Exploring immunoglobulin and metabolomic biomarkers of subfertility in *Bos taurus* beef heifers

By

Anna Grace Holliman

A thesis submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Master of Science

Auburn, Alabama
August 5, 2023

Keywords: Heifer, Subfertility, Immunoglobulins, Follicular Fluid, Plasma, Metabolome

Approved by
Paul W. Dyce, Chair, Associate Professor of Animal Sciences
Soren P. Rodning, Extension Specialist and Professor of Animal Sciences
Marko Rudar, Assistant Professor of Animal Sciences

Abstract

Unexplained heifer subfertility is a resource drain on beef production, negatively effecting production efficiencies. Animals that do not conceive within a breeding season and those conceive late result in lighter calves and less return on investment for producers upon weaning and marketing. The ability to detect and avoid heifers that fail to become pregnant, or become pregnant late, would improve the efficiency of beef production.

In biomarker discovery, highly accessible animal samples are preferable such as in blood, saliva, and urine. The objectives of this study were to identify the differences in plasma immunoglobulin levels and metabolomic signatures at different stages of heifer development. To accomplish this, the relative immunoglobulin G (IgG) levels in the blood plasma were tested using SDS-PAGE and Western blot between *Bos taurus* beef heifers that conceived to first service AI and those that conceived late or not at all in the breeding season (1stAI, n=12; LBred, n=12). There was no significant difference in the levels of IgG1 between heifers that established pregnancy to first-service AI and those that established pregnancy late in the breeding season. However, IgG2 levels were significantly higher in heifers that established pregnancy to first service AI.

In the second study, blood plasma metabolomic profiles generated by gas chromatography-mass spectrometry (GC-MS) were compared for *Bos taurus* beef heifers that conceived to AI (fertile, n=6) and heifers that conceived late or not at all (subfertile, n=6) at weaning and for a subset of the same heifers 30 days prior to AI (fertile n=3, subfertile n=4). At weaning, six metabolites were present at significantly lower levels in fertile heifers compared to subfertile heifers, including arabitol, glycolic acid, indole-3-lactate, indole-3-propionic acid, and palmitoleic acid. Three metabolites were present at significantly higher levels in fertile heifers, including lactic

acid, maltose, and nicotinamide. Thirty days prior to AI, four metabolites were differentially abundant. In fertile heifers, tryptophan was at higher levels, and palmitoleic acid, triethanolamine, and 9-myristoleate were at lower levels. Five metabolites changed levels significantly over time within cohorts, including palmitoleic acid, 1,5-anhydroglucitol, capric acid, myristic acid, and isolinoleic acid.

For the third study, metabolomic profiles were generated for blood plasma and follicular fluid from beef heifers that either conceived at AI or remained non-pregnant following fixed time artificial insemination and 60-day exposure to fertile bulls (fertile, n=8; subfertile, n=5). Fourteen differentially abundant metabolites were identified in follicular fluid, including arachidonic acid and ascorbic acid and four were identified in blood plasma, including fucose and lactic acid. Fucose and pipecolinic acid showed medium correlation between blood plasma and follicular fluid and high ROC-AUC values in blood plasma. In plasma and follicular fluid of post-breeding season animals, identified metabolites were related to reactive oxygen species, energy metabolism, and cell growth and proliferation. Correlation of plasma and follicular fluid profiles suggests that two differentially abundant metabolites in plasma may be tightly correlated enough to follicular fluid to serve as diagnostic of the follicular state; however, these statistical results need to be specifically and carefully validated with a targeted study.

Acknowledgements

Dr. Dyce, thank you for taking a chance on me. I struggle to put into words how grateful I am for that. Over the last two years you've shown the patience of a saint. You've answered my silly and random questions; given good advice; pushed me out of my comfort zone; challenged, trusted, and supported me. You've been a mentor, role model, and a friend when I really needed one. I can only hope that one day I can be even a fraction of the researcher and educator that you are.

Dr. Rudar and Dr. Rodning, thank you so much for being part of my graduate experience. I have learned so much from each of you. Dr. Rodning, I loved being out on projects with you. They were all learning experiences and I have immense respect for your knowledge, teaching ability, and throwing arm. Dr. Rudar, I really enjoyed working with you when I could. The first time I was in your lab, I had absolutely no idea what was going on but still managed to learn from you. Any time we spoke, you managed to ask a question I hadn't thought of, usually opening up a whole new set of considerations and challenges for me to work on solving.

The rest of my lab mates, I couldn't have done this without you all. Dr. Banerjee, you make me a better researcher every time we talk. You posed questions and challenges that made me realize that 'what' isn't enough to know, I also have to know 'how' and 'why'. If I ever try to tackle bioinformatics again, you will be my first in-case-of-emergency call. Rachel, I'm not exaggerating when I say that you helped keep me…well, sane is the wrong word, but less insane than I might have been. You were the bold one that I got to hide behind when I first got here and suffered through the heifer project, SAS, and anything forage related with me. Thank you for being the

reason I passed the SAS class. Hector, I really wish that I could stick around. I think you are going to do great things and I can't wait to hear about them. If you ever get stuck, I'm always just a text message or call away. Sarah, thank you so much for the wisdom, jokes, and friendship over the last two years. You've been a joy to work with.

To my family; Mom, Dad, Adrian, Maddie, Aaron, Cheyna, Austin, Amy, Amos, and Asa. I couldn't have done what I've done without ya'll. Whether it was telling me to get my ducks in a row, mocking me for attending a Cow College, or six hour phone calls- you all were there for me when I needed you.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

30DP-AI	30 day prior to artificial insemination
ADG	average daily gain
AI	artificial insemination
ART	assisted reproductive technologies
BCA	bicinchoninic acid
BCS	body condition score
BiW	birth weight
BSA	bovine serum albumin
BW	body weight
CIDR	controlled internal drug release
COC	cumulus-oocyte-complex
CV	coefficient of variability
DU	densitometric units
EBV	estimated breeding value
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
FDR	false discovery rate
FF	follicular fluid
FSH	follicle stimulating hormone
GC-TOF-MS	gas chromatography-time of flight-mass spectrometry

GnRH	gonadotropin releasing hormone
IDO	indoleamine 2,3-dioxygenase
IFNγ	interferon gamma
IFNτ	interferon tau
IgG	immunoglobulin G
IL6	interleukin 6
IVD	*in vivo* derived
IVF	*in vitro* fertilization
IVM	*in vitro* maturation
LC	loading control
LPS	lipopolysaccharide
miRNA	micro RNA
NAD+	nicotinamide adenine dinucleotide
PA	pelvic area
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCA	principal component analysis
PCOS	polycystic ovary syndrome
PLS-DA	partial least squares discriminant analysis
POI	protein of interest
PVDF	polyvinylidene difluoride
pWBCs	peripheral white blood cells
ROC-AUC	receiver operating characteristics area under the curve

ROS	reactive oxygen species
RTS	reproductive tract score
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SNP	single nucleotide polymorphism
Th1	T helper cell 1
Th2	T helper cell 2
TNFα	tumor necrosis factor α
WW	weaning weight
YW	yearling weight

Chapter I: Review of Literature

I.I. Cattle production and production systems in the United States

The livestock industry in the US was reported to employ upwards of 263,551 people in direct or support roles on livestock farms [https://www.ers.usda.gov/topics/farm-economy/farm-labor/#size]. The USDA (United States Department of Agriculture) reported in 2022 that the population of cattle (including both beef and dairy animals) numbered 98.8 million head. In beef and dairy systems, some animals are retained and developed for breeding, as bulls and replacement heifers, while others are developed for meat production. Animals culled for health and production reasons also are used for meat production (USDA, 2022a). In 2022, 4.15 million beef heifers and 3.75 dairy heifers were retained for breeding (USDA, 2022a). Overall cash receipts for the marketing of cattle and calves in the United States, as reported by the USDA in 2021, totaled $72.9 billion (USDA, 2022b). Additionally, the USDA Agriculture Resource Management Survey (ARMS) reported that farms showed an overall loss of $812 (USD) yearly from the sale of breeding livestock, representing unrecovered financial investment when selling breeding animals (USDA, 2021) (https://my.data.ers.usda.gov/arms/data-analysis).

In the United States there are defined differences and separation between beef production and dairy production but this is not globally true. Culled dairy animals and male calves from dairy bloodlines are the main source of beef production in many other parts of the world (Berry, 2021). Experiments have found that cross-breeding dairy and beef breeds does not compromise the quality of meat produced, and it has been suggested that crossing dairy into beef dams could result in greater weaning weights for calves, potentially due to the higher milk production of the cross-bred dams (Coleman et al., 2023). It has also been suggested that weaning rate and calving rate may be greater in dairy x beef cross animals, though a reason for this is not entirely clear (Nelson et al.,

1982). However this process of cross-breeding is highly dependent on the benefit it can provide to a dairy system, namely increased income over cost of breeding (Pereira et al., 2022). As such, research into cattle in the US is segmented along these production lines. Dairy cattle are intensively managed and selected for milk production while beef breeds are selected and managed for growth and carcass quality. This high selective intensity, especially in breeds specialized for dairy production, has greatly increased yields for production. However, this selection intensity may have also increased the fertility issues that cattle face at a commiserate rate (Kadri et al., 2014; NAHMS, 2014). While both sectors of the industry struggle with these fertility issues, the research presented here represents specifically research within the beef sector.

In the US, an average of 40.6% of beef cows are culled for being non-pregnant or for having lost a confirmed pregnancy (NAHMS, 2020) and 15% of heifers will fail to conceive during the breeding season and are culled [reviewed by (Moorey and Biase, 2020)]. This would suggest that between 0.623 million head of beef heifers will reproductively fail while 0.563 million head of dairy heifers will reproductively fail. While some of the financial resources used to develop and maintain these animals can be recouped when the animal is culled/sold, it is overall more likely that there is a net loss to a producer when a breeding animal is removed (USDA, 2021). While input costs for heifer development will dictate the net amount of loss an individual producer will suffer with each reproductive failure, it has been suggested that for every 1% increase in pregnancy failure rate there is a loss of $6.25 per head exposed (Mercadante et al., 2020). Heifers, retained and developed for replacement into beef herds, are one of the largest assets a production system can have and also represent one of the largest losses a production system can suffer when heifers fail to conceive (Moorey and Biase, 2020). Additionally, this reproductive failure represents not

just economic loss but the loss of other inputs such as feed, forage, and pharmaceuticals. By reducing the incidence of reproductive failure, these resource losses could be reduced, increasing efficiency and sustainability of the beef sector.

I.II. Heifer development and selection

Studies have shown a link between the mechanics of a first breeding season for heifers and for her lifetime productivity, thus development of a heifer from birth to first breeding is important to ensuring her fitness for breeding (Moorey and Biase, 2020). Herd levels fluctuate over time, due to changes in cattle prices and environmental changes like droughts and drought-easing. Heifer retainment rates also change in response to these factors, as well as in response to low performance of older breeding cows in a herd (Hughes, 2013). Heifers must be selected from calf crops for further development, be subjected to the correct nutritional plane to reach between 55-70% of mature body weight prior to their first breeding (Dickinson et al., 2019), and have the correct health program applied before her first breeding season (ACES; Mejia et al., 1999). The long generational interval of cattle means that under optimum conditions, heifers will be 24 months on average (*Bos taurus)* (Engelken, 2008) or potentially older (*Bos indicus*) (Cardoso et al., 2020) prior to their first parturition. Costs for developing heifers vary depending on the geographical region and production system (Hughes, 2013; Boyer et al., 2020). Depending upon the analysis, it has been calculated that a heifer must undergo three to four successful breeding seasons (Hindman et al., 2022) before the cost of her development is matched by the income generated. Other studies have suggested that six seasons are preferable to actually produce profit from a breeding female (Moorey and Biase, 2020). Boyer et al., in 2020 published an analysis of the profitability of beef cattle, raised on endophyte infected tall fescue, over an assumed 11-year production life span, noting a cost of $889-$894 (USD) to develop a heifer from dam-breeding to heifer breeding in

Tennessee. This review also included analysis of how many calves were necessary for a heifer to recover the cost of her development. Boyer et al. notes that if a heifer does not miss a breeding season, ~6 calves would recoup her development costs. However, loss of even one breeding season increased the necessary number of calves to ~8 (Boyer et al., 2020; Moorey and Biase, 2020).

Animal selection is a multifaceted decision, dependent on many factors. Phenotypic factors like age, weight, body condition score, and reproductive tract score are often considered for selection. Estimated breeding value (EBV) and expected progeny difference (EPD) are two potential measures of animal value to a breeding system. EBV is the relative value of a particular animal in a particular trait (i.e. milking ability or RFI) half of which can hypothetically be passed on to their offspring (Martin et al., 2021). EBVs are generated from both the animal in question and its relatives. An EPD is a measure of how a particular animal's offspring are expected to perform in a trait when compared to a second animal's offspring in the same trait in relation to the mean of the same trait within the breed. A producer may know the EBV of a particular heifer or know the EPD of the dam or sire that she came from and make a decision to retain her because of those measures.

I.II.I. Age

Age, maturity, and environment play a large role in reproduction and it is commonly accepted that females must reach a particular percentage of mature body size before initiating puberty and regular reproductive cycling (Senger, 2012). To an extent, a producer can influence onset of puberty with diet, feeding, and average daily gain (Schubach et al., 2019) although there is also a breed dependent effect on puberty that has not been fully elucidated (Wolfe et al., 1990; Canovas et al., 2014). It is also important to avoid excess adiposity (also called over-conditioning) which can lead to reduced fertility (Gonzalez-Maldonado et al., 2017; Silvestris et al., 2018).

Certain environmental factors can also effect pubertal attainment, as demonstrated by the effect of parasite load and ivermectin treatment in dairy cattle (Mejia et al., 1999).

It has been suggested that heifers allowed to pass through 3 successive estrous cycles have greater chance of establishing pregnancy than heifers bred upon first or pubertal estrus (Byerley et al., 1987). More recent studies have suggested that a greater number of estrous cycles passed prior to first breeding may be reflected by greater rebreeding rates in the second breeding season and not necessarily breeding success in the first season (Roberts et al., 2019). Dickinson et al. demonstrated that heifers over 368 days old had 87.5% chance of successful conception while those that were younger than 368 days old had only a 12.5% chance of the same (Dickinson et al., 2019). Conception to artificial insemination (AI) was closely linked with age, with increasing age in days being correlated with increasing odds of successful pregnancy to AI (Hindman et al., 2022). This is in agreement with a study by Marella et al. (2021) which showed that AI-sired heifers had greater first-service pregnancy rates than their bull-sired sisters, most likely due to the greater maturity of AI-sired females though age at conception was not affected by sire type. A study performed in dairy cattle specifically bred for low or high fertility phenotypes found that high fertility heifers achieved puberty earlier than low fertility heifers (Meier et al., 2021). In some cases, a heifer's reproductive maturity can be manipulated with exposure to reproductive hormones such as progestins or prostaglandins (Wood-Follis et al., 2004; Monn et al., 2019).

I.II.II. Weight

The amount of mass that an animal must attain prior to puberty may be controlled by many factors, but in cattle it is suggested that puberty will be attained at 50% of body weight (Senger, 2012). However, animals will achieve puberty at a range of weights and ages (Canovas et al., 2014). Weight at breeding for heifers is recommended to be between 55-65% mature cow body

weight (ACES; Berger and Funston, 2013; Arthington and Moriel, 2021). Heifers managed to be at ~50% at the start of the breeding season compared to those managed to be ~60% are less likely to pubertal at the start of the breeding season (Funston and Deutscher, 2004). However, these lighter heifers may be equally fertile, as there are no significant differences in pregnancy rates between the two cohorts at the end of the breeding season (Funston and Deutscher, 2004). There are likely genetic factors involved in puberty attainment age as a study of dairy heifers found that despite not showing differences in body weight, heifers with greater fertility achieved puberty at younger ages (Meier et al., 2021). It is believed that signaling molecules such as leptin (Senger, 2012; Cardoso et al., 2020) insulin like growth factor 1, and insulin may play a role in signaling that an animal has reached appropriate body mass for puberty (Cardoso et al., 2020).

Dickinson et al. found that weight at weaning had very little effect on breeding outcomes (pregnant to AI, pregnant to natural service, not pregnant at end of season), when considered as a selection tool for replacement heifers (Dickinson et al., 2019). However, Hindman et al. that found that average daily gain (ADG) and weight at breeding were significantly linked to success of first AI (Hindman et al., 2022). These results support the important role of nutritional management post-weaning to achieve puberty onset prior to the first breeding season. Meier et al. as well found that body weight was significantly different between high-fertility EBV heifers and low-fertility EBV heifers by 21 months of age, due to differences in ADG between the two cohorts (Meier et al., 2021). While not measured as often as factors such as BCS and RTS, frame score and pelvic measurements are important metrics for maturity and must be considered with weight, as animals with larger frames require more time to achieve sufficient body weight for cycling (Hindman et al., 2022).

Weight can negatively impact reproduction and there are linkages between obesity and inflammatory states. Adipose tissue acts as an endocrine organ, secreting factors called adipokines, amongst which are TNFα, IL-6 (Silvestris et al., 2018) and leptin (Cardoso et al., 2020). Phillips et al. (2018) found that low-performing *Bos taurus* beef heifers had higher expression of mRNA transcripts for TNFα and IL-6 in their peripheral white blood cells (pWBCs) when compared to high-performing beef heifers though they did not note any significant differences in body weight and BCS. Vailati-Riboni et al. (2016) found that dairy cattle with higher BCS in the periparturient phase also had higher expression of genes for IL-6 and TNFα in subcutaneous adipose tissue. Studies in humans have linked obesity to inflammation as well, and reduction in reproductive efficiency (Silvestris et al., 2018). High percentage body fat has been linked to dysmenorrhea and oligomenorrhea in humans, as well as altered steroidogenesis at the ovarian levels and reduced feed intake [as reviewed by (Silvestris et al., 2018)]. Another study in humans linked obesity to higher levels of high-sensitivity C-reactive protein (hsCRP) and high-sensitivity interleukin 6 (hsIL-6), leading to chronic low-grade inflammation (Cussotto et al., 2020). In the same study, hsCRP and hsIL-6 was negatively correlated with plasma levels of tryptophan and indole-related metabolites (Cussotto et al., 2020).

I.II.III. Body condition score

Along with body weight, body condition scoring (BCS) is a common metric for judging health and fitness of cattle. In dairy cattle, the scale is 1 to 5, with increments of 0.25 (Ishler et al., 2016). In beef cattle the score is from 1 to 9 and usually whole numbers are assigned, though occasionally increments of 0.5 are seen (Wilke et al., 2023). BCS is a judgement of relative ‘fatness’ of an animal based upon the exposure of certain skeletal markers, such as pin bones and

ribs (Ishler et al., 2016). In both scoring systems, a score of 1 means an animal is emaciated, while a high score means an animal is over-conditioned or obese (Ishler et al., 2016;Wilke et al., 2023).

It has been widely shown that animals are most reproductively successful when their BCS is not at either extreme, being over- or under-conditioned. Marques et al. (2022) found that in beef heifers and cows a BCS less than 4 had a significantly negative effect on conception rates. This is in agreement with Guzman et al. (2016) that found beef females were more reproductively successful when BCS was between 4-6. Marrella et al. (2021) is also in agreement with this data, showing that animals with a BCS of 5-6 have a greater chance at conception following a first parturition and lactation. Liles et al. (2022) also agrees with the positive effect of BCS on pregnancy rates, as there was a positive correlation of BCS in their study with pregnancy attainment to AI breeding. On the contrary, BCS is not a good prognostic indicator for breeding success when the BCS 5-6 guideline is met (Dickinson et al., 2019) and was not different between high-fertility EBV heifers and low-fertility EBV dairy heifers (Meier et al., 2021).

I.II.IV. Reproductive tract score

Reproductive tract scoring (RTS), like BCS, is a measure of the maturity and cyclic status of structures relating to reproduction in females. As described by Rosenkrans and Hardin (2003), a score of 1 for an RTS describes a female that is reproductively immature, having no uterine tone or palpable ovarian structures. A RTS 2 heifer would have no uterine tone and follicles of less than 8mm; an RTS of 3 would have slight uterine tone and follicles of 8-10 mm. Heifers with a score of 1-3 are considered prepubertal. RTS 4 describes a female with good uterine tone, horn diameter of 30 mm or more, and palpable follicles of greater than 10 mm on the ovary; and RTS 5 has a palpable corpus luteum on the ovary (Rosenkrans and Hardin, 2003).

Gutierrez et al. (2014) showed in a several year study that heifers with an RTS of 3 had a 7.6% higher chance of establishing pregnancy following AI when compared to heifers with an RTS of 2 or lower. Heifers of RTS 4 and 5 were 16.9% and 23.9%, respectively, more likely to establish pregnancy to AI than heifers of RTS 2 or lower (Gutierrez et al., 2014). In the same study, it was shown that animals with RTS of 1-2 had greater chances of establishing pregnancy when exposed to a bull and that heifers with higher RTS earlier in the breeding season were less likely to be non-pregnant at the end of the breeding season (Gutierrez et al., 2014). Dickinson et al. (2019) showed that RTS failed to fully predict animals that conceived to AI compared to those that conceived to natural service bulls or non-pregnant at the end of the breeding season, this along with BCS and age at AI. This is supported by Hindman et al. (2022) who found no differences between RTS in heifers that conceived to AI and all others in their study, however this similarity was suggested to be due to producer selection. Holms et al. (2015) showed that reproductive tract score is predictive of success in the first breeding season and in the second breeding season but may not be predictive of long term reproductive success. In this same study, body weight and age were not different between RTS categories (Holm et al., 2015).

I.III. Reproductive management programs for beef and dairy

With the advent of assisted reproduction technologies there are several possibilities for how a heifer can become pregnant, such as AI, natural service (NS) by a bull, or embryo transfer (ET). The extent to which these technologies are utilized is highly dependent on type of production system (i.e. dairy versus beef) and upon the herd size in question (small herds, 1-49 head; medium herds, 50-199 head; large herds, 200+ head) as larger herds are more likely to utilize assisted reproductive technologies (ARTs) (NAHMS, 2020). The USDA National Animal Health Monitoring System (NAHMS) reported that only 15.1% of first-calf heifers in beef production

were bred using both AI and NS bulls. It reported that 95.5% of beef cows were bred solely to bulls and 5.2% were bred to AI and to NS bulls (NAHMS, 2020). Additionally, 58.7% of production systems did not identify as using a defined breeding season (separation of males and females for at least 30 days out of a year) (NAHMS, 2020). Without a defined breeding season many ARTs are not accessible to producers. In beef production systems, only 37.5% of production systems use at least one reproductive technology such as ES, AI, pregnancy diagnosis, pelvic measurements, BCS, semen evaluation, or embryo transfer (NAHMS, 2020). This is in contrast to the majority of dairy systems, in which 89.3% of systems use any AI, 46% use strictly AI, and 43.4% use AI and natural service (NAHMS, 2014). Additionally, dairy systems are much more likely to utilize pregnancy diagnosis to ensure conception of cows and heifers, 98.8% of dairy production systems in 2014 reported using some kind of pregnancy diagnostics, 70.6% by rectal palpation, 44.1% by ultrasound, and 91.7% either rectal palpation or ultrasound (NAHMS, 2014).

I.III.I. Bull breeding

Bull breeding, as the 'traditional' breeding system for cattle production is mostly defined by how it is not an ART. Notably, many production systems use natural service and do not have defined breeding seasons (58.7% in beef production), suggesting that in these systems the bulls are maintained with the breeding cows throughout the year and allowed to breed whenever a cow or heifer is in estrus regardless of season. If this system is well-managed, there is no reason it is not a viable option for producers. However, bull power must be carefully managed to avoid decreases in calf crops due to poor bull to cow ratios, and inbreeding depression must be avoided by either changing the bull or in not retaining replacement females from the producer's own dams (NAHMS, 2020). Bull breeding can also be combined with defined breeding seasons, simply by maintaining bulls and females separately for at least 30 days out of the year (NAHMS, 2020). This

system conveys the benefits of a defined breeding season on calf crop (more uniform calf crops at sale and more uniform nutritional and health management programs for the herd) without the added cost of AI equipment, labor, and facilities (NAHMS, 2017). Bull breeding can be leveraged with synchronization protocols as well, where females are exposed to progestins and prostaglandins to synchronize estrous cycles prior to a period of exposure to a bull with the intent of increasing earlier conception in the breeding season (Baruselli et al., 2018). However, this practice is not widespread within the US.

I.III.II Artificial insemination and estrous synchronization

AI is more widely used in the dairy industry than the beef industry (98.8% in dairy, 11.6% in beef). This wide gap is created mostly by the more intensive handling of dairy cattle, higher culling rates (30% in dairy, 13% in beef), and the overall greater infrastructure available on dairy farms (NAHMS, 2017). AI requires greater labor and higher skill from a producer, however it can improve breeding and production programs by reducing bull power, integrating sires of great genetic merit and the introduction of specific traits (maternal traits or terminal traits) (NAHMS, 2017). It should be noted however that financial comparisons between natural service systems and ART systems (ES-AI) can be a net zero, especially if there are no genetic gains by using AI (such as increased weaning weight or increased carcass quality) (Hall, 2019).

Estrous synchronization (alternatively called estrus synchronization, ES) involves exogenous manipulation of the natural hormone cycle of female animals. There are numerous ways to synchronize estrous, some using only one factor and some using numerous. Exogenous hormones used in estrous synchronization include prostaglandins like PGF2a, progestins (progesterone or analogs), and GnRH or analogs (Marques et al., 2022). ES can be utilized in natural service systems but most usually is combined with AI (Xu et al., 1998). Types of AI include

fixed-time AI (FTAI), estrus detection and AI (E-AI) and a combination of estrus detection and timed AI (E-TAI) (Xu et al., 1998). Estrus detection can be done simply, using observation, detector animals, or detection technologies like painting, mounting patches, or with more sophisticated and technological methods like pressure sensors and radio transmissions (Xu et al., 1998). Baruselli et al. (2018) suggests that use of estrous synchronization in conjunction with timed AI followed by exposure to a natural service bull (ES-TAI-NS) can increase pregnancy rates at the end of the breeding season when compared to estrous synchronization with heat detect and AI (ES-ED-AI) and natural service only (NS). This is due to the fact that ES increases the number of cycles an animal can complete within a breeding season and TAI removes human error in diagnosing estrus behavior (Baruselli et al., 2018).

In addition to estrus detection, other determinations can be made at breeding or AI to determine the likelihood an animal is in estrus, such as elevated rectal temperature which in two studies was positively correlated with pregnancy to AI rates in both *Bos taurus* and *Bos indicus* cattle (Liles et al., 2022).

I.IV. Cattle reproductive failure

Reproductive success in production animals can be defined as establishment of pregnancy and production of a live offspring following gestation. Depending on species (puberty attainment, gestation length) and production system (dairy, beef), the definition of reproductive efficiency changes. However, reproduction and failures of reproduction are complex and multi-trait processes.

I.IV.I Endogenous causes of reproductive failure

Endogenous factors affecting reproductive success can arise from numerous factors including genetic, epigenetic, and metabolic. Arishima et al. showed a linkage between repeat

breeding (failure to establish pregnancy after repeated confirmed inseminations) and a single-nucleotide polymorphism (SNP) upstream of the *FOXP3* gene on the maternal X chromosome in Japanese Black cattle (Arishima et al., 2017) which was hypothesized to be due to the interaction of *FOXP3* with the appropriate function of Treg cells. In humans, polycystic ovary syndrome (PCOS)is a complex and multifaceted endocrine-related dysfunction that can alter fertility (Zhao et al., 2012; Jozkowiak et al., 2022; Tian-Min et al., 2022). Symptoms and etiologies of PCOS are highly variable, including hyperinsulinemia, hyperandrogenism, ovarian resistance to gonadotropins, and altered ratios of LH and FSH (Jozkowiak et al., 2022). Interestingly, a small population of cattle have been discovered with high androgen levels present in follicular fluid, with altered expression of AMH and genes relating to cell proliferation (McFee et al., 2021). In this same subpopulation of animals, it was judged that they tended to be less fertile, but the reduction was not statistically significant (Summers et al., 2014). Indoleamine 2,3-deoxygenase (IDO) is a rate limiting enzyme in the tryptophan-kynurenine pathway and this pathway is suggested to be impacted in obesity and PCOS (Zhao et al., 2012; Cussotto et al., 2020) as well as in infection or inflammation (Cussotto et al., 2020; Zhao et al., 2020). IDO is also linked to pregnancy establishment, specifically within the MRP period (maternal recognition of pregnancy) and alters trophoblastic proliferation, migration, and invasion (Zong et al., 2016). Dysregulation of IDO has been linked to recurrent spontaneous abortion in humans (Zong et al., 2016) and repeat breeding in Holstein dairy cattle (Funeshima et al., 2021). Other amino acids and amino acid metabolism pathways have been linked to successful reproduction, such as lysine in cattle (Kusama et al., 2022) and histidine in human follicular fluid (Huang et al., 2022a). It has been suggested that levels of amino acids within the uterine lumen is important for successful pregnancy attainment (Meier et al., 2014). It has also been suggested that size of the corpus luteum formed in

ovulation may have an effect (via produced levels of progesterone) on these amino acid levels within the uterine lumen as well as affecting expression of genes relating to amino acid metabolism and transport (Franca et al., 2017).

I.IV.II. Exogenous causes of reproductive failure

Reproduction as a high-energy process is affected by the environment and exogenous factors like heat stress (Diaz et al., 2021), toxins (Henley et al., 2021; Sabry et al., 2022), and infectious disease (Singh et al., 2004; NAHMS, 2020).

Plant based estrogens (phytoestrogens) and estrogenic homologs (such as bisphenyl A) can act as endocrine disrupting chemicals (EDC) and have been found to affect reproduction in male and female subjects (Jozkowiak et al., 2022). Bisphenols (BPA, BPF, BPS) are a class of chemicals commonly used in plastics and plastic products (Jozkowiak et al., 2022; Sabry et al., 2022) and were shown to induce higher levels of mitochondrial stress and activation of antioxidant genes in bovine granulosa cells, though protein expression of antioxidant enzymes was not as heavily effected (Sabry et al., 2022). Studies have also shown the disrupting effect of chemicals like phthalates, also used in plastics, and glyphosate, a chemical commonly found in pesticides (Jozkowiak et al., 2022).

In cattle, one of the most common toxicological and environmental factors encountered is tall fescue (*Schedonorus arundinaceus*) and its symbiotic endophyte *Epichloë coenophiala* (Lucas et al., 2021). Tall fescue is one of the most common cool season forages grown in the Eastern regions of North America and commonly used globally in temperate climates (Stuedemann and Hoveland, 1988). However, endophyte colonization of tall fescue can have severe negative effects on animal performance. A long noted and studied phenomenon called fescue toxicosis (Stuedemann and Hoveland, 1988) caused by the production of ergot alkaloids by the endophyte,

which are more highly produced when a stand is stressed (Lucas et al., 2021). Clinical symptoms include exacerbating heat stress due to altered hair coat shedding, vasoconstriction and necrosis of extremities and visceral adipose tissue (Stuedemann and Hoveland, 1988; Lucas et al., 2021).

Heat stress is another well-documented deregulator of reproduction, that results in lower pregnancy rates. Cattle under heat stress have been shown to produce oocytes of lower overall quality when under heat stress compared to being at temperature neutral conditions (Diaz et al., 2021). As previously stated, heat stress can also be exacerbated by interactions with tall fescue grazing (Lucas et al., 2021).

Cattle can also suffer reproductive failure from infection or exposure to disease. *Tritrichomonas foetus* is a protozoan parasite that bulls can become permanently infected with that they then spread through sexual contact to females (NAHMS, 2020), which then can lead to female infertility and late-term abortion (Singh et al., 2004) a condition termed trichomoniasis. Piersanti et al. (2019) showed in an *in vitro* study that exposure of granulosa cells and cumulus-oocyte-complexes to LPS (lipopolysaccharide) had a significant effect on the transcript levels of 92 genes related to oocyte health.

I.V. Predictive biomarkers

Discussed above are numerous and multi-faceted ways that endogenous and exogenous factors can affect fertility, however many of them do not have apparent and readily visible effects outside of reproductive failure. When a heifer suffers even her first reproductive failure, she has already reached 13-14 months old (*Bos taurus*) and has already significantly cost the developer resources and time (Boyer et al., 2020). Thus, it is desirable to discover and validate biomarkers for high-performing and fertile female animals to avoid some of the losses accrued with conceptive failure. The World Health Organization (WHO) has defined a biomarker as “any substance,

structure or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease." (2001). Biomarkers can be directly related to genetic structures, such as in SNPs (Arishima et al., 2017; Stegemiller et al., 2021; Mora-Palazuelos et al., 2022), related to transcript expression of genes, expression of genes into functional proteins (Kusama et al., 2022), and related to circulating levels of metabolites (Tian-Min et al., 2022). The rest of this review will discuss various studies in light of potential biomarker discovery for reproductive failure.

I.VI. Plasma immunoglobulins as predictive of reproductive performance

I.VI.I. Interactions of reproduction and immunity

Reproduction and fertility are intrinsically linked with immune response and inflammation. Inflammatory signals are integral to the regular cycling of females (such as the activity of the cytokine TNFα in luteolysis) (Senger, 2012) and maternal immune recognition of an allogenic fetus for correct establishment of pregnancy (Tsuda et al., 2021).

Innate immunity is the first line of defense of an organism and is 'un-targeted' and 'un-adaptive' as cells of the innate immune system respond to commonly expressed molecular patterns present on the surface of common pathogens (Janeway et al., 2001). Upon activation, cells of the innate immune system secrete cytokines to signal for continuing immune response (Janeway et al., 2001). Cytokines are small molecules that act as cellular messengers and trigger inflammatory and immune responses (Berger, 2000). Cytokines can be broadly grouped into Th1 (inflammatory, such as IFNy, IL6, and TNFa) and Th2 (anti-inflammatory, IL10, IL4, IL13) (Berger, 2000).

Some pathogens can rapidly evolve to escape the pattern recognition ability of the innate immune system, or like viruses, do not express such patterns at all (Janeway et al., 2001). The adaptive immune system has evolved to capture and react to these pathogens. This process begins

with dendritic cells encountering and capturing a pathogenic cell. The dendritic cell then undergoes maturation into an antigen-presenting cell (APC) and begins to alter lymphocytes. Lymphocytes arise from bone-marrow (B-lymphocytes/B-cells) and the thymus (T-lymphocytes, T-cells). B-cells are more closely linked with antibody-mediated response and humoral immune response and T-cells are more closely linked with cell-mediated immune response. Mature B and T cells are programmed by dendritic cells to be specifically reactive to whatever antigen they are presented with and secrete cytokines[as covered by (Janeway et al., 2001)].

B-cells produce and secrete glycoprotein antibodies upon activation, antibodies that are highly specific for the same antigens as their parent B-cell (Janeway et al., 2001). These secreted antibodies are free versions of B-cell antigen receptors (Janeway et al., 2001). Antibodies are capable of binding to pathogens and toxins and marking them for opsonization (destruction) or simply blocking them from binding to cells. The FC chain of the antibody can also trigger complement activation to remove the pathogen via renal podocytes (Janeway et al., 2001).

Cytokines are broadly grouped by activity, either being pro-inflammatory or anti-inflammatory (Berger, 2000). Th1 cytokines are broadly speaking inflammatory (IL6, TNFa, IFNy) and Th2 cytokines are broadly speaking anti-inflammatory (IL10, IL13, IL4) (Berger, 2000). If there is too much of one set of cytokines present, side effects of tissue damage (Th1) or the blocking of microbicidal activity (Th2) can be observed (Berger, 2000). Thus, a balance of Th1/Th2 response is desirable.

I.VI.II. The Th1/Th2 response in pregnancy

In early pregnancy, the Th1/Th2 cytokine environment is altered, with downregulation of the Th1 response and an upregulation of the Th2 response (Corpron et al., 2021). Pre-eclampsia, a hypertension related syndrome of human pregnancy, has been related back to alterations in the

Th1/Th2 cytokine balance and inflammatory cytokine production, such as SNPs present in inflammatory cytokines like TNFα and IL-6 (Mora-Palazuelos et al., 2022). Th1-dominated immune response (inflammatory) is implicated in poor placentation and the exacerbated inflammatory response that characterizes pre-eclampsia (Mora-Palazuelos et al., 2022). Progesterone levels may be important to controlling the Th1/Th2 balance, as progesterone supplementation to PBMC (peripheral blood mononuclear cells) cultures reduced the production of inflammatory signals and increased the production of anti-inflammatory ones (Raghupathy et al., 2005; Raghupathy, 2013).

In cattle, it has been shown that PBMC under the influence of interferon tau (IFNτ) increased production of IL-10 (Th2) in animals that correctly established pregnancy compared to animals with early embryonic death and animals that did not conceive (Shirasuna et al. 2012). In this same study, production of TNFα (Th1) by PBMC was steady in animals that established pregnancy, suffered early embryonic death, or did not conceive (Shirasuna et al., 2012). Phillips et al. (2018) found increased levels of IL6 and TNFα mRNA in the pWBCs of beef heifers that failed to conceive when compared to fertile controls. In humans, adipocyte-produced TNFα has been related to obesity-impacted fertility issues (Silvestris et al., 2018).

I.VI.III. Immunoglobulin relation to Th1/Th2 response

As previously discussed, B-cells (as cells of the adaptive immune system) produce antibodies upon activation by cytokines (Janeway et al., 2001). Antibodies produced by B-cells can then bind to antigens and activate the complement system, tag antigens for opsonization, or prevent antigens from binding to target cells (Janeway et al. 2001). Immunoglobulin G (IgG) occurs in four subclasses (IgG1, IgG2, IgG3, IgG4) and is the most abundant antibody in serum (Vidarsson et al., 2014). IgG1 is related to the Th2 response and IgG2 to the Th1 response (Corpron

et al., 2021; Hernandez-Davies et al., 2022). As the IgG subclasses maintain relatively stable fractions to one another in a healthy state (Vidarsson et al., 2014) an IgG2/IgG1 ratio of greater than 1 can be defined as activation of a Th1 response and increased TNFα and INFγ increased the ratio of IgG2/IgG1 (Rittipornlertrak et al., 2022). A higher ratio of IgG2 to IgG1 was demonstrated by Corpron et al. to be negatively correlated to establishment of pregnancy to AI service in primiparous and multiparous beef cattle (Corpron et al., 2021). Ratios of IgG2 to IgG1 have also been suggested to be prognostic of disease risk in the periparturient period, due to the altered IgG1/IgG2 balance created by onset of lactation (Tsai et al., 2021).

I.VII. Metabolomics

I.VII.I. What is a metabolome?

-*Omics* is the study of global populations of biologically significant molecules (Conesa and Beck, 2019) and includes the fields of genomics, metagenomics, epigenomics, transcriptomics, proteomics, metabolomics, and lipidomics (Chakraborty et al., 2022). At the conception of the technique, usually only one form of assay was the research focus (for example, metabolomics) and created single-omics data, but in recent years multi-omics data, or the combination of assays (for example transcriptomics and metabolomics (Tian-Min et al., 2022)) is more commonly generated (Conesa and Beck, 2019). Metabolomics is the global study of small molecular weight molecules present in biofluids like plasma, urine, or serum, or within tissues or cells (Wishart, 2005). Metabolomics can be applied either in untargeted discovery studies (Phillips et al., 2018) or in highly targeted studies focused on a specific chemical class of metabolites (Cussotto et al., 2020). There are a number of potential platforms that can be used in a metabolomics study each with advantages and disadvantages that must be taken into account [as reviewed by (Blaurock et al., 2022)]. Proton nuclear magnetic resonance (^{1}H-NMR) analyzes metabolites by the characteristic

shifts of functional groups and provides quantitative data but is insensitive to molecules present in very small quantities. High performance liquid chromatography mass spectrometry (HPLC-MS) is the most commonly used system in metabolomics and utilizes a solvent and stationary phase to separate metabolites and mass spectrometry to detect metabolites. HPLC-MS is broadly sensitive as well, with a wide linear dynamic range. However, the solvent phase can theoretically interfere with proper observation of metabolites. Gas chromatography mass spectrometry (GC-MS) uses small sample sizes and does not require high volumes of liquid solvent like HPLC-MS. GC-MS is also highly sensitive to small concentrations of metabolites and can be applied in targeted and untargeted studies, however it only provides qualitative information. (Blaurock et al., 2022).

I.VII.II. Metabolome use in biomedical sciences

Metabolomics has been utilized in numerous fields and types of study, and has been often applied to studies of disease and healthy states (Wishart, 2005; Zhao et al., 2012; Hailemariam et al., 2014; Broughton-Neiswanger et al., 2020; Arshad et al., 2022; Tian-Min et al., 2022; Zhang et al., 2022b); toxicological studies (Broughton-Neiswanger et al., 2020; Gaskins et al., 2021; Hou et al., 2022; Hwang et al., 2022); and studies of biological states (Guo and Tao, 2018; Bai et al., 2020; Funeshima et al., 2021; Huang et al., 2022a).

As discussed by Wishart, metabolomic profiling to diagnose the failure of transplantation has shown diagnostic promise as changes in metabolites may be precursors to other signals of rejection (Wishart, 2005). Similarly Tian-Min et al. performed a study of miRNA profiles and metabolomic profiles in patients with PCOS and found 157 miRNAs and 31 metabolites changed between case profiles when compared to healthy controls (Tian-Min et al., 2022). Hailemariam et al. showed metabolomic profile changes related to the development of postpartum diseases like metritis and mastitis in dairy cattle (Hailemariam et al., 2014). In this study, changes in

metabolomic profiles were noted as early as 4 weeks prepartum which was suggested to be used as a marker of risk for the development of such conditions (Hailemariam et al., 2014).

In women undergoing ART (assisted reproduction techniques) environmental exposure to nitrous oxide, particulate matter, and black carbon (common air pollutants in urban areas) were correlated to changes in metabolite profiles of follicular fluid and success of ART procedures (Hwang et al., 2022). Gaskins et al. utilized serum metabolomics to examine the chance of live birth from ART with air pollution as a factor, finding changes in metabolite profiles related to exposure to air pollution, including alterations in tryptophan metabolism (Gaskins et al., 2021).

Fatty liver disease has severe impacts on dairy cattle production in the postpartum period and is difficult to diagnose (Zhang et al., 2022b). Using urine, feces, and serum metabolomic profiling in conjunction with liver biopsies it was shown that 10 metabolites were significantly changed between fatty liver disease animals and healthy animals (Zhang et al., 2022b). It has been suggested that metabolomic profiles and changes in profiles may be affected by breed or breed-type, as shown in Hu et al. (2022) that studied metabolomic changes in Inner-Mongolia Sanhe cattle and Holstein cattle exposed to acute cold stress.

I.VII.III. Metabolomics in female fertility

Biomarkers are of the most use when present in an easily accessible location. Blood is the most readily available tissue and has been shown to be descriptive of certain tissues in dysbiosis (Basu et al., 2021). Previously, transcriptomic analysis of pWBC populations has been discriminatory of heifer reproductive potential both at weaning and at AI (Dickinson et al., 2018; Banerjee et al., 2023). Expression of IDO in pWBC has been linked to reduced kynurenine levels in plasma of subfertile cows (Funeshima et al., 2021) and a well-known metabolite-organ interaction that can be used for diagnostics would be blood glucose levels be descriptive of

pancreatic β-cell health. The blood-follicle barrier (BFB) shows differences in permeability throughout development and may be altered in reproductive disease and dysfunction (Siu and Cheng, 2012). The BFB is passively selective against molecules greater than 500 kDa (Siu and Cheng, 2012) but also actively selective against some proteins, such as inter-alpha-trypsin inhibitor protein which is not found inside the follicle prior to the ovulatory GnRH surge even though it is 225 kDa (Powers et al., 1995). This was shown to be due to the activity of NO (nitric oxide) that can also be increased under oxidative stress conditions (Abdoon et al., 2023) that may suggest that increased stress and increased nitric oxide could affect the permeability and accessibility of the ovarian follicle. Metabolite passage over the BFB is most likely passive due to the similarities of electrolytes between plasma/serum and follicular fluid (Gosden et al., 1988) with a blockade of diffusion of metabolites back out of the follicle. However, there may also be active transport of some metabolites over the BFB, such as boron which is greater in follicular fluid than in serum despite its low molecular weight (10 Da) (Zhang et al., 2022a). It has been suggested that there are structural and permeability/selectivity differences in the BFB in a mouse model of PCOS (Zhou et al., 2008).

I.VII.III.a. Blood plasma

Polycystic ovary syndrome (PCOS) is a multi-trait, multi-system endocrine disruption of normal ovarian function. Zhao et al. (2012) explored blood plasma metabolite profiles of women with PCOS compared to healthy controls. This study showed upregulation of lactate, ornithine, tryptophan, and low-density lipoprotein, suggesting alterations to carbohydrate, protein, and lipid metabolisms in PCOS (Zhao et al., 2012). Also, in humans, the relationship of indole metabolites (tryptophan, serotonin, kynurenine, indole-3-lactate, etc.) in blood plasma were explored in

relation to obesity and chronic low-grade inflammation, showing a negative correlation between inflammation and tryptophan in plasma (Cussotto et al., 2020).

I.VII.III.b. Plasma and follicular fluid

Follicular fluid is formed by extrusion of plasma through the basement membrane of the follicle, secretions from the granulosa, and from the oocyte itself, and is an important medium for oocyte health and development (Horn et al., 2022). As such, follicular fluid is a common medium for metabolomic studies in humans, less for biomarker discovery and more for elucidating underlying changes in metabolism. Depending on the platform utilized, the number of identified features and the number of overlapped features varies. Also dependent on platform is which biofluid is more feature dense (Hood et al., 2022). Hood et al. (2022) utilized two types of platforms and identified 1,928 features and 1,149 features that overlapped between follicular fluid and serum. In cattle, a study utilized ultra-high performance liquid chromatography procedures and identified 38 features in follicular fluid and 50 features in serum and 32 overlapped (Horn et al., 2022). More direct studies of particular metabolites and ions such as β-hydroxybutyrate, sodium, and NEFAs have been performed between plasma and follicular fluid as well (Leroy et al., 2004). In human studies, Xia et al. found differences in the follicular fluid of women with successful IVF cycles when compared to women with repeated failure of IVF, including elevated levels of cysteine, lysine, and ornithine (Xia et al., 2014). Huang et al. applied follicular fluid metabolomics to study the differences between a younger cohort of women undergoing ART and an older cohort; amongst their findings was an elevated level of histidine in older women's follicular fluid (Huang et al., 2022a).

I.VII.IV. Metabolomics in agriculture and livestock sciences

Phillips et al. (2018) used plasma metabolomic profiling to examine the differences between high-performing (fertile) and low-performing (infertile) cross-bred beef heifers. In this study, levels of the amino acids tryptophan, kynurenine, lysine, histidine, cystine, and ornithine were lower in infertile animals when compared to fertile animals at the time of AI (Phillips et al., 2018). Kusama et al. (2022) used linked proteomic analysis with serum metabolomic profiling to study successful or non-successful fetal implantation in cattle, finding an alteration in the expression levels of a protein related to intracellular trafficking and in some amino acids, like serine, phenylalanine, alanine, and lysine. On day 20 of this study, serum levels of lysine were higher in successful animals (Kusama et al., 2022).

As failure to breed in livestock species, especially cattle can have high economic consequences for producers (Boyer et al., 2020), there is considerable research into predicting, preventing, and selecting high performing females. Fernandez-Hernandez et al. (2022) compared equine follicular fluid from post-mortem ovaries to two common IVM medias using metabolomic processes with intent to improve the makeup of IVM/IVF media to be more similar to the intrinsic growth media of follicular fluid. Bai et al. (2020) used follicular fluid analysis of dairy cattle to study the difference between cows in lactational anestrous and healthy controls. Fourteen metabolites were significantly altered between groups, including glutamine and arachidonic acid (Bai et al., 2020). Repeat breeding is an issue in dairy cattle (multiple confirmed inseminations but no pregnancy) and has been linked by plasma metabolomic profiling to tryptophan metabolism via the metabolite kynurenine that was decreased in repeat breeders (Funeshima et al., 2021) which is in agreement with Phillips et al. (2018) that also found decreased levels of kynurenine in low-performing beef heifers. In the same study, arachidonic acid levels were found at lower levels in repeat breeding cohort compared to the healthy controls (Funeshima et al., 2021). Guo and Tao

(2018) showed metabolomic changes in bovine plasma in the early part of pregnancy, with changes in profiles between AI and Day 17 of pregnancy, AI and Day 45 of pregnancy, but no significant difference between Day 17 and Day 45 by OPLS-DA modeling.

Obesity has been shown to be a disrupter of female fertility (Silvestris et al., 2018) and studies in cattle have shown that high BCS can have negative effects on pregnancy rates (Guzman et al., 2016; Marrella et al., 2021; Marques et al., 2022). Horn et al. studied the differences in serum and follicular fluid metabolomes in lactating beef cattle with divergent BCS, discovering changes in serum relating to BCS, but perhaps a buffering effect of the follicle against those changes as there were no significant changes in follicular fluid in response to BCS (Horn et al., 2022).

In swine, Fletcher et al. (2022) used saliva, urine, and serum metabolomic profiles to study differences between high-fertility sows (high litter numbers) and low-fertility sows (low litter numbers), showing altered amino acid, fatty acid, and steroid hormone metabolism between high-fertility animals and low- fertility animals including down-regulated kynurenine in saliva and down regulated histamine in serum of high fertility sows, as well as upregulated tryptophan in serum of highly fertile sows. This study also suggests that urine metabolite profiles may out-perform salivary or serum metabolite profiles for predictive accuracy based on PLS-DA modelling (Fletcher et al., 2022). Mo et al. (2021) reported 18 metabolites at different levels in follicular fluid from atretic and healthy follicles, including altered levels of amino acids and lipids.

I.VIII. Impacts conclusions, and implications

It has been shown that, while useful tools for making selection decisions, age, weight, BCS, and RTS are not perfect prognostic tools to select the most fertile females and as such, biomarkers and selection tools with greater predictive accuracy are needed in animal production. Identification of biomarkers at early time points (i.e. weaning) in heifer development would benefit producers

by identifying fertile heifers prior to investment in her development, growth, and first breeding season. Along with biomarkers for development and fertility, biomarkers for health and wellness could provide benefit to cattle producers by identifying animals with subclinical disease and dysbiosis.

Numerous studies have linked immune response and inflammation to pregnancy, validating the study of immunological markers as biomarkers for fertility. Highly available antibodies in circulation are desirable as metrics of fertility and subclinical disease but have not yet been validated in beef heifers.

Metabolite profiles in both plasma and follicular fluid have been shown to be altered under healthy and disease states, and between animals that are fertile and those that are subfertile. However, many of these studies have been done at or after breeding, reducing the utility of their findings as biomarkers due to the late time point, though the findings are still biologically relevant. Ideally, easily testable biomarkers for fertility can be found at earlier time points, such as weaning or some months prior to AI. Biomarkers that were accurate and specific in such time points could help to reduce waste in animal development, as producers can cull less fertile heifers, and increase sustainability by increasing the number of animals produced with the same resources.

Chapter II: Plasma Immunoglobulins as Biomarkers of Pregnancy to First Service AI in *Bos taurus* Beef Heifers

II.I. Abstract

Immune status is closely and intimately linked to the establishment of pregnancy. Activation of the innate immune system or the adaptive immune system and dysregulation of the Th1/Th2 cytokine balance has been linked to reproductive failure in humans and animals. This study sought to determine whether highly abundant plasma immunoglobulins can serve as easily accessible biomarkers for reproductive performance in beef heifers when tested shortly before AI. Sixty-two virgin heifers were estrous synchronized and bred by heat detection and AI, and blood samples were taken at the start of the estrous synchronization period. Twelve heifers each were chosen for further testing due to establishing pregnancy to first service AI or for conceiving late or not at all during the breeding season. Blood plasma levels of IgG1 and IgG2 were tested using SDS-PAGE and Western blotting methods. In heifers that established pregnancy to first AI, there were significantly higher levels of IgG2. There was no significant difference in levels of IgG1 between cohorts. Receiver operating characteristic (ROC) analysis of IgG1 returned area under the curve (AUC) values of less than 0.75. ROC analysis of IgG2 returned AUC of 0.8125. These results suggest that heifers more likely to establish pregnancy to AI have greater antibody levels in plasma. Considering previous research, this may indicate that high-performing heifers may have greater immune response ability than low-performing heifers.

II.II. Introduction

Heifer development in beef and dairy cattle production systems can have large financial impacts (Boyer et al., 2020). Should a cow or heifer fail to conceive, it extends the number of successful breeding seasons that she must complete before she becomes a net profit for a producer (Boyer et al., 2020). While most beef animals in the US are bred by natural service bulls (NAHMS, 2020) estrous synchronization and artificial insemination in conjunction with natural service bulls can be utilized to increase overall pregnancy rates (Hall, 2019). This is due to the effect of estrous synchronization hormones 'kick-starting' estrous cyclicity in non-cyclic and prepubertal animals prior to the breeding season (Monn et al., 2019; Boyer et al., 2020). Increased pregnancy rates are desirable to decrease the cost of each pregnancy whether bred by natural service bulls, AI, or a combination (Hall, 2019).

Correct estrous cycling and conception are closely linked with inflammation (Senger, 2012) and immune response (Tsuda et al., 2021). Alterations in the maternal immune response are critical for proper recognition and subsequent tolerance of allogenic fetuses (Tsuda et al., 2021) and dysbiosis in this system of immunomodulation has been linked to reproductive syndromes like preeclampsia (Mora-Palazuelos et al., 2022).

Immunity in mammals is formed by interactions of the innate and adaptive immune systems (Janeway et al., 2001). Cells of the innate immune system secrete cytokines upon activation which then alters the activity of cells of the adaptive immune system (Janeway et al., 2001). Cytokines are proteinaceous cellular messengers broadly classified as pro-inflammatory or anti-inflammatory (Berger, 2000). Upon activation by cytokines of the innate immune system, plasma B-cells (also called B-lymphocytes) secrete antibodies with high specificity to antigens (Janeway et al., 2001). Immunoglobulins are glycoprotein-based antibodies secreted by B-cells

and Immunoglobulin G (IgG) is the most common antibody in circulating blood (Vidarsson et al., 2014).

Cytokines are produced by cells of the immune system and canonically are grouped by being Th1 (pro-inflammatory) and Th2 (anti-inflammatory) (Berger, 2000). The balance of Th1 and Th2 cytokines is important for health and immune response as alterations of this balance can cause tissue damage (as in an unchecked Th1 response) and block anti-microbial activity (an unchecked Th2 response) (Berger, 2000). Pregnancy is a Th2-dominated state (Corpron et al., 2021) and high Th1 response is undesirable in reproduction (Corpron et al., 2021; Mora-Palazuelos et al., 2022).

IgG occurs in four isoforms (IgG1, IgG2, IgG3, IgG4) numbered in order of prevalence; immunoglobulin isotypes also maintain steady ratios to one another in healthy states (Vidarsson et al., 2014). IgG1 has been shown to be related to the Th2-cytokine response and IgG2 to the Th1-cytokine response (Corpron et al., 2021; Rittipornlertrak et al., 2022). Plasma immunoglobulin testing is a frequent metric to show competence and activation of an antibody-mediated immune response (Reverter et al., 2021; Rittipornlertrak et al., 2022). It has previously been suggested that measurements of relative levels of IgG1 and IgG2 can be descriptive of animal disease risk (Tsai et al., 2021) and of post-partum fertility (Corpron et al., 2021). By measuring the altered ratio of IgG1 to IgG2, it is proposed that an altered Th1/Th2 response can be inferred (Corpron et al., 2021). A ratio of IgG2/IgG1>1 is suggestive of a switch to a Th1 immune response (Rittipornlertrak et al., 2022) and is undesirable for pregnancy establishment (Corpron et al., 2021). Immunoglobulins would make ideal biomarkers as they are prevalent in general circulation and IgG1 and IgG2 have 21-day half-lives (Vidarsson et al., 2014), the same as average cattle estrous cycles (Senger, 2012).

It is hypothesized that heifers with increased levels of immunoglobulins will show a low-performing reproductive phenotype compared to heifers with reduced levels of immunoglobulins. The objective was to determine whether or not plasma levels of IgG1 and IgG2 were correlated with reproductive outcomes in *Bos taurus* beef heifers. Plasma was tested from heifers that established pregnancy to first service AI (1stAI) and those that did not conceive on first service (LBred). Western blotting was utilized to quantitate IgG1 and IgG2 in the plasma from beef heifers of both groups.

II.III. Materials and Methods

II.III.a. Animal care and use

All animal-related activities were reviewed and approved by the Institutional Animal Care and Use Committee of Auburn University (IACUC protocol #2021-3968). Heifers were maintained at the North Auburn Beef Unit managed by the Auburn University College of Veterinary Medicine.

II.III.b. Phenotypic measures

Heifer age was collected by unit staff and later accessed by researchers. On Day 0 of the experimental timeline, heifer's body weight (BW), reproductive tract score (RTS), and pelvic measurements (PA) were collected. RTS was measured via transrectal palpation by a single trained technician. The pelvic area was measured as pelvic height and pelvic width using calipers inserted transrectally. Pelvic area and RTS were measured by a single, trained technician on all animals.

II.III.c. Estrous synchronization and artificial insemination

Figure 2.1: Experimental timeline and timeline of 7+7 estrous synchronization and artificial insemination protocol. AI- artificial insemination, BW-body weight, CIDR-controlled internal drug release, GnRH-gonadotropin releasing hormone, HD- heat detect, PA- pelvic area, PC-pregnancy check, PG- prostaglandin, RTS- reproductive tract score.

Heifers were estrous synchronized utilizing a 7 + 7 synchronization protocol. On Day 5 of the study, heifers were injected with a prostaglandin analog (Lutalyse®, Zoetis) and a controlled internal drug release device (EZI-Breed CIDR®, Zoetis) containing progesterone was transvaginally inserted. On Day 12, heifers were injected with a GnRH analog (Cystorelin®, Boehringer Ingelheim). On Day 19, heifers had CIDRs removed, an injection of prostaglandin analog, and estrus detection devices (Estrotect^{TM} Breeding Indicators) were applied. Heifers were observed for activation of estrus patches. On Day 21, artificial insemination began. On Day 22, due to management reasons, heifers that had not activated estrus patches were AI-bred and injected with an GnRH analog. Heifers were bred by four trained inseminators using one straw of semen from two bulls of proven fertility.

II.III.d. Pregnancy determination

On day 72 of the experiment, heifers were checked for pregnancy to AI via transrectal ultrasound. Ultrasound was performed by trained technicians, and animals were categorized as conceived to first service AI (1stAI) or not-pregnant to first service AI (LBred). Heifers had been briefly exposed to natural service bulls for 60 days starting 14 days after the end of AI the period.

II.III.e. Sample collection

Samples were collected at the start of the estrous synchronization protocol (Day 5). Whole blood samples were collected by jugular venipuncture with an 18G needle into a 10 mL EDTA

vacutainers (BD, Franklin Lakes, NJ, USA). Tubes were immediately inverted ten times and placed on ice until further processing. Blood plasma was separated from the whole blood via centrifugation at 1500 *g* for 10 minutes at 4°C. Plasma was then stored at -80°C until further handling.

II.III.f. Protein quantification

Blood plasma was thawed at 4°C, followed by dilution using 1x PBS into a 10x working dilution (10 µl of plasma and 90 µl of PBS) and into a 100x quantification dilution (10 µl of 10x working dilution with 90 µl of PBS).

To determine the total protein content of plasma samples a Pierce™ BCA Protein Assay Kit (ThermoFisher catalog #23227) was utilized. For quantification, 10 µl of 100x dilution was combined with 200 µl of working reagent in a clear bottomed 96-well plate (Greiner Bio One catalog #655 180). The plate was agitated briefly to ensure a complete combination of sample and working reagent and then incubated at 37°C for 30 minutes. Plate was read with a 456 nm wavelength on an automated plate reader (EMax® Plus, Molecular Devices) using a pre-loaded BCA assay program (SoftMaxPro 6.5.1, Molecular Devices). Samples were run in duplicate, and the assay was repeated until intra-sample CVs were less than 5%. Assay R^2s were required to be above 0.995.

II.III.g. SDS-PAGE sample preparation, blot transfer, total protein staining

Plasma samples for SDS-PAGE were prepared by diluting 10X in 1x PBS and SDS-Laemli loading buffer (4X) was added. Samples were then incubated at 95°C for 7 minutes and then vortexed briefly. A total of 15 µg of protein from each plasma sample was then loaded per well on an 8% SDS-PAGE gel. Gel makeup and set up protocols are available in **Appendix 1**. Inclusion of 2,2,2-trichloroethanol in 8% SDS-PAGE gel allowed for total protein visualization. Gels were

then electrophoresed at 80 mA for 2 hours (20 mA/gel) in 1x running buffer (25 mmol/L Tris, 192 mmol/L glycine, and 0.1% w/v SDS). Upon completion, gels were removed from sandwich and placed in ddH_2O until activated by ultraviolet exposure. Images of protein load were captured using a ChemiDoc XRS+ imager. Gels were then equilibrated in transfer buffer until loaded into transfer sandwich.

Gels were transferred using wet transfer techniques onto low-fluorescence PVDF membranes activated in methanol. Transfer took place at room temperature for 16 hours in 1x transfer buffer (25 mmol Tris, 192 mmol glycine, and 10% v/v methanol). Post-transfer, membranes were moved to ddH_2O and exposed to ChemiDoc XRS+ imager to determine transfer of protein from gel to membrane. Upon capture of total protein images, membranes were moved to wash buffer [1x TBS-T (0.1% Tween-20 v/v)] and stored at 4°C until use.

II.III.h. Western Blot- blocking, probe, quantification

Membranes were blocked overnight at 4°C in 5% BSA in 1x TBS-T (0.1% Tween-20 v/v) and exposed to antibodies diluted in blocking buffer for 1 hour at room temperature with gentle agitation. IgG1-HRP (NovusBio catalog # NB783) was diluted 1:10000 (v/v) and IgG2-HRP (NovusBio catalog #NB788) was diluted 1:2000 (v/v) in the blocking buffer.

Upon finishing exposure to antibodies, membranes were washed 3-4 times in wash buffer for 5 minutes each time with gentle agitation. Membranes were incubated with ECL reagent (Azure Biosystems catalog #AC2103) for 30 seconds (IgG1) and 1 minute (IgG2). Images were captured using the auto-exposure feature of cSeries Capture Software (Azure Biosystems) on an Azure Biosystems c400 imager with sensitivity set to normal and a marker (colorimetric) image captured for each membrane.

II.III.i. Statistical analysis

Phenotypic measures of age at AI, body weight, pelvic area, and RTS were analyzed using non-paired Student's t-test with a Welch's correction in Prism v6.

Densitometry of images was captured using ImageJ (NIH). Measures of immunoglobulins were normalized to total protein with the equation POI_{lane}/LC_{lane}=normalized $value_{lane}$ as suggested by Aldridge et al. (2008). A control sample was used on each blot to normalize samples between membranes. Prism v6 was used to perform statistical tests and generate graphs. Two-tailed Mann-Whitney non-parametric test was used to determine statistical significance between median ranks of cohorts. Significance was set at $p \leq 0.05$.

Receiver operating characteristics (ROC) curves were generated for antibody levels to determine their diagnostic ability, with curves determined to be significant at $p \leq 0.05$.

II.IV. Results

II.IV.a. Pregnancy determination

Pregnancy was determined for cohort of 62 head via transrectal ultrasound by a trained technician. From animals pregnant to first service AI (1stAI) (n=25) and those not detected as pregnant at first pregnancy checking (n=36), 24 animals (1stAI, n=12; LBred n=12) were randomly selected for plasma immunoglobulin testing.

II.IV.b. Phenotypic measures

There were no significant differences in body weight, pelvic area, RTS, or age at AI when tested by Student's t-test ($p > 0.05$, **Table 2.1**). Graphs of phenotypic measures are in **Figure 2.2**. All sampled animals were of RTS 4-5.

Table 2.1: P-value and standard deviation of phenotypic measures of 1stAI (n=12) and LBred (n=12) animals by Student's t-test. BW- body weight in kilograms; RTS- reproductive tract score. (*) denotes values with p-value ≤ 0.05.

Phenotype.	BW (kg)	Pelvic Area (cm^2)	RTS	Age at AI (days)
LBred	387.73 ± 28.55	191.08 ± 15.50	4.58 ± 0.52	419.75 ± 13.45
1stAI	403.86 ± 42.91	186.33 ± 10.99	4.5 ± 0.52	422.75 ± 13.60
P-value	0.290	0.396	0.698	0.592

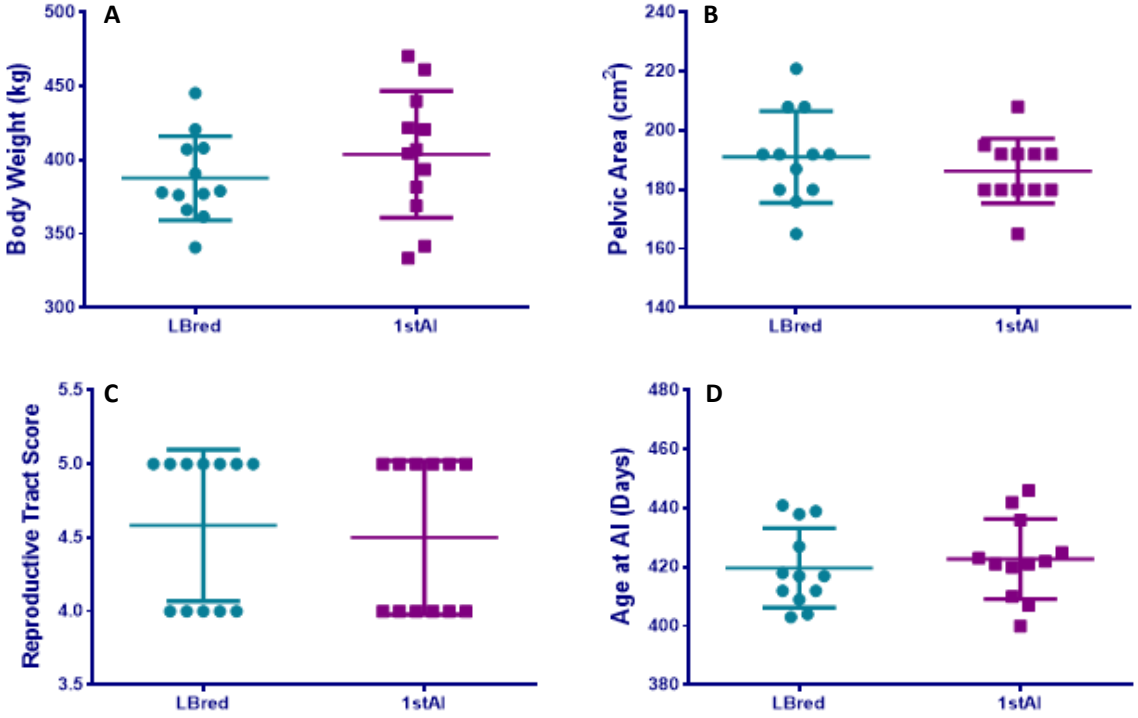

Figure 2.2: Phenotypic measures of 1stAI (n=12) and LBred (n=12) animals. A) Body weight in kilograms; B) Pelvic Area in centimeters squared; C) RTS- reproductive tract score; D) Age at AI in days. (*) represents p-value ≤ 0.05 by Student's t-test.

II.IV.c. Protein quantification

Levels of total protein in bovine blood plasma ranged from 72.40 µg/µl to 119.8 µg/µl with a mean and standard deviation of 88.92 ± 12.55 µg/µl.

Total protein content of plasma samples was checked by BCA assay and was not significantly different between 1stAI and LBred animals (p = 0.884). The LBred cohort had mean total protein content of 88.53 µg/µl with a standard deviation in content of ± 10.82 µg/µl. The

1stAI cohort had a mean total protein content of 89.31 µg/µl with a standard deviation of ± 14.56 µg/µl. The total protein levels of cohorts are shown in **Figure 2.3**.

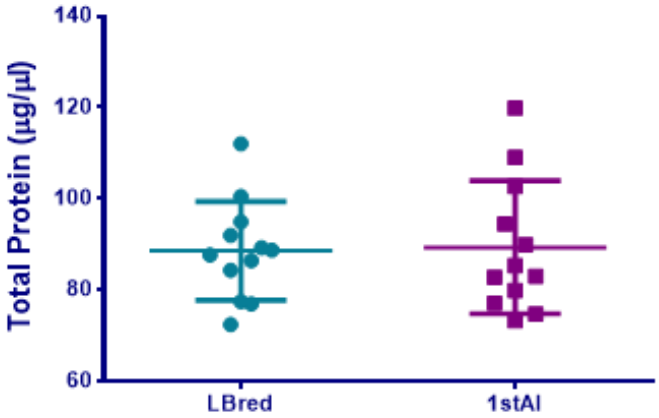

Figure 2.3: Mean and standard deviation of total plasma protein measures for 1stAI (n=12) and LBred (n=12) animals in µg/µl. (*) denotes p-value ≤ 0.05 by Student's t-test.

II.IV.d. Relative Comparison of IgG levels

Representative images of total protein staining, chemiluminescent, and colorimetric staining are in **Appendix 2**. Protein of interest was normalized by total protein and to a loading control sample and levels are expressed as relative densitometric units (DU). There was no significant difference between IgG1 levels between cohorts, with 1stAI having 1.06 ± 0.59 DU and LBred having 1.35 ± 0.55 DU (p=0.239). IgG2 levels were significantly higher in 1stAI heifers (2.54 ± 0.84) when compared to LBred heifers (1.78 ± 0.36, p = 0.008). Relative levels of IgG1 and IgG2 in cohorts are shown in **Figure 2.4**.

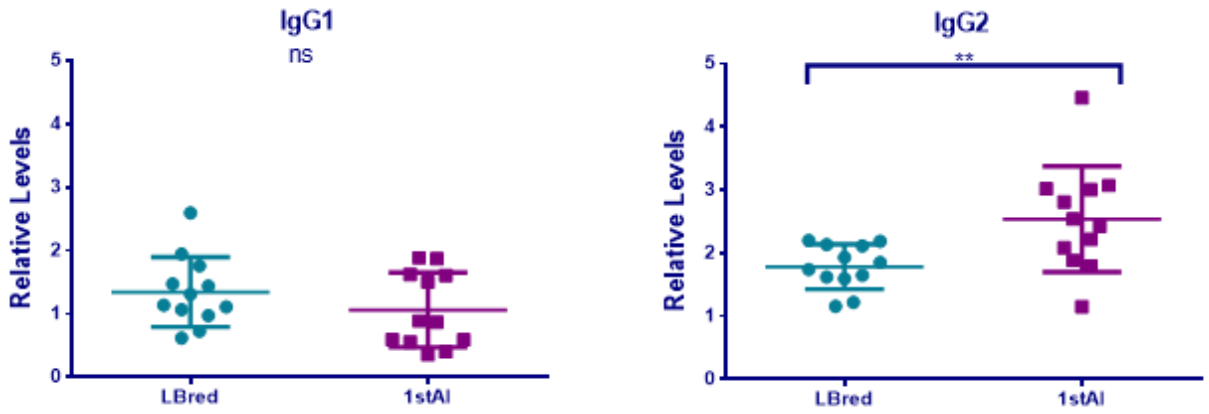

Figure 2.4: Relative levels of IgG1 and IgG2 in LBred (n=12) and 1stAI (n=12) cohorts. (**) denotes $p \leq 0.01$ by Mann-Whitney U test.

Levels of IgG1 and IgG2 were examined using ROC analysis. AUC for IgG1 was 0.646 and non-significant ($p > 0.05$). AUC for IgG2 was 0.813 and was significant ($p = 0.009$) with a 95% confidence interval of 0.625 to 0.9998. ROC curves for IgG1 and IgG2 are shown in **Figure 2.5**.

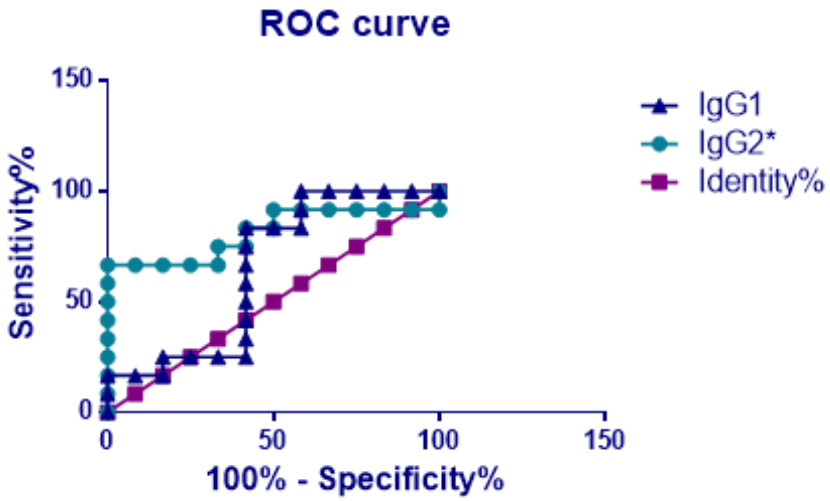

Figure 2.5: ROC curve of IgG1 and IgG2 levels. (*) denotes a $p \leq 0.05$ of ROC curve.

II.V. Discussion

There was no significant difference in measures of body weight, pelvic area, age at AI or reproductive tract score between 1stAI and LBred cohorts. These phenotypic results agree with numerous other studies that could not discriminate between high-performing and low-performing heifers based solely on phenotypic measures (Gutierrez et al., 2014; Dickinson et al., 2019; Hindman et al., 2022). Plasma total protein levels were not significantly different between cohorts in this study.

The data collected does not support the stated hypothesis. IgG2 levels were higher in the 1stAI cohort when compared to the LBred heifers. Additionally, AUC value for IgG2 was 0.8125, suggesting that increased IgG2 (total immunoglobulin) levels could serve as a decent diagnostic marker for fertility when measured prior to estrous synchronization. Immunoglobulin levels following estrous synchronization and antibody reaction to exogenous hormones was not examined in this study. IgG1 levels were not significantly different between cohorts.

Previously, it has been suggested that immunoglobulin ratios in general circulation could be utilized to determine the postpartum disease risk (Tsai et al., 2021) and rebreeding chance (Corpron et al., 2021) of cattle. These studies were predicated on the preferential transfer of IgG1 to mammary secretions in early lactation (Corpron et al., 2021) and utilized a gold nanoparticle sensor (Tsai et al., 2021) and ELISA (Corpron et al., 2021). As virgin heifers are not in lactation, the alterations in IgG1/IgG2 ratios are likely to be less extreme than post-partum animals. Testing of immunoglobulins is traditionally how activation of the adaptive immune system is quantified (Reverter et al., 2021; Rittipornlertrak et al., 2022) and could be utilized to determine the immune challenge a heifer might be under in the periconception period.

Previous research has suggested differences in animal performance based on the measure of immune competence, defined as the interaction of antibody mediated immune response and cell

mediated immune response (Reverter et al., 2021). Reverter et al. suggested a negative relationship between carcass traits and immune competence traits in steers and non-bred heifers (Reverter et. Al., 2021). A study in Holstein heifers bred for low or high fertility breeding value (EBV) showed that animals with high immune competency values had higher ADG and lower age at puberty (Grala et al., 2022). The same study suggested that animals with low immune response measures had a greater calving to first ovulation interval when compared to cows with higher immune response measures (Grala et al., 2022). Another study saw a significantly higher ADG with high antibody mediated immune response in Holstein heifers raised on grazing system (Aleri et al., 2015). These results may suggest that the higher IgG2 levels in 1stAI heifers are representative of a greater antibody mediated immune response.

Further validation of this theory should be pursued. Immune competency has been suggested to be somewhat heritable (Reverter et al., 2021) which might mean it could be utilized as a selection tool for high-performing animals. Utilized in conjunction with fertility selection factors and phenotypic factors (like ADG and age within a cohort), immune response testing could prove to be a powerful tool to help combat idiopathic infertility in heifers. However, for this to be readily available to producers, simple chute-side tests should be developed and made available to producers. This study utilized SDS-PAGE and western blotting which are qualitative rather than quantitative, so absolute levels of immunoglobulins assayed could not be determined. Further testing and validation utilizing larger cohorts and quantitative processes such as ELISA should be pursued.

II.VI. Implications and Conclusions

In this study, IgG2 was higher in fertile heifers compared to subfertile heifers and there was no difference in levels of IgG1 between cohorts. With the relationship of IgG2 to inflammatory

response, it can be theorized that fertile heifers have greater pro-inflammatory response than subfertile animals. IgG2 also showed high AUC values, suggesting it can serve as a prognostic factor of fertility in heifers tested before estrous synchronization.

Unexplained heifer infertility could greatly benefit from the diagnostic ability of immunoglobulin levels. Use of immunoglobulins could also be utilized, potentially, to determine subclinical disease or dysregulation of cytokine balances increasing disease-risk outside of the breeding season. Additionally, it has been suggested that immune response and competence may be moderately heritable and has been linked to production traits. Further study to determine heritability of immune competence and its effect on reproduction should be pursued. Continued study and validation should be pursued in more nulliparous animals and larger cohorts with different technologies. Research focus should be on development of colorimetric, chute side assays for producers and discovery of important detection time points as earlier detection of subfertility will benefit producers.

Chapter III: Plasma Metabolomics at Weaning and 30 Days Prior to Breeding in Subfertile and Fertile *Bos Taurus* Beef Heifers

III.I. Abstract

Subfertility in beef heifers is a major drain of resources for producers and total costs for heifer development cannot be fully recouped if she fails to conceive. Metabolomics is the study of populations of small molecules in tissues and biofluids. These small molecules are precursors and products of metabolic processes and changes in metabolites have been suggested to describe alterations in metabolic enzymes. Metabolomic profiles can potentially discriminate between healthy and disease states and fertile and subfertile states. In this study, we aimed to identify plasma metabolomic differences between fertile and subfertile beef heifers at weaning and one month prior to breeding (30DP-AI). For this purpose, blood samples were collected from twelve Angus-Simmental heifers (n=6 fertile; n=6 subfertile) at weaning and seven heifers (n=3 fertile; n=4 subfertile) at 30DP-AI. Blood plasma samples were analyzed by gas chromatography coupled to time-of flight mass spectrometry (GC-TOF-MS) for primary metabolism metabolites. Multiple Student's t-tests identified metabolites that were present at significantly different levels between the cohorts. At weaning, nine metabolites including arabitol, nicotinamide, and palmitoleic acid were at significantly different levels in fertile animals when compared to subfertile. At 30DP-AI, four metabolites, including palmitoleic acid and tryptophan were significantly different between the subfertile and fertile animals. Time series analysis was performed between weaning and 30DP-AI, with phenotype (subfertile or fertile) as a covariate. Five metabolites were identified as changing significantly between weaning and 30DP-AI in one of the phenotypes, including 1,5-anhydroglucitol and palmitoleic acid. Metabolites found to be significant at weaning are linked to energy metabolism and stress metabolism. One metabolite discovered to be at significantly

different levels in the fertile and subfertile group at weaning remained at different levels at the 30DP-AI. These results suggest that metabolites may be able to serve as biomarkers for reproductive potential or reproductive success in beef cattle when tested prior to breeding or at weaning. Additionally, analysis of changes in metabolomic profiles between time points could help inform of changes in animal metabolism over time.

III.II. Introduction

It has been discussed previously that phenotypic measures, such as BCS and RTS, and growth measures such as weaning weight and age at AI do not provide complete predictive power of reproductive ability (Dickinson et al., 2019). A heifer or a cow failing to conceive exacts a financial toll on producers as it increases the time needed for her to fully recoup the cost of her development (Boyer et al., 2020). Easily acquired biomarkers could supplement phenotypic metrics and combat reproductive inefficiency in production animals. Studies have examined genomic variation (Arishima et al., 2017; Stegemiller et al., 2021), transcriptomic variation (Dickinson et al., 2018), metabolomic variation (Phillips et al., 2018; Funeshima et al., 2021), and physiological variation at the moment of AI (Liles et al., 2022) in an attempt to determine intrinsic and extrinsic factors leading to reproductive success or failure.

Metabolomics, as the study of global populations of small molecules in biofluids and tissues, often representing precursors and products of important metabolic processes (Wishart, 2005), has shown potential in discriminating differences between fertile and subfertile states in humans and production species (Blaurock et al., 2022; Fletcher et al., 2022).

In women, plasma metabolomic profiling had the ability to differentiate between different phenotypes of polycystic ovary syndrome (PCOS) and between PCOS and a healthy control state (Zhao et al., 2012). Gaskins et al. leveraged serum metabolites to judge the effect of nitrous oxide, particulate matter, and black carbon upon chance of live birth in women undergoing assisted reproduction techniques (ART), finding alterations in amino acid metabolism, oxidant and antioxidant balance, and vitamin B3 metabolism (niacin) (Gaskins et al., 2021). In cattle, metabolite profiles in plasma found significant changes between Day 0 (insemination) and Day 17 and Day 45 of pregnancy (Guo and Tao, 2018). A study that combined serum metabolite profiles

with global proteomics also found altered metabolite profiles between cattle that established a successful pregnancy following embryo transfer and those that suffered an early embryonic loss (Kusama et al., 2022). Phillips et al. showed metabolomic profiles differed in virgin beef heifers at AI depending on AI success and establishment of pregnancy (Phillips et al., 2018). In high producing dairy cattle, repeat breeding (failure to establish pregnancy after repeated confirmed inseminations) is a major drain of resources in dairy production (Funeshima et al., 2021). In a recent study, plasma metabolomic profiles were found to have differences between repeat breeding animals and healthy control animals, as well as altered expression of genes related to amino acid metabolism (Funeshima et al., 2021). Furthermore, Hailemariam et al. explored metabolomic differences between healthy dairy cattle and those that developed post-partum disorders like metritis and mastitis, finding that differences in metabolite profiles could be detected as early as 4 weeks prepartum (Hailemariam et al., 2014). Fletcher et al. showed serum, urine, and salivary metabolome differences between sows with high-litter capacity when compared to sows with low litter capacity (Fletcher et al., 2022).

From previous research, it is hypothesized that there will be differences in metabolomic profiles between subfertile and fertile heifers when generated at weaning and at a time point prior to breeding. The objective of the current study was to generate plasma metabolomic profiles in *Bos taurus* beef heifers at weaning and 30 days prior to AI. Using the same animals in both time points can determine if metabolites differentially expressed at weaning may remain differentially abundant at a time prior to breeding. As well, whether differentially abundant metabolites can predict fertility or whether there are differences in animal development shown by metabolomic profiles.

III.III. Materials and methods

III.III.a. Animal care and use

All animal-related protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Auburn University (IACUC protocol #2019-3591).

III.III.b. Growth and development

Heifers were born to Angus and Angus-Simmental dams at the Auburn University Black Belt Research and Extension Center between in the fall of 2019 and were weaned in spring of 2020. Following weaning, heifers were maintained on pastures with tall fescue, dallis grass, and clover while being offered soyhull:corn gluten (50:50) supplement and ryegrass hay.

III.III.c. Phenotypic assessment

Measurements of adjusted birth weight (BiW), adjusted weaning weight (WW), and adjusted yearling weight (YW) were collected and archived by staff of the Auburn University Research and Experiment Center and accessed later by researchers. Heifer age at AI was calculated from records maintained by the staff of the experiment station. Body condition score (BCS) was collected on sampling days and judged by a single, trained observer. Reproductive tract score (RTS) was determined by rectal palpation by a trained veterinarian 30 days prior to AI breeding (30DP-AI).

III.III.d. Estrous synchronization, artificial insemination, bull exposure

Heifers were subjected to a Select Synch + CIDR® with TAI for breeding. Briefly, heifers were injected with an analog of GnRH (Cystorelin®, Boehringer Ingelheim, Ingelheim am Rhein, Germany) on Day 0 of the protocol and had a progestin infused CIDR (controlled internal drug release) inserted transvaginally. On Day 7 of the protocol, CIDR® (Zoetis, Parsippany-Troy Hills, NJ, USA) was removed and each heifer was injected with an analog for prostaglandin F2α (Lutalyse®, Zoetis, Parsippany-Troy Hills, NJ, USA). Heifers were bred on detection of standing

estrus between Day 7 and Day 10, those heifers that had not responded to estrous synchronization by Day 10 were bred at 84 hours post prostaglandin shot and received an injection of GnRH at 84 hours. Heifers were inseminated by one trained technician, each with a single straw of semen from a bull of proven fertility. Fourteen days after artificial insemination, heifers were exposed to natural service bulls for 81 days. Bulls were examined for breeding soundness thirty days prior to the breeding season and deemed 'satisfactory'.

III.III.e. Phenotypic categorization, pregnancy checking

At 75 days and 168 days post-artificial insemination, heifers were examined by trained veterinarian for pregnancy using transrectal ultrasound and transrectal palpation, respectively. Animals were classified as AI-conception or Recheck at 75 days post-artificial insemination. In this work, these groups are classified as Fertile and Subfertile, respectively.

III.III.f. Blood draw and plasma isolation

Samples were collected from heifers at weaning and then from a subset of heifers at 30DP-AI. Whole blood was collected with 18G needle (BD, Franklin Lakes, NJ, USA) by jugular venipuncture into 10mL EDTA tubes (BD, Franklin Lakes, NJ, USA), immediately inverted 10 times and placed on ice until transport back to laboratory. EDTA tubes were centrifuged at 1500 *g* at 4° C for 10 minutes to isolate plasma and buffy coat from red blood cells. 500 µL of plasma was collected into a cryotube and stored at -80° C until further testing. Samples were submitted to West Coast Metabolomics Center (UC-Davis) for primary metabolism, untargeted metabolomics.

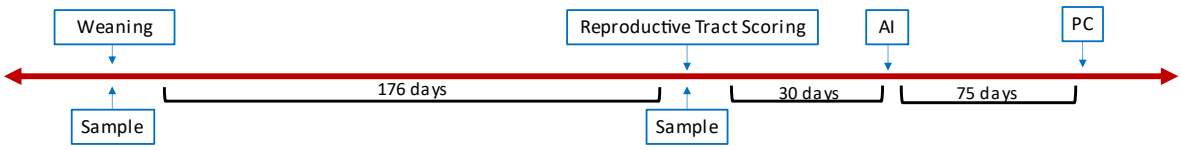

Figure 3.1: Timeline of cohort handling and sampling points for breeding herd. AI- artificial insemination; PC- pregnancy check.

III.III.g. Metabolomic profiling

Samples (total n=19) were submitted for metabolomic profiling of primary metabolism metabolites using untargeted gas chromatography-time of flight-mass spectrometry (GC-TOF-MS) by the West Coast Metabolomics Center at UC-Davis. This process was completed on an Agilent 6890 GC equipped with a Gerstel automatic liner exchange system (ALEX; Gerstel, Muehlheim, Germany) and a cold injection system (CIS) also produced by the Gerstel corporation. Injection volume and speed was 0.5 µl and 10 µl/s, respectively. The chromatographic parameters were: column was a Restek corporation Rtx-5Sil MS (30m length x 0.25mm internal diameter) with a film of 0.25µm film of 95% dimethyl/ 5% diphenylpolysiloxane; mobile phase was helium with a 1mL/min flow rate. Oven temperatures were held constant at 50° C for 1 minute and then ramped at 20° C/ min to 330° C and held constant for 5 minutes. Mass spectrometry data was collected with a Leco Pegasus IV mass spectrometer with unit mass resolution at 17 spectra/sec from 80-500 Da at -70 eV ionization energy. Raw data was processed using ChromaTOF v2.32 and absolute spectra intensities were further processed using filtering algorithms in BinBase, a metabolomics database. Quantification is reported as peak height with the unique ion as the default rather than peak area. Binned data was finally normalized and scaled to reduce potential bias caused by sample handling and run variability.

III.III.h. Statistical analysis

From raw data, annotated metabolites were selected for further analysis. The online tool MetaboAnalyst 5.0 was utilized for analysis of metabolomic profiles (Pang et al., 2021). To reduce variability created by sample preparation and to weight metabolites equally despite inherent

differences in concentration data was normalized by sum and scaled by mean centering and dividing by the square root of the standard deviation of each variable. In MetaboAnalyst 5.0, this scaling option is referred to as Pareto-scaling. The module Statistical Analysis [one-factor] performed Student's t-tests between groups with correction for multiple tests by false discovery rate (FDR). Results of the t-tests were deemed significant if raw $p \leq 0.05$. Principal Component Analysis plots (PCA) were generated to determine overall structure of the data and Partial Least Squares-Discriminant Analysis (PLS-DA) plots were utilized to visualize separation capability of metabolomic profiles. PLS-DA models were verified by the accuracy of 5 components and Q^2 values.

The Biomarker Analysis module of MetaboAnalyst 5.0 was used to generate ROC-AUC (receiver operating characteristic-area under curve) analysis for metabolites. Data was again sum normalized and Pareto-scaled within MetaboAnalyst 5.0. Univariate ROC curve analysis was used to determine AUC values of each metabolite alone with 95% confidence intervals and was only reported for t-tests with a p-value ≤ 0.05.

The Pathway Analysis module was utilized to determine pathway hits of significant metabolites. Due to low differentially abundant metabolites at 30DP-AI, metabolites from both time points were compiled and submitted to pathway analysis together. This module combines enrichment analysis and pathway topology analysis. Fisher's Exact Test was utilized for enrichment and the pre-loaded *Bos taurus* pathway library was used for reference. KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis was utilized to determine downstream fate of identified metabolites. Additionally, KEGG Pathway Database (https://www.genome.jp/kegg/pathway.html) was utilized to specifically query metabolites that did not present resulting pathways in MetaboAnalyst 5.0.

For analysis of metabolites significantly impacted between weaning and RTS time points, the MetaboAnalyst module Statistical Analysis [metadata table] was utilized. This module allows for the definition of data tables by multiple factors. For this analysis, data was defined by phenotype (subfertile or fertile), collection point (weaning or 30DP-AI), and subject ID (animal 1-7). Metabolomic profiles were compiled for animals (n=7) that were represented in both time points and then sum normalized and Pareto-scaled. Instead of Student's t-test, this module utilizes a linear regression model with consideration of the metadata of interest, in this case for phenotype, to identify differentially abundant metabolites. Significant metabolites were declared at a raw $p \leq 0.05$.

III.III.i. Prism procedures

Prism v6.01 was utilized to analyze phenotypic data. Adjusted birth weight, adjusted weaning weight and adjusted yearling weight were transformed into kilograms, and age at AI was calculated from birth date and date of AI. RTS and BCS were reported as categorical variables (score of 1 to 5; and score of 1 to 9; respectively). Data were checked for normal distribution using a Shapiro-Wilk test. To determine differences between study cohorts, a Student's t-test was utilized. Significance of phenotypic measures was declared if $p \leq 0.05$. Metabolites determined to be significant in MetaboAnalyst 5.0 were imported to Prism v6.01 to generate graphs of expression level.

III.IV. Results

III.IV.a. Phenotypic characterization and pregnancy checking

At 75 days post-AI, heifers were checked for pregnancy. Overall pregnancy rates for the breeding herd were 7.14% of heifers non-pregnant and 92.86% heifers were pregnant at the end of the breeding season. Of the samples collected at weaning, 6 animals were chosen as being pregnant

to AI (fertile) and 6 animals were chosen for being non-pregnant at 75 days (subfertile). From the twelve heifers collected at weaning, a subset of seven were further tested at 30DP-AI (n=4 subfertile; n=3 fertile).

For adjusted birth weight, adjusted weaning weight, adjusted yearling weight, age at AI, RTS, and BCS there was no significant difference ($p > 0.05$) between groups when examined with an unpaired t-test. BCS range for selected heifers was from 5-6 and RTS range for selected heifers was 3-5. Mean and standard deviations of phenotypic measures are presented in **Table 3.1** and **Figure 3.2**.

Table 3.1: Mean and standard deviation and p-value from t-test for phenotypic and growth characteristics of fertile and subfertile animals (subfertile n=6; fertile n=6). Adj BiW- adjusted birth weight (kg); Adj WW- adjusted weaning weight (kg), Adj YW- adjusted yearling weight (kg), age at AI (days), RTS- reproductive tract score, BCS- body condition score. Columns marked with * (subfertile n=4, fertile n=3). (†) denotes p-value ≤ 0.05.

Phenotype	Adj. BiW (kg)	Adj. WW (kg)	Adj. YW* (kg)	Age at AI* (days)	BCS* (1-9)	RTS* (1-5)
Subfertile	32.89 ± 8.13	263.84 ± 27.62	375.66 ± 50.76	438.50 ± 9.75	5.25 ± 0.50	4.50 ± 0.58
Fertile	31.83 ± 5.12	266.26 ± 23.45	355.06 ± 46.60	445.67 ± 7.02	5.17 ± 0.29	3.67 ± 0.58
P-Value	0.793	0.873	0.573	0.333	0.809	0.117

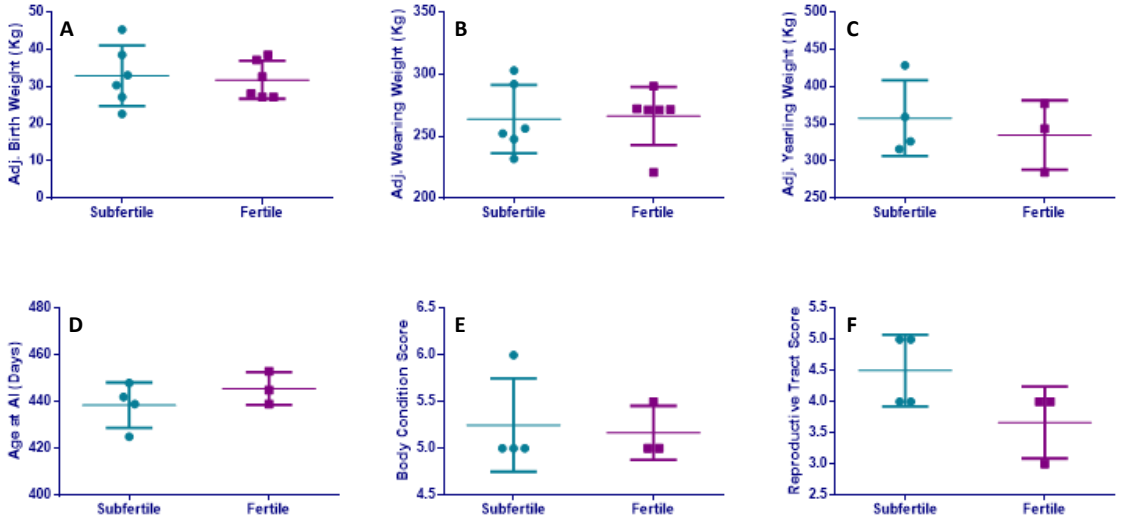

Figure 3.2: Mean and standard deviation of phenotypic measures. Adjusted birth weights in kilograms (A), 205-day adjusted weaning weight in kilograms (B), and adjusted yearling weight in kilograms (C), age at AI in days (D), body condition score (E) and reproductive tract score (F)

for fertile and subfertile cohorts. Significant differences between cohorts denoted by (*) when tested by Student's t-test. Panel A-B, subfertile n=6 and fertile n=6. Panel C-F, subfertile n=4 and fertile n=3.

III.IV.b. Metabolomic profiling

In raw data, 423 unique metabolite peaks were identified and quantifiable. 146 metabolites were annotated. For further analysis, the 277 non-annotated metabolites were removed from the data and only annotated metabolites were examined for differential abundance.

III.IV.c. Metabolite differences at weaning

When analyzed by Student's t-test, nine metabolites were found to be significantly different between subfertile (n=6) and fertile (n=6) groups by raw p-value ($p \leq 0.05$). No metabolites were significant by false discovery rate (FDR). Arabitol, indole-3-propionic acid, glycolic acid, lyxitol, indole-3-lactate, and palmitoleic acid were present at lower levels in fertile animals at weaning. Lactic acid, maltose, and nicotinamide were present at higher levels in fertile animals at weaning. Significant metabolites and p-values are listed in **Table 3.2** and visualized in **Figure 3.3**.

Table 3.2: Significant metabolites at weaning in subfertile heifers (n=6) when compared to fertile heifers (n=6) when analyzed by t-test ($p < 0.05$). FDR- false discovery rate; AUC- area under the curve; Log2(FC)- log2 fold change.

Metabolites	Raw p-value	FDR	Log2(FC)	AUC	In Fertile Heifers
Arabitol	0.006	0.673	0.279	1	Down
Glycolic acid	0.016	0.673	0.289	0.861	Down
Indole3-lactate	0.030	0.787	0.675	0.833	Down
Indole3-propionic acid	0.018	0.673	0.867	0.972	Down
Lactic acid	0.011	0.673	1.110	0.917	Up
Lyxitol	0.033	0.787	0.339	0.778	Down
Maltose	0.049	0.790	0.786	0.778	Up
Nicotinamide	0.043	0.787	0.284	0.806	Up
Palmitoleic acid	0.041	0.787	0.864	0.833	Down

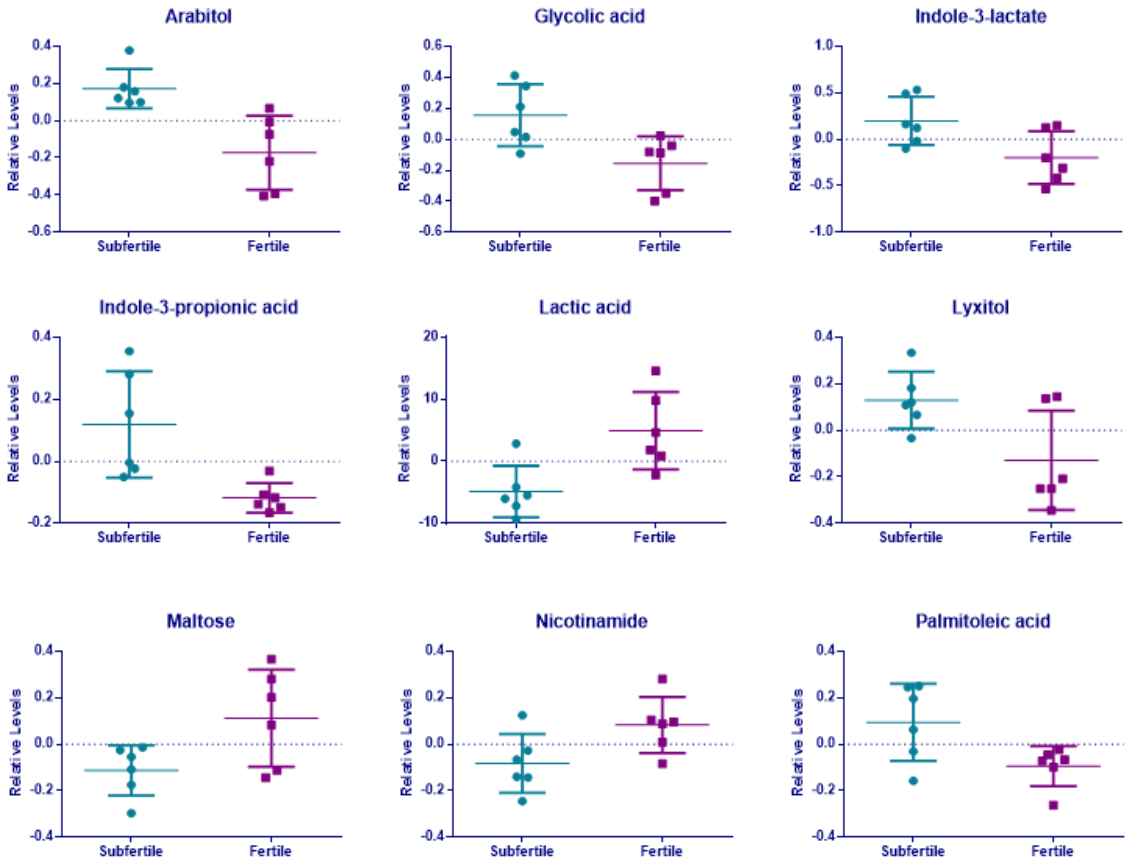

Figure 3.3: Relative levels of significant metabolites at weaning in subfertile (n=6) and fertile (n=6) heifers when analyzed by t-test ($p \leq 0.05$).

Principle component analysis was utilized to show the intrinsic separation tendency of metabolomic profiles while partial least squares discriminant analysis was performed to increase the separation of cohorts and identify highly discriminatory metabolites. PCA score plots and PLS-DA score plots were generated to visualize the grouping of phenotypic cohorts, as shown in **Figure 3.4**. PCA failed to fully separate subfertile and fertile groups while PLS-DA showed greater separation between subfertile and fertile cohorts with little overlap.

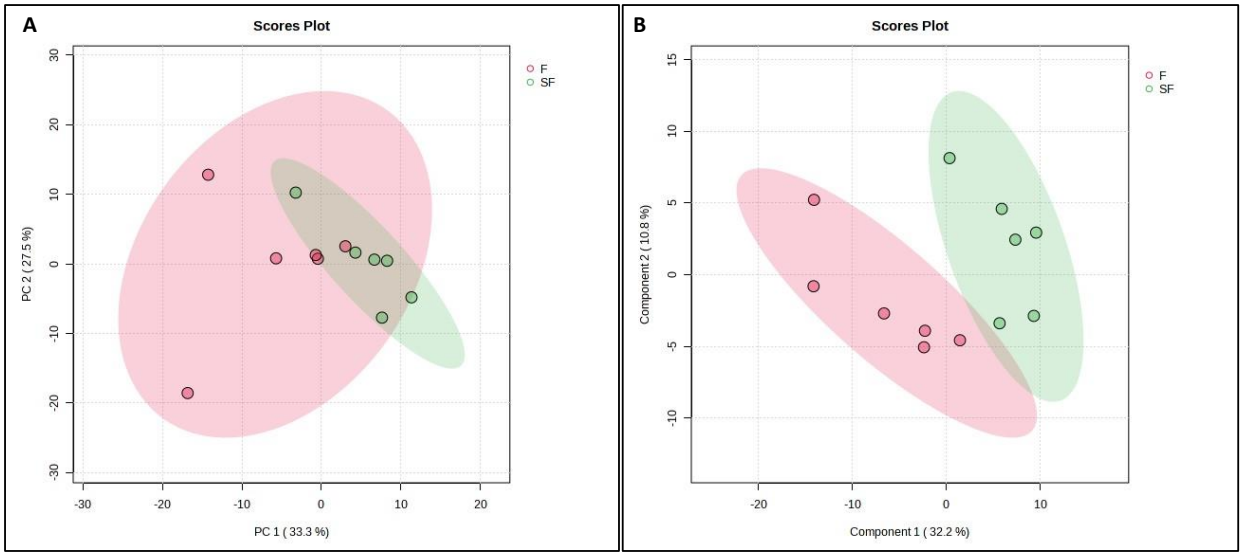

Figure 3.4: Score plots from Principal Component Analysis (Panel A) and Partial Least Squares-Discriminant Analysis (Panel B) of subfertile (SF, n=6) and fertile (F, n=6) animals at weaning.

To determine the fit characteristics of the PLS-DA model, cross-validation was reported from five components (**Figure 3.5**). The averaged accuracy of the five components was 0.73333, R^2 of the same was 0.8846 and Q^2 was 0.1451.

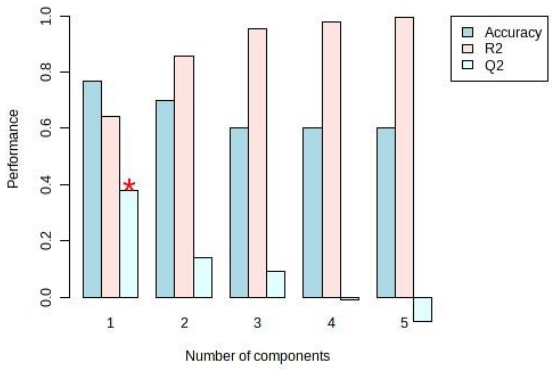

Figure 3.5: Cross-validation statistics presented as a bar plot of R^2 and Q^2 and accuracy values.

To determine potential diagnostic ability of metabolites at weaning, ROC-AUC analysis was performed. AUC values of significant metabolites ranged between 0.778 (lyxitol and maltose) to 1 (arabitol). AUC values for all metabolites are in **Table 3.3**, representative graphs of AUC curves in **Figure 3.6**.

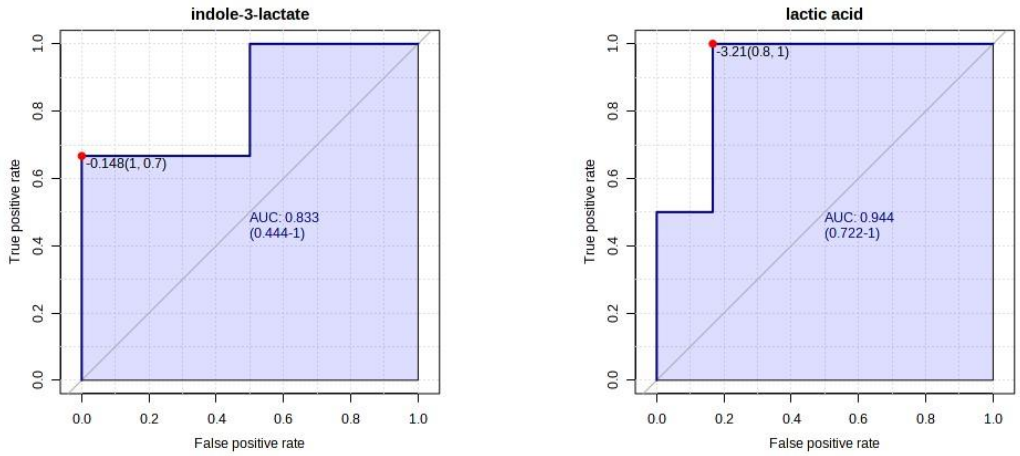

Figure 3.6: Representative ROC curves of indole-3-lactate and lactic acid from profiles at weaning.

III.IV.d. Differences in metabolite levels at 30DP-AI

Reproductive tract scoring was performed 30 days prior to artificial insemination (30DP-AI). Four metabolites were discovered at significantly different levels in the fertile and subfertile heifers, tryptophan, palmitoleic acid, triethanolamine, and 9-myristoleate when analyzed by t-test and considered by raw p-value ($p \leq 0.05$). No metabolite was significant by FDR. Tryptophan was present at higher levels in fertile animals. Palmitoleic acid, triethanolamine, and 9-myristoleate were present at lower levels in fertile animals. Significant metabolites and p-values of t-tests are listed in **Table 3.3**, relative levels of significant metabolites are in **Figure 3.7**.

Table 3.3: Significant metabolites at 30DP-AI in subfertile heifers (n=4) when compared to fertile heifers (n=3) when analyzed by t-test ($p < 0.05$). FDR- false discovery rate; AUC- area under the curve; log2(FC)- log2 fold change.

Metabolites	Raw p-value	FDR	Log2(FC)	AUC	In Fertile Heifers
Palmitoleic acid	0.009	0.936	1.051	1	Down
Tryptophan	0.019	0.936	0.764	1	Up
Triethanolamine	0.032	0.936	0.927	0.917	Down
9-myristoleate	0.039	0.936	0.236	1	Down

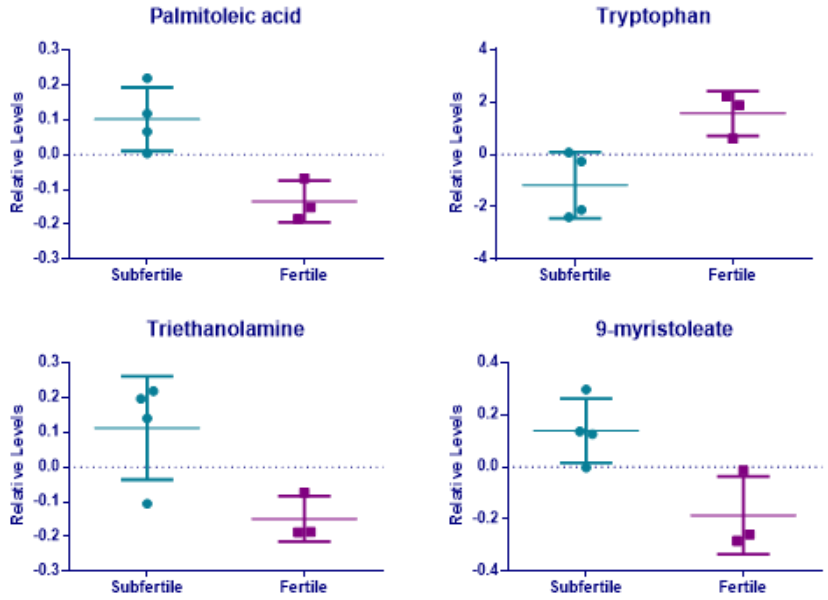

Figure 3.7: Relative levels of significant metabolites at 30DP-AI in subfertile (n=4) and fertile (n=3) heifers by t-test ($p \leq 0.05$).

PCA score and PLS-DA score plots (**Figure 3.8**) were generated to visualize separation between subfertile and fertile cohorts. PCA failed to separate cohorts and PLS-DA had no overlap in cohorts when compared.

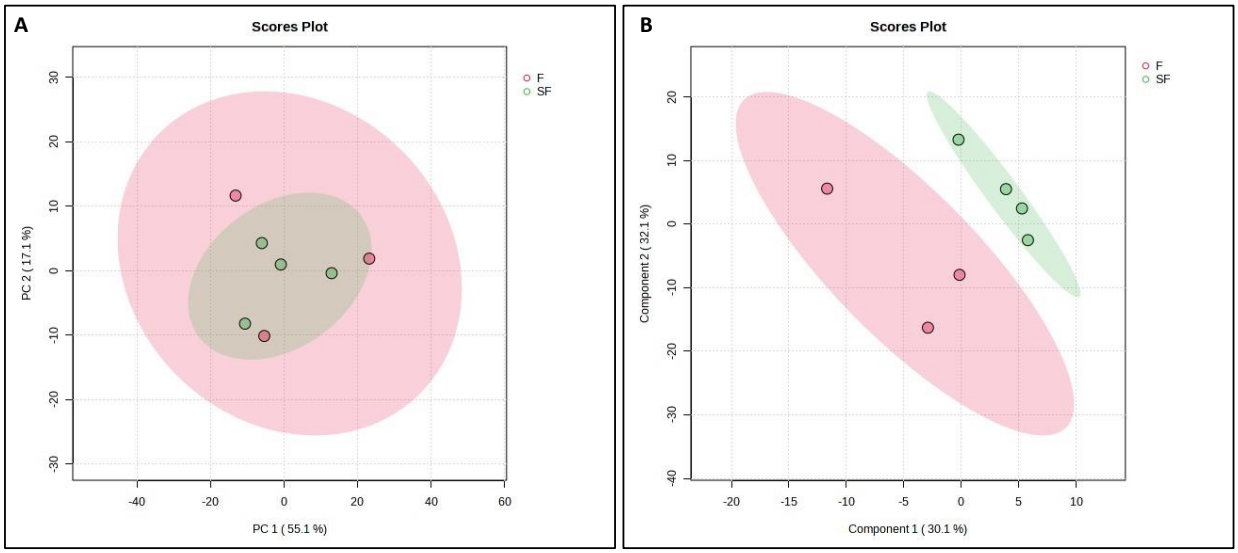

Figure 3.8: Principal Component Analysis (Panel A) and Partial Least Squares- Discriminant Analysis (Panel B) of subfertile (SF, n=4) and fertile (F, n=3) cohorts tested at 30DP-AI.

Fit diagnostics of PLS-DA model were examined using cross-validation of 3 components due to low sample numbers. The accuracy of this test was 0.375, R^2 was 0.8244 and Q^2 was -2.5511. Visualization of the fit diagnostics is shown in **Figure 3.9**.

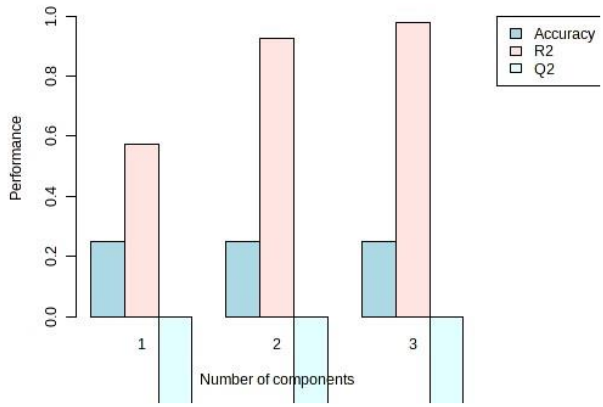

Figure 3.9: Cross-validation statistics of PLS-DA generated from 30DP-AI profiles presented as a bar plot of R2 and Q2 and accuracy values.

Diagnostic capability of metabolites was determined by ROC-AUC analysis. AUC values ranged from 0.917 to 1. All AUC values are listed in **Table 3.3**, representative graphs are shown in **Figure 3.10**.

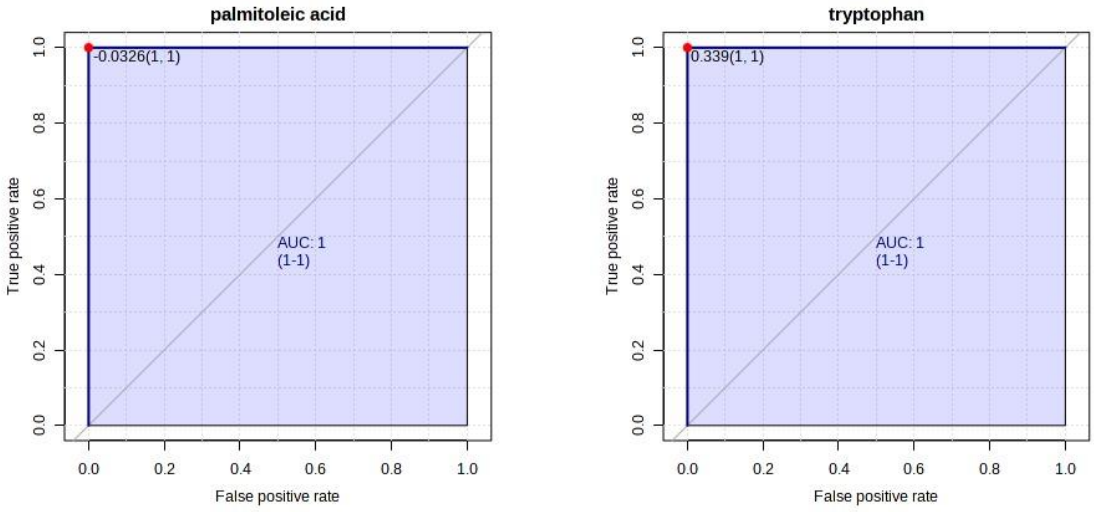

Figure 3.10: Representative ROC curves of tryptophan and palmitoleic acid from 30DP-AI profiles.

III.IV.e. Pathway analysis

Due to the low number of differentially abundant metabolites found at 30DP-AI and weaning the metabolites were compiled and submitted for pathways analysis together. No pathway was significantly impacted when considered by Holm's adjusted p-value (> 0.05). Pentose and glucuronate interconversions was the only pathway to have more than one metabolite associated with it. Tryptophan was involved with two pathways, tryptophan metabolism and aminoacyl-tRNA biosynthesis. The full list of pathways identified through mapping is in **Table 3.4**.

Table 3.4: Pathways from weaning and 30DP-AI metabolites, with raw-p value, Holm's adjusted p-value, and metabolite mapped within the pathway.

Pathways	Raw p-value	Holms adjusted	Metabolites
Nicotinate and nicotinamide metabolism	0.042	1	Nicotinamide
Pentose and glucuronate interconversions	0.058	1	Arabitol, lyxitol
Pyruvate metabolism	0.071	1	Lactic acid
Glycolysis/Gluconeogenesis	0.083	1	Lactic acid
Fatty acid degradation	0.123	1	Palmitoleic acid
Tryptophan metabolism	0.129	1	Tryptophan
Aminoacyl-tRNA biosynthesis	0.149	1	Tryptophan

III.IV.f. Changes in metabolite levels from weaning to 30DP-AI

A linear model was utilized for time series analysis. This model analyzes changes in metabolites within cohorts over time before comparing those changes between cohorts. A metabolite is labeled as significant if there is a difference in the changes over time between cohorts. In the time series analysis (subfertile n=4, fertile n=3), 5 metabolites were identified as being significantly changed between weaning and 30DP-AI within either the subfertile or fertile cohorts, including palmitoleic acid, 1,5-anhydroglucitol, capric acid, myristic acid, and isolinoleic acid. No metabolite was significant by adjusted p-value. Significant metabolites are listed in **Table 3.5**.

Table 3.5: Raw p-value, adjusted p-value and log2 fold change of metabolites significant in time series (subfertile, n=4; fertile, n=3) ($p < 0.05$).

Metabolites	Raw p-value	Adj. p-value	Log(FC)
Palmitoleic acid	0.001	0.168	0.261
1,5-anhydroglucitol	0.002	0.168	0.954
Capric acid	0.031	0.943	0.280
Myristic acid	0.033	0.943	0.417
Isolinoleic acid	0.040	0.943	0.270

Palmitoleic acid was also significantly altered in weaning and 30DP-AI timepoints. Visualization of metabolite changes over time is shown in **Figure 3.11**. Palmitoleic acid, 1,5-anhydroglucitol, capric acid, and myristic acid were greater in subfertile animals at both time points; isolinoleic acid was higher in fertile heifers at both time points. Capric acid and palmitoleic acid decreased in subfertile heifers between timepoints while remaining at steady relative levels in fertile heifers. 1,5-anhydroglucitol remained at steady levels in subfertile heifers while increasing in fertile heifers between timepoints. Myristic acid decreased in both cohorts over time. Isolinoleic acid decreased in fertile heifers and increased in subfertile heifers between timepoints.

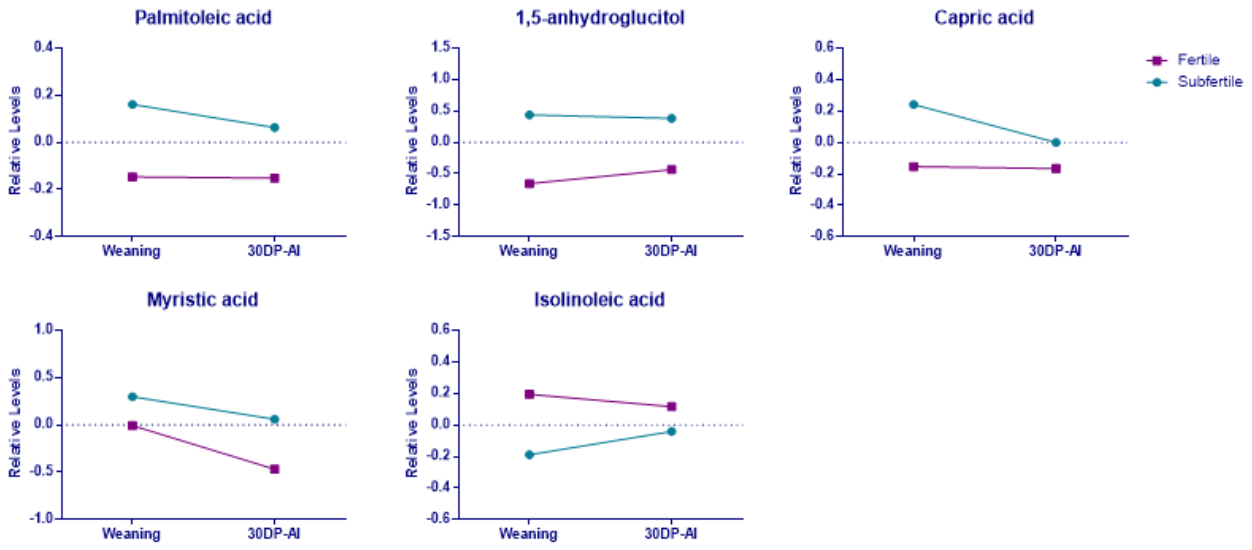

Figure 3.11: Relative levels at weaning and 30DP-AI of significant metabolites in subfertile (n=4) and fertile (n=3) in time series analysis (raw $p \leq 0.05$).

III.V. Discussion

III.V.a. Phenotypics

In this study, phenotypic measures of BCS, RTS, age at AI (days), and adjusted weights for birth, weaning, and AI were analyzed and there was no significant difference in measures for subfertile or fertile animals. This agrees with numerous other studies that found little diagnostic power to phenotypic measures. Dickinson et al. found that age at weaning and weaning weight did not affect heifer fertility (Dickinson et al., 2019). RTS, as a measure of maturity of the reproductive tract, is an important metric for determining fertility potential and is ranked from 1 to 5 (1, immature and non-cycling; 5, mature and cycling) (Gutierrez et al., 2014). In this study, all animals had a range of RTS of 3-5. Additionally, there was no difference in BCS between fertile (4.17 ± 0.75) and subfertile animals (5.50 ± 0.55), $p = 0.809$. This is in agreement with Meier et al. 2021 which found that in animals bred for diverging fertility, there was no significant interaction of BCS with fertility (Meier et al., 2021). Because there were no significant differences in phenotypic

measures, they were not considered covariates for metabolite profile analysis and excluded from further analysis.

III.V.b. Weaning metabolites and pathways

At weaning, nine metabolites were altered between fertile and subfertile animals. The significant metabolites were enriched for five metabolic pathways. None of the pathways was considered significantly impacted by alterations of the metabolite in question.

Nicotinamide, maltose, and arabitol have all been related to weaning stress in swine at weaning (Li et al., 2018) (Jiang et al., 2020).

Nicotinamide is involved with the nicotinate and nicotinamide metabolism pathway and was lower in subfertile animals at weaning. Nicotinamide is related to niacin and is a precursor to NAD+ and NADP (Pollard et al., 2022) which are central factors of cellular metabolism (Li et al., 2018). Nicotinamide biosynthesis is related to tryptophan metabolism as *de novo* synthesis from tryptophan is the primary pathway for synthesis in vertebrates (Belenky et al., 2007). The conversion rate of tryptophan to nicotinamide has been suggested to be altered in periods of feed restriction, even if tryptophan is present in sufficient levels in food (Shibata et al., 2014). Post puberty, higher levels of NAD+ are linked to improved oocyte quality and embryonic development in some livestock species (Pollard et al., 2022). Interestingly, plasma levels of two other tryptophan metabolites, indole-3-propionic acid and indole-3-lactic acid, were increased in subfertile animals at weaning. In monogastric models, bacterium production of indole-3-lactic acid had anti-inflammatory effects, reducing the production of IL-8 (Meng et al., 2020). Additionally, indole-3-propionic acid is suggested to be anti-inflammatory, and higher serum levels are suggested to be related to survival of septic patients (Huang et al., 2022b). Indole metabolism has been suggested to be related to inflammation; indole-3-lactic acid and indole-3-propionic acid are (in

monogastrics) dietary indoles that can interact with the aryl-hydrocarbon receptor (AHR), potentially affecting inflammation in the gut (Stone and Williams, 2023). A study performed in adult, obese humans found indole-3-lactic acid and indole-3-propionic acid in plasma to be decreased compared to healthy weight controls (Cussotto et al., 2020).

From review of KEGG pathways, lactic acid is related to pyruvate metabolism, glycolysis/gluconeogenesis, and nicotinate and nicotinamide metabolism. In ruminant animals, lactic acid is a natural product of rumen metabolism and often is produced in rapidly acidifying conditions (Mackenzie, 1967). In the present study, lactic acid was present at higher levels in the fertile animals. In piglets exposed to chronic LPS at weaning, lactic acid was increased in serum (though this was attributed to higher gut leakage) (Yu et al., 2022).

Palmitoleic acid, a long chain fatty acid, was higher in subfertile animals and was mapped to occur within the fatty acid degradation pathway. Palmitoleic acid was also found to be higher in the colonic digesta of weaning stressed piglets (Li et al., 2018) and was also present in higher amounts in the serum of dairy cows in the early stages of fatty liver disease, suggesting a dysregulation of fatty acid synthesis pathways in negative energy balance (Zhang et al., 2022b). While not significant, palmitoleic acid was amongst the fatty acids in serum queried in a study of high fertility dairy cattle in comparison to low fertility dairy cattle; ROC analysis of fatty acid profiles of serum returned a 0.879 AUC value in this study (Moore et al., 2017).

III.V.c. Metabolites and pathways altered at 30DP-AI

At 30DP-AI, four metabolites were different between subfertile and fertile animals. Only three pathways were indicated by mapping metabolites, none of which was significantly impacted by the metabolites in question. Palmitoleic acid was enriched for fatty acid degradation and tryptophan was associated with tryptophan metabolism and aminoacyl-tRNA biosynthesis.

Previously, our lab created metabolomic profiles for fertile and subfertile heifers at AI, finding fifteen metabolites significantly altered between cohorts (Phillips et al., 2018). Amongst the fifteen metabolites altered between fertile and subfertile animals, tryptophan was present at lower levels in subfertile animals (Phillips et al. 2018) which is in agreement with the current study performed 30 days prior to AI. Amino acid levels in the periconception period have been linked to successful conception and implantation in cattle (Meier et al., 2014; Franca et al., 2017) and amino acid metabolism is closely associated with immune response and activation of innate immunity (Zhao et al., 2020).

Tryptophan metabolism has been implicated to be altered in cases of exposure to atmospheric contamination like nitrous oxide, particulate matter, and black carbon in human women (Gaskins et al., 2021). In swine, higher tryptophan in serum was noted in a cohort of high fertility sows when compared to a cohort of low fertility sows (Fletcher et al., 2022). Indoleamine-2,3-deoxygenase (IDO) is a rate limiting enzyme that occurs in tryptophan metabolism, governing the breakdown of tryptophan into kynurenine and is also responsive to IFNτ in PBMCs (Funeshima et al., 2021). Kynurenine levels were found to significantly lower in repeat breeding dairy cattle compared to healthy dairy cattle, suggesting that dysregulation of IDO activity and tryptophan metabolism may affect conception and pregnancy establishment (Funeshima et al., 2021). IDO-regulated breakdown of tryptophan is also implicated to have immunomodulatory activity in the peri-implantation period (Mohapatra et al., 2020). Levels of tryptophan metabolites (tryptophan, kynurenine, serotonin, indole-3-lactic acid, and indole-3-propionic acid) were found to be decreased in cases of obesity-linked chronic inflammation and negatively correlated to plasma levels of inflammatory markers (Cussotto et al., 2020).

Palmitoleic acid was lower in fertile animals at 30DP-AI as well as at weaning. As previously described, palmitoleic acid has been linked to fatty liver disease in dairy cattle (Zhang et al., 2022b). Palmitoleic acid *in vitro* has also been suggested to increase adipogenesis in bovine skeletal muscle satellite cells (Zhang et al., 2021).

9-myristoleate (also myristoleic acid) is a medium-chain fatty acid and part of fatty acid biosynthesis and degradation, present at lower levels in fertile samples. Previously, 9-myristoleate has been found to be lower in the follicular fluid of dairy cattle with higher fertility EBVs however it was not altered in blood plasma (Moore et al., 2017). There are linkages between levels of myristoleic acid and bone metabolism and activation of NFATc1 levels (Kwon et al., 2015). *GH* (growth hormone) polymorphism has been linked to fatty acid profile in subcutaneous fat in Japanese black cattle (Matsuhashi et al., 2011), and depending on protein source in feed, intramuscular fat profile of PUFA can be affected (Segers et al., 2011). NFATc1 has linkages to age at first calving in Nellore animals (Dubon et al., 2021) and may be linked to the effect of GnRH on cells (Armstrong et al., 2009).

III.V.d. Time series analysis

To examine the changes in subfertile and fertile heifers over time, a time series analysis was performed on the seven animals that appeared in both time points (n=4 subfertile, n=3 fertile). Palmitoleic acid was again differentially abundant in this analysis and was always higher in subfertile animals. Subfertile animals showed a decrease in levels between weaning and 30DP-AI while fertile animals did not show a large change between the two timepoints.

Capric acid (also decanoic acid) was consistently higher in subfertile animals but showed a substantial decrease in subfertile animals between timepoints. Myristic acid was more elevated in subfertile animals in both timepoints and showed a decrease in both cohorts over time.

Isolinoleic acid was higher in fertile animals in both time points but decreased in fertile animals and increased in subfertile animals over time. Capric and myristic acid are medium chain fatty acids; isolinoleic acid and palmitoleic acid are long-chain fatty acids. Capric acid is a medium chain fatty acid that in monogastrics is supplied from the diet (Lee et al., 2016). Capric acid supplementation has been suggested to alter profiles of steroidogenic hormones and steroidogenic enzymes in a rat model of PCOS (Lee et al., 2016). Fatty acid profiles in milk production have been suggested to be affected by genetic background of animals (Shi et al., 2020) and supplementation of MCFA (medium chain fatty acids) to transition dairy cattle has been suggested to affect immune response in the transition period (Wang et al., 2023). Supplementation of MCFAs also changed bile acid profiles in the transition period, which could be linked to decreased liver function (Wang et al., 2023). This change in fatty acids could be linked to diet. Supplementation of fatty acids may have an effect on reproduction, as suggested by changes in follicular size and potential embryonic loss (Elis et al., 2016).

Interestingly, palmitoleic acid is present in both time points (weaning and 30DP-AI). Combined with the results from time series analysis, this may suggest that free fatty acid metabolism is linked to the fertility phenotypes observed in this study. Whether this difference in fatty acids is due to external factors, such as a difference in animal choice of diet, or internal factors like differences in liver metabolism and creation of fatty acids cannot be answered in the current data set. Tryptophan has been previously identified as also differentially abundant at AI by (Phillips et al., 2018). While tryptophan was not differentially abundant in the weaning time point, nicotinamide was differentially abundant at weaning and the two pathways are closely linked. Two other tryptophan metabolites, indole-3-lactic acid and indole-3-propionic acid were also

differentially abundant at weaning. Tryptophan and its metabolites plays a role in immune response and inflammation.

III.VI. Conclusion and Implications

This study demonstrates fertile and subfertile beef heifers have differentially abundant metabolites in blood plasma at weaning. Early selection of highly fertile heifers would greatly benefit producers, so exploring these metabolites as being descriptive of later performance would be beneficial. At 30 days prior to AI, heifers were tested again, and one metabolite was again differentially abundant, and the essential amino acid tryptophan showed differential abundance, which agrees with previous studies. With consideration of other differentially abundant metabolites in this study discovered in time series analysis, liver function and metabolism of fatty acids is implicated in development of fertility. However, these results require more intensive study in larger cohorts over more sampling time periods.

Chapter IV: Metabolomic Analysis of Bovine Plasma and Ovarian Follicular Fluid Post-Breeding in Subfertile and Fertile Cross-bred Beef Heifers

IV.I. Abstract

Unexplained subfertility in cattle is a widespread phenomenon that affects up to 15% of cattle bred within a single season. While the mechanisms behind this lowered fertility are not yet fully elucidated, one theory is a high rate of early embryonic loss in cattle. Early embryonic loss can arise from a wide range of issues, including uterine receptivity and embryonic competence. As the *in vivo* maturation medium of oocyte development, follicular fluid is a prime candidate for detecting and exploring mechanisms for oocyte competence and development. This study aimed to identify differentially abundant metabolites in blood plasma and ovarian follicular fluid collected from beef heifers of differing fertility status, either fertile or subfertile. To gather this information, *Bos taurus* crossbred heifers were subjected to estrous synchronization and fixed-time artificial insemination (AI) protocol (7-Day CO-synch + CIDR). Heifers pregnant to AI were classified as fertile (n=8). Any heifers that remained non-pregnant following AI were exposed to a bull of proven fertility for 61 days and evaluated for pregnancy 34 days after the end of bull exposure. The non-pregnant heifers following AI and natural service were classified as subfertile (n=5). Pregnancies were terminated in fertile heifers, and heifers from both groups were allowed to resume normal cyclicity prior to collection of blood plasma and follicular fluid from ovaries. Plasma and follicular fluid were subjected to untargeted metabolomic profiling utilizing a GC-TOF-MS platform. After normalization, metabolomic profiles were analyzed via Principal Component Analysis and Student's t-test to show underlying data structure and to elucidate significant differences in metabolite abundance. Fourteen metabolites were differentially abundant in the follicular fluid of fertile and subfertile animals ($p \leq 0.05$) including deoxycholic acid, alpha-

tocopherol, arachidonic acid, and pimelic acid. In blood plasma, 4 metabolites were at different levels in subfertile and fertile animals ($p \leq 0.05$), including lactic acid and fucose. Additionally, Pearson correlation procedures were performed to determine whether there was a significant relationship between metabolites in plasma and those in follicular fluid. As the medium of oocyte growth and maturation *in vivo* follicular fluid represents an opportunity to explore the mechanisms behind differential oocyte competence and potentially early embryonic loss. This study shows differences in follicular fluid in un-synchronized animals, suggesting underlying physiological processes unrelated to point in the estrous cycle. However, further study with larger cohorts and integration of profile information with oocyte development studies is required to fully validate these metabolites and pathways.

IV.II. Introduction

The problem of idiopathic and unexplained subfertility can affect the economic sustainability of beef production (Boyer et al., 2020). Missed pregnancies increase development costs and number of parturitions required for a cow to produce profit or to 'break even' on her development costs (Boyer et al., 2020). A considerable amount of research has been conducted to address the phenomenon of unexplained heifer subfertility.

The ovarian follicle is the location of development for the oocyte. It comprises the developing oocyte, granulosa cells (mural and cumulus), a basement membrane, and theca cells that abut the ovarian stromal tissue (Senger, 2012). As a follicle develops, it begins to accumulate fluid in an antrum, aptly referred to as follicular fluid (Senger, 2012). The follicular fluid is the *in vivo* maturation matrix for oocytes, produced as an extrudate from general circulation and from granulosa cell secretions within the follicle (Leroy et al., 2004). Follicular fluid may also be representative of the crosstalk between granulosa (mural and cumulus) and the oocyte itself, as follicular fluid is rich in extracellular vesicles (Andronico et al., 2019). Hormone levels (including steroid hormones, glycoprotein hormones, and growth factors), biochemical properties, and amino acid content of follicular fluid have all been explored in search of descriptive factors of oocyte competence [reviewed by (Revelli et al., 2009)]. However, no highly descriptive and easily accessible factors have yet been identified, leading to embryo 'over-production' in clinical settings, and reliance on visual features to grade embryo quality (Revelli et al., 2009).

Signaling factors within the follicle (presumably mediated by follicular fluid) are altered by metabolic and physiological states. miRNA populations within follicular fluid have been noted to be altered by lactation status in dairy cattle (Hailay et al., 2019) and similar studies have shown differences in follicular fluid metabolite profiles due to lactational status (Forde et al., 2016). Horn

et al. (2022) also explored follicular fluid metabolomic profiles of beef cattle in thin (BCS=4), moderate (BCS=6), and fat (BCS>8) conditions (Horn et al., 2022).

Throughout the estrous cycle, changing levels of progesterone from the corpus luteum have been suggested to alter the milieu of general circulation, oviductal fluids, and uterine luminal fluids, including fatty acids and amino acids (Hugentobler et al., 2010). Correlation between certain plasma-borne metabolites and follicular fluid metabolites has been shown to be related to the size of the follicle (Leroy et al., 2004). Follicular fluid from dairy cows noted to have low fertility potential had altered amino acid levels compared to dairy cows of higher fertility potential (Moore et al., 2017). Additionally, follicular fluid has been noted to be a dynamic fluid with changes related to the state of the follicle (healthy or atretic) (Mo et al., 2021) and state of the estrus cycle (Orsi et al., 2005).

Numerous studies have examined the relationship between follicular fluid and reproductive outcomes, both in humans and in animal species. Amino acid profiles have been compared between follicular fluid and IVF media; as well as oocyte interaction with those amino acids when supplemented in IVF media (Hong and Lee, 2007). Another study utilized amino acid supplementation in IVF media to study the metabolic differences between highly competent oocytes and lowly competent oocytes (Hemmings et al., 2012). PCOS (polycystic ovary syndrome) pathogenesis has been suggested to alter the amino acid profiles of follicular fluid (Zhang et al., 2014) and integrated -omics study of PCOS patients follicular fluid (miRNA-Seq and metabolomics) found 31 metabolites significantly different between control and PCOS patients (Tian-Min et al., 2022). Metabolomic profiling of follicular fluid from patients undergoing IVF showed discriminatory separation based on successful outcomes of the IVF procedure (Xia et al., 2014).

It is hypothesized that there will be metabolites differentially abundant between the follicular fluid of subfertile heifers and fertile heifers. The objective of this study was to utilize tandem blood plasma and follicular fluid samples from *Bos taurus* beef heifers (subfertile n=5, fertile n=8) to explore global and ovarian metabolomic profile differences between subfertile and fertile cohorts. By utilizing blood plasma and follicular fluid collected at the same time from the same animals, correlation of metabolites between biofluids was possible, to determine whether or not blood metabolites could be descriptive of follicular environment.

IV.III. Materials and methods

IV.III.a. Animal care and use

All procedures with live animals were reviewed and approved by the Institutional Animal Care and Use Committee of Auburn University (IACUC protocol #2021-3591). Heifers were maintained on the project for a total of 117 days.

IV.III.b. Estrous synchronization, breeding, pregnancy check

Experimental Cohort 1:

Heifers (n=3) originated from the breeding herd maintained at the North Auburn Beef Unit (NABU). NABU heifers were born in the spring of 2020 and managed for initial breeding in spring of 2021 at ~14 months old. Estrous synchronization was Select Synch + CIDR® protocol with estrus detection and AI for 72 ± 2 hr. Briefly, at Day 0, heifers were administered exogenous GnRH analog (Cystorelin®, Boehringer Ingelheim) and a controlled internal drug release device containing progesterone (EZI-Breed CIDR®, Zoetis) was applied. On Day 7 of the protocol, CIDRs were removed, and exogenous prostaglandin F2α analog (Lutalyse®, Zoetis) was injected. Heifers were observed for standing estrus for 72 ± 2 hr. and bred to AI 12 hours after first observed estrus behavior. Following AI, heifers were exposed to bulls of proven fertility for 60 days. Following

spring breeding season, heifers were checked for pregnancy and found to be non-pregnant but were retained for fall breeding. Procedures for fall breeding season were the same as for spring, with heifers synchronized utilizing a Select Synch + CIDR® protocol with estrus detection. Following AI breeding, heifers were again exposed to a bull for 60 days and checked for pregnancy at the end of the breeding season. Upon being classified as non-pregnant following the second breeding season, heifers were enrolled as subfertile in the current study and moved from the North Auburn Beef Unit to the Wilson Beef Teaching Center. Heifers were maintained without exposure to bulls on concrete flooring and cardboard shavings with access to pasture. Heifers were maintained on Bermuda grass hay and 30% soyhull pellet, 30% corn gluten meal pellet, 20% cracked corn, and 20% distiller's grains diet near ad libitum until harvest.

Experimental Cohort 2:

Forty-eight cross-bred *Bos taurus* beef heifers were born in the spring of 2021 at four private production systems in North Alabama and purchased by Auburn University researchers post-weaning. Heifers were brought to the Auburn University Wilson Beef Teaching Center pastures in December 2021 and maintained until transported to E.V. Smith Research Center (Macon County, Alabama) in January 2022. Heifers were maintained at E.V. Smith pastures on research trial from January until April when they were estrous synchronized and bred by FTAI. Heifers were estrous synchronized utilizing a 7-day CIDR® +PG protocol. Briefly, heifers were injected with Fertagyl® (Merck Animal Health) on Day 0 of the protocol, and a controlled internal drug release device containing progesterone (EZI-Breed CIDR®, Zoetis) was inserted. On Day 7 of the protocol, CIDRs were removed, and Lutalyse® (Zoetis) was injected. At 54 ± 2 hours post-PG injection, heifers were bred by fixed-time artificial insemination (FTAI). AI was performed by a single trained technician utilizing a single straw of semen from two bulls of proven fertility. Bulls were

evenly distributed amongst heifers. Following FTAI, heifers were removed to pastures at the Wilson Beef Teaching Center until pregnancy checking at 41 days after first service AI. Heifers were checked for pregnancy via transrectal ultrasound by a trained technician and classified as pregnant to first service or non-pregnant. Heifers classified as pregnant were either retained as fertile controls (n = 8) or sold as pregnant replacement heifers. Fertile control pregnancies were aborted on the day of pregnancy checks with Lutalyse® (Zoetis). Heifers classified as non-pregnant were retained for natural service bull exposure and were kept on concrete flooring, bare dirt corral, and cardboard bedding. Heifers and bulls were provided twice a day with Bermuda grass hay. Bull exposure lasted from May 2022 until July 2022, providing 61 days with bulls of proven fertility. At the end of the bull-exposure period, heifers were switched to a 30% soyhull pellet, 30% corn gluten meal pellet, 20% cracked corn, and 20% distiller's grains diet and fed for near ad libitum access until harvest. Fertile controls and infertile animals from Experimental Cohort 1 were not exposed to bulls for the rest of the experimental period. Heifers (n=20) that were not pregnant at first check were checked for pregnancy at days 20, 40, and 60 of bull exposure. Additionally, heifers were pregnancy checked 13 and 34 days after the end of bull exposure. Two heifers were deemed subfertile from this cohort at the end of the experiment, being open at all pregnancy checks.

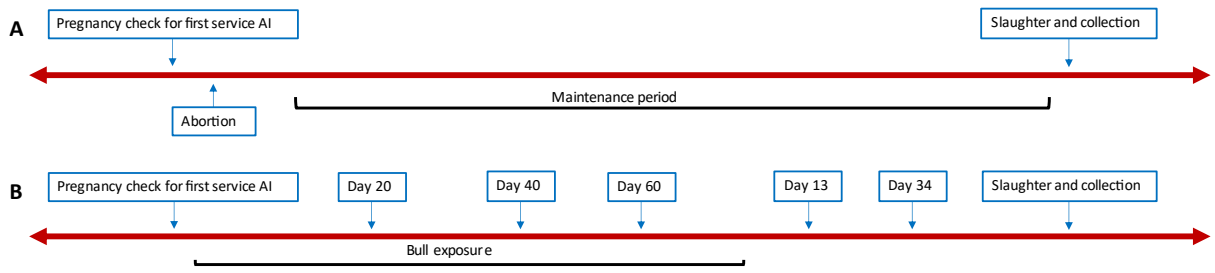

Figure 4.1: Experiment timeline from first pregnancy check for the first AI. A) is the timeline for fertile controls from Cohort 2 and subfertile heifers from Cohort 1; B) for the subfertile heifers in Cohort 2. Days marked in Panel B indicate pregnancy checks.

IV.III.c. Harvest

Heifers were separated into three cohorts and slaughtered on three separate days. In Slaughter 1, two fertiles and two subfertiles were sacrificed. Slaughter 2 had four fertiles and one subfertile. Slaughter 3 had the last two fertiles and two subfertiles.

IV.III.d. Phenotypic characterization

Heifer body weights, BCS, and RTS were collected prior to first-service AI. Final body weights were collected prior to slaughter and feed withdrawal.

IV.III.e. Sample collection, submission to WCMC

Whole blood was captured in EDTA blood vacutainers (BD, Franklin Lakes, NJ, USA) from jugular directly following exsanguination. Tissues relevant to study design were collected by kill floor workers and delivered to researchers no more than 45 minutes after completion of exsanguination. Before sample collection, some factors were collected about reproductively relevant tissues: uterine body length, uterine horn length, and uterine horn diameter were measured by two technicians. Ovary weight was measured prior to aspiration.

Blood samples

Whole blood samples were immediately inverted at least 10 times after collection and stored on ice until further processing. Samples were centrifuged at 1500 *g* for 15 minutes at 4° C to separate plasma which was aliquoted and frozen at -80° C until further analysis.

Follicular fluid

Ovaries were maintained at room temperature in 0.8% saline from the kill floor to the laboratory. Upon decanting from saline, ovaries were examined by a practiced investigator for abnormalities or pathologies. Using a short-bevel, 18 G needle (BD, Franklin Lakes, NJ, USA), follicular fluid from all follicles 2mm to 6mm were collected via aspiration into a 10 mL syringe.

Follicular fluid was centrifuged at 500 *g* for 5 minutes. The supernatant was then collected into cryovials and snap frozen in liquid nitrogen. Samples were stored at -80ºC until used for metabolomic profiling.

Generation of metabolomic profiles

Follicular fluid samples and plasma samples (n=8 fertile, n=5 subfertile) were submitted to West Coast Metabolomics Center (UC-Davis; Davis, California) for untargeted, primary metabolism via GC-TOF-MS. At WCMC, samples were processed on an Agilent 6890 gas chromatograph linked with a Leco Pegasus IV mass spectrometer with mass resolution set at 17 spectra/sec from 80-500 Da at -70 eV ionization energy. The column was an Rtx-5Sil MS (Restek corporation) that was 30 m long with an 0.25 µm interior diameter and a 0.25 µm film (95% dimethyl/5%diphenylpolysiloxane). The chromatograph was equipped with an Gerstel automatic liner exchange system (ALEX; Gerstel, Muehlheim, Germany) and a cold injection system (CIS) also produced by the Gerstel corporation. Injection volume was 0.5 µL. Column temperature was 50° C to 330° C with a flow rate of 1 mL/min and oven temperature 50° C for 1 minute and ramped by 20° C per minute until 330° C and then held constant for 5 minutes. Data was collected by Leco ChromaTOF v2.32. Data was normalized by peak heights to reduce variation caused by run differences. Metabolites were identified using the BinBase algorithm.

IV.III.f. Statistical analysis

Relative levels of metabolites are reported as peak heights. Metabolomic profiles were submitted to the online metabolomics software MetaboAnalyst v5.0 (Pang et al., 2021). To control for discrepancies created by different slaughter dates, data was batch corrected in MetaboAnalyst using the ComBat procedure (Johnson et al., 2007).

The module Statistical Analysis [one factor] was used to perform statistical analysis. Batch corrected data was submitted to MetaboAnalyst and not filtered due to low sample numbers. Sum normalization and Pareto-scaling (mean centered and divided by the square root of standard deviation of each variable) were used to reduce variability between metabolite concentrations. Student's t-test was used to search for different levels between metabolites with $p \leq 0.05$ being declared significant. Principal Component Analysis (PCA) was utilized for data structure visualization and Partial Least Squares Discriminant Analysis (PLS-DA) models were generated to increase the differences between cohorts. PLS-DA models were validated by cross validation utilizing 5 components and analysis of Q2 values. Receiver operating characteristics-area under the curve (ROC-AUC) analysis was performed to determine the potential diagnostic ability of metabolites. AUC values were calculated from the biofluid of discovery (i.e. only in follicular fluid for metabolites discovered in follicular fluid). Utilizing R version 4.2.2, plasma profiles and follicular fluid profiles were subjected to Pearson correlation analysis using the R package, *metabolomicsR* (Han and Liang, 2022). For correlation analysis, data was Pareto-scaled but not sum normalized. Prism v6 was utilized for statistical tests of phenotypic measures and visualization of metabolites. Phenotypic measures of body weight, tract length, horn diameter, largest follicle size, and cumulus-oocyte-complex (COC) count were analyzed using an unpaired Student's t-test with Welch's correction for unequal standard deviation with significance set at $p \leq 0.05$. Metabolites for visualization were either Pareto-scaled and sum normalized for graphs of relative levels between cohorts, or only sum normalized for visualizations of biofluid correlation. The venn diagram was generated using Microsoft PowerPoint (Microsoft Corporation).

IV.IV. Results

IV.IV.a. Phenotypic measures

From the 48 heifers of Experimental Cohort 2, 95.8% were pregnant and 4.2% were non-pregnant at the end of AI and NS breeding.

The body weight of heifers was collected prior to sacrifice. Tract length and horn diameter were measured prior to sample collection and after examination for gross pathologies by two technicians. Largest follicle size was measured prior to aspiration and did not include potentially cystic follicles. Number of COCs collected was reported by two technicians during searching. There was no statistically significant difference in phenotypic measures collected for subfertile and fertile animals. Mean ± SD and p-values of phenotypic measures are shown in **Table 4.1**.

Table 4.1: Mean and standard deviation of phenotypic measures in subfertile (n=5) and fertile (n=8) animals and p-value of t-test. BW at harvest- body weight at harvest, COCs- cumulus oocyte complex.

Phenotype	BW at harvest (kg)	Tract Length (cm)	Horn Diameter (cm)	Follicle Size (mm)	COCs
Subfertile	563.6 ± 50.19	78.59 ± 11.63	17.22 ± 1.1	10.06 ± 2.71	19.0 ± 4.74
Fertile	556.5 ± 19.30	80.89 ± 8.09	17.40 ± 2.17	13.53 ± 4.64	21.25 ± 4.77
P-value	0.7731	0.6787	0.8694	0.1614	0.4249

IV.IV.b. Plasma and follicular fluid metabolomic profiles

Metabolomic profiles were generated for plasma and follicular fluid from subfertile (n=5) and fertile (n=8) heifers. In plasma profiles, 284 unique metabolite peaks were identified and 126 metabolites were annotated. In follicular fluid, 491 peaks were identified and 182 metabolites were annotated. When annotated metabolites were considered, 7 metabolites were unique to plasma, 119 metabolites were shared between plasma and follicular fluid, and 63 metabolites were unique to follicular fluid (**Figure 2**).

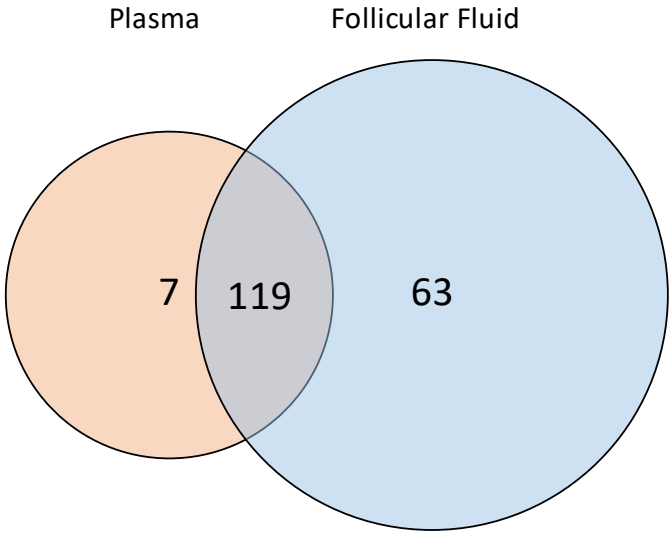

Figure 4.2: Distribution of annotated metabolites between plasma and follicular fluid.

Prior to analysis, metabolites were normalized to remove batch effect. Visualization of correction is in **Appendix 3**.

Fourteen metabolites were present at differential levels in follicular fluid and four metabolites in plasma ($p \leq 0.05$) (**Table 4.2**). In follicular fluid, 3-amino-2-piperidone, ascorbic acid, N-methylethanolamine, and parabanic acid were lower in fertile animals compared to subfertile animals. 5-methylthioadenosine, alpha-aminoadipic acid, alpha-tocopherol, aminomalonic acid, arachidonic acid, deoxycholic acid, glucose-6-phosphate, methanolphosphate, pimelic acid, and trans-4-hydroxyproline were higher in follicular fluid of fertile animals. In plasma, lactic acid was higher in fertile animals; fucose, glycerol-alpha-phosphate, and pipecolinic acid was lower in fertile animals. Metabolites, p-value, false discovery rate (FDR), area under the

curve (AUC), log_2 fold change, and biofluid of discovery are shown in **Table 4.2**. Relative levels of significant metabolites are shown in **Figure 4.3**, in both biofluids.

Table 4.2: Metabolites significant by Students t-test in follicular fluid, p-value, false discovery rate, log fold change, and direction of expression in fertile controls.

Metabolites	Raw p-value	FDR	AUC	Log2(FC)	Biofluid	In Fertile Heifers
3-amino-2-piperidone	0.013	0.590	0.925	0.249	Follicular fluid	Down
5-methylthioadenosine	0.040	0.590	0.800	0.370	Follicular fluid	Up
Alpha-aminoadipic acid	0.023	0.590	0.850	0.524	Follicular fluid	Up
Alpha-tocopherol	0.019	0.590	0.900	0.551	Follicular fluid	Up
Aminomalonic acid	0.013	0.590	0.925	0.487	Follicular fluid	Up
Arachidonic acid	0.042	0.590	0.875	0.356	Follicular fluid	Up
Ascorbic acid	0.028	0.590	0.800	0.877	Follicular fluid	Down
Deoxycholic acid	0.044	0.590	0.900	0.890	Follicular fluid	Up
Glucose-6-phosphate	0.005	0.590	0.950	0.782	Follicular fluid	Up
Methanolphosphate	0.021	0.590	0.875	0.342	Follicular fluid	Up
N-methylethanolamine	0.035	0.590	0.800	0.197	Follicular fluid	Down
Parabanic acid	0.040	0.590	0.900	0.335	Follicular fluid	Down
Pimelic acid	0.041	0.590	0.925	0.806	Follicular fluid	Up
Trans-4-hydroxyproline	0.037	0.590	0.850	0.519	Follicular fluid	Up

Table 4.3: Metabolites significant by Students t-test in blood plasma, p-value, false discovery rate, log fold change, and direction of expression in fertile controls.

Metabolites	Raw p-value	FDR	AUC	Log2(FC)	Biofluid	In Fertile Heifers
Fucose	0.040	0.999	0.850	0.433	Plasma	Down
Glycerol-alpha-phosphate	0.040	0.999	0.850	0.419	Plasma	Down
Lactic acid	0.027	0.999	0.800	0.798	Plasma	Up
Pipecolinic acid	0.017	0.999	0.900	0.588	Plasma	Down

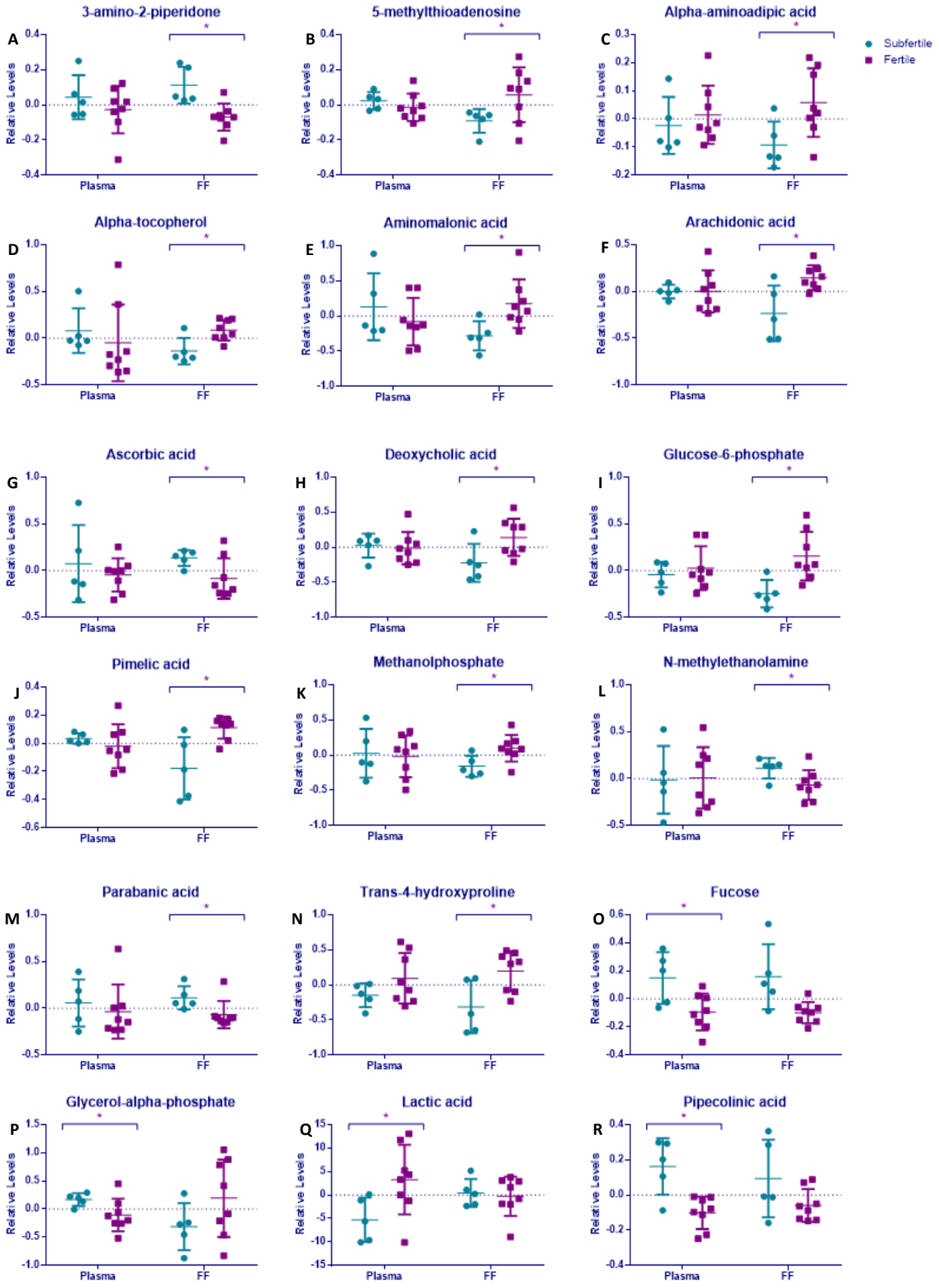
3-amino-2-piperidone
5-methylthioadenosine
Alpha-aminoadipic acid
Alpha-tocopherol
Aminomalonic acid
Arachidonic acid
Ascorbic acid
Deoxycholic acid
Glucose-6-phosphate
Pimelic acid
Methanolphosphate
N-methylethanolamine
Parabanic acid
Trans-4-hydroxyproline
Fucose
Glycerol-alpha-phosphate
Lactic acid
Pipecolinic acid
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
Relative Levels
Plasma
FF
*
Subfertile
Fertile

Figure 4.3: Relative expression levels of significant metabolites by t-test (p-value ≤ 0.05) in subfertile (circle) and fertile (square) animals. Biofluid (follicular fluid or plasma) shown on x-axis. Panel A-M; metabolites identified at significantly different levels in follicular fluid; Panel N-R; metabolites identified at significantly different levels in plasma.

For pathway analysis, metabolites from plasma and follicular fluid were combined for mapping and submitted to MetaboAnalyst Pathway Analysis module. 12 pathways were identified from 18 metabolites. 9 metabolites did not associate to any pathways in MetaboAnalyst and were explored through other resources. Pathways identified from the significant metabolites are shown in **Table 4.3**.

Table 4.4: Pathways identified to be impacted by significant metabolites in follicular fluid and plasma. Raw p-value and Holm's adjusted p-value shown for each pathway.

Pathways	Raw p-value	Holms adjusted	Metabolites
Lysine degradation	0.007	0.581	Alpha-aminoadipic acid, pipecolinic aci
Neomycin, kanamycin, and gentamycin biosynthesis	0.011	0.876	Glucose-6-phosphate
Starch and sucrose metabolism	0.092	1	Glucose-6-phosphate
Fructose and mannose metabolism	0.101	1	Fucose
Pyruvate metabolism	0.111	1	Lactic acid
Glycolysis/Gluconeogenesis	0.130	1	Lactic acid
Inositol phosphate metabolism	0.148	1	Glucose-6-phosphate
Cysteine and methionine metabolism	0.162	1	5-methylthioadenosine
Biosynthesis of unsaturated fatty acids	0.176	1	Arachidonic acid
Amino sugar and nucleotide sugar metabolism	0.180	1	Fucose
Arachidonic acid	0.180	1	Arachidonic acid
Arginine and proline metabolism	0.185	1	Trans-4-hydroxyproline

Unsupervised Principal Component Analysis was utilized to determine underlying structures or separation tendency of the metabolomic profiles. For both follicular fluid and plasma, PCA plots failed to separate subfertile and fertile cohorts (**Figure 4.4A, 4.4B**). Partial Least Squares-Discriminant Analysis modeling was utilized to increase separation tendency of the data.

For the follicular fluid, cohorts grouped with no overlap of samples (**Figure 4.4C**) but plasma samples showed considerable overlap even in the PLS-DA model (**Figure 4.4D**).

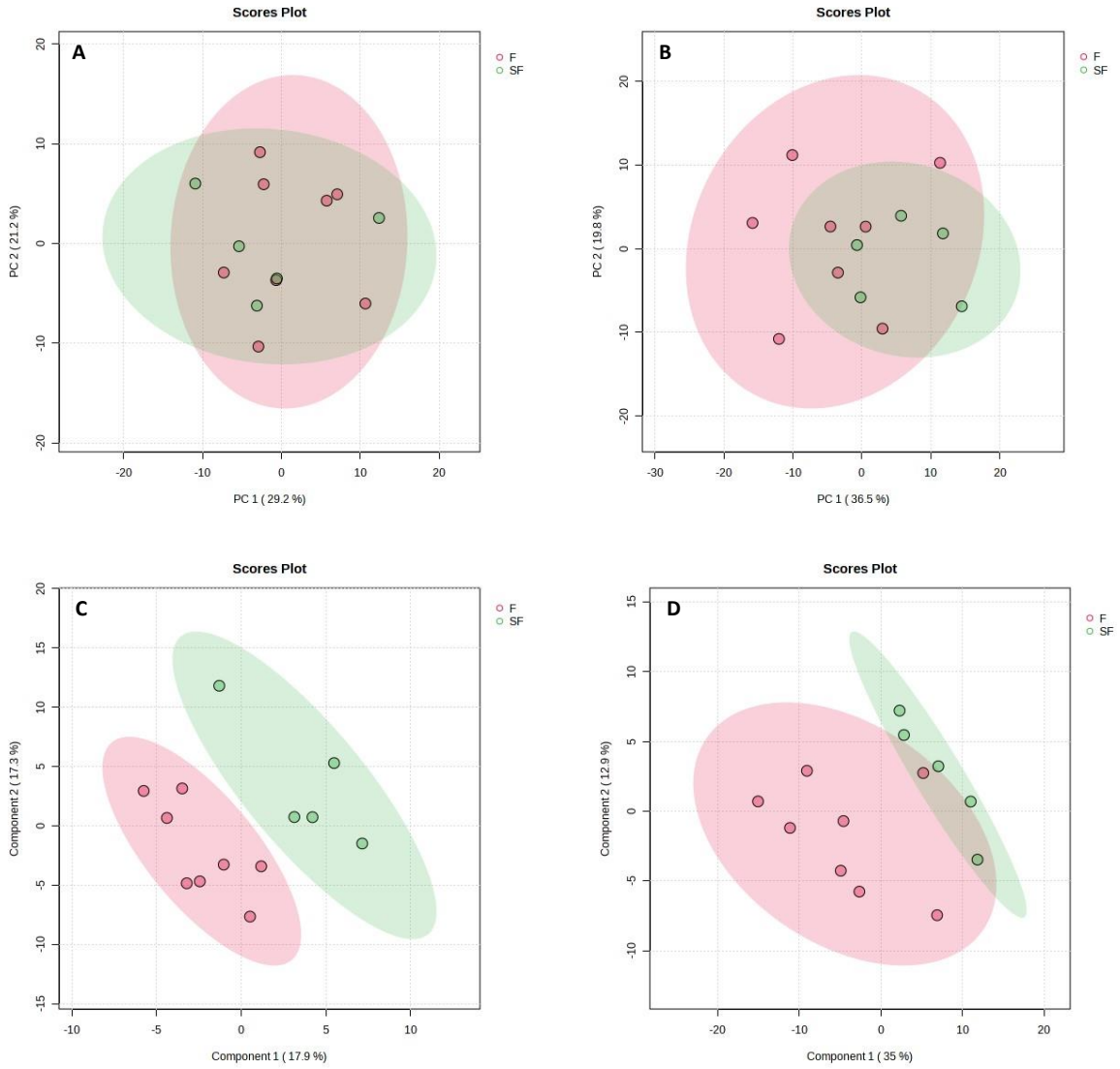

Figure 4.4: PCA score plot for follicular fluid (Panel A) and plasma (Panel B). PLS-DA score plots for follicular fluid (Panel C) and for plasma (Panel D).

PLS-DA models were checked by cross-validation and fit statistics are reported for 5 components. In the follicular fluid PLS-DA accuracy averaged from 5 components was 0.36667, R^2 of the same was 0.8859 and Q^2 was -0.6328. In the PLS-DA for plasma profiles, accuracy

averaged from 5 components was 0.5867, R^2 was 0.7648 and Q^2 was -0.0539. Results of cross validation shown in **Figure 4.5**.

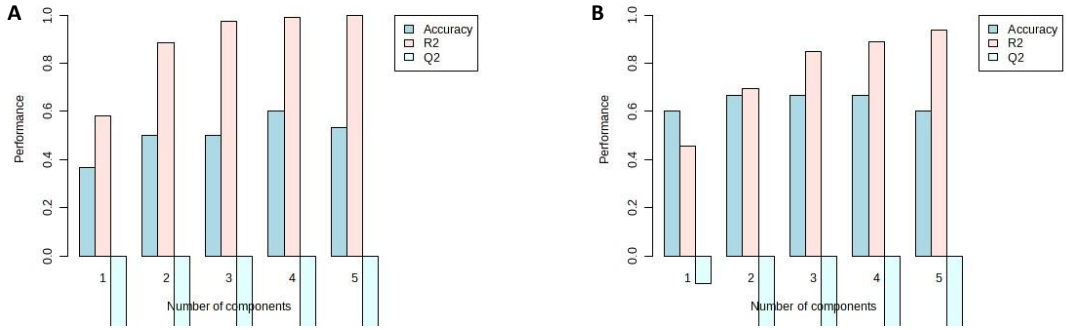

Figure 4.5: Cross-validation results of PLS-DA models generated by plasma and follicular fluid profiles. Panel A) follicular fluid; Panel B) plasma.

IV.IV.c Correlation

Using the RStudio package *metabolomicsR*, Pearson correlation of metabolites between plasma and follicular fluid was performed. From 126 metabolites in plasma and 182 metabolites in follicular fluid, 119 were present in both biofluids and correlated. Sixteen metabolites showed a significant correlation between plasma and follicular fluid ($p \leq 0.05$). Two metabolites were negatively correlated and fourteen were positively correlated. **Table 4.4** shows metabolites with significant correlations and correlation patterns of significant metabolites identified at different levels.

Table 4.5: Metabolites with significant correlation between plasma and follicular fluid and correlation coefficient, by p-value.

Correlated Metabolites	R-value	P-value
Phenylethylamine	0.896	< 0.001
Citric acid	0.853	< 0.001
Isocitricacid	0.833	< 0.001
Oleic acid	0.792	0.001
2-hydroxyvaleric acid	0.764	0.002
P-tolyl glucuronide	0.738	0.004
Pipecolinic acid	0.688	0.009
Fucose	0.678	0.011
Ornithine	0.670	0.012
N-acetylglycine	0.652	0.016
Aconitic acid	0.645	0.017
Methionine sulfone	0.634	0.020
Kynurenine	0.629	0.021
Sorbitol	0.569	0.042
Dehydroascorbic acid	-0.618	0.024
Succinic acid	-0.711	0.006

For visualization, representative metabolites were sum normalized without scaling to rank metabolites equally between follicular fluid and plasma and plotted using Prism v6. Representative graphs of plasma and follicular fluid levels are shown in **Figure 4.6**.

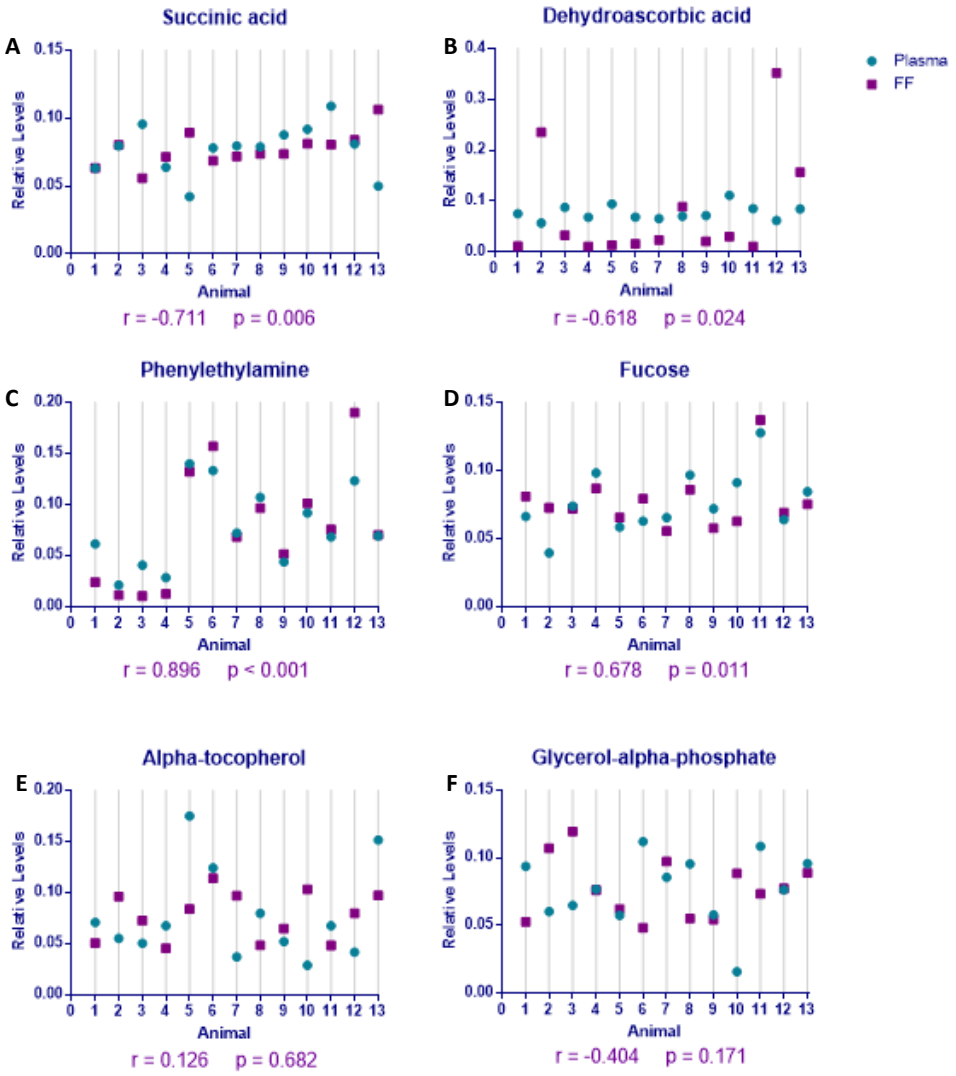

Figure 4.6: Representative graphs of metabolites with significant correlations (Panel A-D) and non-significant correlations (Panel E-F). Plasma values (circle) and follicular fluid values (square).

IV.V. Discussion

IV.V.a. Phenotypic variables

Phenotypic measures of body weight, tract length, horn diameter, largest follicle size, and number of collected COCs were compared for subfertile and fertile animals. There were no significant differences between subfertile and fertile animals.

IV.V.b. Metabolites

Plasma and follicular fluid profiles were analyzed independently of one another but utilizing identical workflows. To correct for variation introduced by samples being collected on separate days, profiles were batch corrected prior to normalization and scaling. The analysis identified four metabolites as being present at significantly different levels in plasma in the fertile and subfertile heifers, those being glycerol-alpha-phosphate, fucose, lactic acid, and pipecolinic acid. Fourteen metabolites were significant in follicular fluid between the two groups. 3-amino-2-piperidone, ascorbic acid, N-methylethanolamine, and parabanic acid were present at lower levels in the fertile heifer's follicular fluid. Glucose-6-phosphate, aminomalonic acid, alpha-tocopherol, methanolphosphate, alpha-aminoadipic acid, trans-4-hydroxyproline, 5-methylthioadenosine, pimelic acid, arachidonic acid, and deoxycholic acid were present at higher levels in the follicular fluid of the fertile animals.

Alpha-tocopherol, also known as Vitamin E, is involved in the processes of vitamin digestion and absorption, ferroptosis, and ubiquinone/terpenoid-quinone biosynthesis and was present at higher levels in the follicular fluid of fertile animals. In IVF experiments, Vitamin E supplementation has been suggested to increase cleavage and blastocyst formation due to antioxidant properties and reduction of ROS (Silva et al., 2015). *In vivo*, an experiment showed dairy cows with Vitamin E supplementation in the peripartum period decreased AI services to conception without altering milk factors (Baldi et al., 2000). This could suggest higher ROS in subfertile animals compared to fertile animals, or greater ability to respond to ROS in fertile animals.

Arachidonic acid is related to the pathways of arachidonic metabolism and biosynthesis of unsaturated fatty acids and is an upstream metabolite to prostaglandins, including prostaglandin F2α and PGE, key hormones in estrous cycling. Arachidonic acid was higher in the follicular fluid

of fertile animals compared to subfertile animals. This is in contrast to Bai et al. (2020) that found arachidonic acid being higher in the follicular fluid of cows in lactational anestrous compared to cyclic controls (Bai et al., 2020). However, this result is roughly in agreement with a study that found arachidonic acid higher in the follicular fluid of virgin heifers and non-lactating post-partum cows compared to lactating cows (Forde et al., 2016). Arachidonic acid levels were also found to be higher in the follicular fluid of women with PCOS compared to healthy age matched controls (Lai et al., 2022). Lai and colleagues also suggested that alterations in fatty acid metabolism in follicular fluid was related to inflammation in granulosa cells. Inflammation is not necessarily a bad thing to find in the ovary, as inflammatory signals like prostaglandins and TNFα are involved in processes like ovulation and luteolysis (Senger, 2012). With such differing results from different studies, it is difficult to determine the ultimate meaning of the differences in arachidonic acid levels in the current study.

Two metabolites significant in this study were linked to the pathway of lysine degradation. Pipecolinic acid (also called pipecolic acid) was significantly lower in the plasma of fertile animals compared to subfertile animals, with the same pattern of presence in follicular fluid, though it was not a significant difference. Interestingly, pipecolinic acid levels were somewhat correlated between plasma and follicular fluid ($r = 0.688$, $p = 0.009$). Pipecolinic acid supplementation to IVF medium is suggested to have improved the developmental competence of mouse oocytes through reduction of mitochondrial activity and reduced ROS species in embryos (Treleaven et al., 2021).

Alpha-aminoadipic acid was also mapped to be part of the lysine degradation pathway and was higher in the follicular fluid of fertile animals with a similar pattern of presence in plasma, but not significant. In contrast, a study performed in lactating cattle found that alpha-aminoadipic acid

was higher in follicular fluid compared to post-partum cows that were dried off immediately after parturition and primiparous heifers (Forde et al., 2016). As well, in a study of PCOS follicular fluid, alpha-aminoadipic acid was higher in patients compared to controls (Chang et al., 2017). These results from published literature suggest that increased levels of alpha-aminoadipic acid might be linked to reduced fertility, while the results gathered by this study would state the opposite. However, it is important to consider that in the current study, fertile animals were harvested three months after phenotypic categorization and may have entered a period of lower fertility, increasing alpha-aminoadipic acid in their follicular fluid.

Ascorbic acid was lower in the follicular fluid of fertile animals and related to the pathways of ascorbate and alderate metabolism, glutathione metabolism, HIF-1 signaling pathway, and vitamin digestion and absorption. This is in contrast to *in vitro* fertilization results on porcine oocytes that found increased ascorbic acid supplementation increased glutathione levels and reduced oxidative stress marker (Kere et al., 2013) and reduced or prevented DNA damage (Tatemoto et al., 2001).

Deoxycholic acid is a bile acid with a structure similar to cholesterol (Sanchez-Guijo et al., 2016) and related to the pathways of bile acid secretion and secondary bile acid synthesis. Deoxycholic acid was higher in the follicular fluid of fertile animals. It has been suggested that bile acids are naturally present in follicular fluid (Sanchez-Guijo et al., 2016) and that their levels may be descriptive of healthy or atretic state (Wei et al., 2022). Deoxycholic acid was noted to be higher in healthy follicles compared to atretic ones (Wei et al., 2022) however it is not clear what function bile acids play in follicular dynamics.

Glucose-6-phosphate is an intermediate energy metabolite present in starch and sucrose metabolism, inositol phosphate metabolism, and glycolysis/gluconeogenesis. Additionally,

glucose-6-phosphate interacts with NAD+ as part of the nicotinamide and nicotinic acid pathway, is involved in glutathione metabolism, and is part of the pentose phosphate pathway. It was present at higher levels in the follicular fluid of fertile animals compared to subfertile animals. Glucose-6-phosphate dehydrogenase (G6PD) catalyzes the reduction of glucose-6-phosphate to D-Glucono-1,5-lactone 6-phosphate in the pentose phosphate pathway and the reaction of NADP+ to NADPH in the glutathione pathway. G6PD activity in cumulus granulosa may impact oocyte maturation, as silencing of this enzyme led to reduced maturation competency and increased oxidative stress markers in cocultured oocytes (Xie et al., 2018). Glucose-6-phosphate dehydrogenase is also implicated in atresia in ewe follicular dynamics, being highest in small healthy follicles and reducing with increased atresia and follicular size (Ortega-Camarillo et al., 2009). Glucose-6-phosphate is part of the inositol phosphate pathway, one that was noted to be changed between day 0 and day 17/day 45 of pregnancy (Guo and Tao, 2018) potentially showing an effect of steroid hormones on this pathway in reproductively important tissues.

Fucose was present at lower levels in the plasma of fertile animals compared to subfertile animals and showed significant correlation between follicular fluid and plasma ($r = 0.678$, $p = 0.011$). Fucose showed the same distribution in follicular fluid as in plasma, however levels were not significant in follicular fluid ($p = 0.067$). Fucose is part of fructose and mannose metabolism as well as amino sugar and nucleotide sugar metabolism. Fucosylation (connection of a fucose to a protein) is a common modification of glycoproteins (Lin et al., 2010). Additionally, fucose levels in urine have been suggested to be related to inflammation in a mouse model of Crohn's Disease (Lin et al., 2010).

5-methylthioadenosine (MTA) was higher in the follicular fluid of fertile animals and is part of cysteine and methionine metabolism. 5-methylthioadenosine may be closely related to cell

cycle activity through the enzyme methylthioadenosine phosphorylase (MTAP) (Sunkara et al., 1985). MTAP is increased in cells shortly before the DNA synthesis stage and loss of MTAP expression in cells is linked to increased tumorigenesis and metastasis (Sunkara et al., 1985). This is in contrast to MTA itself which can limit proliferation and increase apoptosis of cells (Li et al., 2019). When mouse ovaries were stimulated by FSH, 5-methylthioadenosine was present at higher levels than in ovaries of unstimulated controls (Sun et al., 2018). This might suggest that there is a difference in granulosa metabolism and apoptotic states between fertile and subfertile animals.

Two metabolites were discovered to be involved in glycerophospholipid metabolism. N-methylethanolamine, which was lower in the follicular fluid of fertile animals, and glycerol-alpha-phosphate, which was lower in the plasma of fertile animals. N-methylethanolamine was found to be higher in the serum of cattle that developed mastitis postpartum (Zandkarimi et al., 2018). This might suggest alterations in N-methylethanolamine could be predictive of future inflammation or infection. In plasma samples, glycerol-alpha-phosphate was present at lower levels in fertile animals. Glycerolipid metabolism in the follicle was suggested to be discriminatory between women with repeated failure of IVF and those considered healthy controls (Batushansky et al., 2020).

Parabanic acid is potentially related to purine metabolism and was lower in the follicular fluid of fertile animals. Parabanic acid may be descriptive of the ROS status of a follicle, as it can be formed from guanine and uracil by exposure to reactive oxygen species (Jena and Mishra, 2012; Iida et al., 2017). Also potentially related to oxidative stress in the follicle is 3-amino-2-piperidone which was present at lower levels in follicular fluid of fertile animals. 3-amino-2-piperidone is potentially related to ornithine metabolism, and in a study of COVID-19 patients was present at higher levels in serum in relation to increased IL-6 and CRP (Li et al., 2021).

Pimelic acid and trans-4-hydroxyproline were higher in the follicular fluid of fertile animals. Pimelic acid is related to biotin metabolism which is related to keratin synthesis. Biotin supplementation to cattle has been suggested to reduce lameness and increase fertility (Wilde, 2006). Trans-4-hydroxyproline is also involved in structural proteins, being a core factor in collagen synthesis and is conditionally related to arginine and ornithine metabolism (Wu et al., 2011). Addition of proline (a metabolic precursor to trans-4-hydroxyproline) to IVF media was suggested to improve embryo health in mice (Treleaven et al., 2021). Trans-4-hydroxyproline has been found to be increased in the follicular fluid of older women undergoing IVF procedures (Huang et al., 2022a). Higher levels of these two factors in follicular fluid could suggest that fertile animals had greater breakdown of connective tissues in the ovary at the time of collection, or greater turnover in connective tissue of the ovary compared to subfertile animals.

Lactic acid levels were present at higher levels in the plasma of fertile animals compared to subfertile animals, while being higher in the follicular fluid of subfertile animals than in the fertile animals. Lactic acid is a core energy substrate of cellular metabolism and is part of glycolysis/gluconeogenesis and pyruvate metabolism. Pyruvate presence in follicular fluid has previously been positively correlated with developmental capability of oocytes (Alves et al., 2019). It has been suggested that lower lactic acid presence in plasma could be diagnostic of subclinical ketosis (Sun et al., 2014). Additionally, lactic acid levels in circulation can easily be increased by high stress (Frese et al., 2016). It is important to recall that this blood plasma sample was collected from the jugular upon exsanguination. Animals had been deprived of food for at least 12 hours prior to slaughter and were in unfamiliar surroundings. These stressors could be the cause of altered levels of lactic acid, considering the broad range of values in both subfertile and fertile animals.

Of the above discussed metabolites, alpha-tocopherol, ascorbic acid, glucose-6-phosphate, parabanic acid, and 3-amino-2-piperidone were related to levels of reactive oxygen species and oxidative stress levels in cells while fucose, alpha-aminoadipic acid, and N-methylethanolamine have been linked to inflammation. Success of *in vitro* fertilization has been suggested to be related to levels of pipecolinic acid and alpha-tocopherol. Glycerolipid metabolism has been suggested to be linked to successful IVF procedures. Two metabolites were potentially linked to cell cycles and cell health and potentially follicular atresia, those being the bile acid deoxycholic acid and 5-methylthioadenosine. Trans-4-hydroxyproline and pimelic acid are linked to connective tissue, either through collagen or keratin homeostasis. Lactic acid was an interesting finding in this study, however due to the circumstances of harvest, the discrepancies in lactic acid could be due to higher numbers of fertile animals, or differences in when samples were collected that was not entirely corrected for by batch correction.

Reactive oxygen species creation is a normal part of cell metabolism as is the production of antioxidants by the cell to counteract ROS effects (Sabry et al., 2022). The balance of antioxidants and ROS species is important for cellular health and continued proper functioning (Sabry et al., 2022). Metabolites related to reactive oxygen species and antioxidant balance could implicate that these processes are dysregulated in subfertile animals, leading to imbalance of these factors in the ovary and decreased production/maturation of fertile oocytes. Inflammatory factors are also key to regulation of reproduction and the ovary. TNFα, an inflammatory cytokine, is part of the luteolytic cascade (Senger, 2012) and IL-6 stimulation in the uterus has been implicated in endometrial remodeling for pregnancy establishment (Schjenken et al., 2021). As such, relation of certain metabolites to inflammatory response is interesting, but needs to be more deeply explored at the ovarian and follicular level.

This study shows interesting results both in follicular fluid and in blood plasma. While no metabolites were significant in both biofluids, pipecolinic acid and fucose showed medium correlation between biofluids ($r > 0.60$, $p \leq 0.05$). When ROC-AUC analysis was performed on batch corrected results, pipecolinic acid had an AUC of 0.900 and fucose had an AUC of 0.850. This result suggests that pipecolinic acid and fucose could serve as descriptive of the fertility state of the ovary but could be tested for in blood plasma. However, this needs to be validated with intensive study and repeated measures. While these results point to interesting processes and physiological states that should be more deeply explored, the limitations of this study should be noted and understood. To ensure enough subfertile animals were collected it was necessary to include three heifers (Experimental Cohort 1) with the two heifers identified from Cohort 2. The heifers from Cohort 1 were older and offered more opportunities for establishing pregnancy than the younger heifers of Cohort 2. However, inclusion of these older heifers could also be a strength of this study, increasing the variability in production systems and bloodlines represented by the animals collected. Additionally, the heifers that were identified as fertile were retained and harvested at the same time as the subfertile animals. While this created greater uniformity in sample collection days by reducing the number of days and increasing the number of samples processed within a day, it should be noted that these samples were collected and tested several months past the point at which they were deemed fertile.

IV.VI. Implications and conclusions

The above results suggest that reproductive failure can begin at the ovarian level in the follicular environment. Metabolites, as precursors and products of cellular metabolism could be descriptive of follicular environments incapable of producing highly fertile oocytes. Metabolite levels are likely descriptive of both circulating metabolites from general metabolism as well as

local metabolism, being secreted by thecal and granulosa cells as well as the oocyte itself. An interesting relationship between some metabolites in plasma and follicular fluid warrants closer study. Potentially, such closer study could be done with ovum pickup procedures and repeated measurements of the same animals, increasing statistical power of correlation and giving a temporal description of the relationship between follicular fluid and plasma as well as a moment to moment one.

These results could also be informative for improving *in vitro* fertilization procedures, if the underlying metabolites critical for proper oocyte maturation are identified. Several studies have utilized parallel analysis of follicular fluid and synthetic maturation medias. *In vitro* matured oocytes often lack the developmental competence of *in vivo* derived or naturally occurring pregnancies. However, natural mating and IVD embryos are not always available or attainable, so increasing the efficiency and success rate of IVM/IVF is desirable, not only in cattle but also in humans and other species.

Literature Cited

2001. WHO International Programme on Chemical Safety Biomarkers in Risk Assessment: Validity and Validation.

Abdoon, A. S. S., A. M. E. Al-Atrash, S. S. Soliman, A. M. El-Sanea, A. A. Gamal El Din, and H. M. Fahmy. 2023. Lyophilized equine platelet-rich plasma (L-GFequina) antagonize the Reproductive toxicity and oxidative stress Induced by Cyclophosphamide in female rats. J Ovarian Res 16doi: 10.1186/s13048-023-01161-x

ACES. Alabama Beef Handbook. https://www.aces.edu/wp-content/uploads/2019/05/ANR-1323_Alabama-Beef-Handbook_090718.pdf (Accessed May 6 2023 2023).

Aldridge, G., D. Podrebarac, W. Greenough, and I. Weiler. 2008. The use of total protein stains as loading controls: an alternative to high-abundance single-protein controls in semi-quantitative immunoblotting. Journal of Neuroscience Methods 172(2):250-254. doi: 10.1016/j.jneumeth.2008.05.003

Aleri, J. W., B. C. Hine, M. F. Pyman, P. D. Mansell, W. J. Wales, B. Mallard, and A. D. Fisher. 2015. Assessing adaptive immune response phenotypes in Australian Holstein-Friesian heifers in a pasture-based production system. J Anim Sci 93(7):3713-3721. doi: 10.2527/jas.2015-9078

Alves, G. P., F. B. Cordeiro, C. B. de Lima, K. Annes, E. C. Dos Santos, J. Ispada, P. K. Fontes, M. F. G. Nogueira, M. Nichi, and M. P. Milazzotto. 2019. Follicular environment as a predictive tool for embryo development and kinetics in cattle. Reprod Fertil Dev 31(3):451-461. doi: 10.1071/RD18143

Andronico, F., R. Battaglia, M. Ragusa, D. Barbagallo, M. Purrello, and C. Di Pietro. 2019. Extracellular Vesicles in Human Oogenesis and Implantation. Int J Mol Sci 20(9)doi: 10.3390/ijms20092162

Arishima, T., S. Sasaki, T. Isobe, Y. Ikebata, S. Shimbara, S. Ikeda, K. Kawashima, Y. Suzuki, M. Watanabe, S. Sugano, K. Mizoshita, and Y. Sugimoto. 2017. Maternal variant in the upstream of FOXP3 gene on the Y chromosome is associated with recurrent infertility in Japanese Black cattle. BMC Genetics 18(1):103. doi: 10.1186/s12863-017-0573-8

Armstrong, S. P., C. J. Caunt, R. C. Fowkes, K. Tsaneva-Atanasova, and C. A. McArdle. 2009. Pulsatile and sustained gonadotropin-releasing hormone (GnRH) receptor signaling: does the Ca2+/NFAT signaling pathway decode GnRH pulse frequency? J Biol Chem 284(51):35746-35757. doi: 10.1074/jbc.M109.063917

Arshad, H., A. Siokis, R. Franke, A. Habib, J. C. L. Alfonso, Y. Poliakova, E. Lücke, K. Michaelis, M. Brönstrup, M. Meyer-Hermann, U. Bilitewski, J. Vila, L. Abel, T. Illig, J. Schreiber, and F. Pessler. 2022. Reprogramming of Amino Acid Metabolism Differs between Community-Acquired Pneumonia and Infection-Associated Exacerbation of Chronic Obstructive Pulmonary Disease. Cells 11(15):2283. doi: 10.3390/cells11152283

Arthington, J., and P. Moriel. 2021. Targeting ADG of Developing Replacement Heifers Using Age and Body Weight. https://edis.ifas.ufl.edu/publication/AN305 (Accessed May 6 2023 2023).

Bai, Y., F. Zhang, H. Zhang, C. Xu, L. Wu, and C. Xia. 2020. Follicular Fluid Metabolite Changes in Dairy Cows with Inactive Ovary Identified Using Untargeted Metabolomics. Biomed Res Int 2020:9837543. doi: 10.1155/2020/9837543

Baldi, A., G. Savoini, L. Pinotti, E. Monfardini, F. Cheli, and V. Dell'Orto. 2000. Effects of vitamin E and different energy sources on vitamin E status, milk quality and reproduction

in transition cows. J Vet Med A Physiol Pathol Clin Med 47(10):599-608. doi: 10.1046/j.1439-0442.2000.00323.x
Banerjee, P., W. J. S. Diniz, R. Hollingsworth, S. P. Rodning, and P. W. Dyce. 2023. mRNA Signatures in Peripheral White Blood Cells Predict Reproductive Potential in Beef Heifers at Weaning. Genes (Basel) 14(2)doi: 10.3390/genes14020498
Baruselli, P. S., R. M. Ferreira, M. F. Sa Filho, and G. A. Bo. 2018. Review: Using artificial insemination v. natural service in beef herds. Animal 12(s1):s45-s52. doi: 10.1017/S175173111800054X
Basu, M., K. Wang, E. Ruppin, and S. Hannenhalli. 2021. Predicting tissue-specific gene expression from whole blood transcriptome. Sci Adv 7(14)doi: 10.1126/sciadv.abd6991
Batushansky, A., A. Zacharia, A. Shehadeh, R. Bruck-Haimson, D. Saidemberg, N. M. Kogan, C. Thomas Mannully, S. Herzberg, A. Ben-Meir, and A. Moussaieff. 2020. A Shift in Glycerolipid Metabolism Defines the Follicular Fluid of IVF Patients with Unexplained Infertility. Biomolecules 10(8)doi: 10.3390/biom10081135
Belenky, P., K. L. Bogan, and C. Brenner. 2007. NAD^+ metabolism in health and disease. Trends in Biochemical Sciences 32(1):12-19. doi: 10.1016/j.tibs.2006.11.006
Berger, A. 2000. Th1 and Th2 responses: what are they? BMJ 321(7258):424. doi: 10.1136/bmj.321.7258.424
Berger, A., and R. N. Funston. 2013. Reducing Replacement Heifer Development Costs Using a Systems Approach. https://extensionpublications.unl.edu/assets/html/g2215/build/g2215.htm (Accessed May 6 2023 2023).
Berry, D. P. 2021. Invited review: Beef-on-dairy-The generation of crossbred beef x dairy cattle. J Dairy Sci 104(4):3789-3819. doi: 10.3168/jds.2020-19519
Blaurock, J., S. Baumann, S. Grunewald, J. Schiller, and K. M. Engel. 2022. Metabolomics of Human Semen: A Review of Different Analytical Methods to Unravel Biomarkers for Male Fertility Disorders. Int J Mol Sci 23(16)doi: 10.3390/ijms23169031
Boyer, C. N., A. P. Griffith, and K. L. DeLong. 2020. Reproductive Failure and Long-Term Profitability of Spring- and Fall-Calving Beef Cows. Journal of Agricultural and Resource Economics 45(1):78-91. doi: 10.22004/ag.econ.298435
Broughton-Neiswanger, L. E., S. M. Rivera-Velez, M. A. Suarez, J. E. Slovak, P. E. Pineyro, J. K. Hwang, and N. F. Villarino. 2020. Urinary chemical fingerprint left behind by repeated NSAID administration: Discovery of putative biomarkers using artificial intelligence. PLoS One 15(2):e0228989. doi: 10.1371/journal.pone.0228989
Byerley, D. J., R. B. Staigmiller, J. G. Berardinelli, and R. E. Short. 1987. Pregnancy rates of beef heifers bred either on puberal or third estrus. J Anim Sci 65(3):645-650. doi: 10.2527/jas1987.653645x
Canovas, A., A. Reverter, K. L. DeAtley, R. L. Ashley, M. L. Colgrave, M. R. Fortes, A. Islas-Trejo, S. Lehnert, L. Porto-Neto, G. Rincon, G. A. Silver, W. M. Snelling, J. F. Medrano, and M. G. Thomas. 2014. Multi-tissue omics analyses reveal molecular regulatory networks for puberty in composite beef cattle. PLoS One 9(7):e102551. doi: 10.1371/journal.pone.0102551
Cardoso, R. C., S. M. West, T. S. Maia, B. R. C. Alves, and G. L. Williams. 2020. Nutritional control of puberty in the bovine female: prenatal and early postnatal regulation of the neuroendocrine system. Domest Anim Endocrinol 73:106434. doi: 10.1016/j.domaniend.2020.106434

Chakraborty, D., N. Sharma, S. Kour, S. S. Sodhi, M. K. Gupta, S. J. Lee, and Y. O. Son. 2022. Applications of Omics Technology for Livestock Selection and Improvement. Front Genet 13:774113. doi: 10.3389/fgene.2022.774113

Chang, A. Y., A. Z. Lalia, G. D. Jenkins, T. Dutta, R. E. Carter, R. J. Singh, and K. S. Nair. 2017. Combining a nontargeted and targeted metabolomics approach to identify metabolic pathways significantly altered in polycystic ovary syndrome. Metabolism 71:52-63. doi: 10.1016/j.metabol.2017.03.002

Coleman, L. W., N. M. Schreurs, P. R. Kenyon, S. T. Morris, and R. E. Hickson. 2023. Growth, carcass and meat quality characteristics of Charolais-sired steers and heifers born to Angus-cross-dairy and Angus breeding cows. Meat Sci 201:109178. doi: 10.1016/j.meatsci.2023.109178

Conesa, A., and S. Beck. 2019. Making multi-omics data accessible to researchers. Sci Data 6(1):251. doi: 10.1038/s41597-019-0258-4

Corpron, M., A. Ahmadzadeh, P. Rezamand, and Q. Huo. 2021. Relationship between beef cow fertility and maternal immunity. Applied Animal Science 37(6):733-737. doi: 10.15232/aas.2021-02214

Cussotto, S., I. Delgado, A. Anesi, S. Dexpert, A. Aubert, C. Beau, D. Forestier, P. Ledaguenel, E. Magne, F. Mattivi, and L. Capuron. 2020. Tryptophan Metabolic Pathways Are Altered in Obesity and Are Associated With Systemic Inflammation. Front Immunol 11:557. doi: 10.3389/fimmu.2020.00557

Diaz, F. A., E. J. Gutierrez-Castillo, B. A. Foster, P. T. Hardin, K. R. Bondioli, and Z. Jiang. 2021. Evaluation of Seasonal Heat Stress on Transcriptomic Profiles and Global DNA Methylation of Bovine Oocytes. Front Genet 12:699920. doi: 10.3389/fgene.2021.699920

Dickinson, S. E., M. F. Elmore, L. Kriese-Anderson, J. B. Elmore, B. N. Walker, P. W. Dyce, S. P. Rodning, and F. H. Biase. 2019. Evaluation of age, weaning weight, body condition score, and reproductive tract score in pre-selected beef heifers relative to reproductive potential. J Anim Sci Biotechnol 10:18. doi: 10.1186/s40104-019-0329-6

Dickinson, S. E., B. A. Griffin, M. F. Elmore, L. Kriese-Anderson, J. B. Elmore, P. W. Dyce, S. P. Rodning, and F. H. Biase. 2018. Transcriptome profiles in peripheral white blood cells at the time of artificial insemination discriminate beef heifers with different fertility potential. BMC Genomics 19(1):129. doi: 10.1186/s12864-018-4505-4

Dubon, M. A. C., V. B. Pedrosa, F. L. B. Feitosa, R. B. Costa, G. M. F. de Camargo, M. R. Silva, and L. F. B. Pinto. 2021. Identification of novel candidate genes for age at first calving in Nellore cows using a SNP chip specifically developed for Bos taurus indicus cattle. Theriogenology 173:156-162. doi: 10.1016/j.theriogenology.2021.08.011

Elis, S., S. Freret, A. Desmarchais, V. Maillard, J. Cognie, E. Briant, J. L. Touze, M. Dupont, P. Faverdin, V. Chajes, S. Uzbekova, P. Monget, and J. Dupont. 2016. Effect of a long chain n-3 PUFA-enriched diet on production and reproduction variables in Holstein dairy cows. Anim Reprod Sci 164:121-132. doi: 10.1016/j.anireprosci.2015.11.020

Engelken, T. J. 2008. Developing replacement beef heifers. Theriogenology 70(3):569-572. doi: 10.1016/j.theriogenology.2008.05.032

Fernandez-Hernandez, P., M. J. Sanchez-Calabuig, L. J. Garcia-Marin, M. J. Bragado, A. Gutierrez-Adan, O. Millet, C. Bruzzone, L. Gonzalez-Fernandez, and B. Macias-Garcia. 2020. Study of the Metabolomics of Equine Preovulatory Follicular Fluid: A Way to

Improve Current In Vitro Maturation Media. Animals (Basel) 10(5)doi: 10.3390/ani10050883
Fletcher, L., N. Akhtar, X. Zhan, M. Jafarikia, B. P. Sullivan, L. A. Huber, and J. Li. 2022. Identification of Candidate Salivary, Urinary and Serum Metabolic Biomarkers for High Litter Size Potential in Sows (Sus scrofa). Metabolites 12(11)doi: 10.3390/metabo12111045
Forde, N., A. O'Gorman, H. Whelan, P. Duffy, L. O'Hara, A. K. Kelly, V. Havlicek, U. Besenfelder, L. Brennan, and P. Lonergan. 2016. Lactation-induced changes in metabolic status and follicular-fluid metabolomic profile in postpartum dairy cows. Reprod Fertil Dev 28(12):1882-1892. doi: 10.1071/RD14348
Franca, M. R., M. I. S. da Silva, G. Pugliesi, V. Van Hoeck, and M. Binelli. 2017. Evidence of endometrial amino acid metabolism and transport modulation by peri-ovulatory endocrine profiles driving uterine receptivity. J Anim Sci Biotechnol 8:54. doi: 10.1186/s40104-017-0185-1
Frese, D. A., C. D. Reinhardt, S. J. Bartle, D. N. Rethorst, J. P. Hutcheson, W. T. Nichols, B. E. Depenbusch, M. E. Corrigan, and D. U. Thomson. 2016. Cattle handling technique can induce fatigued cattle syndrome in cattle not fed a beta adrenergic agonist. Journal of Animal Science 94(2):581-591. doi: 10.2527/jas.2015-9824
Funeshima, N., R. Miura, T. Katoh, H. Yaginuma, T. Kitou, I. Yoshimura, K. Konda, S. Hamano, and K. Shirasuna. 2021. Metabolomic profiles of plasma and uterine luminal fluids from healthy and repeat breeder Holstein cows. BMC Vet Res 17(1):54. doi: 10.1186/s12917-021-02755-7
Funston, R. N., and G. H. Deutscher. 2004. Comparison of target breeding weight and breeding date for replacement beef heifers and effects on subsequent reproduction and calf performance. J Anim Sci 82(10):3094-3099. doi: 10.2527/2004.82103094x
Gaskins, A., Z. Tang, R. Hood, J. Ford, J. Schwartz, D. Jones, F. Laden, D. Liang, and E. S. Team. 2021. Periconception air pollution, metabolomic biomarkers, and fertility among women undergoing assisted reproduction. Environment International 155:106666. doi: 10.1016/j.envint.2021.106666
Gonzalez-Maldonado, J., R. Rangel-Santos, R. Rodriguez-de Lara, and O. Garcia-Pena. 2017. Effect of injectable trace mineral complex supplementation on development of ovarian structures and serum copper and zinc concentrations in over-conditioned Holstein cows. Anim Reprod Sci 181:57-62. doi: 10.1016/j.anireprosci.2017.03.015
Gosden, R. G., R. H. Hunter, E. Telfer, C. Torrance, and N. Brown. 1988. Physiological factors underlying the formation of ovarian follicular fluid. J Reprod Fertil 82(2):813-825. doi: 10.1530/jrf.0.0820813
Grala, T. M., B. Kuhn-Sherlock, M. Crookenden, C. G. Walker, J. R. Roche, M. D. Price, C. R. Burke, and S. Meier. 2022. Adaptive immune response ranking is associated with reproductive phenotypes in grazing dairy cows divergent in genetic merit for fertility traits. Journal Of Dairy Science 105(6):5519-5533. doi: 10.3168/jds.2021-21538
Guo, Y. S., and J. Z. Tao. 2018. Metabolomics and pathway analyses to characterize metabolic alterations in pregnant dairy cows on D 17 and D 45 after AI. Sci Rep 8(1):5973. doi: 10.1038/s41598-018-23983-2
Gutierrez, K., R. Kasimanickam, A. Tibary, J. M. Gay, J. P. Kastelic, J. B. Hall, and W. D. Whittier. 2014. Effect of reproductive tract scoring on reproductive efficiency in beef

heifers bred by timed insemination and natural service versus only natural service. Theriogenology 81(7):918-924. doi: 10.1016/j.theriogenology.2014.01.008
Guzman, A., E. Gonzalez-Padilla, P. Garces-Yepez, J. Rosete-Fernandez, R. Calderon-Robles, W. Whittier, D. Keisler, and C. Gutierrez. 2016. Increased body condition score through increased lean muscle, but not fat deposition, is associated with reduced reproductive response to oestrus induction in beef cows. Animal 10(10):1706-1713. doi: 10.1017/S175173111600063X
Hailay, T., M. Hoelker, M. Poirier, S. Gebremedhn, F. Rings, M. Saeed-Zidane, D. Salilew-Wondim, C. Dauben, E. Tholen, C. Neuhoff, K. Schellander, and D. Tesfaye. 2019. Extracellular vesicle-coupled miRNA profiles in follicular fluid of cows with divergent post-calving metabolic status. Sci Rep 9(1):12851. doi: 10.1038/s41598-019-49029-9
Hailemariam, D., R. Mandal, F. Saleem, S. M. Dunn, D. S. Wishart, and B. N. Ametaj. 2014. Identification of predictive biomarkers of disease state in transition dairy cows. Journal Of Dairy Science 97:2680-2693. doi: 10.3168/jds.2013-6803
Hall, J. B. 2019. Capturing the Value of Artificial Insemination in Commercial Herds. In: The Range Beef Cow Symposium XXVI, Mitchell, Nebraska
Han, X., and L. Liang. 2022. metabolomicsR: a streamlined workflow to analyze metabolomic data in R. Bioinformatics Advances 2(1)
Hemmings, K. E., H. J. Leese, and H. M. Picton. 2012. Amino Acid Turnover by Bovine Oocytes Provides an Index of Oocyte Developmental Competence In Vitro. Biology of Reproduction 86(5)doi: 10.1095/biolreprod.111.092585
Henley, P. A., F. A. Ireland, I. F. Canisso, J. L. Edwards, and D. W. Shike. 2021. Effects of management system on beef heifer growth and reproductive performance. Transl Anim Sci 5(1):txaa209. doi: 10.1093/tas/txaa209
Hernandez-Davies, J., E. Dollinger, E. Pone, J. Felgner, L. Liang, S. Strohmeier, S. Jan, T. Albin, A. Jain, R. Nakajima, A. Jasinskas, F. Krammer, A. Esser-Kahn, P. Felgner, Q. Nie, and D. Davies. 2022. Magnitude and breadth of antibody cross-reactivity induced by recombinant influenza hemagglutinin trimer vaccine is enhanced by combination adjuvants. Scientific Reports 12(1):9198. doi: 10.1038/s41598-022-12727-y
Hindman, M. S., B. Huedepohl, G. A. Dewell, T. A. Brick, G. S. Silva, and T. J. Engelken. 2022. Physical Traits and Reproductive Measurements Associated with Early Conception in Beef Replacement Heifers. Animals (Basel) 12(15)doi: 10.3390/ani12151910
Holm, D. E., M. Nielen, R. Jorritsma, P. C. Irons, and P. N. Thompson. 2015. Evaluation of pre-breeding reproductive tract scoring as a predictor of long term reproductive performance in beef heifers. Preventive Veterinary Medicine 118(1):56-63. doi: 10.1016/j.prevetmed.2014.10.016
Hong, J., and E. Lee. 2007. Intrafollicular amino acid concentration and the effect of amino acids in a defined maturation medium on porcine oocyte maturation, fertilization, and preimplantation development. Theriogenology 68(5):728-735. doi: 10.1016/j.theriogenology.2007.06.002
Hood, R. B., D. Liang, Y. Tan, J. Ford, I. Souter, D. P. Jones, R. Hauser, and A. J. Gaskins. 2022. Characterizing the follicular fluid metabolome: quantifying the correlation across follicles and differences with the serum metabolome. Fertil Steril 118(5):970-979. doi: 10.1016/j.fertnstert.2022.07.023
Horn, E. J., C. C. Read, J. L. Edwards, F. N. Schrick, J. D. Rhinehart, R. R. Payton, S. R. Campagna, J. L. Klabnik, H. M. Clark, P. R. Myer, K. J. McLean, and S. E. Moorey.

2022. Preovulatory follicular fluid and serum metabolome profiles in lactating beef cows with thin, moderate, and obese body condition. J Anim Sci 100(7)doi: 10.1093/jas/skac152

Hou, M., C. Xu, X. Zou, Z. Xia, L. Su, N. Qiu, L. Cai, F. Yu, Q. Wang, X. Zhao, Y. He, C. Wang, and J. Wang. 2022. Long-term exposure to microplastics induces intestinal function dysbiosis in rare minnow (Gobiocypris rarus). Ecotoxicol Environ Saf 246:114157. doi: 10.1016/j.ecoenv.2022.114157

Hu, L., L. Brito, H. Zhang, M. Zhao, H. Liu, H. Chai, D. Wang, H. Wu, J. Cui, A. Liu, Q. Xu, and Y. Wang. 2022. Metabolome profiling of plasma reveals different metabolic responses to acute cold challenge between Inner-Mongolia Sanhe and Holstein cattle. Journal Of Dairy Science 105(11):9162-9178. doi: 10.3168/jds.2022-21996

Huang, Y., M. Tu, Y. Qian, J. Ma, L. Chen, Y. Liu, Y. Wu, K. Chen, J. Liu, Y. Ying, Y. Chen, Y. Ye, L. Xing, F. Zhang, Y. Hu, R. Zhang, Y. C. Ruan, and D. Zhang. 2022a. Age-Dependent Metabolomic Profile of the Follicular Fluids From Women Undergoing Assisted Reproductive Technology Treatment. Front Endocrinol (Lausanne) 13:818888. doi: 10.3389/fendo.2022.818888

Huang, Z. B., Z. Hu, C. X. Lu, S. D. Luo, Y. Chen, Z. P. Zhou, J. J. Hu, F. L. Zhang, F. Deng, and K. X. Liu. 2022b. Gut microbiota-derived indole 3-propionic acid partially activates aryl hydrocarbon receptor to promote macrophage phagocytosis and attenuate septic injury. Front Cell Infect Microbiol 12:1015386. doi: 10.3389/fcimb.2022.1015386

Hugentobler, S. A., J. M. Sreenan, P. G. Humpherson, H. J. Leese, M. G. Diskin, and D. G. Morris. 2010. Effects of changes in the concentration of systemic progesterone on ions, amino acids and energy substrates in cattle oviduct and uterine fluid and blood. Reprod Fertil Dev 22(4):684-694. doi: 10.1071/RD09129

Hughes, H. 2013. Raised replacement heifers: some economic considerations. Vet Clin North Am Food Anim Pract 29(3):643-652. doi: 10.1016/j.cvfa.2013.07.013

Hwang, S., R. Hood, R. Hauser, J. Schwartz, F. Laden, D. Jones, D. Liang, and A. Gaskins. 2022. Using follicular fluid metabolomics to investigate the association between air pollution and oocyte quality. Environment International 169:107552. doi: 10.1016/j.envint.2022.107552

Iida, S., Y. Ohkubo, Y. Yamamoto, and A. Fujisawa. 2017. Parabanic acid is the singlet oxygen specific oxidation product of uric acid. Journal of Clinical Biochemistry And Nutrition 61(3):169-175. doi: 10.3164/jcbn.17-24

Ishler, V. A., J. Heinrichs, and C. M. Jones. 2016. Body Condition Scoring as a Tool for Dairy Herd Management. https://extension.psu.edu/body-condition-scoring-as-a-tool-for-dairy-herd-management (Accessed 02/12/2023 2023).

Janeway, C. A. J., P. Travers, M. Walport, and e. al. 2001. Immunobiology: The Immune System in Health and Disease. 5th Edition ed. New York: Garland Science.

Jena, N. R., and P. C. Mishra. 2012. Formation of ring-opened and rearranged products of guanine: mechanisms and biological significance. Free Radic Biol Med 53(1):81-94. doi: 10.1016/j.freeradbiomed.2012.04.008

Jiang, X., N. Lu, H. Zhao, H. Yuan, D. Xia, and H. Lei. 2020. The Microbiome-Metabolome Response in the Colon of Piglets Under the Status of Weaning Stress. Front Microbiol 11:2055. doi: 10.3389/fmicb.2020.02055

Johnson, W. E., C. Li, and A. Rabinovic. 2007. Adjusting batch effects in microarray expression data using empirical Bayes methods. Biostatistics 8(1):118-127. doi: 10.1093/biostatistics/kxj037

Jozkowiak, M., H. Piotrowska-Kempisty, D. Kobylarek, N. Gorska, P. Mozdziak, B. Kempisty, D. Rachon, and R. Z. Spaczynski. 2022. Endocrine Disrupting Chemicals in Polycystic Ovary Syndrome: The Relevant Role of the Theca and Granulosa Cells in the Pathogenesis of the Ovarian Dysfunction. Cells 12(1)doi: 10.3390/cells12010174

Kadri, N. K., G. Sahana, C. Charlier, T. Iso-Touru, B. Guldbrandtsen, L. Karim, U. S. Nielsen, F. Panitz, G. P. Aamand, N. Schulman, M. Georges, J. Vilkki, M. S. Lund, and T. Druet. 2014. A 660-Kb deletion with antagonistic effects on fertility and milk production segregates at high frequency in Nordic Red cattle: additional evidence for the common occurrence of balancing selection in livestock. PLoS Genet 10(1):e1004049. doi: 10.1371/journal.pgen.1004049

Kusama, K., R. Bai, Y. Matsuno, A. Ideta, T. Sakurai, K. Nagaoka, M. Hori, and K. Imakawa. 2022. Characterization of Serum Metabolome and Proteome Profiles Identifies SNX5 Specific for Pregnancy Failure in Holstein Heifers. Life (Basel) 12(2)doi: 10.3390/life12020309

Kwon, J. O., W. J. Jin, B. Kim, H. H. Kim, and Z. H. Lee. 2015. Myristoleic acid inhibits osteoclast formation and bone resorption by suppressing the RANKL activation of Src and Pyk2. Eur J Pharmacol 768:189-198. doi: 10.1016/j.ejphar.2015.10.053

Lee, B. H., I. R. Indran, H. M. Tan, Y. Li, Z. Zhang, J. Li, and E. L. Yong. 2016. A Dietary Medium-Chain Fatty Acid, Decanoic Acid, Inhibits Recruitment of Nur77 to the HSD3B2 Promoter In Vitro and Reverses Endocrine and Metabolic Abnormalities in a Rat Model of Polycystic Ovary Syndrome. Endocrinology 157(1):382-394. doi: 10.1210/en.2015-1733

Leroy, J. L. M. R., T. Vanholder, J. R. Delanghe, G. Opsomer, A. Van Soom, P. E. J. Bols, and A. de Kruif. 2004. Metabolite and ionic composition of follicular fluid from different-sized follicles and their relationship to serum concentrations in dairy cows. Animal Reproduction Science 80(3-4):201-211. doi: 10.1016/S0378-4320(03)00173-8

Li, T., N. Ning, B. Li, D. Luo, E. Qin, W. Yu, J. Wang, G. Yang, N. Nan, Z. He, N. Yang, S. Gong, J. Li, A. Liu, Y. Sun, Z. Li, T. Jia, J. Gao, W. Zhang, Y. Huang, J. Hou, Y. Xue, D. Li, Z. Wei, L. Zhang, B. Li, and H. Wang. 2021. Longitudinal Metabolomics Reveals Ornithine Cycle Dysregulation Correlates With Inflammation and Coagulation in COVID-19 Severe Patients. Front Microbiol 12:723818. doi: 10.3389/fmicb.2021.723818

Li, Y., Y. Guo, Z. Wen, X. Jiang, X. Ma, and X. Han. 2018. Weaning Stress Perturbs Gut Microbiome and Its Metabolic Profile in Piglets. Sci Rep 8(1):18068. doi: 10.1038/s41598-018-33649-8

Li, Y., Y. Wang, and P. Wu. 2019. 5'-Methylthioadenosine and Cancer: old molecules, new understanding. J Cancer 10(4):927-936. doi: 10.7150/jca.27160

Liles, H., L. Schneider, K. Pohler, R. Oliveira Filho, F. Neal Schrick, R. Payton, J. Rhinehart, K. Thompson, K. McLean, and J. Edwards. 2022. Positive relationship of rectal temperature at fixed timed artificial insemination on pregnancy outcomes in beef cattle. Journal of Animal Science 100(7)(Research) doi: 10.1093/jas/skac100

Lin, H. M., M. P. Barnett, N. C. Roy, N. I. Joyce, S. Zhu, K. Armstrong, N. A. Helsby, L. R. Ferguson, and D. D. Rowan. 2010. Metabolomic analysis identifies inflammatory and

noninflammatory metabolic effects of genetic modification in a mouse model of Crohn's disease. J Proteome Res 9(4):1965-1975. doi: 10.1021/pr901130s

Lucas, K. M., D. A. Koltes, L. R. Meyer, J. D. Tucker, D. S. Hubbell, 3rd, J. G. Powell, J. K. Apple, and J. E. Koltes. 2021. Identification of Breed Differences in Known and New Fescue Toxicosis Associated Phenotypes in Charolais-and Hereford-Sired Crossbred Beef Cows. Animals (Basel) 11(10)doi: 10.3390/ani11102830

Mackenzie, D. D. 1967. Production and utilization of lactic acid by the ruminant. A review. J Dairy Sci 50(11):1772-1786. doi: 10.3168/jds.S0022-0302(67)87715-4

Marques, J., G. Cadima, A. Faria, E. Guimaraes, F. Oliveira, and R. Dos Santos. 2022. Application of PGF2a at the moment of fixed-time artificial insemination in crossbred beef cows. Animal Reproduction 19(4):e20220012. doi: 10.1590/1984-3143-AR2022-0012

Marrella, M. A., R. R. White, N. W. Dias, C. Timlin, S. Pancini, J. Currin, S. Clark, J. L. Stewart, V. R. G. Mercadante, and H. L. Bradford. 2021. Comparison of reproductive performance of AI- and natural service-sired beef females under commercial management. Transl Anim Sci 5(3):txab114. doi: 10.1093/tas/txab114

Martin, N., L. Coleman, N. López-Villalobos, N. Schreurs, S. Morris, H. Blair, J. McDade, P. Back, and R. Hickson. 2021. Estimated Breeding Values of Beef Sires Can Predict Performance of Beef-Cross-Dairy Progeny. Front Genet 12doi: 10.3389/fgene.2021.712715

Matsuhashi, T., S. Maruyama, Y. Uemoto, N. Kobayashi, H. Mannen, T. Abe, S. Sakaguchi, and E. Kobayashi. 2011. Effects of bovine fatty acid synthase, stearoyl-coenzyme A desaturase, sterol regulatory element-binding protein 1, and growth hormone gene polymorphisms on fatty acid composition and carcass traits in Japanese Black cattle. J Anim Sci 89(1):12-22. doi: 10.2527/jas.2010-3121

McFee, R. M., S. M. Romereim, A. P. Snider, A. F. Summers, W. E. Pohlmeier, S. G. Kurz, R. A. Cushman, J. S. Davis, J. R. Wood, and A. S. Cupp. 2021. A high-androgen microenvironment inhibits granulosa cell proliferation and alters cell identity. Mol Cell Endocrinol 531:111288. doi: 10.1016/j.mce.2021.111288

Meier, S., L. R. McNaughton, R. Handcock, P. R. Amer, P. R. Beatson, J. R. Bryant, K. G. Dodds, R. Spelman, J. R. Roche, and C. R. Burke. 2021. Heifers with positive genetic merit for fertility traits reach puberty earlier and have a greater pregnancy rate than heifers with negative genetic merit for fertility traits. J Dairy Sci 104(3):3707-3721. doi: 10.3168/jds.2020-19155

Meier, S., M. D. Mitchell, C. G. Walker, J. R. Roche, and G. A. Verkerk. 2014. Amino acid concentrations in uterine fluid during early pregnancy differ in fertile and subfertile dairy cow strains. J Dairy Sci 97(3):1364-1376. doi: 10.3168/jds.2013-6954

Mejia, M., A. Gonzalez-Iglesias, G. S. Diaz-Torga, P. Villafane, N. Formia, C. Libertun, D. Becu-Villalobos, and I. M. Lacau-Mengido. 1999. Effects of continuous ivermectin treatment from birth to puberty on growth and reproduction in dairy heifers. J Anim Sci 77(6):1329-1334. doi: 10.2527/1999.7761329x

Meng, D., E. Sommella, E. Salviati, P. Campiglia, K. Ganguli, K. Djebali, W. Zhu, and W. Walker. 2020. Indole-3-lactic acid, a metabolite of tryptophan, secreted by Bifidobacterium longum subspecies infantis is anti-inflammatory in the immature intestine. Pediatric Research 88(2):209-217. doi: 10.1038/s41390-019-0740-x

Mercadante, V. R. G., N. W. Dias, C. Timlin, and S. Pancini. 2020. Economic Consequences of Pregnancy Loss in Beef Cattle. Journal of Animal Science 98(124)doi: doi: 10.1093/jas/skaa278.226

Mo, J., L. Sun, J. Cheng, Y. Lu, Y. Wei, G. Qin, J. Liang, and G. Lan. 2021. Non-targeted Metabolomics Reveals Metabolic Characteristics of Porcine Atretic Follicles. Front Vet Sci 8:679947. doi: 10.3389/fvets.2021.679947

Mohapatra, S. K., B. S. K. Panda, A. K. Verma, R. Kapila, and A. K. Dang. 2020. Implantation associated changes in expression profile of indoleamine-2, 3-dioxygenase 1, Th1-Th2 cytokines and interferon-stimulated genes on neutrophils and peripheral blood mononuclear cells of crossbred cows. J Reprod Immunol 142:103188. doi: 10.1016/j.jri.2020.103188

Monn, R. E., R. K. Poole, J. C. Mackey, K. J. Mayberry, H. B. Dudley, M. Alley, and D. H. Poole. 2019. A two-injection prostaglandin F(2alpha) presynchronization treatment decreases pregnancy rates of cycling replacement beef heifers. Transl Anim Sci 3(1):456-463. doi: 10.1093/tas/txy136

Moore, S. G., A. O'Gorman, L. Brennan, T. Fair, and S. T. Butler. 2017. Follicular fluid and serum metabolites in Holstein cows are predictive of genetic merit for fertility. Reprod Fertil Dev 29(4):658-669. doi: 10.1071/RD15182

Moorey, S. E., and F. H. Biase. 2020. Beef heifer fertility: importance of management practices and technological advancements. J Anim Sci Biotechnol 11:97. doi: 10.1186/s40104-020-00503-9

Mora-Palazuelos, C., M. Bermudez, M. Aguilar-Medina, R. Ramos-Payan, A. Ayala-Ham, and J. G. Romero-Quintana. 2022. Cytokine-polymorphisms associated with Preeclampsia: A review. Medicine (Baltimore) 101(39):e30870. doi: 10.1097/MD.0000000000030870

NAHMS. 2014. Trends in Dairy Cattle Health and Management Practices in the United States, 1991-2014, USDA APHIS.

NAHMS. 2020. Beef 2017, USDA APHIS.

Nelson, L. A., G. D. Beavers, and T. S. Stewart. 1982. Beef X beef and dairy X beef females mated to Angus and Charolais sires II. Calf growth, weaning rate and cow productivity. J Anim Sci 54(6):1150-1159. doi: 10.2527/jas1982.5461150x

Orsi, N. M., N. Gopichandran, H. J. Leese, H. M. Picton, and S. E. Harris. 2005. Fluctuations in bovine ovarian follicular fluid composition throughout the oestrous cycle. Reproduction 129(2):219-228. doi: 10.1530/rep.1.00460

Ortega-Camarillo, C., A. Gonzalez-Gonzalez, M. Vergara-Onofre, E. Gonzalez-Padilla, A. Avalos-Rodriguez, M. E. Gutierrez-Rodriguez, L. Arriaga-Pizano, M. Cruz, L. A. Baiza-Gutman, and M. Diaz-Flores. 2009. Changes in the glucose-6-phosphate dehydrogenase activity in granulosa cells during follicular atresia in ewes. Reproduction 137(6):979-986. doi: 10.1530/REP-08-0463

Pang, Z., J. Chong, G. Zhou, D. de Lima Morais, L. Chang, M. Barrette, C. Gauthier, P. É. Jacques, S. Li, and J. Xia. 2021. MetaboAnalyst 5.0: narrowing the gap between raw spectra and functional insights. Nucleic Acids Res 41(W1):W388-W396. doi: 10.1093/nar/gkab382.

Pereira, J. M. V., D. Bruno, M. I. Marcondes, and F. C. Ferreira. 2022. Use of Beef Semen on Dairy Farms: A Cross-Sectional Study on Attitudes of Farmer Toward Breeding Strategies. Front Anim. Sci 2doi: 10.3389/fanim.2021.785253

Phillips, K. M., C. C. Read, L. A. Kriese-Anderson, S. P. Rodning, T. D. Brandebourg, F. H. Biase, M. L. Marks, J. B. Elmore, M. K. Stanford, and P. W. Dyce. 2018. Plasma metabolomic profiles differ at the time of artificial insemination based on pregnancy outcome, in Bos taurus beef heifers. Sci Rep 8(1):13196. doi: 10.1038/s41598-018-31605-0

Piersanti, R. L., J. E. P. Santos, I. M. Sheldon, and J. J. Bromfield. 2019. Lipopolysaccharide and tumor necrosis factor-alpha alter gene expression of oocytes and cumulus cells during bovine in vitro maturation. Mol Reprod Dev 86(12):1909-1920. doi: 10.1002/mrd.23288

Pollard, C. L., Z. Gibb, A. Swegen, and C. G. Grupen. 2022. NAD(+), Sirtuins and PARPs: enhancing oocyte developmental competence. J Reprod Dev 68(6):345-354. doi: 10.1262/jrd.2022-052

Powers, R. W., L. Chen, P. T. Russell, and W. J. Larsen. 1995. Gonadotropin-stimulated regulation of blood-follicle barrier is mediated by nitric oxide. Am J Physiol 269(2 Pt 1):E290-E298. doi: 10.1152/ajpendo.1995.269.2.E290

Raghupathy, R. 2013. Cytokines as key players in the pathophysiology of preeclampsia. Medical Principles And Practice: International Journal of the Kuwait University, Health Science Centre 22:8-19. doi: 10.1159/000354200

Raghupathy, R., E. Al Mutawa, M. Makhseed, F. Azizieh, and J. Szekeres-Bartho. 2005. Modulation of cytokine production by dydrogesterone in lymphocytes from women with recurrent miscarriage. BJOG 112(8):1096-1101. doi: 10.1111/j.1471-0528.2005.00633.x

Revelli, A., L. Delle Piane, S. Casano, E. Molinari, M. Massobrio, and P. Rinaudo. 2009. Follicular fluid content and oocyte quality: from single biochemical markers to metabolomics. Reprod Biol Endocrinol 7:40. doi: 10.1186/1477-7827-7-40

Reverter, A., B. C. Hine, L. Porto-Neto, Y. Li, C. J. Duff, S. Dominik, and A. B. Ingham. 2021. ImmuneDEX: a strategy for the genetic improvement of immune competence in Australian Angus cattle. J Anim Sci 99(3)doi: 10.1093/jas/skaa384

Rittipornlertrak, A., B. Nambooppha, A. Muenthaisong, N. Apinda, P. Koonyosying, W. Srisawat, P. Chomjit, K. Sangkakam, V. Punyapornwithaya, S. Tiwananthagorn, N. Yokoyama, and N. Sthitmatee. 2022. Immunization of Cattle With Recombinant Structural Ectodomains I and II of Babesia bovis Apical Membrane Antigen 1 [BbAMA-1(I/II)] Induces Strong Th1 Immune Response. Front Vet Sci 9:917389. doi: 10.3389/fvets.2022.917389

Roberts, A. J., J. N. Ketchum, and R. N. Funston. 2019. Developmental and reproductive characteristics of beef heifers classified by number of estrous cycles experienced by start of first breeding. Transl Anim Sci 3(1):541-548. doi: 10.1093/tas/txy118

Rosenkrans, K. S., and D. K. Hardin. 2003. Repeatability and accuracy of reproductive tract scoring to determine pubertal status in beef heifers. Theriogenology 59:1087-1092.

Sabry, R., M. Nguyen, S. Younes, and L. A. Favetta. 2022. BPA and its analogs increase oxidative stress levels in in vitro cultured granulosa cells by altering anti-oxidant enzymes expression. Mol Cell Endocrinol 545:111574. doi: 10.1016/j.mce.2022.111574

Sanchez-Guijo, A., C. Blaschka, M. F. Hartmann, C. Wrenzycki, and S. A. Wudy. 2016. Profiling of bile acids in bovine follicular fluid by fused-core-LC-MS/MS. J Steroid Biochem Mol Biol 162:117-125. doi: 10.1016/j.jsbmb.2016.02.020

Schjenken, J. E., D. J. Sharkey, E. S. Green, H. Y. Chan, R. A. Matias, L. M. Moldenhauer, and S. A. Robertson. 2021. Sperm modulate uterine immune parameters relevant to embryo

implantation and reproductive success in mice. Commun Biol 4(1):572. doi: 10.1038/s42003-021-02038-9
Schubach, K., R. Cooke, A. Brandoa, T. Schumaher, K. Pohler, D. Bohnert, and R. Marques. 2019. Impacts of postweaning growth rate of replacement beef heifers on their reproductive development and productivity as primiparous cows1. Journal of Animal Science 97(10):4171-4181. doi: 10.1093/jas/skz262
Segers, J. R., R. L. Stewart, Jr., C. A. Lents, T. D. Pringle, M. A. Froetschel, B. K. Lowe, R. O. McKeith, and A. M. Stelzleni. 2011. Effect of long-term corn by-product feeding on beef quality, strip loin fatty acid profiles, and shelf life. J Anim Sci 89(11):3792-3802. doi: 10.2527/jas.2011-4154
Senger, P. L. 2012. Pathways to Pregnancy and Parturition. Third Edition ed. Current Conceptions, Inc., Oregon.
Shi, L., L. Liu, X. Lv, Z. Ma, C. Li, Y. Li, F. Zhao, D. Sun, and B. Han. 2020. Identification of genetic effects and potential causal polymorphisms of CPM gene impacting milk fatty acid traits in Chinese Holstein. Anim Genet 51(4):491-501. doi: 10.1111/age.12936
Shibata, K., C. Nakata, and T. Fukuwatari. 2014. Moderate food restriction suppresses the conversion of L-tryptophan to nicotinamide in weaning rats. Biosci Biotechnol Biochem 78(3):478-481. doi: 10.1080/09168451.2014.890025
Shirasuna, K., H. Matsumoto, E. Kobayashi, A. Nitta, S. Haneda, M. Matsui, C. Kawashima, K. Kida, T. Shimizu, and A. Miyamoto. 2012. Upregulation of interferon-stimulated genes and interleukin-10 in peripheral blood immune cells during early pregnancy in dairy cows. J Reprod Dev 58(1):84-90. doi: 10.1262/jrd.11-094k
Silva, E., A. F. Greene, K. Strauss, J. R. Herrick, W. B. Schoolcraft, and R. L. Krisher. 2015. Antioxidant supplementation during in vitro culture improves mitochondrial function and development of embryos from aged female mice. Reprod Fertil Dev 27(6):975-983. doi: 10.1071/RD14474
Silvestris, E., G. de Pergola, R. Rosania, and G. Loverro. 2018. Obesity as disruptor of the female fertility. Reproductive biology and endocrinology: RB&E 16(1):22. doi: 1477-7827
Singh, B. N., J. J. Lucas, G. R. Hayes, I. Kumar, D. H. Beach, M. Frajblat, R. O. Gilbert, U. Sommer, and C. E. Costello. 2004. Tritrichomonas foetus induces apoptotic cell death in bovine vaginal epithelial cells. Infect Immun 72(7):4151-4158. doi: 10.1128/IAI.72.7.4151-4158.2004
Siu, M. K., and C. Y. Cheng. 2012. The blood-follicle barrier (BFB) in disease and in ovarian function. Adv Exp Med Biol 763:186-192. doi: 10.1007/978-1-4614-4711-5_9
Stegemiller, M. R., G. K. Murdoch, T. N. Rowan, K. M. Davenport, G. M. Becker, J. B. Hall, and B. M. Murdoch. 2021. Genome-Wide Association Analyses of Fertility Traits in Beef Heifers. Genes (Basel) 12(2)doi: 10.3390/genes12020217
Stone, T. W., and R. O. Williams. 2023. Modulation of T cells by tryptophan metabolites in the kynurenine pathway. Trends Pharmacol Sci doi: 10.1016/j.tips.2023.04.006
Stuedemann, J. A., and C. S. Hoveland. 1988. Fescue Endophyte: History and Impact on Animal Agriculture. Journal of Production Agriculture 1(1):39-44. doi: 10.2134/jpa1988.0039
Summers, A. F., W. E. Pohlmeier, K. M. Sargent, B. D. Cole, R. J. Vinton, S. G. Kurz, R. M. McFee, R. A. Cushman, A. S. Cupp, and J. R. Wood. 2014. Altered theca and cumulus oocyte complex gene expression, follicular arrest and reduced fertility in cows with

dominant follicle follicular fluid androgen excess. PLoS One 9(10):e110683. doi: 10.1371/journal.pone.0110683
Sun, L., L. Chen, Y. Jiang, Y. Zhao, F. Wang, X. Zheng, C. Li, and X. Zhou. 2018. Metabolomic profiling of ovary in mice treated with FSH using ultra performance liquid chromatography/mass spectrometry. Biosci Rep 38(6)doi: 10.1042/BSR20180965
Sun, L. W., H. Y. Zhang, L. Wu, S. Shu, C. Xia, C. Xu, and J. S. Zheng. 2014. (1)H-Nuclear magnetic resonance-based plasma metabolic profiling of dairy cows with clinical and subclinical ketosis. J Dairy Sci 97(3):1552-1562. doi: 10.3168/jds.2013-6757
Sunkara, P. S., C. C. Chang, and P. J. Lachman. 1985. Cell proliferation and cell cycle dependent changes in the methylthioadenosine phosphorylase activity in mammalian cells. Biochem Biophys Res Commun 127(2):546-551. doi: 10.1016/s0006-291x(85)80194-7
Tian-Min, Y., L. Suxia, D. Shufang, C. Dandan, L. Long-Dan, and Y. W. Shu Biu. 2022. Combined Transcriptomic and Metabolomic Analysis of Women with Polycystic Ovary Syndrome. Dis Markers 2022:4000424. doi: 10.1155/2022/4000424
Treleaven, T., M. L. M. Hardy, M. Guttman-Jones, M. B. Morris, and M. L. Day. 2021. In Vitro Fertilisation of Mouse Oocytes in L-Proline and L-Pipecolic Acid Improves Subsequent Development. Cells 10(6)doi: 10.3390/cells10061352
Tsai, C., R. Hassan, H. Hung, T. Weber, W. Price, P. Rezamand, and Q. Huo. 2021. A rapid blood test to monitor the immune status change of dairy cows and to evaluate their disease risk during the periparturient period. Sensors International 2:100078. doi: 10.1016/j.sintl.2020.100078
Tsuda, S., A. Nakashima, K. Morita, T. Shima, S. Yoneda, H. Kishi, and S. Saito. 2021. The role of decidual regulatory T cells in the induction and maintenance of fetal antigen-specific tolerance: Imbalance between regulatory and cytotoxic T cells in pregnancy complications. Hum Immunol 82(5):346-352. doi: 10.1016/j.humimm.2021.01.019
USDA. 2021. USDA Agricultural Resource Management Survey.
USDA. 2022a. Cattle Report 07/2022, Department of Agriculture National Agricultural Statistics Service.
USDA. 2022b. Meat Animals Production, Disposition, and Income 2021 Summary, Department of Agriculture National Agricultural Statistics Service.
Vailati-Riboni, M., M. Kanwal, O. Bulgari, S. Meier, N. V. Priest, C. R. Burke, J. K. Kay, S. McDougall, M. D. Mitchell, C. G. Walker, M. Crookenden, A. Heiser, J. R. Roche, and J. J. Loor. 2016. Body condition score and plane of nutrition prepartum affect adipose tissue transcriptome regulators of metabolism and inflammation in grazing dairy cows during the transition period. J Dairy Sci 99(1):758-770. doi: 10.3168/jds.2015-10046
Vidarsson, G., G. Dekkers, and T. Rispens. 2014. IgG subclasses and allotypes: from structure to effector functions. Front Immunol 5:520. doi: 10.3389/fimmu.2014.00520
Wang, Z., Q. Wang, C. Tang, J. Yuan, C. Luo, D. Li, T. Xie, X. Sun, Y. Zhang, Z. Yang, C. Guo, Z. Cao, S. Li, and W. Wang. 2023. Medium chain fatty acid supplementation improves animal metabolic and immune status during the transition period: A study on dairy cattle. Front Immunol 14:1018867. doi: 10.3389/fimmu.2023.1018867
Wei, Y., J. Cheng, M. Luo, S. Yang, Q. Xing, J. Cheng, J. Lv, C. Yu, L. Sun, D. Shi, and Y. Deng. 2022. Targeted metabolomics analysis of bile acids and cell biology studies reveal the critical role of glycodeoxycholic acid in buffalo follicular atresia. J Steroid Biochem Mol Biol 221:106115. doi: 10.1016/j.jsbmb.2022.106115

Wilde, D. 2006. Influence of macro and micro minerals in the peri-parturient period on fertility in dairy cattle. Anim Reprod Sci 96(3-4):240-249. doi: 10.1016/j.anireprosci.2006.08.004
Wilke, K., T. Mulliniks, and K. McCarthy. 2023. Body Condition Scoring Your Beef Cow Herd. https://beef.unl.edu/learning/condition1a.shtml (Accessed 2/12/2023 2023).
Wishart, D. S. 2005. Metabolomics: the principles and potential applications to transplantation. Am J Transplant 5(12):2814-2820. doi: 10.1111/j.1600-6143.2005.01119.x
Wolfe, M. W., T. T. Stumpf, P. L. Wolf, M. L. Day, R. M. Koch, and J. E. Kinder. 1990. Effect of selection for growth traits on age and weight at puberty in bovine females2. Journal of Animal Science 68(6):1595-1602. doi: 10.2527/1990.6861595x
Wood-Follis, S. L., F. N. Kojima, M. C. Lucy, M. F. Smith, and D. J. Patterson. 2004. Estrus synchronization in beef heifers with progestin-based protocols. I. Differences in response based on pubertal status at the initiation of treatment. Theriogenology 62(8):1518-1528. doi: 10.1016/j.theriogenology.2004.02.017
Wu, G., F. W. Bazer, R. C. Burghardt, G. A. Johnson, S. W. Kim, D. A. Knabe, P. Li, X. Li, J. R. McKnight, M. C. Satterfield, and T. E. Spencer. 2011. Proline and hydroxyproline metabolism: implications for animal and human nutrition. Amino Acids 40(4):1053-1063. doi: 10.1007/s00726-010-0715-z
Xia, L., X. Zhao, Y. Sun, Y. Hong, Y. Gao, and S. Hu. 2014. Metabolomic profiling of human follicular fluid from patients with repeated failure of in vitro fertilization using gas chromatography/mass spectrometry. Int J Clin Exp Pathol 7(10):7220-7229.
Xie, H. L., S. Zhu, J. Zhang, J. Wen, H. J. Yuan, L. Z. Pan, M. J. Luo, and J. H. Tan. 2018. Glucose metabolism during in vitro maturation of mouse oocytes: An study using RNA interference. J Cell Physiol 233(9):6952-6964. doi: 10.1002/jcp.26484
Xu, Z. Z., D. J. McKnight, R. Vishwanath, C. J. Pitt, and L. J. Burton. 1998. Estrus detection using radiotelemetry or visual observation and tail painting for dairy cows on pasture. J Dairy Sci 81(11):2890-2896. doi: 10.3168/jds.S0022-0302(98)75849-7
Yu, J., C. Zheng, J. Zheng, G. Duan, Q. Guo, P. Zhang, M. Wan, and Y. Duan. 2022. Development of Intestinal Injury and Restoration of Weaned Piglets under Chronic Immune Stress. Antioxidants (Basel) 11(11)doi: 10.3390/antiox11112215
Zandkarimi, F., J. Vanegas, X. Fern, C. S. Maier, and G. Bobe. 2018. Metabotypes with elevated protein and lipid catabolism and inflammation precede clinical mastitis in prepartal transition dairy cows. J Dairy Sci 101(6):5531-5548. doi: 10.3168/jds.2017-13977
Zhang, C. M., Y. Zhao, R. Li, Y. Yu, L. Y. Yan, L. Li, N. N. Liu, P. Liu, and J. Qiao. 2014. Metabolic heterogeneity of follicular amino acids in polycystic ovary syndrome is affected by obesity and related to pregnancy outcome. BMC Pregnancy Childbirth 14:11. doi: 10.1186/1471-2393-14-11
Zhang, G., A. Wang, L. Zhuang, X. Wang, Z. Song, R. Liang, M. Ren, M. Long, X. Jia, Z. Li, S. Su, J. Wang, N. Zhang, G. Shen, and B. Wang. 2022a. Enrichment of boron element in follicular fluid and its potential effect on the immune function. Environ Pollut 304:119147. doi: 10.1016/j.envpol.2022.119147
Zhang, J., Q. Li, K. M. C. Nogoy, J. Sun, B. Sun, Y. Wang, L. Tang, J. Yu, X. Jin, X. Li, and S. H. Choi. 2021. Effect of palmitoleic acid on the differentiation of bovine skeletal muscle satellite cells. J Anim Sci Technol 63(4):919-933. doi: 10.5187/jast.2021.e78
Zhang, X., T. Liu, X. Hou, C. Hu, L. Zhang, S. Wang, Q. Zhang, and K. Shi. 2022b. Multi-Channel Metabolomics Analysis Identifies Novel Metabolite Biomarkers for the Early Detection of Fatty Liver Disease in Dairy Cows. Cells 11(18)doi: 10.3390/cells11182883

Zhao, H., L. Raines, and S. C. Huang. 2020. Carbohydrate and Amino Acid Metabolism as Hallmarks for Innate Immune Cell Activation and Function. Cells 9(3):562. doi: 10.3390/cells9030562

Zhao, Y., L. Fu, R. Li, L. N. Wang, Y. Yang, N. N. Liu, C. M. Zhang, Y. Wang, P. Liu, B. B. Tu, X. Zhang, and J. Qiao. 2012. Metabolic profiles characterizing different phenotypes of polycystic ovary syndrome: plasma metabolomics analysis. BMC Med 10:153. doi: 10.1186/1741-7015-10-153

Zhou, H., N. Ohno, N. Terada, S. Saitoh, I. Naito, and S. Ohno. 2008. Permselectivity of blood follicle barriers in mouse ovaries of the mifepristone-induced polycystic ovary model revealed by in vivo cryotechnique. Reproduction 136(5):599-610. doi: 10.1530/REP-08-0022

Zong, S., C. Li, C. Luo, X. Zhao, C. Liu, K. Wang, W. Jia, M. Bai, M. Yin, S. Bao, J. Guo, J. Kang, T. Duan, and Q. Zhou. 2016. Dysregulated expression of IDO may cause unexplained recurrent spontaneous abortion through suppression of trophoblast cell proliferation and migration. Scientific Reports 6(1):19916. doi: 10.1038/srep19916

Appendix 1: SDS-PAGE procedures

SDS-PAGE sample preparation, gel preparation, and blot transfer

Plasma samples were diluted 10x in 1x PBS and combined with a 4x Laemli loading buffer (42% glycerol; 16.8% 1.5 M Tris-HCl, pH 6.8; 41.1% ddH_20; 8.42% SDS (w/v); bromophenol blue) and incubated at 95° C for seven minutes and then vortexed briefly. Samples were prepared to load 15 µg of total protein. An 8% SDS-PAGE was prepared within a Hoefer sandwich set up (4 gels) utilizing a 1 mm spacer between plates and a comb with 20 wells. The gels were prepared following the protocol given in **Table A.1.1** for resolving and stacking gel, respectively.

Table A.1.1: 8% SDS-PAGE resolving and stacking gel.

Reagent	Resolving gel	Stacking gel
Acrylamide/bis, 40% (29:1)	20%	----------
Acrylamide/bis, 40% (37.5:1)	----------	10%
4x resolving buffer (1.5 M Tris-HCl, pH 8.8)	25%	----------
4x stacking buffer (0.5 M Tris -HCl, pH 6.8)	----------	25%
20% SDS	0.5%	0.5%
2,2,2-trichloroethanol	0.5%	----------
ddH20	53%	64.5%
10% ammonium persulfate	1%	1%
TEMED	0.1%	0.1%

Once the resolving gel was poured into the cast, water-saturated isobutanol was placed over the resolving gel to prevent dehydration of the upper gel. The resolving gels were matured for 45 minutes at room temperature. The stacking gels were matured at room temperature for 1 hour before the combs were removed and samples were loaded.

Gels were run at 80 mA for 2 hours (20 mA/gel) in 1x running buffer (25 mmol/L Tris, 192 mmol/L glycine, and 0.1% w/v SDS). Upon completion of the run, gels were removed from the sandwich

and placed in ddH_2O. Total protein load was quantified by 2,2,2- trichloroethanol hybridization with tryptophan and images were captured using a ChemiDoc XRS+ imager after five minute ultraviolet exposure. Gels were then equilibrated in 1x Tris-glycine/methanol until loaded into a transfer sandwich.

Gels were transferred using wet transfer techniques onto low-fluorescence PVDF membranes activated in methanol. The transfer took place at room temperature for 16 hours in 1x transfer buffer (25 mmol/L Tris, 192 mmol/L glycine, and 10% v/v methanol). Post-transfer, membranes were moved to ddH_2O and exposed to a ChemiDoc XRS+ imager to determine protein transfer from the gel to the membrane. Upon capturing total protein images, membranes were moved to wash buffer [1x TBS-T (0.1% Tween-20 v/v)] and stored at 4°C until further work.

Appendix 2: Representative blot images

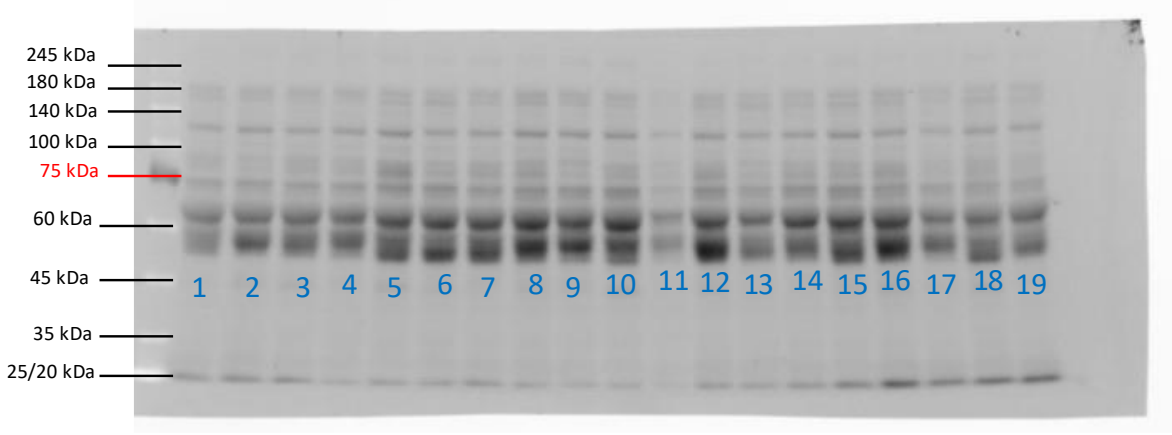

Figure A.2.1: Representative image of total protein staining via TCE inclusion and UV activation. Protein ladder on far left; Lane 1, control sample; Lane 2-10, LBred heifers; Lane 11-19, 1stAI heifers.

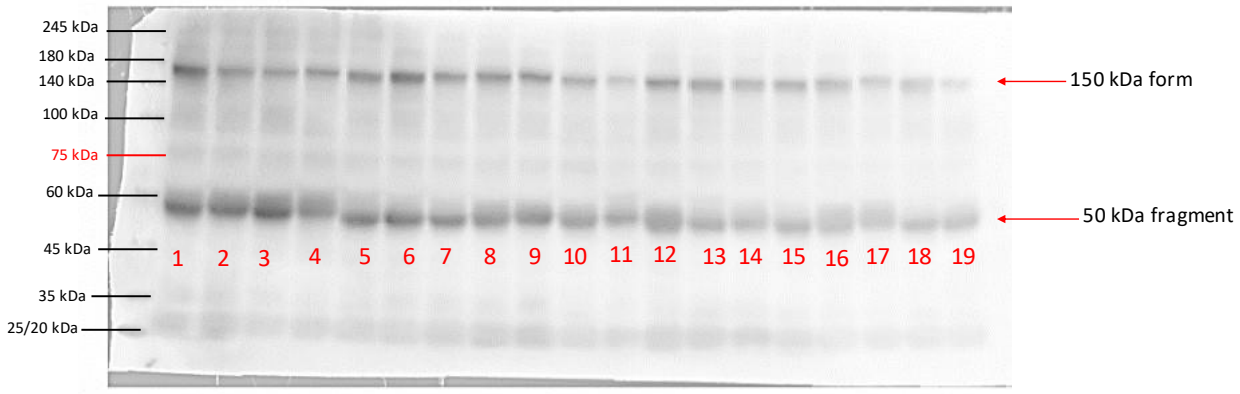

Figure A.2.2: Representative merged image of chemiluminescent IgG1 visualization and colorimetric ladder visualization. 150 kDa form and 50 kDa fragment combined for densitometric analysis. Protein ladder on far left; Lane 1, control sample; Lane 2-10, LBred heifers; Lane 11-19, 1stAI heifers.

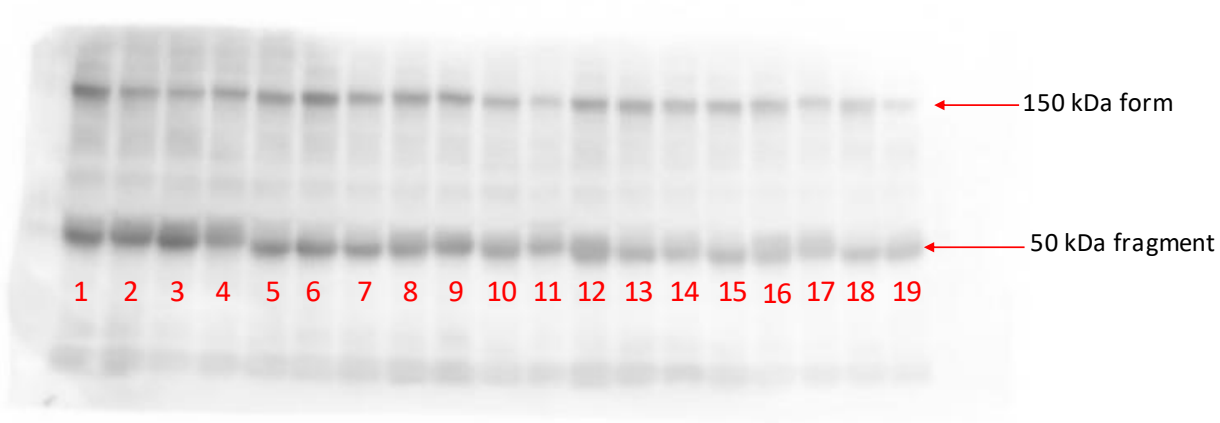

Figure A.2.3: Representative image of chemiluminescent IgG1 visualization. 150 kDa form and 50 kDa fragment combined for densitometric analysis. Lane 1, control sample; Lane 2-10, LBred heifers; Lane 11-19, 1stAI heifers.

Appendix 3: Batch correction images for follicular fluid and plasma

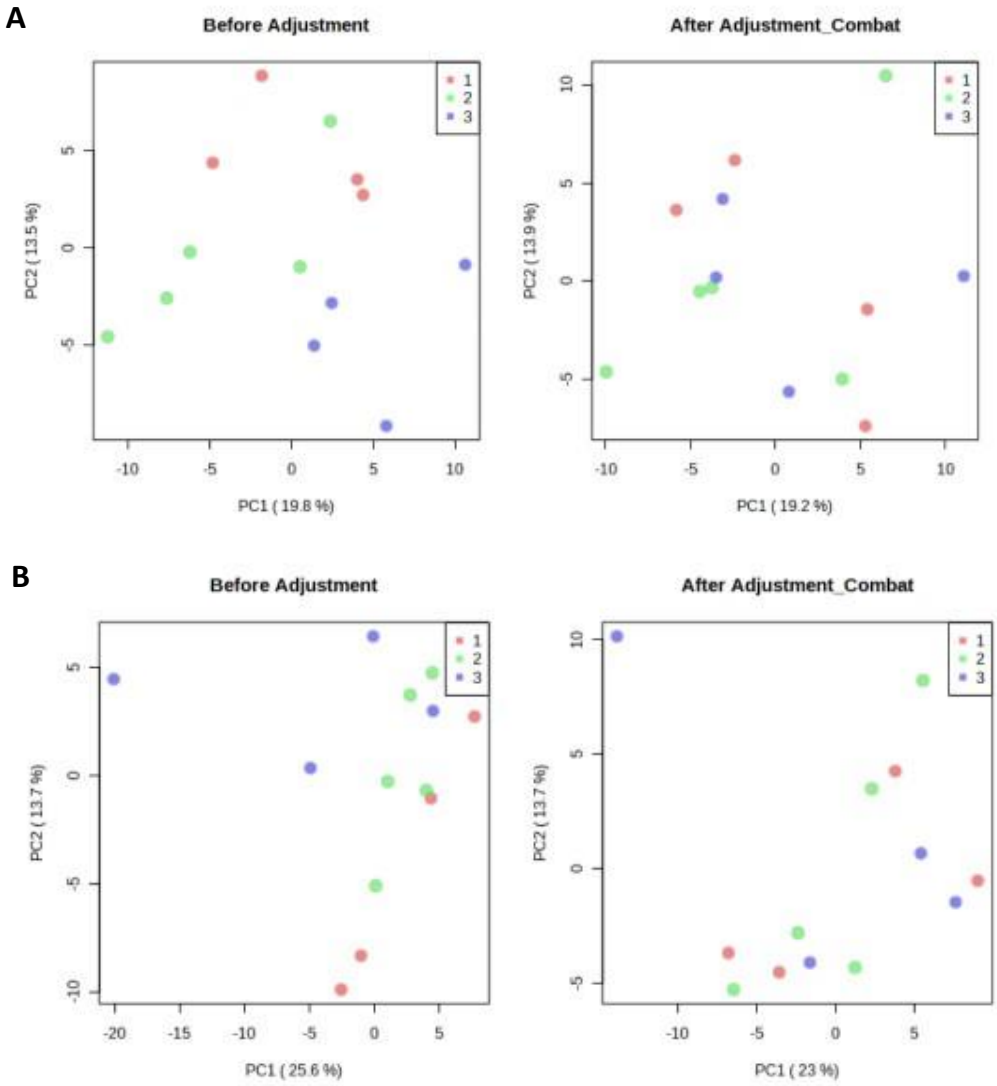

Figure A.3.1: PCA plots of before normalization and after batch correction for follicular fluid (Panel A) and blood plasma (Panel B).