:0046933	8	0	0.017784
oxidoreductase activity, acting on NAD(P)H	MF	GO:0016651	17	4	0.029692
hydro-lyase activity	MF	GO:0016836	11	5	0.03786
motor activity	MF	GO:0003774	4	28	0.03786
proton transmembrane transporter activity	MF	GO:0015078	23	4	0.038296
peptide binding	MF	GO:0042277	14	26	0.044591

[1]BP = biological process, CC = cellular component, MF = molecular function

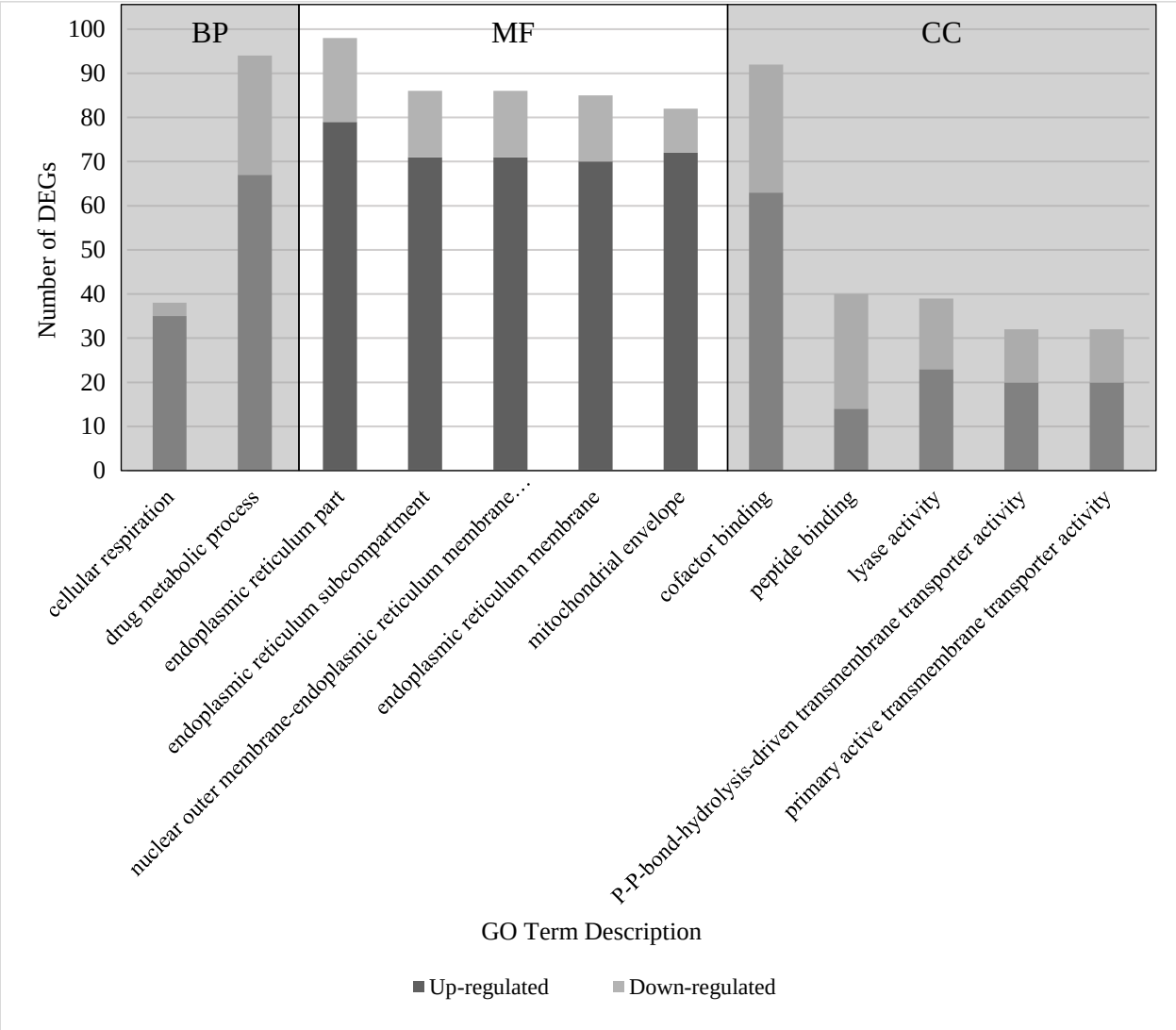


Figure 5. Differentiated GO terms by top five terms with highest number of DEGs per category (BP = biological process, CC = cellular component, MF = molecular function), with *P*-adjusted < 0.05.

DISCUSSION

The present study provides the first data on the effects of late-gestation maternal overnutrition on reproductive health in neonatal male foals. While male-specific responses to fetal programming remain understudied, research is beginning to describe sex-specific responses and the potential impact of maternal nutritional insults on male offspring health.

Diets were validated as a maternal overnutrition model through maternal BW, BCS, leptin, and NEFA. Expected maternal weight gain between d 234 and 319 (final BW measurement) was 26 kg (NRC, 2007). Mares on the CON dietary treatment gained 24.80 ± 5.51 kg whereas HIGH mares gained 45.84 ± 5.51 kg (Bradbery et al., 2021b). Additionally, mares fed the HIGH diet increased BCS while CON mares decreased BCS, but there was no difference in change in RFT (Bradbery et al., 2021b). There tended to be a treatment × gestational age interaction in maternal plasma leptin concentrations. At the start of dietary treatments, there was no difference in maternal leptin; however, by the end of gestation, HIGH mares had greater circulating concentrations of leptin (CON 6.43 ± 1.52 ng/mL; HIGH 10.92 ± 1.52 ng/mL; Bradbery et al., 2021b). There was no effect of dietary treatment for maternal plasma NEFA concentrations; however, there was an effect of gestational age with circulating NEFA concentrations decreasing with increasing gestational age (Bradbery et al., 2021b).

In the present work, testes were 61% larger in neonatal foals from overfed dams. This contrasts with work in other species which recorded no change or a decrease in size of testes in response to maternal overnutrition (Da Silva et al., 2001; Da Silva et al., 2003; Rodriguez-Gonzalez et al., 2015; Kalem et al., 2018; Mossa et al., 2018; Meneghini et al., 2022). Horses undergo a period of hyperplasia of interstitial tissue from day 40 to day 250 of gestation. It

remains to be determined whether the larger size of testes of HIGH foals at birth is due to greater cell proliferation and growth during intrauterine development, or due to limited apoptosis and cell degeneration following this hyperplasia. A reasonable hypothesis is that testes of both groups achieved a similar size during this hyperplasia event *in utero*, but due to the timing of nutritional insults applied in this study at day 235, the HIGH group may have failed to undergo a similar degree of hypotrophy, apoptosis, and cell regulation after day 250 which is needed to return to normal peripartum organ size. Future work examining the mechanisms causing this size difference of male equine gonads at birth would strengthen understanding of how maternal overnutrition impacts reproductive tract development.

Despite the increase in testicular size, number of seminiferous tubules and ratio of seminiferous tubules to interstitial tissue was less in testes of foals from overfed mares, indicating the potential for decreased reproductive capacity compared to colts from control-fed mares. While testes from HIGH foals were 1.61× larger than testes from CON foals, they had 2.3× fewer seminiferous tubules and subsequently lower ratios of seminiferous tubules compared to interstitial tissue. The increase in testes size does not effectively compensate for the decrease in spermatogenic tissue in HIGH foals and may correlate with the previously suggested alterations in cell regulation leading to decreased apoptosis and retention of interstitial tissue after day 250 of gestation. Seminiferous tubules are developed and distributed by day 60 of gestation in horses (Scarlet et al., 2021), and seminiferous tubules change in size, fluid fill, and organization postnatally (Jones and Lopez, 2014). Changes to initial number or degree of elongation and development of seminiferous tubules in the neonatal foal could negatively impact future reproductive potential. Considering this timing of seminiferous tubule development in the

horse, it is likely that the dietary changes applied during late gestation impacted interstitial tissue more than functional tissue organization, which may explain why a change in tubule area or Sertoli cell number was not detected.

Differences in seminiferous tubule area and Sertoli cell number per tubule were not detected in the present work, which parallels previous maternal over- and under- nutrition studies in sheep (Da Silva et al., 2003; Andrade et al., 2013), but contrasts with maternal overnutrition work in cattle and rats (Rodriguez-Gonzalez et al., 2015; Weller et al., 2016). Species, timing of dietary insults, and timing of sample collection may impact the presence of changes in seminiferous tubule or Sertoli cell number. No changes were seen in seminiferous tubule area or Sertoli cell number in sheep at day 103 and 110 of gestation following whole-gestation maternal overnutrition or undernutrition, respectively (Da Silva et al., 2003; Andrade et al., 2013). Seminiferous tubule area and spermatocyte number was lower in rats at postnatal days 110 and 650 following maternal overnutrition throughout pre-pregnancy, pregnancy, and lactation (Rodriguez-Gonzalez et al., 2015), while diameter, length, and proportion of seminiferous tubules was lower in cattle at mid and late gestation following maternal overnutrition starting in early gestation (Weller et al., 2016). While changes in seminiferous tubule area or Sertoli cell number were not seen in the present work, the decreased number of seminiferous tubules, along with smaller proportion of seminiferous tubules compared to interstitial tissue observed in testes from HIGH foals leads to the conclusion that overall spermatogenic/functional tissue was decreased in foals from overfed dams.

DEG enrichment analysis from RNA sequencing data detected eleven KEGG pathways altered by maternal overnutrition, with most notable changes to oxidative phosphorylation, fatty

acid metabolism, thermogenesis, and natural killer cell mediated cytotoxicity pathways. Additionally, GO enrichment analysis detected changes involving the broad themes of mitochondrial function, endoplasmic reticulum function, cellular respiration, and energy metabolism. Within cellular component GO terms, multiple endoplasmic reticulum and mitochondria related terms were different between HIGH and CON groups.

In stallions, oxidative phosphorylation provides the primary source of energy for sperm (Darr et al., 2016; Davila et al., 2016). Oxidative phosphorylation occurs within the mitochondria and is the final step of cellular respiration, used to produce large amounts of adenosine triphosphate (ATP) from glucose to supply energy for biological functions. In the testes, mitochondria are involved in steroid hormone synthesis, spermatogenesis, and sperm motility. On an individual spermatozoa, highly concentrated mitochondria at the midpiece are primarily responsible for providing energy for sperm motility (Meyers et al., 2019). However, oxidative phosphorylation in all cell types, not just germ cells, can contribute to ROS levels in the testes. Sertoli cells rely heavily on glycolysis rather than oxidative phosphorylation for their nutritional support of spermatogenesis (Oliveira et al., 2015), while Leydig cells rely on oxidative phosphorylation and properly functioning mitochondria for normal testosterone synthesis (Allen et al., 2006). Although ROS are a necessary by-product of mitochondrial metabolism, increased levels of ROS leading to oxidative stress can cause accelerated aging of reproductive capacity and/or infertility (Varner et al., 2015). Primary upregulation of genes in this pathway indicates the potential for mitochondrial dysfunction and/or excess oxidative stress in the testes. Increased cellular respiration in the testes would increase the production of ROS, with excess oxidative stress negatively affecting fertility and reproduction. In stallions, the impacts of ROS on sperm

viability have been debated. Horses present a paradoxical relationship where increased levels of ROS are associated with highly motile sperm directly after ejaculation, but these sperm are proposed to “live fast and die young”, rapidly declining in viability with time and semen preservation (Aitken et al., 2012; Gibb et al., 2014; Varner et al., 2015). The upregulation of cellular respiration, oxidative phosphorylation, and mitochondrial-related terms from RNA sequencing enrichment analyses likely relates to retained interstitial cell volume following hyperplasia, including increased Leydig cell numbers which primarily rely on oxidative phosphorylation for proper function.

While the effect of mitochondrial dysfunction on spermatogenesis and fertility may be more apparent, endoplasmic reticulum (ER) stress also plays a key role in testicular function (Liu et al., 2016; Karna et al., 2020). As environmental insults disrupt proper protein folding in the ER, it responds by activating the unfolded protein response (UPR). Under normal conditions, the UPR acts to maintain homeostasis, however under chronic or extreme levels of stress, the UPR can induce apoptosis (Cao and Kaufman, 2014). Other conditions that cause testicular dysfunction, such as varicocele and cryptorchidism, act through the ER stress pathway. Varicocele is characterized by dilation of the pampiniform plexus, the network of blood vessels above and around the testes that aid in temperature regulation and oxygen exchange. Recently, ER stress has been shown to occur in varicocele rats, contributing to apoptosis in the testes (Hosseini et al., 2020). Cryptorchidism has also been related to changes in endoplasmic reticulum gene and protein expression as well as morphological changes in endoplasmic reticulum components (Lunstra and Schanbacher, 1988; Zhou et al., 2019).

It is suggested that ER stress and oxidative stress are closely linked (Figure 6; Malhotra and Kaufman, 2007; Chong et al., 2017). Protein folding produces ROS as a byproduct, while excess ROS levels can disrupt normal protein folding, leaving unfolded or misfolded proteins that induce the ER stress response. The ER mediates reduction-oxidation (redox) balance yet is also easily influenced by redox state (Cao and Kaufman, 2014). To expand upon the relationship between ER and oxidative stress, ER stress markers are upregulated while antioxidant levels progressively decline as mice age. This imbalance of oxidative and ER stress is accompanied by decreased steroidogenesis and Leydig cell function (Huang et al., 2018). Increased oxidative phosphorylation as well as mitochondrial and endoplasmic reticulum function, which are commonly seen in physical and chemical testicular insults, indicate premature aging and impaired function of testes from the HIGH group in the present work.

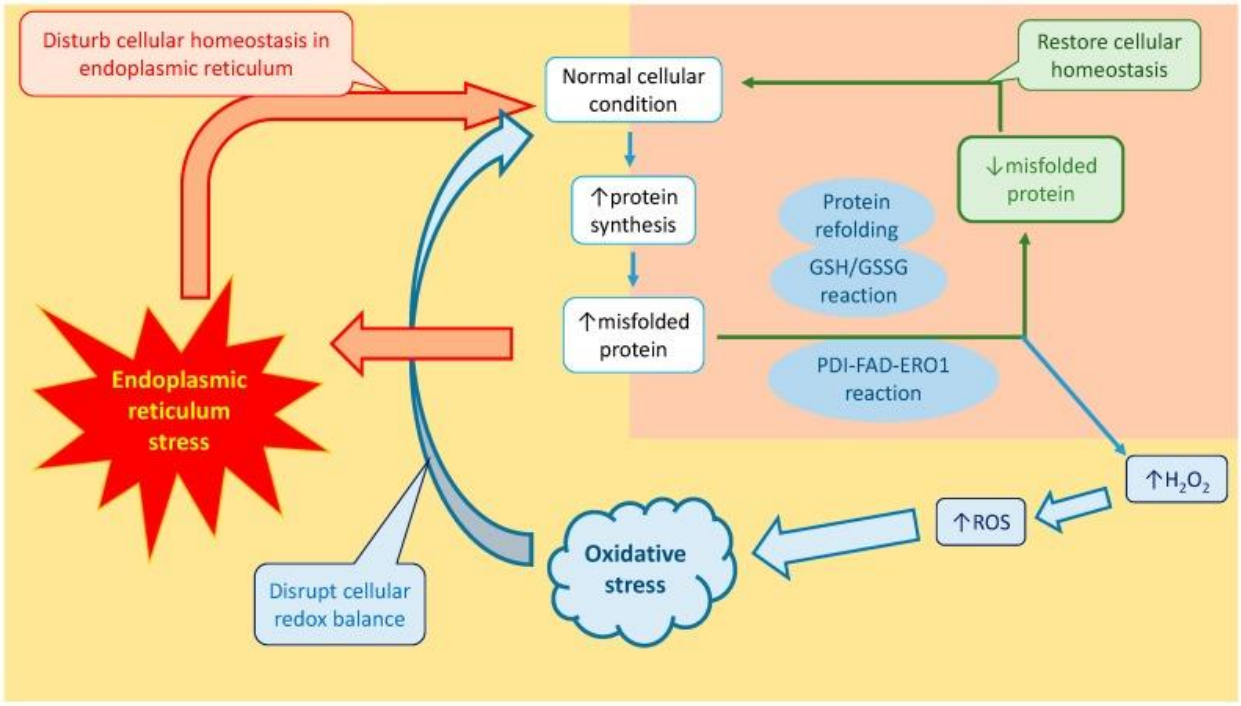


Figure 6. Diagram of the impacts of ER stress on oxidative stress and vice versa. (Chong et al., 2017).

Many individual DEGs were related to cancer, immune, or neuronal function. A majority of DEGs were downregulated in HIGH compared to CON (8 up, 48 down). Select DEGs of interest to testicular and reproductive function are luteinizing hormone beta subunit (*LHB*), *AMH*, cadherin 3 (*CDH3*), SPOC domain-containing protein 1 (*SPOCD1*), choline dehydrogenase (*CHDH*), and cytochrome P450 family 26 subfamily B member 1 (*CYP26B1*).

LHB is involved in the formation of luteinizing hormone (LH). It is a glycoprotein hormone forming a beta subunit which confers biological specificity when in combination with a common gonadotropin alpha subunit to synthesize LH. LH stimulates Leydig cells to secrete testosterone, the male sex hormone, which in turn stimulates spermatogenesis. Without proper formation of LH, hormonal signaling involving testosterone is disrupted. Clinical case studies in humans report that *LHB* deficiency or mutations/polymorphisms of *LHB* can lead to

hypogonadism and delayed onset of puberty (Potorac et al., 2016; Yang et al., 2018; Barnabas et al., 2023). *Lhb*-knockout mice displayed smaller testes, abnormal Leydig cell development, decreased testosterone levels, hypogonadism, defects in steroid biosynthesis pathways, and were infertile, with spermatogenesis arresting at the round spermatid stage (Ma et al., 2004). Downregulation of *LHB* in the HIGH group (log_2foldchange = -2.39) suggests the potential for delayed onset of puberty, hypogonadism, and subfertility in male offspring from maternal overnutrition.

While primarily expressed in the pituitary gland of most species, testes-specific *LHB* transcripts have been detected in rat, mouse, and human testes, along with expression of the common alpha subunit (Berger et al., 1994; Zhang et al., 1995a; Zhang et al., 1995b; Hee Soo and Sung-Ho, 2017). *LHB* is also expressed in normal yak testes and increases in mRNA and protein expression as the animal matures and spermatogenic potential develops (Kalwar et al., 2020). While *LHB* transcription was detected throughout the seminiferous tubules, LHB translation determined by immunohistochemistry was localized to the cytoplasm of elongated spermatids (Zhang et al., 1995a; Hee Soo and Sung-Ho, 2017). Interestingly, GnRH expression has also been localized in the seminiferous tubules of both rat and human testes, with GnRH receptor (GnRHR) localized in interstitial tissue (Bahk et al., 1995). *LHB*, *GnRH*, and *GnRHR* expression is detected in the ovaries of rats and fish, suggesting a gonadal GnRH-gonadotropin axis (Wong and Zohar, 2004; Schirman-Hildesheim et al., 2005; Schirman-Hildesheim et al., 2008). *LHB* is most commonly and highly expressed in the pituitary, a logical location of expression for its role in the formation of LH. While *LHB* function in the testes is not fully understood, perhaps the expression of *LHB* in testes tissue indicates autocrine or paracrine action

within the testes or support for mature spermatids (Zhang et al., 1995b; Hee Soo and Sung-Ho, 2017).

The *AMH* gene encodes for a glycoprotein hormone by the same name, which is secreted by Sertoli cells and has roles in sex differentiation, testicular descent, and male sexual development and behavior (Matuszczak et al., 2013). In horses, AMH is highly expressed in Sertoli cells of fetal, neonatal, and prepubertal testes, but not mature testes (Ball et al., 2008). In post-pubertal animals, increased AMH (circulating or immunolabeled) is considered a marker of sexual immaturity, however in prepubertal animals AMH should be highly expressed to support proper sexual development. For example, AMH is expressed in the testes of cryptorchid stallions, signifying decreased reproductive function due to this physical testicular insult, despite maturity of the animal (Ball et al., 2008). In normal mature animals, the decline of AMH is initiated by testosterone production and the beginning of spermatogenesis at puberty (Josso et al., 2001). In animals with hypogonadism or cryptorchidism, the lack of normal spermatogenesis and testosterone secretion by Leydig cells may contribute to the maintained expression of AMH after puberty. Deficiency of AMH due to changes in gene expression have been linked to hypogonadism and Sertoli cell dysfunction in humans (Matuszczak et al., 2013). *Amh* deficiency in mice is correlated with Leydig cell hyperplasia, suggesting its role in Leydig cell regulation (Behringer et al., 1994). Leydig cell hyperplasia may contribute to the increased testes size in HIGH foals in the current work, further supporting that sustained interstitial cell hyperplasia may explain larger testes size at birth in foals from overfed dams. Serum AMH concentrations can be used to assess gonadal function and understand endocrine changes in offspring due to maternal overnutrition. Downregulation of *AMH* expression in the neonatal testes (log_2foldchange = -1.48)

indicates the potential for impaired sexual development and testicular descent. A corresponding decrease in circulating AMH in neonatal foals would validate the systemic effects of decreased *AMH* gene expression, and evaluation of circulating AMH as animals mature would give insights into the function of testicular tissue.

Cadherins, including *CDH3* (also known as P-cadherin), are involved in cell interactions, cell adhesions, and barrier integrity. *CDH3* is also involved in various cancers, indicating a role in cell proliferation and apoptosis (Vieira and Paredes, 2015; Zhou et al., 2020). In mouse testes, CDH3 is expressed in Sertoli cells during embryonic development until postnatal day 8, after which it is expressed in peritubular cells surrounding the seminiferous tubules (Lin and DePhilip, 1996). When expressed in peritubular cells, CDH3 is accompanied by the expression of beta-catenin and actin filaments in-between peritubular cells, displaying the role of CDH3 in cell adhesion and junction formation (Lin and DePhilip, 1996). Additionally, primordial germ cells express CDH3 during and after migration to the genital ridges for embryonic development (Bendel-Stenzel et al., 2000). However, the absence of *Cdh3* may be compensated for by other mechanisms in the testes, as *Cdh3*-deficient and *Cdh3*-null mice were fertile (Radice et al., 1997). *CDH3* was downregulated in testes from HIGH foals ($\log_2$foldchange = -2.17). Downregulation of *CDH3* in the HIGH group may influence cell adhesion, interactions, and the blood-testis barrier in foals from overfed dams, but it ultimately remains to be determined whether this downregulation would impact fertility at breeding age.

During early development, *SPOCD1* is predominantly expressed in spermatogonial stem cells (SSCs), the precursors for future spermatogenesis in mature testes. Implicated in cell proliferation, apoptosis, and male infertility, *SPOCD1* affects germ cell survival. *SPOCD1*-

knockdown decreased proliferation and increased apoptosis in human SSCs *in vitro* (Zhou et al., 2022), while *Spocd1*-knockout mice had smaller testes with abnormal seminiferous tubules, an absence of spermatozoa in the epididymis, and male-specific infertility. Spermatids in these mice were arrested in the pachytene stage of meiosis, with higher levels of apoptosis detected in the testes (Zoch et al., 2020). *Spocd1* is also implicated in various cancers, where knockdown decreases the proliferation, migration, and invasion of cancer cells in mice (Zhu et al., 2017). *SPOCD1* is not detected in Leydig cells (Zhou et al., 2022), so likely is not involved in the Leydig cell hyperplasia mechanisms suggested for the change in testes size in the present study. While SSC numbers were not evaluated in the present study, downregulation of *SPOCD1* in testes from HIGH foals (log_2foldchange = -3.10) has potential impacts to testicular histoarchitecture and cell regulation, with increased apoptosis of SSCs leading to future infertility in these foals.

CHDH is a catalyst for the conversion of choline to betaine and is also a key gene influencing male fertility. *Chdh* knockout mice had significantly decreased fertility, primarily due to impaired sperm motility. *Chdh*-null mice had abnormal mitochondrial morphology in the sperm and decreased ATP content, mitochondrial dehydrogenase activity, and inner mitochondrial membrane polarization. Mitochondrial abnormalities were also seen in other tissues, including the testes (Johnson et al., 2010). In humans, polymorphisms of the *CHDH* gene were correlated with altered sperm concentrations (Lazaros et al., 2012). In the current study, *CHDH* was downregulated in testes from HIGH foals (log_2foldchange = -2.09). Decrease in expression of the *CHDH* transcript, impacting activity of the CHDH enzyme, could negatively affect fertility through compromised energy metabolism, motility, and concentration of sperm.

CYP26B1 encodes a cytochrome P540 enzyme that is involved in the catabolism of retinoic acid (RA). RA is necessary for the entry into meiosis in germ cells, so *CYP26B1* effectively blocks entry and reentry into meiosis in both male and female germ cells. *CYP26B1* is expressed in the somatic cells of the testes, and Sertoli-cell specific expression holds germ cells in an undifferentiated state, preventing them from entering meiosis during embryonic development (Li et al., 2009). *Cyp26b1*-null mice had smaller testes, with depletion of germ cells in some seminiferous tubules, accompanied by a decrease in tubule diameter, compared to wild-type counterparts. Following entry into meiosis initiated by a lack of *CYP26B1*, germ cells will undergo premature apoptosis (MacLean et al., 2007; Li et al., 2009). Prenatally, germ cells prematurely entered meiosis in *Cyp26b1*-null mice, followed by a postnatal depletion of germ cells within seminiferous tubules. While Sertoli cell numbers were not different in *Cyp26b1* knockout mice, Leydig cells displayed greater hyperplasia (Li et al., 2009). These findings may help to explain the changes in histoarchitecture of the testes seen in the current work, with larger testes but decreased proportion of seminiferous tubules compared to interstitial tissue in testes from HIGH foals. *CYP26B1* was downregulated in testes from HIGH foals ($\log_2$foldchange = -2.12). Perhaps *CYP26B1* plays a role in the altered cell regulation in the testes, maintaining hyperplasia of Leydig cells leading to increased interstitial tissue contributing to larger testes size in the HIGH group.

Overall, RNA sequencing data suggests important changes in energy metabolism, oxidation/antioxidant systems, proliferation, and apoptosis within the testes, with the potential to impact spermatogenesis, steroidogenesis, cell regulation, and sperm health parameters. Future work using immunofluorescence markers such as GATA-binding factor 1 (GATA1, Sertoli cell

marker), 3β-hydroxysteroid dehydrogenase (3βHSD, Leydig cell marker), and TRA98 (germ cell marker) can determine changes in specific testes cell numbers and presence, allowing greater understanding of the mechanisms that affect changes in testicular histoarchitecture. Apoptosis assays such as annexin V, caspase, or terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) would also be useful in determining patterns of cell regulation in testes from overfed dams. Other DEGs related to cancer and immune function that were not described in an in-depth analysis likely contribute to dysregulation of cell proliferation and apoptosis patterns leading to altered histoarchitecture of the testes. Single-cell or single-nuclei RNA sequencing may be a valuable tool to assess the cell-specific mechanisms of change in prepubertal testes in response to maternal overnutrition. Determining cell-type-specific changes in gene expression would aid in guiding further studies of the mechanisms impacting reproductive capacity in male offspring.

Histological and gene expression changes of the testes observed in this study were in response to maternal overnutrition during third trimester gestation in horses. The HIGH diet used in the present work was not an extreme or unrealistic plane of nutrition, but rather represented upper-level feeding recommendations of an industry-standard mare and foal concentrate. While the needs of each individual horse and breeding operation should be considered when formulating a diet, after further research it may be appropriate to re-evaluate industry feeding recommendations for the late-gestating mare.

Fertility and reproductive success are not the focus of selection or breeding soundness exams for either mares or stallions. Unlike other livestock species which are selected directly or indirectly based on reproductive traits, horses are often selected based on performance traits or

appearance, leading to a large disparity in fertility levels among stallions. While mares are expected to readily conceive, maternal obesity and overnutrition may not be considered, inadvertently impacting the health of future foals. These factors of both maternal and paternal influence will compound and continue to create challenges for equine fertility, breeding success, and advancement of the species. Male offspring from overfed dams show changes in reproductive parameters at birth that may persist to puberty and be exacerbated by other environmental factors such as nutrition, stress, disease, and management. These stallions with suboptimal fertility may be used for breeding later in life, with the potential to again breed to overfed mares, further compounding the impacts of maternal and/or paternal overnutrition as well as the lack of reproductive selection in horses. The increase in testicular size with a corresponding decrease in seminiferous tubules and altered reproductive-related gene transcripts and pathways provides evidence against a positive correlation between size and functionality of the testes. Due to this observation, the use of scrotal circumference as an indicator of fertility may be ineffective in estimating reproductive health and efficiency of equine breeding stock.

Overall, the findings of this study indicate that late-gestation maternal overnutrition in horses leads to compromised histomorphology and gene expression in neonatal foal testes. Despite their larger size, testes from foals of overfed dams may be less efficient, have lower spermatogenic potential, and display disrupted reproductive function at puberty. Future work should continue to examine the effects of maternal overnutrition on reproductive health of equine offspring. Considerations should be made to adjust breeding soundness exams for both mares and stallions to include measures of obesity and plane of nutrition, to reevaluate scrotal

circumference as an indicator of fertility, and to modify current broodmare management practices to optimize feeding for offspring health.

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