Investigating plasma metabolomics profiles at the time of artificial insemination, based on pregnancy outcome, in *Bos taurus* heifers.

by

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Abstract

The development of biomarkers for the identification of infertile heifers has the potential to improve the efficiency of cow-calf production. In this study, we utilized metabolomic profiling to look for biomarkers in the blood plasma that may be useful in identifying infertile heifers at the time of artificial insemination (AI). Angus and Angus-cross heifers, undergoing a 7 - day estrous synchronization protocol, were utilized for analysis. The heifers were housed at three separate Research and Extension Centers (Black Belt, Gulf Coast, and Wiregrass) located across the state of Alabama, U.S.A. and compared across two breeding seasons (2015 – 2016 and 2016 – 2017).

We compiled data on a total of 166 heifers (N = 166) from three locations across two breeding seasons for phenotypic parameters including body condition score (BCS), weight at time of weaning, reproductive tract score (RTS), and age at AI to determine the utility of using "traditional" heifer assessment metrics to predict reproductive outcome. These phenotypic parameters proved to not be significantly different ($p > 0.05$ for BCS, RTS, Weight at Weaning, and Age at AI) in heifers undergoing fixed-time artificial insemination that became pregnant by AI or those remaining open. These parameters also proved to not be significant (except for BCS across locations) when compared across two separate breeding seasons, three separate locations, and based on pregnancy outcome.

Heifers were compared to determine if there were metabolomic profile differences between heifers housed at two separate Research and Extension Centers [Black Belt (N = 8) and Wiregrass (N = 8)] across two breeding seasons (2015 – 2016 and 2016 – 2017). Analysis revealed six metabolites present at differential levels (T-test; $p < 0.05$; fold change > 2, FDR = 0.05) between Black Belt heifers and Wiregrass heifers. Tocopherol-alpha, Ornithine, Myristic Acid, P-tolyl Glucuronide, Sulfuric Acid, and Alpha-ketoglutarate were all found to be differentially expressed by at least 2-fold in Black Belt heifer's blood plasma compared to Wiregrass heifer's blood plasma.

In a second study, a total of N = 20 heifers were analyzed for phenotypic heifer assessment, as well as metabolomic profiling, to identify metabolite differences in infertile (open) heifer blood plasma compared to fertile (AI) heifer blood plasma. Heifers were deemed fertile (AI) if they conceived from the artificial insemination following estrous synchronization. Heifers were deemed infertile if they did not maintain a conceptus following estrous synchronization, fixed-time AI, and three consecutive estrus cycles in the presence of a fertile bull. Phenotypic parameters were determined to not be significantly different ($p > 0.05$ for BCS, RTS, Weight at Weaning, and Age at AI) between categorized fertile and infertile heifers.

Metabolomics profiles of N = 20 heifers revealed seven metabolites present at different levels (T-test; $p < 0.05$; fold change > 2; FDR = 0.05) between infertile and fertile heifers. Tryptophan, Cystine, Histidine, Ornithine, Asparagine, Glutamine, and Lysine were all found to be at least 2-fold less in the infertile heifer's blood plasma compared to the fertile heifer's blood plasma. We further characterized the utility of using the levels of these metabolites in the blood plasma to discriminate between fertile

and infertile heifers. In order to identify the predictive ability of the significant metabolites discovered ($P < 0.05$; >2-fold change), we calculated the Receiver Operating Characteristic (ROC) area under the curve (AUC) value. We tested the models on the blood plasma metabolomes of the 20 selected heifers to determine their ability to predict pregnancy outcomes. Glutamine and Histidine alone, and in combination, predicted the correct pregnancy outcome in 90% of the animals. They did not incorrectly categorize a fertile heifer as infertile.

Finally, we investigated the potential role that inflammation might play by comparing the expression of inflammatory cytokines in the white blood cells of infertile heifers to that of fertile heifers. We found significantly higher expression ($p < 0.05$) of the proinflammatory cytokines Tumor Necrosis Factor alpha (*TNFα*), C-X-C Motif Chemokine Ligand 5 (*CXCL-5*), and Interleukin 6 (*IL-6*) in infertile heifers when compared to fertile heifers. The study presented offers potentially valuable information regarding the identification of fertility problems in heifers undergoing AI.

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List of Abbreviations

AA	Amino Acid
AI	Artificial Insemination
ANOVA	Analysis of Variance
AUC	Area Under Curve
BB	Black Belt Research and Extension Center
BCS	Body Condition Score
BP	Base Pair
BSE	Breeding Soundness Exam
CI	Confidence Interval
CIDR	Controlled Internal Drug Release
cm	Centimeter
CXCL-5	C-X-C Motif Chemokine Ligand five
EPD	Expected Progeny Difference
FDR	False Discovery Rate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC	Gas Chromatography
GC	Gulf Coast Research and Extension Center
GC-TOF	Gas Chromatography Time-of-Flight
GNRH	Gonadotropin Releasing Hormone

IACUC	Institutional Animal Care and Use Committee
IL-6	Interleukin six
LC	Liquid Chromotography
MCP1	Monocyte Chemoattractant Protein-one
mm	Millimeter
mL	Milliliter
MS	Mass Spectrometry
NASS	National Agriculture Statistic Service
NMR	Nuclear magnetic Resonance
PA	Pelvic Area
PCA	Principal Component Analysis
PGF2α	Prostaglandin-F2α
PLSDA	Partial Least Squares Discriminant Analysis
POSTN	Periostin
RBC	Red Blood Cell
ROC	Receiver Operating Characteristic
RTS	Reproductive Tract Score
TNFα	Tumor Necrosis Factor alpha
µL	Microliter
USDA	United States Department of Agriculture
WBC	White Blood Cell
WG	Wiregrass Research and Extension Center
WT	Weight

WW	Weaning Weight

CHAPTER I.

REVIEW OF LITERATURE

I. Cattle Production

I.I. *The Cattle Industry*

The cattle industry is one of the most profitable and expansive global industries in the agricultural field. In 2017, the USDA-NASS (United States Department of Agriculture – National Agriculture Statistic Service) reported the global census of cattle production at 998.3 million head of cattle across the top eighteen leading production countries (USDA-NASS, 2018). The United States was ranked the fourth largest cattle industry in the world following India, Brazil, and China. On January 1, 2018, the U.S. annual inventory of cattle was observed at 94.4 million head (USDA-NAAS, 2018). Out of the 94.4 million head of cattle, 41.4 million head (44%) were heifers and cows that calved in the previous breeding season (USDA-NASS, 2018).

Cattle production in the southern region of the United States is comprised primarily of cow-calf production systems. This region has a longer grazing season with less need for supplemental forages. With less supplemental forages needed in the winter, the end result is a lower feed cost for all operations involved (McBride et al., 2011). In 2017, there was a reported 2.05 million farms located throughout the continental U.S. (USDA-NASS, 2017). Total land in farms was equivalent to 910 million acres, with the average sized farm being 444 acres in size (USDA-NASS, 2017). According to the USDA census in 2017, the majority of the beef cow herd in the United

States reside in the thirteen southeastern states (Alabama, Arkansas, Florida, Georgia, Kentucky, Louisiana, Mississippi, North Carolina, Oklahoma, South Carolina, Tennessee, Texas, and Virginia) (Scaglia et al., 2016). Out of the 2.05 million farms reported in 2017, 40% (811,400) of these farms are located in these thirteen southeastern states (Scaglia et al., 2016). In particular, Alabama reported 43,600 farms in 2017 over a span of 8,900 acres, with each farm averaging 204 acres in size and 59 head per farm (McBride et al., 2011 and USDA-NAAS, 2017).

I.II. *Production Systems*

In the United States, there are three primary phases of beef cattle production: cow-calf, stocker, and feedlot (McBride et al., 2011). Cow-calf is composed of maintenance during breeding, gestation, calving, and weaning of calves when they are 6 - 9 months of age, or 400-700 lbs. respectively. "Stocker" entails backgrounding and putting an additional 200 - 400 lbs. of weight to calves over a span of 3 - 8 months (McBride et al., 2011). This timeframe and backgrounding is used with the intention of increasing animal weight and maturity before putting the calves on a feedlot. "Feedlot" is the final step of the production cycle, where the "finishing" of calves occurs. Finishing refers to the addition of grains to the diet, and calves will leave the production cycle with a processing weight of 1,000 – 1,500 lbs. (McBride et al., 2011).

In order for a production system to be successful, the profitability of calves must be higher and outweigh any production costs associated with the maintenance of a given herd (Funston, 2004). In 2015, the gross income for cattle and calves in the United States totaled $78.8 billion in cash receipts, making cattle production the largest value production in agriculture from agricultural commodities (USDA-NASS, 2016).

Profitability of a cow-calf production system is dependent upon the percentage of heifers and/or cows in the herd that can consistently calve at 12-month intervals. In order to have a 12-month calving interval, the cow or heifer must be re-bred within 80 days after the birth of her calf (Lowman et al., 1976). Studies show that heifers who become pregnant early on in the breeding season and calve in the first three weeks of calving season remain in the herd for a greater amount of time compared to heifers who calve at a later date (Cushman et al., 2013). Proper development of the herd on cow-calf operations ensures low-cost heifer development without sacrificing associated heifer performance (Funston et al., 2004).

II. Heifer Development and Selection

The development of heifers is one of the most critical components of the beef cattle production industry (Grings et al., 2007). Heifers should be managed and developed in such a way that they can reach puberty early, conceive in their first breeding season, calve without assistance, and breed back early for the subsequent breeding season (Funston et al., 2004). In order to have a productive, profitable, and efficient breeding herd, nulliparous heifers must become pregnant early in their first breeding season and deliver a live calf, while primiparous heifers must become pregnant early during the following breeding season (Larson et el., 2016). Proper heifer development for reproductive success involves selecting for heifers that have optimal nutrition while encompassing ideal maturity and puberty during the pre-breeding period. The condition of heifers at breeding can affect calving intervals, conception, percentage of open or non-pregnant cows, and overall reproductive performance (Larson et al., 2016).

II.I. *Age*

In order for a heifer to conceive, she must be pubescent. Age at puberty is influenced by the heifer's breed, plane of nutrition and diet, and season (Herd et al., 1998). The onset of puberty is primarily induced by the weight of the heifer and her reproductive status. The age at which heifers reach puberty is reported between 292 and 678 days, or 9.5 – 22 months respectively (Larson et al., 2016). In North America, the average age for the onset of puberty in *Bos taurus* heifers ranges from 303 – 429 days, or 10 – 14 months of age (Larson et al., 2016). Ideally most producers require heifers to calve between 23 – 24 months of age, heifers should be bred at the time of the onset of puberty (Herd et al., 1998). The age at first calving at or near 2 years (23 – 25 months) is optimum for heifer performance, as it maintains the seasonal calving pattern (Wathes et al., 2014). In order for a low age at first calving to be maintained, proper management and adequate growth is required to ensure an appropriate body weight at conception and calving (Wathes et al., 2014).

II.II. *Weight*

Weight is one of the major factors that will determine the onset of puberty in a heifer. In order to produce a successful breeding season, the nutrition program of the herd must ensure heifers are meeting their average daily gain requirements from the time of weaning to the time of breeding (Larson et al., 2016). Target weight is said to be the threshold weight for puberty in heifers (Holm et al., 2015). If a heifer is below target weight, her nutrition and growth rate are limiting factors for the onset of puberty (Holm et al., 2015). Research shows that the target weight to reach puberty is calculated by taking the ratio of the average weight of heifers in the herd divided by the average weight

of the multiparous cows in the herd (Patterson et al., 1992). Using this calculation, heifers who have been fed a diet to reach 55% to 65% of their mature weight have better reproductive performance at time of breeding than those heifers fed to reach a lower target weight ratio (Patterson et al., 1992).

II.III. *Body Condition Score*

Body Condition Scores (BCS) are essentially the visual appraisal of muscle and adipose tissues on a given herd of cattle (Swecker et al., 2014). In commercial farms, BCS can be practiced regularly with satisfactory scoring in a situation where weighing heifers may be impractical (Herd et al., 1998). Changes in body condition, compared to changes in weight, give a more reliable understanding of the nutritional status of the cow or heifer (Herd et al., 1998). Body condition scoring provides producers a measure of the animal's nutritional reserves, which is much more useful than live weight or body weight alone (Herd et al., 1998). Body fat percentage in beef cattle at different stages of the production cycle assists with not only reproductive performance, but overall productivity of the individual (Herd et al., 1998). Many reproductive failures in a beef heifer can be attributed to either improper nutrition, or a sub-par percentage of body fat. Without an adequate amount of body fat on a heifer or cow, she will not breed at an acceptable or beneficial rate for the producer (Herd et al., 1998). Many Body Condition Scoring systems utilize a 9-point scale, with a BCS score of 1 – 3 reflecting extremely thin conditions, a BCS of 4 reflecting borderline conditions, a BCS of 5 – 7 reflecting average or moderate conditions, and a BCS score of 8 – 9 reflecting extremely obese conditions (Herd et al., 1998).

A BCS of 1 – 3 reflects extremely thin conditions. A heifer with a BCS of 1 will show easily-visible and pin-sharp bone structures of the shoulder, ribs and back, with little evidence of fat deposition or muscling (Lowman et al., 1976). A heifer with a BCS of 2 will show little evidence of fat deposition but will have some muscling in their hindquarters. The heifer's spinous process will feel sharp to the touch and is easily seen with space between them. A heifer with a BCS of 3 will show the beginning of fat coverage over her loin, back, and foreribs. Her backbone will still be slightly visible, and spinous process can be identified by touch and sight. The spaces between her spinous process will be less pronounced than a heifer with a BCS of 2 (Lowman et al., 1976).

A BCS of 4 reflects borderline and unfavorable conditions. Her foreribs are not noticeable, and the transverse spinous processes can be identified (by slight pressure palpation) and feel rounded instead of sharp. Hindquarter muscling is full (Lowman et al., 1976). Heifers who average a BCS of 4 or lower during breeding have poor reproductive performance in comparison to heifers who have a BCS of 5 or above (Herd et al., 1998).

A BCS of 5 – 7 reflects average or moderate conditions. A heifer with a BCS of 5 will show visibility of the 12^{th} and 13^{th} ribs. Her transverse spinous process can only be felt with firm pressure and will feel rounded (not noticeable to the eye). The spaces between the spinous processes will not be visible, and only distinguishable by firm pressure. Areas near the tail and the head are well filled, but do not show excess accumulation (Lowman et al., 1976). A heifer with a BCS of 6 will show fully covered ribs, unnoticeable to the eye. She will have full and plump hindquarters, and a noticeable "sponginess" to her foreribs, tail, and head. Firm pressure needs to be applied to feel the spinous processes (Lowman et al., 1976). A heifer with a BCS of 7 will show non-

distinguished spaces between the spinous process, and the spinous process can only be felt with very firm pressure. There is abundant fat coverage on both sides of the tail and neck (Lowman et al., 1976).

A BCS of 8 – 9 reflects extremely obese conditions. A heifer with a BCS of 8 will show a smooth and blocky appearance, disappearance of visual bone structure, and thick fat coverage throughout the body. A heifer with a BCS of 9 shows no visible bone structure, tail head buried in fat, and declined or halted mobility due to excess fat impairment (Lowman et al., 1976) (Appendix 1).

Low body conditions can lead to low pregnancy rates. Herd and Sprott (1998) conducted a study involving over 1,000 heifers and compared their BCSs during breeding season to resulting pregnancy rates (Herd and Sprott, 1998). Six hundred and nineteen heifers in this study had a BCS of 6 or higher, and the resulting pregnancy rate after 150 days was 95%. Three hundred heifers had a BCS of 5, and the resulting pregnancy rate after 150 days was 85%. Four hundred and twenty-two heifers in this study had a BCS of 4 or less during breeding, and the resulting pregnancy rate after 150 days was 58% (Herd et al., 1998). The trial supports the idea that a BCS of less than 5 is not suitable to conceive or maintain pregnancy, thus enforcing the importance of nutrition and proper body conditioning (Herd and Sprott, 1998).

II.IV. *Reproductive Tract Score*

Pre-breeding exams on heifers can provide useful information on the up-to-date reproductive status of the breeding herd, which can allow producers to have a better prediction of the success of their upcoming breeding season (Larson et al., 2016). Palpation of the reproductive tract is used to examine all reproductive structures present

in the heifer. Evaluation of heifers six weeks before the breeding season gives producers more time to identify any potential changes or concerns regarding keeping a heifer in the breeding program, or culling due to poor reproductive status.

In 1991, Anderson et al. created the reproductive tract score (RTS) scoring method that is used throughout the beef cattle industry and is an effective method for evaluating heifers (Anderson et al., 1991). Reproductive Tract Scores can be evaluated on a scoring system with the ranking of 1 through 5. Uterine horns, uterine tone, ovarian structures, and ovarian length (L), height (H), and width (W) are then evaluated and scored (Anderson et al., 1991). Listed are the parameters for a RTS of 1 through 5: RTS of 1: immature uterine horns with a < 20 mm diameter, no uterine tone, ovary parameters = 15 mm L x 10 mm H x 8 mm W, and no palpable follicles on the ovarian structure; RTS of 2: 20 – 25 mm uterine horn diameter, no uterine tone, ovary parameters = 18 mm L x 12 mm H x 10 mm W, and 8 mm follicles on the ovarian structure; RTS of 3: 20 – 25 mm uterine horn diameter, slight uterine tone, ovary parameters = 22 mm L x 15 mm H x 10 mm W, and 8 – 10 mm follicles on the ovarian structure; RTS of 4: 30 mm uterine horn diameter, good uterine tone, ovary parameters = 30 mm L x 16 mm H x 12 mm W, and > 10 mm follicles + corpus luteum possible on the ovarian structure; and RTS of 5: > 30 mm uterine horn diameter, good uterine tone, ovary parameters = > 32 mm L x 20 mm H x 15 mm W, and corpus luteum present on the ovarian structure (Appendix 2).

Studies have shown a relative relationship between reproductive tract score, estrous synchronization response, and synchronized pregnancy rates (LeFever et al., 1987). For heifers that scored a RTS of 1 or 2, their estrus response was 50% and 79.9%, and synchronized pregnancy rates were 0% and 17%, respectively (LeFever et al., 1987).

When scored between 12 and 14 months of age, heifers with a RTS of 1 or 2 show poor reproductive performance during the breeding season (Hall et al., 2005). Heifers that scored a RTS of 3 had a 93.1% estrous response and 62.1% synchronized pregnancy rate. Heifers that scored a RTS of 4 and 5 had an estrous response rate of 93.1% and 96.8%, respectively. These same heifers had a synchronized pregnancy rate of 62% for a RTS of 4 and 54% for a RTS of 5 (LeFever et al., 1987). The authors noted that heifers who were 12 – 14 months of age at RTS, and had a score of 4 or 5, had a higher probability of reaching puberty early, responding to estrous synchronization, and increased pregnancy rates (LeFever et al., 1987). Overall, reproductive tract scoring is a valid management tool for culling or keeping heifers at the beginning of the breeding season (Holm et al., 2015). Culling that is based off of pre-breeding RTSs enhances the longevity of a successful breeding herd and program.

II.V. *Pelvic Measurements*

Pelvic Area (PA) measurement is recorded at or around one year of age, and it is predominately associated with either calving ease or calving difficulty. Pelvic area is an important aspect of heifer selection in regard to dystocia, or difficult and obstructed labor (Neville et al., 1978). A major cause of dystocia in a cow or heifer is a large calf during parturition in comparison to the heifer's pelvis (Larson et al., 2016). The reported correlation between a yearling's pelvic area compared to a 2-year-old cow's pelvic area is 0.7. Measuring a yearling's pelvic area is therefore a better predictor of pelvic size at the time of parturition (Neville et al., 1978). Rather than selecting for a "maximum" pelvic size on a yearling heifer, a sliding caliper is used to assess a standard "minimum" pelvic size as a culling criterion (130 – 150 cm^2 at one year of age) (Neville et al., 1978). Studies

indicate that selection for an increased pelvic area results in a larger frame size. Pelvic area (PA) is used to eliminate heifers that have a misshapen or unusually small pelvic opening (Hall et al., 2005), with its main benefit aiding in the reduction of dystocia.

II.VI. *Temperament*

Heifer temperament is described as the reaction characteristics of cattle when they are exposed to human handling (Kasimanickam et al., 2014). Cattle that remain calm (not stressed) in the chute and during routine handling perform better than cattle who become agitated (very stressed). A study conducted by Kasimanickam et al. (2014) compared heifer temperament to pregnancy rates after estrous synchronization and fixed-time artificial insemination. A total of 967 beef heifers from 8 locations were evaluated for temperament on a 2-point scoring system (0 = calm, slow exit; 1 = excitable; fast exit at jump/trot/run) and evaluated for pregnancy 70 days after AI. Any heifers that required external stimuli to exit the chute were excluded from the study (Kasimanickam et al., 2014). AI pregnancy status differed by 8.4% when comparing calm heifer pregnancy rates (51.9%) to excitable heifer pregnancy rates (60.3%). Calm heifers also displayed a higher number of class 1, 2, and 3 follicles (Class 1: n = 5.6; 3 – 5 mm, Class 2: n = 1.4; 6 – 9 mm, and Class 3: n = 1.2; > 9 mm, respectively) compared with excitable heifers with class 1, 2, and 3 follicles (Class 1: n = 4.4; 3 – 5 mm, Class 2: n = 1.2; 6 – 9 mm, and Class 3: n = 0.60; > 9 mm, respectively) (Kasimanickam et al., 2014). Authors then compared three different designs of cattle handling facilities to heifer pregnancy rates and temperament. Facility designs ranged from alleyway with acute bends and turns, long and straight alleyway, and semi-circular alleyway. The acute bends and turns alleyway pregnancy rate was 53.5%, the long and straight alleyway pregnancy rate was 56.3%, and

the semi-circular alleyway pregnancy rate was 67.0%. Between the two most extreme handling facility designs (acute bends and turns vs. semi-circular), the pregnancy rate difference was 13.5% (Kasimanicham et al., 2014). Overall, heifers who had a more excitable temperament had lower AI pregnancy rates (8.4%) and a smaller number of follicles compared to heifers who had calmer temperaments. Results from this study demonstrate temperament greatly affects reproductive performance following estrous synchronization and fixed-time artificial insemination in beef heifers (Kasimanickam et al., 2014).

Heifer selection for a variety of traits can impact her age at puberty and subsequent fertility (Hall et al., 2005). Producers must use a combination of reproductive exams, body weights, body conformation, and temperament modules that meet their farm's requirements for efficiency and growth, while also maintaining a high rate of reproductive success (Hall et al., 2005).

III. Common Heifer Breeding Management Programs

III.I. *Natural Bull Service*

Bulls used for natural service should be evaluated to ensure their EPDss (expected progeny differences) for parameters such as birthweight and calving ease are consistent with the producer's breeding program goals (Larson et al., 2016). A BSE, or Breeding Soundness Exam, is performed prior to the start of the breeding season to evaluate structural soundness, semen quality, and the overall health of the bull. Producers should observe bull activity at the start of breeding season, ensuring he is searching out females in heat, appropriately mounting, and copulating Statistics show if > 80% of heifers are

cycling at the beginning of breeding season, an average of 4% – 5% of heifers will be natural bred each day (Larson et al., 2016).

III.II. *Estrous Synchronization and Artificial Insemination*

One of the most widely used and impactful reproductive technologies for cattle is estrous synchronization and artificial insemination (Youngs, 2016). The successful utilization of both of these technologies facilitates precisely timed control of the expression of heifer estrus and artificial insemination (Youngs, 2016). If a properly administered and monitored estrous synchronization protocol is performed, 70% – 90% of cycling heifers are expected to express estrus and ovulate a viable oocyte within the time window predicted by the estrous synchronization system (Wood-Follis et al., 2004 and Day et al., 2005). AI is traditionally performed following the AM/PM rule, which involves inseminating heifers that are observed in estrus in the morning on the same evening of the same day, and vice versa (Youngs, 2016). Beef heifers that are bred through an appropriately-timed AI program, in relation to ovulation of a fertile oocyte, have a 60% – 80% probability of establishing a pregnancy that can be detected < 50 days of gestation (Tauck et al., 2007 and Bon Durant, 2007).

Estrous synchronization involves exogenous administration of hormones already naturally produced in the heifer, such as Prostaglandin F2α, progesterone, and gonadotropin-releasing hormone (Youngs, 2016). If heifers are synchronized, artificially inseminated, and then bred with a bull, heifers should be held out of the breeding pasture with the bull for at least two weeks following the last day of artificial insemination. This ensures AI pregnancy rates can be accurately determined in early gestation via ultrasound examination or palpation (Tauck et al., 2007). If bred correctly, 60% – 70% of heifers

identified in estrus should become pregnant by artificial insemination (Larson et al., 2016).

IV. Heifer Reproductive Failure

Reproductive failure is one of the major factors that affects the profitability of the beef cattle production industry (Diskin, 1980). Fertility is defined as a heifer that shows the desire to mate, the capacity to conceive, nourish a growing embryo, and successfully expel a live calf at 12-month intervals (Abraham, 2017). Heifers can be classified as fertile, infertile (reduced fertility or failure to maintain pregnancy), or sterile (absolute inability to produce offspring) (Abraham, 2017). In beef cattle, studies show the estimated fertilization rate (natural or AI) for oocytes is 90%. However, the estimated calving rate from a single service is between 40% – 55%. This suggests an overall embryonic and fetal mortality rate (not including fertilization failure) of 35% – 50%, with the majority of embryonic losses (70% – 80%) occurring during the first three weeks of pregnancy (Diskin et al., 2006).

Functional causes of infertility tend to affect individual heifers, but when combined, these infertile heifers can make a large impact on the overall herd (Abraham, 2017). Most functional causes can occur from an endocrinological abnormality, which can reflect fertility issues. These abnormalities include, but are not limited to: non-detected estrous (silent estrous), anestrus, ovulatory defects, persistent corpus lutea, luteal deficiencies, cystic ovaries, and repeat breeders (Abraham, 2017). Infertility that results in the failure of a cow or heifer to conceive a calf is the single-largest economic loss to beef producers (Lamb, 2014).

Poor fertility remains the major reason for culling in the beef cattle industry, which reduces the longevity of the herd (Wathes et al., 2014). Longevity is a desirable trait, as it relates to total profit of the producer and optimizing economic returns (Wathes et al., 2014). Lamb et al. (2016) calculated an average ratio for the economic impact one infertile heifer can have on a herd. The average loss due to failure to become pregnant during the breeding season is ~$165/heifer (Lamb, 2014). Taking this into consideration, the NASS estimated total heifer infertility in the United States exceeding $4.7 billion annually (Lamb, 2014).

In a logical sense, producers cannot pin-point all infertile heifers that might come their way or know the exact reasons why they may be infertile. However, understanding the costs and roles associated with heifer infertility may push management practices to explore new realms of technology to improve livestock reproductive efficiency (Lamb, 2014).

V. Metabolomics

V.I. *The Metabolome*

Metabolomics is a rapidly growing field that has the potential to play a major role in improving the diagnosis and treatment of complex issues in health and disease. The term "metabolome" was first defined by Oliver et al. in 1998 as the "quantitative complement of all of the low-molecular-weight molecules present in cells in a particular physiological or developmental state" (Oliver et al., 1998). Essentially, metabolomics involves the quantitative measurement of the global set of low-molecular-weight metabolites in a biological fluid. It is thought that changes in quantities of individual

enzymes can have little to no effect on fluxes of metabolites. However, these individual enzymatic changes can have significant effects on the concentrations of multiple stand-alone metabolites that make up the "metabolome" (Oliver et al., 1998). Metabolomics reflects events that are well down-stream of gene expression, and it gives valuable information about the metabolism of cells that other "-omics" technologies cannot accomplish (Bracewell-Milnes et al., 2017).

The field of metabolomics is gaining increased interest and application across multiple disciplines, including integrative and systems biology, functional genomics, and biomarker discovery (Oliver et al., 1998). The study of metabolomics is a relatively new and novel practice in the many "-omics" realms. The field and its sub-fields are commonly known among many names, such as metabolomics, metabolic fingerprinting, metabolic profiling, metabolite target analysis, metabolite profiling, and metabolic footprinting. There has been much skepticism about why exactly metabolomics is a useful tool, in comparison to proteomics, genomics, and transcriptomics. An example by Goodacre et al. in 2004 explains the simple and precise advantageous reason to use metabolomics over other "-omics" studies, and it strives from the stem of evolution. Goodacre fabricates a scenario involving a specific biomarker or metabolite, such as fructose 1,6-bisphosphatase, from multiple organisms. In order to measure the amount of this biomarker, one must know the presumptive DNA and protein sequences from each individual organism in order to design correct and suitable oligonucleotides (to capture mRNA on a nucleotide array) and effectively identify proteins (via 2-D gel electrophoresis and mass spectrometry) (Goodacre et al., 2004). Inversely, the substrate and product of fructose 1,6-bisphosphatase are fructose 1,6-bisphosphate and fructose 6-

phosphate. This substrate and product have the same basic chemical structure throughout, regardless of the organism they are extracted from. From this knowledge, one can quantify the metabolites in various samples, thus creating a more universal approach that can span and adapt to the species barrier (Goodacre et al., 2004).

From a conceptual level, the genome gives rise to the transcriptome and the transcriptome gives rise to the proteome. The proteome acts on small molecules within an organism (both endogenous and exogenous) known as the metabolome. There is evidence that feedback interactions exist at all levels, and these levels are sensitive to environmental cues, environmental influences, nutrition, disease states, toxicants, etc. The physiological status of an organism is ultimately affected by the varying combination of feedback interactions throughout the genome, transcriptome, proteome, and metabolome.

As far as technology is concerned, the increased interest and knowledge of metabolomics has arrived at precisely the right time. Generally speaking, metabolomics analysis consists of four steps that include sample preparation, data collection, data processing, and biological interpretation. MS (Mass Spectrometry) and NMR (Nuclear Magnetic Resonance) are two major technologies that are used to gather metabolomics data, and they have an inverse relationship involving quantification and specificity (Veenstra, 2012). MS has the main advantage of heightened sensitivity, as it can detect metabolites from femtomolar (10^{-15} moles/liter) to attomolar ranges (10^{-18} moles/liter). MS is typically coupled with either GC (gas chromatography) or LC (liquid chromatography), as both can define the measurement of hundreds of individual species within one single sample. Mass spectrometry, combined with increased metabolomics

databases and software systems, is allowing researchers to identify metabolomes of whole organisms as a routine practice (Veenstra, 2012). The main weakness of mass spectrometry is associated with quantification. Signal intensity measured by a mass spectrometer is affected by the type of sample used and the molecular environment the sample was established and maintained in. However, the major weaknesses of MS are accurately defined as the major strength of NMR spectrometry.

Compound peaks in NMR spectrums are directly related to the concentration of specific nuclei in a given sample. With this correlation, nuclear magnetic resonance makes quantification of compounds in complex mixtures extremely accurate and precise (Veenstra, 2012). NMR can detect metabolites with increased abundance by measuring their local resonance positioning of nuclei in the NMR spectrum. The samples typically used and associated with NMR are liquid-state samples (blood, plasma, serum, urine, etc.), tissue samples (tumors), and *in-vivo* cell lines.

Evidence suggests that global metabolomics profiles are useful for the identification of diagnostic biomarkers located in easily-accessible biofluids (Dutta et al., 2012). Alterations in the biofluid, cells, or cell culture environments can induce a response to environmental and developmental stimuli. This can ultimately result in changes to intermediate pathway metabolites and leads to a change in the accumulation of metabolites at the terminal ends of a given pathway (Oliver et al., 1998). Biofluids are the most easily obtained sample and can be analyzed with little or no sample preparation via NMR (Nuclear Magnetic Resonance) (Oliver et al., 1998). Recent studies have shown that measurements of global sets of low molecular weight metabolites are important

indicators of various diseased states, reinforcing the idea of metabolomics being an effective tool for monitoring disease progression (Dutta et al., 2012).

V.II. *Biomedical Metabolomics*

While it is known that metabolomics is a relatively new area of study compared to proteomics and genomics, it has been making major impact in a wide variety of scientific research fields. Since the completion of the human genome, functional genomics has been used to identify the links between protein and gene expression profiles in both normal and diseased states. Transcriptomics and proteomics have made remarkable breakthroughs in the identification of therapeutic targets, disease subtypes, newborn screenings, toxicology reports, food safety, and biomarker discovery for disease (Veenstra, 2012 and Yang et al., 2007). However, scientists have concluded that these technologies alone are falling short regarding the "big picture" of differential cellular networks and pathways.

Within the last decade, the use of metabolomics has sparked much interest involving biomarker discovery for certain types of cancers. Metabolomics can provide comprehensive profiling of thousands of metabolites in multiple tumor types (Yan 2018). Comprehensive profiling has resulted in the discovery of filtering for differential cancer metabolites, evaluating the effects of treatment drugs, revealing underlying mechanisms of oncogenes, and the discovery of novel drug targets (Yan 2018). Yang et al. (2007) used comparative metabolomics profiling to characterize cancerous vs. normal cells to improve the underlying mechanisms of tumorigenesis. By using metabolomics analysis, they were able to identify information about both the metabolite pools and metabolite fluxes associated with cancer-cell line and normal-cell line metabolic pathways. The use

of metabolic profiling is an important aspect of cancer biology due to the compound changes in the central metabolism of the host, as well as the metabolism associated with the expansion of the tumor itself (Yang et al., 2007). An additional cancer study was conducted by Sreekumar et al. (2009) in regard to prostate cancer. The authors used LC-MS and GC-MS spectrometry on tissue, urine, and plasma samples from patients with three types of prostate disease: benign prostate disease, clinically localized prostate disease, and metastatic prostate disease. Not only were the authors able to classify, distinguish, and identify these three conditions in a blind study by the use of NMR, but they were also able to detect Sarcosine. Sarcosine is a tumor-specific metabolite that is highly increased in concentration when clinically localized prostate cancer progresses towards and develops into metastatic prostate cancer. The significance of Sarcosine discovery is how it is detected. The authors discovered that Sarcosine can be detected in patients non-invasively through a simple urine sample (Sreekumar et al.,2009). Urinary metabolites are downstream products of cellular processes. Because of this, they can provide complementary information in relation to plasma and tissue metabolome analysis (Zhang et al., 2012). Urinary metabolome analysis provides an affordable, convenient, and clinical approach to aid in the introduction of cancer biomarker discovery.

Previous work by Suliman et al. (2005) looked at the levels of amino acids in the blood plasma of kidney disease patients (with and without inflammation). They discovered that the patients with inflammation had significantly lower levels of Asparagine, Serine, Glutamine, Glycine, Arginine, Alanine, Histidine, and Threonine (Suliman et al., 2005). In another study investigating the plasma amino acid levels of cats with chronic gastrointestinal (GI) disease, it was shown that Arginine, Histidine, Lysine,

Methionine, Phenylalanine, Taurine, and Tryptophan (along with several non-essential amino acids) were lower in cats with chronic GI diseases (Sakai et al., 2018). The authors discovered that Histidine and Tryptophan levels were inversely correlated to symptom severity, and Histidine could suppress inflammatory cytokine release by their macrophages (Sakai et al., 2018).

Metabolic diseases are referred to as a disease caused by a disordered metabolism (Yan 2018). Metabolic diseases are the direct response of the combination of changes in the genetic makeup of an organism and changes associated with environmental cues. During a diseased state, the response of an organism's cells are to modify the concentrations of numerous metabolites, with the end goal of maintaining homeostasis within the host (Bracewell-Milnes et al., 2017). The metabolites within an organism are in constant communication and display dynamic balance with the biofluids that profuse within cells. Because of this relationship, the direct result should be reflected in the organism's biofluid composition (Bracewell-Milnes et al., 2017). It is feasible that the future of metabolomics will be able to distinguish multiple physiological states, such as diseased and non-diseased states, based entirely on the host's metabolomics fingerprint alone (Bracewell-Milnes et al., 2017).

V.III. *Metabolomics in Agriculture*

Previous studies in humans have shown that differential levels of metabolites has been able to detect various difficult-to-diagnose ailments including diabetic kidney disease (Kloet et al., 2012), Parkinson's disease (Bogdanov et al., 2008), myocardial ischemia (Sabstine et al., 2005), ovarian cancer (Zhang et al., 2012), and endometriosis

(Dutta et al., 2012). However, the use of metabolomics in the agriculture sector has remained largely underutilized.

Cheng et al. (2014) investigated tilapia and tilapia-associated bacterial infections, such as *Streptococcus iniae*. With a broad host-range impacting over 27 species, *S. iniae* is becoming a challenging bacterial infection that has emerged as the leading fish pathogen affecting global aquaculture operations. Current antibiotics and vaccines lack practicality and have their own sets of limitations and drawbacks. With the use of metabolomics and metabolomic profiling, Cheng et al. used a GC/MS-based metabolomic profile of the tilapia liver to compare the differential metabolomes of survivor tilapia livers and post-mortem tilapia livers infected with *S. iniae.* Proceeding their study, they identified the metabolite N-acetylglucosamine to be elevated in the surviving group compared with the post-mortem group. This metabolite is essential for differentiating those tilapias that will survive after exposure to *S. iniae* and those tilapias that will not survive following exposure to *S. iniae.* The exogenous circulation of N-acetylglucosamine in tilapia was shown to significantly increase the "survival abilities" of those fish exposed to the bacterial infection. In order to test the survival frequency, exogenous N-acetylglucosamine was injected into *S. iniae* infected tilapia. Survival rates of those tilapias injected with N-acetylglucosamine ranged from 75% - 85%, compared with the 40% survival rate in the control untreated tilapia group. Through the use of metabolomics, N-acetylglucosamine (a potential modulator of *S. iniae*) can elevate the survival of tilapia infected with bacterial pathogens via reverse regulation of metabolites and proteins. Through these studies, there is potential for functional metabolomics to develop treatments for pathologies (Cheng et al., 2014).

Chen et al. (2017) conducted a study in which acute liver failure was induced in pigs, and the aim of this study was to identify plasma biomarkers for acute liver failure (ALF). They utilized continuous collection of samples, which allowed the evaluation and association between varying degrees of liver damage in comparison to increased or decreased metabolite levels. In humans, ALF presents itself as multiple organ failure, jaundice, and complete deterioration of all liver functions. Liver injury is typically assessed by concentrations of hepatic enzymes, albumin, bilirubin, ALT (alanine aminotransferase) and prothrombin (Chen et al., 2017). Many of these metabolites are present and circulating throughout multiple areas of the body, which decreases their overall sensitivity and specificity for evaluating the "standard" concentration level in regard to liver assessment. Three upregulated amino acids (Phenylalanine, Tryptophan, and Methionine), two upregulated bile acids [Glycoursodeoxycholic acid (GCDCA) and Tauroursodeoxycholic acid (TCDCA)], and two downregulated metabolites [Lysophosphatidylcholines (LPC) and Phosphatidylcholines (PC)] were identified. Amino acids identified as biomarkers for the severity of liver impairment, conjugated bile acids identified predictive stages of early stage liver damage, and the LPC/PCs are relative for the overall early prognosis of ALF. Chen and his team discovered new biomarkers with greater specificity and greater sensitivity in the pig model for the early diagnosis and prognosis of acute liver failure in humans (Chen et al., 2017).

V.IV. *Metabolomics and Male Infertility*

Infertility is a complex disorder that leaves patients (human and animals alike) with significant physiological, medical, and financial complications pre- and post-diagnosis. There have been countless areas of research conducted world-wide over the

last century interested in diagnosing the pathology of unexplained infertility. However, infertility still remains the number one reproductive-associated disorder. Metabolomics techniques are providing useful information for working with multiple species to investigate the causes of unexplained infertility at the molecular and metabolite level (Bracewell-Milnes et al., 2017).

Male factor infertility contributes to ~ 40% of all human infertility cases. Male infertility can be caused by an array of issues, including sperm morphology, motility, and sperm count. Semen analyses are an important factor in regard to the diagnosis of male factor infertility, but they are not specific enough to identify the root cause of the infertility problem and dictate accurate treatment or therapy for the patient. Recent studies have identified shifts in male reproductive hormone levels, as well as shifts associated with the metabolome of infertile men (Mendiola et al., 2012). DEHP (di-2-ethylhexyl) is a compound known as a phthalate. Phthalates are man-made compounds primarily used in industrial environments. These compounds emit anti-androgenic properties related to adverse reproduction and development in males. Properties of phthalates have been reported to cause complete disruption of reproductive development in male rodents, as well as decreased serum FT (free testosterone) levels post-exposure. Mendiola et al. (2012) investigated the relationship of urinary phthalate metabolite concentrations in relation to serum reproductive hormone levels in both fertile and fertile men. Their results suggest that exposure to phthalates (at normal environmental concentrations) is associated with significantly reduced levels of FT (free testosterone) and serum estradiol. These exposures highlight the androgenic effects of phthalates. The use of metabolomics can assist and aid in the discovery of decreased semen quality,

changes in circulating levels of male sex steroids, and reproductive failure from increased urinary phthalate concentrations (Mendiola et al., 2012).

In addition to phthalates leading to reproductive failure and male factor infertility, oligozoospermia places an increased risk on the success rate or decline of male fertility. Oligozoospermia is the leading cause of male sub-fertility and infertility, and refers to a patient with substantially low sperm concentration in semen compared with the average male (Zhang et al., 2014). Relative sperm concentration is a typical clinical diagnosis of oligozoospermia but remains inadequate in relation to assessing infertility as a whole. Zhang et al. (2014) conducted a LC/TOF-MS metabolomic study to identify differential urinary metabolic patterns of infertile men with oligozoospermia, with the goal to discover biomarkers indicative of oligozoospermic infertility. The authors discovered several biomarkers such as acyl-carnitines (C3:1, C8, and C10:2), aspartic acid, and adenine that are associated with energy consumption and anti-oxidant defenses against spermatogenesis. Acyl-carnitines from previous studies have been associated with decreased sperm concentrations, decreased sperm amplitude of lateral head displacement, and diagnostic parameters related to oligozoospermic infertility. Aspartic acid from previous studies plays a major role in spermatogenesis by decreasing energy supply and hormone metabolisms in infertile men. Through the use of urinary metabolomics analysis, oligozoospermic biomarkers can have the potential to reflect responses of reduced fertility in the male (Zhang et al., 2014).

V.V. *Metabolomics and Female Infertility*

Female infertility contributes to ~50% of all infertility cases (American Pregnancy Association, 2017). Infertility can be caused from a number of complex issues

that range from ovulation disorders, uterine disorders, and hormonal disorders (American Pregnancy Association, 2017). The pathology of unexplained infertility remains the number-one reported reproductive disorder. Metabolomics techniques are providing useful information for working with multiple species to investigate the causes of unexplained infertility at the molecular and metabolite level (Bracewell-Milnes et al., 2017).

The follicular fluid in females is defined as the *in-vivo* micro-environment for a developing oocyte. Essentially, the follicular fluid must contain many components necessary for oocyte growth, maturation, and survival. The necessary components housed in the follicular fluid are metabolites secreted by the oocyte, granulosa cells, and the surrounding vasculature (Revelli et al., 2009). There is evidence that suggests the metabolic makeup of the follicular fluid itself can differentiate between developmentally poor and competent oocytes.

Within the agriculture sector, metabolite concentrations in follicular fluid and blood were used in attempt to explain differences in fertility between heifers and lactating cows (Bender et al., 2010). Within the last three decades, there has been a rapid decline in the fertility of dairy cows. Bender et al.(2010) utilized metabolomics to investigate metabolic differences between the follicular fluid of the dominant follicle of lactating cows and the follicular fluid of the dominant follicle of heifers. Follicular fluid was collected over three phases of follicular development: newly selected dominant follicles, pre-ovulatory follicles prior to oestrous, and post-LH surge follicles (Bender et al., 2010). Twenty-four fatty acids and nine aqueous metabolites were found significantly different when comparing cows to heifers. Palmitic Acid and Stearic Acid (saturated fatty acids)

were higher in the follicular fluid of cows, and Docosahexaenoic Acid (saturated fatty acid) was higher in the follicular fluid of heifers. Bender discovered that if there is a higher concentration of saturated fatty acids in cows, oocyte maturation and early embryo development will be negatively impacted. Results suggested that the overall follicular microenvironment in cows places oocytes at a developmental disadvantage when compared with the microenvironment for heifers. This overall conclusion could contribute to fertility differences in heifers and cows alike (Bender et al., 2010).

Pre-eclampsia, or PE, is the leading complication during pregnancy in women, as it presents a risk for both fetal and maternal morbidity and mortality (Austdal et al., 2014). Affecting approximately three-percent of women worldwide, PE contributes to substantial health problems later in life such as chronic hypertension, cardiovascular disease, and diabetes mellitus type-2 up to eight-fold (Kuc et al., 2014). PE is typically diagnosed early-mid pregnancy (first through second trimester) by clinical symptoms such as spontaneous high blood pressure and proteinuria, or increased and abnormal amounts of protein in the urine. However, when these parameters are present, PE has already progressed in the patient and cannot be prevented or stopped. Currently, there are no leads on the actual pathophysiology and complex gene mechanisms related to the onset of pre-eclampsia. Kuc et al. in 2014 introduced a novel LC-MS-based metabolomics approach with interest in discovering signature patterns of metabolites that are significantly altered in patients diagnosed with PE during their first trimester of pregnancy, compared with serum of patients undergoing a healthy pregnancy (Kuc et al., 2014). The authors discovered decreased levels of taurine, asparagine, and glycylglycine (amino acids and amino-acid derivatives) in first-trimester patients diagnosed with PE

compared with healthy non-PE patients. Reduced taurine levels are associated with impaired trophoblast invasion, reduced trophoblast-placenta communication, and reduced placentation. However, previous studies indicated that taurine, asparagine, and glycylglycine supplementation reduced hypertension in rats and reduced peripheral vessel resistance. Similarly, Austdal et al. (2014) investigated the serum and urinary metabolomics profile of women diagnosed with PE compared with women with a healthy pregnancy. They discovered that patients diagnosed with PE have significantly lower serum concentrations of histidine (amino acid) compared with healthy pregnant women. Significantly higher levels of glycerol (amino acid) were also discovered in the serum of PE women compared to healthy pregnant women. Early detection of pre-eclampsia would provide clinicians with targeted intervention for patients diagnosed with PE. The use of serum and urinary metabolomics profiling during the first trimester of pregnancy could create and provide an early-detection assay that has the potential to detect specific maternal metabolite serum levels, aiding in the prevention of pre-eclampsia (Kuc et al., 2014).

VI. Inflammation

V.I. *The Immune System*

The maternal immune system is plays a critical role in the establishment and maintenance of pregnancy and parturition (Prins et al., 2012). The immune system is a host defense system composed of a vast network of cells, tissues, and organs that are designed to protect a host or an organism against disease. The immune system must defend against pathogens in order to maintain a healthy state within the host organism. The immune system can typically be classified into two sub-systems: the innate immune

system (humoral immunity) and the adaptive immune system (cell-mediated immunity) (Parkin, 2001). Throughout evolution, pathogens have rapidly adapted and evolved, which have enabled the avoidance of detection and neutralization by the immune system.

The bovine innate immune system produces an immediate, or native response, to the pathogen affecting the host. The innate immune system functions through a combination of natural barriers that defend the heifer, such as skin, neutrophils, phagocytes, natural killer cells, and cytokines (BioRad, 2016; Rainard et al., 2006). After an initial microbial invasion, macrophages and dendritic cells secrete cytokines to increase the total amount of white blood cell production, thus aiding in fighting the present infection.

Many reproductive failures in females of all species can be immunologically determined (Mahdi, 2010). Studies suggest that many cytokines are crucially important for reproductive processes and aid in reproductive success. Over-production of inflammatory cytokines has been suggested to cause multiple reproductive maladies such as fetal growth restriction in response to hypoxia by means of decreased amino-acid uptake (Briana et al., 2009).

V.II. *Inflammatory Cytokines in Relation to Infertility*

The term “fetal allograft” is defined as the unique immunological mechanism at which fetal survival is achieved, despite modifications of the host organism’s immune response. Placental invasion has been described similarly to that of tumorigenicity, or the growth of carcinomas, thus invoking principles and mechanisms similar to tumor immunology (Koumantaki et al., 2001).

During pregnancy, the female reproductive system is in constant communication with the endocrine system and the immune system. The immune system plays a significant role in the processes of folliculogenesis, oogenesis, embryo implantation, preparation of the endometrium, and maintaining pregnancy (Ostanin et al., 2007). The fluid associated with the female reproductive tract contains cytokines that assist in interactions between multiple cell types. Cytokines play a significant role in multiple reproductive processes, as reproductive success is dependent upon interactions between cytokines and the fetal-maternal interface (Austgulen et al., 2016).

Cytokines are immunoregulatory molecules that are responsible for determining the nature and severity of immune response in a host organism (Mahdi, 2010). Inflammatory cytokines are known by multiple names, such as monokines (cytokines made by monocytes), lymphokines (cytokines made by lymphocytes), chemokines (cytokines with chemotactic activities), and interleukins (cytokines acting on other leukocytes and made by a singular leukocyte) (Zhang et al., 2007). Cytokines can act on cells that secrete them (autocrine action), distant cells (endocrine action), or on nearby cells (paracrine action). Cytokines can be categorized as pro-inflammatory or anti-inflammatory. Pro-inflammatory cytokines are produced by active macrophages and are typically involved in the up-regulation of inflammatory reactions (Zhang et al., 2007). Pro-inflammatory cytokines are predominately active during early and late events of gestation (pregnancy establishment and parturition), as both events can be described as inflammatory-like events. Inflammation is essentially a defense-mechanism in which multiple varieties of WBC (white blood cells) and their cytokines travel to the damaged area and act to repair any potential injuries.

Tumor Necrosis Factor alpha (*TNFα*), also known as cachectin, is a pro-inflammatory cytokine that is secreted by primarily monocytes and macrophages. Studies show that *TNFα* affects endothelial function in the uterus. Hunt et al. (1996) demonstrate the expression of *TNFα* in embryo and placental tissues, thus re-enforcing its beneficial roles in the development of early pregnancy. However, *TNFα* has also been shown to inhibit blastocyst growth in murine pregnancy. Additionally, the administration of *TNFα* in normal pregnant mice results in fetal resorption (Raghupathy, 1999). Higher serum levels of *TNFα* are seen in females who frequently experience reproductive failure termed RSA, or recurrent spontaneous abortions (Reid et al., 2001). *TNFα* in women is essential for conception and early pregnancy, but retained and high levels are dangerous during gestation. A reasonable explanation for this contradiction comes from Guilbert et al. (1996) suggesting that the timing, localization, and regulatory signaling cascades of *TNFα* are responsible for the up and down-regulation of *TNFα* itself throughout conception and maintenance of pregnancy.

IL-6, or Interleukin-6, is also a pro-inflammatory cytokine that is secreted from cells such as helper T-cells and macrophages (Prins et al., 2012). *IL-6* is a stimulating factor in response to mediating the innate and adaptive immune response of an individual, as well as aiding in acute-phase responses to chronic inflammation. *IL-6* has three distinct receptor-binding sites. *IL-6* is widely expressed in gestational tissues and in the female reproductive tract, and it regulates functions for placental development and embryo implantation (Prins et al., 2012). Studies show that overall elevated levels of *IL-6* result in pre-eclampsia, pre-term delivery, and unexplained infertility. Decreased local *IL-6* levels associated with the endometrium suggest a contribution to fetal loss, as recurrent

miscarriages are correlated with reduced and insufficient *IL-6* expression in the endometrium (Prins et al., 2012). Consistent with the reproductive role of *IL-6*, *IL-6* knockout mice exhibit delayed parturition and elevated fetal absorption (Prins et al., 2012). Additionally, the *IL-6* concentration of plasma and cervical mucous is reported to be higher in women experiencing unexplained infertility compared with healthy, fertile women.

CXCL5, or C-X-C Motif Chemokine Ligand 5 [also known as epithelial-derived neutrophil-activating peptide-78 (*ENA-78*)], is a protein-coded neutrophil-activating peptide that is generated in response to stimulation of inflammatory cytokines such as *TNF*α. It is a member of the chemokine family and aids in recruitment of neutrophils, promotion of angiogenesis, and connective tissue remodeling. *MCP1*, or Monocyte Chemoattractant Protein-one [also known as *CCL-2* (chemokine C-C motif ligand-2)], is a part of the chemokine family and aids in the inflammatory response to damaged tissue or infection. *MCP1* aids in the recruitment of monocytes to sites of inflammation. *MCP1* is pertinent for the pathogenesis of many diseases such as psoriasis, rheumatoid arthritis, and Alzheimer's disorder.

CXCL-5 is secreted from white adipose tissue and highly increased during obesity. During this secretion, insulin-signaling pathways are inhibited and blocked, thus promoting insulin resistance. Zohrabi et al. in 2017 utilized a PCOS, or polycystic ovarian syndrome, model to assess the serum levels and contributing concentrations of *CXCL-5* for PCOS diagnosis (Zohrabi et al., 2017). PCOS affects between 4% - 8% of women during their reproductive age and remains a major cause of infertility in women. Studies indicated that women diagnosed with PCOS had higher serum concentration of

CXCL-5 and higher concentrations of fasting blood sugar (FBS) than those in the control group (non-PCOS). Chavey et al. (2009) discovered a positive correlation between serum levels of *CXCL-5* and body weight; the inhibition of *CXCL-5* secretion reduced the risk of the individual developing an obesity-related pathogenesis (such as PCOS). The presence of *CXCL-5* could potentially be used as a biomarker for the inhibition of early-onset PCOS and associated obesity/metabolic syndromes in women (Zohrabi et al., 2017).

POSTN, or Periostin, is an extracellular protein that is expressed throughout multiple areas of the body. It is found most prevalently in the stomach, aorta, lower gastrointestinal tract, uterus, and placenta (Freis et al., 2017). During an injury, *POSTN* levels rise due to its role in inflammatory response. In recent years, studies have found *POSTN* playing a role in allergic reactions, skin, bone, heart, kidney, and cancer pathways. *POSTN* has been shown to be essential in metastatic cell maintenance, and knockout *POSTN* studies suggest prevention of metastasis completely. Freis et al. (2017) conducted a study investigating the changes in *POSTN* serum concentrations of women suffering from recurrent spontaneous abortions (miscarriage) during their first trimester of pregnancy. The relative expression of *POSTN* between four and six weeks of gestation was significantly higher in patients who experienced recurrent spontaneous abortions than those with maintaining a healthy pregnancy. This study suggests that increased Periostin levels could become a promising biomarker and early-screening method for pregnancy outcome assessment or risk of miscarriage during the first trimester (Freis et al., 2017).

VII. The Impact on Agriculture

Reproductive failure and unexplained heifer infertility are major factors that negatively impact the profitability of beef cattle production (Diskin, 1980). Heifer infertility remains a difficult to diagnose pathology resulting in inefficiencies within the cow-calf sector. Functional causes of infertility tend to affect individual heifers, but when combined these infertile heifers can make a large impact on the overall herd (Abraham, 2017). Poor fertility remains the major reason for culling in the beef cattle industry, which reduces herd longevity (Wathes et al., 2014). Metabolomics techniques are providing research breakthroughs with regards to investigating the causes of unexplained infertility at the molecular and metabolite level (Bracewell-Milnes et al., 2017). The pathology of unexplained infertility remains the number-one reported reproductive disorder in females and males alike.

Successful pregnancy establishment in a heifer is associated with multiple factors coming into play and working together to establish and maintain a healthy pregnancy. These factors can include but are not limited to precise regulation of circulating inflammatory cytokines, relative up- or down-regulation of biomarker metabolites needed for the maintenance of pregnancy, and efficient heifer selection based upon decisive phenotypic parameters. The ability to detect heifers that are unable to produce offspring would provide a mechanism to remove them from the breeding herd prior to investing valuable time, money, and resources. Utilizing efficient heifer selection, metabolomics detection of biomarkers for fertility, and understanding the diagnostic mechanisms of cattle innate immunity can lead researchers closer to understanding, diagnosing, and treating the phenomenon of unexplained heifer infertility.

CHAPTER II.

PHENOTYPIC HEIFER ASSESSMENT AND ANALYSIS

II.I. ABSTRACT

Heifer management and development remains one of the most critical components of efficient beef production (Grings et al., 2007). Current common management practices include utilizing BCS, RTS, Weight, and Age to discriminate reproductively-sound heifers from non-reproductively sound heifers. Proper heifer development for reproductive success involves selecting heifers that have optimal nutrition, phenotypic characteristics, and have reached maturity prior to breeding. The condition of heifers at breeding can affect calving intervals and rates, thereby affecting the overall reproductive performance (Larson et al., 2016).

Angus and Angus-cross heifers, undergoing a 7 - day estrous synchronization protocol, were utilized for this study. The heifers were housed at three Research and Extension Centers (Black Belt, Gulf Coast, and Wiregrass) located across the state of Alabama, U.S.A. and compared across two breeding seasons (2015 – 2016 and 2016 – 2017).

We initially measured a total of 166 heifers (N = 166) for phenotypic parameters including Body Condition Score, Weight at time of Weaning, Reproductive Tract Score

(RTS), and Age at AI to determine the utility of using the "traditional" heifer assessment metrics to predict reproductive outcomes. Across two breeding seasons and three locations, a total of 64 heifers were pregnant by AI, 27 heifers were pregnant by bull, and 28 heifers remained open (infertile). BCS proved to be significantly different ($p < 0.05$) when comparing Black Belt, Wiregrass, and Gulf Coast Research and Extension Centers across two combined breeding seasons. However, RTS, Weight at time of Weaning, and Age at AI were proven to not be significantly different when compared across the three locations and two combined breeding seasons. When the two separate breeding seasons (2015 – 2016 and 2016 – 2017) were compared, only BCS was proven to be significantly different ($p < 0.05$) across combined locations. BCS, RTS, Age at AI, and Weight at time of Weaning were not significantly different ($p > 0.05$), when compared based on reproductive outcomes, in heifers undergoing fixed-time artificial insemination.

II.II. INTRODUCTION

Heifer infertility remains a difficult to diagnose pathology resulting in inefficiencies within the cow-calf sector. The development of replacement heifers represents a major economic burden on the cattle industry. The ability to identify heifers with high reproductive potential for recruitment into the breeding stock is one of the keys to efficient cattle production. Currently, due to a lack of informative biomarkers, replacement heifers are selected based on phenotypic and genetic background information (Calus et al., 2005 and Liu et al., 2008). Current management practices utilize phenotypic methods to attempt to minimize heifers remaining open following the breeding season. However, selection efficiency remains limited due to the low heritability of reproductive performance and results in the recommendation to select ~25% more heifers than required (Kuhn et al., 2006). Analysis of breeding data in the U.S. over two breeding seasons found an artificial insemination (AI) conception rate of 40 – 70% in first-service heifers (Kuhn et al., 2006).

Heifers should be managed and developed in such a way that they can reach puberty early, conceive in their first breeding season, calve without assistance, and breed back early in the subsequent breeding season (Funston et al., 2004). Several reproductive parameters are analyzed prior to each breeding season to determine if the heifer is reproductively sound for conception and pregnancy.

We approached this study with the goal to compare phenotypic heifer variations [BCS, RTS, Weight at Weaning, and Age at AI] across pregnancy outcomes [fertile (AI) and infertile (open)], detect any phenotypic heifer variations across breeding seasons (2015 – 2016 and 2016 – 2017), and detect any phenotypic heifer variations across locations (Black Belt, Wiregrass, and Gulf Coast Research and Extension Centers).

A total of 166 Angus and Angus-cross heifers undergoing a 7-day estrous synchronization protocol were utilized for phenotypic assessment and analysis. Measurements included phenotypic parameters such BCS, RTS, Weight at Weaning, and Age at AI. Across two breeding seasons and three locations, a total of 64 heifers were pregnant by AI, 27 heifers were pregnant by bull, and 28 heifers remained open (infertile). Phenotypic parameters were compared by One-way ANOVA. Variables of condensed breeding seasons (2015 – 2016 and 2016 – 2017) and location were used to determine if any discrepancies were present in relation to differences in phenotypic parameters. Phenotypic heifer assessment (RTS, BCS, Weight at Weaning, and Age at AI) was then cross-compared to determine if pregnancy outcome could be predicted. Pregnancy outcome was compared by breeding season and type of pregnancy obtained. Heifers that remained open (infertile) following AI and three consecutive estrous cycles (in the presence of a fertile bull) were compared to heifers that became pregnant immediately following artificial insemination.

II.III. MATERIALS AND METHODS

Animal Use

All procedures involving animals were approved by the Auburn University Institutional Animal Care and Use Committee (IACUC). Heifers utilized for this study (N = 166) originated from and were housed at the Black Belt (Marion Junction, AL, U.S.A.), Wiregrass (Headland, AL, U.S.A.), and Gulf Coast (Fairhope, AL, U.S.A.) Research and Extension Centers of the Alabama Agricultural Experiment Station.

Reproductive Management

Angus and Angus-cross heifers underwent an estrus synchronization and fixed-time artificial insemination program (TAI) [7-day CO-Synch + CIDR® (Whittier et al., 2013)] spanning the two fall breeding seasons of 2015 – 2016 and 2016 – 2017. Briefly, at the initiation of the estrus synchronization protocol, all heifers received 100 μg GnRH via intramuscular injection (CYSTORELIN®, Merial Animal Health, Duluth, GA, USA), and a controlled internal drug release (CIDR®) device containing 1.38 g of progesterone was placed intravaginally (EAZI-BREED™ CIDR® Cattle Insert, Zoetis, Kalamazoo, MI, USA). Each CIDR® was removed following 7 days, and an intramuscular injection of 25 mg of dinoprost tromethamine (LUTALYSE®, Zoetis, Kalamazoo, MI, USA) was administered at the same time. Heifers were then artificially inseminated with a single

straw of semen originating from selected Angus sires 54 ± 2 hrs. following CIDR® removal. A second intramuscular injection of 100 μg GnRH was administered at the time of artificial insemination (AI). Fourteen days following AI, heifers were exposed to an intact sire for three consecutive estrous cycles. Bulls at each research station were all proven breeders. All bulls passed a standard BSE (Breeding Soundness Exam) with semen quality having < 10% abnormality, and all were cleared for any reproductive discrepancies before each breeding season. Bulls were placed at an average density of 1 bull per 33 heifers for 60 days following artificial insemination.

Heifer Nutrition Management

Heifers at Black Belt were placed on Fescue pasture and had free-choice Ryegrass hay available. Heifers at Wiregrass were placed on Bermudagrass pasture and had free-choice Bermudagrass hay available. Heifers at Gulf Coast were placed on Bahia grass pasture and had free-choice Ryegrass hay available. Throughout all locations, all heifers received 5 – 7 lbs. of Soyhull + Corn-Gluten supplementation per heifer per day, and trace minerals were available *ad libitum*.

Phenotypic Observations

A total of 166 (N = 166) heifers, split between two breeding seasons (2015 – 2016 and 2016 – 2017), were used for this study and analyzed for phenotypic conditions by a trained veterinarian including body condition score (BCS) (Appendix 1), reproductive tract score (RTS) (Appendix 2), Weight at time of Weaning, and Age at AI. BCS was determined as previously described (Herd et al., 1998). The BCS scale ranged from 1-9, with 1 being emancipated and 9 being obese. Reproductive tract score (RTS) evaluation was performed by veterinarians via transrectal palpation. Heifers were assigned a RTS

ranging from 1-5 based on uterine size, uterine tone, ovarian size, and ovarian structure, as previously described (Cushman et al., 2013). Heifer weight was determined at the time of weaning for all animals. The RTS was collected one month prior to AI. Weight was determined at time of weaning, age was determined by counting the days between day of birth and day of artificial insemination, and BCS was determined at the time of AI.

Pregnancy Determination

Pregnancy was determined at 45 and 65 days post AI via transrectal palpation by a trained veterinarian. Heifers were identified as pregnant (AI), pregnant (Bull) or non-pregnant based on the size of the conceptus.

Statistics

PRISM-6 software was used for all analyses in this study. Statistical analyses included one-way ANOVAs with 95% confidence intervals, Tukey's multiple comparison's tests, and unpaired parametric two-tailed T-tests with 95% confidence intervals. Arcsine transformation was performed to analyze multiple pregnancy outcomes across breeding seasons. All data are analyzed and presented as mean ± standard deviation of the mean. Black bars in figures represents standard error. Significance is noted as $p < 0.05$.

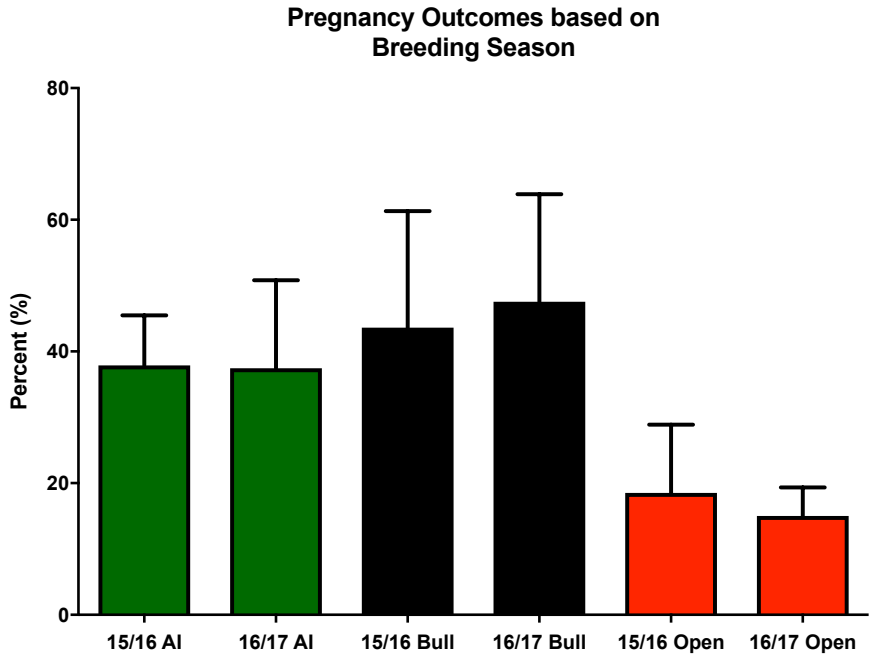
II.IV. RESULTS

Pregnancy outcomes did not differ between breeding seasons

The proportion of heifers that became pregnant by AI, natural service (Bull) and those that remained open were compared between the 2015 - 2016 and 2016 - 2017 breeding seasons (Figure 1). A total of 166 heifers were compared over two breeding seasons (2015 – 2016 and 2016 – 2017) and housed at three locations (Black Belt, Wiregrass, and Gulf Coast Research and Extension Centers) for this study. N = 87 heifers were analyzed for pregnancy outcomes during the 2015 – 2016 breeding season. N = 79 heifers were analyzed for pregnancy outcomes during the 2016 – 2017 breeding season.

No significant difference ($p = 0.946$) was determined when comparing AI rates from the 2015 – 2016 breeding season (37.87 ± 7.60%) to AI rates from the 2016-2017 breeding season (37.44 ± 13.39%, Figure 1). Additionally, no significant difference ($p = 0.796$) was determined when comparing Bull (natural service) rates from the 2015 – 2016 breeding season (43.61 ± 17.72%) to Bull (natural service) rates from the 2016-2017 breeding season (47.53 ± 16.34%, Figure 1). Furthermore, no significant difference ($p = 0.717$) was found when comparing Open (infertile) rates from the 2015 – 2016 breeding season (18.52 ± 10.35%) to Open (infertile) rates from the 2016-2017 breeding season (15.02 ± 4.32%, Figure 1).

Figure 1.



Graph comparing pregnancy outcomes from the 2015 – 2016 and 2016 – 2017 breeding seasons. No significant difference was found between the proportion of heifers becoming pregnant by AI ($p > 0.05$), pregnant by bull (natural service, $p > 0.05$), or failing to become pregnant (open, $p > 0.05$) when comparing the breeding seasons.

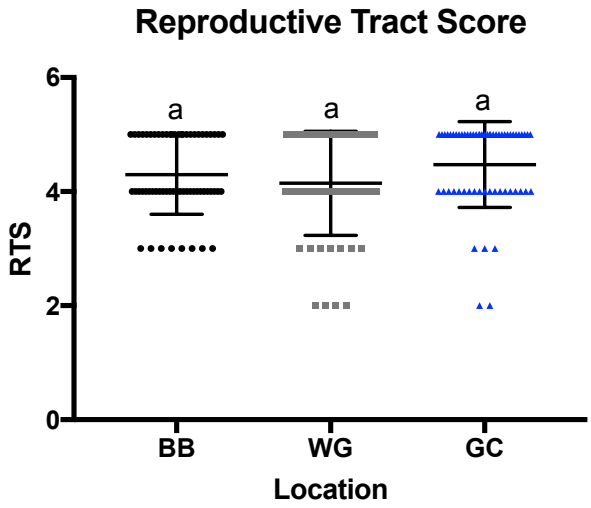
Phenotypic Heifer Assessment based upon Location

Phenotypic parameters (RTS, BCS, Weight at Weaning, and Age at AI) were collected at three locations: Black Belt (BB) N = 62, Wiregrass (WG) N = 50, and Gulf Coast (GC) N = 54 Research and Extension Centers.

Reproductive Tract Scores did not differ between locations

Heifer RTSs (N = 166) from two breeding seasons were compared across locations. No significant difference (p = 0.332) was found between locations: BB = 4.30 ± 0.70; WG = 4.15 ± 0.91; and GC = 4.478 ± 0.75 (Figure 2).

Figure 2.

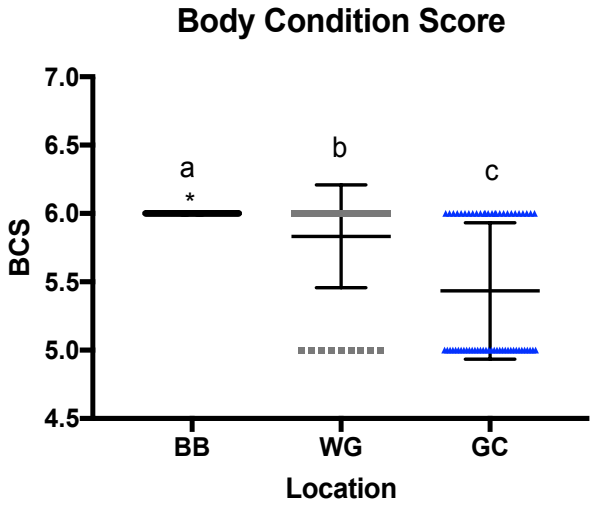


Graph depicting the RTSs of Black Belt (BB), Wiregrass (WG), and Gulf Coast (GC) heifers across two breeding seasons (2015 – 2016 and 2016 – 2017). Data are mean ± standard deviation of the mean. Different letters represent significant differences ($p < 0.05$). No significant difference was found in RTSs between locations ($p > 0.05$).

Body Condition Scores differed depending upon location

Heifer BCSs (N = 166) from two breeding seasons were compared across locations. A significant difference ($p < 0.001$) was determined between locations: BB = 6.00 ± 0.00; WG = 5.83 ± 0.376; and GC = 5.433 ± 0.50 (Figure 3).

Figure 3.

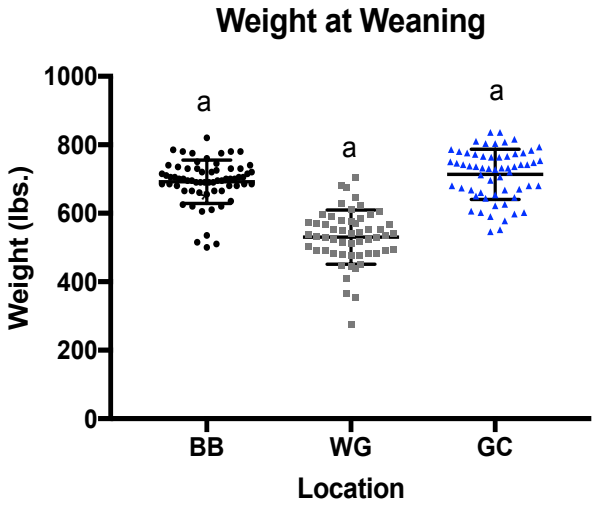


Graph depicting the BCSs of Black Belt (BB), Wiregrass (WG), and Gulf Coast (GC) heifers across two combined breeding seasons (2015 – 2016 and 2016 – 2017). Data are mean ± standard deviation of the mean. Different letters represent significant differences ($p < 0.05$). A significant difference was found in BCSs between locations ($p < 0.05$).

Weights at Weaning did not differ between locations

Heifer Weaning Weights (N = 166) over two breeding seasons were compared across locations. No significant difference ($p = 0.112$) was found among the weaning weights at different locations: BB = 692.20 ± 63.15 lbs.; WG = 530.30 ± 78.95 lbs.; and GC = 713.90 ± 73.33 lbs. (Figure 4).

Figure 4.

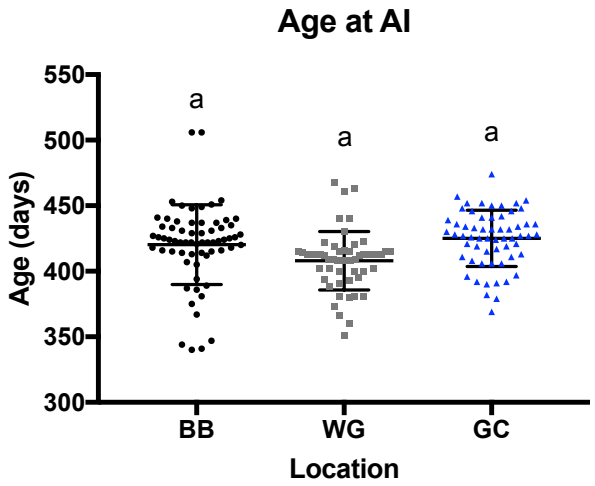


Graph depicting the Weights at time of Weaning of Black Belt (BB), Wiregrass (WG), and Gulf Coast (GC) heifers across two combined breeding seasons (2015 – 2016 and 2016 – 2017. Data are mean ± standard deviation of the mean. Different letters represent significant differences ($p < 0.05$). No significant difference was found in weight at weaning between locations ($p > 0.05$).

Ages at AI did not differ between locations

Heifer Ages at AI (Artificial Insemination) (N = 166) were compared over two breeding seasons across locations. No significant difference ($p = 0.368$) was found between locations: BB = 420.30 ± 30.47 days of age; WG = 408.0 ± 22.30 days of age; and GC = 425.10 ± 21.37 days of age (Figure 5).

Figure 5.



Graph depicting the Ages at AI of Black Belt (BB), Wiregrass (WG), and Gulf Coast (GC) heifers across two combined breeding seasons (2015 – 2016 and 2016 – 2017). Data are mean ± standard deviation of the mean. Different letters represent significant differences ($p < 0.05$). No significant difference was found in age at AI between locations ($p > 0.05$).

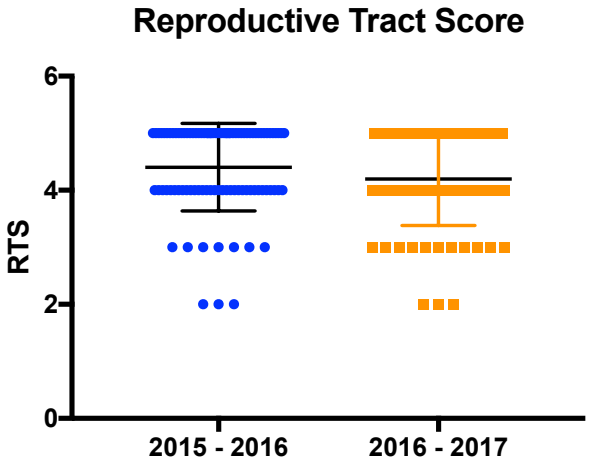
Phenotypic Heifer Assessment based upon Breeding Season

Phenotypic parameters (RTS, BCS, Weight at Weaning, and Age at AI) were determined for (N = 166) heifers across three locations [Black Belt (BB), Wiregrass (WG), and Gulf Coast (GC) Research and Extension Centers]. Phenotypic parameters were then compared between two separate breeding seasons (2015 – 2016 and 2016 – 2017).

Reproductive Tract Score did not differ across breeding seasons

Heifer RTSs (N = 166) were compared between two separate breeding seasons (2015 – 2016 and 2016 – 2017). There was no significant difference (p = 0.085) between RTS from different breeding seasons: 2015 – 2016 = 4.40 ± 0.77 and 2016 – 2017 = 4.20 ± 0.81 (Figure 6).

Figure 6.



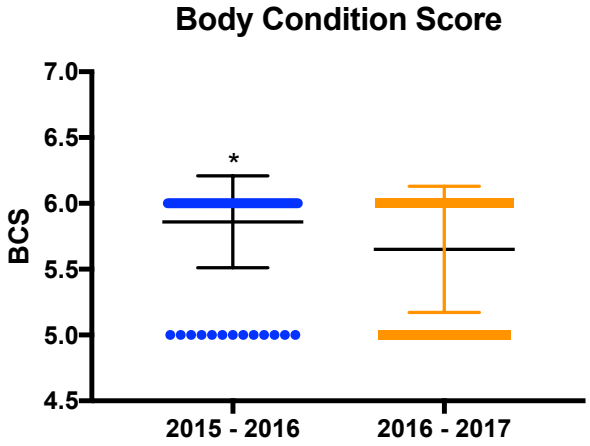
Graph depicting heifer RTSs from three combined locations (BB, WG, and GC) and compared across two separate breeding seasons. Data are mean ± standard deviation of

the mean ($p < 0.05$). No significant difference was found between the RTSs of heifers in the 2015 – 2016 or the 2016 – 2017 breeding seasons ($p > 0.05$).

Body Condition Scores differed between breeding seasons

Heifer BCSs (N = 166) were compared across two separate breeding seasons (2015 – 2016 and 2016 – 2017). There was a significant difference ($p = 0.001$) in BCSs between breeding seasons: 2015 – 2016 = 5.86 ± 0.35 and 2016 – 2017 = 5.65 ± 0.48 (Figure 7).

Figure 7.

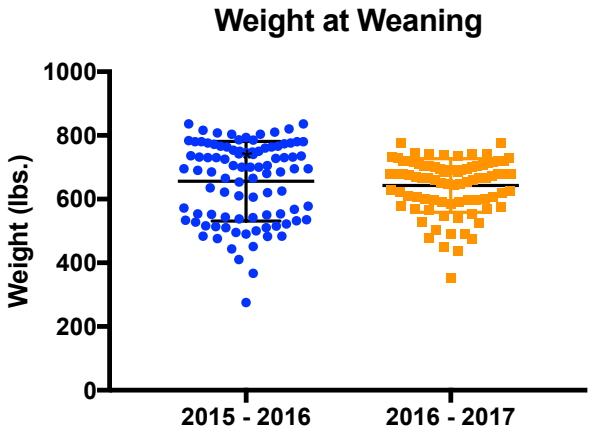


Graph depicting heifer BCSs from three combined locations (BB, WG, and GC) and compared across two separate breeding seasons Data are mean ± standard deviation of the mean ($p < 0.05$). A significant difference was found between the BCSs of heifers in the 2015 – 2016 or the 2016 – 2017 breeding seasons ($p < 0.05$).

Weights at Weaning did not differ between breeding seasons

Heifer weaning weights (N = 166) were compared across two separate breeding seasons (2015 – 2016 and 2016 – 2017). No significant difference ($p = 0.406$) was found between breeding seasons: 2015 – 2016 = 656.30 ± 124.80 lbs. and 2016 – 2017 = 643.00 ± 84.09 lbs. (Figure 8).

Figure 8.

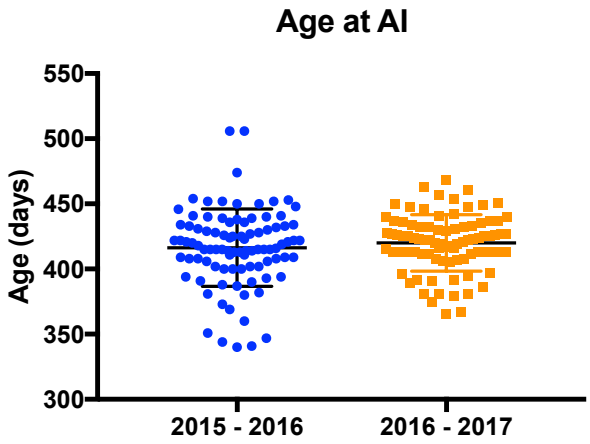


Graph depicting heifer Weights at time of weaning from three locations (BB, WG, and GC) and compared across two separate breeding seasons Data are mean ± standard deviation of the mean ($p < 0.05$). No significant difference was found between the weaning weight of heifers in the 2015 – 2016 or the 2016 – 2017 breeding seasons ($p > 0.05$).

Ages at AI did not differ across breeding seasons

Heifer Age at AI (artificial insemination) (N = 166) were compared between two separate breeding seasons (2015 – 2016 and 2016 – 2017). No significant difference ($p = 0.346$) was found in the age at AI between breeding seasons: 2015 – 2016 = 416.4 ± 29.70 days of age and 2016 – 2017 = 420.10 ± 21.63 days of age. (Figure 9).

Figure 9.



Graph depicting heifer Ages at AI from three combined locations (BB, WG, and GC) and compared across two separate breeding seasons. Data are mean ± standard deviation of the mean ($p < 0.05$). No significant difference was found between the age at AI of heifers in the 2015 – 2016 or the 2016 – 2017 breeding seasons ($p > 0.05$).

Phenotypic Heifer Assessment based upon Pregnancy Outcome and Location

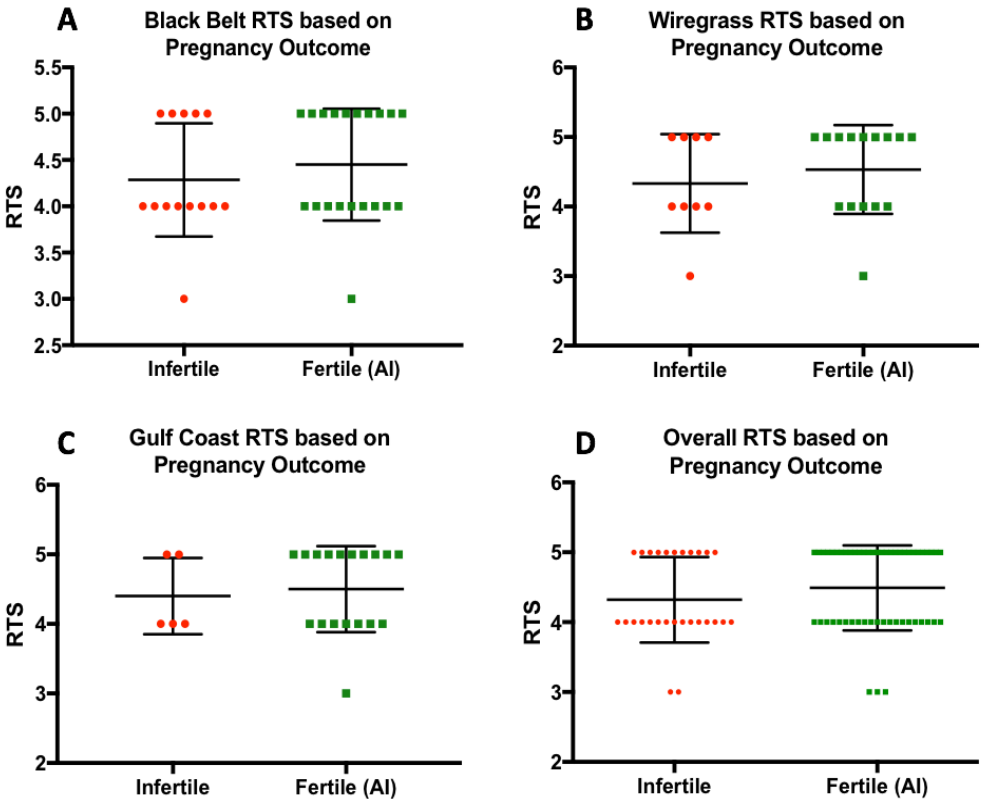
Phenotypic parameters (RTS, BCS, Weight at Weaning, and Age at AI) were compared between heifers (N = 166) from three locations [Black Belt (BB), Wiregrass (WG), and Gulf Coast (GC) Research and Extension Centers] across two breeding

seasons (2015 – 2016 and 2016 – 2017). Heifers becoming pregnant from the AI were categorized as fertile and those remaining open following AI and three rounds of natural service were categorized as infertile. Because we are looking at the two most extreme ends of heifer fertility [(pregnant by AI and open (infertile)], we did not include heifers pregnant by bull for these analyses.

RTS of fertile and infertile heifers did not differ by location

Black Belt, Wiregrass, and Gulf Coast heifer RTSs were compared between fertile and infertile heifers across two combined breeding seasons (2015 – 2016 and 2016 – 2017) (Figure 10). There was no significant difference between RTSs at the Black Belt Research and Extension Center when comparing fertile with infertile heifers: BB Infertile = 4.29 ± 0.61 and BB Fertile (AI) = 4.45 ± 0.60. ($p = 0.443$, Figure 10-A). Additionally, there was no significant difference between RTS at the Wiregrass Research and Extension Center when comparing fertile with infertile heifers: WG Infertile = 4.33 ± 0.71 and WG Fertile (AI) = 4.53 ± 0.64. ($p = 0.483$, Figure 10-B). Similarly, there was no significant difference between RTS at the Gulf Coast Research and Extension Center when comparing fertile with infertile heifers: GC Infertile = 4.40 ± 0.55 and GC Fertile (AI) = 4.50 ± 0.62. ($p = 0.747$, Figure 10-C). Finally, there was no significant difference overall between the RTS of fertile (4.49 ± 0.61) and infertile (4.32 ± 0.61) heifers ($p = 0.239$, Figure 10-D).

Figure 10.

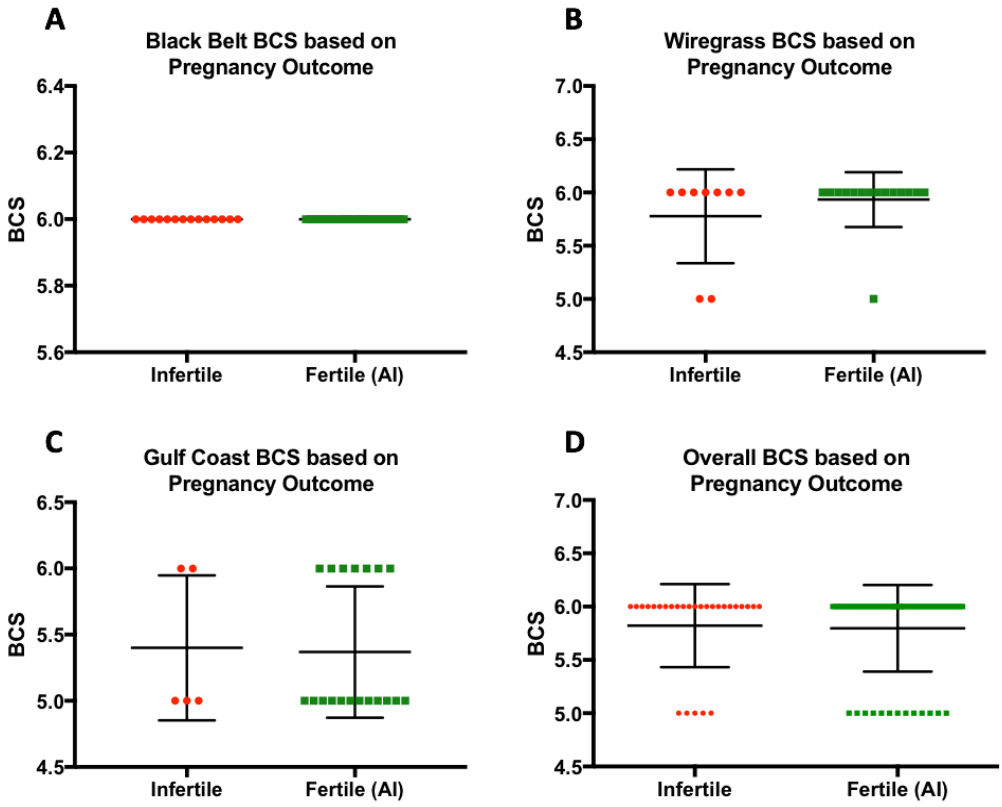


Graphs displaying RTSs of infertile or fertile heifers at different locations (A = Blackbelt, B = Wiregrass, and C = Gulf Coast, and D = Overall RTS). Data are mean ± standard deviation of the mean ($p < 0.05$). No significant difference between RTSs of fertile and infertile heifers was found at the Black Belt, Wiregrass, or Gulf Coast Research and Extension Centers ($p > 0.05$).

BCS of fertile and infertile heifers did not differ by location

Black Belt, Wiregrass, and Gulf Coast heifer BCS were compared between fertile and infertile heifers across two combined breeding seasons (2015 – 2016 and 2016 – 2017) (Figure 11). There was no significant difference between BCSs at the Black Belt Research and Extension Center when comparing fertile with infertile heifers: BB Infertile = 6.00 ± 6.00 and BB Fertile (AI) = 6.00 ± 6.00 ($p = 0.000$, Figure 11-A). Additionally, there was no significant difference between BCSs at the Wiregrass Research and Extension Center when comparing fertile with infertile heifers: WG Infertile = 5.78 ± 0.44 and WG Fertile (AI) = 5.93 ± 0.26 ($p = 0.285$, Figure 11-B). Similarly, there was no significant difference between BCSs at the Gulf Coast Research and Extension Center when comparing fertile with infertile heifers: GC Infertile = 5.40 ± 0.55 and GC Fertile (AI) = 5.37 ± 0.50 ($p = 0.902$, Figure 11-C). Finally, there was no significant difference overall between the BCSs of fertile (5.80 ± 0.41) and infertile (5.82 ± 0.39) heifers ($p = 0.788$, Figure 11-D).

Figure 11.

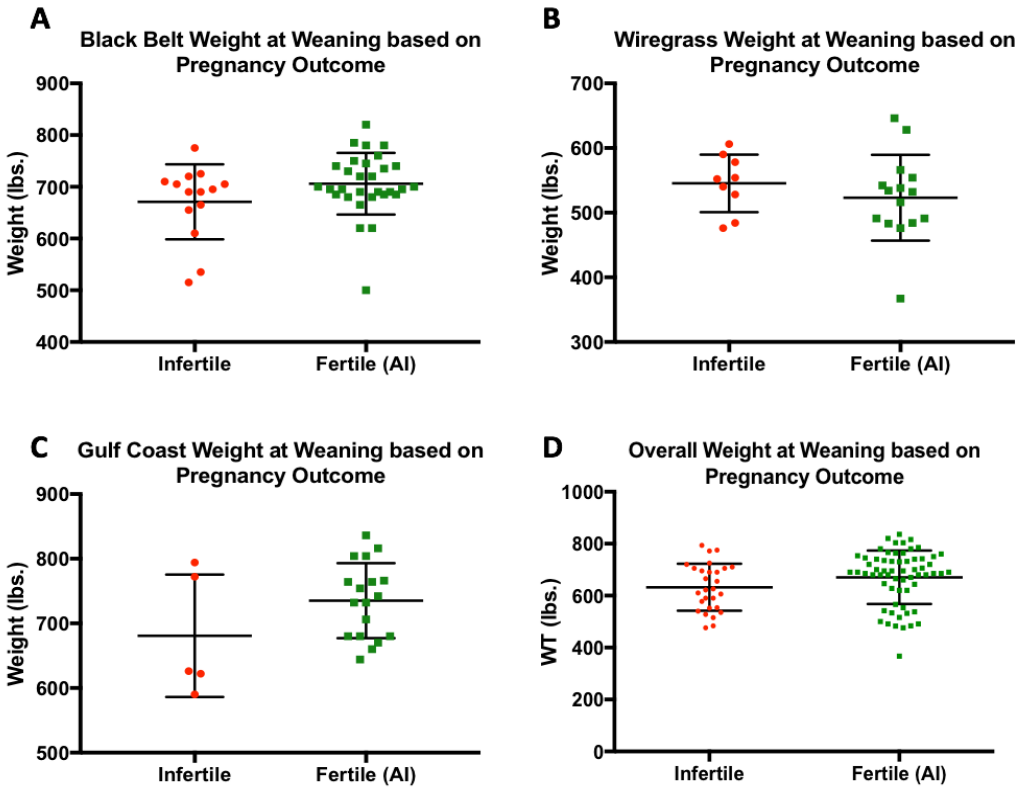


Graphs depicting BCSs of infertile or fertile heifers at different locations (A = Black Belt, B = Wiregrass, and C = Gulf Coast, D = Overall BCS. Data are mean ± standard deviation of the mean ($p < 0.05$). No significant difference between BCSs of fertile and infertile heifers was found at the Black Belt, Wiregrass, or Gulf Coast Research and Extension Centers ($p > 0.05$).

Weight at time of weaning between fertile and infertile heifers did not differ by location

Black Belt, Wiregrass, and Gulf Coast heifer weaning weights were compared between fertile and infertile heifers across two combined breeding seasons (2015 – 2016 and 2016 – 2017) (Figure 12). There was no significant difference between weaning weights at the Black Belt Research and Extension Center when comparing fertile with infertile heifers: BB Infertile = 671.10 ± 72.38 lbs. and BB Fertile (AI) = 706.00 ± 59.73 lbs. ($p = 0.099$, Figure 12-A). Additionally, there was no significant difference between weaning weights at the Wiregrass Research and Extension Center when comparing fertile with infertile heifers: WG Infertile = 545.30 ± 44.38 lbs. and WG Fertile (AI) = 523.20 ± 66.29 lbs. ($p = 0.385$, Figure 12-B). Similarly, there was no significant difference between weaning weights at the Gulf Coast Research and Extension Center when comparing fertile with infertile heifers: GC Infertile = 680.00 ± 94.65 lbs. and GC Fertile (AI) = 735.20 ± 58.11 lbs. ($p = 0.121$, Figure 12-C). Finally, there was no significant difference overall between the weaning weight of fertile (670.80 ± 103.30 lbs.) and infertile (632.40 ± 90.39 lbs.) heifers ($p = 0.093$).

Figure 12.

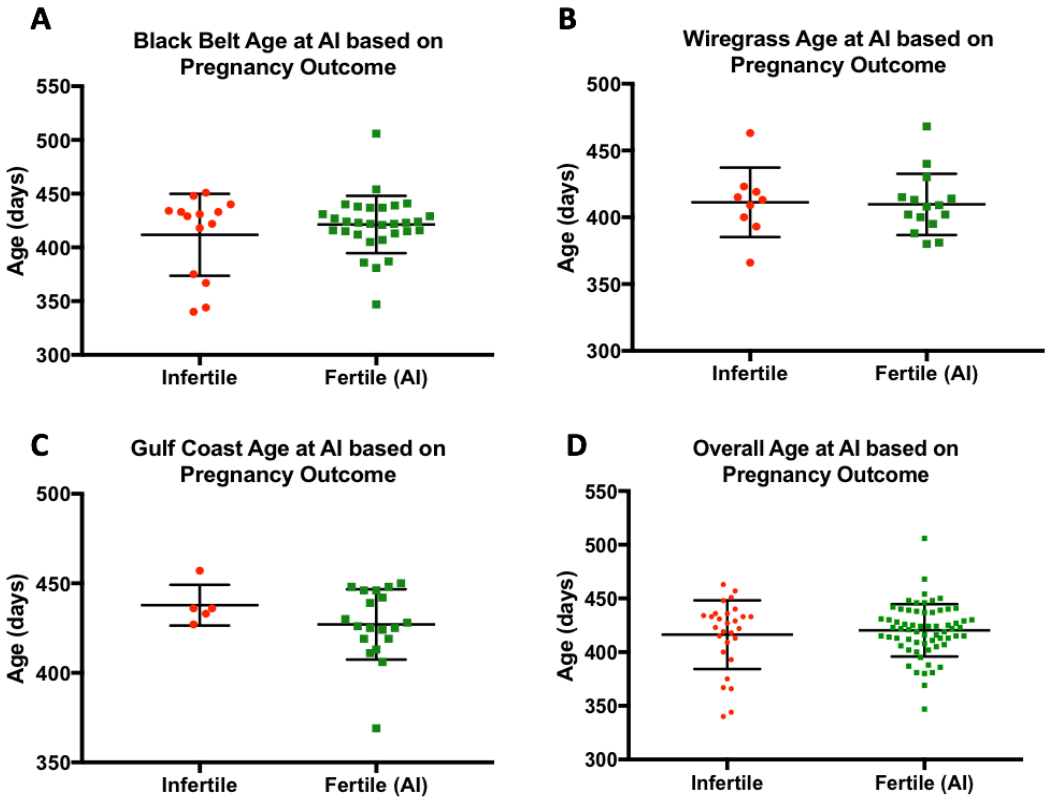


Graphs displaying weaning weights of infertile and fertile heifers (A = Blackbelt, B = Wiregrass, and C = Gulf Coast, D = Overall Weaning Weights). Data are mean ± standard deviation of the mean ($p < 0.05$). No significant difference between weaning weights of fertile and infertile heifers was found at the Black Belt, Wiregrass, or Gulf Coast Research and Extension Centers ($p > 0.05$).

Age at AI of fertile and infertile heifers did not differ by location

Black Belt, Wiregrass, and Gulf Coast heifer Age at AI were compared between fertile and infertile heifers across two combined breeding seasons (2015 – 2016 and 2016 – 2017) (Figure 13). There was no significant difference between ages at AI at the Black Belt Research and Extension Center when comparing fertile with infertile heifers: BB Infertile = 411.80 ± 38.18 days and BB Fertile (AI) = 421.30 ± 26.65 days ($p = 0.345$, Figure 13-A). Additionally, there was no significant difference between ages at AI at the Wiregrass Research and Extension Center when comparing fertile with infertile heifers: WG Infertile = 411.20 ± 25.99 days and WG Fertile (AI) = 409.70 ± 22.98 days ($p = 0.880$, Figure 13-B). Similarly, there was no significant difference between ages at AI at the Gulf Coast Research and Extension Center when comparing fertile with infertile heifers: GC Infertile = 437.80 ± 11.34 days. and GC Fertile (AI) = 427.10 ± 19.60 days ($p = 0.257$, Figure 13-C). Finally, there was no significant difference overall between the age at AI of fertile (420.30 ± 24.40 days) and infertile (416.30 ± 32.03 days) heifers ($p = 0.512$) (Figure 13-D).

Figure 13.



Graphs displaying Age at the time of AI of fertile and infertile heifers at various locations (A = Blackbelt, B = Wiregrass, and C = Gulf Coast, D = Overall Ages at AI). Data are mean ± standard deviation of the mean ($p < 0.05$). No significant difference between ages at AI of fertile and infertile heifers was found at the Black Belt, Wiregrass, or Gulf Coast Research and Extension Centers ($p > 0.05$).

II.V. DISCUSSION

Replacement heifers are a major economic burden on the beef cattle industry. Heifer infertility remains a difficult to diagnose pathology resulting in inefficiencies within the cow-calf sector. The ability to detect heifers that are unable to produce offspring would allow their removal from the breeding herd prior to investing valuable time, money, and resources.

We approached this study with the goal to compare and detect traditional heifer phenotypic variables (BCS, RTS, Weight at Weaning, and Age at AI) across pregnancy outcomes [fertile (AI) and infertile (open)], detect any phenotypic heifer variations across breeding seasons (2015 – 2016 and 2016 – 2017), and detect any phenotypic heifer variations across locations (Black Belt, Wiregrass, and Gulf Coast Research and Extension Centers).The ability to detect phenotypic variations across pregnancy outcome can greatly aid a producer in the culling decisions for a breeding herd. Additionally, identifying any trends across locations or breeding seasons can ensure the producer has a consistent herd without any discrepancies present.

Current management practices utilize phenotypic methods to attempt to minimize heifers remaining open following the breeding season. One commonly used method is to conduct Reproductive Tract Scoring (RTS) prior to the breeding season (Appendix 2). The RTS system involves transrectal palpation of the ovaries and uterine horns in order to

estimate the pubertal status and presence of ovarian cyclicity (Rosenkrans et al., 2003). The system classifies heifers based on the size of the reproductive tract and the presence of a corpus luteum, resulting in a range of scores from 1 (immature; anestrus) to 5 (mature; cycling) (Rosenkrans et al., 2003). In a previous study, heifers were then grouped based on their RTSs, and then went through AI and natural breeding service (Gutierrez et al., 2014). The RTS was shown to minimize the number of heifers remaining open following the breeding season from 20.3% in the heifers scored 1 and 2 to 9.8% in the heifers scored 5 (Gutierrez et al., 2014). In our studies, we did not see a statistically significant difference between the RTSs of heifers among different locations (Figure 2), during different breeding seasons (Figure 6), or between pregnancy outcomes (Figure 10). The most interesting result is that we were unable to detect a difference in the RTS of fertile and infertile heifers at any of the locations (Figure 10). This suggests that RTS is a useful tool to minimize heifers remaining open following the breeding season, but is not able to identify all problem breeders.

Body condition score (BCS) is an often-utilized parameter for evaluating the reproductive potential of replacement heifers. BCSs range from 1-9 with 1 being extremely thin and 9 being extremely obese (Appendix 1). A previous study compared Holstein heifers with a BCS below the median to those above the median with regards to their rate of conception at first service (Donovan et al., 2003). They did not see a significant correlation between BCS and ability to become pregnant at first service (Donovan et al., 2003). In our study, we did not see a statistically significant difference between the BCSs of heifers when predicting pregnancy outcomes (Figure 11). We did, however, see a statistically significant difference between the BCSs of heifers when

comparing locations (Figure 3) and breeding seasons (Figure 7). The potential reasoning and specifics behind the significant differences in BCSs across location and breeding seasons, but not pregnancy outcomes, could be due to the varying management practices of the heifers. These results highlight the importance of environment and management practices on the BCSs of replacement heifers. They also however support the caveat that BCS, while useful in limiting problematic breeders, is not a consistent way of identifying reproductive outcomes. It must also be stated that the limited animal numbers and variations in BCSs limits the scope of the results.

Heifer weight can assist in determining the onset of puberty, thus increasing the overall reproductive performance of the heifer. Target weight is said to be the threshold weight for puberty in heifers (Holm et al., 2015). If a heifer is below target weight, her nutrition and growth rate are limiting factors for the onset of puberty (Holm et al., 2015). Research shows that the target weight to reach puberty is calculated by taking the ratio of the average weight of heifers in the herd divided by the average weight of the multiparous cows in the herd (Patterson et al., 1992). Using this calculation, heifers who have been fed a diet to reach 55% to 65% of their mature weight have better reproductive performance at time of breeding than those heifers fed to reach a lower target weight ratio (Patterson et al., 1992). In our studies, we compared the weaning weight of animals to determine if it played a role in reproductive outcomes following heifer development. We did not see a statistically significant difference between weights at weaning in heifers when comparing pregnancy outcomes (Figure 12), breeding seasons (Figure 8) or locations (Figure 4). An item of particular interest during this study was the weaning weights of heifers at Wiregrass Research and Extension Center. From the data, one can

speculate that there is an ongoing trend of low weaning weights at Wiregrass compared to Black Belt of Gulf Coast Research and Extension Centers. Although a significant difference was not determined between locations, the low weaning weights of these heifers could in fact be attributed to lower heifer birth weights. Lower birth weight could be correlated with the nutrition of the heifer's dam during gestation. Further studies would need to be conducted in order to indicate a symbiotic relationship of low birthing weights relating to low weaning weights, and the nutritional status and management of the dam during gestation.

Age is an important factor that comes into play during the process of determining the pubescent status of the heifer prior to breeding. Age at puberty is influenced by the heifer's breed, plane of nutrition and diet, and season (Herd et al., 1998). In North America, the average age for the onset of puberty in *Bos taurus* heifers ranges from 303 – 429 days, or 10 – 14 months of age (Larson et al., 2016). Because most producers require heifers to calve between 23 – 24 months of age, heifers should be bred at the time of the onset of puberty for optimum breeding performance (Herd et al., 1998). When comparing the heifer's Ages at AI for our studies, we did not see a statistically significant difference between the days of age of heifers when comparing pregnancy outcomes (Figure 13), breeding seasons (Figure 9), or locations (Figure 5). These results highlight the variation in age at which puberty onset occurs in heifers, which makes age not an ideal marker for reproductive outcomes. Although increased variations in age could contribute to the correlation of successful reproductive outcomes, a larger data set would be needed to aid this.

Heifer infertility that results in the failure of a cow or heifer to conceive a calf is the single-largest economic loss to beef producers. Phenotypic parameters such as RTS, BCS, Age at AI, and Weight at Weaning are all relevant and important pieces of information needed to produce a successful and productive breeding herd. Many producers establish minimum and maximum requirements, or values, in order to create and maintain a relative breeding herd "standard." However, reproductive failure is still an element of concern for the beef cattle industry not only locally, but nationally and internationally alike. Functional causes of infertility tend to effect individual heifers, but when combined, these infertile heifers can make a large impact on the overall herd (Abraham, 2017). This study highlights the limitations in traditional phenotypic approaches for identifying productive heifers.

CHAPTER III.

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

III.I. ABSTRACT

Biomarker development for the identification of infertile heifers has the potential to improve the efficiency of cow-calf production. In this study, we utilized metabolomics profiling to identify metabolites in the blood plasma that may be useful in identifying infertile heifers at the time of artificial insemination (AI). Twenty Angus and Angus-cross heifers, undergoing a 7-day estrous synchronization protocol, were utilized from three locations (Black Belt: N = 8; Wiregrass: N= 8; Gulf Coast: N = 4). Prior to artificial insemination, phenotypic parameters including Body Condition Score (BCS), Weight at time of Weaning, Reproductive Tract Score (RTS), and Age at time of AI were measured. These phenotypic parameters were determined to not be significantly different between fertile and infertile heifers. Analysis of the resulting metabolomics profiles revealed seven metabolites (Tryptophan, Cystine, Histidine, Ornithine, Asparagine, Glutamine, and Lysine) present at significantly different levels (T-test; $p < 0.05$; Fold Change > 2; FDR < 0.05) between infertile and fertile heifers. Additionally, we

performed a metabolomics analysis to determine if heifer metabolomes differed by location and breeding season. We discovered six metabolites (Tocopherol-alpha, Ornithine, Myristic Acid, Sulfuric Acid, Alpha-ketoglutarate, and P-tolyl Glucuronide) identified as significantly different ($p < 0.05$; Fold Change > 2; FDR 0.05) between Black Belt and Wiregrass Research and Extension Centers.

We further characterized the utility of using the levels of these metabolites in the blood plasma to discriminate between fertile and infertile heifers. Based on the ROC-AUC values for each of the seven significant metabolites based on fertility outcome (Tryptophan, Cystine, Histidine, Ornithine, Asparagine, Glutamine, and Lysine), we tested their predictive abilities in categorizing fertile and infertile heifers. By testing predictive models, metabolites did not categorize any fertile heifer as infertile, and vice versa. Finally, we investigated the potential role inflammation may play by comparing the expression of inflammatory cytokines in the white blood cells of infertile heifers to that of fertile heifers. We found significantly higher expression of the proinflammatory cytokines, Tumor Necrosis Factor alpha (*TNFα*), Interleukin 6 (*IL-6*), and neutrophil activating peptide C-X-C Motif Chemokine 5 (*CXCL5*) in infertile heifers compared with fertile heifers (T-test; $p < 0.05$; Fold Change > 2). The work in this study offers potentially valuable information regarding diagnosis of fertility problems in heifers undergoing AI.

III.II. INTRODUCTION

Unexplained infertility remains a significant source of inefficiency within the cow-calf production sector. The ability to identify heifers with high reproductive potential for recruitment into the breeding stock is one of the keys to efficient cattle production. Currently, due to a lack of informative biomarkers, replacement heifers are selected based on phenotypic and genetic background information (Calus et al., 2005; Liu et al., 2008). However, selection efficiency remains limited due to the low heritability of reproductive performance, which results in the recommendation to select ~25% more heifers than required (Kuhn et al., 2006). Analysis of breeding data in the U.S. over two years found an artificial insemination (AI) conception rate of 40-70%, in first service heifers (Kuhn et al., 2006). Overall pregnancy rates in heifers range from 70-90% utilizing AI and natural breeding programs (Schatz et al., 2008). Poor fertility accounts for the majority of cows culled and remains largely unmanageable due to a lack of informative biomarkers for fertility (Wathes et al., 2008). The development of an early detection assay utilizing biomarkers will minimize costs associated with over-selecting heifers and the resulting cull.

Metabolomics, or metabolomics profiling, involves the quantitative measurement of the global set of low-molecular-weight metabolites in a biological fluid (Goodacre et al., 2004). Metabolite levels can be compared among different phenotypic states and

can potentially be used as health indicators. Mass spectrometry-based metabolite analysis has been applied in the development of many informative biomarkers to help identify hard-to-diagnose disorders (van der Kloet et al., 2012; Günther, 2015; Jafarzadeh et al., 2015; Zhou et al., 2007). Recent studies have utilized metabolomics analysis in assessing embryo and oocyte quality (Singh et al., 2007; Nagy et al., 2009; Revelli et al., 2009). Within cattle, metabolomics analysis of follicular fluid has been used to potentially explain differences in fertility between heifers and lactating cows (Bender et al., 2010). Authors in this study discovered metabolites significantly differed in the blood serum between heifers and cows (Bender et al., 2010).

Furthermore, inflammation has been shown to perturb the growth and steroidogenic potential of the preovulatory follicle and negatively impact conception rates in cattle (Price et al., 2013; Lavon et al., 2011; Hertl et al., 2010). In fact, bovine granulosa cells express functional Toll-like receptors (TLRs) that respond to lipopolysaccharide (LPS) and Pam3CSK4 (PAM) exposure, leading to an upregulation of proinflammatory cytokines such as interleukins (Price et al., 2013). Limited knowledge remains regarding a potential relationship between inflammatory status and heifer infertility.

Although metabolomics approaches have been extensively used within the biomedical field, there are limited studies in farm animals. In the present study, we have conducted comprehensive metabolomics profiling of the blood plasma of heifers at the time of artificial insemination (AI). 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). Heifers that remained open following

AI and three consecutive estrous cycles, in the presence of a fertile bull, were compared to heifers that became pregnant following AI. Metabolomics profiling was also analyzed by location and breeding season to reveal any metabolome differences of heifers participating in the research study. In addition, we analyzed and compared the expression levels of proinflammatory cytokines in the white blood cells of heifers with differing pregnancy outcomes following AI and natural breeding.

III.III. MATERIALS AND METHODS

Animal Use

All procedures involving animals were approved by the Auburn University Institutional Animal Care and Use Committee (IACUC). Heifers utilized for this study originated from and were housed at the Black Belt (Marion Junction, AL, U.S.A.), Wiregrass (Headland, AL, U.S.A.), and Gulf Coast (Fairhope, AL, U.S.A.) Research and Extension Centers of the Alabama Agricultural Experiment Station.

Reproductive Management

Angus and Angus-cross heifers underwent an estrus synchronization and fixed-time artificial insemination program (TAI) [7-day CO-Synch + CIDR® (Whittier et al., 2013)], spanning the two fall breeding seasons of 2015 – 2016 and 2016 – 2017. Briefly, at the initiation of the estrus synchronization protocol, all heifers received 100 μg GnRH via intramuscular injection (CYSTORELIN®, Merial Animal Health, Duluth, GA, USA), and a controlled internal drug release (CIDR®) device containing 1.38 g of progesterone was placed intravaginally (EAZI-BREED™ CIDR® Cattle Insert, Zoetis, Kalamazoo, MI, USA). Each CIDR® was removed following 7 days, and an intramuscular injection of 25 mg of dinoprost tromethamine (LUTALYSE®, Zoetis, Kalamazoo, MI, USA) was administered at the same time. Heifers were then artificially inseminated with a single straw of semen originating from selected Angus sires 54 ± 2 hrs. following CIDR® removal. A second intramuscular injection of 100 μg GnRH was administered at the time

of artificial insemination (AI). Fourteen days following AI, heifers were exposed to an intact sire for three consecutive estrous cycles. Bulls at each research station were all proven breeders. All bulls passed a standard BSE (Breeding Soundness Exam) with semen quality having < 10% abnormality, and all were cleared for any reproductive discrepancies before each breeding season. Bulls were placed at an average density of 1 bull per 33 heifers for 60 days following artificial insemination.

Heifer Nutrition Management

Heifers at Black Belt were placed on Fescue pasture and had free-choice Ryegrass hay available. Heifers at Wiregrass were placed on Bermudagrass pasture and had free-choice Bermudagrass hay available. Heifers at Gulf Coast were placed on Bahia grass pasture and had free-choice Ryegrass hay available. Throughout all locations, all heifers received 5 – 7 lbs. of Soyhull + Corn-Gluten supplementation per heifer per day, and trace minerals were available *ad libitum*.

Phenotypic Observations

A total of 166 (N = 166) heifers, split between two breeding seasons (2015 – 2016 and 2016 – 2017), were used for this study and analyzed for phenotypic conditions by a trained veterinarian including body condition score (BCS) (Appendix 1), reproductive tract score (RTS) (Appendix 2), Weight at time of Weaning, and Age at AI (artificial insemination). BCS was determined as previously described (Herd et al., 1998). The BCS scale ranged from 1-9, with 1 being emaciated and 9 being obese. Reproductive tract score (RTS) evaluation was performed by veterinarians via transrectal palpation. Heifers were assigned a RTS ranging from 1-5 based on uterine size, uterine tone, ovarian size, and ovarian structure, as previously described (Cushman et al., 2013). Heifer weight was

determined at the time of weaning for all animals. The RTS was collected one month prior to AI. Weight was determined at time of weaning, age was determined by counting the days between day of birth and day of artificial insemination, and BCS was determined at the time of AI.

Blood Collection and Processing

At the time of artificial insemination, 10 mL of blood was collected via jugular vein of each heifer using an 18G needle into an EDTA blood collection tube (BD Vacutainer). The sample was immediately inverted 10 times, immersed on ice, and transported to the Reproductive Biology and Development Laboratory at the Center for Advanced Science, Innovation, and Commerce in Auburn, AL. Once in the laboratory, the blood tubes were sprayed with 70% ETOH to ensure elimination of contamination from the onsite farm location. Samples were centrifuged at 2,000 x *g* for 15 minutes at 4 degrees Celsius. Two 500-µl samples of blood plasma were removed and stored at -80 degrees Celsius for metabolomics data analysis. Samples were stored at -80 degrees Celsius until further processing.

Pregnancy Determination

Pregnancy was determined at 45 and 65 days post AI via transrectal palpation by a trained veterinarian. Heifers were identified as pregnant (AI), pregnant (Bull) or non-pregnant (Open) based on the size of the conceptus or lack thereof. In this study, only samples from heifers remaining open following AI and natural breeding exposure (infertile), and those impregnated through AI (fertile) were analyzed for metabolite levels.

Heifer selection for metabolomics analysis

Twenty heifers (10 AI-pregnant and 10 non-pregnant) were randomly selected for metabolomics analysis. Heifers were selected based on their similarities in age, phenotypic characteristics, and puberty status. In the 10 AI-pregnant group, two heifers were from the Black Belt 2015 – 2016 breeding season, two heifers from the Black Belt 2016 – 2017 breeding season, two heifers were from the Wiregrass 2015 – 2016 breeding season, two heifers were from the Wiregrass 2016 – 2017 breeding season, and two heifers were from the Gulf Coast 2016 – 2017 breeding season. Parameters were the same for choosing the 10 non-pregnant samples.

Metabolomics Data Collection

Blood plasma samples from 20 animals collected at the time of AI (N = 10 pregnant by AI and N = 10 non-pregnant) were used to identify metabolites at different levels. A further 20 blood plasma samples collected at the time of AI (N = 13 pregnant by AI and N = 7 non-pregnant) were used to determine the predictive potential of the metabolites found at different levels in the first sample set. Samples (N = 40) 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 5^{th}-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 122 metabolites from 10 fertile (Pregnant by AI) and 10 infertile (Open) heifer plasma samples. Data were normalized by sum in order to minimize concentration differences. Following normalization, scaling (mean-centering and division by the square root of standard deviation of each variable) was performed to equally weight each variable regardless of absolute value. T-tests were performed with an FDR cutoff of 0.05. Metabolites were considered at significantly different levels when $P \leq 0.05$ Data are presented as mean ± standard deviation of the mean.

Multivariate statistical analysis

Multivariate analysis was applied to a total of 122 metabolites from 10 fertile and 10 infetile heifer's plasma samples. Data were normalized by sum in order to minimize concentration differences. Following normalization, scaling (mean-centering and division by the square root of standard deviation of each variable) was performed to equally weight each variable regardless of absolute value. 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) in order to maximize class discrimination. Model robustness was assessed using Receiver operating characteristic – Area Under Curve (ROC-AUC) analysis using MetaboAnalyst software. Classification models were built based on metabolites showing significant differential levels ($p < 0.05$; FDR > 0.05) with at least a 2-fold difference. Twenty blinded samples were used to test the robustness of the models to characterize heifers as fertile and infertile. Further validation was performed with MetaboAnalyst using permutation tests.

Metabolic Pathway Analysis

Metabolic Pathway Analysis was performed using MetaboAnalyst 3.0. Pathway Analysis combined results from Pathway Enrichment Analyses and Pathway Topology Analyses to correctly identify relative pathways involved in both fertile and infertile samples. 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). KEGG-metabolic pathways were utilized to determine the course of each individual metabolite. To analyze the effect of 15

differential metabolites identified for biological pathways, a Fisher's Exact Test was performed.

Buffy Coat Isolation

Samples were centrifuged in an EDTA blood collection tube at 2,000 × *g* for 15 minutes at 4 degrees Celsius to separate plasma and buffy coat layers. Following centrifugation, a 500-μl band of buffy coat was aseptically pipetted and re-suspended into a sterile 15-mL centrifuge tube containing 12 mL ice-cold lysis solution (0.15 mM ammonium chloride, 10 μM sodium bicarbonate, and 1.3 μM EDTA). Tubes were inverted every two minutes for a total of ten minutes. Tubes were then centrifuged at 250 × *g* for 10 minutes at 4 degrees Celsius in order to form a visible pellet. The supernatant was discarded, and pellets were gently re-suspended in a 1.5-mL micro-centrifuge tube containing 1-mL wash buffer (PBS with 2% fetal bovine serum). Samples were centrifuged in a tabletop centrifuge at 250 x *g* for 10 minutes at 4 degrees Celsius. The supernatant was discarded, and pellets were stored at - 80 degrees Celsius until further processing.

RNA Isolation and cDNA Synthesis

Total buffy coat RNA was isolated from the pelleted 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. The total RNA extracted was then quantified using a Qubit Fluorometer (Thermo Fisher Scientific). One μg of isolated RNA was then reverse-

transcribed (RT) into cDNA using qScript cDNA Supermix (Quanta BioSciences Inc., Beverly, MA).

Real-time PCR

To account for the variations in RNA concentrations, C_q values from the PCR data of the samples were normalized to the C_qs of the reference gene GAPDH using the ΔΔCq method. For fertile and infertile samples, four total isolations were used. The isolated RNA was then 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 4). A Roche LightCycler 480 Real-time qPCR machine was utilized to compare the expression levels of the target transcripts using the delta-delta C_q method (Schmittgen and Livak, 2008). *GAPDH* was used as an 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.

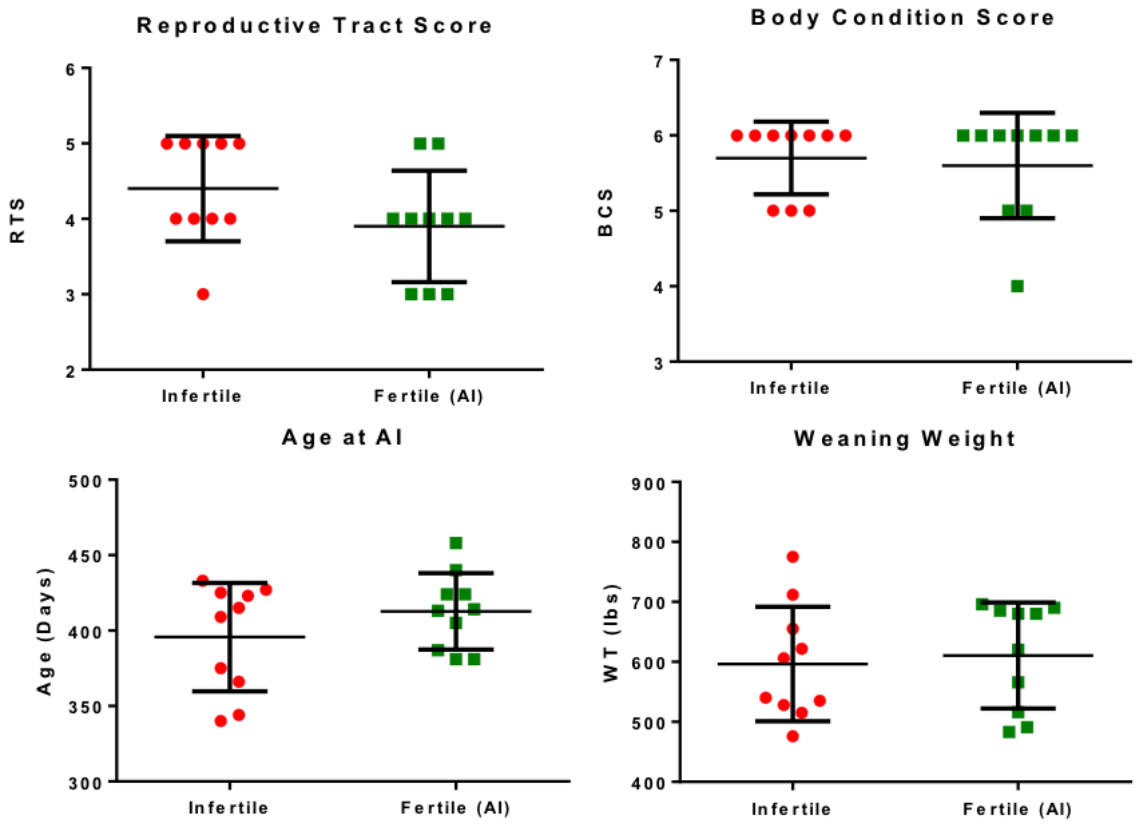
III.IV. RESULTS

Phenotypic Heifer Assessment

Phenotypic Parameters did not differ based upon Fertility Outcome

In order to determine if phenotypic differences could differentiate between fertile and infertile heifers, we collected RTS, Weight at Weaning, Age at AI, and BCS on N = 20 heifers. No significant difference ($p = 0.137$) was seen in Reproductive Tract Scores (RTS) between heifers becoming pregnant by AI (3.90 ± 0.74) or those remaining open (4.40 ± 0.71). No significant difference ($p = 0.714$) was seen in body condition scores (BCS) between heifers becoming pregnant by AI (5.60 ± 0.71) and those remaining open (5.70 ± 0.48). Furthermore, heifer age at AI was not significantly different ($p = 0.237$) between heifers becoming pregnant by AI (412.70 ± 25.35 days) or those remaining open (395.70 ± 35.92 days). Weight (WT) at Weaning was also found to be not significantly different ($p = 0.732$) between heifers becoming pregnant by AI (610.70 ± 88.43 lbs.) or those remaining open (596.40 ± 95.57 lbs.) (Figure 14). Data are presented as mean ± standard deviation of the mean.

Figure 14.



Phenotypic comparisons between fertile and infertile heifers. Data are mean ± standard deviation of the mean ($p < 0.05$). No significant difference was seen in RTSs, Body Condition Scores (BCSs) Age at AI, or Weight at Weaning between fertile and infertile heifers ($p > 0.05$).

Metabolome Assessment and Analysis based upon Pregnancy Outcome

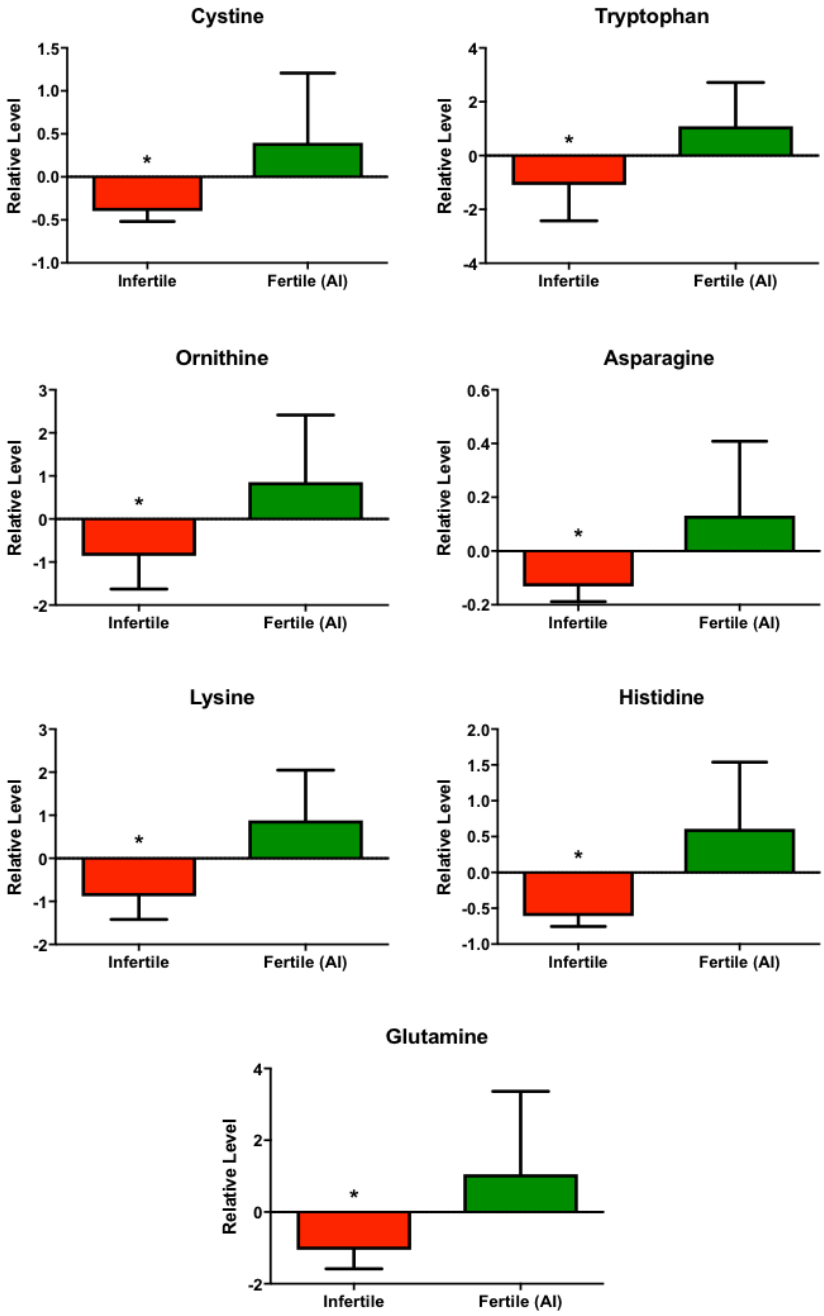
Fifteen metabolites were identified at different levels between fertile and infertile heifers

Univariate T-test analysis found 15 differentially expressed metabolite levels between the fertile and infertile (N = 20) plasma samples (Table 1). The metabolites Tryptophan ($p = 0.0042$), Cystine ($p = 0.0066$), Histidine ($p = 0.0007$), Ornithine ($p = 0.0059$), Asparagine ($p = 0.0085$), Glutamine ($p = 0.0117$), and Lysine ($p = 0.0004$) were identified as significantly different ($p < 0.05$, FDR 0.05) between fertile and infertile groups following filtering with at least a 2-fold change (Table 1; Figure 15). PLS-DA (Partial Least Squares Discriminant Analysis) displayed significant group separation between fertile (2 – green) and infertile (1 – red) samples ($P = 0.05$) (Figure 16). A Heat Map depicts the top twenty metabolites at differential levels (as identified via T-test) showing a trend of being down regulated in infertile heifers (1 – red) compared with fertile heifers (2 – green) (Figure 17).

Table 1: Metabolites found at significantly different levels in the infertile heifers when compared with the fertile heifers. Tryptophan, Cystine, Histidine, Ornithine, Asparagine, Glutamine, and Lysine were identified as significantly different between fertile and infertile groups ($p < 0.05$).

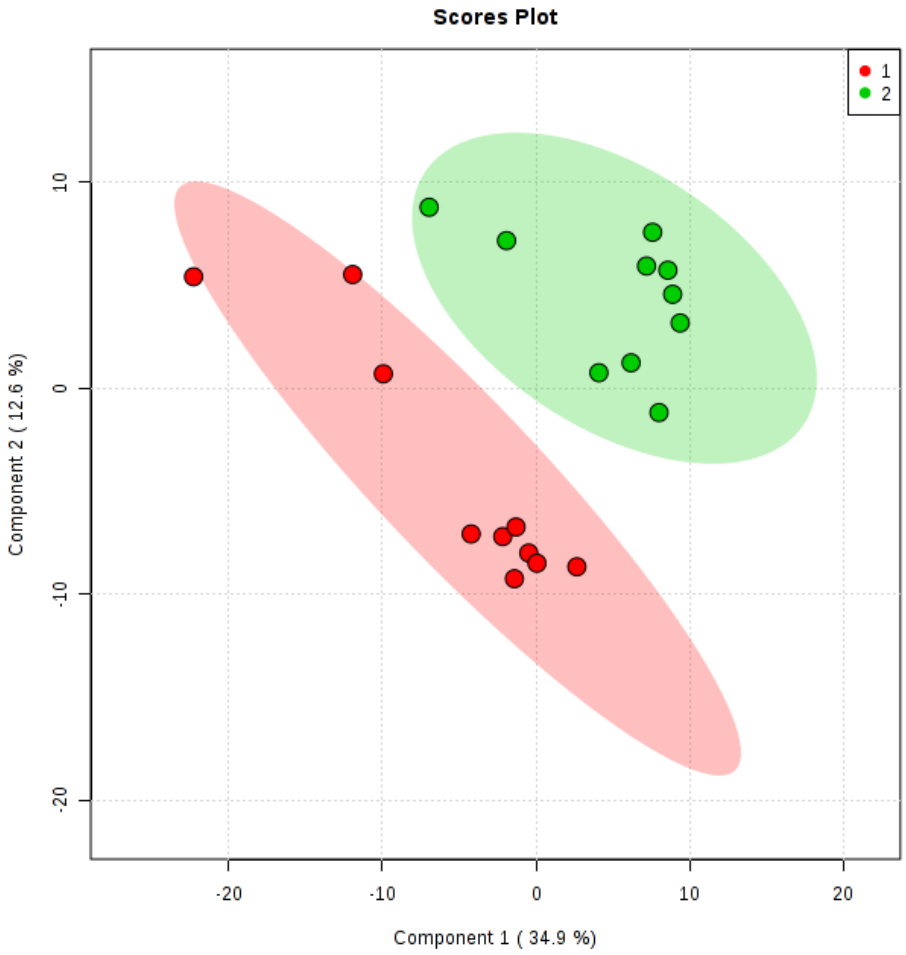
	Metabolite	P-Value	Fold Change	log2 (FC)	ROC AUC
1	Asparagine	0.0085415	0.48387	-1.0473	0.89
2	Lysine	0.00037698	0.44538	-1.1669	0.89
3	Ornithine	0.0059493	0.42779	-1.225	0.85
4	Glutamine	0.011729	0.3577	-1.4832	0.94
5	Histidine	0.00068495	0.18904	-2.4032	0.91
6	Cystine	0.0065674	0.14492	-2.7867	0.80
7	Tryptophan	0.0042144	0.51232	- 0.96488	0.86
8	Hydrocinnamic acid	0.0043435	0.52634	- 0.92594	0.86
9	2-aminobutyric acid	0.012595	0.54995	- 0.86262	0.87
10	Cysteine	0.047278	0.59574	- 0.74725	0.74
11	Phenylethylamine	0.020158	0.60189	- 0.73242	0.83
12	Methionine	0.014004	0.6119	- 0.70863	0.87
13	Kynurenine	0.018156	0.63053	- 0.66535	0.80
14	N-acetylornithine	0.022932	0.6444	- 0.63397	0.79
15	Allantoic acid	0.037071	0.66502	- 0.58854	0.75

Figure 15.



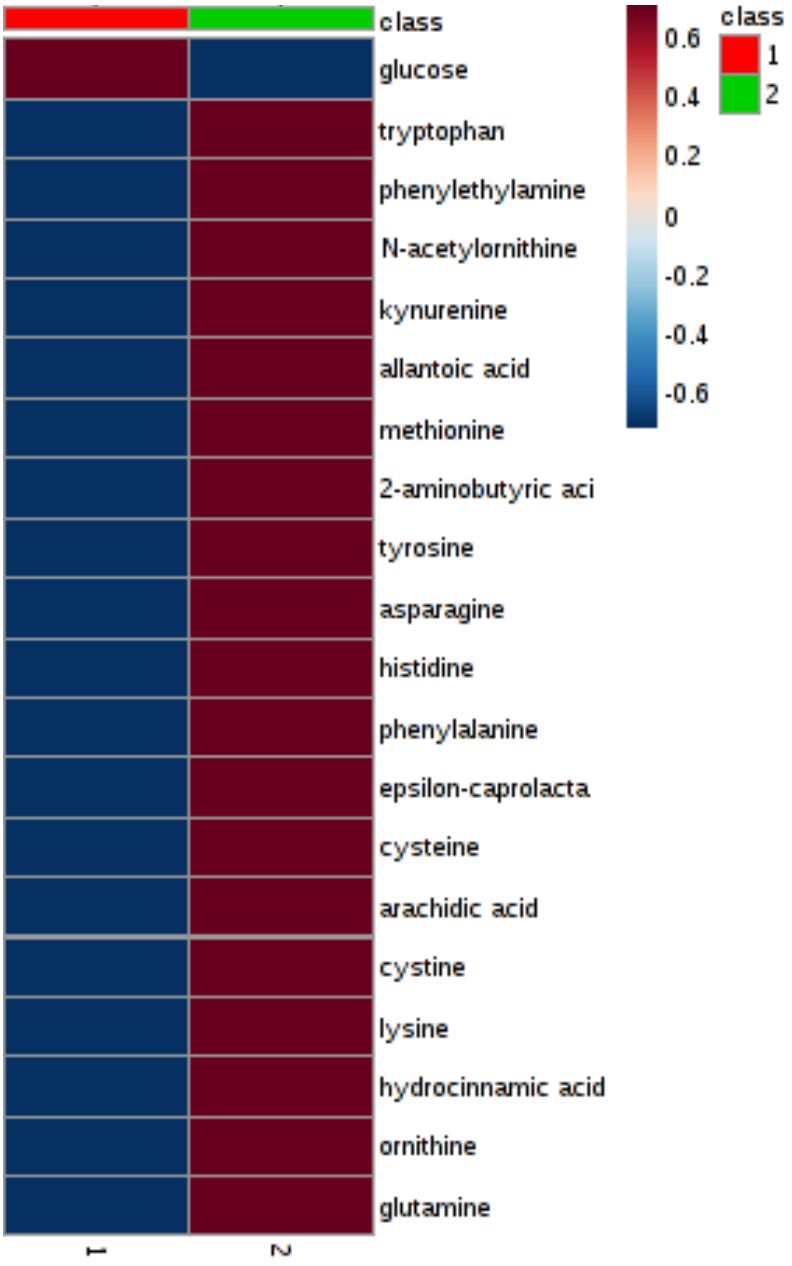
Relative levels of marker metabolites identified at significantly different levels in infertile heifers compared with fertile heifers ($p < 0.05$; > 2-fold change).

Figure 16.



PLS-DA scores plot displaying a significant separation between infertile heifers (red – 1) and fertile heifers (green – 2).

Figure 17.



Heat map depicting top 20 metabolites at differentially expressed levels. Samples were grouped as infertile heifers (red – 1) and fertile heifers (green – 2).

Predictive Ability Identified for Significant Metabolites

In order to identify the predictive ability of the significant metabolites discovered ($P < 0.05$; >2-fold change), we calculated the Receiver Operating Characteristic (ROC) area under the curve (AUC) value. Previous studies indicate that a metabolite with an ROC-AUC value of 0.80 or higher has a high predictive ability and success rate of correctly categorizing samples compared to a lower ROC-AUC value. The ROC-AUC values were 0.86 for Tryptophan, 0.80 for Cystine, 0.91 for Histidine, 0.85 for Ornithine, 0.89 for Asparagine, 0.93 for Glutamine, and 0.87 for Lysine (Table 2). The seven identified metabolites had ROC-AUC values of 0.80 – 0.94, suggesting they can predict fertility problems at the time of AI better than chance. We next analyzed the metabolome generated from fertile (N = 13) and infertile heifers (N = 7) using the identified metabolites individually, and in combination, to determine their accuracy in identifying infertile heifers. Metabolites with the highest ROC-AUC value in the logistical regression model were Glutamine (0.91), Asparagine (0.89), and Histidine (0.88). We tested the models on the blood plasma metabolomes of the 20 selected heifers to determine their ability to predict pregnancy outcomes. Glutamine and Histidine alone, and in combination, predicted the correct pregnancy outcome in 90% of the animals. They did not incorrectly categorize a fertile heifer as infertile (Table 2).

Table 2: ROC-AUC analysis of the top seven metabolites found at significantly different levels in infertile heifers compared to fertile heifers (AUC (95% CI)).

Metabolite Tested	AUC	Sensitivity	Specificity	% Correctly Categorized	% Fertile Categorized as Infertile
Tryptophan	0.820 (0.625 - 1.000)	0.800 (0.800 - 1.000)	0.700 (0.416 - 0.984)	80	10
Cystine	0.720 (0.456 - 0.984)	0.700 (0.700 - 0.984)	0.800 (0.552 - 1.000)	70	15
Histidine	0.880 (0.727 - 1.000)	0.700 (0.700 - 0.984)	1.000 (1.000 - 1.000)	90	0
Ornithine	0.840 (0.664 - 1.000)	0.800 (0.800 - 1.000)	0.700 (0.416 - 0.984)	70	0
Asparagine	0.890 (0.694 - 1.000)	0.900 (0.900 - 1.000)	0.900 (0.714 - 1.000)	70	0
Glutamine	0.910 (0.773 - 1.000)	0.800 (0.800 - 1.000)	1.000 (1.000 - 1.000)	90	0
Lysine	0.860 (0.659 - 1.000)	0.800 (0.800 - 1.000)	0.900 (0.714 - 1.000)	75	0
His, Glut, Asp	0.735 (0.465 - 1.000)	0.800 (0.800 - 1.000)	0.900 (0.714 - 1.000)	85	0
His, Glut	0.860 (0.681 - 1.000)	0.900 (0.900 - 1.000)	0.800 (0.552 - 1.000)	90	0

Pathway Analysis reveals amino acid significance

Following the Holm adjustment of p values (FDR < 0.05), aminoacyl-tRNA biosynthesis was found to be a significantly perturbed pathway of the fifteen differentially expressed metabolites between fertile and infertile heifers. To analyze the effect of fifteen differential metabolites identified for biological pathways, a Fisher's Exact Test was performed (Table 3). Expected hits are identified as the percentage of the fifteen differentially expressed metabolites that are intermediately involved in a given pathway.

Table 3: Pathway Analysis for selected significant metabolites. Aminoacyl-tRNA was the most significant pathway affected when comparing fertile with infertile heifer metabolomes.

	Total	Expected	Hits	Raw p-value	-log(p)	Holm Adjusted p-value
Aminoacyl-tRNA Biosynthesis	64	0.69015	7	1.51E-06	13.401	0.00012259
Cysteine and Methionine Metabolism	28	0.30194	3	0.00283	5.8676	0.22637
Nitrogen Metabolism	9	0.097052	2	0.003743	5.588	0.29566
Arginine and Proline Metabolism	44	0.47448	3	0.010304	4.5752	0.80373
Alanine, Aspartate, and Glutamate Metabolism	23	0.24802	2	0.024103	3.7254	1
Glutathione Metabolism	26	0.28037	2	0.030389	3.4937	1
D-Glutamine and D-Glutamate Metabolism	5	0.053918	1	0.052842	2.9404	1
Biotin Metabolism	5	0.053918	1	0.052842	2.9404	1
Tryptophan Metabolism	41	0.44213	2	0.069861	2.6612	1
Taurine and Hypotaurine Metabolism	7	0.075485	1	0.07324	2.614	1
Thiamine Metabolism	7	0.075485	1	0.07324	2.614	1
Phenylalanine Metabolism	9	0.097052	1	0.093227	2.3727	1
Histidine Metabolism	14	0.15097	1	0.14145	1.9558	1
Pantothenate and CoA Biosynthesis	15	0.16175	1	0.1508	1.8918	1
Purine Metabolism	68	0.73329	2	0.16435	1.8057	1
Glycine, Serine and Threonine Metabolism	32	0.34508	1	0.29594	1.2176	1

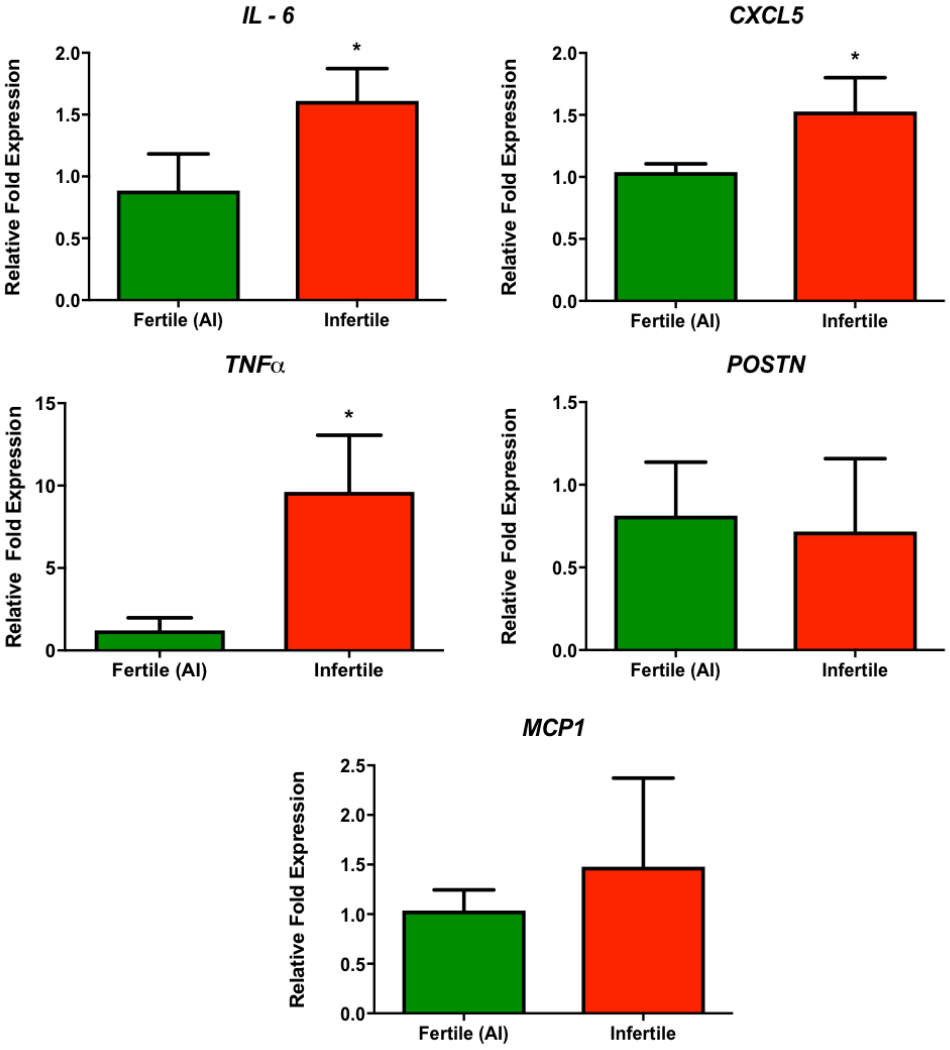
Inflammatory cytokines identified as significantly different

In order to investigate the possibility that infertile heifers could have asymptomatic inflammation, we isolated the mRNA from white blood cells and compared the transcript level of inflammatory cytokines with that of fertile (N = 20) heifers (Table 4). The transcript level of the inflammatory response regulator, Tumor Necrosis Factor-alpha (*TNFα*), was found to be significantly higher (p = 0.003) in infertile heifers (9.62 ± 3.43-fold) when compared to fertile heifers (1.223 ± 0.76-fold) (Figure 18). Similarly, transcripts for the pro-inflammatory cytokine Interleukin 6 (*IL-6*) were found to be significantly higher in the infertile heifers (p = 0.010; 1.61 ± 0.26-fold) when compared with fertile heifers (0.89 ± 0.30-fold) (Figure 18). We also found the neutrophil activating peptide C-X-C Motif Chemokine 5 (*CXCL5*) to be significantly higher in the infertile heifers (p = 0.040, 1.53 ± 0.27-fold) when compared with the fertile heifers (1.04 ± 0.07-fold (Figure 18). Conversely, we did not see a significant difference in the expression of the interleukin-induced Periostin (*POSTN*) gene when we compared infertile (p = 0.775; 0.72 ± 0.44-fold) with fertile heifers (0.81 ± 0.32-fold) (Figure 18). Moreover, we did not see a difference in the expression of the pro-inflammatory chemokine Monocyte Chemoattractant Protein 1 (*MCP1*) when we compared the infertile (P = 0.371; 1.48 ± 0.89-fold) with the fertile heifers (1.04 ± 0.21-fold) (Figure 18).

Table 4: Primer Sequences used for identification of inflammatory cytokine expression.

Primer	Sequence (5→ 3')	NCBI Accession Number	Efficiency (%)	Product Length (BP)
TNFα	F: TCAAGCCTCAAGTAACAAGCC R: GTTGTCTTCCAGCTTCACACC	NM_173966.3	93	123
IL-6	F: TGAGTGTGAAAGCAGCAAGGA R: TCGCCTGATTGAACCCAGAT	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

Figure 18.



Comparison of transcript levels of inflammatory cytokines in the white blood cells from fertile and infertile heifers. Data are mean ± standard deviation of the mean ($p < 0.05$).

Metabolome Assessment based upon Location

Metabolomics profiling over two breeding seasons was analyzed on samples collected at two locations on a total of sixteen heifers (N = 16, Black Belt Research and Extension Center N = 8 and Wiregrass Research and Extension Center N = 8).

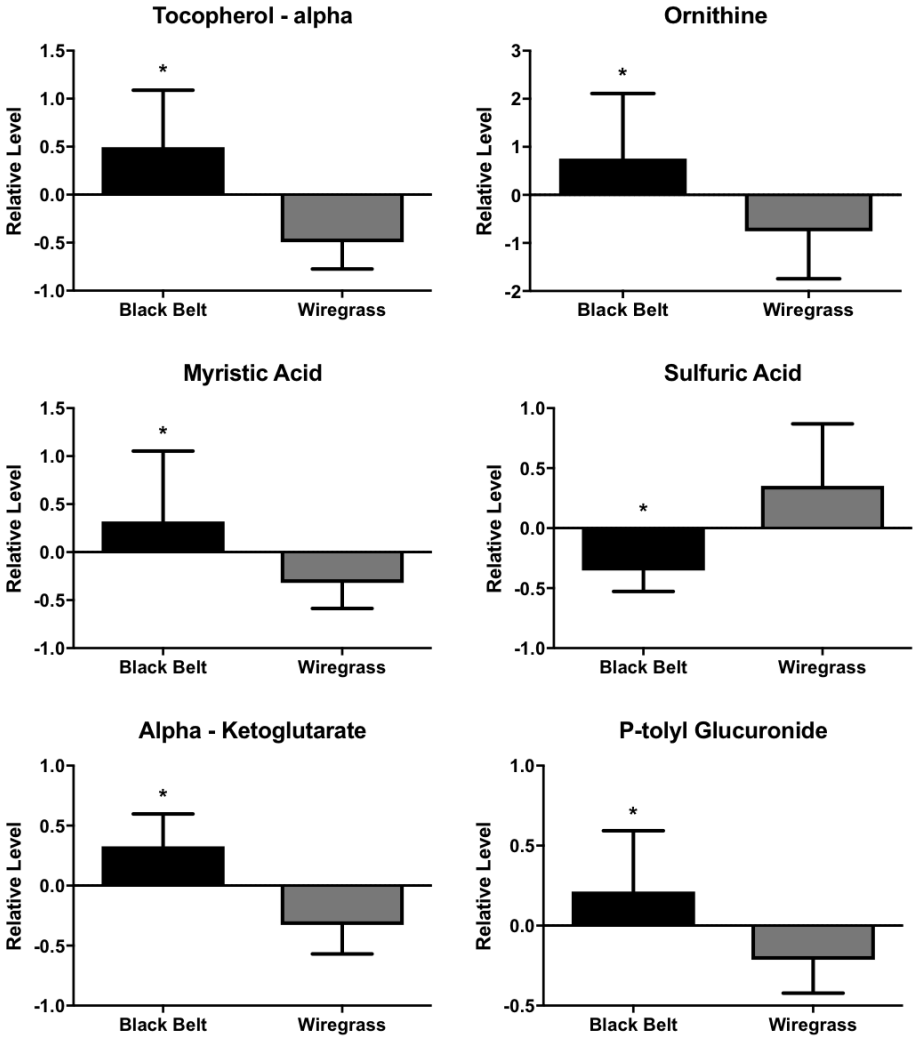
Six Metabolites were identified as differentially expressed

Six metabolites were present at significantly different levels between the Black Belt Research and Extension Center heifers compared with the Wiregrass Research and Extension Center heifers (Table 5). The metabolites Tocopherol-alpha ($p = 0.0008$), Ornithine ($p = 0.0227$), Myristic Acid ($p = 0.0355$), Sulfuric Acid ($p = 0.0026$), Alpha-ketoglutarate ($p = 0.0002$), and P-tolyl Glucuronide ($p = 0.0145$) were identified as significantly different ($p < 0.05$, FDR 0.05) between Black Belt and Wiregrass following filtering with at least a 2-fold change (Table 5; Figure 19). PLS-DA (Partial Least Squares Discriminant Analysis) displayed overlapping and group separation between Wiregrass (green) and Black Belt (red) heifer metabolome analysis ($p = 0.05$ by Permutation Test) (Figure 20). A Heat Map depicts the top twenty-five metabolites at differential levels (as identified via T-test) showing a trend of being down-regulated in Wiregrass heifers (green) compared with Black Belt heifers (red) during a combined breeding season metabolome analysis (Figure 21).

Table 5: Metabolites found at significantly different levels in heifers at Black Belt Research and Extension Center compared to heifers at Wiregrass Research and Extension Center during a combined breeding season analysis (2015 – 2016 and 2016 – 2017).

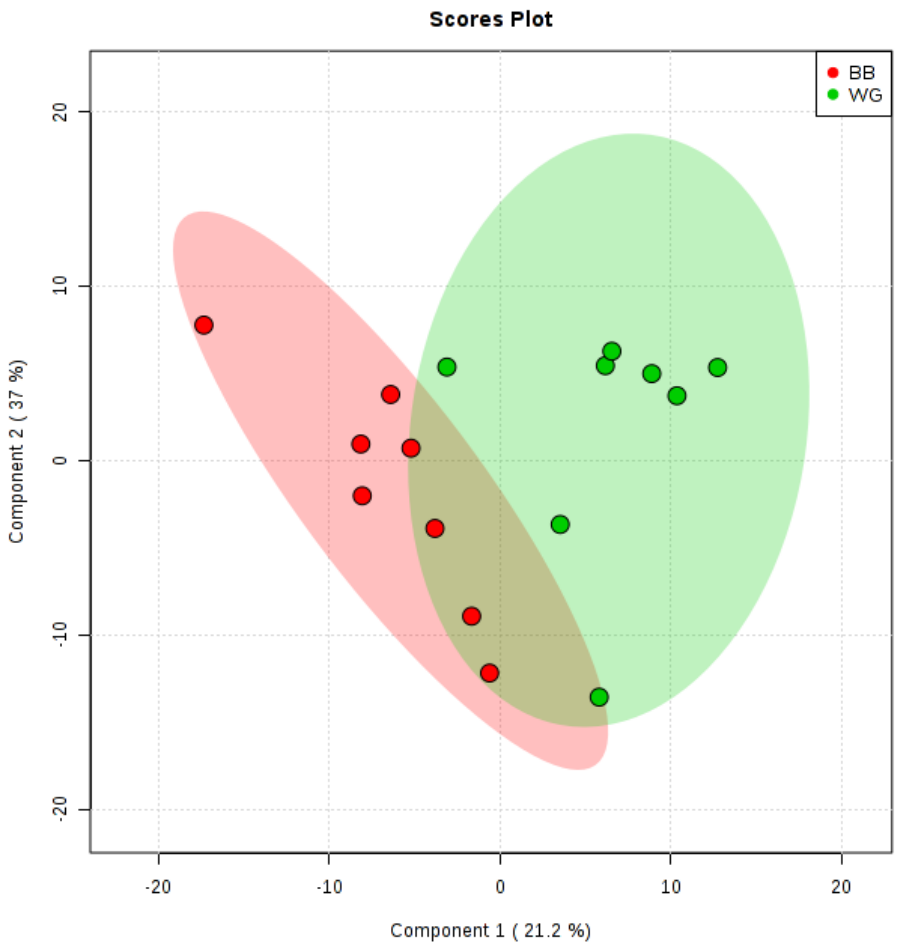
	Metabolite	P-Value	Fold Change	log2 (FC)
1	Ornithine	0.0227	2.2575	1.1747
2	Tocopherol-alpha	0.0008	2.6644	1.4138
3	Myristic Acid	0.0355	2.2513	1.1708
4	P-tolyl Glucuronide	0.0145	2.2098	1.1439
5	Sulfuric Acid	0.0026	0.4614	-1.1159
6	Alpha-ketoglutarate	0.0002	2.0124	1.0089

Figure 19.



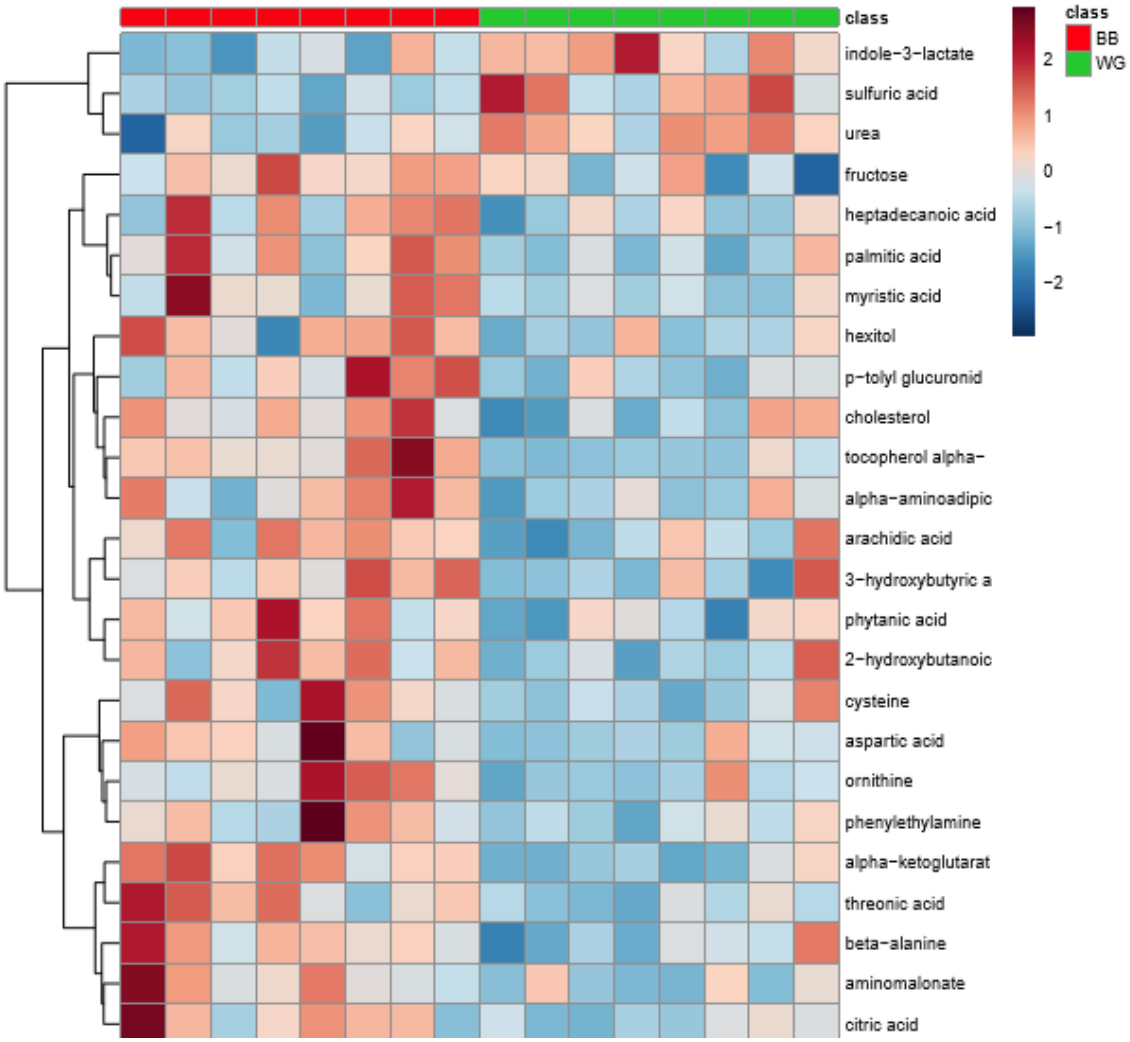
Relative levels of marker metabolites identified at significantly different levels in Black Belt heifers compared with Wiregrass heifers during a combined breeding season metabolome analysis. Tocopherol-alpha, Ornithine, Myristic Acid, Sulfuric Acid, Alpha-ketoglutarate, and P-tolyl Glucuronide were identified as significantly different between Black Belt and Wiregrass heifers (2-fold; $p < 0.05$).

Figure 20.



PLS-DA scores plot displaying group separation between Wiregrass (green) and Black Belt (red) heifer metabolome analysis.

Figure 21.



Heat Map depicting differential levels of metabolites showing a trend of being down regulated in Wiregrass heifers (green) compared to Black Belt heifers (red) during a combined breeding season metabolome analysis.

III.V. DISCUSSION

Replacement heifers are a major economic burden on the beef cattle industry. Heifer infertility remains a difficult-to-diagnose pathology resulting in inefficiencies within the cow-calf sector. The ability to detect heifers that are unable to produce offspring would provide a mechanism to remove them from the breeding herd prior to investing valuable time, money, and resources. Therefore, biomarkers able to detect heifers with fertility problems would be highly utilized by cow-calf producers to minimize the costs associated with heifer production and development.

Previous studies have shown that differential levels of metabolites has been able to detect various difficult-to-diagnose ailments including diabetic kidney disease (Kloet et al., 2012) Parkinson's disease (Bogdanov et al., 2008), myocardial ischemia (Sabatine et al., 2005), ovarian cancer (Zhang et al., 2012), and endometriosis (Dutta et al., 2012). Moreover, within the agriculture sector, metabolite concentrations in follicular fluid and blood were used to potentially explain differences in fertility between heifers and lactating cows (Bender et al., 2010). We approached this study to detect metabolite differences in the blood plasma of heifers managed at multiple research stations across the state of Alabama, U.S.A. Current management practices utilize phenotypic methods to attempt to minimize heifers remaining open following the breeding season. Therefore, we compared common phenotypic parameters between the heifers included in our metabolomic analysis.

We did not see a statistically significant difference between the RTSs of heifers remaining open or those becoming pregnant by AI (Figure 14). Similarly, no significant difference was seen in body condition scores (BCSs) between heifers becoming pregnant by AI and those remaining open (Figure 14). Furthermore, heifer age at AI was not significantly different between heifers becoming pregnant by AI or those remaining open (Figure 14). Weight (WT) at weaning was also found to be not significantly different between heifers becoming pregnant by AI or those remaining open (Figure 14). Collectively, these data highlight the limitations of phenotypic parameters to identify infertile heifers at the time of AI. Heifer infertility that results in the failure of a cow or heifer to conceive a calf is the single largest economic loss to beef producers. Phenotypic parameters such as RTS, BCS, age at AI, and weight at weaning are all relevant and important pieces of information needed to produce a successful and productive breeding herd. Many producers establish minimum and maximum requirements, or values, in order to create and maintain a relative breeding herd "standard". However, reproductive failure is still an element of concern for the beef cattle industry not only locally, but nationally and internationally alike. Functional causes of infertility tend to affect individual heifers, but when combined, these infertile heifers can make a large impact on the overall herd (Abraham, 2017). The utilization of biomarkers that are able to detect heifers with fertility problems could be highly adopted by cow-calf producers to minimize the costs associated with heifer production and development. The inability to detect infertile heifers using traditional phenotypic methods highlights the importance of identifying biomarkers using innovative new approaches including metabolomics.

In order to detect biomarkers for fertility assessment in replacement heifers, we utilized metabolomic analysis of the blood plasma of heifers becoming pregnant at first service through AI (fertile) and those failing to become pregnant following AI and three natural breeding cycles (infertile). The present two-year study identified seven metabolites that had a greater than 2-fold difference between the two groups. The seven identified metabolites had ROC-AUC values ranging from 0.80 – 0.94, suggesting they can predict fertility problems at the time of AI better than chance (Table 1; Figure 15). We then used each of the identified metabolites individually, and in combination, to develop a logistic regression model to predict pregnancy outcomes in a blind study. We found the percentage of animals correctly categorized ranged from 70% using Asparagine, Ornithine, or Cysteine to 90% using Glutamine or Histidine. Interestingly, utilizing Histidine in combination with Glutamine did not improve the detection of infertile heifers, with the accuracy remaining at 90%. An element of this study that was particularly problematic was the labelling of "fertile" heifers as "infertile". This would run the risk of potentially removing valuable heifers from the herd as a result of a false fertility diagnosis. In all cases of false identification, we observed infertile heifers being categorized as fertile (Table 2). Using Histidine or Glutamine individually, and in combination, did not result in any fertile heifers being identified as infertile.

Pathway analysis revealed a significant effect on the aminoacyl-tRNA biosynthesis pathway (Table 3). This is not surprising, as of the fifteen metabolites shown to be at differential levels in the infertile heifer's blood plasma, seven were amino acids (Table 1; Table 3). The seven amino acids were all present at a lower level in the infertile heifers than the fertile heifers. Previous work looking at the levels of amino acids in the

blood plasma of kidney disease patients (with and without inflammation) found those with inflammation had significantly lower levels of Asparagine, Serine, Glutamine, Glycine, Arginine, Alanine, Histidine, and Threonine (Suliman et al., 2005). In another study investigating the plasma amino acid levels of cats with chronic gastrointestinal (GI) disease, it was shown that Arginine, Histidine, Lysine, Methionine, Phenylalanine, Taurine, and Tryptophan (along with several non-essential amino acids) were lower in cats with chronic GI diseases (Sakai et al., 2018). They also found Histidine and Tryptophan levels to be inversely correlated to symptom severity, and Histidine could suppress inflammatory cytokine release by their macrophages (Sakai et al., 2018).

In our study, we found significantly lower levels of Asparagine, Lysine , Glutamine, Histidine, Tryptophan, Cysteine, and Ornithine. The decreased level of Histidine found in the infertile heifer's blood plasma was of particular interest due to its well-described antioxidant and anti-inflammatory functions (Sakai et al., 2018; Yu et al., 2015). Studies have shown that the presence of low plasma Histidine is associated with inflammation (Watanabe et al., 2008). Furthermore, studies in humans have shown supplementation with histidine interestingly suppressed inflammatory cytokines such as *TNFα* and *IL-6* (Feng et al., 2013). An additional study investigated the effect of Histidine supplementation on other metabolites in the blood. Inversely, the authors discovered that supplementation of Histidine increased Glutamine, Aspartate, Glycine, Choline, and Trimethylamine-*N*-oxide in humans (Du et al., 2017). In comparison to our study, we observed decreased Glutamine and Histidine in the infertile heifers (Figure 15).

Because of the high amount of significant amino acids related to inflammation in our metabolomics analysis, we therefore investigated the expression of various

inflammatory cytokines in the white blood cells from the fertile and infertile heifers. In order to investigate the possibility that infertile heifers could have asymptomatic inflammation, we isolated the mRNA from white blood cells and compared the transcript level of inflammatory cytokines (Figure 18) to fertile heifers. We observed a significant upregulation in the transcripts for the inflammatory cytokines *TNFα*, *IL-6*, and *CXCL5*, suggesting a potential relationship between the fertility and the inflammatory status in heifers (Figure 18). The specifics of this relationship remains to be studied.

The metabolome, or the metabolomics profile, of an organism can vary by location, species, life stage, and environment. Because of varying metabolomics profiles, we therefore became interested in how heifer metabolomes (as a whole) differed based upon location. Metabolomics profiling and analysis was performed on heifer blood plasma samples from two separate locations across Alabama, U.S.A. All locations in our study carefully documented and reported heifer nutrition, and all heifers fell under the same broad-spectrum management programs.

The metabolomics profiles of blood plasma from eight heifers housed at the Black Belt Research and Extension Center and eight heifers housed at the Wiregrass Research and Extension Center across two breeding seasons were analyzed. The present two-year location study identified six metabolites that had at least a 2-fold difference between heifers housed at the two locations (Table 5; Figure 19). Five of the six metabolites were all present at a lower level in the Wiregrass heifers when compared to the Black Belt heifers. The only metabolite that was upregulated in Wiregrass heifers when compared to Black Belt heifers was Sulfuric Acid. This could be due to the low amount of sulfur in the soil of Black Belt Research and Extension Center. Interestingly, only one of the

metabolites detected at different levels (Ornithine) overlapped with those identified as correlated to pregnancy outcome. Differences in the metabolomes of heifers across multiple locations can be due to management practices, type of feed, feed intake, and overall environmental influence. This data highlights the sensitivity of metabolomic analysis to detect differences in animals under different environmental conditions. The implications of these metabolites being at different levels and possible effects on reproductive outcomes remain to be determined.

CHAPTER IV.

CONCLUSION AND IMPLICATIONS

In conclusion, we utilized metabolomics to investigate the differential levels of metabolites in the blood plasma of fertile and infertile heifers, as well as compare the metabolomes of heifers from various locations across the state of Alabama. Traditional phenotypic heifer selection parameters such as RTS, BCS, age, and weight were compared across locations, breeding seasons, and pregnancy outcomes. We identified seven metabolites with significant differential levels (> 2-fold) between fertile and infertile heifers, and six metabolites with significant differential levels from separate locations. The predictive ability of the metabolites was assessed and used to predict the correct pregnancy outcomes at the time of AI. Furthermore, a relationship between the inflammatory status of the animals and pregnancy outcome was identified.

As discovered in this study, there is confirmed differential levels of selected metabolites from both infertile and fertile heifers at the time of artificial insemination. The question that remains is if these metabolites (or metabolomics profiles) differ at various stages of a heifer's life cycle. The current focus of ongoing research is to establish a metabolomics profile from the weaning date of heifers through the date of artificial insemination. If the opportunity presents itself, would it be possible that metabolomics profiles stay consistent at multiple time points throughout a heifer's life?

Does the metabolomics profile change at the onset of puberty, or does the metabolomics profile change from the onset of stress? Is it possible to pin-point specific up-regulated or down-regulated metabolites in heifers that could identify her potential infertility, before she even hits puberty? Multiple questions remain unanswered in regard to exactly how the metabolome of replacement heifers changes, or lack thereof.

Reproductive failure and unexplained heifer infertility are the major factors that affect the profitability of beef cattle production industries (Diskin, 1980). Heifer infertility remains a difficult to diagnose pathology resulting in inefficiencies within the cow-calf sector. If a producer has the correct set of tools and resources available, it is possible that a single blood test from each heifer could determine the predictive fertility of his or her herd for the upcoming breeding season. With the use of metabolomics, and further research into studies like the one we have presented, the beef cattle industry could have the potential to detect infertility in phenotypically sound heifers. Heifer infertility that results in the failure of a cow or heifer to conceive a calf is the single-largest economic loss to beef producers. With the development of biomarkers for the identification of infertile heifers, there is outstanding potential to improve the world-wide efficiency of cow-calf production.

Literature Cited:

Abraham F. An Overview on Functional Causes of Infertility in Cows. J Fertilization Vitro - Ivf-Worldwide Reproductive Medicine Genetics Stem Cell Biology 2017; 05.

American Pregnancy Association. Female Infertility: Causes, Treatment and Prevention; 2017. http://americanpregnancy.org/infertility/female-infertility/. Accessed May 10, 2018.

Anderson, K.J., D.G. Lefever, J.S., Brinks, and K.G. Odde. 1991. The use of reproductive tract scoring in beef heifers. Agri-Practice. 12(4):19.

Austdal M et al. Metabolomic biomarkers in serum and urine in women with preeclampsia. PLoS One 2014; 9(3):e91923.

Austgulen R et al. (2016) Circulating levels of cytokines and cytokine receptors in normal human pregnancy, Scandinavian Journal of Rheumatology; 27sup107.

Bender K, Walsh S, Evans AC, Fair T, Brennan L. Metabolite concentrations in follicular fluid may explain differences in fertility between heifers and lactating cows. Reproduction 2010; 139:1047–1055.

Bio-Rad Laboratories, Inc. Innate Immune Responses in Cattle LIT.IIR. 2016 Bio-Rad Laboratories, Inc., Endeavour House, Kidlington.

Bogdanov M, Matson WR, Wang L, Matson T, Saunders-Pullman R, Bressman SS, Flint Beal M. Metabolomic profiling to develop blood biomarkers for Parkinson's disease. Brain 2008; 131: 389-396.

Bon Durant RH. Selected diseases and conditions associated with bovine conceptus loss in the first trimester. Theriogenology 2007;68:461–73.

Bracewell-Milnes et al. Metabolomics as a tool to identify biomarkers to predict and improve outcomes in reproductive medicine: a systematic review. Hum Reprod Update 2017; 23:723–736.

Briana DD, Malamitsi-Puchner A: Intrauter- ine growth restriction and adult disease: the role of adipocytokines. Eur JEndocrinol 2009; 160:337–347.

Calus MPL, Windig JJ, Veerkamp RF. Associations Among Descriptors of Herd Management and Phenotypic and Genetic Levels of Health and Fertility. J Dairy Sci 2005; 88:2178–2189.

Chavey C et al. *CXCL5* is an adipose tissue derived factor that links obesity to insulin resistance. Cell Metab 2009; 9: 339-349.

Chen E et al. Dynamic changes of plasma metabolites in pigs with GalN-induced acute liver failure using GC–MS and UPLC–MS. Biomed Pharmacother 2017; 93:480–489.

Cheng Z, Ma Y, Li H, Peng X. N-acetylglucosamine enhances survival ability of tilapias infected by streptococcus iniae. Fish and Shellfish Immunology 2014;40(2): 524-530.

Cushman, R.A., L.K. Kill, R.N. Funston, E.M. Mousel and G.A. Perry. 2013. Heifer calving date positively influences calf weaning weights through six parturitions. J. Anim. Sci. 91:4486- 4491

Day ML, Grum DE. Breeding strategies to optimize reproductive efficiency in beef herds. Vet Clin North Am Food Anim Pract 2005;21:367–81.

Diskin, Sreenan. Fertilization and embryonic mortality rates in beef heifers after artificial insemination. J Reprod Fertil 1980; 59:463–468

Diskin MG, Murphy JJ, Sreenan JM (2006) Embryo survival in dairy cows managed under pastoral conditions. Ani Reprod Sci 96: 297-311.

Donovan GA, Bennett FL, Springer FS. Factors associated with first service conception in artificially inseminated nulliparous Holstein heifers. Theriogenology 2003; 60(1):67-75.

Du S et al.. Effects of Histidine Supplementation on Global Serum and Urine 1H NMR-based Metabolomics and Serum Amino Acid Profiles in Obese Women from a Randomized Controlled Study. J Proteome Res 2017; 16(6): 2221-2230.

Dutta M, Joshi M, Srivastava S, Lodh I, Chakravarty B, Chaudhury K. A metabonomics approach as a means for identification of potential biomarkers for early diagnosis of endometriosis. Molecular Biosyst 2012; 8(12): 3281-3287.

Feng RN et al. Histidine supplementation improves insulin resistance through suppressed inflammation in obese women with the metabolic syndrome: a randomised controlled trial. Diabetologia 2013; 56(5): 985-994.

Freis A. Serum periostin levels in early in pregnancy are significantly altered in women with miscarriage. Reproductive Biology and Endocrinology 2017; 15.

Funston, Deutscher. Comparison of target breeding weight and breeding date for replacement beef heifers and effects on subsequent reproduction and calf performance. J Anim Sci 2004; 82:3094.

Goodacre, R., Vaidyanathan, S., Dunn, W.B., Harrigan, G.G. & Kell, D.B. Metabolomics by numbers: acquiring and understanding global metabolite data. Trends Biotechnol. 22(5), 245-252 (2004)

Grings, Geary, Short, MacNeil. Beef heifer development within three calving systems. J Anim Sci 2007; 85:2048.

Guilbert LJ: There is a bias against type 1 (inflammatory) cytokine expression and function in pregnancy. J Reprod Immunol 1996; 32:105–110.

Günther, U.L. Metabolomics Biomarkers for Breast Cancer. Pathobiology. 82, 153–65 (2015)

Gutierrez K et al.Effect of reproductive tract scoring on reproductive efficiency in beef heifers bred by timed insemination and natural service versus only natural service. Theriogenology 2014; 81(7): 918-924.

Hall, John B. 2005. Reproductive Evaluation of Heifers. Proceedings, Applied Reproductive Strategies in Beef Cattle. Virginia Tech, Blacksburg, VA. http://www.sites.ext.vt.edu/newsletter-archive/livestock/aps-98_07/aps-935.html

Herd, D. and L.R. Sprott. 1998. Body Condition, Nutrition and Reproduction of Beef Cows. Texas Agricultural Extension Services. B-1526.

Hertl JA, Gröhn YT, Leach JD, et al. Effects of clinical mastitis caused by Gram-positive and Gram-negative bacteria and other organisms on the probability of conception in New York State Holstein dairy cows. J Dairy Sci . 2010;93:1551–1560.

Holm DE, Nielen, Jorritsma, Irons PC, Thompson PN. Evaluation of pre-breeding reproductive tract scoring as a predictor of long term reproductive performance in beef heifers. Prev Vet Med 2015; 118:56–63.

Holm DE, Thompson PN, Irons PC. The value of reproductive tract scoring as a predictor of fertility and production outcomes in beef heifers. J Anim Sci 2009: 87:1934-1940.

Hunt JS, Chen HL, Miller L: Tumor necrosis factors: pivotal components of pregnancy? Biol Reprod 1996;54:554–562.

Jafarzadeh N et al. Metabolomics fingerprinting of seminal plasma from unexplained infertile men: a need for novel diagnostic biomarkers. Mol Reprod Dev 2015; 82:150.

Kasimanickam, Schroeder, Assay, Kasimanickam, Moore, Gay, Whittier. Influence of Temperament Score and Handling Facility on Stress, Reproductive Hormone Concentrations, and Fixed Time AI Pregnancy Rates in Beef Heifers. Reprod Domest Anim 2014; 49:775–782.

Kloet, V.F.M. et al. Discovery of early-stage biomarkers for diabetic kidney disease using ms-based metabolomics (FinnDiane study). Metabolomics. 8(1), 109-119 (2012)

Koumantaki Y. Detection of interleukin-6, interleukin-8, and interleukin-11 in plasma from women with spontaneous abortion. European Journal of Obstetrics & Gynecology and Reproductive Biology 2001; 98:66–71

Kuc S et al. Metabolomics profiling for identification of novel markers in early prediction of preeclampsia. PLoS One 2014; 9(5):e98540.

Kuhn, M.T., Hutchison, J.L. & Wiggans, G.R. Characterization of Holstein heifer fertility in the United States. J Dairy Sci. 89(12), 4907-4920 (2006)

Lamb GC. What Is the Impact of Infertility in Beef Cattle? IFAS Extension - University of Florida 2014. http://edis.ifas.ufl.edu/an208

Larson RL, White BJ, Laflin S. Beef Heifer Development. Vet Clin North Am Food Anim Pract. 2016;32(2):285–302

Lavon Y et al. Subclinical, chronic intramammary infection lowers steroid concentrations and gene expression in bovine preovulatory follicles. Dom An Endocrinol . 2011;40:98–109.

LeFever, D.G. and K.G. Odde. 1987. Predicting reproductive performance in beef heifers by reproductive tract evaluation before breeding. CSU Beef Research Report. pp. 13-15.

Liu, Jaitner, Reinhardt, Pasman, Rensing, Reents. Genetic Evaluation of Fertility Traits of Dairy Cattle Using a Multiple-Trait Animal Model. J Dairy Sci 2008; 91:4333–4343.

Lowman, B. G., N. A. Scott, and S. H. Somerville. 1976. "Condition Scoring of Cattle," East of Scotland College of Agr. Bull. 6.

Mahdi, B. Role of some cytokines on reproduction. Middle East Fertility Society Journal 2011; 16:220 – 223.

Mendiola J, et al. Urinary concentrations of di(2-ethylhexyl) phthalate metabolites and serum reproductive hormones: pooled analysis of fertile and infertile men. J Androl 2012; 33:488–98.

McBride W, Mathews K. The Diverse Structure and Organization of U.S. Beef Cow-Calf Farms. Ssrn Electron J 2011.

Nagy ZP et al. Metabolomic assessment of oocyte viability. Reprod Biomed Online 2009; 18(2): 219-225.

Neville WE, Mullinix BG, Smith JB, et al. Growth patterns for pelvic dimensions and of their body measurements of beef females. J Anim Sci 1978;47:1080–8.

Oliver, S.G. et al. (1998) Systematic functional analysis of the yeast genome. Trends Biotechnol. 16, 373–378

Ostanin, et al. Role of cytokines in the regulation of reproductive function. B Exp Biol Med+ 2007; 143:75–79

Parkin J. An overview of the immune system. The Lancet 2001; 357:1721-1812

Patterson DJ, Perry RC, Kiracofe GH, et al. Management considerations in heifer development and puberty. J Anim Sci 1992;70:4018–35.

Price JC, Bromfield JJ, Sheldon IM.Pathogen-associated molecular patterns initiate inflammation and perturb the endocrine function of bovine granulosa cells from ovarian dominant follicles via TLR2 and TLR4 pathways. Endocrinology. 2013 Sep;154(9):3377-86. doi: 10.1210/en.2013-1102. Epub 2013 Jul 3.

Prins JR. Interleukin-6 in pregnancy and gestational disorders. Journal of Reproductive Immunology 2012; 95:1–14.

Rainard et al. Innate immunity of the bovine mammary gland. Vet. Res. (2006) 37(3):369-400. http://www.ncbi.nlm.nih.gov/pubmed/16611554

Raghupathy R (ed): Th1 and Th2 Cytokine Profiles in Successful and Unsuccessful Pregnancy. New Delhi, Narosa Publishing, 1999, pp 149–158.

Reid JG, et al. The carriage of pro-inflamma- tory cytokine gene polymorphisms in recurrent pregnancy loss. Am J Reprod Immunol 2001;45:35–40.

Revelli A et al. Follicular fluid content and oocyte quality: from single biochemical markers to metabolomics. Reprod Biol Endocrinol 2009; 7:40.

Rosenkrans, K.S., & Hardin, D.K. Repeatability and accuracy of reproductive tract scoring to determine pubertal status in beef heifers. *Theriogenology.* **59(5-6)**, 1087-92 (2003)

Sabatine MS et al. Metabolomic identification of novel biomarkers of myocardial ischemia. Circulation 2005; 112(25): 3868-3875.

Sakai K, Maeda S, Yonezawa T, Matsuki N. Decreased plasma amino acid concentrations in cats with chronic gastrointestinal diseases and their possible contribution in the inflammatory response. Vet Immunol Immunopathol 2018; 195: 1-6.

Scaglia G, Beck P, Lalman D, Rouquette FM. Invited Review: Issues affecting research and extension programs on cow-calf and stocker cattle production in the Southeast region of the United States. American Society of Animal Science Southern Section, February 7, 2016, San Antonio, Texas. The Professional Animal Scientist 2017; 33:310 – 319.

Schatz, T.J. & Hearnden M.N. Heifer fertility on commercial cattle properties in the Northern Territory. Australian Journal of Experimental Agriculture. 48, 940-944 (2008)

Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc 2008; 3(6): 1101-1108.

Singh R, Sinclair KD. Metabolomics: approaches to assessing oocyte and embryo quality. Theriogenology 2007; 68 Suppl 1:S56–62.

Sreekumar A et al. Metabolomic profiles delineate potential role for sarcosine in prostate cancer progression. *Nature* 2009, 457:910-914

Suliman ME et al. Inflammation contributes to low plasma amino acid concentrations in patients with chronic kidney disease. American J Clin Nutr 2005; 82(2): 342-349.

Swecker W. Bovine Reproduction. Wiley; **29,** 276 – 282 (2014).

Tauck SA, Wilkinson JRC, Olsen JR, et al. Comparison of controlled internal drug release device and melengesterol acetate as progestin sources in an estrous synchronization protocol for beef heifers. Theriogenology 2007;68:162–7.

USDA-NASS (National Agricultural Statistics Service). 2018. Cattle. Accessed Apr. 2018, http://usda.mannlib.cornell.edu/usda/nass/Catt/2010s/2018/Catt-01-31-2018.pdf

USDA-NASS (National Agricultural Statistics Service). 2017. Farm and Land in Farms 2017 Summary – February 2018. Accessed Apr. 2018. http://usda.mannlib.cornell.edu/usda/current/FarmLandIn/FarmLandIn-02-16-2018.pdf

USDA-NASS (National Agricultural Statistics Service). 2016. Overview of the United States Cattle Industry. Accessed Apr. 2018. http://usda.mannlib.cornell.edu/usda/current/USCatSup/USCatSup-06-24-2016.pdf

van der Kloet et al. Discovery of early-stage biomarkers for diabetic kidney disease using ms-based metabolomics (FinnDiane study). Metabolomics 2012; 8:109–119.

Veenstra T. Metabolomics: the final frontier? Genome Medicine 2012; 4:1–3.

Watanabe M et al. Consequences of low plasma histidine in chronic kidney disease patients: associations with inflammation, oxidative stress, and mortality. Am J Clin Nutr 2008; 87(6): 1860-1866.

Wathes, D.C. et al. Factors influencing heifer survival and fertility on commercial dairy farms. Animal. 2(8), 1135-1143 (2008)

Wathes, Pollott, Johnson, Richardson, Cooke. Heifer fertility and carry over consequences for life time production in dairy and beef cattle. Animal 2014; 8:91–104.

Whittier WD, Currin JF, Schramm H, Holland S, Kasimanickam RK. Fertility in Angus cross beef cows following 5-day CO-Synch+ CIDR or 7-day CO-Synch+ CIDR estrus synchronization and timed artificial insemination. Theriogenology 2013; 80(9): 963-969.

Wood-Follis SL, Kojima FN, Lucy MC, et al. Estrus synchronization in beef heifers with progestin-based protocols I. Differences in response based on pubertal status at the initiation of treatment. Theriogenology 2004;62:1518–28.

Xia J, Sinelnikov IV, Han B, Wishart DS. MetaboAnalyst 3.0—making metabolomics more meaningful. Nucleic Acids Research 2015; 43(W1): W251-257.

Xia J, Psychogios N, Young N, Wishart DS. MetaboAnalyst: a web server for metabolomic data analysis and interpretation. Nucleic Acids Research 2009; 37: W652-660.

Yan M, Xu G. Current and future perspectives of functional metabolomics in disease studies–A Review. Anal Chim Acta 2018.

Yang C, Richardson A, Smith J, Osterman A. Comparative metabolomics of breast cancer. Pac Symposium Biocomput Pac Symposium Biocomput 2007:181–92.

Youngs C. Modern Reproductive Technologies to Improve Cattle Production. Ceiba 2 016; 54:31–40.

Yu B et al. Association of rare loss-of-function alleles in HAL, serum histidine levels and incident coronary heart disease. Circ Cadiovasc Genet 2015; 8(2): 351-355.

Zhang J.M. et al. Cytokines, Inflammation, and Pain. International Anesthesiology Clinics. 2007; 45(2): 27 0 37.

Zhang T et al. Identification of potential biomarkers for ovarian cancer by urinary metabolomic profiling. Journal of Proteome Research 2012; 12(1): 505-512.

Zhang J, et al. Urinary metabolome identifies signatures of oligozoospermic infertile men. Andrology 2014; 102(1):44-53

Zhou X et al. A potential tool for diagnosis of male infertility: Plasma metabolomics based on GC–MS. Talanta 2016; 147: 82-89.

Zohrabi M, Rahmani E. CXC Ligand 5 cytokine serum level in women with polycystic ovary syndrome and normal body mass index: A case-control study. International Journal of Reproductive BioMedicine 2017; 15:619–624.

Appendix 1: Body Condition Score 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 12th and 13th 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 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 Score Scoring System. Data adapted from Lowman et al., 1976.

RTS	Uterine Horns (diameter, mm)	Ovarian Length (mm)	Ovarian Height (mm)	Ovarian Width (mm)	Ovarian Structures
1	Immature < 20 mm No uterine tone	15	10	8	No palpable follicles
2	20 – 25 mm No uterine tone	18	12	10	8 mm follicles
3	20 – 25 mm Slight uterine tone	22	15	10	8 – 10 mm follicles
4	30 mm Good uterine tone	30	16	12	> 10 mm follicles CL possible
5	> 30 mm	> 32	20	15	CL present