Investigating the ability of traditional and molecular methods to differentiate between reproductive potentials in *Bos taurus* heifers

by

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Abstract

Within the livestock industry, one of the main problems that a producer faces is infertility. Infertility in cattle can lead to an animal being removed from cow-calf operations earlier in their lifetime. Reproductive technologies have helped to improve the efficiency of cattle production, however, limitations still remain. Characteristics such as phenotype and genetic background are what producers normally base their decisions off of when deciding on replacement heifers. Currently, there is a lack of informative biomarkers that would help to identify replacement heifers. The levels of the metabolites present within the blood plasma can be compared between heifers of different phenotypes and could provide a basis to discriminate heifers based on their reproductive potentials.

The objective of this study was to identify differences in metabolomic profiles between heifers at the time of weaning based on their pregnancy outcomes. Angus/Simmental-cross heifers (n=36) housed at the Black Belt Research and Extension Center were assessed at weaning and 10 ml of blood was collected. The blood plasma and white blood cells (WBCs) were isolated. Heifers were then assessed 30 days prior to artificial insemination (AI) for weight, pelvic area (PA), body condition scores (BCS) and reproductive tract scores (RTS). Reproductively mature heifers underwent a fixed-time AI program with estrus detection and were then exposed to a bull two weeks after AI. Pregnancy was determined via rectal palpation and heifers were categorized into three groups: pregnant by AI, pregnant by natural breeding or open after AI and 60 days of bull exposure.

9 heifers pregnant by AI and 11 open were chosen for metabolomic profiling. Of the 20 heifers selected, there was no difference in age at weaning, weaning weight, BCS, RTS or pelvic area ($p > 0.05$). Ten metabolites were found to be significantly up or down regulated in the heifers who remained open after AI and 60 days of bull exposure, when compared to those pregnant by AI. Alanine, cystine, lysine, methionine, tyrosine, tryptophan and valine were down regulated. Glycerol, frustose-6-phosphate and ribulose-5-phosphate were found to be upregulated in open heifers ($p<0.05$).

RNA isolated from white blood cells was used to determine the expression of five inflammatory cytokines in the open and pregnant by AI groups (*TNFα, IL6, CXCL5, POSTN and MCP1*). Inflammatory cytokines were increased in all heifers that remained open after AI and 60 days of bull exposure ($p < 0.05$). Lastly, ELISAs were used to detect proteins for TNF*α* and IL6. The differences between heifers pregnant by AI and open was not significant with $p > 0.05$. In summary, the quantity of specific metabolites present within the blood plasma are different at weaning between heifers with differing reproductive potentials. This could potentially be used to develop an assay to aid in selecting replacement females to incorporate into an existing herd.

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List of Abbreviations

ADG	Average Daily Gain
AI	Artificial Insemination
BB	Black Belt Research and Extension Center
BCS	Body Condition Score
BSE	Breeding Soundness Exam
CIDR	Controlled Internal Drug Release
CL	Corpus Luteum
CXCL-5	C-X-C Motif Chemokine Ligand five
EPD	Expected Progeny Difference
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
IL-6	Interleukin six
LH	Luteinizing Hormone
MCP1	Monocyte Chemoattractant Protein-one
MS	Mass Spectrometry
NMR	Nuclear Magnetic Resonance
PA	Pelvic Area
PGF2α	Prostaglanddin-F2α
POSTN	Periostin
RBC	Red Blood Cell
RTS	Reproductive Tract Score

TNFα	Tumor Necrosis Factor alpha
WBC	White Blood Cell
WT	Weight

CHAPTER I.

REVIEW OF LITERATURE

I. Cattle Production

I.I. The Cattle Industry

One of the most commercially successful aspects of the agricultural industry is livestock production. Cattle remain one of the top agricultural commodities in the United States. As of July 1, 2020, there were a total of 103 million head of cattle and calves in the United States, increasing slightly from 2019 (USDA-NASS, 2020). Forty-one million head consisted of all cows and heifers that had calved, with 32.1 million of those being beef cows (USDA-NASS, 2020). Heifers five hundred pounds and over totaled 16.5 million head, while beef replacement heifers totaled 4.40 million head, unchanged from the previous year (USDA-NASS, 2020). All calves weighing in under five hundred pounds was slightly lower than in 2019, coming in at 28 million head (USDA-NASS, 2020). As far as the 2020 calf crop, there was a slight decrease from the year before in 2019. Nationally, the state of Alabama ranks 17th in the production of all livestock. Within the U.S., Alabama ranks 29th in cattle and calf production. In 2019, cattle and calves were the state's second most produced commodity (USDA-NASS, 2020).

There is slightly over 2 million farms in the United States, which is decreasing as larger commercial farms take the place of small farms. Farms are operating on fewer acres and production still continues to rise. Out of 882,692 cattle farms in the United States, 729,046 farms focus on the production of beef cows (OCEX, NCBA, 2020). Eight hundred

ninety-seven million acres make up the farms, which is decreasing from year to year. On average, a farm consists of 444 acres, which has increased from 2018 (USDA-NASS, 2019). There is 22 million acres of land within the state of Alabama. In 2019, 8.3 million acres in Alabama were used for farmland, consisting of over 38,000 farming operations, which is down from 2018 (USDA-NASS, 2019).

In the year of 2020, the beef cow inventory decreased slightly due to the nation being challenged by a worldwide pandemic. The effects of the beef industry being challenged by the COVID-19 pandemic are still occurring and will continue into the coming months. The local cattle producer has felt the direct effects of the pandemic and the U.S. beef supply is threatened. In total, the beef industry has taken on a loss of over 13 million dollars. The economic loss per head is between $146 and $216 (OCEX, NCBA, 2020). In relation to beef cow and heifer numbers, this would result in an $8 million loss with cow-calf operations representing almost 60% of the total impact. As of April of 2020, it is expected for there to be over 32 million beef cows and heifers on cow-calf operations with an average loss of $247.15 per head of each reproductively mature animal to the producer (OCEX, NCBA, 2020). The prices of cattle have dropped tremendously, encouraging producers to hold onto their cattle instead of selling to make a profit. If producers are having to retain the majority of their calf crop due to price decline, this could affect the future calf crop, depending on farm size and pasture capacity. Most cow-calf operations are small-scale and any economic impacts could have a great effect.

I.II. Production Systems

In the United States, there are three major cattle production systems: cow-calf, stocker (or backgrounding) and the feedlot (Gadberry et al., 2016). Cow-calf operations

are one of the primary methods for the production of beef cattle in the United States. Cows and heifers are expected to be productive over the course of multiple years throughout their lifetime. A producer's goal in a cow-calf operation is to have one calf on the ground, per cow, every 365 days (Stewart et al., 2017). This type of operation would be considered a long-term investment for the producer. The stocker phase consists of raising cattle to a certain goal weight before being sold to a feedlot for finishing. Cattle included in the stocker phase weigh between 450 and 850 pounds and includes the time from weaning until entering the feedlot (Gadberry et al., 2016). Cattle will have a high average daily gain (ADG) ratio, will be fed a high quality forage and would ideally be placed into uniform groups with other cattle of the same size and structure. The feedlot phase consists of cattle weighing between 700 lbs. and 1400 lbs. They will be fed a high quality ration including roughage and grain concentrate and will gain around 3 pounds or more per day (Gadberry et al., 2016). Cattle are normally retained in this phase for around 180 days and will then be sent for slaughter.

Since the main focus of a cow-calf operation relies heavily on reproduction, the focus on raising replacement heifers has to be well managed in order for a heifer to be an ideal candidate and be successfully incorporated into the breeding herd. The two main areas of concentration in a cow calf system are calf crop percentage and calf weaning weight, which accurately determines the reproductive efficiency of the operation as a whole. During the cow-calf production phase, heifers need to be developed to reach puberty at an early age in order to be able to conceive earlier within their first breeding season and increase stayability within the herd. A tightly managed calving season is one of the keys to producing a uniform calf crop. Cows and heifers must be rebred within 80 days after

calving and have a 60 to 90 day calving season in order to be profitable to the producer (Gadberry et al., 2016 and Stewart et al., 2017). One of the overall goals of a cow-calf operation is to raise replacement heifers with decreased development costs without compromising the overall performance of the heifer (Funston et al., 2004).

II. Heifer Development and Selection

When it comes to heifer development, one of the most important aspects is the close management of replacement heifers. Three characteristics of a good management program with high reproductive success are a high percentage of heifers becoming pregnant early in the breeding season, delivering a live calf and those same heifers conceiving early in the season of their second pregnancy (Larson et al., 2016). Along with successful heifer development, an operation must appropriately manage other aspects of a successful breeding season such as adequate nutrition, bull fertility and a successful AI program if it applies.

II.I. Age

One of the most important time points in a heifer's growth and development stage is the age in which she will reach puberty. Puberty in beef heifers is defined as the time point where she is able to ovulate a fertile oocyte and display estrus behavior (Larson et al., 2016). Heifers should reach this point at least 21 days before a scheduled breeding, whether by AI or bull exposure. Close management of heifers and evaluation of yearling heifers before breeding can be indicative of pubertal status and predictive of the future breeding season (Larson et al., 2016). If the heifer reaches puberty earlier in her lifetime, the more likely she is to remain in the herd and will be more productive over the course of her lifetime (Patterson et al., 2002).

The onset of puberty in beef heifers is highly dependent on the age and weight of the animal. A study done by Gunn et al. (2015) indicated that the mean age for reaching puberty in Angus-Simmental heifers was 303 days, plus or minus ten days, which is around 10 months. Studies have shown that crossbred heifers will reach puberty earlier in their lifetime compared to purebred heifers that may lack heterosis (Martin et al., 1992). Bos taurus breeds as a whole tend to reach puberty between ten and fourteen months of age (Larson et al., 2016). Close management and knowing the heifer's age up until and after they reach puberty can help a producer more adequately determine the candidates which should be more closely evaluated to be retained in the herd as replacement heifers.

II.II. Weight

The weight of heifers is directly related to the age at which they reach the onset of puberty. Studies have shown that heifers fed to reach 55% to 65% of their body weight will perform better reproductively compared to lower weight heifers (Funston et al., 2012). This being said, producers should aim to have a higher ADG from weaning to breeding in order to reach a goal weight and have a larger number of heifers to select herd replacements from. A study by Dickinson et al (2019) demonstrated that while weaning weight is likely not associated with pregnancy outcome, the age at which heifers reach at least 53% of their mature body weight is influential on reproductive potential. Depending on the uniformity of the group of heifers, frame size can impact the weight that a heifer will experience her first estrus and what her mature weight will be. A study done by Clanton et al (1983) showed that weight gain does not have to be consistent in order to reach puberty and have successful reproductive performance.

II.III. Body Condition Score

In order to improve reproductive performance, all cows and heifers need to maintain a higher level of body condition at the time of breeding and calving. The percentage of fat deposition in beef cows has been shown to be directly related to reproductive performance and onset of puberty (Rossi et al., 2014). Being able to body condition heifers has served as an economical way for producers to determine overall body fat percentage with the possibility to help estimate reproductive success. The order for distribution of nutrient partitioning to different systems is body maintenance, fetal development, lactation, growth and breeding (Rossi et al., 2014). Body fat is seen as the storage of excess nutrients in the body, where a certain amount of body fat is required for the reproductive system to function correctly. Body scores range from a value of 1 to 9, 1 being very thin to 9 having excessive fat cover. These ranges have been categorized into four groups as follows: thin (BCS 1-3), borderline (BCS 4), optimum (BCS 5-7) and fat (BCS 8-9) (Herd and Sprott, 1986).

Heifers with a body condition score of 1 to 3 are considered very thin with a clearly defined bone structure. Thin heifers will vary between 3.77% to 11.3% of total body fat (Herd and Sprott, 1986). A BCS of 1 is where bones such as the shoulder, ribs, back, hooks and pins will be easily identifiable to the eye and will be covered with very little, if any, muscle or fat. A BCS of 2 consists of there being a small amount of muscling in the hindquarters with very little fat. Space between the spinous process can be seen and could feel sharp to the touch. A BCS of 3 can be classified as fat beginning to cover the loin, back and foreribs. The average pregnancy rate of a heifer with a BCS of 3 is 43% (Kunkle

et al., 1998). The upper skeletal structures and spinous process can still be easily identified at this stage.

A BCS of 4 is considered a borderline condition with around 15% total body fat (Herd and Sprott, 1986). At this stage, the fore ribs are no longer visible and only the 12th and 13th rib can been easily seen. The transverse spinous process can be felt, but no longer seen. The area around the head of the tail can also serve as a good indicator of fat deposition. At this stage, the tail head can be seen filling out, but not yet completely filled. The average pregnancy rate of a heifer with a BCS of 4 is 61% (Kunkle et al., 1998).

A BCS of 5, 6 and 7 are considered optimum composition for beef cows and heifers and vary between 18.19% to 26.38% total body fat (Herd and Sprott, 1986). At a BCS of 5, the 12th and 13th rib are no longer visible, unless the animal has been shrunk off feed, and each side of the tail head will continue to fill. The spinous process and space in between can only be identified by palpation. With a BCS of 6, all of the ribs are no longer visible to the eye, despite being shrunk or not. The hindquarter of the animal will appear more muscular and fat deposition will be noticeable on the fore ribs and around the tail head. Heifers with a BCS of 5 or 6 will have a pregnancy rate between 86% and 93%. At a BCS of 7, there is an abundant amount of fat surrounding the tail head. At this stage, the animal will appear more full, muscled and healthy.

BCS 8 and 9 is where an animal would be considered as fat or obese with 30% or more total body fat (Herd and Sprott, 1986). At a BCS of 8, the animal will have a blocky appearance with bones not visible to the eye. Fat can be seen covering the majority of the animal. A BCS of 9, being overly fat and obese, is where fat is covering most of the surface

area on the body. The tail head will no longer be visible and the mobility of the animal may be impaired by the excessive amount of fat deposition.

In relation to reproductive performance, a low body condition score can affect the calf just as much as the heifer. If a heifer has a low body condition score, it can contribute to a longer post-partum interval, low quality and quantity of colostrum and in turn weaker calves (Rossi et al., 2014). Cows with a moderate body composition of 5 to 6 tend to have a shorter calving interval and will be more reproductively successful in terms of calving ease and pounds of calves weaned. If cows and heifers are nutrient deficient and their BCS drops from 5 to 4, pregnancy rates can drop as much as 30%. Likewise, if cattle drop from a BCS 4 to a 3, pregnancy rates can drop by an additional 30% (Rae et al, 1993). Any cows or heifers above or below optimal BCS can expect to have reduced pregnancy and conception rates. It is profitable to the producer to maintain heifer BCS between 5 to 7, or at least 14% body fat, throughout pregnancy (Rossi et al., 2014). A heifer's BCS will drop when nursing a calf. The producers should be feeding heifers to meet their nutritional needs in order to ensure that they remain healthy and can breed back within 80 days of calving. This will benefit the producer by increasing reproductive success and minimizing supplemental feed costs.

II.IV. Reproductive Tract Score

One of the ways that a producer can predict reproductive performance in young heifers is by reproductive tract scoring. Studies have shown that heifers with more mature reproductive tracts tend to have a higher pregnancy rate and calve earlier in the season (Anderson et al., 1991). This can also be a useful method for producers to group heifers more appropriately for breeding purposes. It is a low cost measurement that can be taken

at the same time as yearling weights, BCS, along with any health management protocols at least one month before breeding.

Reproductive tract scores are on a scale of one to five, one being an immature tract to 5 being the most mature. Measurements that are taken into consideration are the length, height and width of the uterine horns as well as structures present on the ovary (Gutierrez et al., 2013). A RTS of 1 represents an immature tract with uterine horns measuring less than 20 mm and no tone. No follicles will be palpable on the ovaries which indicates that a heifer is pre-pubescent and far from cycling (Patterson et al., 2002). A RTS of 2 consists of uterine horns between 20 to 25 millimeters in diameter, but no uterine tone is present. 8 millimeter follicles will also be present on the ovary (Anderson et al., 1991). A RTS of 3 reveals slight tone of the uterine horns that are 25 to 30 millimeters in diameter. 8 to 10 millimeter follicles will be present on the ovary (Anderson et al., 1991). These heifers will be on the verge of cycling. Uterine horns with good tone and at least 30 millimeters in diameter will be a RTS of 4. Follicles over 10 millimeters will be present with a CL possible (Anderson et al., 1991). A RTS of 5 reflects the most mature reproductive tract. The uterine horns will measure over 30 millimeters and have great erect tone. 10 millimeter follicles and bigger will be present with a CL being able to be felt on the ovary (Anderson et al., 1991).

A study done by Rosenkrans and Hardin (2002) evaluated heifer puberty status by randomized rectal palpation, RTS by ultrasonography as well as measuring serum progesterone levels. The diagnosis of pubertal and cycling status showed to be accurate 79% to 82% of the time validating the reproductive tract scoring system as a valid way to evaluate young heifers. As far as studying whether reproductive tract scoring can be

indicative of long term reproductive performance, a study by Holm et al., (2014) evaluated heifers based on their RTS until they weaned their fifth calf. Heifers with a RTS of 1 or 2 remained anestrus for longer and many failed to become pregnant compared with heifers with a RTS of 4 or 5. They concluded that RTS is a useful way for producers to determine long term reproductive success and can be used as a tool for culling heifers that are unable to become pregnant or calve late in their first calving season (Holm et al., 2014). RTS is even more useful when being combined with other measurements in relation to puberty. Heifers with a BCS of 6 and a RTS of 5 are more likely to have a higher pregnancy rate, around 89%, at the end of a particular breeding season (Dickinson et al., 2019). Likewise, heifers with a BCS of 6 and a RTS of at least 4 had the greatest chance of becoming pregnant by AI at first service (Dickinson et al., 2019).

II.V. Pelvic Measurements

Along with BCS and RTS, pelvic measurements can be just as predictive of a tool to determine a heifers' reproductive success and calving difficulty especially when replacing other successful brood cows within the herd. Measurements such as pelvic area, pelvic width and pelvic height can help to identify heifers that could have future calving difficulties due to having a small pelvic canal. Dystocia is the common term used that describes calving difficulty. Dystocia has been shown to extend the postpartum period in cows while also delaying estrus and contributing to low conception rates (Grussing, 2020). A pelvimeter can be used to take these measurements while inserted into the rectum to measure the height and width of the pelvis. For a 600 lb. heifer, the average pelvic area should be around 140 cm^2, big enough to deliver a 67 lb. calf, and should proceed to grow at 0.27 cm^2 per day until the heifer reaches 2 years of age (Grussing, 2020).

A study done by Johnson et al. (1988) evaluated 186 Herford heifers by measuring their pelvic area, body measurements, calving difficulty and live calf weight. They concluded that pelvic area measurements and calf birth weight were the most predictive of calving ease with a heifer's first calving. Knowing that a heifer has a small pelvic area could help a producer with the decision to breed that heifer to an easy calving bull and knowing to watch her a little more careful around the time of calving or if she does not need to be selected as a replacement heifer (Donkersgoed et al., 1990).

II.VI. Temperament

Temperament is not a common characteristic that producers seem to consider when selecting heifers with the highest reproductive potentials. Temperament can be measured by evaluating chute score and exit velocity (Cooke et al., 2012) A study conducted by Kasimanickam et al. (2014) evaluated heifers on their temperament leaving the chute by 0 = a slow, calm, walk exit and 1 = excited, fast exit at a jump, trot or run after AI. They also evaluated the temperament of the heifers depending on the type of working facility that was used. This study concluded that Angus beef heifers that exited the head gate calm after AI and became pregnant had a predictive value of 87%, whereas heifers that exited the head gate excited and failed to become pregnant was 76%. They also found that an alley with small bends and turns, as well as the long and straight alley way, had lower pregnancy rates compared to the semi-circular alley way (Kasimanickam et al., 2014). *Bos taurus* heifers have been shown to have impaired reproductive performance if they have a more aggressive temperament in the chute (Cooke et al., 2012). The same study also showed that working the heifers through the chute more often acclimated them more to being handled

and can result in a calmer temperament and higher reproductive performance, independent of breed type (Cooke et al., 2012).

III. Common Heifer Breeding Management Programs

While a portion of a heifer's reproductive potential is dependent on genetics, a reproductively successful heifer depends mainly on the operation's breeding management program. Calf crop is just as influenced by herd management than it is by animal trait characteristics. There are traditionally two ways of breeding that producers normally use: natural bull service, artificial insemination (AI), or both.

III.I. Natural Bull Service

Natural bull service is one of the most used breeding standards in the livestock industry today. In order for there to be a successful breeding season, bulls used must be critically evaluated for fertility by breeding soundness exams (BSE). These must be conducted at least 30 to 60 days prior to the breeding season (Troxel). Some of the characteristics that are examined when evaluating a bull's fertility are semen quality, sex drive, scrotal circumference and possibly social interactions with other animals in the pasture (Chenoweth et al., 1984). A bull should be carefully evaluated for any structural unsoundness or health concerns. External features such as eyes, feet, external genetalia and scrotal circumference should be examined. An increased scrotal circumference, hence testicular volume, has been shown to be related to a decreased age in which their daughters reach puberty (Martin et al., 1992). Semen assessment is important because sperm need to be seen as at least 30% progressively motile and at least 70% morphologically normal (Parkinson, 2004).

Along with physical characteristics, producers should consider a bull's expected progeny difference (EPDs) before being introduced into a herd. Depending on the producer's breeding goals, two of the more reproductively important EPDs are birthweight and calving ease direct (Larson et al., 2016). Even if a bull passes as BSE, the producer will still need to ensure that the bull is seeking out females in the pasture, mounting correctly and is able to complete the breeding process. One way to do this instead of constantly watching the herd is to make use of mounting detection aids that can help a producer identify the number of matings that are taking place and which heifers could have been bred. Studies show that if at least 80% of the breeding herd is cycling at the start of the breeding season, that at least 4% of the herd should be bred each day (Larson et al., 2016).

III.II. Estrous Synchronization and Artificial Insemination

In order to utilize advanced reproductive technologies, it is beneficial to use estrus synchronization in order to have all females cycling at one time. Cows and heifers typically exhibit signs of estrus every 21 days (Youngs, 2016). Estrus can be synchronized in cows and heifers by the introduction of exogenous hormones that are chemically similar to the ones that the animal would produce herself. Some hormones used include Prostaglandin-F2α (PGF2α), Progesterone, and Gonadotropin Releasing Hormone (GnRH) (Youngs, 2016). PGF2α can be administered to lyse the corpus luteum (CL) present on the ovary and progesterone can be given to promote follicle growth on the ovary which would in turn produce estrogen. GnRH can be administered to force the ovulation of the dominant follicle to release the oocyte that is to hopefully be fertilized through AI (Youngs, 2016). There are a number of estrus synchronization protocols that a producer could choose from for

artificial insemination. The protocol chosen would need to reflect the facilities available, time and labor that an operation is willing to put into a breeding management program. Some farms would rather not work their cattle as many times as certain protocols would require, so there is flexibility in which protocol a producer chooses. Studies show that if heifers that have reached puberty and a synchronization program was implemented correctly, 70% to 90% of heifers should display estrus within the time window predicted (Larson et al., 2016).

Artificial Insemination (AI) has been shown to be one of the most widely utilized reproductive technologies for cattle (Youngs, 2016). AI involves collecting semen from genetically superior bulls and placing it into the reproductive tract of an estrous synchronized heifer or cow. AI provides a producer with an opportunity to introduce superior genetics within a herd without having to deal with keeping a potentially dangerous bull in the pasture. An AI program can also shorten calving season tremendously. AI is normally performed using the AM/PM breeding rule (Trimberger, 1944). This means that any females in heat in the morning will be bred in the afternoon, ideally 12 hours later. Any females in heat in the afternoon will be bred the following morning. Paired with a successful estrus synchronization protocol, 60% to 70% of heifers should become pregnant by AI (Larson et al., 2016). If a bull is introduced to heifers after AI, it is recommended that the producer should wait two weeks before bull exposure to ensure correct fetal aging whether by ultrasound or rectal palpation (Larson et al., 2016). Any discrepancies in meeting a successful reproductive rate could be due to inability to detect estrus, inexperienced AI technician, poor semen quality or poor condition of the heifers (Larson et al., 2016).

IV. Heifer Reproductive Failure

Heifer reproductive failure is one of the most important factors that effects profitability on cow-calf operations. The goal for most operations is to breed heifers by 15 months of age and calve for the first time at 24 months. At this age, heifers will have grown enough to be able to calve without difficulty and receive a good lactation yield after their first parturition (Wathes et al., 2014). While there are numerous studies related to cow fertility, there is considerably less research done investigating heifer fertility. It is hard to find adequate research that lays a foundation for heifer fertility. There is also a lack of a database for heifer breeding data (Kuhn et al., 2006). There are many aspects that go into a successful pregnancy and live birth. Factors such as genetic, environmental and managerial along with their complex interaction make pin pointing the exact reason for reproductive failure difficult (Abraham, 2017). Not only do all of the previously mentioned factors have to work in together in unison, but nutrition, stress and hereditary factors play a secondary role as well. Fertility is a term used to describe an animal that has the desire and ability to mate, can conceive and nourish a growing embryo and to expel a healthy calf with the fetal membranes (Abraham, 2017).

Both dairy and beef industries rely heavily on a successful reproductive performance in order to meet their production goals, whether for milk or beef. Studies have indicated that for beef cattle, fertilization rates for oocytes is 90% and calving rates with single service are between 40% and 55%. This results in either embryonic or fetal mortality between 35% to 50% of the time (Diskin et al., 2006). A study by Berg et al. (2010) showed that 70% to 80% of embryonic loss occurred in the first 3 weeks of pregnancy between

days 7 and 16. Likewise, a study by Diskin and Sreenan (1980) determined that most embryonic mortality in beef heifers after AI occurred between days 8 and 16.

There are many hormones that play a role in reproduction. It is the combination of correct hormone production and metabolism that help to maintain the correct hormone balance during the follicular phase, luteal phase and estrus. Some of the most common causes of heifer infertility are non-detected estrus, anestrus, ovulatory defects, persistent CL, cystic ovaries, luteal deficiency and repeat breeders (Abraham, 2017). Many of these issues are caused by an endocrinological abnormality which can be hard to detect when only a single sample of blood is taken (Abraham, 2017). When stress of the season's changing weather is considered, heifers that are artificially inseminated in the summer are four times less likely to become pregnant compared to heifers bred at other times of the year (Donovan et al., 2003). The same study also showed that breeding by the presence of secondary signs of estrus rather than standing estrus reduced the odds of conception to first service (Donovan et al., 2003).

Overall, a heifer is deemed to be very fertile throughout her lifetime if she conceives earlier in her breeding season and has a live healthy calf at first parturition (Wathes et al., 2007). By utilizing measured fertility traits wisely within an operation, decreased fertility in beef heifers can be stopped or reversed and improve the longevity and stayability of all heifers in the herd (Liu et al., 2008).

V. Metabolomics

V.I. The Metabolome

Over the years, there has been a need for a comprehensive list of biomarkers that could serve as an indicator for possible diseases or the function of bodily systems in all

living species. For years, researchers have used transcriptomics to analyze gene expression and proteomics to study protein translation in an effort to bridge the genotype to phenotype gap (Goodacre et al., 2004). The term "metabolome" was introduced by Oliver et al. (1998) as "the quantitative complement of all of the low molecular weight molecules present in cells in a particular physiological or developmental state". These can also be defined as small molecules such as amino acids, carbohydrates, organic acids, nucleic acids and vitamins that are required for the maintenance, growth and normal function of a cell (Goodacre et al., 2004). Scientific researchers have gained interest in using comprehensive metabolomic profiling to understand complex biological systems (Kell and Oliver, 2016). Most metabolite research was first carried out in yeast and there was found to be 600-700 metabolites in the yeast cell (Goffeau et al., 1996).

Many have associated the genome with the metabolome, but have found that there may not be a direct link between the two as multiple genes could change the synthesis and turnover of a single metabolite (Kell and Oliver, 2016). Researchers knew that metabolites were directly linked to the function of bodily systems. This was able to provide some explanation for why changes in the levels of gene expression had a minimal effect on metabolic fluxes, but have a large influence on metabolite concentrations (Kell and Oliver, 2016). The initial study by Oliver et al. in 1998 determined that in order to maintain these fluxes of the metabolic networks, the cells would be required to change the concentrations of the metabolites.

A challenge faced by clinical researchers was in efforts to accurately quantify metabolites within different systems in the body, whether in tissues or cells, at a given point in time. The metabolites seemed to be made up differently, making them variable in their

extraction method leading to a discrepancy in identification. While there are still a large number of metabolites that are identifiable in cells and tissues, there is still a large number that have yet to be identified due to chemical complexity, heterogeneity and variation of extraction protocols (Goodacre et al., 2004). The classification of metabolites can be identified by several terminologies including: metabolite target analysis, metabolite profiling, metabolomics, metabolic fingerprinting, metabolic profiling and metabonomics (Goodacre et al., 2004). Besides using the already successful transcriptomics and proteomics, Goodacre at al. (2004) gives an example of why metabolomics can be seen as a more universal approach to answering common research questions. If one wanted to measure the amount of a specific metabolite in a sample, such as fructose 1,6-bisphosphatase, they would have to know the DNA and/or protein sequences from that specific species in order to design a complementary oligonucleotide for mRNA to recognize it. The product of fructose 1,6-biophosphatase can be either be fructose 1,6 biphosphate or fructose 6-phosphate. These will both have the same chemical structure despite the species or organism and have to opportunity to break species barriers by becoming a more universal tool used to understand cell function.

There are two main technologies that are commonly used to quantify and identify metabolites in various samples: mass spectrometry (MS) and nuclear magnetic resonance (NMR). Each method of quantification have advantages and disadvantages. MS is commonly used because it can be coupled with liquid chromatography or gas chromatography to measure hundreds of metabolites within a single sample in the femtomolar to attomolar range (Veenstra, 2012). The identification of metabolites only strengthens the identification databases that are currently being formed. As with NMR

spectroscopy, the area of concentration of the metabolite is related to the concentration of a specific nuclei. This method of quantification will utilize both position of the nuclei or pulse-sequences to identify the metabolite in specific samples (Veenstra, 2012). Another huge advantage of NMR spectroscopy is its ability to quantify metabolites in liquids such as blood plasma, serum and urine, tissues as well as *in vivo* samples (Veenstra, 2012). MS has been seen to be more sensitive than NMR when detecting metabolites, but both can be seen making huge strides in the fields of toxicology, drug discovery, biomarker discovery as well as others (Veenstra, 2012).

Overall, metabolites can be quantified in various tissues, biological fluids or even cell culturing media. Metabolite concentration can be easily influenced by outside factors such as environmental and physiological factors, even more than the genome and proteome. The continuing research and identification of metabolites is an important and challenging task in the field of reproductive biology. While the defined definition of metabolites and pathways involved in pregnancy are lacking, they can potentially serve as a reproductive tool to determine fertility potential in a number of species. Future research needs to be conducted to determine if metabolites can serve as biomarkers in different species, breeds and environments. Despite the species, each metabolite research project can provide a foundation for future research related to infertility in the livestock industry.

V.II. Biomedical Metabolomics

While the term metabolomics is relatively new to the field of research, understanding intracellular metabolite alterations is making head way in the biomedical field of disease diagnosis. There is thought to be approximately 3000 to 5000 detectable metabolites in the human body (Wishart et al., 2007). Most studies in biomedical

metabolomics have concentrated on finding disease related biomarkers, investigation of phenotypes, toxicology as well as molecular mechanisms (Yan et al., 2018). Researchers have found that metabolites have various functions within the biological systems of the body. Some of these functions include synthesizing macromolecules, producing energies as well as signaling molecules and various hormones (Yan et al., 2018). Most importantly, it was discovered that interrupted metabolism can increase the chance of disease as well as disease progression. Researchers are still working on understanding the function of metabolites, increasing the methods to quantify potential biomarkers and discovering the underlying cause of metabolite fluctuations.

One of the more obvious areas of study in biomedical metabolomics is the study of metabolic diseases. The main causes of metabolic diseases are dysregulation in small molecule metabolism (Vinayavekhin et al., 2009). A study by Shaham et al. (2008) investigated metabolites in plasma on individuals that were having imbalanced glucose levels. They discovered changes in 18 metabolites that were linked to enhanced glucose metabolism, decreased lipolysis, ketogenesis and proteolysis, which are all related to insulin pathways. These metabolites served as biomarkers and were shown to be highly predictive of the variation of insulin levels in these patients.

There has been a lot of metabolite research done in patients with various types of cancer such as breast, colon, oral and prostate. More often than not, cancer is detected during its late stages where patient treatment options are limited. Using metabolites for biomarkers to detect cancers offers a unique opportunity that a patient might be able to be diagnosed earlier in their cancer growth phase.

Ovarian cancer is one of the leading causes of death in women with gynecological cancers. 90% of patients diagnosed with stages four and five ovarian cancer have a less than 30% chance of living (Jacobs et al, 2004). A study by Zhang et al. (2012) studied urinary metabolites as potential biomarkers for ovarian cancer. Not only are urinary samples noninvasive and inexpensive, it also represents to downstream processes of various bodily systems. They evaluated urine samples from patients with ovarian cancer, benign ovarian tumors and healthy controls with liquid chromatography and mass spectrometry. Twenty-two biomarkers were found to be predictive of ovarian cancer and represented disturbed metabolic pathways. Eighteen of the ovarian cancer patients had previously underwent surgery to remove the cancer. These patient's urinary metabolites had changed compared to their preoperative condition. In addition, several of the patient's urinary metabolites suggested recovery back to their normal metabolite levels. This study lays a great foundation for the diagnosis of other diseases by urinary metabolic profiling.

Breast cancer is another disease that is associated with the death of over 40,000 women a year (American Cancer Society, 2009). A study by Asiago et al. (2010) investigated blood serum metabolites in relation to the recurrence of breast cancer. 56 previously diagnosed and surgically treated breast cancer patients, as well as patients who had never been diagnosed with the disease, were included in the study. 257 total blood samples were collected and blood serum was analyzed via NMR and MS methods. They found that 55% of patients could be accurately predicted to have a recurrence of breast cancer within thirteen months before a clinical diagnosis. Not only have we seen that metabolites could potentially serve as biomarkers for early stage cancer, but they also have

a possible platform to be used in determining the recurrence of breast cancer and hopefully others.

There have been several studies focusing on one specific metabolite: histidine. Histidine is commonly considered an anti-inflammatory factor and a dietary essential amino acid for humans. A study by Watanabe et al. (2008) investigated histidine levels in blood plasma in patients with chronic kidney disease. They found that histidine levels were significantly lower in patients with chronic kidney disease along with having cardiovascular disease. It was concluded that low plasma concentration of histidine was associated with inflammation, stress, as well as contributing to a higher mortality rate in chronic kidney disease patients. A study by Yu et al. (2015) investigated histidine as well in relation to coronary heart disease. 1,152 African Americans were studied based on loss-of-function variants in the gene *HAL.* This gene is encodes histidine and is necessary for histidine catabolism. After metabolic analysis of blood samples, they found that patients with loss-of-functions in the *HAL* gene had increased histidine levels which put them more at risk for coronary heart disease. Each of these studies can be seen as a huge breakthrough in the medical industry if differing biomarkers can be seen though collecting noninvasive samples such as blood or urine from patients.

V.III. Metabolomics in Agriculture and Livestock Fertility

Metabolomics is becoming more common in the agricultural industry as more studies are being conducted in efforts to improve overall heard health and reproductive success. Diseases in animals are normally not found until the animal starts to exhibit symptoms that are noticeable to the producer. Metabolomics allows researchers and producers a unique opportunity understand system-wide metabolism of a certain livestock

species. Metabolomics has been more commonly used in crop trait, breeding and evaluation compared to livestock species (Goldansaz et al., 2017). Within the livestock industry, metabolomics can serve as a minimally invasive way to detect subtle phenotypic changes, innate phenotypic propensities and dietary responses (Fihn, 2002). It can also serve as a tool for research, breeding and overall assessment of livestock (Fihn, 2002). More recently, metabolomics has been used to research feed efficiency, disease, carcass merit, fertility, milk quality and other traits in livestock. Currently, there have been 1070 metabolites that have been detected, but possibly not identified, in cattle, sheep, goats, horses and pigs (Goldansaz et al., 2017). Researchers are constantly working to identify each metabolite and compile them into a livestock metabolome data base in an effort to understand fluctuations in livestock metabolites.

As discussed, one of the main reasons that an animal is removed from the production herd is infertility. A study by Phillips et al. (2018) investigated the potential for metabolites to be used as biomarkers to determine fertility potential in beef heifers. Heifers underwent an AI program and were exposed to a bull fourteen days after breeding. Blood was collected, and plasma isolated, at the time of AI. Parameters such as BCS, weight and RTS were recorded one month prior. Pregnancy data was collected 45 and 65 days post AI. Blood plasma was isolated and used for metabolomic analysis. They found fifteen metabolites that were significantly different between the fertile and infertile heifers at the time of AI. This study lays a foundation for continued research using metabolites as biomarkers for heifer fertility.

Along with blood plasma metabolites, researchers have even gone as far as evaluating the metabolite concentrations of follicular fluid to investigate if it could explain

differences in fertility. A study by Bender et al. (2010) investigated follicular fluid in heifers and lactating cows. Follicular fluid surrounds the oocyte in a follicle and is thought to be reflective of the intrafollicular environment that could affect oocyte maturation as well as embryo growth and development. In this study, follicular fluid from the dominant follicle was taken and metabolomic analysis by MS was used. Nine metabolites were found to be significantly different between heifers and cows. Through the metabolite analysis, they found that there was a higher amount of fatty acids in follicular fluid from lactating cows compared to heifers. The follicular fluid with a higher fatty acid content placed lactating cow oocytes at a developmental disadvantage that could possibly explain the fertility differences between the two groups.

In order to understand the metabolism of a growing embryo, Perkel and Madan (2017) took it upon themselves to study the culture medium that bovine embryos were being cultured in. This study aimed to help understand the metabolomic dynamic of the bovine preimplantation embryo in correlation with developmental rates. They hypothesized that slow growing embryos would differ in this metabolic rates and would consume or secrete metabolites differently than fast growing embryos. Oocytes were collected and matured and then underwent in-vitro fertilization. The embryos that successfully fertilized were placed in a culture media that consisted of specific amounts of oviductal fluid, non-essential amino acids, essential amino acids, sodium pyruvate and gentamicin. Each embryo was evaluated at different growth stages and the culture media was evaluated for the loss and gain of certain metabolites compared to the previously known amount. Sixteen metabolites were found in the spent culture medium and six differed significantly between the fast growing and slow growing embryos at the same

developmental stage. The metabolic signatures of embryos obtained can provide a platform for a non-invasive assessment of embryo health as well as the viability of mammalian embryos.

Metabolomics has also been used to discover biomarkers for bull fertility. Proven bull fertility is just as important as female reproductive success when introducing a bull to a herd. Bull's semen can vary in sperm number, motility and morphology. While some bulls will still pass a BSE, some still experience unexplained infertility. A study by Menezes at al. (2019) investigated the metabolomic profile of spermatozoa. Bulls of high and low fertility were evaluated and twenty-two metabolites were reported and five were found to be significantly different between the two groups. An additional study by Velho et al. (2018) aimed to identify metabolomic biomarkers in seminal plasma of bulls in relation to fertility. Sixty-three metabolites were identified in seminal plasma. Two metabolites, 2-oxoglutaric acid and fructose, were significantly different between the two groups. 2-oxoglutaric acid was decreased and fructose was increased in higher fertility bulls compared to bulls of lower fertility.

V.IV. Metabolomics and Male Infertility

When the word infertility is mentioned, many tend to think of reproductive issues related to the female. It has been shown that 30-55% of infertility cases are related to the male (Sharlip et al., 2002). Male infertility is classified by either having erectile dysfunction, semen abnormalities or both (Zhou et al, 2015). Researchers have been actively trying to find a way to diagnose infertility in men as well as develop a treatment. Currently there is a lack of rapid, noninvasive tests to evaluate semen quality and an inability to predict gamete quality and embryo viability (Deepinder et al., 2007). Infertility

in men is clinically diagnosed by semen analysis based on sperm number, motility and morphology. Metabolites in men can be analyzed in fluids such as seminal plasma, urine and blood. To date, there have been studies relating antioxidants and reactive oxygen species as biomarkers of infertility in men. Metabolomics offers a unique platform to analyze body fluids reflective of phenotype of men and can be very informative of their reproductive potential at a lower cost compared to genomics, transcriptomics or proteomics.

One of the main obstacles that researchers are facing right now is attaining a list of metabolite biomarkers that are related to fertility, and more specifically infertility, in men. A study by Xu et al (2020) used metabolic profiling on seminal plasma in men that were healthy and were experiencing infertility with various semen abnormalities by liquid chromatography-mass spectrometry. 63 potential biomarkers in seminal plasma were found to be directly related to infertility. They were also able to group different biomarkers in relation to different forms of infertility. Every patient had their sperm parameters analyzed including sperm concentration, deformity rate and motility in hopes to associate metabolomic changes with specific abnormalities. They found seventeen metabolites that were directly linked to abnormalities in various sperm parameters. Expression levels of acylcarnitine were found to be related to sperm concentration and deformity. Antioxidant expression was related to sperm deformity rate and motility. Expression of isopentenyl pyrophosphate, 2-phosphoglyceric acid and y-glutamyls-methyl selenocysteine was found to be linked to sperm deformity and creatine ribose was linked to sperm concentration. After pathway analysis of the seventeen metabolites, the biomarkers were shown to be

involved in energy production, antioxidation, hormone regulation as well as affecting the sperm membrane.

Blood plasma is also shown to be an effective way to analyze metabolites close to the phenotype of an individual. A study by Zhou et al. (2015) compared healthy and fertile men with men who experienced erectile dysfunction or semen abnormalities via gas chromatography-mass spectrometry. The goal of this study was to discover biomarkers that would be able to group healthy men from the infertile men, no matter the reason. They found that healthy men could be differentiated from men with semen abnormalities 80% of the time and men with erectile dysfunction 87% of the time just by looking at metabolites as biomarkers. 1,5-Anhydro-sorbitol and a-hydroxyisovaleric acid were biomarkers for patients experiencing infertility. Other metabolites such as lactate, glutamate and cholesterol were shown to be biomarkers that differentiate semen abnormality patients from erectile dysfunction patients. This study was one of many to introduce the possibility that male infertility could possibly be determined by a simple blood test.

Lastly, analyzing metabolites present in urine can be a low cost and non-invasive way to potentially determine infertility in men. While many infertility cases in men can be determined by a simple semen analysis, this does not reflect the health and function of reproductive organs as a whole, which is something that metabolomic analysis could possibly reflect. A study by Zhang et al. (2013) investigated men's urinary metabolites that could in indicative of oligozoospermic infertile men. Oligozoospermic is defined as a low total concentration of sperm. 135 men with a low sperm count were compared to 158 men who were deemed fertile by mass spectrometry as well as performing pathway analysis. Ten metabolites were found to accurately differentiate the fertility potential between the

two groups. Acylcarnitine, aspartic acid and leucylproline were down regulated and adenine and methylxanthine were up regulated in urinary samples from patients with low sperm counts. After pathway analysis was performed, the previously mentioned biomarkers were shown to be linked to sperm concentration as well as sperm head displacement. Overall, metabolomic analysis of urine, blood plasma or seminal plasma in men could be useful method in pinpointing the cause of why they are experiencing infertility.

V.V. Metabolomics and Female Infertility

Fifteen to twenty percent of couples that are planning to conceive experience infertility (Deepinder et al., 2007). Infertility is defined as the "inability to conceive after 12 months of regular intercourse" (Callister et al., 2010). When someone is deemed "infertile" they tend to turn towards assisted reproductive technologies (ART). Metabolomics has been more commonly used recently to help identify the cause of subfertility by analyzing the imbalance of metabolism in the body in relation to reproduction (Bracewell-Milnes et al., 2017).

There are numerous possibilities as to why an imbalanced metabolism could have even a small effect on reproduction. A lot of bodily systems have to work together in unison in order to successfully grow and sustain an embryo. Endometriosis is commonly defined as the "proliferation of endometrial glands and stroma outside of the uterus" (Dutta et al., 2012). Women diagnosed with endometriosis have been shown to experience infertility, but there is a lot about the disease that remains unknown. It has been shown that 10% of women of reproductive age are diagnosed with the gynecological disorder (Giudice and Kao, 2004). A study by Dutta et al. (2012) used metabolomics as a means for identification

of potential biomarkers for endometriosis in its early stages. Blood samples were collected from twenty-two women of reproductive age and serum was separated and analyzed by NMR. They found that serum alone was able to differentiate between patients diagnosed with endometriosis 80% to 90% of the time. Lactate, 3-hydroxybutrate, alanine, leucine, valine, threonine, lysine, glycerophosphatidylcholine, succinic acid and 2-hydroxybutyrate were all up regulated in women with endometriosis compared to the healthy controls.

While body fluids such as urine and blood plasma can be tested, another way researchers have studied subfertility in women is though their follicular fluid. Follicular fluid creates a very unique and specialized microenvironment for developing oocytes. The fluid contains all of the necessary components to grow, nourish and mature an oocyte until a possible ovulation (Bracewell-Milnes et al., 2017). Researchers have investigated the metabolic makeup of follicular fluid to determine if the concentration of certain metabolites are reflective of oocyte quality. A study by Wallace et al. (2012) investigated the metabolic profile of follicular fluid and its impact on oocyte developmental potential and viability as well as implantation outcome. Fifty-eight women who were undergoing IVF had transvaginal ultrasound-guided needle aspiration performed on mature follicles twelve to twenty-four millimeters in size. After fertilization, the embryos were classified based on their morphologic criteria and cleavage rate. The patient was able to choose between a single, double, or triple embryo transfer. This research group found that metabolites identified as indicative of oocyte competence were choline, lactate, glucose, proline phosphocholine, glutamine and leucine. Glucose was downregulated in pregnant women compared with the women that did not get pregnant. Lactate, choline, leucine and phosphocholine were decreased in non-pregnant women. These metabolites had a high

ability to differentiate pregnancy outcome where the differences correlate with the developmental competence of the human oocyte. This study creates a platform for the same analysis to possibly be carried out in livestock follicular fluid.

The metabolomic profile of embryo culture medium can also be of use when determining the possibility of fertility. With a normal IVF protocol, embryos are chosen for transfer based on their morphology and cleavage rates (Bracewell-Milnes et al., 2017). While this is a quick solution, it takes a skilled embryologist with a good eye to be able to determine which embryo might be "the best" in relation to pregnancy outcome. Researchers have turned to studying the media that oocytes and embryos are cultured in to see it could possibly be reflective of the quality of the embryo itself. A study by Nagy et al. (2008) investigated the metabolic profile of the culture media that the oocytes were matured in to see if it would correlate with maturity status as well as embryo potential. 412 samples were analyzed by near-infrared (NIR) spectroscopy. The metabolite concentrations of oocytes in growth phases metaphase I and metaphase II differed significantly from each other. The metabolic profile of oocytes that resulted in a positive pregnancy were highly variable and could differentiate between day 3 and day 5 embryos. This study showed that the metabolic profile of oocyte culture medium could be indicative of the oocyte's viability as well as embryo growth and development of that same oocyte.

VI. Inflammation

VI.I. The Immune System

The immune system acts as the main defense mechanism against foreign material in the body. It is composed on lymphoid organs, cells, humoral factors and cytokines (Parkin and Cohen, 2001). These factors work together to aid against foreign bodies by

either innate or adaptive responses. Innate response is defined as the body's immediate defense mechanisms such as the physical, chemical and microbiological barriers which would include neutrophils, monocytes, macrophages, cytokines and acute phase proteins (Parkin and Cohen, 2001). Adaptive immunity is referred to as a higher form of immunity which consists of antigen-specific reactions through lymphocytes. The adaptive response is very concise and can take ten to fourteen days to develop after being exposed to a foreign body. Interestingly, the adaptive immune response has a "memory" where if the body is exposed again, it will have a larger and more rapid response (Parkin and Cohen, 2001).

Immunity plays a large role in infertility in women and in animals. In fact, immune infertility has been shown to affect approximately 1 out of 5 couples of reproductive age that are struggling to conceive (Brazdova et al, 2016). This can include when a female's immune system rejects the semen leading to a systemic immune response which could induce high levels of anti-seminal sperm antibodies. Immune infertility can be diagnosed in women when their antibodies bind to the antigens that are present on the male or female gametocytes (Brazdova et al, 2016). Antibody-binding proteins in semen are supposed to protect the sperm against the female immune system leading to the successful passage of sperm through the female reproductive tract to the site of fertilization.

Inflammation has been considered to be highly related to reproductive disorders and the normal function of a menstrual cycle. An increased immune response can affect normal ovulation and hormone production, which could lead to a reproductive disorder (Weiss et al., 2009). Immune cells involved in normal cycle function produce a variety of different inflammatory cytokines. A reproductive disorder that can be a result of disrupted immunity is endometriosis which has been previously defined. Studies have found that

inflammatory cytokines such as IL-1, IL-6, and TNFα are increased in women with endometriosis whereas IL-6 and TNFα both promote endometrial cell proliferation, adhesion and angiogenesis (Weiss et al., 2009). Likewise, inflammation can play a large role in proper ovulation and corpus luteum function. The presence of estradiol will lead to a surge of luteinizing hormone (LH) for proper ovulation of the dominant follicle on the ovary. LH has been shown to stimulate granulosa cells to secrete cytokines at the time of ovulation (Weiss et al., 2009).

In cattle, the defense mechanisms to the immune system are largely the same with adaptive and innate responses. Innate immunity, like mentioned previously, is the body's immediate reaction after exposure to a foreign microbe. The cow's body will quickly work through sentinel cells such a macrophages and dendritic cells to secrete cytokines (Bio-Rad, 2016). These cytokines can include TNFα and IL6 along with many others. Toll-like receptors can also initiate immune responses through exposure to foreign microbes. A common toll-like receptor is TLR4 which can bind to lipopolysaccharide (LPS) leading to increased cytokines due to an immune response (Bio-Rad, 2016).

VI.II. Inflammatory Cytokines in Relation to Infertility

Cytokines are defined as "small secreted proteins released by cells that have a specific effect on the interactions and communications between cells" (Zhang and An, 2009). There are other names to define cytokines such as lymphokine, monokine, chemokine and interleukin and each can act on its host cells or surrounding and nearby cells to create an immune response. (Zhang and An, 2009). Cytokines can also fall into two different categories: pro-inflammatory cytokines and anti-inflammatory cytokines. Certain inflammatory cytokines can be linked to different biological processes and systems within

the body in relation to pain or injury. One specific cytokine can be released by different cells types as well as one cytokine can have an effect of different cells types (Zhang and An, 2009). When a cytokine is produced from a certain cell line, it will then act on other cells to produce different cytokines as a result. They can also work synergistically by multiple cytokines being able to induce the same inflammatory response. Cells lines that are more commonly known for producing cytokines are T cells and macrophages which are located close to peripheral nerve tissue, spinal cord, or inflamed skin (Zhang and An, 2009).

There are two more common pro-inflammatory cytokines: TNFα and IL6. TNFα, which is also referred to as cachectin, acts through several different pathways on two different cell surface receptors, TNFR1 and TNFR2 (Zhang and An, 2009). It is mainly secreted through monocytes and macrophages (Mahdi, 2011) TNFα has been shown to be an integral part of cycle regulation within the ovary as well as contributing to the growth and development of the follicle (Vital et al., 2005). A study done by Reid et al. (2001) investigated the role of inflammatory cytokines on recurrent pregnancy loss. They found that levels of TNFα, along with other cytokines, were directly related to pregnancy loss in women of reproductive age. A similar study also showed that cytokines, such as TNFα, are involved in inflammation within the maternal uteroplacental blood vessels in mice (Mahdi, 2011). While TNFα is required in small amounts for reproduction, it has also been shown that increased TNFα levels in human amniotic fluid have been linked to infection and preterm labor (Romero et al., 1989).

IL-6 is the other pro-inflammatory cytokine that has been extensively studied in relation to peripheral and nerve injury as well as the subsequent pain (Zhang and An, 2009).

It normally plays a role in adaptive immunity. IL-6 is very commonly seen in the female reproductive tract and in gestational tissues and plays a large role in embryo implantation as well as placental development (Prins et al., 2012). Studies have shown that increased levels of IL-6 have been seen in women with infertility, miscarriage, preeclampsia as well as premature delivery (Prins et al., 2012). A female reproductive tract undergoes a lot of stress when pregnant. The success of that pregnancy is dependent on the body's toleration to pregnancy in general. A study by Prins et al. (2012) concluded that too much or too little IL-6 present in the endometrium and fetal-placental tissue could lead to pregnancy loss or infertility. Granulosa cells are located within the follicle of the ovary and create a unique microenvironment for the growth and potential of the oocyte. Price et al. (2013) showed that when granulosa cells were treated with LPS, a source of inflammation, within 30 minutes IL-6 had significantly increased which could affect the endocrine function the cells have in reproductive health. Lastly, IL-6 has been shown to be involved in abortions in women within the first trimester of pregnancy. A study by Koumantaki et al. (2001) found reduced blood plasma levels of IL-6 in women who had experienced miscarriages compared to healthy pregnant and non-pregnant women.

CXCL5, also named CXC motif chemokine ligand 5, is a member of a larger family of CXC chemokines. It can be secreted from cells such as monocytes, neutrophils, epithelial cells and muscle cells (Zohrabi et al., 2017). The cytokine has one receptor, CXCR2, and aids in promoting angiogenesis as well as tissue remodeling as it is essential for cell adhesion and migration (Sun et al., 2020). It has been shown that increased CXCL5 could interfere with intrauterine adhesion or endometrial damage. Sun et al. (2020) found that CXCL5 expression was much lower in rats with intrauterine-adhesions compared to

reproductively healthy rats. It was previously mentioned that the amount of fat on an animal can affect the timing of puberty. Increased levels of CXCL5 can be secreted from adipose tissues and can block the insulin signaling pathway on obese animals. A study by Chavey et al. (2009) showed that CXCL5 was significantly increased in obese humans and levels decreased after weight reduction. Later studies have shown that increased levels of CXCL5 in women could lead to polycystic ovary syndrome (Zohrabi et al., 2017).

Monocyte Chemotatic Protein-1, commonly known as MCP-1, is a cytokine that predominantly produced by monocytes and macrophages and has proven to have roles in adaptive and innate immunity (Gmyrek et al., 2005). Specific to reproduction, MCP-1 expression has been shown to effect processes such as fetal allograft, pregnancy maintenance as well as parturition (Denison et al., 1998). It has also been hypothesized that an increased level of MCP-1 in amniotic fluid could lead to pregnancy loss or early parturition (Chaiworapongsa et al., 2002). Lastly, increased MCP-1 has been seen in women with endometriosis. The macrophage system has been shown to be an integral component in the maintenance of cell-mediated immunity (Gmyrek et al., 2005). A study by Kalu et al. (2007) investigated cytokine profiles in blood serum and peritoneal fluid in infertile women that were diagnosed with endometriosis. Their results indicated that MCP-1 and IL-6 levels were higher in peritoneal fluid, but not blood serum, in women with endometriosis compared to healthy women (Kalu et al., 2007). Similarly, a study by Tao et al. (2010) found that MCP-1 levels were elevated in patients diagnosed with endometriosis compared with women with differing infertility reasonings.

Lastly, POSTN, or Periostin, is a cytokine that can be found in multiple places within the body including the placenta and the uterus. Anh et al. (2009) conducted a study

in which they concluded that POSTN gene expression in the bovine endometrium is dependent on progesterone. Researchers have found that POSTN expression can effect embryo implantation by increasing Wnt signaling, a pathway that plays a crucial role in embryo development (Tepekoy et al., 2014). It has also been found in amniotic membranes and in the neonate umbilical cord (Dobreva et al., 2012). Freis et al. (2017) concluded that women who had recently experienced a miscarriage had increased levels of POSTN compared to healthy, pregnant women. This could mean that POSTN could serve as a potential biomarker for pregnancy outcome in women and livestock in the future.

VII. The Impact on Agriculture

One of the most important factors that effects beef production on a cow-calf operation is heifer reproductive failure. Heifer infertility results in costing the producer time and money by keeping her on farm in hopes that she will get pregnant and eventually being removed from the operation at a young age. One of the key components of a cow-calf operation needs to be being able to discriminate fertile from infertile heifers before incorporating them into an profitable existing herd. Carefully evaluating heifers and their traits representative of fertility can improve the longevity and stayability of all heifers within a herd, increasing the producers profits (Liu et al., 2008).

Reproductive technologies are constantly being evaluated and changed in order to improve livestock performance in the cow-calf sector. Metabolomics is becoming a topic of interest within reproductive and developmental biology to help understand the mechanisms of pregnancy and embryo growth and development. Being able to understand the metabolism of an animal can help to detect phenotypic changes and serve as a tool for research, breeding and overall assessment of heifer development. While not all metabolites

have been identified, researchers are constantly working to create a complete metabolome database for livestock species. Being able to understand fluctuations in livestock metabolites in relation to fertility and taking phenotypic characteristics into consideration have the possibility to help producers determine whether to retain heifers as replacement animals.

Heifer infertility, for the most part, is a unexplained phenomenon in the agricultural sector and one of the biggest challenges a cow-calf producer will likely face. The direct cause of interfilty in heifers can be hard to diagnose as there are many factors that have to work in unison to create and maintain a healthy pregnancy and offspring. In our study, we investigated the relationship between metabolite and inflammatory cytokine levels, at weaning, and pregnancy outcomes following a typical breeding season. These were compared with the accuracy of traditional phenotypic parameters in determining reproductive potential in heifers. We hypothesized that, at weaning, metabolites and/or inflammatory cytokine levels may prove useful as molecular indictors of reproductive potential in heifers. By utilizing both metabolites and inflammatory markers as a source of biomarkers to determine heifer fertility at an earlier age, researchers can be one step closer to identifying the cause of heifer infertility and producers can avoid investing valuble resources into infertile heifers.

CHAPTER II.

PHENOTYPIC HEIFER ASSESSMENT AND ANALYSIS

II.I. ABSTRACT

Developing a reproductively successful heifer is highly dependent on an operations growth and development program. One of the vital components of raising replacement heifers is optimizing nutrition to meet her needs in order to reach puberty (Larson et al., 2016). Selecting and managing potential replacement beef heifers can affect the future productivity of a herd as a whole (Patterson et al., 2002). Producers have utilized physiological characteristics that influence puberty such as weaning weight, BCS, RTS, PA and age to determine and more accurately group heifers based on their reproductive potential. The development of a heifer to the time that she reaches puberty is pivotal on her lifetime productivity as well as her stayability in the herd.

Angus/Simmental cross heifers (N = 104) were used for this study. They were housed at the Black Belt Research and Extension Center in Marion Junction, Alabama, U.S.A. Heifers underwent a fixed-time AI program with estrus detection over the course of two breeding seasons (2018-2019 and 2019-2020). Phenotypic characteristics such as age, weaning weight, body condition score, reproductive tract score and pelvic area were compared between heifers pregnant by AI and those that failed to conceive after 60 days with bull exposure. Over the course of two breeding seasons at one location, 35 heifers became pregnant by AI (fertile), 31 heifers were bull bred, and 38 heifers remained open after 60 days with bull exposure (infertile). All previously mentioned phenotypic

parameters, other than weaning weight, were shown to be significantly different ($p < 0.05$) over the course of two breeding seasons compared. For age, the 2019-2020 breeding season was slightly higher than the 2018-2019 breeding season. For body condition score, reproductive tract score and pelvic area, the 2018-2019 breeding season was slightly higher than the 2019-2020 breeding season. Weaning weight was not significant ($p > 0.05$) when comparing the two breeding seasons. While the phenotypic parameters differed between breeding seasons, they did not prove to be an accurate in determining reproductive potential in heifers.

II.II. INTRODUCTION

One of the main problems that a producer faces today in the livestock industry is infertility, especially within replacement beef heifers. Infertility not only has an effect on reproductive performance, but it can also increase the cost of developing replacement heifers (Perry et al., 2009). When selecting replacement heifers, decisions are normally based off of characteristics such as phenotype and genetic background. While traditional methods of selection are most commonly used, there has also been a drastic change in the industry as a whole over the last few decades. Breed genetics, supply of inexpensive feeds and management practices are just a few of the changes the industry has experienced (Endecott et al., 2013). Each of these can have an overall effect on the lifetime production efficiency of a heifer. The overall goal is to raise replacement heifers with decreased development costs without compromising the overall performance of the heifer (Funston et al., 2004). It has been shown that after the beginning of a breeding season, for each day that passes after the herds first calving, 2.4 pounds of weaning weight is loss in calves born later in the season (Perry et al., 2009). This could affect the timing of puberty as well as reproductive performance. In order to increase the efficiency of raising beef heifers, it is important for producers to develop heifers to conceive and calve early in the breeding season. To do this, parameters such as weaning weight, age at weaning, BCS, RTS and PA are used to make a decision in the selection of beef replacement heifers.

The goal of this portion of the study was to compare traditional methods of selecting replacement beef heifers. Weaning weight, age at weaning, BCS, RTS and PA were compared between heifers with various pregnancy outcomes [fertile (AI) and infertile

(open)]. The data was collected over two breeding seasons (2018-2019 and 2019-2020) at one location (Black Belt Research and Extension Center).

A total of 104 Angus-Simmental cross heifers where phenotypically evaluated and analyzed according to the previously mentioned parameters. Over the course of two breeding seasons, 35 heifers were pregnant by AI (fertile), 31 heifers were bull bred, and 38 heifers remained open after 60 days with bull exposure (infertile). Phenotypic measurements were compared using One-way ANOVA. Pregnancy outcome is defined by the type of pregnancy obtained. Infertile heifers that remained open following AI and presence of a fertile bull were compared to those that became pregnant following artificial insemination.

II.III. MATERIAL AND METHODS

Animal Use

All studies utilizing animals were approved by the Auburn University Institutional Animal Care and Use Committee (IACUC). Heifers used for this study (N = 104) were originated from and housed at the Black Belt Research and Extension Center of the Alabama Agricultural Experiment Station in Marion Junction, AL, U.S.A.

Reproductive Management

Angus-Simmental cross heifers participated in an estrous synchronization and artificial insemination program by destrus detection [7-day CO-Synch + CIDR © (Whttier et al., 2013)] over the course of two breeding seasons (2018-2019 and 2019-2020). Seven days before artificial insemination, heifers were given 2 cc of GnRH intramuscularly (CYSTORELIN, Boehringer Ingelheim Animal Health, Athens, GA, USA). At the same time of injection, a controlled internal drug release (CIDR ©) consisting on 1.38 g of progesterone was inserted intravaginally (EAZI-BREEDTM CIDR © Cattle Insert, Zoetis, Kalamazoo, MI, USA). After seven days, the CIDR © was removed and 5 cc of dinoprost thromethamine (LUTALYSE ©, Zoetis, Kalamazoo, MI, USA) was intramuscularly injected. Over the next 48 hours, heifers were observed for signs of estrus and artificially inseminated 12 hours later following the AM/PM rule. Heifers were artificially inseminated with a single straw of semen from Angus sures of provent fertility. Heifers that did not show signs of estus 72 hours after CIDR © removal were bred via AI and injected with 2 cc of GnRH intramuscularly (CYSTORELIN, Boehringer Ingelheim

Animal Health, Athens, GA, USA). Fourteen days following AI, heifers were exposed to a clean up bull of proven fertility for 60 days. Bulls exposed to heifers had previously passed a BSE (Breeding Soundness Exam) and had <10% semen abnormalities. The 104 heifers were exposed to one bull for 60 days following artificial insemination.

Heifer Nutrition Management

Heifers housed at the Black Belt Research and Extension Center were grazed on fescue pasture with unlimited access to ryegrass hay. 5-7 lbs of Soyhull + Corn Gluten supplementations was given to each heifer per day. Trace minerals were available *ad libitum*.

Phenotypic Observations

Phenotypic conditions of 104 heifers (N = 104) over the course of two breeding seasons (2018-2019 and 2019-2020) were used and analyzed for this study. A trained veterinarian evaluated body condition score (BCS) (Appendix 1), reproductive tract score (RTS) (Appendix 2), Weaning Weight, Age at Weaning as well as Pelvic Area. Weight was determined at the time of weaning on all heifers. Age was calculated by the number of days between birth and day of weaning. BCS was determined at the time of weaning as previously described and ranged on a scale of 1-9 with 1 being very thin and 9 being very obese (Herd and Sprott, 1986). A trained veterinarian evaluated reproductive tract score (RTS) one month prior to breeding. Scores ranged from 1-5 with 1 being an immature tract and 5 being a very mature tract with signs of cycling (Anderson et al., 1991).

Pregnancy Determination

A trained veterinarian determined pregnancy vis rectal palpation 45 and 65 days post artificial insemination. Heifers were grouped based on pregnancy outcome as follows: pregnant (AI), pregnant (Bull) or infertile if not pregnant following AI and bull exposure.

Statistics

Analysis of collected data was done by PRISM-6 software. Statistical analyses included unpaired parametric two-tailed tests (t-test) with 95% confidence intervals. Data analyzed is shown as mean ± standard deviation of the mean. Standard error is represented by the black bars in the figures. Significance is noted as $p < 0.05$.

II.IV. RESULTS

Phenotypic Heifer Assessment Over Three Breeding Seasons

Phenotypic body condition and reproductive parameters such as BCS, Weaning Weight, Age at Weaning, RTS, and PA were determined in 104 heifers (N = 104) housed at the Black Belt Research and Extension Center in Marion Junction, Alabama, U.S.A. All measurements were taken over the 2018-2019 and 2019-2020 breeding seasons. The percentage of heifers that became pregnant by AI, natural service (Bull) and those that remained open after 60 days with bull exposure were compared over the course of two breeding seasons (Figure 2.1). N = 53 heifers were analyzed for pregnancy outcomes during the 2018-2019 breeding season. N = 51 heifers were analyzed for pregnancy outcomes during the 2019-2020 breeding season.

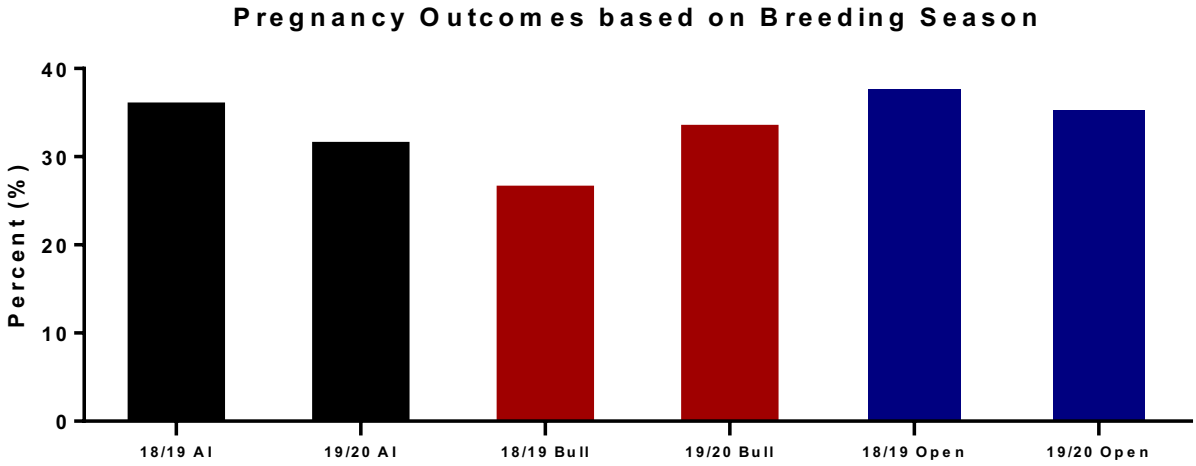


Figure 2.1. Graph comparing pregnancy outcomes from the 2018-2019 and 2019-2020 breeding seasons.

Phenotypic Heifer Assessment based upon Breeding Season

Phenotypic parameters (Age at Weaning, Weaning Weight, BCS, RTS and PA) were collected on heifers 30 days prior to artificial insemination over the course of two breeding seasons (2018-2019 and 2019-2020). All heifers were housed that the Black Belt Research and Extension Center in Marion Junction, AL, U.S.A.

Age at Weaning differed between breeding seasons

Heifer Ages at Weaning (N = 104) were compared between two breeding seasons (2018-2019 and 2019-2020). There was a significant difference ($p = 0.0068$) between age at weaning between breeding seasons: 2018 – 2019 = 222.1 ± 12.66 days of age, 2019 – 2020 = 228.9 ± 12.31 days of age.

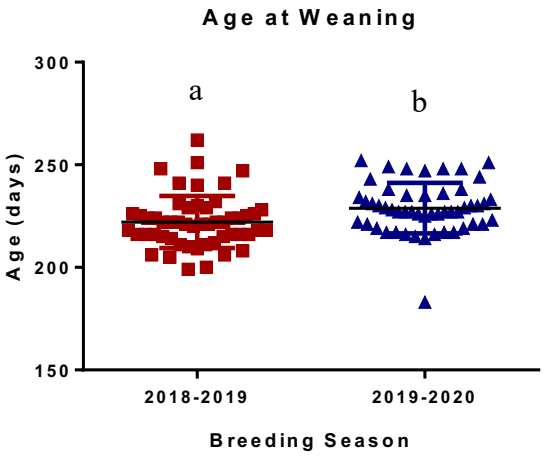


Figure 2.2. Graph depicting heifer Ages at Weaning from two breeding seasons 2018-2019, 2019-2020). Data are mean ± standard deviation of the mean ($p < 0.05$). The two breeding seasons were shown to be significantly different from each other.

Weaning Weight did not differ between breeding seasons

Heifer Weights at Weaning (N = 104) were compared across two breeding seasons (2018-2019 and 2019-2020). There was not a significant difference (p = 0.0504) between weight at weaning between the breeding seasons: 2018 – 2019 = 277.4 ± 21.94 kg, 2019 – 2020 = 268.7 ± 23.03 kg.

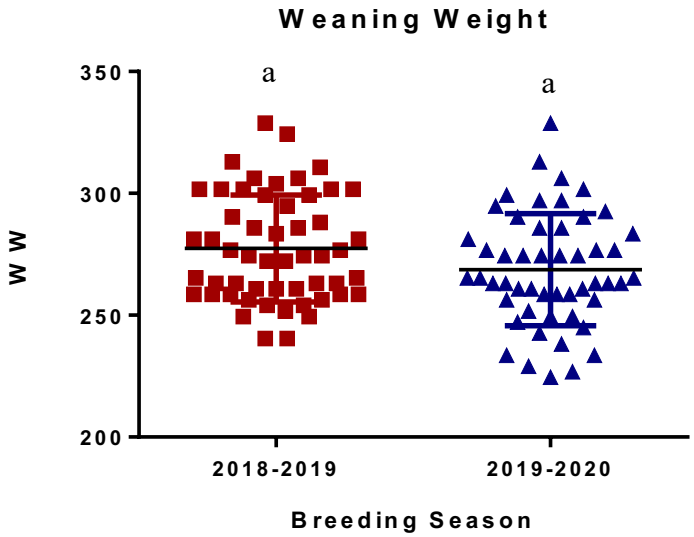


Figure 2.3. Graph depicting heifer Weights at the time of weaning from two breeding seasons (2018-2019 and 2019-2020). Data are mean ± standard deviation of the mean ($p < 0.05$).

Body Condition Score differed between breeding seasons

Heifer Body Condition Scores (N = 104) were compared across two breeding seasons (2018-2019 and 2019-2020). There was a significant difference ($p = 0.0021$) between body condition scores between breeding seasons: 2018 – 2019 = 5.991 ± 0.2498, 2019 – 2020 = 5.784 ± 0.4032. For body condition score, the two breeding seasons were shown to be significantly different ($p < 0.05$) from each other.

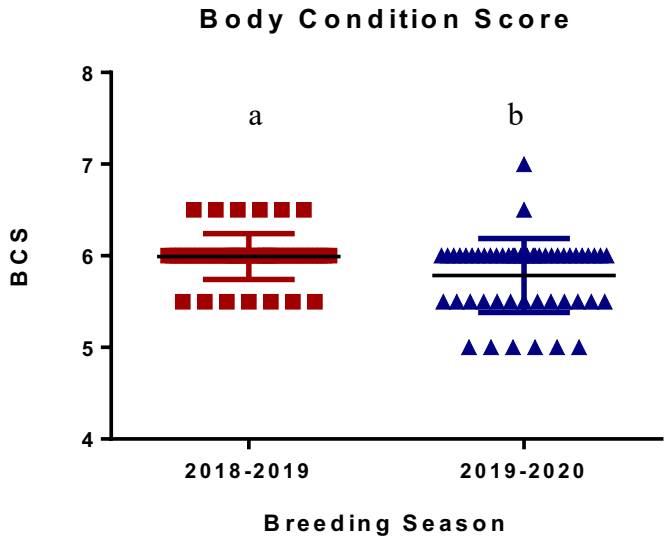


Figure 2.4. Graph depicting heifer BCSs from two breeding seasons (2018-2019, 2019-2020). Data are mean ± standard deviation of the mean ($p < 0.05$). For body condition score, the two breeding seasons were shown to be significantly different ($p < 0.05$) from each other.

Reproductive Tract Score differed between breeding seasons

Reproductive Tract Scores (N = 104) were compared across two breeding seasons (2018-2019 and 2019-2020). There was a significant difference ($p = 0.0111$) between reproductive tract scores between breeding seasons: 2018 – 2019 = 4.255 ± 0.6326, 2019 – 2020 = 3.902 ± 0.7551.

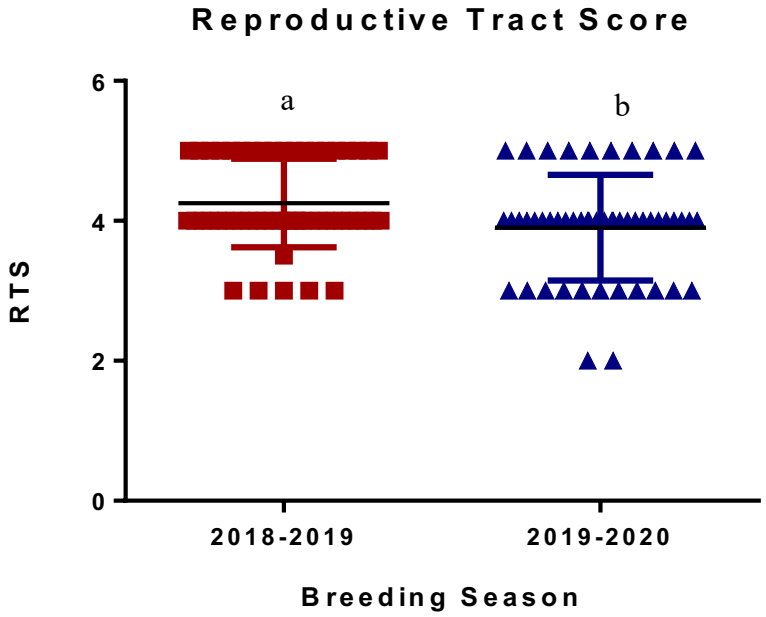


Figure 2.5. Graph depicting heifer RTSs from two breeding seasons (2018-2019, 2019-2020). Data are mean ± standard deviation of the mean ($p < 0.05$). For reproductive tract score, the two breeding seasons were shown to be significantly different ($p < 0.05$) from each other.

Pelvic Area differed between breeding seasons

Pelvic Area (N = 104) were compared across two breeding seasons (2018-2019 and 2019-2020). There was a significant difference ($p = 0.0002$) between pelvic areas between breeding seasons: 2018 – 2019 = 189.1 ± 16.58 cm^2, 2019 – 2020 = 176.7 ± 11.09 cm^2.

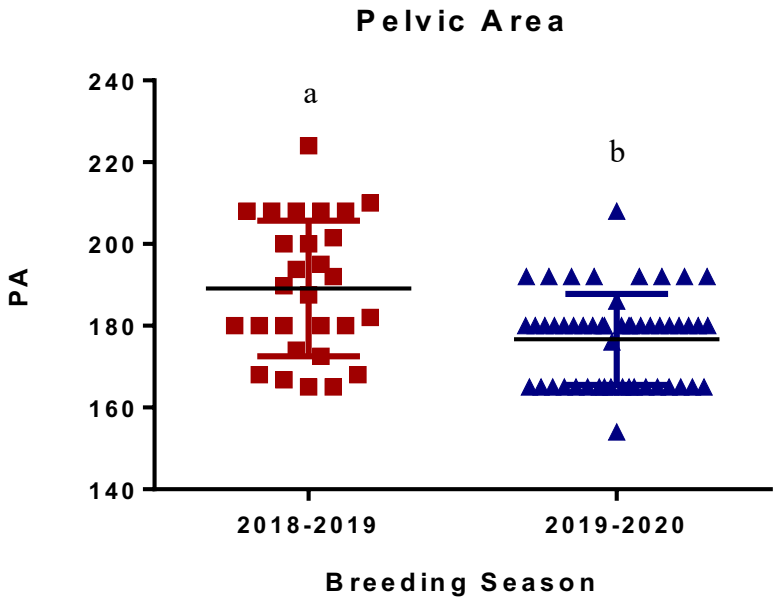


Figure 2.6. Graph depicting heifer Pelvic Area from two breeding seasons (2018-2019, 2019-2020). Data are mean ± standard deviation of the mean ($p < 0.05$). For pelvic area, the two breeding seasons were shown to be significantly different ($p < 0.05$) from each other.

Phenotypic Heifer Assessment based upon Pregnancy Outcome

Phenotypic parameters (Age at Weaning, Weaning Weight, BCS, RTS and PA) were collected on heifers either at weaning (age and weight) or 30 days prior to artificial insemination over the course of two breeding seasons (2018-2019 and 2019-2020). All heifers were housed that the Black Belt Research and Extension Center in Marion Junction, AL, U.S.A. Heifers were categorized by pregnant by artificial insemination (AI) or those remaining open following AI and 60 days of bull exposure. Heifers pregnant by bull were not included for the analyses. Overall, phenotypic parameters alone were not sufficient enough to accurately determine reproductive potential in heifers.

Age at Weaning of AI and Open heifers did not differ by breeding season

Age at weaning was compared between pregnant by AI and Open heifers across two breeding seasons (2018-2019 and 2019-2020) (Figure 2.7). There was no difference between ages at weaning at the Black Belt Research and Extension Center when comparing AI and Open heifers: 2018-2019 AI = 223.8 ± 12.48 days, Open = 219.3 ± 12.88 days ($p = 0.2710$, Figure 2.7A) ; 2019-2020 AI = 226.9 ± 14.04 days, Open = 230.8 ± 11.07 days ($p = 0.3798$, Figure 2.7B). Additionally, there was no significant difference overall between the age at weaning of AI (days) and Open (days) heifers ($p = 0.8666$).

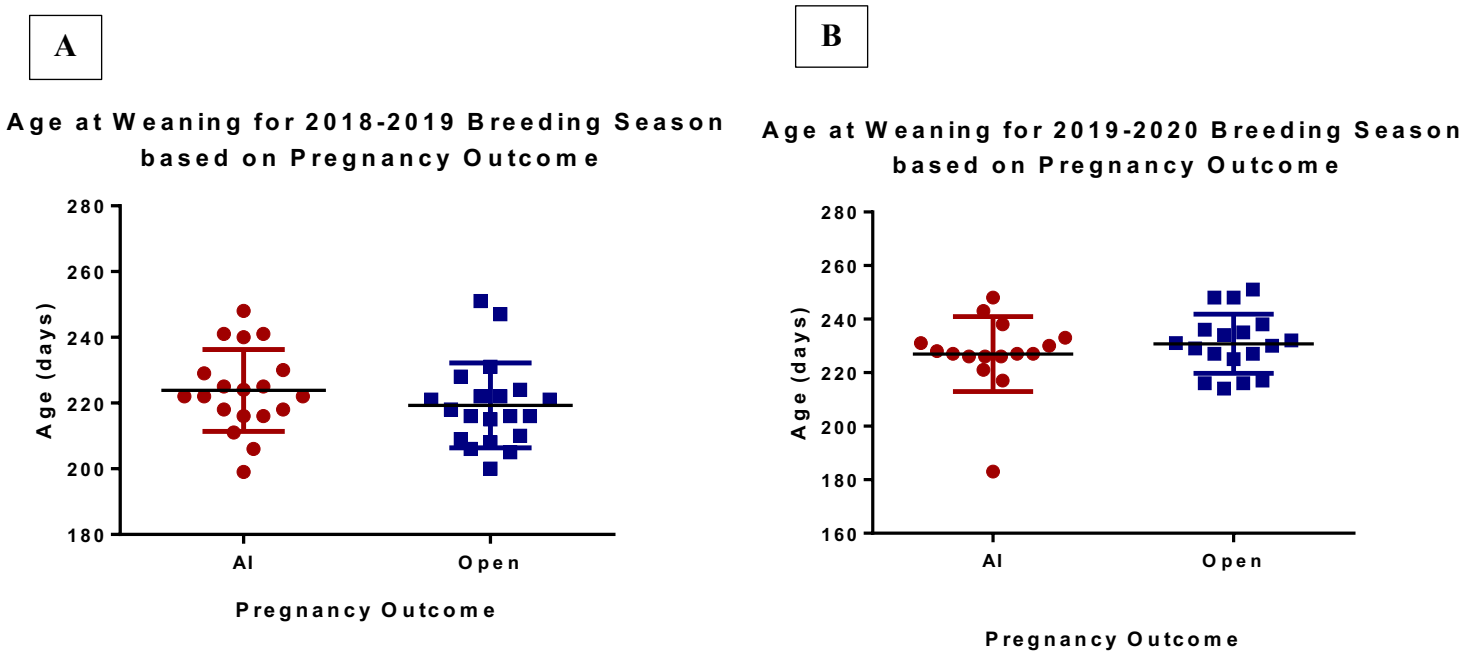


Figure 2.7. Graphs displaying Age at the time of Weaning of pregnant by AI and Open heifers over the course of two breeding seasons (2018-2019, 2019-2020). Data are mean ± standard deviation of the mean. No significant difference between ages at weaning of AI and Open heifers was found in either of the two breeding seasons ($p > 0.05$).

Weight at weaning of AI and Open heifers did not differ by breeding season

Weight at weaning was compared between pregnant by AI and Open heifers across two breeding seasons (2018-2019, 2019-2020) (Figure 2.8). There was no difference between weights at weaning at the Black Belt Research and Extension Center when comparing AI and Open heifers: 2018-2019 AI = 279.8 ± 18.58 kg, Open = 273.6 ± 18.91 kg ($p = 0.3114$, Figure 2.8A) ; 2019-2020 AI = 264.8 ± 25.34 kg, Open = 269.1 ± 25.48 kg ($p = 0.6221$, Figure 2.8B). Additionally, there was no significant difference overall between the weight at weaning of AI (kg) and Open (kg) heifers ($p = 0.7860$).

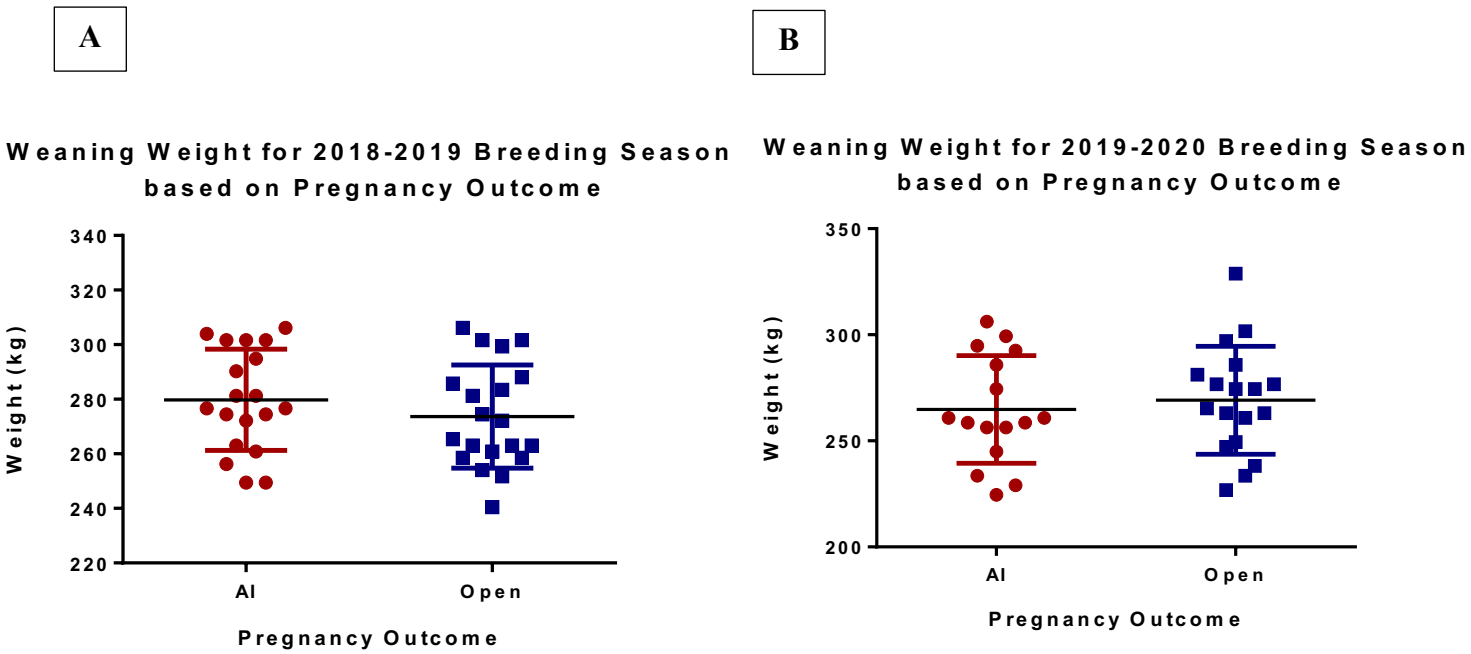


Figure 2.8. Graphs displaying Weight at the time of Weaning of pregnant by AI and Open heifers over the course of two breeding seasons (2018-2019, 2019-2020). Data are mean ± standard deviation of the mean. No significant difference between weight at weaning of AI and Open heifers was found in either of the two breeding seasons ($p > 0.05$).

BCS of AI and Open heifers did not differ by breeding season

Body Condition Score was compared between pregnant by AI and Open heifers across two breeding seasons (2018-2019 and 2019-2020) (Figure 2.9). There was no difference between BCS at weaning at the Black Belt Research and Extension Center when comparing AI and Open heifers: 2018-2019 AI = 6.026 ± 0.2621, Open = 6.0 ± 0.2294 ($p = 0.7402$, Figure 2.9A) ; 2019-2020 AI = 5.750 ± 0.4472, Open = 5.722 ± 0.3919 ($p = 0.8481$, Figure 2.9B). Additionally, there was no significant difference overall between the BCS of AI and Open heifers ($p = 0.7098$).

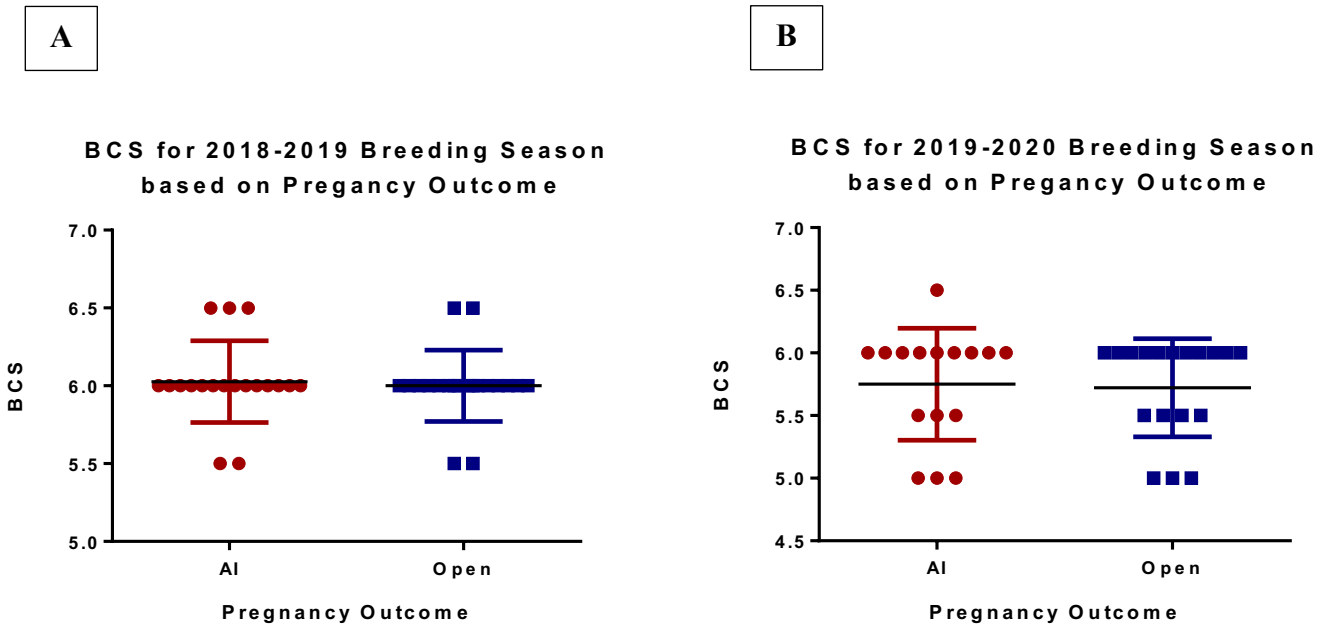


Figure 2.9. Graphs displaying Body Condition Score of pregnant by AI and Open heifers over the course of two breeding seasons (2018-2019 and 2019-2020). Data are mean ± standard deviation of the mean. No significant difference between body condition scores of AI and Open heifers was found in either of the two breeding seasons ($p > 0.05$).

RTS of AI and Open heifers did not differ by breeding season

Reproductive Tract Score was compared between pregnant by AI and Open heifers across two breeding seasons (2018-2019, 2019-2020) (Figure 2.10). There was no difference between RTS at weaning at the Black Belt Research and Extension Center when comparing AI and Open heifers 2018-2019 AI = 4.263 ± 0.6534, Open = 4.275 ± 0.7518 ($p = 0.9585$, Figure 2.10A) ; 2019-2020 AI = 3.875 ± 0.8851, Open = 3.778 ± 0.6468 ($p = 0.7149$, Figure 2.10B). Additionally, there was no significant difference overall between the RTS of AI and Open heifers ($p = 0.7957$).

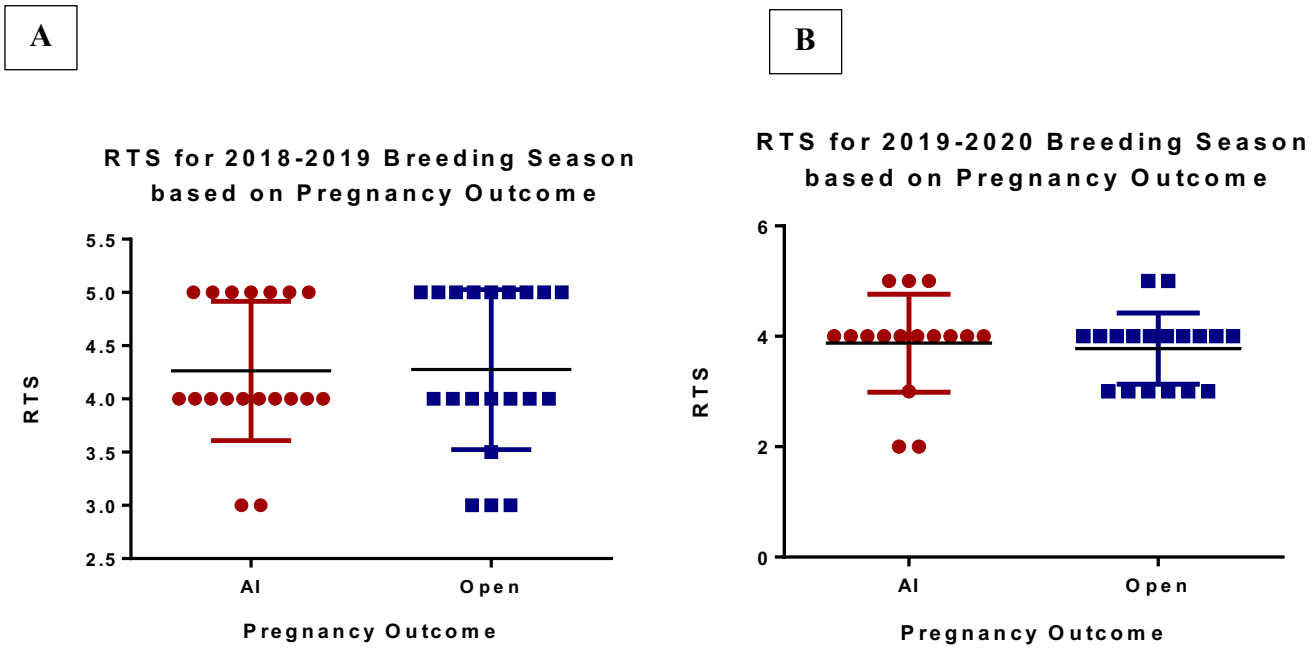


Figure 2.10. Graphs displaying Reproductive Tract Scores of pregnant by AI and Open heifers over the course of two breeding seasons (2018-2019 and 2019-2020). Data are mean ± standard deviation of the mean. No significant difference between reproductive tract scores of AI and Open heifers was found in either of the two breeding seasons ($p > 0.05$).

Pelvic Area of AI and Open heifers did not differ by breeding season

Pelvic Area was compared between pregnant by AI and Open heifers across two breeding seasons (2018-2019 and 2019-2020) (Figure 2.11). There was no difference between PA at weaning at the Black Belt Research and Extension Center when comparing AI and Open heifers: 2018-2019 AI = 190.9 ± 19.94 cm^2 Open = 191.6 ± 11.91 cm^2 ($p = 0.9324$, Figure 2.11A) ; 2019-2020 AI = 172.8 ± 11.43 cm^2 Open = 177.3 ± 7.340 cm^2 ($p = 0.1690$, Figure 2.11B). Additionally, there was no significant difference overall between the pelvic area of AI (cm^2) and Open (cm^2) heifers ($p = 0.7046$).

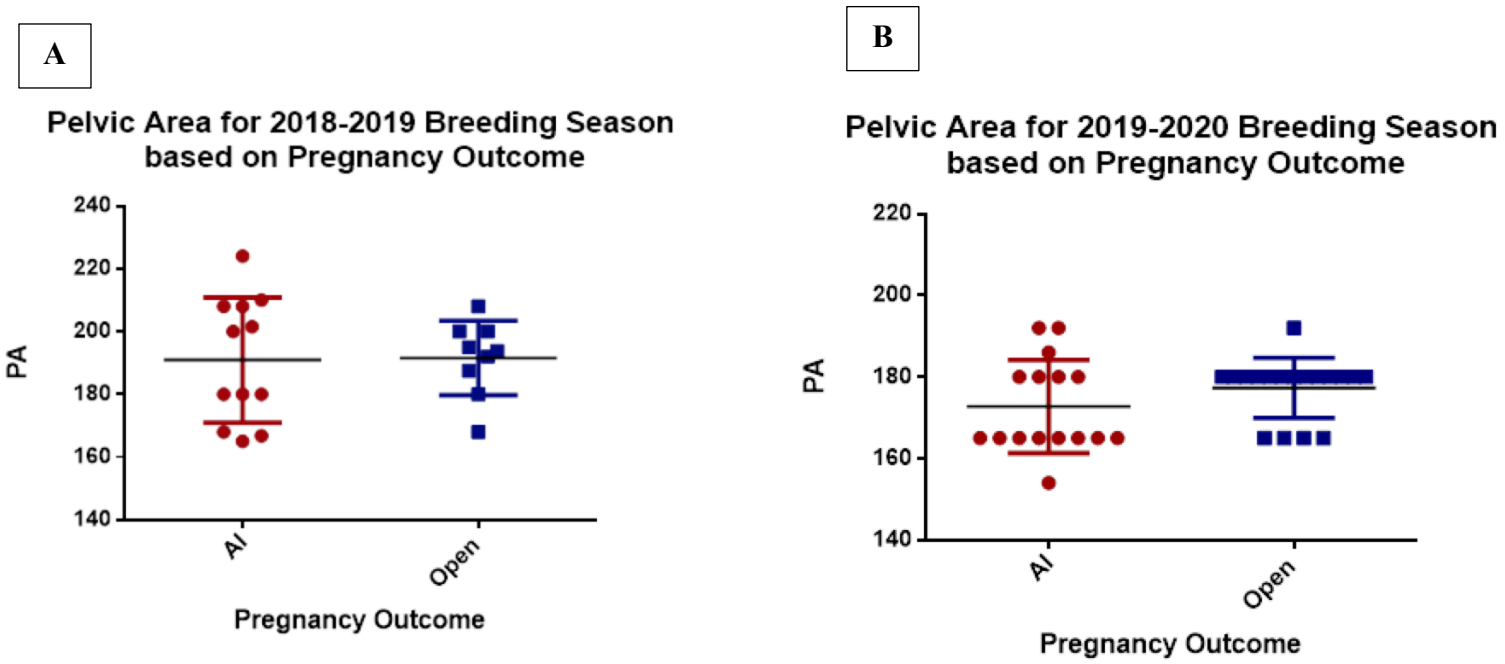


Figure 2.11. Graphs displaying Pelvic Area of pregnant by AI and Open heifers over the course of two breeding seasons (2018-2019 and 2019-2020). Data are mean ± standard deviation of the mean. No significant difference between pelvic area of AI and Open heifers was found in either of the two breeding seasons ($p > 0.05$).

II.V. DISCUSSION

Heifer reproductive failure is one of the main factors that effects the profitability and success of a cow calf operation. The ability to evaluate and identify heifers with the most potential for reproductive success is one of the more challenging tasks a producer is faced with when raising replacement heifers. While there are newer reproductive technologies that are being used to improve heifer development, producers still tend to lean more towards traditional methods such as evaluations in age at weaning, weaning weight, BCS, RTS and PA. The careful evaluation of heifers based on phenotypic characteristics that could have an effect on fertility has the potential to improve the longevity and stayability of all heifers within a herd.

One of the goals of our study was to compare traditional methods of selecting replacement heifers. Evaluations in phenotypic characteristics such as age at weaning, weaning weight, BCS, RTS and PA were compared at one location (Black Belt Research and Extension Center) across two breeding seasons (2018 – 2019 and 2019 – 2020) as well as across pregnancy outcomes [pregnant by artificial insemination (AI) or those remaining open following AI and 60 days of bull exposure (Open)]. While traditional phenotypic characteristics can be useful in minimizing reproductive inefficiencies, as can be seen in our data, they are not able to completely identify heifers with reproductive issues.

One of the first characteristics that is evaluated at the time of weaning is the age of the heifer. Not only does her age determine the time at which she should be weaned, it also plays a role into when she will reach puberty. It has been previously determined that, on average, the age at which Angus-Simmental cross heifers reach puberty was 303 days ± 10

days, or 10 months with most *Bos taurus* breeds reaching puberty by 14 months (Gunn et al, 2015). When evaluating the age of heifers at weaning included in this study, we did not see a statistically significant difference between the days of age in heifers when comparing pregnancy outcome (Figure 2.7), but we did see a statistically significant difference in age across the two breeding seasons (Figure 2.2). When looking at the age at weaning between AI and Open heifers, it can be concluded that age is not an ideal characteristic to be used alone in determining reproductive success in heifers.

Heifer's weight and percentage body fat at is related to the age at which she will reach the onset of puberty. It has been previously shown that while weaning weight is not highly correlated with pregnancy outcome, the age at which heifers reach 53% of their mature body weight can have the most influence on reproductive potential (Dickinson et al., 2019). To reach this target weight of at least 53% of mature body weight, producers calculate the ratio between the average weight of heifers in a group divided by the average mature weight of the multiparous cows in the herd that produced the heifers (Larson et al., 2016). This calculation results in a target body weight that is specific to the cow-calf operation instead of acquiring a target weight from a statistical number not specific to the operation. This is also useful as it has been proven that heifers that are fed to reach 55% to 65% of their mature body weight have better reproductive performance. When evaluating weaning weight in the heifers included in this study, we did not see a statistically significant difference between weaning weight in heifers when comparing pregnancy outcome (Figure 2.8) or breeding season (Figure 2.3).

In order to maintain body weight and reach puberty at an early age, heifers need to maintain a higher level of body condition. A study by Rossi et al. (2014) has shown that percentage of fat deposition in beef cows is directly related to onset of puberty and, in turn, successful reproductive performance. Body condition scores range from 1 to 9 with 1 being very thin to 9 having excessive fat cover (Appendix 1). A study by Dickinson et al. (2019) concluded that beef heifers with a body condition score of 6, combined with their reproductive tract score, presented the greatest pregnancy rate at the end of the breeding season. In our study, we did not see a statistically significant difference between BCS in heifers when comparing pregnancy outcome (Figure 2.9), but we did see a statistically significant difference in BCS across the two breeding seasons (Figure 2.4). While all heifers included in our study were housed at one location under similar management year to year, it is important to point out that heifers that are managed differently and located in different farm environments are subject to different BCSs due to different management practices. While body condition scoring heifers can help to identify those that may be undernourished prior to breeding, BCS alone is shown to not be enough to accurately determine reproductive potential of a replacement heifer.

Reproductive tract scoring is an inexpensive and useful way for producers to be able to determine puberal status within replacement heifers while also helping producers to group heifers for breeding purposes. Anderson et al. (1991) has concluded that heifers with more mature reproductive tracts have shown to have higher pregnancy rates and will also calve earlier in the breeding season. Measurements that are taken in order to come up with a RTS include the length, height and width of the uterine horns as well as structures being present on the ovary. RTSs range on a scale from 1 to 5 with 1 being an immature

tract to 5 being a mature tract with a CL present (Appendix 2). A previous study by Rosenkrans and Hardin (2002) evaluated heifer puberty status by RTS in relation to pregnancy outcome. Their study showed that reproductive tract scoring was 79% to 82% accurate in determining cycling status in relation to puberty. It has also been shown that heifers with a RTS of at least a 4 were able to conceive and calve earlier in the breeding season (Holm et al. 2014 and Dickinson et al. 2019). In our study, we did not see a statistically significant difference between RTS in heifers when comparing pregnancy outcome (Figure 2.10), but we did see a statistically significant difference in RTS across the two breeding seasons (Figure 2.5). All of the data presented validated that reproductive tract scoring is an accurate system to determine puberal status in replacement heifers, but not reproductive potential.

Lastly, evaluations of pelvic area can serve as a predictive tool in determining a heifers reproductive success as well as being indicative of potential calving difficulty. A producer should aim to develop heifers that not only calve early in the calving season, but also do so without difficulty. Pelvic area is calculated by multiplying pelvic width by pelvic height. Theoretically, the larger the pelvic area is, the less calving difficulty she is expected to have. A previous study in 1988 by Johnson et al. concluded that pelvic area measurements alone were the most predictive of calving ease with a heifer's first calf. This measurement can also serve as an important tool in deciding which bull to breed heifers to since a low pelvic area might sway a producer to breed that heifer to a low birth weight bull. When comparing heifer PAs in our study, we did not see a statistically significant difference between PA in heifers when comparing pregnancy outcome (Figure 2.11), but we did see a statistically significant difference in PA across the two breeding seasons

(Figure 2.6). The heifer's in the 2019-2020 breeding season had a significantly lower PA compared to the other breeding season. While PA measurements aid in identifying heifer with possible calving difficulty, they were not able to accurately determine which heifers would conceive by AI or remain Open.

The beef industry relies heavily on successful reproductive performance in order to be profitable and reach production goals. By evaluating and using phenotypic traits wisely, infertility in beef heifers can be avoided and will help to improve the longevity and stayability of all heifers in the herd. Evaluating phenotypic parameters such as age at weaning, weaning weight, BCS, RTS and PA can aid in replacement heifer development, but together cannot accurately determine the reproductive success of specific heifers. Limitations still remain in being able to determine the reproductive success of a beef heifer at the time of weaning.

CHAPTER III.

INVESTIGATING PLASMA METABOLOMIC PROFILES AT THE TIME OF WEANING, BASED ON PREGNANCY OUTCOME, IN BOS TAURUS HEIFERS

III.I. ABSTRACT

There is currently a need for a comprehensive list of biomarkers that can serve as indicators of disease or reproductive potential in livestock species. Metabolomics offers a unique opportunity to understand system-wide metabolism of a certain livestock species. Overall, the use of metabolomics is becoming more common in agriculture in an effort to improve overall herd health and reproductive efficiency. For this study, heifer blood plasma underwent metabolomic profiling to identify potential metabolites that could serve as biomarkers for fertility potential at the time of weaning. Thirty-four Angus-Simmental cross heifers housed at Black Belt Research and Extension Center in Marion Junction, AL were included in the study. Whole blood was collected at weaning and plasma was isolated. Phenotypic parameters such as BCS, RTS, Weaning Weight, Age at Weaning and Pelvic Area were measured before artificial insemination. All phenotypic parameters were not significantly different between heifers pregnant by AI and those that remained open after 60 days of bull exposure over two breeding seasons.

Heifer metabolomic profiles identified ten metabolites (Alanine, Cystine, Lysine, Tyrosine, Valine, Tryptophan, Methionine, Glycerol, Frusctose-6-Phosphate, Ribulose-5-Phosphate) that were shown to be significantly different (T-test; $p < 0.05$; FDR < 0.05) between heifers pregnant by AI and those that remained open after AI and 60 days of bull

exposure. To be able to determine the accuracy of predication in using these metabolites as biomarkers for reproductive potential, we calculated the receiver operating characteristic-area under the curve (ROC-AUC) values for the ten significantly different metabolites. Six metabolites (Alanine, Methionine, Valine, Lysine, Tyrosine and Cystine) had a ROC-AUC above our cut off of 0.80. Four metabolites (Tryptophan, Fructose-6-Phosphate, Ribulose-5-Phosphate and Glycerol) had a ROC-AUC value of 0.78 or lower meaning they would have the least accuracy when determining reproductive potential in heifers.

The role of inflammation in relation to fertility was also considered in this study. The levels of inflammatory cytokines were compared at the time of weaning between heifers pregnant by AI and those that remained open after AI and 60 days of bull exposure. We found significantly higher expression of proinflammatory cytokine transcripts such as Tumor Necrosis Factor alpha (*TNFα*), Interleukin 6 (*IL-6*), neutrophil activating peptide C-X-C Motif Chemokine 5 (*CXCL5*), monocyte chemoattractant protein-one (MCP1) and Periostin (POSTN) in infertile heifers compared with fertile heifers (T-test; $p < 0.05$; Fold Change > 2). Lastly, ELISAs were performed to detect cytokine proteins within the blood plasma for TNFα and IL6. The differences between heifers pregnant by AI and open was not significant with ($p > 0.05$). In summary, the quantity of specific metabolites present within the blood plasma are different at weaning between heifers with differing reproductive potentials. This could potentially be used to develop an assay to aid in selecting replacement heifers.

III.II. INTRODUCTION

With phenotypic characteristics not serving as an accurate indicator of reproductive potential in heifers, producers need a reliable method to discriminate between fertile and infertile heifers, ideally before the start of the breeding season. Fertility is defined as an animal that has the desire and ability to mate, conceive and nourish an embryo and to expel a healthy calf with the fetal membranes (Abraham, 2017). While there has been many studies focusing on cow fertility, there has only been a handful of studies that focus strictly on the challenges of heifer fertility and ways to improve conception rates. When heifer infertility is discussed, there are a plethora of reasons why a heifer could be deemed infertile including incorrect management, genetics and the environment which make pin pointing one cause for infertility a difficult task.

Currently, there is a need for a list of potential biomarkers that could serve as an indicator of reproductive potential in beef cattle. Metabolomics is a relatively new method that is being used in the biomedical, and more recently, the livestock industry to be able to detect possible diseases and describe the overall function of bodily systems in all living species. The metabolome is commonly described as the measurement of low molecular weight molecules that are present in cells in a particular physiological or developmental state (Oliver et al., 1998). These low molecular weight molecules can be amino acids, carbohydrates, organic acids and vitamins found in various tissues, biological fluids or cell culture media. The most attractive thing about metabolomics is that it is serving as a tool to evaluate complex biological systems that are required for the maintenance, growth and normal function of a cell (Goodacre et al., 2004). Metabolomics has been used in human

medicine to detect metabolomic diseases (Shaham et al., 2009), various cancer types (Zhang et al, 2012; Asiago et al., 2010) infertility in men (Xu et al., 2020; Zhou et al., 2015; Zhang et al., 2013) as well as female infertility (Dutta et al., 2012; Wallace et al,. 2012; Nagy et al., 2008). Within the livestock industry, metabolite concentrations have been utilized to study crops, phenotypic changes in animals as well as dietary responses in livestock species. In relation to fertility, it has been used to evaluate metabolites present in blood plasma (Phillips et al., 2018), follicular fluid (Bender et al., 2010), embryo development (Perkel and Madan, 2017), along with determining bull fertility (Menezes et al., 2019). Researchers are still working to generate a list of metabolites and pathways involved in pregnancy. This growing list will be useful in determining reproductive potential in a number of species. Metabolomic profiling is an attractive method to investigate heifer fertility at the time of weaning.

In addition to metabolomic profiling, we also investigated the role of inflammation and the impact it may have on pregnancy outcome. Infertility related to immune regulation has been shown to affect 1 out of 5 couples who are experiencing challenges trying to conceive. (Brazdova et al., 2016). When there is a heightened immune response in the body, it can have an effect on normal ovulation as well as hormone production, which could lead to infertility (Weiss et al., 2009). A previous study by Phillips et al. (2018) investigated the role of inflammation on fertility by comparing the transcript expression of inflammatory cytokines in white blood cells between fertile and infertile heifers. This study showed that, at the time of artificial insemination, proinflammatory cytokines such as Tumor Necrosis Factor alpha (*TNFα*), Interleukin 6 (*IL-6*), neutrophil activating peptide C-X-C Motif Chemokine 5 (*CXCL5*), monocyte chemoattractant protein-one (MCP1) and

Periostin (POSTN) were significantly higher in infertile heifers. Current research is limited on linking heifer infertility to inflammation which is why we decided to investigate these inflammatory cytokines earlier in the heifer's life time at the time of weaning. If a producer is able to identify infertile heifers before the time of artificial insemination, it will save them time and money in the end by investing in a heifer that will not become a productive member of the herd.

Studies linking differential metabolites and inflammation to possible heifer infertility are lacking. We hypothesized that there is a relationshop between the inflammatory status of the heifers and their metabolomic profiles. In our study, we analyzed heifer blood plasma at the time of weaning by conducting comprehensive metabolomic profiling as well as determining the role of inflammatory cytokines present in white blood cells. Blood plasma samples were analyzed via untargeted profiling of primary metabolism by automatic linear exchange/cold injection gas chromatography time-of-flight mass spectrometry (GC-TOF-MS). We compared heifers that were pregnant by AI to those that remained open after AI and 60 days of bull exposure. Metabolomic profiling was analyzed from one breeding season (2019-2020) from heifers housed at the Black Belt Research and Extension Center in Marion Junction, AL. Lastly, we analyzed and compared the expression levels of proinflammatory cytokine mRNA expression in white blood cells as well as the plasma cytokine levels of heifers pregnant by AI and those that remained open after AI and 60 days of bull exposure.

III.III. MATERIAL AND METHODS

Animal Use

All studies utilizing animals were approved by the Auburn University Institutional Animal Care and Use Committee (IACUC). Heifers used for this study (N = 34) were originated from and housed at the Black Belt Research and Extension Center of the Alabama Agricultural Experiment Station in Marion Junction, AL, U.S.A.

Reproductive Management

Angus-Simmental cross heifers participated in an estrus synchronization and artificial insemination program by estrus detection {7-day CO-Synch + CIDR® (Whittier et al., 2013)] over the course of one breeding season (2019-2020). Seven days before artificial insemination, heifers were given 2 cc of GnRH intramuscularly (CYSTORELIN®, Boehringer Ingelheim Animal Health, Athens, GA, USA). At the same time of injection, a controlled internal drug release (CIDR®) consisting of 1.38 g of progesterone was inserted intravaginally (EAZI-BREEDTM CIDR® Cattle Insert, Zoetis, Kalamazoo, MI, USA). After seven days, the CIDR® was removed and 5 cc of dinoprost tromethamine (LUTALYSE®, Zoetis, Kalamazoo, MI, USA) was intramuscularly injected. Over the next 48 hours, heifers were observed for signs of estrus and artificially inseminated 12 hours later following the AM/PM rule. Heifers were artificially inseminated with a single straw of semen from Angus sires of proven fertility. Heifers that did not show signs of estrus 72 hours after CIDR® removal were bred via AI and injected with 2 cc of GnRH intramuscularly (CYSTORELIN®, Boehringer Ingelheim Animal Health, Athens, GA,

USA). Fourteen days following AI, heifers were exposed to a clean-up bull of proven fertility for 60 days. Bulls exposed to heifers had previously passes a BSE (Breeding Soundness Exam) and had < 10% semen abnormalities. The 34 heifers were exposed to one bull for 60 days following artificial insemination.

Heifer Nutrition Management

Heifers housed at the Black Belt Research and Extension Center were grazed on fescue pasture with unlimited access to ryegrass hay supplemented 50:50 with Soyhull + Corn Gluten at 1% of BW per day to achieve a target gain of 1 kg/heifer/day. Trace minerals were available *ad libitum.*

Phenotypic Observations

Phenotypic conditions of 104 heifers (N = 104) over the course of two breeding seasons (2018-2019 and 2019-2020) were used and analyzed for this study. A trained veterinarian evaluated body condition score (BCS) (Appendix 1), reproductive tract score (RTS) (Appendix 2), Weaning Weight, Age at Weaning as well as Pelvic Area. Weight was determined at the time of weaning on all heifers. Age was calculated by the number of days between birth and day of weaning. BCS was determined at the time of weaning as previously described and ranged on a scale of 1-9 with 1 being very thin and 9 being very obese (Herd and Sprott, 1986). A trained veterinarian evaluated reproductive tract score (RTS) one month prior to breeding. Scores ranged from 1-5 with 1 being an immature tract and 5 being a very mature tract with signs of cycling (Anderson et al., 1991).

Blood Collection and Processing

At the time of weaning, an 18G needle was used to collect 10 mL of blood into an EDTA blood collection tube (BD Vacutainer) via tail vein on each heifer. The blood tube was inverted 10 times and placed on ice upon being transferred back to the Reproductive and Developmental Biology Lab at the Center for Advanced Science, Innovation and Commerce in Auburn, AL. After arrival, blood samples were sprayed with 70% ETOH to rid of possible on farm contamination. Samples were centrifuged for 10 minutes at 1,500 x *g* at 4°C. Two 500 µl plasma samples were placed into cryogenic tubes and stored at -80°C.

Buffy Coat Isolation

While avoiding red blood cells and remaining plasma, 500 of µl the buffy coat (leukocytes) were aspirated and added to a sterile 15 ml centrifuge tube containing 10 ml of red blood cell lysis buffer (0.15 mM ammonium chloride, 10 µM sodium bicarbonate, and 1.3 µM EDTA) and inverted periodically. After 10 minutes of incubation, samples were spun down at 4°C for 5 minutes at 500 x *g.* Supernatant was poured off and the pellet was moved to a 1.5 ml micro-centrifuge tube containing 1 ml ice cold PBS supplemented with 2% fetal bovine serum. Pellet was additionally spun for 5 min at 500 x *g,* Supernatant was poured off and the pellet was stored at -80°C until further processing.

Pregnancy Determination

A trained veterinarian determined pregnancy via rectal palpation 45 and 65 days post artificial insemination. Heifers were grouped based on pregnancy outcome as follows: pregnant (AI), pregnant (Bull) or infertile if not pregnant following AI and bull exposure. Heifers that were pregnant by AI and Open after 60 days of bull exposure were analyzed for metabolite levels.

Heifer selection for metabolomic analysis

9 heifers pregnant by AI (fertile) and 11 open (infertile) were chosen for metabolomic profiling. Heifers were grouped for the 2019-2020 breeding season based parameters such as similarities in age, phenotypic characteristics and status of puberty. All heifers were housed at the Black Belt Research and Extension Center from the time of weaning until calving.

Metabolomics Data Collection

Blood plasma isolated from whole blood samples at the time of weaning were collected and used to identify different levels of metabolites from 20 animals (N = 9 pregnant by AI and N = 11 non-pregnant). Samples had metabolomic profiles generated via untargeted profiling of primary metabolism by automatic linear exchange/cold injection at the West Coast Metabolomics Center (Davis, California, U.S.A.). An Agilent 6890 GC equipped with a Gerstel automatic liner exchange system (ALEX) that includes a multipurpose sample (MPS2) dual rail, and a Gerstel CIS cold injection system (Gerstel, Muehlheim, Germany) was used to collect GC-TOF. Temperature program was as follows:

50°C to 275°C final temperature at a rate of 12 °C/s and hold for 3 minutes. Injection volume is 0.5 µl with 10 µl/s injection speed on a splitless injector with purge time of 25 seconds. Liner (Gerstel #011711-010-00) is changed after every 10 samples (using the Maestro1 Gerstel software vs. 1.1.4.18). Before and after each injection, the 10-µl injection syringe is washed three times with 10 µl ethyl acetate. Data were acquired with the following chromatographic parameters: column used Rtx-5Sil MS (30 m X 0.25 mm diameter Restek corp.) with a 0.25-µm 95% dimethyl/5% diphenylpolysiloxane film; mobile phase Helium with a 1 mL/min flow rate; injection volume 0.5 µL [18] . The oven temperature is held constant at 50°C for 1 min and then ramped at 20°C/min to 330°C at which it is held constant for 5 min. A Leco Pegasus IV time of flight mass spectrometer is controlled by the Leco ChromaTOF software vs. 2.32 (St. Joseph, MI). The transfer line temperature between gas chromatograph and mass spectrometer is set to 280°C. Electron impact ionization at 70V is employed with an ion-source temperature of 250°C. Acquisition rate is 17 spectra/second, with a scan mass range of 85-500 Da. Raw data files were preprocessed directly using ChromaTOF vs. 2.32 without smoothing, 3-s peak width baseline subtraction just above the noise level, and automatic mass spectral deconvolution and peak detection at signal to noise levels of 5:1. Absolute spectra intensities were further processed by a filtering algorithm implemented in the metabolomics BinBase database. The BinBase algorithm used the following settings: validity of chromatogram (< 10 peaks with intensity >10^7 counts/s), unbiased retention index marker detection (MS similarity > 800, validity of intensity range for high m/z marker ions), retention index calculation by 5th-order polynomial regression. Spectra are cut to 5% base peak abundance and matched to database entries from most to least abundant spectra using the following matching filters:

retention index window ± 2,000 units (equivalent to about ±2 s retention time), validation of unique ions and apex masses (unique ion must be included in apexing masses and present at >3% of base peak abundance), mass spectrum similarity must fit criteria dependent on peak purity and signal/noise ratios and a final isomer filter. Failed spectra are automatically entered as new database entries if s/n >25, purity < 1.0 and presence in the biological study design class was >80%. All thresholds reflect settings for ChromaTOF v. 2.32. Quantification is reported as peak height using the unique ion as default, unless a different quantification ion is manually set in the BinBase administration software BinView. A quantification report table is produced for all database entries that are positively detected in more than 10% of the samples of a study design class (as defined in the miniX database) for unidentified metabolites. The data were then prepared as peak heights for the quantification ion at the specific retention index. Binned data were normalized and scaled to remove potential bias arising due to sample handling and variability. Normalization by sum was performed followed by scaling (mean-centering and division by the square root of standard deviation of each variable), to give all variables equal weight regardless of their absolute value.

Univariate Statistical Analysis

Univariate analysis was applied to a total of 155 chemically recognized metabolites from 9 fertile (Pregnant by AI) and 11 infertile (Open) plasma samples from heifers. In order to minimize concentration differences, data was normalized by sum. Following normalization, scaling (mean-centering and division by the square root of standard deviation of each variable) was performed to equally weigh each variable, regardless of

absolute value. T-tests were formed with an FDR cutoff of 0.05. Metabolites were considered significantly different when $p \leq 0.05$. Data is presented as mean ± standard deviation of the mean.

Multivariate Statistical Analysis

A total of 155 chemically recognized metabolites from 9 fertile (Pregnant by AI) and 11 infertile (Open) heifer's plasma samples was analyzed via multivariate analysis. In order to minimize concentration differences, data was normalized by sum. Following normalization, scaling (mean-centering and division by the square root of standard deviation of each variable) was performed to equally weigh each variable, regardless of absolute value. In order to maximize class discrimination, Partial Least Squares Discriminant Analysis (PLS-DA) was then performed using MetaboAnalyst [accessible at http://metaboanalyst.com (Sabatine et al., 2005)] using functions from the R and Bioconductor packages (Zhang et al., 2012). Model robustness was assessed using ROC-AUC analysis using MetaboAnalyst software. Classification models were built based on metabolites showing significantly different levels ($p < 0.05$; FDR > 0.05) with at least a 2-fold difference.

Metabolic Pathway Analysis

Metabolic Pathway Analysis was performed using MetaboAnalyst 5.0. To correctly identify relative pathways involved in both fertile and infertile heifers, Pathway Analysis combined results from Pathway Enrichment Analyses and Pathway Topology Analyses. Parameters for Metabolic Pathway Analysis included normalization by sum and Pareto

data scaling (mean-centered and divided by the square root of the standard deviation of each variable presented).

RNA Isolation and cDNA Synthesis

Total buffy coat RNA was isolated from the pelleted white blood cell sample using the illustra™ RNAspin Mini RNA Isolation Kit (GE Healthcare, Buckinghamshire, UK) following the manufacturer's instructions. Samples were subjected to DNase treatment for 15 minutes at room temperature. After extraction, RNA was then quantified using a Qubit Fluorometer (Termo Fisher Scientific). RNA was additionally analyzed by the Bioanalyzer RNA 6000 Nano (Agilent Technologies) to obtain an objective measurement of RNA quality with RIN (RNA Integrity Number). Only RNA samples with a RIN greater that 9 were further analyzed. Two µl of isolated RNA was then reverse-transcribed (RT) into cDNA using qScript cDNA Supermix (Quanta BioSciences Inc., Beverly, MA).

Real-time PCR

C_q values from the PCR data of the samples were normalized to the C_{qs} of three reference genes, *GAPDH, B2M and TBP,* using the ΔΔCt method to account for the variations in RNA concentrations. RNA isolations from eight animal, four top performers (pregnant by AI) and four poor performers (Open) were used to determine inflammatory status. The isolated RNA was reverse-transcribed (RT) to cDNA using qScript cDNA Supermix (Quanta BioSciences Inc., Beverly, MA) according to the manufacturer's recommended protocol. Primers for *GAPDH, TNFα, IL-6, CXCL5, POSTN,* and *MCP1* were validated for product specificity and efficiency tested prior to use (Table 3.5). A

Roche Light Cycler 480 Real-time qPCR machine was utilized to compare the expression levels of the target transcripts using the delta-delta Cq method (Schmittgen and Livak, 2008). *GAPDH* was used as in internal loading control (Dutta et al., 2012). The qPCR reactions were ran using PerfeCTa SYBR Green Supermix (Quanta Biosciences Inc., Beverly, MA) according to the manufacturer's protocol.

TNFα Enzyme Linked Immunosorbent Analysis (ELISA)

An Invitrogen TNFα ELISA kit (Thermo Fisher, Catalog #: EBTNF) was utilized according to the manufacturer's protocol to detect TNFα proteins within in heifer blood plasma. Standard diluent B was used in order to generate a standard curve. Samples were diluted 2-fold before addition to the pre-coated well. After binding the antigen, biotin conjugate was added, followed by Streptavidin-HRP, a TMB substrate and lastly the stop solution. Wells were washed accordingly between steps with 1 x wash buffer. Upon the addition of the stop solution, the plate was analyzed using an EMax® Plus microplate reader at a 450 nm absorbance.

IL-6 Enzyme Linked Immunosorbent Analysis (ELISA)

A IL-6 Bovine ELISA Kit (G-Biosciences, Catalog #: IT1159) was utilized according to the manufacturer's protocol to detect IL-6 proteins within heifer blood plasma. The sample/standard dilution buffer was used in order to generate a standard curve. Samples were diluted 2-fold before addition to the pre-coated well. After binding to the antigen, biotin-labeled antibody was added, followed by HRP-Streptavidin Conjugate (SABC), and TMB substrate and lastly the stop solution. Wells were washed accordingly

between steps with 1 x wash buffer. Upon the addition of the stop solution, the plate was analyzed using an EMax® Plus microplate reader at a 450 nm absorbance.

III.IV RESULTS

Phenotypic Heifer Assessment

Phenotypic Parameters did not differ based upon Fertility Outcome

Phenotypic body condition and reproductive parameters such as BCS, Weaning Weight, Age at Weaning, RTS, and PA were determined in 104 heifers (N = 104) housed at the Black Belt Research and Extension Center in Marion Junction, Alabama, U.S.A. All measurements were taken over the 2018-2019 and 2019-2020 breeding seasons in order to determine if phenotypic characteristics could differentiate between heifers pregnant by AI and those that remained open after 60 days with bull exposure. No significant difference ($p = 0.8666$) was seen in age at weaning between heifers becoming pregnant by AI (225.3 ± 13.11 days) or those remaining open (224.7 ± 13.24 days) (Figure 3.1A). No significant difference ($p = 0.7860$) was seen in weight at weaning between heifers becoming pregnant by AI (272.9 ± 22.89 kg) or those remaining open (271.5 ± 22.07 kg) (Figure 3.1B). No significant difference ($p = 0.7098$) was seen in body condition scores (BCS) between heifers becoming pregnant by AI (5.900 ± 0.3796) or those remaining open (5.868 ± 0.3426) (Figure 3.1C). No significant difference ($p = 0.7957$) was seen in reproductive tract scores (RTS) between heifers becoming pregnant by AI (4.086 ± 0.7811) or those remaining open (4.039 ± 0.7387) (Figure 3.1D). No significant difference ($p = 0.7046$) was seen in pelvic area (PA) between heifers becoming pregnant by AI (180.5 ± 17.85 cm^2) or those remaining open (182.1 ± 11.21 cm^2) (Figure 3.1E). Data are presented as mean ± standard deviation of the mean.

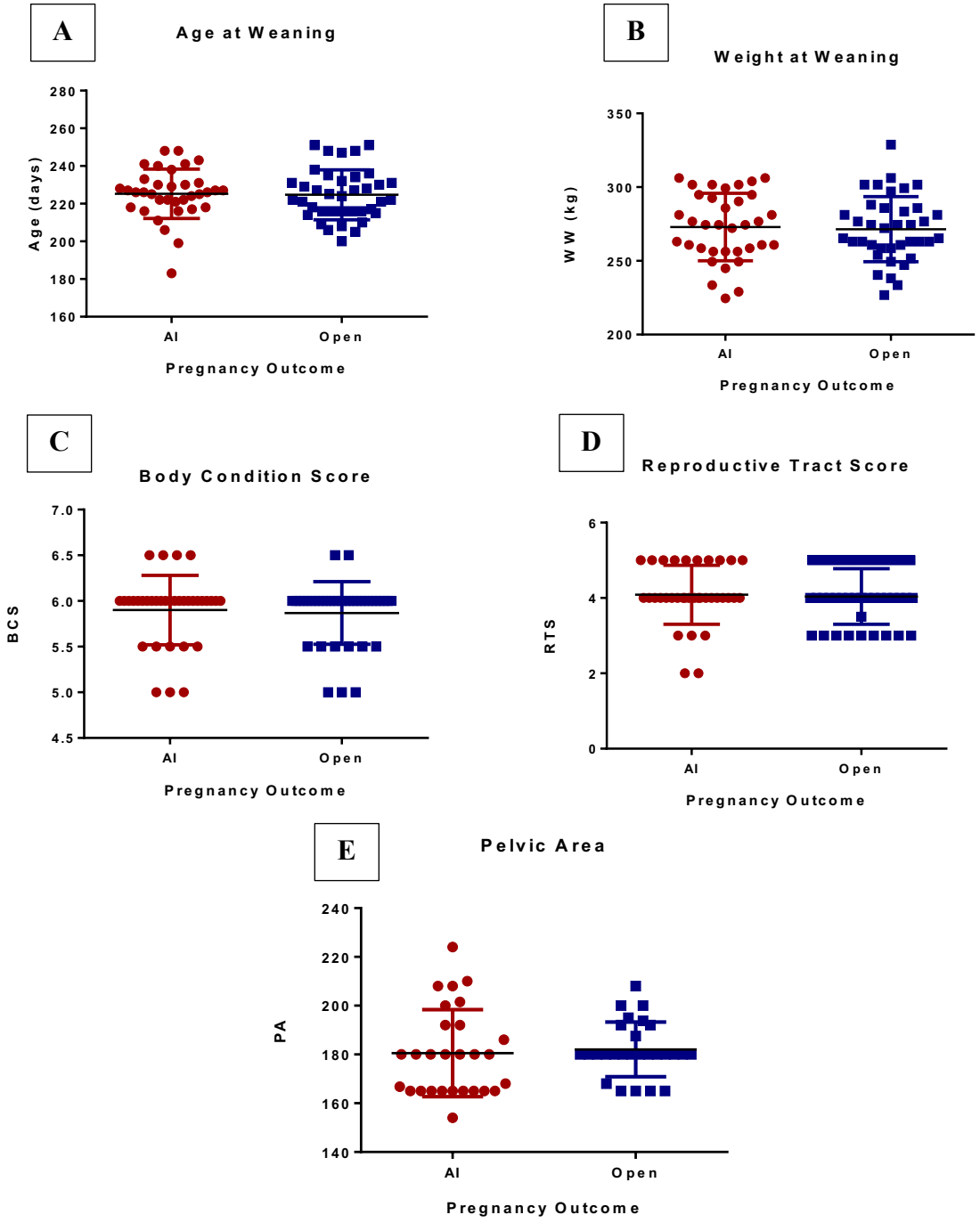


Figure 3.1. Phenotypic comparisons between heifers pregnant by AI and those that remained open. No significant difference was seen in Age at Weaning, Weight at Weaning, BCSs, RTSs, or PAs ($p > 0.05$). Data are mean ± standard deviation of the mean.

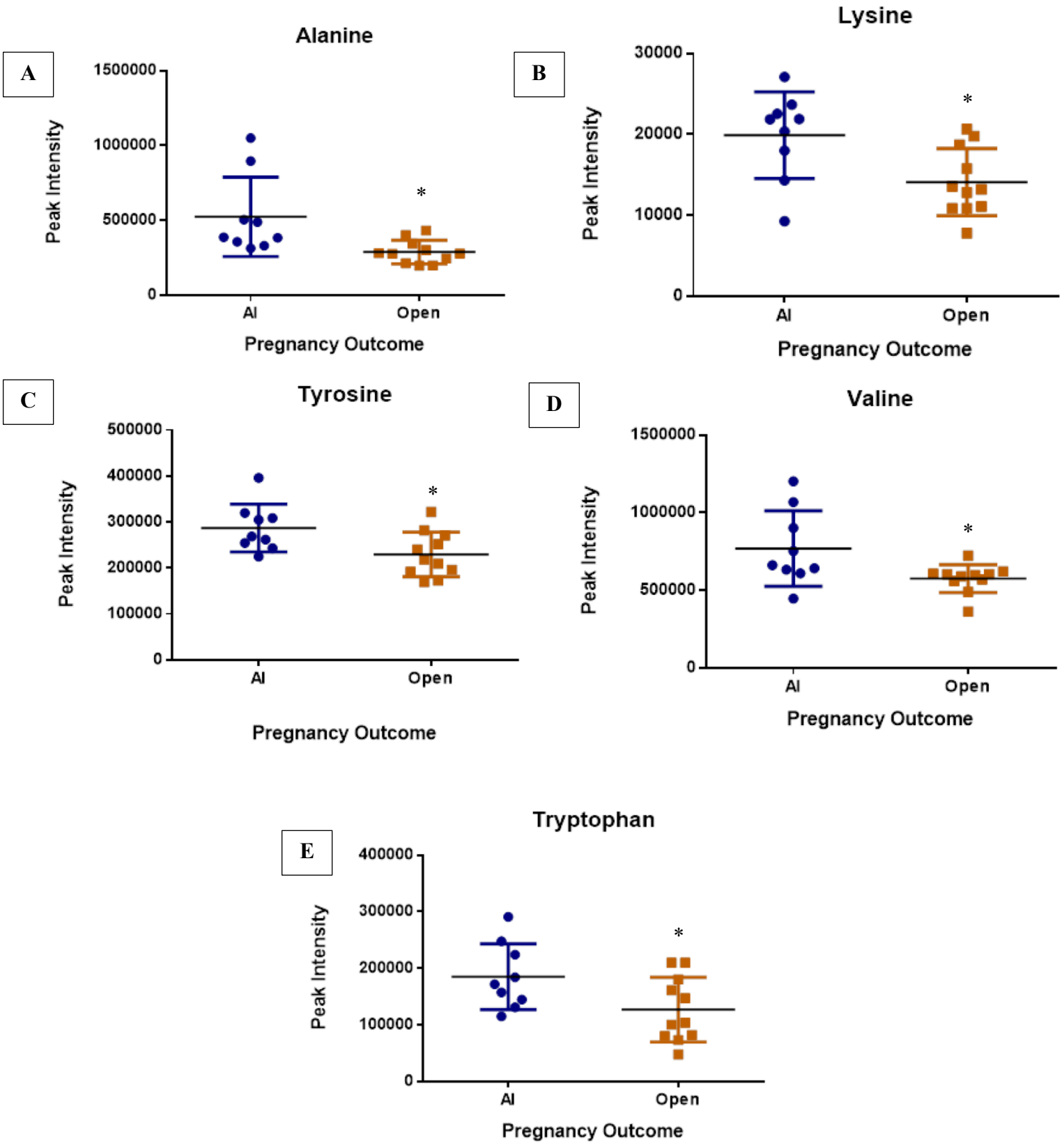
Metabolome Assessment and Analysis based upon Pregnancy Outcome

Ten metabolites were found to be at different levels between fertile and infertile heifers

Univariate T-test analysis found 10 metabolites significantly different ($p < 0.05$) between the pregnant by AI (N = 9) and Open (N = 11) plasma samples (Table 1). The metabolites Alanine ($p = 0.0132$, Figure 3.2A), Methionine ($p = 0.0272$, Figure 3.2F), Valine ($p = 0.0229$, Figure 3.2D), Lysine ($p = 0.0105$, Figure 3.2B), Tyrosine ($p = 0.0181$, Figure 3.2C), Cystine ($p = 0.0177$, Figure 3.2G), Tryptophan ($p = 0.0354$, Figure 3.2E), Fructose-6-Phosphate ($p = 0.0208$, Figure 3.2H), Ribulose-5-Phosphate ($p = 0.0433$, Figure 3.2I), Glycerol ($p = 0.0474$, Figure 3.2J) were identified as significantly different ($p < 0.05$, FDR 0.05) between AI and Open groups. PLS-DA (Partial Least Squares Discriminant Analysis) displayed group separation between samples from heifers pregnant by AI and those that remained Open (Figure 3.3). A Heat Map depicts the top twenty-five differential levels of metabolites (as identified via T-test) showing a trend of being up or down regulated in heifers that remained Open compared with those pregnant by AI (Figure 3.4).

Table 3.1.: Metabolites found at significantly different levels ($p < 0.05$) in heifers that remained Open compared with those pregnant by AI. Alanine, Methionine, Valine, Lysine, Tyrosine, Cystine, Tryptophan, Fructose-6-Phosphate, Ribulose-5-Phosphate and Glycerol were identified as significantly different between heifers pregnant by AI and those that remained Open.

	Metabolite	P-Value	Log2 (FC)	ROC AUC
1	Alanine	0.0132	0.86042	0.84
2	Methionine	0.0272	0.35706	0.81
3	Valine	0.0229	0.41843	0.83
4	Lysine	0.0105	0.49665	0.85
5	Tyrosine	0.0181	0.32214	0.85
6	Cystine	0.0177	0.60524	0.86
7	Tryptophan	0.0354	0.54428	0.78
8	Fructose-6-Phosphate	0.0208	-0.50567	0.62
9	Ribulose-5-Phosphate	0.0433	-1.4448	0.68
10	Glycerol	0.0474	-0.25997	0.50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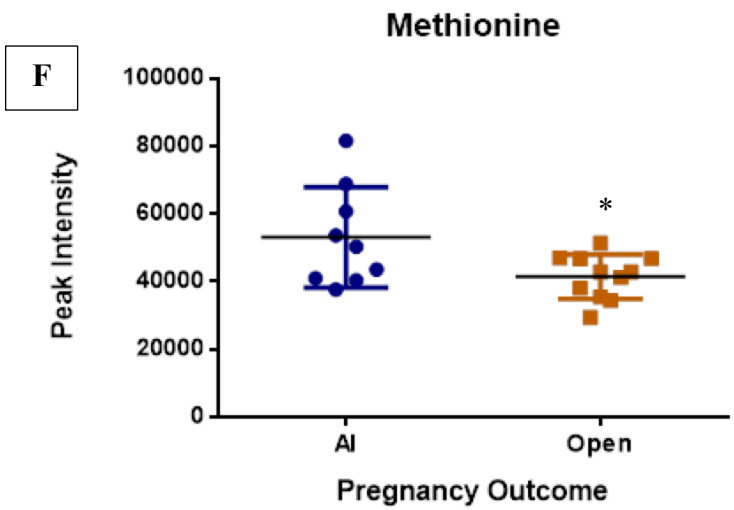
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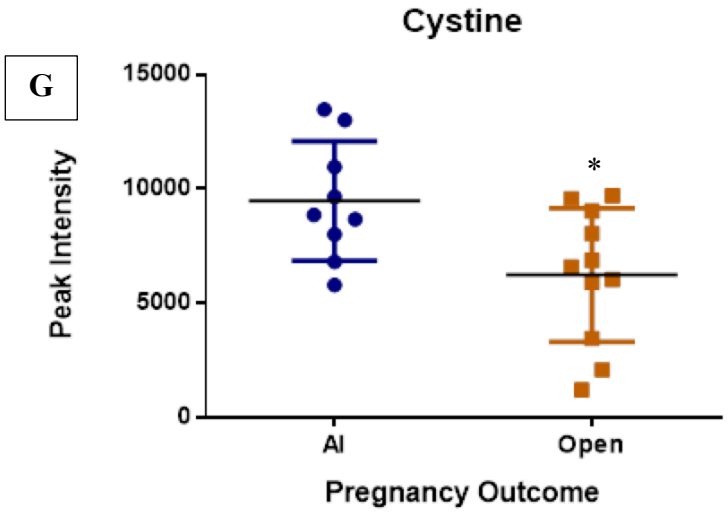
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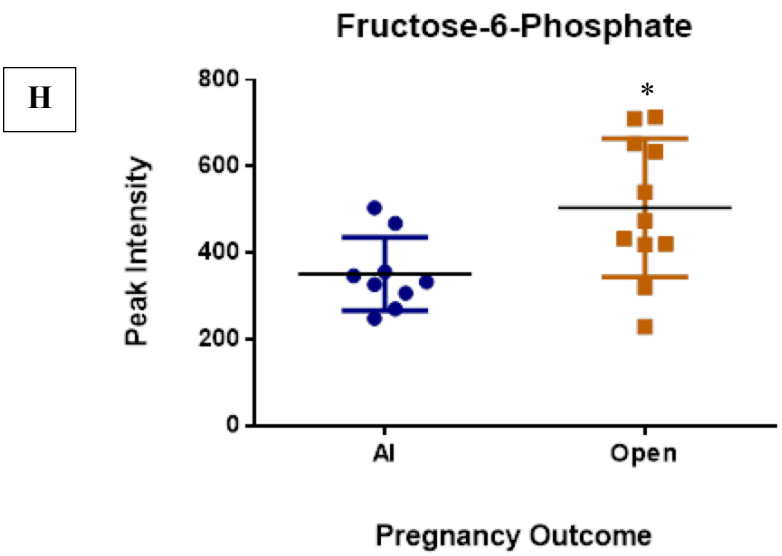
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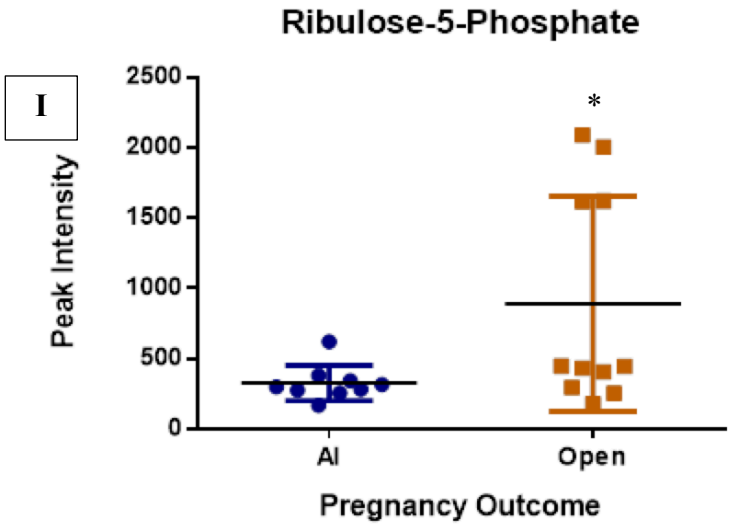
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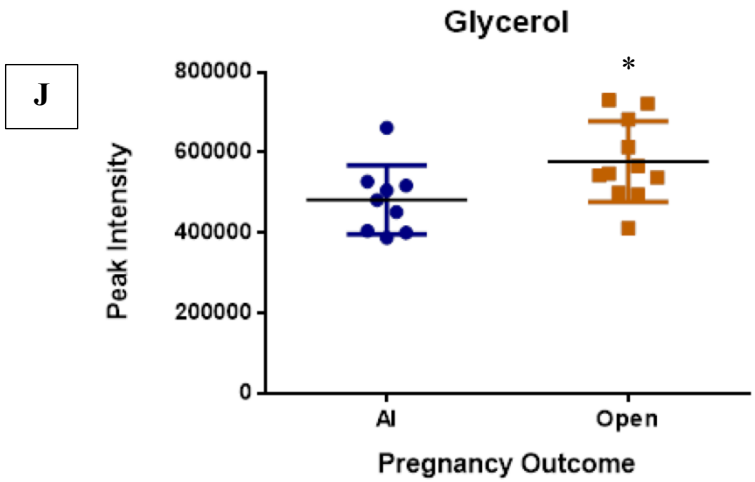
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AI
Open
Pregnancy Outcome

Figure 3.2. Peak intensity of marker metabolites identified at significantly different levels in Open heifers compared with those pregnant by AI (*, $p < 0.05$).

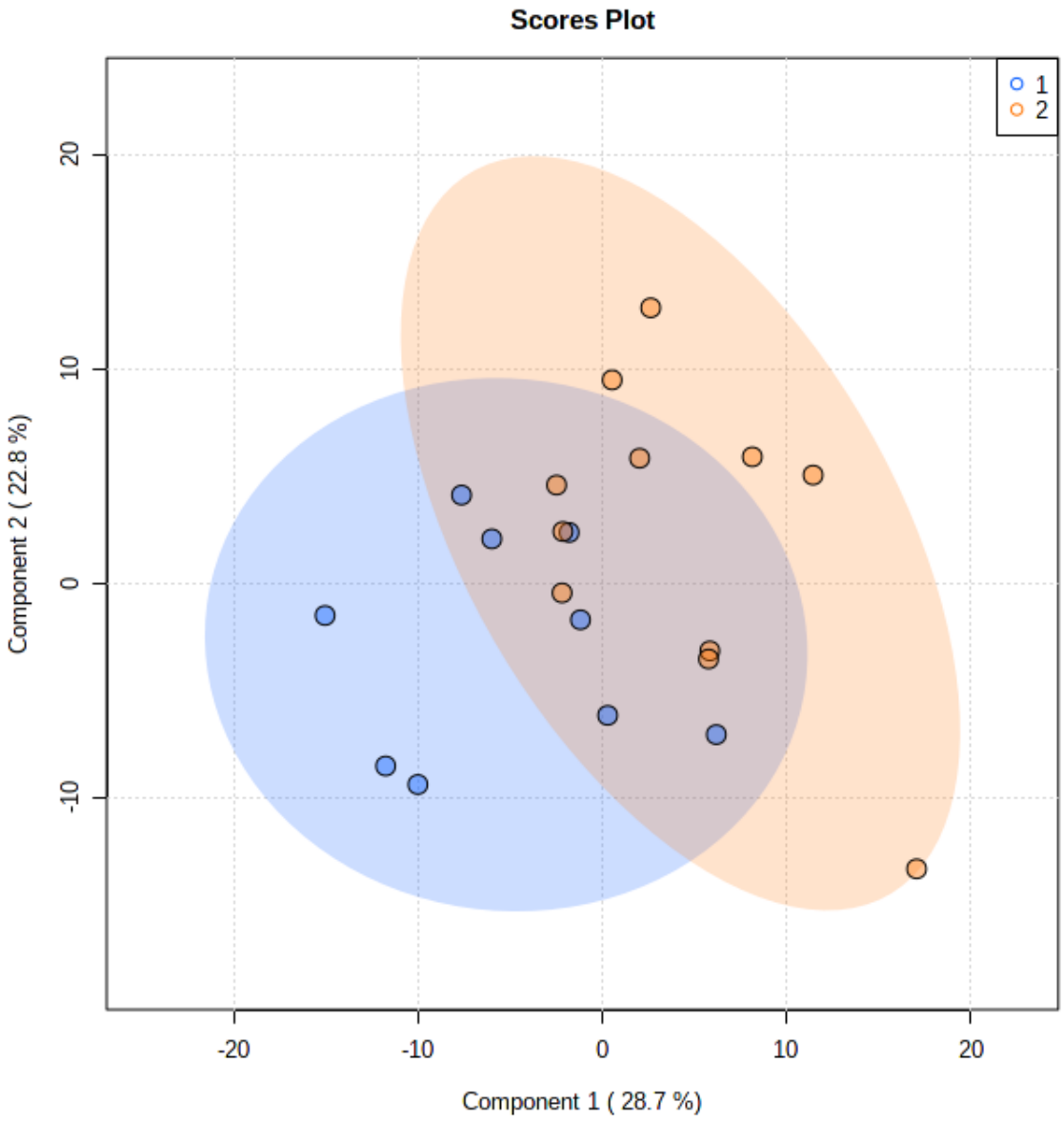

Figure 3.3. PLS-DA scores plot displaying a significant separation between Open heifers (Orange and #2) and those pregnant by AI (Blue and #1).

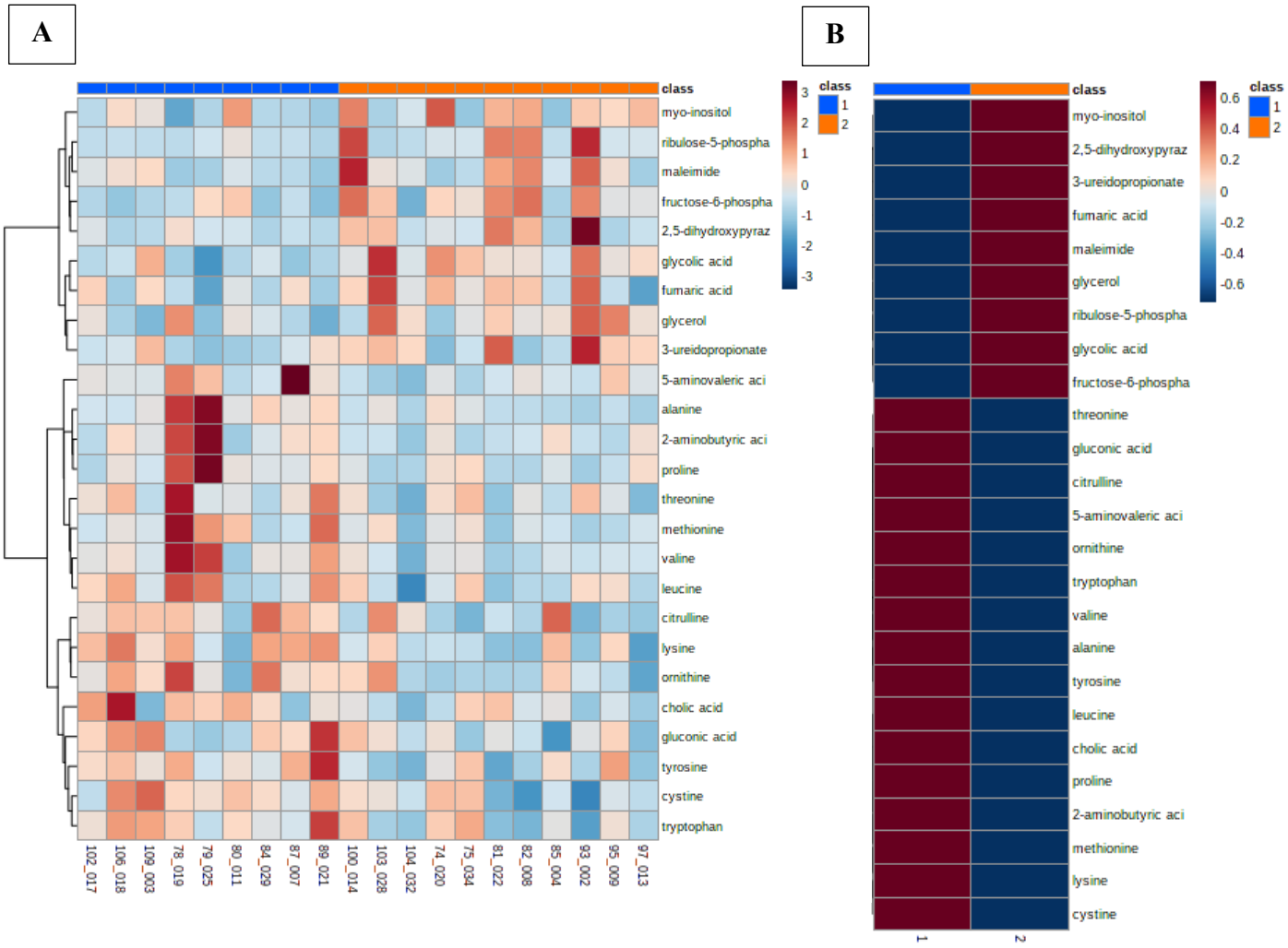

Figure 3.4. Heat map depicting top 25 metabolites at differentially expressed levels. Samples are represented individually at Open (Orange and #2) and those pregnant by AI (Blue and #1).

Predictive Ability Identified for Significant Metabolites

The Receiver Operating Characteristic (ROC area under the curve (AUC) values were calculated in order to identify the predictive ability of the significantly different metabolites between the two groups. Based on previous studies, metabolites with a 0.80 or higher ROC-AUC value had a higher chance of success in correctly categorizing samples compared to a lower ROC-AUC value (Mandrekar, 2010). The ROC-AUC values for our study were 0.83 for Valine, 0.85 for Tyrosine, 0.78 for Tryptophan, 0.81 for Methionine, 0.85 for Lysine, 0.50 for Glycerol, 0.62 for Frucose-6-Phosphate, 0.86 for Cystine, 0.84 for Alanine and 0.68 for Ribulose-5-Phosphate. This indicated that there was six metabolites that had a ROC-AUC value above 0.80 suggesting that they had a better chance of correctly categorizing heifers at pregnant by AI or Open. The metabolome of heifers pregnant by AI (N = 9) and those that remained Open (N = 11) were compared with the previously listed metabolites to determine their accuracy in categorizing heifers based on their reproductive outcome. All of the listed metabolites, except Ribulose-5-Phosphate, had ROC-AUC values over 0.80 in the logistical regression model.

Table 3.2.: ROC-AUC analysis of the metabolites found at significantly different levels in Open heifers compared with those pregnant by AI (AUC (95% CI)).

Metabolite	AUC	Sensitivity	Specificity	% Correctly Categorized	%AI Pregnant Categorized as Open
Valine	0.885 (0.760-1.000)	0.909 (0.909-1.000)	0.789 (0.606-0.973)	80	10
Tyrosine	0.880 (0.759-1.000)	0.727 (0.727-0.990)	0.842 (0.678-1.000)	70	10
Tryptophan	0.861 (0.731-0.992)	0.727 (0.727-0.990)	0.789 (0.606-0.973)	65	15
Methionine	0.847 (0.706-0.987)	0.818 (0.818-1.000)	0.789 (0.606-0.973)	70	20
Lysine	0.847 (0.687-1.000)	0.909 (0.909-1.000)	0.789 (0.606-0.973)	75	10
Glycerol	0.847 (0.675-1.000)	0.818 (0.818-1.000)	0.842 (0.678-1.000)	70	15
Fructose-6-Phosphate	0.813 (0.617-1.000)	0.818 (0.818-1.000)	0.895 (0.757-1.000)	70	10
Cystine	0.842 (0.687-0.997)	0.818 (0.818-1.000)	0.789 (0.606-0.973)	75	10
Alanine	0.885 (0.729-1.000)	0.818 (0.818-1.000)	0.842 (0.678-1.000)	75	10
Ribulose-5-Phosphate	0.789 (0.601-0.978)	0.727 (0.727-0.990)	0.895 (0.757-1.000)	75	5

Pathway Analysis reveals amino acid significance

Pathway analysis was performed following the Holm adjustment of the p-values (FDR < 0.05). Aminoacyl-tRNA biosynthesis revealed itself as the most aggravated pathway followed closely by Valine, leucine and isoleucine degradation, Tryptophan metabolism and Tyrosine metabolism pathways. A Fisher's Exact Test was performed to determine the effects of the 10 significantly differential metabolites (Table 3.3). Expected hits are identified as the percentage of the fifteen significantly different metabolites that are involved in the given pathway.

Table 3.3. Pathway Analysis for selected significant metabolites.

Pathway	Total	Expected	Hits	Raw p-value	-log(p)	Holm Adjusted p-value
Aminoacyl-tRNA biosynthesis	48	0.31746	6	1.42E-07	6.8466	1.20E-05
Cysteine and methionine metabolism	33	0.21825	2	0.018638	1.7296	1
Phenylalanine, tyrosine and tryptophan biosynthesis	4	0.026455	1	0.026219	1.5814	1
Valine, leucine and isoleucine biosynthesis	8	0.05291	1	0.051819	1.2855	1
Ubiquinone and other terpenoid-quinone biosynthesis	9	0.059524	1	0.058123	1.2357	1
Biotin metabolism	10	0.066138	1	0.06439	1.1912	1
Phenylalanine metabolism	12	0.079365	1	0.076811	1.1146	1
Glycerolipid metabolism	16	0.10582	1	0.10121	0.99479	1
Pentose and glucuronate interconversions	18	0.11905	1	0.11319	0.9462	1
Starch and sucrose metabolism	18	0.11905	1	0.11319	0.9462	1
Pantothenate and CoA biosynthesis	19	0.12566	1	0.11912	0.924	1
Selenocompound metabolism	20	0.13228	1	0.12502	0.90301	1
Pentose phosphate pathway	22	0.1455	1	0.13672	0.86418	1
Lysine degradation	25	0.16534	1	0.15399	0.8125	1
Galactose metabolism	27	0.17857	1	0.16534	0.78163	1
Alanine, aspartate and glutamate metabolism	28	0.18519	1	0.17096	0.76711	1
Amino sugar and nucleotide sugar metabolism	37	0.24471	1	0.22003	0.65751	1
Valine, leucine and isoleucine degradation	40	0.26455	1	0.2358	0.62746	1
Tryptophan metabolism	41	0.27116	1	0.24099	0.618	1
Tyrosine metabolism	42	0.27778	1	0.24615	0.6088	1

Inflammatory cytokines identified as significantly different

RNA isolated from white blood cells was used to determine the expression of five inflammatory cytokines in the open and pregnant by AI groups (*TNFα , IL6, CXCL5, POSTN and MCP1*) to determine if inflammation may have played a role in heifers remaining open. Inflammatory cytokines were normalized to three different housekeeping genes: GAPDH, B2M and TBP. Inflammatory cytokines were increased in all heifers that remained open after 60 days with bull exposure ($p < 0.05$). The p-values and mean ± standard deviations of the mean are listed in Table 3.4 for each inflammatory cytokines normalized to each of the three housekeeping genes.

Table 3.4. P-values and Standard Deviations for Inflammatory Cytokines to Each housekeeping gene *(GAPDH, B2M AND TBP)*

PREGNANT BY AI vs. OPEN	TNFα	IL-6	CXCL5	MCP1	POSTN
GAPDH	p = <0.0001 4.063 ± 0.4176 vs. -0.2400 ± 0.8147	p = 0.0002 8.850± 1.964 vs. -3.870 ± 2.548	p = 0.0017 3.758 ± 1.971 vs. -4.893 ±2.555	p = <0.0001 11.25 ± 1.390 vs. -5.830 ± 2.152	p = <0.0001 10.59 ±1.903 vs. -5.610 ± 2.552
B2M	p = <0.0001 9.750 ± 0.6817 vs. 0.7150 ± 0.3897	p = <0.0001 14.54 ± 1.428 vs. -2.863 ± 3.031	p = 0.0003 9.445 ± 2.141 vs. -3.933 ± 2.935	p = <0.0001 16.93 ± 0.7696 vs. -4.825 ± 2.699	p = <0.0001 17.57 ± 2.295 vs. -4.603 ± 3.117
TBP	p = 0.0022 0.8700 ± 0.3943 vs. -0.2825 ± 0.2202	p = 0.0023 5.660 ± 1.638 vs. -3.860 ± 3.380	p = 0.0302 0.5625 ± 2.064 vs. -4.930 ± 3.297	p = 0.0001 8.053 ± 0.9489 vs. -5.820 ± 2.992	p = 0.0039 7.398 ± 1.466 vs. -7.448 ± 6.345

Table 3.5.: Primer Sequences used for identification of inflammatory cytokine expression.

Primer	sequence (5→3')	NCBI Accession Number	Efficie-ncy (%)	Product Length (BP)
TNFα	F:TCAAGCCTCAAGTAACAAGCC R:GTTGTCTTCCAGCTTCACACC	NM_173966.3	93	123
IL-6	F:TGAGTGTGAAAGCAGCAAGGA R: TCGCCTGA TTGAACCCAGAT	NM_173923.2	100	100
CXCL5	F: AAAGTTGCCCAGTTCTTCAG R: CAAGCATAGATTCCCTCTTCC	BC142108.1	95	146
POSTN	F:TGTGTTATATGAATGCTGCCCT R: ATCCCTTTCCTTCAATCTCCTC	AY445072.2	91	169
MCP1	F:CTCAGCCAGATGCAATTAACTC R: AAATCACAGCCTCTTTAGGAC	NM_174006.2	91	128
GAPDH	F: CGTAACTTCTGTGCTGTGCC R: ATTGATGGCGACGATGTCCA	NM_001034034.2	107	136
TBP	F: GCCTTGTGCTTACCCACCAACAGTTC R: TGTCTTCCTGAAACCCTTCAGAATAGG	NM_001075742.1		182
B2M	F: CACGCTGAGTTCACTCCCAA R: ATGGACATGTAGCACCCAAGG	NM_173893.3		275

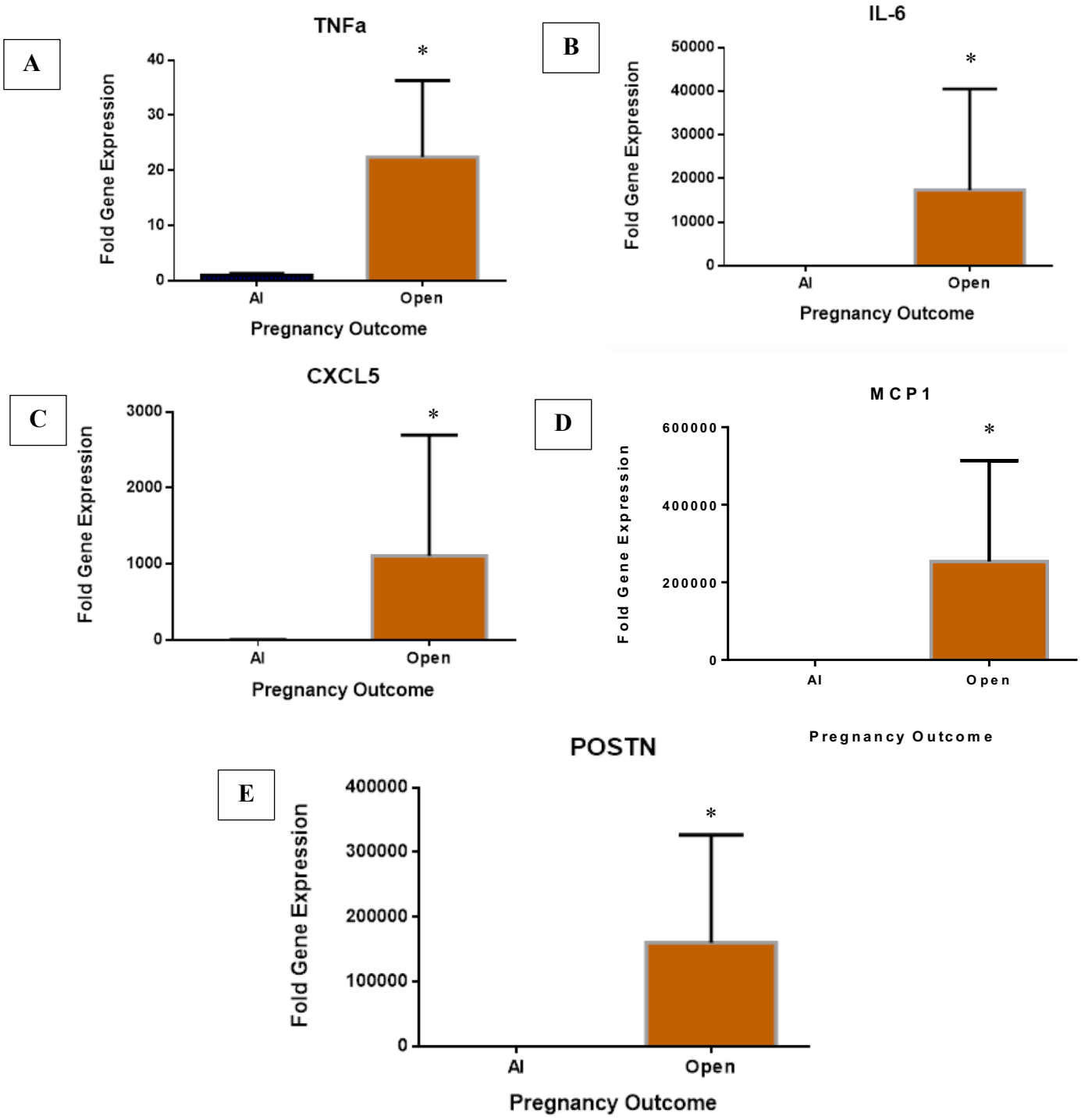

Figure 3.5. Comparison of transcript levels of inflammatory cytokines in the white blood cells from Pregnant by AI and Open heifers normalized to GAPDH. Data are mean ± standard deviation of the mean (*, $p < 0.05$).

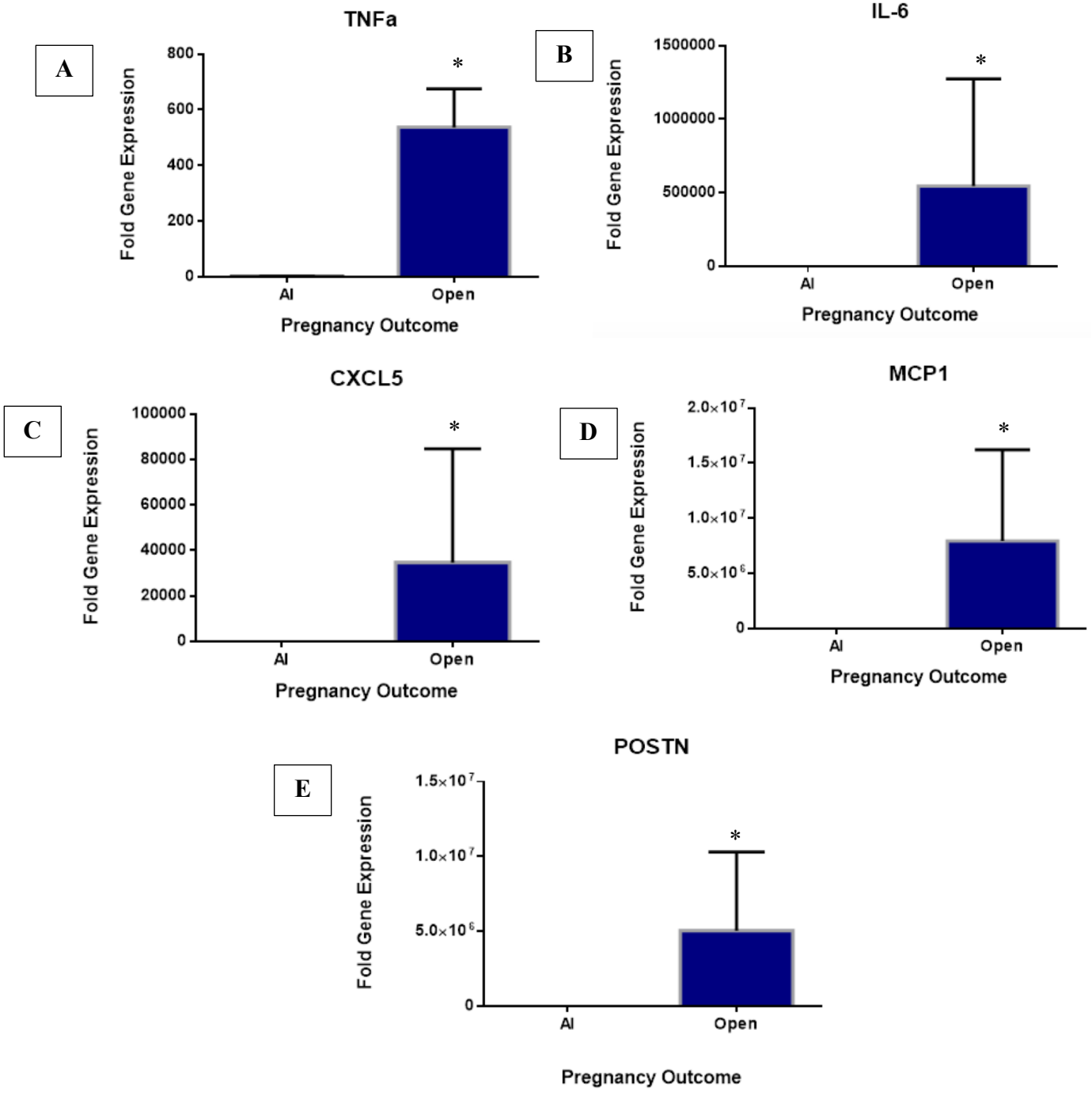

Figure 3.6. Comparison of transcript levels of inflammatory cytokines in the white blood cells from Pregnant by AI and Open heifers normalized to B2M. Data are mean ± standard deviation of the mean (*, $p < 0.05$).

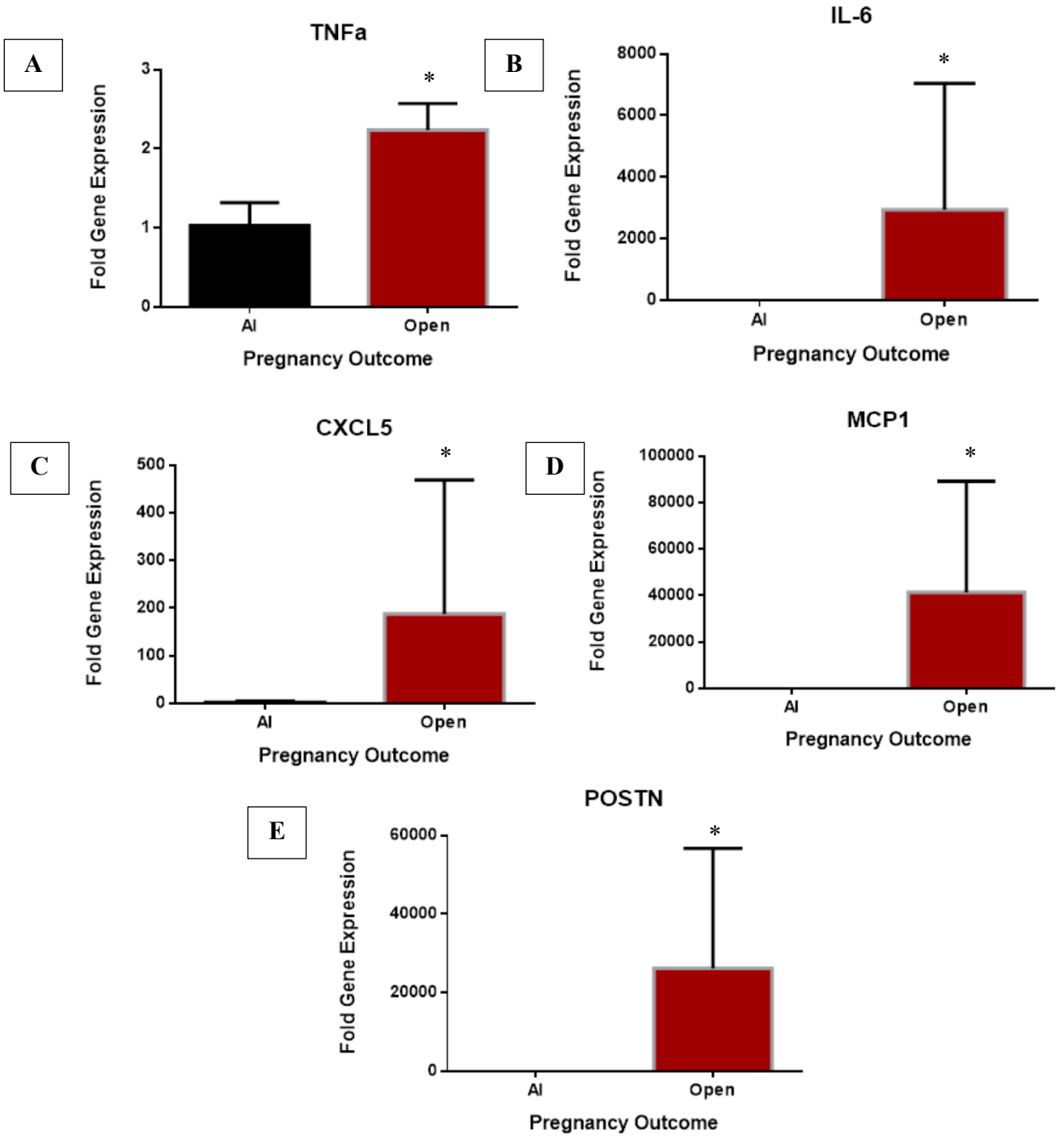

Figure 3.7. Comparison of transcript levels of inflammatory cytokines in the white blood cells from Pregnant by AI and Open heifers normalized to TBP. Data are mean ± standard deviation of the mean (*, $p < 0.05$).

ELISAs

Lastly, ELISAs were used to detect proteins for TNFα and IL6 within blood plasma from heifers that were pregnant by AI and those that remained open after 60 days of bull exposure. Controversial to the inflammatory marker results, the differences of the ELISAs for TNFα and IL-6 between heifers pregnant by AI and open was not significant with ($p > 0.05$). For the TNFα ELISA, it was not significantly different ($p = 0.7636$) when heifers pregnant by AI (0.03186 ± 0.003168 ng/ml) were compared to those that remained Open (0.03267 ± 0.002973 ng/ml). For the IL-6 ELISA, results were not significantly different ($p = 0.6976$) when heifers pregnant by AI (1058 ± 889.2 pg/ml) were compared to those that remained Open (847.9 ± 523.7 pg/ml).

Concentration of TNFα and IL-6 proteins in heifer blood plasma

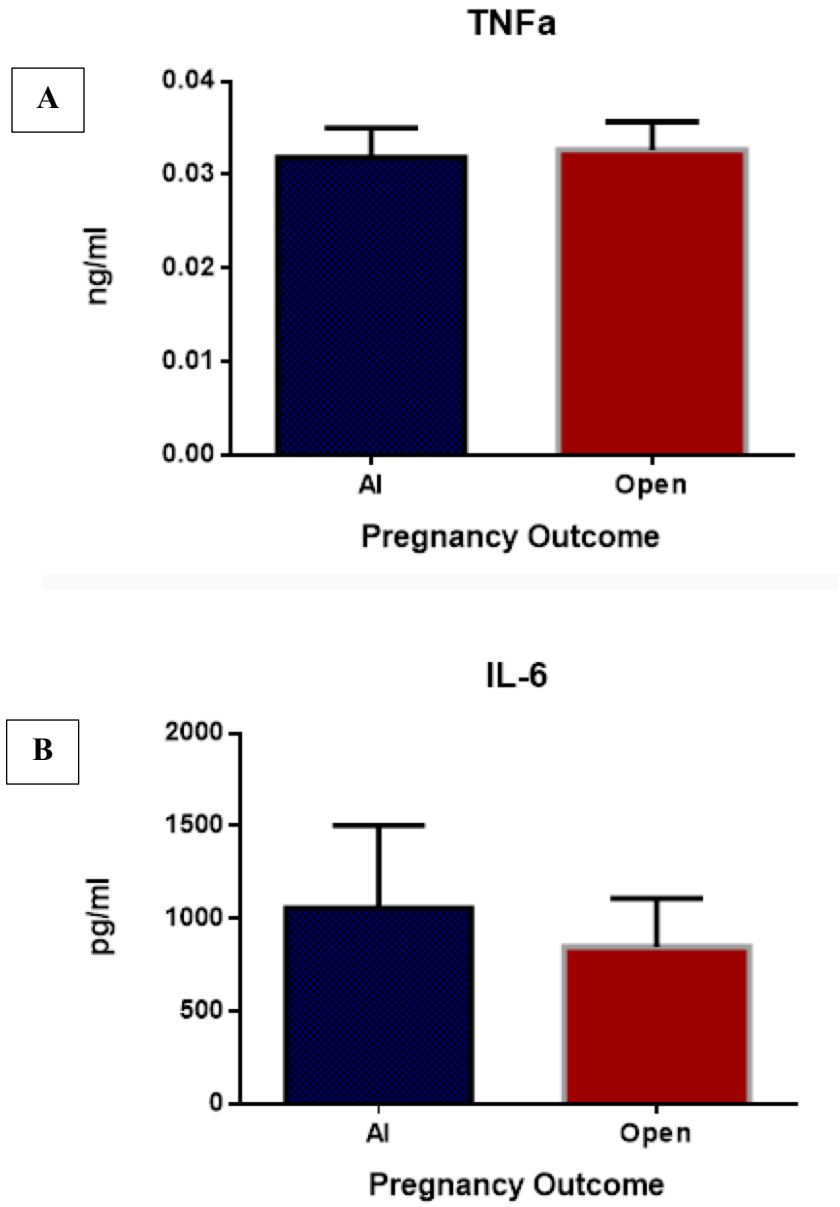

Figure 3.8. ELISA measured concentrations of TNFα and IL-6 proteins in blood plasma of heifers pregnant by AI or those that remained Open. There was no significant difference seen between the two groups. Data are mean ± standard deviation of the mean (*, $p < 0.05$).

III.V. DISCUSSION

While infertile heifers affect the profitability of the operation they reside on, they also have a larger impact on the livestock industry as a whole. Overall, the cost of developing infertile heifers costs the producer, on average, is $43.00 per heifer (USDA). For the 2019-2020 breeding season, there were approximately 5.77 million replacement heifers in the United States which correlated to $4.8 million lost in revenue due to infertility (USDA). Overall, heifer reproductive failure is hard to diagnose due to there being so many factors involved in a successful pregnancy. While there are numerous studies addressing causation of cow infertility, there is significantly less research addressing the unknowns related to heifer infertility. The goal of our study was to have a systematic approach to improve heifer fertility as well as the sustainability of cow-calf operations as a whole. We wanted to provide a method for producers to be able to accurately determine a heifers reproductive potential earlier in her development in order to avoid investing unnecessary money, time and resources into a heifer that will not become a productive member of the herd. In our study, we investigated metabolites as biomarkers to be able to accurately determine the reproductive potential of a single heifer.

While the term “metabolome” has been tossed around for a while, it is not until recently that metabolites have been studied to determine their potential to serve as biomarkers for possible diseases or to understand whole biologic systems. Presently, clinical researchers are still working to be able to accurately quantify and identify all metabolites in bodily tissues and fluids, as only some are currently detectable due to chemical complexity. While research is still ongoing, metabolites still present themselves

as a more universal tool to understand cell function in different species. Research on metabolites as biomarkers has been more commonly seen in human and biomedical research. Previous studies have shown that differential levels of metabolites can serve as biomarkers to diagnose diseases such as metabolic diseases (Shaham et al., 2008), ovarian cancer (Zhang et al., 2012) and breast cancer (Asiago et al., 2010). In relation to male and female infertility, metabolites have served as biomarkers to study semen quality (Xu et al., 2020), erectile dysfunction (Zhou et al., 2015), sperm count (Zhang et al., 2013), endometriosis in women (Dutta et al., 2012) and metabolic makeup of follicular fluid in relation to oocyte quality (Wallace et al., 2012). Lastly, Phillips et al. (2018) was the first to discuss the potential for metabolites to be used as biomarkers to determine reproductive potential in beef heifers at the time of AI.

Our study consisted of analyzing metabolite concentrations from blood plasma in crossbred heifers at the time of weaning to determine differential metabolites and the potential for them to serve as biomarkers in relation to reproductive potential. We also analyzed the use and accuracy of traditional phenotypic methods to be able to accurately discriminate between heifers pregnant by AI and those that remained open after three estrous cycles and bull exposure.

Over the course of two breeding seasons, no significant difference was seen in age at weaning between heifers becoming pregnant by AI or those remaining open (Figure 3.1A). Additionally, there was no significant difference seen in weight at weaning between heifers becoming pregnant by AI or those remaining open (Figure 3.1B). Similarly, there was no significant difference seen in body condition scores (BCS) between heifers becoming pregnant by AI or those remaining open (Figure 3.1C). There was also no

significant difference seen in reproductive tract scores (RTS) between heifers becoming pregnant by AI or those remaining open (Figure 3.1D). Lastly, there was no significant difference seen in pelvic area (PA) between heifers becoming pregnant by AI or those remaining open (Figure 3.1E). Here, we have shown that phenotypic parameters such as age at weaning, weaning weight, BCS, RTS and PA are useful in beef heifer development, but when analyzed together, cannot accurately determine the pregnancy outcome of individual heifers. Since the beef industry relies on reproductively successful replacement heifers to be profitable, it is necessary that other methods to determine fertility potential at the time of weaning be investigated.

Metabolomic analysis was used on heifer blood plasma to detect biomarkers that could be indicative of reproductive potential. Heifers were grouped based on pregnancy outcome as AI (heifers pregnant by AI) or Open (heifers that remained open after AI and 60 days of bull exposure). Over the course of one breeding season at one location, we identified ten significantly different ($p < 0.05$) metabolites at the time of weaning between the two groups. Six of the ten differential metabolites had a ROC AUC value of 0.81 or above which is indicative of their better chance to accurately determine reproductive outcome (Table 3.1). When determining the number of animals that were correctly categorized as pregnant by AI or Open, most of the metabolites alone were able to correctly categorize heifers 70% to 80% of the time. In aims to develop an assay to be able to differentiate between the two groups, producers need to be able to trust that they would not be removing fertile heifers from the herd because of a false result. After analyzing metabolomic predictions, we calculated the percent of pregnant by AI heifers that were falsely categorized as Open (Table 3.2). When pathway analysis was evaluated for the ten

significantly different metabolites, Aminoacyl-tRNA biosynthesis pathway was shown to be the most affected (Table 3.3). Part of the reason for this is that seven of the ten significantly different metabolites are amino acids. All seven of these metabolites that are amino acids were upregulated in heifers pregnant by AI compared to those remaining open.

Inflammation has been shown to play a large role of infertility in men, women and animals. An increased immune response can affect normal hormone production which will in turn affect ovulation. Studies have shown increased levels of TNFα and IL-6 being related to endometriosis in women (Weiss et al., 2009). Inflammatory cytokines were investigated in this study due to the fact that immune cells that are known to be involved in a normal estrous cycle in beef cattle produce a variety of different cytokines. The amount of inflammatory cytokines present in white blood cells can be indicative of the amount of inflammatory cells present in an individual heifer. mRNA was isolated from white blood cells of AI and Open heifers in order to determine the presence of inflammatory cytokines. We found significantly higher expression of proinflammatory cytokines such as Tumor Necrosis Factor alpha (*TNFα*), Interleukin 6 (*IL-6*), neutrophil activating peptide C-X-C Motif Chemokine 5 (*CXCL5*), monocyte chemoattractant protein-one (MCP1) and Periostin (POSTN) in infertile heifers compared with fertile heifers. The inflammatory cytokines were normalized against three different house keeping genes *(GAPDH, B2M and TBP),* making the data that much more significant (Figures 3.5, 3.6 and 3.7). These results suggest that there could be a cause and effect relationship between asymptomatic inflammation and infertility, but exact cause has yet to be identified. Lastly, ELISAs were used to help detect proteins for TNFα and IL-6. While the results were shown to be not significant between heifers pregnant by AI and those that remained open, there could be a

couple reasons that may explain these surprising results. The discrepancy seen between the mRNA transcript and cytokine levels could be explained a number of ways. It is possible that the mRNA is not being translated through post transcriptional control mechanisms such as miRNA. Another explanation would be that the cytokines are at different levels in the white blood cells, but not being secreted into the plasma which is where we measured the cytokine levels. Further research will be needed to clarify these results.

Overall, producers raising beef replacement heifers need a reliable way to determine the future reproductive outcome of heifers. If identification of infertile heifers could be done at the time of weaning, this would save the producer from investing valuable time and money into a heifer that will not become a productive member of the herd. Metabolites serve a new tool that could serve as a low cost and noninvasive way to be able to discriminate heifers based on reproductive potential.

CHAPTER IV.

CONCLUSION AND IMPLICATIONS

In closing of this study, we were able to identify differences in metabolic profiles of heifers pregnant by AI and those that remained Open at the time of weaning. Phenotypic parameters including Weaning Weight, Age at Weaning, RTS, BCS and PA were evaluated to determine the accuracy of traditional methods to be able to differentiate heifers based on their reproductive potential. We found that using traditional phenotypic parameters alone, and in combination, was not sufficient enough to accurately determine heifer fertility outcome. Metabolite analysis revealed ten metabolites at significantly different levels at the time of weaning in heifers pregnant by AI and those that remained Open after 60 days with bull esposure. We then evaluated the inflammatory status of individual heifers to determine if asymptomatic inflammation may have played a role in infertility. We found that there was significantly more mRNA of inflammatory cytokines *(TNFα, IL-6, CXCL5, MCP1 and POSTN)* present in the heifers that remained open compared to those that conceived by AI. Lastly, we investigated the presence of proteins for *TNFα* and *IL-6* within heifer blood plasma at the time of weaning. While this protein work resulted as not being statistically significant, it raises some very valid questions to carry on research related to this project.

The metabolomic data and results generated in this study raise excitement and ongoing curiosity on the metabolomic state of heifers at the time of weaning. Now that we have established that metabolites are different at the time of weaning based future reproductive performance, what is the reasoning for this? Could this be due to the heifer

still being in the growing phase or acute stress that is being taken on by cow-calf separation and sudden change in diet? Can several metabolites together be used to create a non-invasive and low-cost test for producers to determine fertile and infertile heifers possibly even earlier than weaning? In future studies, I would also like temperament to be considered in traditional phenotypic parameters. Over the course of my research, I have noticed that heifers that are more calm coming out of the chute are more likely to be pregnant by AI. Lastly, what affect does miRNA regulation have on mRNA transcripts and protein levels post-translation that made our ELISA data not correlate with our inflammatory results?

Heifer infertility is the top reason that beef replacement heifers are removed from the herd and has a direct effect on the profitability of the operation and livestock industry as a whole. New reproductive technologies are constantly being invented and used in order to study reproduction in beef cows. This study offers a unique option to use metabolomics in order to improve fertility in beef heifers. If a producer is able to understand metabolite fluctuations in animals, this can help to detect subtle phenotypic changes and serve as a tool for research, breeding and overall assessment of heifer development.

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Appendix 1: Body Condition Scoring System. Data adapted from Anderson et al., 1991.

BCS	Overall Condition	Bone Structure	Fat Deposition
1	Extremely thin	Easily visible; Pin – sharp structures of shoulder, ribs and back	Little deposition or muscling
2	Extremely thin	Sharp spinous process with spaces between	Little deposition; some muscling in hind-quarters
3	Extremely thin	Slightly-visible backbone and easily-identified spinous process	Minimal fat coverage on loin, back and foreribs
4	Borderline and Unfavorable	Foreribs not noticeable; Transverse spinous process identified by palpation	Hindquarter muscling is full
5	Average or Moderate	Show visibility of the 12^{th} and 13^{th} ribs; Transverse spinous process felt with firm pressure	Areas near tail and head are well filled; Do not show excess accumulation
6	Average or Moderate	Show fully covered ribs, unnoticeable to the eye	Full and plump hindquarters; Noticeable "sponginess" to foreribs, tail and head
7	Average or Moderate	Non-distinguished spaces between the spinous process	Abundant fat coverage on both sides of the tail and neck

8	Extremely obese	Smooth and blocky appearance, Disappearance of visual bone structure	Thick fat coverage throughout the body
9	Extremely obese	Shows no visible bone structure	Tail head buried in fat; Declined or halted mobility due to excess fat impairment

Appendix 2: Reproductive Tract Scoring System. Data adapted from Anderson et al., 1991)

Reproductive Tract Score	Uterine Horns	Length	Height	Width	Ovarian Structures
1	Immature <20 millimeters diameter, no tone	15 mm	10 mm	8 mm	No palpable follicles
2	20-25 mm, no uterine tone	18 mm	12 mm	10 mm	8 mm follicles
3	20-25 mm, slight uterine tone	22 mm	15 mm	10 mm	8-10 mm follicles
4	30 mm, good uterine tone	30 mm	16 mm	12 mm	>10 mm follicles, CL possible
5	>30 mm	>32 mm	20 mm	15 mm	CL present