Phenotypic and Genetic Aspects of Fertility in Beef Heifers
by

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Abstract

Reproductive success of a heifer's first breeding season is highly critical to the sustainability of beef cattle production systems. Therefore, multiple management practices exist to ensure heifers are properly developed for a successful first breeding season. However, first breeding season pregnancy rates might not exceed 85-90%. Increased understanding of the nature of beef heifer fertility is essential to further improve heifer pregnancy rates. Therefore, we sought to further characterize phenotypes and genetic characteristics of replacement heifers with varied fertility potential. We performed 2 studies to test the hypothesis that production records and bloodborne RNA profiles would differ among beef heifers that conceived to first service artificial insemination (AI-pregnant), conceived to natural breeding (NB-pregnant), or failed to become pregnant (Not-pregnant) in the first breeding season. In our first study, we curated records for age, weaning weight, reproductive tract score (RTS; scale of 1-5 where 1=prepubertal and 5=pubertal, luteal phase), and body condition score (BCS; scale of 1-9 where 1=emaciated and 9=obese) on 259 heifers that were pre-selected at BCS≥4 and RTS≥3 at the start of their first breeding season. None of the parameters tested displayed predictive ability to discriminate among AI-pregnant, NB-pregnant, and Not-pregnant heifers ($P>0.05$). The results highlight the need for additional methods to identify heifers of different reproductive potential before the start of the first breeding season. Therefore, in study 2 we generated RNA-sequencing data from peripheral white blood cells (PWBC) collected at the time of AI from 23 heifers and determined

differential gene expression profiles for 12,538 genes. We detected 18 differentially expressed genes (DEGs) between AI-pregnant and NB-pregnant heifers and 6 DEGs between AI-pregnant and Not-pregnant heifers. Then, we utilized to top scoring pair technique to classify heifers of different pregnancy outcomes based upon the expression ratios of all possible gene pairs. There were 88 and 1,520 pairs of genes whose expression ratios categorized AI-pregnant heifers separately from Not-pregnant and NB-pregnant heifers, respectively. Additionally, relative expression levels from 2 gene pairs correctly classified 10 of 12 AI-pregnant heifers separately from NB-pregnant and Not-pregnant heifers. Therefore, we conclude that differential expression of specific genes in PWBC at the time of AI is associated with beef heifer fertility. Circulating transcript profiles have potential to classify beef heifers according to first breeding season pregnancy outcome.

Acknowledgments

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List of Abbreviations

Adj WW	Adjusted Weaning Weight
AFC	Antral Follicle Count
AgeAI	Age at Artificial Insemination
AgeD	Age of Dam
AgeW	Age at Weaning
AI	Artificial Insemination
AIC	Akaike Information Criteria
BCS	Body Condition Score
BP	Base Pair
BS	Breeding Season
°C	Degrees Celsius
CI	Confidence Interval
CIDR	Controlled Internal Drug Release
CT	Cycle Threshold
DEG	Differentially Expressed Gene
Df	Degrees of Freedom
DNA	Deoxyribonucleic Acid
eFDR	Empirical False Discovery Rate
EPD	Expected Progeny Difference

ES	Estrous Synchronization
FPKM	Fragments per Kilobase per Million Reads
GnRH	Gonadotropin Releasing Hormone
GWAS	Genome Wide Association Study
ID	Identification
kb	Kilobase
KEGG	Kyoto Encyclopedia of Genes and Genomes
kg	Kilogram
lb	Pound
LogFC	Logarithm Fold Change
LR	Likelihood Ratio
MGA	Melengestrol Acetate
miRNA	Micro Ribonucleic Acid
ml	Milliliter
ml	Microliter
mm	Millimeter
mRNA	Messenger Ribonucleic Acid
N	Number
NAHMS	National Animal Health Monitoring System
NB	Natural Breeding
NCBI	National Center for Biotechnology Information
ng	Nanogram
NK	Natural Killer

NS	Natural Service
O	Open
PGF	Prostaglandin F2α
PM	Pelvic Measurements
Pr	Probability
Preg	Pregnant
PWBC	Peripheral White Blood Cells
QTL	Quantitative Trait Loci
RNA	Ribonucleic Acid
RNA-seq	Ribonucleic Acid Sequencing
ROS	Reactive Oxygen Species
qPCR	Quantitative Polymerase Chain Reaction
RTS	Reproductive Tract Score
SD	Standard Deviation
SNP	Single Nucleotide Polymorphism
TSP	Top Scoring Pair
USA	United States of America
USDA	United States Department of Agriculture
WW	Weaning Weight

I. Review of Literature

Beef Heifer Fertility: Importance of Management Practices and Technological Advancements

Importance of reproductive efficiency in beef cattle production

A great portion of the expenses in cow-calf production systems is dedicated to the maintenance of healthy cows in productive condition (Notter et al., 1979). At the same time, approximately 1/3 of cows removed from the beef herd are eliminated because of reproductive failure (~33%, NAHMS 2007-2008). Thus, reproductive inefficiency is a limiting factor for the sustainability of beef cattle production systems that leads to financial losses to cattle producers (Notter et al., 1979; Bellows et al., 2002).

In cattle, female reproductive failure is assumed when animals do not become pregnant within the breeding season or do not maintain pregnancy to calving (Lamb, 2013). Major female related causes of reproductive failure include improper health, reproductive, and nutritional management, reproductive disorders, and genetics (Houghton et al., 1990; BonDurant, 2007; Bolormaa et al., 2015; Larson and White, 2016). To mitigate some negative factors that impact reproduction, practices associated with cow herd nutrition, healthcare, and reproductive management have been established.

One key component of a successful reproductive management program involves increasing the percentage of cows that calve within the first 21 days of the calving season. This strategy produces older and heavier calves at weaning and allows additional days postpartum for cows to resume estrous cyclicity and become pregnant in the subsequent breeding season (Funston et al., 2012). Heifer reproductive success in the first calving season is highly linked with lifetime reproductive efficiency (Morris and Cullen, 1994; Mwansa et al., 2000; Cushman et al., 2013). Therefore, the selection and management of heifers for reproductive success is essential for the sustainability of the global beef cattle industry.

Importance of first breeding season success in replacement heifers

A compilation of data from multiple studies demonstrated that first breeding season pregnancy rates in beef heifers range from 64% to 95% under natural breeding (NB) alone or the combination of artificial insemination (AI) followed by NB [(Patterson et al., 1989; Lynch et al., 1997; Funston and Deutscher, 2004; Bormann et al., 2006; Grings et al., 2007; Martin et al., 2008; Roberts et al., 2009; Funston and Larson, 2011; Mallory et al., 2011; Peters et al., 2013; Gutierrez et al., 2014; Dickinson et al., 2019) Fig. 1]. Altogether, an average of 85% of heifers become pregnant by the completion of the breeding season. By comparison, pregnancy rates to first service artificial insemination range from 36-69% (Diskin and Sreenan, 1980; Lynch et al., 1997; Rorie et al., 1999; Bormann et al., 2006; Funston and Larson, 2011; Mallory et al., 2011; Peters et al., 2013; Gutierrez et al., 2014; Thomas et al., 2017; Dickinson et al., 2019). Our recent analysis of breeding records from 7 years (2011-2017) indicated that 43%, 42%, and 15%

of heifers became pregnant by AI, NB, or failed to become pregnant during their first breeding season, respectively (Dickinson et al., 2019).

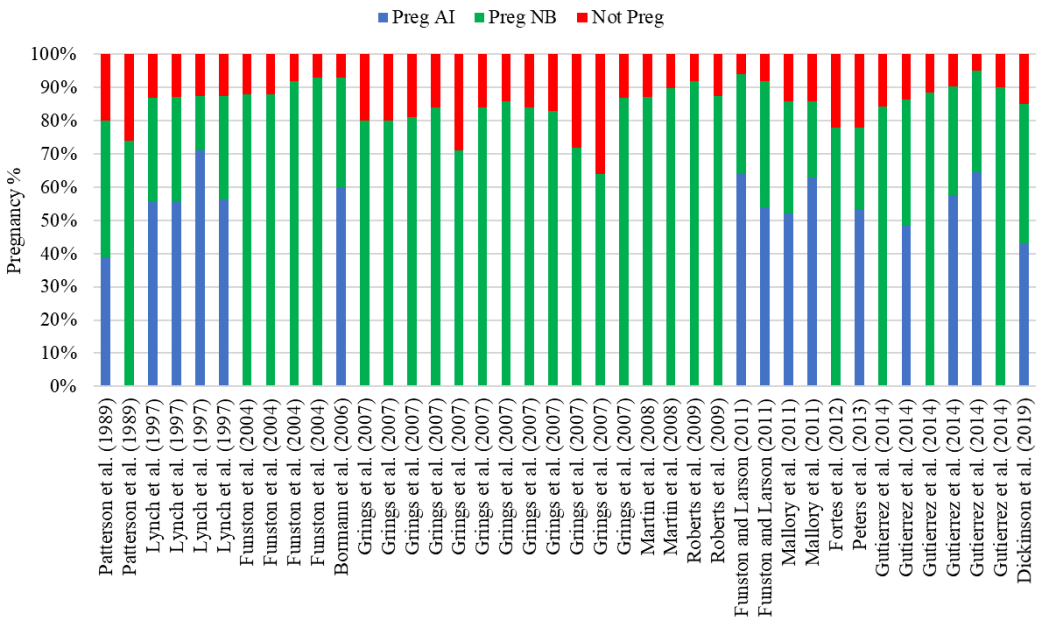


Fig. 1. Pregnancy rates in beef heifers. Y-axis denotes percentage of pregnancy outcome, and x-axis indicates studies referenced. Multiple bars for the same reference indicate different experimental treatment within the same report.

Under current production systems, the most efficient scenario occurs when replacement heifers conceive early in the breeding season. During their first breeding season, ~67%, ~26%, and ~7% of heifers that become pregnant are likely to calve during the first, second, and third 21 day windows of the subsequent calving season, respectively (Cushman et al., 2013). Heifers calving within the first 21 days of their first calving season remain in the productive herd longer and wean more total pounds of calf than their later calving counterparts (Cushman et al., 2013). By contrast, late breeding heifers contribute to a less efficient cow-calf production system due to reduced days postpartum to resume estrous cyclicity, reduced pregnancy rates in the subsequent calving season,

and reductions in calf age and weight at weaning (Lesmeister et al., 1973; Marshall et al., 1990; Cushman et al., 2013; Damiran et al., 2018).

Considering the average pregnancy rate (85%) obtained from compiled data aforementioned, and accounting for 5.9 million heifers being developed as replacements in 2019 (data from National Agricultural Statistics Service, February 2019), one can estimate that approximately 3.4 million heifers will conceive in the first 21 days of the breeding season. Approximately 1.7 million heifers will conceive later in the breeding season, and nearly 900 thousand heifers will not produce a calf by ~23-27 months of age. These numbers underscore a critically large number of heifers that receive important farming resources but do not contribute to a long-term sustainable production system.

Heifers that fail to become pregnant or become pregnant late in their first breeding season lead to significant economic impacts on the beef cattle industry. Losses experienced from non-pregnant replacements are the result of opportunity costs of failing to market infertile heifers as feeder calves, wasted nutritional resources, and expenses of breeding and healthcare. Cattle producers must account for extra costs for heifer development due to losses endured when some heifers fail to become pregnant (Hughes, 2013). Depending on replacement heifer management system, these prices can equate to ~$243 per replacement heifer developed to the time of pregnancy examination (Hughes, 2013). Considering the 5.9 million heifers expected to enter replacement development in 2019, such cost might exceed $1.4 billion nationwide. It must be pondered, however, that the financial losses caused by infertility can be reduced if the initial investment in heifer development is not extreme (Roberts et al., 2009).

The economic impact of the reduced age of calves from late breeding heifers is also considerably high. Considering market prices of ~$1.50 per pound (USDA, Agriculture Marketing Service; Joplin Regional Stockyards; May 20, 2019; average prices of steer and heifer calves of 450 lb) and an average daily gain of ~1.90 lb per day (Grings et al., 2005; Liu et al., 2015), calves born at the midpoint of the second and third 21 days of the calving season would be worth approximately $120 less than calves born on the first day of the calving season. When this number is multiplied by the 1.7 million heifers expected to conceive late in their first breeding season, one can account for an over $204 million lost to beef cattle producers due to late breeding heifers. These numbers underscore reproductive inefficiency among the major limiting biological functions significantly affecting the beef cattle industry.

The yearly cost of female infertility varies with the commodity value but remains unacceptably high under the current economic scenario. Since the early 1970s, it has been established that improving pregnancy rates is paramount for the development and maintenance of efficient and sustainable beef cattle production (Dickerson, 1970). Since then, there have been major advancements to our understanding of the reproductive physiology of beef heifers and the identification of means to address reproductive inefficiency. Next, we will discuss several practices that can be adopted to improve reproductive efficiency in replacement heifers for beef production systems.

Management practices to improve beef heifer reproductive success

The proper selection and development of replacement heifers enhances the likelihood that heifers entering development programs will conceive early in the breeding season followed by increased stayability (Snelling et al., 1995).

Management strategies aimed at increasing first breeding season reproductive success are discussed below, and many are targeted towards increasing the percentage of heifers reaching puberty before the start of the breeding season. Such practices include the selection of older and heavier heifers at weaning (Funston et al., 2012), nutritional management of heifers to reach a defined percentage of their mature bodyweight by the start of the breeding season (Patterson et al., 1989; Funston and Deutscher, 2004), reproductive tract scoring to screen heifers for puberty ~30 days before the start of the breeding season (Gutierrez et al., 2014), the implementation of a progestin-based estrous synchronization protocol (Patterson et al., 2013; Gutierrez et al., 2014), and the incorporation of expected progeny differences (EPDs) to select heifers with increased genetic merit for fertility.

Age of heifers

The selection of replacement heifers that are born early in the calving season is an essential step to optimizing overall reproductive success. It is expected that early born heifers will enter the breeding season with increased morphological and physiological maturity than their younger herd mates.

In a study by Funston and colleagues, heifers born in the first 21 days of the calving season were heavier at pre-breeding than heifers born in the second or third period of the calving season [296, 292 and 276kg, respectively, (Funston et al., 2012)]. Additionally, 70% of early born heifers were cycling by the start of their first breeding season, compared to 58% and 30% of heifers born in the second and third 21-day period, respectively. As a consequence, older heifers presented greater pregnancy rates (90%) compared to 86% and 78% for heifers that were born in consecutive 21 day windows of

the calving period, respectively (Funston et al., 2012). Our analysis of breeding records from Angus x Simmental crossbred heifers indicated that heifers older than 368 days of age at the beginning of the breeding season had 87.5% chance of becoming pregnant within 90 days compared to a 12.5% chance if the heifer was younger than 368 days of age (Dickinson et al., 2019).

Heifers from different breeds reach puberty at different ages, ranging from 10 to 14 months, with crossbred heifers usually displaying estrus at an earlier age than purebreds (Byerley et al., 1987; Freetly and Cundiff, 1997; Freetly et al., 2011; Cardoso et al., 2014; Gunn et al., 2015). These investigations also revealed that within a cohort of heifers of similar genetic make-up, some individuals will reach puberty early or late relative to their counterparts. Directly related to their age and physiological maturity, among cycling heifers, older heifers that are bred on their third estrous cycle present greater pregnancy rates (78%) relative to counterparts that are bred on their first estrous cycle [57%; (Byerley et al., 1987)]. Additionally, heifers entering the breeding season before reaching puberty or after one estrous cycle had reduced calving rates within the first 21 days of their first calving season compared to heifers experiencing at least 2 cycles before the onset of breeding (Roberts et al., 2019).

Older heifers have a greater chance to become pregnant in their first breeding season. Nonetheless, it is critical that an appropriate balance is achieved for heifers to calve around 24 months of age, as these individuals will have a greater overall calving output relative to later breeding heifers (Patterson et al., 1992; Cushman et al., 2009).

Nutritional management of heifers

Appropriate nutritional status is essential for reproductive success in cattle (Lamond, 1970). Energy restriction delays the onset of puberty in beef replacement heifers (Gonzalez-Padilla et al., 1975b; Day et al., 1984). Furthermore, inadequate energy consumption, as exhibited by low body condition score, reduces pregnancy success in beef cows throughout their productive lifespan (Rae et al., 1993). By contrast, heifers experiencing higher levels of nutrition and adequate weight gain prior to the first breeding season experience increased reproductive success in their first and subsequent calving seasons (Milagres et al., 1979; Fleck et al., 1980). To this end, heifer development programs have been established for beef cattle producers to provide adequate nutrition for heifers to attain puberty and high reproductive success in their first breeding season. Cattle farms in different regions have varied sources of nutritional resources available for heifer development, and these feedstuffs have seasonal availability. Thus, the impact of the timing of weight gain on first breeding season pregnancy outcome has been evaluated.

No statistical differences in the percentage of heifers reaching puberty, becoming pregnant in the first or second 21 days of the breeding season, or conceiving by the end of the breeding season were observed among heifers managed to gain at a steady rate (0.45 kg/day), to gain none and then rapidly (0.91 kg/day), or to gain rapidly (0.91 kg/day) and then none during development from 45 days post-weaning to the start of the breeding season (Clanton et al., 1983). However, heifers developed at a steady rate had first service pregnancy rates of 62% as compared to 47% and 35% in fast-slow or slow-fast gaining heifers, respectively (Clanton et al., 1983). In a similar study, heifers that gained 0.11 kg/day initially, followed by 0.91 kg/day had similar first service conception rates

and overall pregnancy rates when compared to heifers developed to gain weight at a constant 0.45 kg/day throughout the peri-pubertal period (Lynch et al., 1997). Nutritional management of heifers to gain weight in a stairstep fashion (fast gain, followed by slow gain, followed by fast gain immediately before breeding) yielded similar breeding season pregnancy rates as developmental programs with consistent gains (Grings et al., 1999; Cardoso et al., 2014).

The timing of weight gain has minimal consequence for heifer fertility, but the weight a heifer reaches by the start of her first breeding season heavily impacts her reproductive success. Patterson and others (1989) demonstrated greater pregnancy rates when heifers reached 65% versus 55% of their mature body weight by the start of the breeding season (Patterson et al., 1989). Since then, reduced rates of puberty, but no difference in breeding season pregnancy rates have been reported in heifers managed to reach 55% versus 58-60% of their mature bodyweight (Funston and Deutscher, 2004; Roberts et al., 2009). However, pregnancy rate to artificial insemination tended to be reduced in heifers developed to 55% of their mature weight (Roberts et al., 2009). The development of heifers to 50% versus 55% of mature bodyweight also yielded no difference in overall 45-day breeding season pregnancy rates, but significantly delayed the date of first calving (Martin et al., 2008).

A large body of data reinforce that heifers should be developed to reach a minimum percentage of their anticipated mature body weight by the start of the breeding season. It must be noted, however, that the target weight depends on heifer genetic makeup (Martin et al., 1992), cow herd size, and breeding protocols utilized.

Implementation of reproductive tract scores

The physiological and morphological maturity of the reproductive system is achieved as heifers attain puberty, but not all animals reach appropriate developmental status by the beginning of the breeding season. A reproductive tract scoring system ranging from 1 (pre-pubertal, infantile tract) to 5 (pubertal, corpus luteum present) was developed to categorize heifers according to uterine and ovarian development as determined by rectal palpation (Anderson et al., 1991). Usually, reproductive tract scoring is performed 4-6 weeks before the start of the heifer's first breeding season and has become a tool to indicate the reproductive readiness in beef heifers.

Several independent reports have demonstrated that there is a strong, nearly linear relationship between reproductive tract score (RTS) and pregnancy rates (Fig. 2). Lower scores (1 and 2) are consistently associated with lower pregnancy rates, whereas scores 4 and 5 indicate heifers that are cycling and therefore have greater pregnancy rates whether bred by AI alone or following a breeding season of AI followed by natural service (Martin et al., 1992; Holm et al., 2009; Gutierrez et al., 2014; Thomas et al., 2017; Dickinson et al., 2019).

Implementation of a progestin-based estrous synchronization program

Progestins can be used to induce puberty in peripubertal heifers and were initially used with estradiol to simulate the hormonal changes associated with the acquisition of puberty (Gonzalez-Padilla et al., 1975a; Short et al., 1976). Such changes begin with the increased progesterone levels associated with pubertal development in heifers (Berardinelli et al., 1979). The utilization of a progestin mimics this rise in progesterone and then allows for increased luteinizing hormone pulse frequency and desensitized negative feedback effects of estradiol on gonadotropin releasing hormone (GnRH)

secretion (Anderson et al., 1996; Hall et al., 1997; Imwalle et al., 1998). Therefore, peripubertal heifers experience increased follicular growth and estradiol production associated with fertile estrus and ovulation (Tanaka et al., 1995; Imwalle et al., 1998).

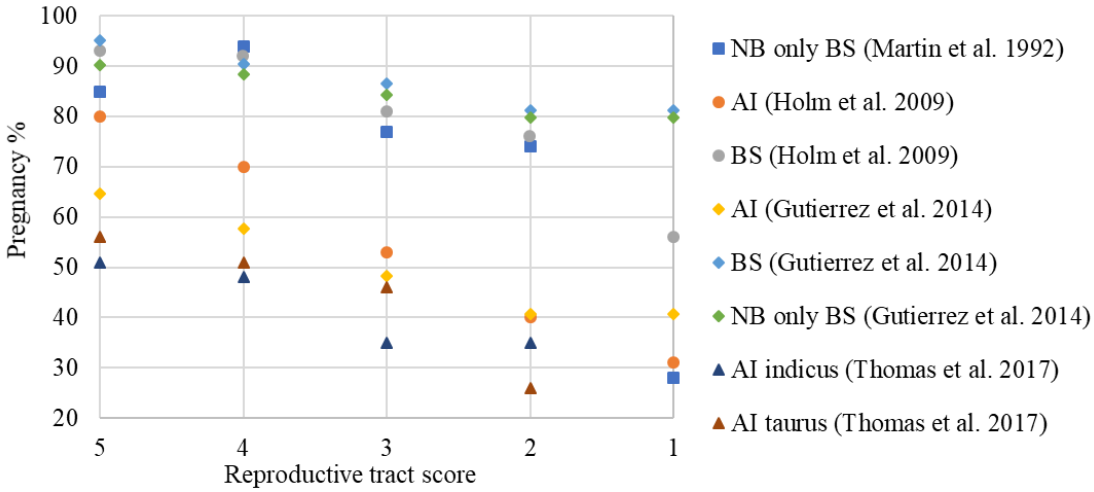


Fig. 2. Pregnancy rates in beef heifers of different reproductive tract scores. Y-axis denotes pregnancy percentage, and x-axis denotes reproductive tract score categories.AI: artificial insemination, BS: breeding season, NB: natural breeding, indicus: *B. indicus*, taurus: *B. taurus*.

There is an additional benefit from progestin-based estrous synchronization programs, whether through the utilization of a controlled internal drug-releasing [CIDR; (Lucy et al., 2001)] insert or melengestrol acetate [MGA; (Patterson et al., 1990)]. Such protocols synchronize ovulation in heifers and allow all heifers to be inseminated on day one of the breeding season. Overall, progestin-based synchronization programs have a positive influence on heifer calving date and breeding season pregnancy rates (Martin et al., 2008; Patterson et al., 2013; Gutierrez et al., 2014; Moriel et al., 2017).

The genetic basis of heifer fertility

Genetic selection is used to improve beef cattle populations for many production related traits. Relatively fast genetic progress can be achieved with traits such as growth rate and carcass quality because of their moderate to high heritability (Fortes et al., 2012b; Peters et al., 2012; Torres-Vázquez et al., 2018). By contrast, the heritability of traits directly related to female reproduction is lower, and thus the rate of genetic change in fertility traits based upon genetic selection is much slower. However, models are being developed utilizing genetic parameters to select beef cattle for the improvement of heifer fertility.

Pregnancy rate is a common trait utilized when evaluating fertility. Interestingly, the genetic correlation between yearling pregnancy rate and lifetime pregnancy rate is high, namely 0.92-0.97 (Morris and Cullen, 1994; Mwansa et al., 2000). These findings support a genetic link between reproductive success in the first breeding season and a productive lifespan, however the genes and genetic models of this correlation are yet to be unveiled.

The genetics of heifer pregnancy rate, or the likelihood of pregnancy within the first breeding season, is valuable to select heifers with increased genetic merit for pregnancy success. However, the heritability of heifer pregnancy rate ranges from 0.07 to 0.20 (Doyle et al., 2000; Bormann et al., 2006; McAllister et al., 2011; Fortes et al., 2012b; Peters et al., 2013; Boddhireddy et al., 2014; Toghiani et al., 2017). First-service conception rate is another trait evaluated when considering heifer genetic merit for fertility. First-service conception rate identifies animals conceiving to their first service separately from animals conceiving later in the breeding season. The heritability of first-service conception in heifers is also low, ranging from 0.03 to 0.18 (Bormann et al.,

2006; Fortes et al., 2012b; Peters et al., 2013). Altogether, diverse reports consistently indicate that the genetic influence on pregnancy in beef heifers is controlled by a small portion of the additive component of a heifer's genetic makeup.

Beef cattle production systems have greatly benefited from heterosis (Gregory et al., 1994). However, the investigations of heterosis on heifer fertility are scarce. Cundiff and others identified that crossbred heifers had 6.6% greater conception rate to natural service followed by 6.4% increase in calf crop weaned (Cundiff et al., 1974). MacNeil and others observed that purebred or linecross heifers presented 76.2 and 79.4% pregnancy rates, respectively, but both groups had similar calf birth rates at 77% (MacNeil et al., 1989). The effect of heterosis on heifer pregnancy is uncertain, but crossbreeding does influence heifer prebreeding weight and anticipated puberty onset (Martin et al., 1992).

Current and emerging technologies for assessing fertility in heifers

The proper development of replacement heifers and the utilization of expected progeny differences to select animals with superior genetics for fertility can improve heifer pregnancy rates. However, the impact of these means of selection and development eventually reach a plateau. Therefore, more detailed analyses of the phenotypic, physiological, and genetic components of heifer fertility are necessary. To this end, studies examining differences in genotypes, transcriptome profiles, and physical indicators of the ovarian reserve have been explored. As such, scientists have begun to reveal deep variations in phenotypically normal heifers of similar genetic background with remarkable contrasts in fertility potential. There is the potential to not only increase

our understanding of heifer fertility, but to identify additional parameters for the selection of highly fertile heifers.

Antral follicle count

There is evidence that the selection of highly fertile heifers as replacement females may be improved with selection based upon antral follicle counts (Cushman et al., 2009; Cushman et al., 2014). In cattle, the oocyte and its surrounding follicle develop during fetal growth, with the presence of primordial follicles occurring by day 74-110 of gestation (Tanaka et al., 2001; Yang and Fortune, 2008; Burkhart et al., 2010). Follicles remain quiescent at the primordial follicle stage until they are activated to the primary follicle stage and progress into the pre-antral and antral stages of follicular development (Tanaka et al., 2001; Burkhart et al., 2010). Antral follicles are then recruited into follicular waves that occur throughout the bovine estrous cycle (Sirois and Fortune, 1988). The number of antral follicles present during a follicular wave can be determined by ultrasonography, in which the number of follicles ≥3mm is reported as the antral follicle count (AFC).

Antral follicle counts are highly variable among animals, yet highly repeatable within an individual animal, allowing animals to be classified according to AFC (Burns et al., 2005). Furthermore, AFC accurately depicts the ovarian reserve of cattle, in which animals with a low AFC possess less healthy primordial, pre-antral, and antral follicles compared to animals with high AFCs (Ireland et al., 2008).

The ovarian reserve is related to fertility in female mammals. Cows with high AFC had higher pregnancy rates and shorter postpartum periods than animals with low AFCs (Mossa et al., 2013). Furthermore, AFC is associated with luteal and uterine

function in cattle, and increased AFC was associated with higher reproductive success in beef heifers (Cushman et al., 2009; Cushman et al., 2014). A study of 47 young adult beef cattle and late lactation dairy cattle revealed that animals with low AFC had poorer endometrial development, followed by progesterone concentrations 30-50% lower than animals with high AFC (Jimenez-Krassel et al., 2015).

Differences in oocyte competence have also been observed between animals with high vs low AFC. Ireland et al. (2008) reported greater abundance of cathepsin mRNA in cumulus cells and higher intrafollicular estradiol concentrations in animals with low AFC, both of which are associated with reduced oocyte competence (Ireland et al., 2008). Antral follicle counts hold great promise for improving replacement heifer selection criteria as they are determined though non-invasive procedures and are correlated with reproductive success in cattle.

DNA polymorphisms

The ability to analyze thousands of single nucleotide polymorphisms (SNPs) allows researchers to investigate complex traits related through genome wide association studies (GWAS.) Multiple studies have identified SNPs significantly associated with traits known to influence reproductive success in beef heifers, such as age at puberty (Fortes et al., 2012a; Hawken et al., 2012).

Peters and others identified 12 chromosomal regions associated with first service conception and 6 regions associated with heifer pregnancy (Peters et al., 2013). Many of the regions containing SNPs associated with first service conception and heifer pregnancy corresponded to previously identified regions related to age at first corpus luteum (Hawken et al., 2012). Additionally, 2 regions on BTA2 and BTA8 were

identified to have a relationship with heifer pregnancy. SNPs identified on chromosome 2 were in close proximity to previously identified quantitative trait loci associated with differences in growth, carcass, lactation, and feed efficiency (Peters et al., 2012). Such results support the importance of systems targeted research that considers the interconnectivity of animal body condition, growth, and reproductive outcome.

McDaneld and others identified SNPs associated with varied levels of reproductive success in *B. taurus* purebred, *B. taurus* x *B. taurus* crossbred, and *B. taurus* x *B. indicus* crossbred animals (McDaneld et al., 2014). Due to the utilization of multiple populations of animals, individuals were either ranked for fertility upon reproductive outcomes in the first 5 breeding seasons or indicated as pregnant or open based on pregnancy success in the first or multiple breeding seasons. A single SNP on BTA29 achieved genome wide significance or nominal significance in some test populations. Interestingly, this SNP was within 786 kb of a SNP previously indicated to be associated with age at first identified corpus luteum in tropically adapted heifers (Hawken et al., 2012). Five additional SNPs on BTA1, BTA5, and BTA25 were identified at a suggestive level of significance in at least one population of animals. SNPs on BTA 5 coincided with previously reported SNPs associated with age at first corpus luteum, length of postpartum anestrous period, and the incidence of corpus luteum before calf weaning (Hawken et al., 2012).

Additionally, presence of DNA from the Y chromosome was identified in low fertility and open classified heifers in the populations described above (McDaneld et al., 2012). Approximately 18-29% of the heifers determined to have low fertility or failing to

become pregnant, respectively, tested positive for segments of the Bovine Y-chromosome.

Quantitative trait loci and gene networks were also identified in beef heifers previously classified as having high or sub-fertility based on day 28 pregnancy outcomes to serial embryo transfer (Neupane et al., 2017). Fourteen *loci* were strongly associated with heifer fertility, while 8 *loci* displayed moderate association. Of these *loci*, 5 had positional candidate genes with previously indicated functions in fertility and uterine receptivity to pregnancy. One remarkable example is the gene kinesin family member 4A (*KIF4A*), which was located within the most significant locus associated with heifer fertility. Previous studies indicated elevated levels of *KIF4A* in endometrium samples collected on day 7 post estrus in Simmental heifers that established pregnancy to embryo transfer following the next observed estrus compared to those that failed to establish pregnancy (Salilew-Wondim et al., 2010).

Investigations have also been conducted to understand fertility in *B. indicus* cattle, with emphasis on Nellore heifers. Many of such studies focused on identifying markers associated with heifers becoming pregnant by 14-16 months of age. Using a targeted approach, Camargo and others identified possible polymorphisms in the gene JY-1, an oocyte specific protein, associated with the probability of pregnancy by 16 months of age (de Camargo et al., 2014). GWAS analyses also unveiled several markers of interest for heifer fertility in zebu cattle. Dias and others identified 3 haplotypes significantly associated with heifer pregnancy, which contained the genes fatty acid binding protein 4 (*FABP4*) and protein phosphatase 3 catalytic subunit alpha (*PPP3CA*) (Dias et al., 2015). Focusing on chromosomic regions, 2 studies identified chromosome regions that

explained as much as 8.91% (Irano et al., 2016) and 12.73% (Junior et al., 2017) of the variance in sexual precocity to become pregnant by 16 months of age and heifer pregnancy, respectively. Of note, both studies identified windows on chromosomes 5, 14, and 18, with a potential overlap on chromosome 14 (Irano et al., 2016; Junior et al., 2017). Takada and others focused on haplotypes encompassing 125 candidate genes and identified 9 haplotypes with significant association with early pregnancy. Those haplotypes were located in the genes pregnancy-associated plasma protein-A2 (*PAPP-A2*), estrogen-related receptor gamma (*ESRRG*), pregnancy-associated plasma protein-A (*PAPP-A*), kell blood group complex subunit-related family (*XKR4*), and mannose-binding lectin [*MBL-1* (Takada et al., 2018)].

The Animal QTL database holds curated and compiled data on hundreds of DNA markers associated with diverse traits in livestock, including cattle (Hu et al., 2013; Hu et al., 2019). The database currently has information on 56 markers associated with heifer pregnancy rate (Table 1). Throughout this selected data from the Animal QTL database, and data from studies not identified in the database, it is important to notice that there is no clear redundancy of markers identified across studies. This observation points to the critical aspect of the replicability of the findings across populations (Marigorta et al., 2018) in addition to the complexity and most likely omnigenic (Boyle et al., 2017) nature of fertility.

Transcriptome profiling of endometrium

Multiple studies have demonstrated the importance of endometrial receptivity to pregnancy in ruminants (reviewed by Bauersachs and Wolf, 2015). Inadequate uterine receptivity, versus reduced oocyte competence, was indicated to explain differences in

day 32-34 pregnancy rates between heifers classified as high or low fertility (Minten et al., 2013). Microarray analysis indicated similar transcriptome profiles in day 14 endometrial samples from heifers with high, low, and infertility (Minten et al., 2013). Other early studies detected 419 differentially expressed genes (DEGs) in endometrial samples collected on day 7 (Killeen et al., 2014) and 430 DEGs in endometrial samples collected on day 14 (Killeen et al., 2016) post-estrus in heifers previously classified to have high or low fertility to 4 rounds of artificial insemination.

As RNA-sequencing technologies emerged, additional studies examined impacts of heifer fertility classification on the transcriptome of endometrial samples collected at various stages of pregnancy. Heifers classified as high or low fertility based on repeated embryo transfer had no or few differences in conceptus recovery on day 14, conceptus morphology, and transcriptome profiling of endometrial samples collected on day 14 of gestation (Geary et al., 2016). However, remarkable differences were observed in heifers of contrasting fertility classification on day 17 of pregnancy (Moraes et al., 2018). Pregnancy rates were markedly higher in heifers of high or sub-fertility than in heifers categorized as infertile (71%, 90%, and 20%, respectively), and conceptus morphology was similar and advanced in heifers of high or sub-fertility compared to heifers of infertility classification. Furthermore, conceptus size was larger in heifers categorized as highly fertile versus sub-fertile, and RNA sequencing revealed 1,287 DEGs in the transcriptome of day 17 conceptuses from heifers of highly fertile versus sub-fertile classification.

Table 1. Quantitative trait *loci* present in the Animal QTL database associated with beef heifer pregnancy [a].

QTL ID	Chr	Range cM	FlankMark A	Peak Mark	FlankMark B	Reference	Candidate gene symbol
137399	1	Na	Na	rs108940570	Na	Regatieri et al. (2017)	*APP*
151129	1	119.14-120.06	rs136647907	Na	rs133111309	Júnior et al. (2017)	Na
22901	2	38.77-39.71	rs42919869	Na	rs43307553	Peters et al. (2013)	Na
151122	2	49.04-49.84	rs42509691	Na	rs134051905	Júnior et al. (2017)	Na
151125	2	52.56-53.43	rs133912634	Na	rs134084039	Júnior et al. (2017)	Na
151131	3	2.94-3.91	rs109945234	Na	rs42368646	Júnior et al. (2017)	Na
22902	4	3.97-4.89	rs110197100	Na	rs110954467	Peters et al. (2013)	Na
151119	5	Na	rs42917128	Na	rs136339681	Júnior et al. (2017)	Na
107840	5	10.23-11.66	Na	Na	Na	Irano et al. (2016)	Na
108449	5	18.49-19.71	Na	Na	Na	Irano et al. (2016)	Na
151128	5	56.06-57.01	rs110797637	Na	rs137576699	Júnior et al. (2017)	Na
151121	5	78.99-80.01	rs137127461	Na	rs109435449	Júnior et al. (2017)	Na
151113	5	80.05-81.09	rs109437025	Na	rs110687761	Júnior et al. (2017)	Na
151114	5	84.41-85.50	rs42561706	Na	rs137385583	Júnior et al. (2017)	Na
151115	5	88.95-89.84	rs110496647	Na	rs136544553	Júnior et al. (2017)	Na
151124	5	89.94-90.94	rs110450288	Na	rs133794376	Júnior et al. (2017)	Na
119777	6	12.13-13.29	Na	Na	Na	Irano et al. (2016)	Na
57465	6	28.43-28.44	rs134077806	Na	rs134383126	Dias et al. (2015)	*PPP3CA*
57466	6	28.54-28.54	rs109697066	Na	rs137526343	Dias et al. (2015)	*PPP3CA*
119778	7	3.77-4.65	Na	Na	Na	Irano et al. (2016)	Na
119779	7	49.92-50.82	Na	Na	Na	Irano et al. (2016)	Na
22903	8	0.40-1.10	rs110007458	Na	rs111021990	Peters et al. (2013)	Na
152647	8	115.08-115.08	rs135042546	Na	rs110990932	Takada et al. (2018)	*PAPPA*
22904	10	103.21-104.31	rs43647342	Na	rs41657367	Peters et al. (2013)	Na
22905	13	99.13-100.27	rs110209373	Na	rs41660868	Peters et al. (2013)	Na
151116	14	28.67-29.98	rs41724652	Na	rs133297141	Júnior et al. (2017)	Na
152648	14	30.64-30.66	rs42646650	Na	rs134214692	Takada et al. (2018)	*XKR4*
119780	14	29.53-30.56	Na	Na	Na	Irano et al. (2016)	Na
151127	14	31.34-32.62	rs135852767	Na	rs42298467	Júnior et al. (2017)	Na
151118	14	36.75-37.97	rs41624840	Na	rs136805030	Júnior et al. (2017)	Na
57464	14	61.17-61.17	rs132819090	Na	rs109077068	Dias et al. (2015)	*FABP4*
152641	16	25.33-25.36	rs136930654	Na	rs132925189	Takada et al. (2018)	*ESRRG*
152642	16	25.65-25.67	rs133536959	Na	rs109979901	Takada et al. (2018)	*ESRRG*
152643	16	73.28-73.35	rs136672059	Na	rs109160879	Takada et al. (2018)	*PAPPA2*
152644	16	73.35-73.38	rs135370722	Na	rs132969356	Takada et al. (2018)	*PAPPA2*
152645	16	73.39-73.41	rs132814943	Na	rs42300953	Takada et al. (2018)	*PAPPA2*
152646	16	73.56-73.66	rs132776805	Na	rs41814719	Takada et al. (2018)	*PAPPA2*
151117	18	Na	rs136460244	Na	rs41891085	Júnior et al. (2017)	Na
119781	18	5.63-6.48	Na	Na	Na	Irano et al. (2016)	Na
22906	20	81.03-82.15	rs41959108	Na	rs110359079	Peters et al. (2013)	Na
137400	21	Na	Na	rs134589009	Na	Regatieri et al. (2017)	Na
137401	21	Na	Na	rs134601255	Na	Regatieri et al. (2017)	*SETD3*
119782	21	0.01-3.77	Na	Na	Na	Irano et al. (2016)	Na
119783	21	77.11-77.86	Na	Na	Na	Irano et al. (2016)	Na
137402	22	Na	Na	rs133503069	Na	Regatieri et al. (2017)	*ARHGEF3*
151123	24	61.41-62.40	rs109329309	Na	rs135881583	Júnior et al. (2017)	Na
151120	24	70.77-71.92	rs136828522	Na	rs137238317	Júnior et al. (2017)	Na
119784	27	1.83-2.91	Na	Na	Na	Irano et al. (2016)	Na
152649	28	50.38-50.38	rs136285814	Na	rs133640737	Takada et al. (2018)	Na
31164	29	18.28-18.48	Na	Na	Na	de Camargo et al. (2014)	*JY-1*
31165	29	18.28-18.48	Na	Na	Na	de Camargo et al. (2014)	*JY-1*
31166	29	18.28-18.48	Na	Na	Na	de Camargo et al. (2014)	*JY-1*
31167	29	18.28-18.48	Na	Na	Na	de Camargo et al. (2014)	*JY-1*
151130	29	33.02-34.37	rs134769207	Na	rs42172278	Júnior et al. (2017)	Na
151126	X	Na	rs134685381	Na	rs137716652	Júnior et al. (2017)	Na
151132	X	64.37-65.63	rs134673004	Na	rs134676523	Júnior et al. (2017)	Na

[a] The completeness of the database is dependent on the submission of data by researchers; Na: not available.

In the same study, uterine response to pregnancy was greater in highly fertile versus sub-fertile heifers. Approximately 20% of genes expressed in endometrium samples were differentially regulated in pregnant highly fertile heifers versus non-pregnant heifers of high fertility, whereas only 6% of transcripts displayed a pregnancy related response in sub-fertile heifers. Differential gene expression analysis of endometrial samples from pregnant highly fertile versus pregnant sub-fertile individuals revealed differences in expression profiles of 168 genes (Moraes et al., 2018). Such results indicate an altered uterine response to pregnancy in sub-fertile heifers, and further investigation of DEGs suggested excessive extracellular matrix in the endometrium of sub-fertile heifers may interfere with intimate conceptus-uterine interactions necessary for pregnancy maintenance.

Promise for the development of circulating indicators of heifer fertility

Recent studies have demonstrated that the profiling of circulating biological features (hormones, metabolites, transcripts, or epigenetic marks on the DNA of circulating cells) is revealing of the physiological state of an individual (Chen et al., 2012; Garrett-Bakelman et al., 2019). The analysis of multiple layers of an individual's molecular blueprint is likely key for the understanding of several complex traits, in a health context and otherwise (Scalici et al., 2015). The systemic profiling of circulating biological features is likely to also contribute to the understanding of infertility (Li-Pook-Than and Snyder, 2013).

Peripheral blood natural killer cells indicate fertility in women

Studies in humans have indicated that women suffering reproductive failure had altered profiles of circulating natural killer monocytes. Women who were infertile, as

well as those who suffered spontaneous abortions, had increased proportions of all peripheral blood natural killer cell types compared to fertile women (Michou et al., 2003). Additionally, infertile women had reduced proportions of endometrial type (CD56+/CD16−/CD3−) peripheral blood natural killer cells relative to the total number of natural killer cells (Michou et al., 2003). Furthermore, women suffering a reduced rate of successful embryo implantation following in-vitro fertilization had higher absolute counts of activated NK cells ($CD56^{dim}CD16^{+}CD69^{+}$) in the peripheral blood compared to women with increased success rates (Thum et al., 2004). High levels of $CD56^{dim}CD16^{+}CD69^{+}$ in peripheral blood natural killer cell absolute counts were also associated with increased miscarriage rates when pregnancy was established (Thum et al., 2004). Most recently, women experiencing failed in-vitro fertilization implantation had reduced levels of $CD69^{+}$ stimulated cells compared to women with successful implantation (Dons'koi et al., 2014).

Bloodborne mRNA profiles

Considering that the physiology of an individual is highly linked to molecular features circulating in the bloodstream and the relationships of peripheral blood natural killer cells with fertility in women, we reasoned that mRNA profiles of peripheral white blood cells (PWBC) may differ among beef heifers who became pregnant to AI, pregnant to NB, and failed to become pregnant in their first breeding season. We profiled mRNA transcripts from heifers from different pregnancy outcomes to reveal 6 DEGs (*ALAS2*, *CNKSR3*, LOC522763, *SAXO2, TAC3, TFF2,* FDR<0.05) between heifers that became pregnant to AI and heifers that did not become pregnant (Dickinson et al., 2018). A natural question is whether one can use gene expression profiles to distinguish

phenotypes (Geman et al., 2004). The analysis of our data using top scoring pair (TSP) approach (Geman et al., 2004) revealed 2 gene pairs (C11orf54, *TAF1B*; *URB2*, ENSTAG00000039129) whose relative expression within heifers discriminated most AI-pregnant (10 out of 12) from the other heifers profiled (Dickinson et al., 2018). The differences in gene expression in PWBC of heifers with differing fertility outcomes suggest circulating mRNA profiles are useful as predictive tools for pregnancy outcome.

Conclusions and outlook

The selection and management of highly fertile replacement heifers will greatly impact the future success of the worldwide beef cattle industry. As technologies allow cattle producers to more effectively identify animals that are sub- or infertile, those animals can be managed as feeder cattle and eliminated from the replacement heifer pool earlier in their productive lifespan. Less capital will be lost on the development costs of infertile individuals, and heifer pregnancy rates early in the first breeding season can be improved.

Multiple strategies exist to aid in the identification and management of heifers to maximize fertility, yet approximately 15% of beef females fail to become pregnant in their first breeding season. We and others have demonstrated that selection and management based on accepted phenotypic parameters has reached a plateau and rarely is inadequate to improve rates of pregnancy success beyond 85-90%.

Scientists must identify parameters beyond phenotypic traits and traditional genetic predictions to improve the producer's ability to retain only the most fertile individuals. To this end, much progress has been made on the identification of differences in AFC, endometrial receptivity to pregnancy, and genotypes of heifers with

contrasting fertility potential. Knowledge of remarkable differences in the endometrium's receptivity to pregnancy in fertility classified heifers highlights the depth of the issue at hand, but currently offers little practicality for improved replacement heifer selection. SNP profiling of certain populations of animals has indicated potential genetic markers of fertility in heifers, however further understanding of differences in the transcription of mutated genes and their outcomes on heifer fertility beg for studies focused at the transcriptome and protein level. While incorporation of AFC into replacement heifer evaluation may increase detection of lowly fertile animals, additional means to further determine heifer fertility potential must be identified.

There is great promise for the utilization of circulating bloodborne molecular features for the detection of heifers with varied fertility at the onset of the first breeding season. Recent studies have demonstrated remarkable differences in bloodborne mRNA of heifers with different reproductive outcome in their first breeding season. Most importantly, we have identified the potential for specific gene transcripts to be successfully utilized to classify heifers by pregnancy outcome. Advancements of such knowledge will fill a gap in current understanding of the physiology of reduced fertility in beef heifers and form a basis from which additional studies aim to develop circulating markers of fertility in beef heifers.

II. Evaluation of Age, Weaning Weight, Body Condition Score, and Reproductive Tract Score in Pre-selected Beef Heifers Relative to Reproductive Potential

Introduction

Reproductive inefficiency is a limiting factor in beef cattle production systems. In females, reproductive failure is assumed when animals do not become pregnant within the breeding season or conceive but do not maintain pregnancy to calving (Lamb, 2013). In beef heifers, first breeding season pregnancy rates range from 64% to 95% (Patterson et al., 1989; Lynch et al., 1997; Funston and Deutscher, 2004; Bormann et al., 2006; Grings et al., 2007; Martin et al., 2008; Roberts et al., 2009; Funston and Larson, 2011; Mallory et al., 2011; Peters et al., 2013; Gutierrez et al., 2014). Furthermore, pregnancy rates to first service artificial insemination range from 36% to 69% (Diskin and Sreenan, 1980; Lynch et al., 1997; Rorie et al., 1999; Bormann et al., 2006; Funston and Larson, 2011; Mallory et al., 2011; Peters et al., 2013; Gutierrez et al., 2014; Thomas et al., 2017). The negative impacts of reduced pregnancy rates in beef heifers contribute to the overall production deficit of the cattle operation that cannot be recovered in the following years (Mathews and Short, 2001). Therefore, the selection and management of replacement heifers to obtain greater reproductive success within their first breeding season is of great importance to beef cattle production systems (Larson et al., 2016).

Many management practices aim at maximizing the percentage of heifers pregnant at the end of the breeding season by increasing the percentage of pubertal heifers entering the breeding season. Most strategies include the selection of heifers that reach appropriate age and 55-65% of the projected mature body weight before the start of the breeding season (Martin et al., 2008; Hall, 2013). Additionally, heifers can be selected based on reproductive tract (Gutierrez et al., 2014; Holm et al., 2015) and body condition scores (RTS and BCS, respectively) prior to breeding. Heifers that do not meet these criteria are usually considered poor replacement candidates. Furthermore, producers can implement a progestin-based estrous synchronization protocol (Martin et al., 2008; Gutierrez et al., 2014) for induction of cyclicity in peripubertal heifers.

Genetic selection has been used extensively to improve production traits in beef cattle, however there are challenges to using genetic selection to improve reproduction. First service conception and pregnancy rate are used to evaluate fertility in heifers. However, unlike growth and carcass traits, the heritability of female reproductive traits is low, for example, 0.03-0.18 (Bormann et al., 2006; Fortes et al., 2012b; Peters et al., 2013) and 0.07-0.20 (Doyle et al., 2000; Bormann et al., 2006; MacNeil et al., 2006; McAllister et al., 2011; Fortes et al., 2012b; Peters et al., 2013; Boddhireddy et al., 2014; Toghiani et al., 2017) for first service conception and pregnancy rate, respectively. The low heritability and polygenic nature of fertility traits make it difficult to utilize statistical models to select animals to improve heifer fertility.

While limited improvement in female fertility can be made through genetic selection, the implementation of appropriate management practices does increase the likelihood of reproductive success in heifers. However, even when the aforementioned

management practices are followed, a percentage of beef heifers still fail to become pregnant or conceive later into the breeding season. Therefore, we aimed to determine if phenotypic parameters differed among heifers of varied pregnancy outcomes. We tested the hypothesis that in a group of heifers managed according to best management practices, records of weaning weight, age at weaning, age at artificial insemination, and age of dam would differ between heifers of varied reproductive outcomes during the first breeding season.

Materials and methods

All animals sourced in this study belonged to Auburn University. All procedures with animals were performed in accordance with the protocols approved by Institutional Animal and Care and Use Committee in Auburn University.

Overall nutritional management of heifers

The dataset used in this study contained the first breeding season pregnancy outcome, phenotypic, and pedigree records for crossbred, beef heifers (Angus x Simmental cross; *n*=259) born in the years 2010 to 2016 at 3 Auburn University Experimental Stations [Black Belt Research and Extension Center (*n*=53); Gulf Coast Research and Extension Center (*n*=136); Wiregrass Research and Extension Center (*n*=70)]. At weaning, a proportion of heifers born each year was retained as potential replacement heifers.

Heifers were managed to reach a target weight of 60% of their mature bodyweight (approximately 381 kg) by the start of their first breeding season, which began in early December of each year. Heifers at the Black Belt Research and Extension Center were weaned and developed on toxic endophyte infected tall fescue pastures and free-choice

ryegrass hay. Heifers were supplemented as needed with a 50:50 mixture of corn gluten pellets and soyhull pellets. Heifers remained on tall fescue pastures from weaning through the winter grazing season and the time of pregnancy diagnosis. Heifers at the Gulf Coast Research and Extension Center were developed from weaning to breeding on bahiagrass pasture alongside free choice ryegrass hay. Heifers were supplemented as needed with a Nutrena NutreBeef® 13% protein pellet. After breeding, heifers were moved to a ryegrass pasture for the remainder of the winter grazing season. At the Wiregrass Research and Extension Center, weaned heifers were managed on bermudagrass pasture with supplementation of 50% pelleted soyhulls and 50% corn gluten feed that was provided as needed. As summer pastures entered dormancy, heifers were fed free-choice Tifton-85 bermudagrass hay and were allowed to graze pastures containing triticale, hairy vetch, and rape seed for the remainder of the winter grazing season.

Classification of heifers based on reproductive outcome

Approximately 30 days before the start of their first breeding season, heifers were evaluated for BCS [scale of 1-9 with 1=emaciated and 9=obese; (Wagner et al., 1988)] and assessed for RTS [scale of 1-5; 1= pre-pubertal, 5= pubertal, luteal phase; (Anderson et al., 1991)] by a single, experienced veterinarian. At approximately 14 months of age (418.7±22.6 days), heifers retained as replacements underwent estrous synchronization for fixed-time artificial insemination utilizing the 7-Day CO-Synch + CIDR protocol (Larson et al., 2006) to begin their first breeding season. Briefly, heifers received an injection of GnRH (i.m.; 100 µg; Cystorelin®; Merial, Duluth, GA) and insertion of a CIDR (intravaginal insert; 1.38 g progesterone; Eazi-Breed® CIDR®; Zoetis Inc.,

Kalamazoo, MI) on day -9, followed by CIDR removal and an injection of prostaglandin F2α (PGF; i.m.; 25 mg; Lutalyse®; Zoetis Inc., Kalamazoo, MI) on day -2. All heifers then received a second GnRH injection (i.m.; 100 µg; Cystorelin®; Merial, Duluth, GA) and were inseminated with a dose of semen of proven fertility on day 0, 54±2 hours after CIDR removal and PGF injection. Two professionals in random rotation were responsible for AI procedures at each experimental station for each year.

Fourteen days after insemination, heifers were exposed to 2 fertile bulls for natural breeding for the remainder of the breeding season. An experienced veterinarian performed initial pregnancy evaluation by transrectal palpation on day 62-89 post insemination, followed by final pregnancy evaluation on day 85-176 post insemination. Presence or absence of a conceptus, alongside morphological features indicating fetal age were recorded, and heifers were classified as ‘‘pregnant to AI’ (Preg AI), ‘pregnant to natural service’ (Preg NS), or ‘not pregnant’ (Not Preg).

Phenotypic dataset

All analytical procedures were carried out in R software (Ihaka and Gentleman, 1996). A schematic diagram representing the phenotypic data, the reproductive data, the merging, and the curation procedures is depicted in Fig. 3. We obtained performance records and pedigree information for all calves born at each station from 2000-2017 from the Alabama Beef Cattle Improvement Association (n=2,530). We then filtered this dataset to include only heifer calves (n=1,240) and merged the performance dataset with records for pre-breeding body condition score, reproductive tract score, artificial insemination date, and pregnancy outcome for all heifers exposed to breeding during

their first breeding season. We computed age of dam by subtracting the year of birth for the dam from the year of birth for each heifer.

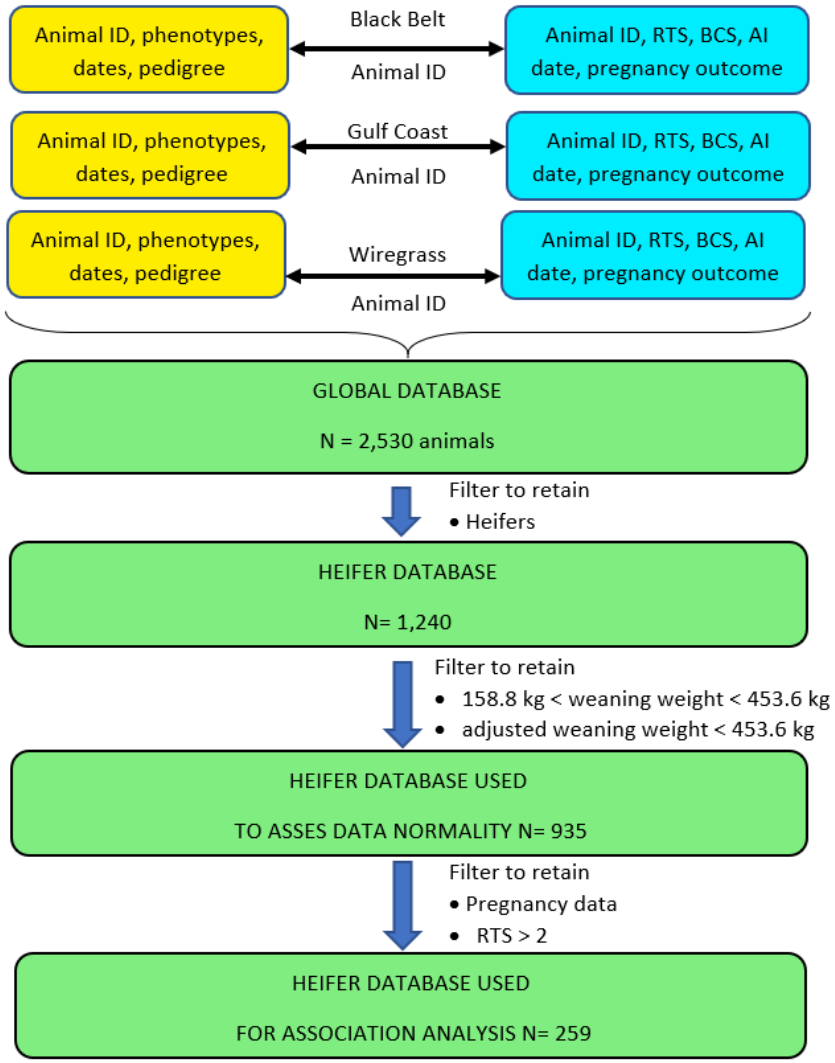


Fig. 3. Flowchart of data origin and filtering. Horizontal, double sided arrows indicate the merging of databases for production records (yellow) and reproductive and breeding related records (blue) for animals at each experimental station (Black Belt, Wiregrass, and Gulf Coast). The records were utilized to create a global database of records from 2,530 animals which was further filtered to retain only heifers creating a heifer database of 1,240 animals. We then filtered the heifer database to remove outliers for weaning weight data and assessed normality of parameters measured. Finally, we filtered the heifer database to retain only animals with records of first breeding season pregnancy outcome and pre-breeding RTS >2 to create the database utilized for analysis in this study. RTS: reproductive tract score; BCS: body condition score; AI: artificial insemination.

We curated the data and eliminated observations that appeared as abnormal data imputation or outliers. We retained records if weaning weight was recorded within 158.8-453.6 kg and adjusted weaning weight was less than 453.6 kg. For analyses of pregnancy outcome, we only retained records for heifers that conceived if the pregnancy was carried out to term and a healthy calf was born. Heifers experiencing pregnancy loss (n=3) were removed from the dataset because conceptus losses were not the focus of this study and analyzing data from these heifers would create a confounding category between pregnant and not pregnant. In addition, heifers presenting RTS <3 (n=5) were removed from the dataset according to consistent data supporting the notion that heifers with an immature reproductive tract are significantly less likely to become pregnant (Gutierrez et al., 2014; Holm et al., 2015).

We assessed normality of the continuous traits by performing a Shapiro-Wilk test and by examining histograms, quantile-quantile, and density plots for each parameter. We utilized the data from all heifers to assess the normality of weaning weight, adjusted weaning weight, and age at weaning, regardless of whether we collected breeding data. We assessed normality of age at AI using the data from the heifers that were artificially inseminated. Amongst heifers included in this dataset, the variables weaning weight (WW) and adjusted weaning weight (adj WW) were normally distributed (P>0.01, Shapiro-Wilk test, Fig. 4). The variables age at weaning and age at AI displayed a deviation when tested from normal distribution (P<0.01, Shapiro-Wilk test, Fig. 4). Nonetheless, visual inspection of the data (Fig. 4) indicated strong resemblance of normal

distribution and the skewness was likely an influence of the varied management strategies at each station.

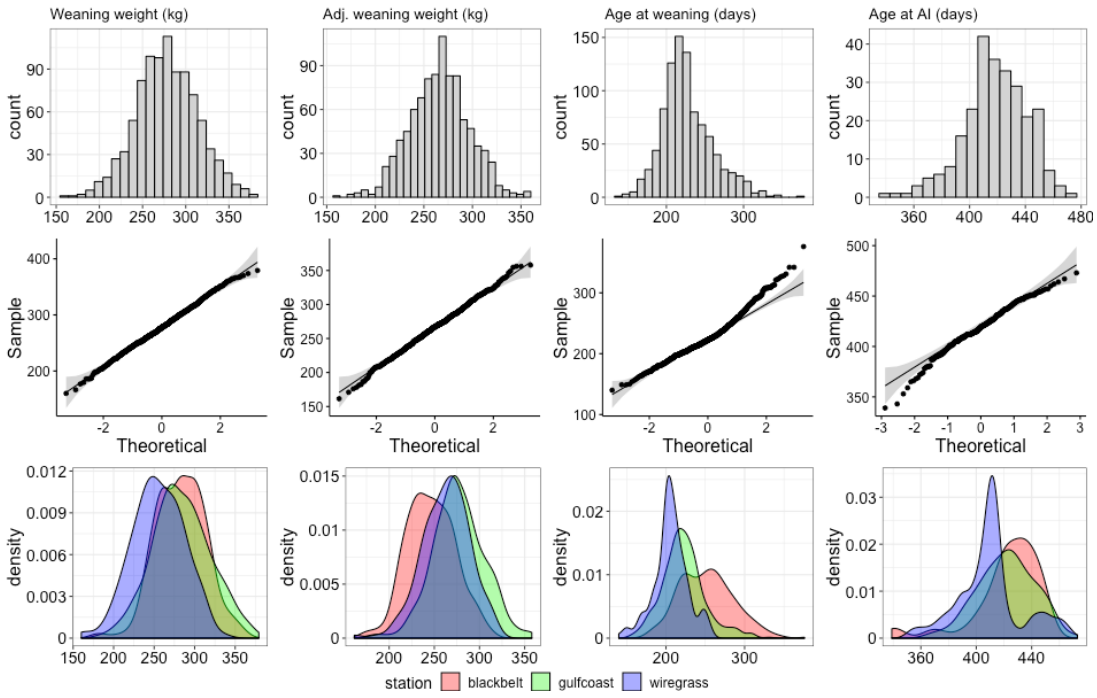


Fig. 4. Distribution of the continuous variables investigated. (top row) Histograms indicating the number of heifers with given performance levels, (middle row) quartile-quartile plots indicating the deviation of the data from theoretical normal distribution, and (bottom row) density of performance levels for records of weaning weight, adjusted weaning weight, age at weaning, and age at artificial insemination. Red shading represents heifers at the Black Belt Research Station, green shading represents heifers at the Gulf Coast Research Station, and blue shading represents heifers at the Wiregrass Research Station. AI: artificial insemination; kg: kilograms.

Analysis of phenotypic parameters relative to pregnancy outcome.

We analyzed the data using a mixed effect multinomial logistic regression model (Hedeker, 2003) because the heifers were categorized according to discrete reproductive outcomes. We modeled the phenotypic parameters relative to the reproductive outcomes according to 2 scenarios.

First, we accounted for the probability of 3 possible reproductive outcomes: Preg AI, Preg NS, or Not preg. The variables station (S_j, j=1,2,3), AI year (Y_k, k= 2011, 2012, 2013, 2015, 2016, 2017), BCS (BCS_l, l=4, 5, 6, 7), RTS (RTS_m, m= 3,4,5), age at weaning (AgeW), age at AI (AgeAI), dam age (AgeD), and weaning weight (WW) were included in the model. Heifer's sires and the bulls used in the breeding programs were not included in the model as they were confounded with stations. The probabilities (Pr) of occurrence of each pregnancy outcome relative to the variables were estimated as follows:

$$ln\left(\frac{\Pr(Preg\ AI)}{\Pr(Not\ preg)}\right) = \beta_{01} + \beta_{11}S_j + \beta_{21}Y_k + \beta_{31}BCS_l + \beta_{41}RTS_m + \beta_{51}AgeAI + \beta_{61}AgeD + \beta_{71}WW + \varepsilon_1$$

, and

$$ln\left(\frac{\Pr(Preg\ NS)}{\Pr(Not\ preg)}\right) = \beta_{02} + \beta_{12}S_j + \beta_{22}Y_k + \beta_{31}BCS_l + \beta_{42}RTS_m + \beta_{52}AgeAI + \beta_{62}AgeD + \beta_{72}WW + \varepsilon_2$$

.

Next, we accounted for the probability of 2 outcomes only: 'pregnant' or 'not pregnant'. The binomial modeling followed the same structure as presented above with exception that the dependent variable was represented by $ln\left(\frac{\Pr(Preg)}{\Pr(Not\ preg)}\right)$.

We used the 'nnet' package (Venables and Ripley, 2002) to fit the multinomial and binomial models. The likelihood of the ratios was calculated with a χ^2 test using the 'Anova' function in the 'car' package. The model was assessed by the Akaike Information Criteria (AIC) (Bozdogan, 1987) using the 'MASS' package. Statistical significance was inferred if $P<0.05$.

Results

Phenotypic description of beef heifers

The initial dataset contained performance data for 1,240 heifer calves born on 3 Auburn University research stations from 2000 to 2017 (Fig. 3). Following data filtering, 935 records indicated a weaning weight of 278.0±35.5 kg, an adjusted weaning weight of 266.2±30.4 kg, and an age at weaning of 227.2±32.6 days for all heifers The 259 records obtained for heifers with pregnancy data demonstrated a weaning weight of 294.7±38.9 kg, an adjusted weaning weight of 278.7±26.6 kg, and an age at weaning of 229.2±34.3 days (Table 2, Fig. 4). At the time of AI, the heifers included in our pregnancy outcome analysis averaged 418.7±22.6 days of age (Table 2, Fig. 4).

Table 2. Descriptive statistics of continuous variables from beef heifers.

Variable	Dataset	No. of Records	Mean	SD	95% CI
Weaning weight,	All heifers[1]	935	278.0	35.5	275.8 - 280.3
kg	Pregnancy heifers[2]	259	294.7	38.9	289.9 - 299.4
Adj weaning	All heifers	935	266.2	30.4	264.2 - 268.1
weight, kg	Pregnancy heifers	259	278.7	26.6	275.5 - 282.0
Age at weaning,	All heifers	935	227.2	32.6	225.1 - 229.3
days	Pregnancy heifers	259	229.2	34.3	225.0 - 233.4
Age at AI, days	Pregnancy heifers	259	418.7	22.6	416.0 - 421.5

[1] All heifer calves recorded from each station that were born between 2000-2017.
[2] Heifer calves recorded from each station that were subjected to AI between 2011-2016.
SD: Standard deviation; CI: Confidence interval; kg: Kilograms.

All 259 heifers in the dataset analyzed for pregnancy outcome had a pre-breeding BCS of 4-7, with 81% of the heifers classified as 6 (Table 3, Fig. 5). The heifers were categorized between 3 and 5 for RTS, with 40 and 52% of the heifers presenting RTS 4

and 5, respectively (Table 3, Fig. 5). Altogether, 41.6% (n=108) of the heifers presented BCS = 6 and RTS = 5, followed by 33.6% (n=87) of the heifers categorized with BCS = 6 and RTS = 4 (Table 4). The heifers were born to dams between 2 and 15 years of age with 53% born to dams 2-4 years old (Fig. 5).

Table 3. Percentages of pregnancy outcome by reproductive tract scoring and body condition scores.

RTS	N	Preg AI, %	Preg NS, %	Not preg, %
3	20	30.0	50.0	20.0
4	104	46.2	35.6	18.3
5	135	42.2	45.2	12.6
BCS				
4	1	100.0	0.0	0.0
5	48	31.3	50.0	18.8
6	209	45.0	40.2	14.8
7	1	100.0	0.0	0.0

RTS: Reproductive tract score; BCS: Body condition score; AI: Artificial insemination; NS: Natural service.

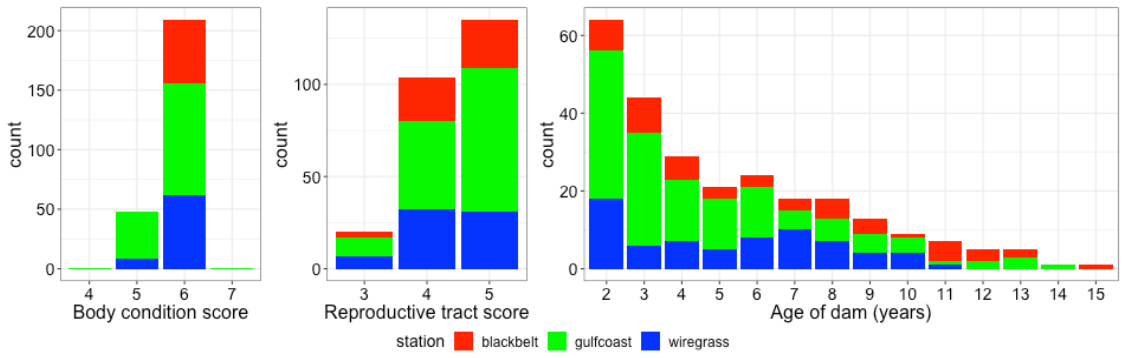


Fig. 5. Distribution of the discrete variables investigated in this study. Number of heifers is depicted on the y-axis and values of each record analyzed are depicted on the x-axis for records of body condition score, reproductive tract score, and age of dam. Each bar depicts the number of heifers in each category from the Black Belt Research Station (red), Gulfcoast Research Station (green), and Wiregrass Research Station (blue).

Table 4. Percentages of heifers distributed on different categories of BCS and RTS.

		RTS		
		3	4	5
BCS	4	0.0	0.0	0.4
	5	2.3	6.6	9.7
	6	5.4	33.6	41.7
	7	0.0	0.0	0.4

RTS: reproductive tract score; BCS: body condition score.

Analysis of phenotypic parameters relative to heifer pregnancy outcome

Assessment of the full model indicated that weight at weaning, age at weaning, age at breeding, BCS, and RTS had insignificant contribution to the variability observed in the response variable, namely reproductive outcome (Table 5, Table 6, Fig. 6). By comparison, the variables location and year presented significant (P<0.05) contribution to the variance (Table 5, Table 6). The variable age of dam, although not significantly associated with pregnancy outcome, also contributed to a model that better fits the variance of the data (Burnham and Anderson, 2004).

Table 5. Analysis of variance for the multinomial logistic regression of pregnancy outcome (Preg AI, Preg NS, Not Preg).

	LR χ^2	Df	Pr(>χ^2)
Station	11.779	4	0.0191
AI year	37.266	10	<0.0001
age at AI	0.138	2	0.9335
age at weaning	0.218	2	0.8967
dam age	3.753	2	0.1532
BCS	3.405	6	0.7565
RTS	2.046	4	0.7273
weaning weight	0.193	2	0.9079

LR: likelihood ratio, Df: degrees of freedom, Pr: probability.

Table 6. Analysis of variance for the binomial logistic regression of pregnancy outcome (Preg, Not Preg).

	LR χ^2	Df	Pr(>χ^2)
Station	7.3549	2	0.0253
AI year	23.7948	5	0.0002
age at AI	0.0241	1	0.8766
age at weaning	0.0366	1	0.8483
dam age	1.4052	1	0.2359
BCS	1.3972	3	0.7062
RTS	0.8231	2	0.6626
weaning weight	0.1682	1	0.6817

LR: Likelihood ratio, Df: degrees of freedom, Pr: probability.

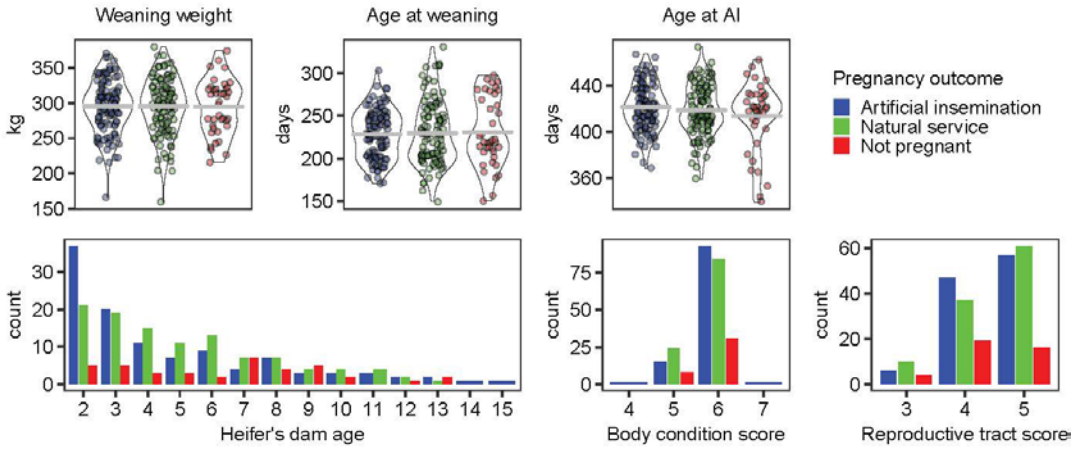


Fig. 6. Distribution continuous and discrete variables evaluated in this study by pregnancy outcome in beef heifers. Each dot represents actual weaning weight in kg, age at weaning in days, or age at AI in days among heifers pregnant to AI (blue), heifers pregnant to natural service (green), and heifers that did not become pregnant (red). Bars represent the number of heifers pregnant to AI (blue), pregnant to natural service (green), or failing to become pregnant (red) that were born to dams of varied ages, heifers that were of different body condition score, or heifers that were of different reproductive tract score.

Fig. 6 shows the distribution of the data for different parameters according to the reproductive outcome. Age or weight at weaning, age at breeding, dam age, BCS (Table 5), and RTS (Table 5) were not significantly associated with the response variable ($P>0.05$), regardless of whether the logistic regression was carried with 3 (Preg AI, Preg NS, Not Preg; Table 5) or 2 (Preg, Not Preg; Table 6) reproductive outcomes. The results are strong indication that these parameters are not predictive of successful reproductive outcome in beef heifers that had been pre-selected as acceptable to enter the breeding season.

It was noteworthy that none of the 6 heifers younger than 368 days at the start of the breeding season became pregnant by AI (Fig. 6). We further categorized our dataset based on heifers younger than 368 days of age, or heifers ≥ 368 days of age. We calculated an 87.5% probability of a heifer to become pregnant if she was 368 days of age or older at the beginning of the breeding season (odds = 7, 95% CI [2.7,17.9], $P<0.001$). However, there was only 12.5% chance of a heifer to become pregnant if she was younger than 368 days (odds = 0.14, 95% CI [0.06, 0.3], $P<0.001$).

Discussion

Appropriate selection of beef replacement heifers is central for enhancing efficiency of the beef industry. Proper management practices serve to eliminate animals from this costly program and increase the likelihood of obtaining greater pregnancy rates early in the first breeding season. In this study, we analyzed key phenotypic and age profiles of Angus × Simmental heifers that were developed to become replacement heifers and were pre-selected for replacement potential prior to entering the breeding

season. Our findings provide evidence-based insights on development and selection of beef heifers relative to their reproductive outcome.

Average weaning weight depicted in this study (294.7±38.9 kg for heifers exposed to breeding) was greater than the average recorded weights of replacement beef heifers across the United States [241.3 kg (USDA, 2008)]. This greater weight can be partly attributed to heifers being weaned, on average, 22 days (average of weaning age = 229.2±34.3) later than the reported national average age at weaning [207 days (USDA, 2008)]. The BCS, RTS, and ages of heifers at AI in this study are in agreement with recommended management practices for replacement heifer development (Rae et al., 1993; Lamb et al., 2014). A greater than expected number of heifers were retained from 2-year-old dams. However, the management practices of each station exclude artificial insemination of the mature cowherd, thus more animals were retained from first parity dams to increase genetic improvement at the experimental stations.

There was no association between age or weight at weaning and pregnancy outcomes. The results corroborate a metanalysis performed on beef heifers by Canellas and others (Canellas et al., 2012). Our results demonstrate that there is potential for heifers of varied weights and ages at weaning to reach reproductive success as long as they are developed to reach a target mature body weight greater than 53% of their mature bodyweight by the start of their first breeding season (Funston and Deutscher, 2004).

Nearly all heifers included in this study were pre-selected prior to entering the breeding season according to general recommendations to increase pregnancy success (Larson et al., 2016). Five heifers that entered the breeding season presenting RTS = 2 (4 became pregnant, 1 remained open) were removed during the data filtering to accomplish

the goal of investigating a data set that adhered to best practices for improving pregnancy success in beef heifers (Larson et al., 2016). Contrary to previous reports (Holm et al., 2009; Gutierrez et al., 2014), our analytical modeling did not detect significant association between RTS and reproductive outcome. Nevertheless, 46% and 42% of heifers presenting RTS 4 and 5, respectively, became pregnant to AI comparatively to the 30% classified with RTS 3. Although not statistically significant, there was a decline in open heifers at the end of the breeding season as the heifers presented greater RTS (Table 3).

Crossbred beef cows presenting BCS ≥ 5 had greater pregnancy rates relative to cows categorized with less fat percentage (Rae et al., 1993). In our study, there was 14% difference in pregnancy rates to AI for heifers categorized with BCS = 6 relative to those classified with BCS = 5, although the difference was not statistically significant. Nonetheless, the final pregnancy rates were very similar in both groups (85 vs 81%). Our results indicate that maintaining a nutritional program that allow heifers to reach BCS=6 at the beginning of the breeding season gave a numerical advantage on pregnancy success to AI (Table 3, Fig. 7). Beyond the quicker changes in the genetic background of the herd, the early conception to AI and early calving are determinant for greater longevity of the heifers in the breeding herd (Cushman et al., 2013; Perry and Cushman, 2013).

Heifers younger than 368 days of age did not become pregnant by AI, and only 1 of these 6 young heifers became pregnant by natural service. It must be noted that the one heifer that became pregnant by natural service had a BCS = 5 and RTS = 3 at pre-breeding examination, while the others that remained open had a BCS = 6 and RTS ≥ 4. Although RTS indicated that these heifers had reached puberty, these data suggest that

age should be carefully assessed within the context of the production systems as a potential criterion for heifer culling.

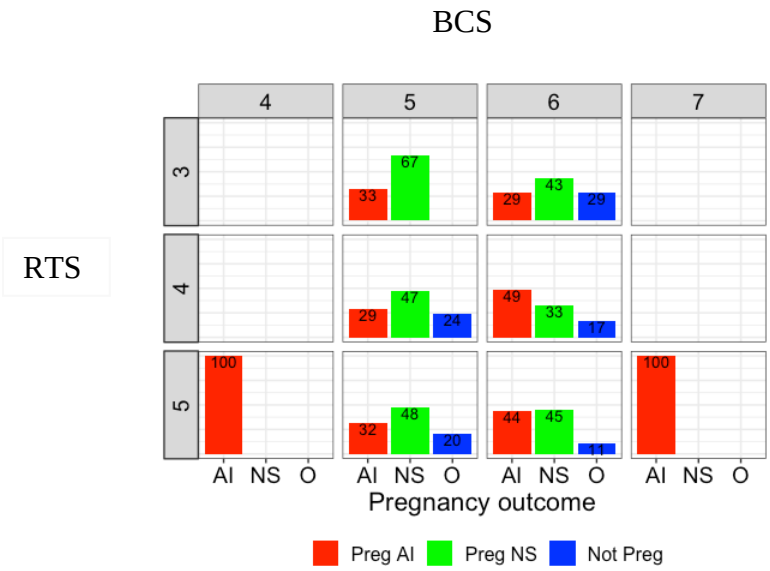


Fig. 7. Percentages of reproductive outcome within different groups of beef heifers categorized by reproductive tract score and body condition score. Reproductive tract scores are depicted on the y-axis and body condition scores are depicted on the x-axis. Number of heifers pregnant to AI (red), pregnant to natural service (green), and not pregnant (blue) for each combination of RTS and BCS are designated by the colored bars with percentage of heifers in each classification written in the top of each bar. RTS: reproductive tract score; BCS: body condition score; AI: heifer pregnant to artificial insemination; NS: heifer pregnant to natural service; O: heifer remained open.

Summary and conclusions

We report phenotypic parameters of beef heifers selected as replacements for development programs in cow-calf production systems. Within this group of pre-selected heifers, our analytical approach did not identify phenotypical or age-related parameters that are predictive of reproductive outcomes. However, developing heifers to BCS = 6 and RTS ≥ 4 might promote a numerical advantage of successful pregnancy to AI, supporting previous management suggestions. Careful risk assessment should be made

when developing replacement heifers if they will not be older than 12 months of age by the start of the breeding season.

The data collected is restricted to *Bos taurus*, crossbred beef heifers (Angus × Simmental) on 3 research stations in the state of Alabama, thus it is difficult to evaluate how representative our results are of beef cow-calf systems of different biological types in different geographic areas in the USA. Nonetheless, our findings provide support for current management guidelines for the development of replacement beef heifers. More importantly, our limited ability to improve heifer pregnancy success from phenotypical parameters emphasizes the need for development of biotechnologies that will serve to reduce infertility in beef heifers.

III. Transcriptome Profiles in Peripheral White Blood Cells at the Time of Artificial Insemination Discriminate Beef Heifers with Different Fertility Potential

Introduction

Female infertility remains a limiting factor in cattle production systems. In beef heifers, pregnancy rates at the conclusion of the first breeding season vary from 64% to 95% (Patterson et al., 1989; Lynch et al., 1997; Funston and Deutscher, 2004; Bormann et al., 2006; Grings et al., 2007; Martin et al., 2008; Roberts et al., 2009; Funston and Larson, 2011; Mallory et al., 2011; Peters et al., 2013; Gutierrez et al., 2014). Pregnancy rates to first service artificial insemination are range from 36-69% (Diskin and Sreenan, 1980; Lynch et al., 1997; Rorie et al., 1999; Bormann et al., 2006; Funston and Larson, 2011; Mallory et al., 2011; Peters et al., 2013; Gutierrez et al., 2014; Thomas et al., 2017). Best management practices in heifer development have been used to increase the probability of reproductive success in a heifer's first breeding season (Larson et al., 2016). For instance, heifers that reach 60% of their mature body weight by the start of the breeding season (Martin et al., 2008), have a body conformation compatible with a healthy and well-nourished animal (Rae et al., 1993; Arango et al., 2002), present reproductive structures indicative of cyclic animals (Holm et al., 2009; Gutierrez et al., 2014; Holm et al., 2015), and are bred on their third versus first estrus (Byerley et al., 1987) may have a greater chance of becoming pregnant early in the breeding season (Larson et al., 2016). Yet, under appropriate management, many of the heifers that are deemed reproductively mature according to morphological assessment and age criteria do not become pregnant. Unexplained infertility of otherwise healthy females impacts the

cattle industry negatively and is a condition of significant importance in other livestock and humans (Quaas and Dokras, 2008).

In addition to the economic losses from infertile animals, heifers that conceive late in their first breeding season to NB are likely not as profitable as early conceiving heifers. Following an unsuccessful AI, heifers that became pregnant to NB and calve after the first 21 days into their first calving season remain productive in the herd for a shorter period of time and wean less total pounds of calf than their early calving counterparts (Cushman et al., 2013). Therefore, improving the selection for heifers that become pregnant by AI at the beginning of the breeding season will reduce economic losses in beef cattle operations.

Genetic selection has been evaluated extensively to improve production and reproductive traits in beef cattle operations. In heifers, fertility is assessed by first service conception and pregnancy rate. Nonetheless, low heritability estimates for pregnancy rate [0.07 – 0.20 (Doyle et al., 2000; Bormann et al., 2006; MacNeil et al., 2006; McAllister et al., 2011; Fortes et al., 2012b; Peters et al., 2013; Boddhireddy et al., 2014; Toghiani et al., 2017)] and first service conception [0.03 - 0.18 (Bormann et al., 2006; Fortes et al., 2012b; Peters et al., 2013)] make it challenging to leverage statistical models to guide the decision making process for sire selection to improve female fertility in cattle. As a consequence, selection for fertility in beef heifers using traditional approaches has not achieved significant progress over generations.

Strategies leveraging molecular genetics biotechnology have added new perspective to understanding the genetic architecture of fertility. Genomic polymorphisms (Fortes et al., 2012b; Hawken et al., 2012; McDaneld et al., 2012; Fortes

et al., 2013; McDaneld et al., 2014), differential gene transcription in the hypothalamus (Fortes et al., 2012b), endometrium (McMillan and Donnison, 1999; Peterson et al., 1999; Salilew-Wondim et al., 2010; Minten et al., 2013; Geary et al., 2016), and metabolites from follicular fluids (Bender et al., 2010) have been associated with fertility in heifers or cows. In humans, investigation of circulating prognostic biomarkers in women have yielded promising candidates that are predictive of infertility (Michou et al., 2003), *in vitro* fertilization success (Thum et al., 2004), or pregnancy outcomes (Thum et al., 2004; Dons'koi et al., 2014). These studies, and the physiological connection between reproduction and the immune system (Fair, 2015), support the rationale that peripheral white blood cells may harbor invaluable molecular information predictive of the physiological state of beef heifers pertaining to their likelihood of pregnancy establishment.

The molecular profile of circulating miRNAs in the bloodstream (Pohler et al., 2017) and gene expression of PWBC (Pugliesi et al., 2014) change during the early stages of pregnancy. Nonetheless, the molecular profiles of gene or protein expression in PWBC prior to fertilization have not been investigated as biomarkers for fertility in cattle. In this study, we tested the hypotheses that at the time of AI in beef heifers on their first breeding season: 1) the transcriptome of PWBC differs between heifers that become pregnant to AI and heifers that become pregnant late in the breeding season by NB or do not become pregnant during the breeding season; and 2) the ratio of transcript abundance in genes expressed in PWBC can be used to classify heifers according to pregnancy by AI, NB, or failure to become pregnant.

Materials and methods

Animal handling and heifer classification according to pregnancy outcome

The experimental scheme of this study is outlined in Fig. 8a. Crossbred beef heifers (Angus-Simmental cross) from 2 Auburn University research stations (Station A: Wiregrass Research and Extension Center, n=27; and Station B: Black Belt Research and Extension Center, n=33) were developed to reach a target weight of 60% of their mature body weight by 13.5 months of age (Patterson et al., 1989; Larson et al., 2016). Pre-breeding examinations were performed approximately 45 days before breeding to evaluate the pubertal status of each heifer. Reproductive tract scores [scale of 1-5; 1= pre-pubertal, 5= pubertal, luteal phase (Anderson et al., 1991; Holm et al., 2009)], pelvic width, and pelvic height were determined by transrectal palpation by a single, experienced veterinarian. Additionally, heifers were evaluated for body condition score [scale of 1-9 with 1=emaciated and 9=obese (Wagner et al., 1988; Rae et al., 1993)]. Fig. 8b depicts the timeline of the experiment from breeding soundness to heifer classification.

Heifers were subjected to estrous synchronization for fixed-time artificial insemination with the 7-Day CO-Synch protocol. Heifers received an injection of GnRH (i.m.; 100 µg; Cystorelin®; Merial, Duluth, GA) and insertion of a CIDR (intravaginal insert; 1.38 g progesterone; Eazi-Breed® CIDR®; Zoetis Inc., Kalamazoo, MI) on day -9, followed by CIDR removal and an injection of prostaglandin F2α (i.m.; 25 mg; Lutalyse®; Zoetis Inc., Kalamazoo, MI) on day -2. All heifers then received a second GnRH injection (i.m.; 100 µg; Cystorelin®; Merial, Duluth, GA) and were inseminated with a dose of semen from a bull of proven fertility on day 0, 54±2 hours after CIDR

removal and PGF injection. Two professionals were responsible for insemination procedures in both research stations, taking turns on random heifers.

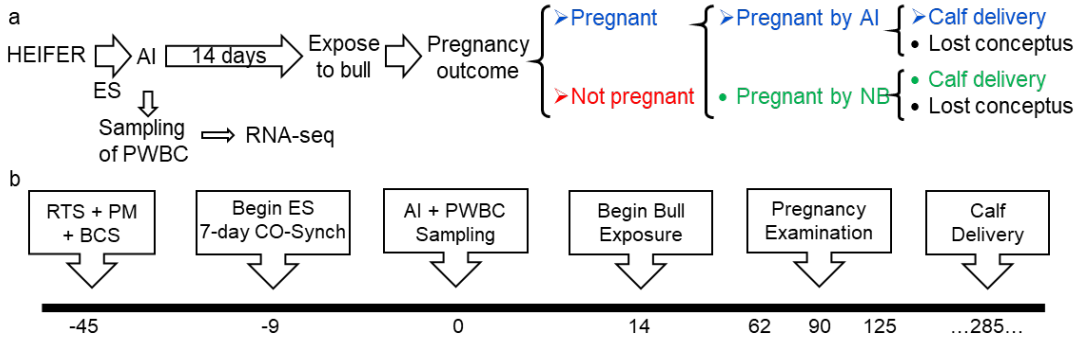


Fig. 8. Overview of the experimental design and heifer classification. (a) General scheme used for the classification of heifers. (b) Depiction of the timeline adopted from breeding soundness evaluation to final heifer classification. See text for details. ES: estrous synchronization; AI: artificial insemination; RTS: reproductive tract scores; PM: pelvic measurements; BCS: body condition score.

Immediately after AI, 10 ml of blood was drawn from the jugular vein into vacutainers containing 18 mg K2 EDTA (Becton, Dickinson and Company, Franklin, NJ). The tubes were inverted for 8-10 times and immersed in ice. Upon arrival in the laboratory, the tubes were sprayed with 10% bleach and rinsed to eliminate contamination from the field. The tubes were centrifuged for 10 minutes at 2000xg at 4°C. The buffy coat was removed and deposited into 14 ml of red blood cell lysis solution (0.15 M ammonium chloride, 10 mM potassium bicarbonate, 0.1 mM EDTA, Cold Spring Harbor Protocols) for 10 minutes at room temperature (24-25°C). A final centrifugation was performed for 5 minutes at 800xg at 4°C, after which the solution was discarded and the pellet was re-suspended in 200 µl of RNAlater® (Lifetechnologies™,

Carlsbad, CA). The PWBCs were then stored at -80°C until RNA extraction. This procedure was reproduced for both experimental stations.

Fourteen days after insemination, heifers were exposed to 2 fertile bulls for natural breeding for the remainder of the 86 day breeding season on station A or 42 day breeding season on station B. An experienced veterinarian performed pregnancy evaluation by transrectal palpation on day 62 and 125 post insemination at station A, and on day 95 post insemination at station B. Presence or absence of a conceptus, alongside morphological features indicating fetal age were recorded, and heifers were classified as pregnant to AI, pregnant to natural service, or non-pregnant. Heifers that became pregnant after the first 21 days of breeding were identified as late breeding for the purpose of this study.

Selection of heifers for RNA-sequencing of PWBC

Eleven heifers (6 AI-pregnant and 5 NB-pregnant) were selected from station A, and 12 heifers (6 AI-pregnant and 6 non-pregnant) were selected from station B for RNA-sequencing of PWBC. Within station, heifers were selected according to their similarities of age and phenotypic parameters. Data for age, weaning weight, pelvic height, pelvic width, and pelvic area were compared between groups using Krustal-Wallis rank sum test. Body condition and reproductive tract scores were tested using Fisher's exact test. Tests were conducted in R software. Selected heifers did not differ for phenotypic traits associated with puberty (Table 7), and all heifers were of pubertal status at the time of breeding. The selection of heifers from different groups that were phenotypically similar, according to trait average and standard deviation, avoided the addition of covariates in the analysis of differential gene expression.

Table 7. Descriptive statistics ($\bar{x} \pm \hat{\sigma}$) of the phenotypical data from the heifers used for the sequencing analysis.

Location	Parameter	Pregnant AI	Pregnant NB	P
A	N	6	5	-
	Age[a]	412 ± 10	402 ± 11	0.36[d]
	WW[b]	234.6 ± 14.3	249.5 ± 21.2	0.14[d]
	Pelvic height[c]	15.3 ± 0.5	15.0 ± 0.7	0.39[d]
	Pelvic width[c]	11.3 ± 0.5	11.6 ± 1.5	1
	Pelvic area[c]	173.8 ± 11.0	174.8 ± 31	0.8[d]
	BCS	5.7 ± 0.5	5.8 ± 0.4	1[e]
	RTS	4.7 ±0.5	4.2 ± 0.8	0.7[e]
Location	Parameter	Pregnant AI	Not Pregnant	P
B	N	6	6	-
	Age[a]	428 ± 8	433 ± 10	0.4[d]
	WW[b]	325.8 ± 15.4	316.4 ± 15.4	0.5[d]
	BCS	6 ± 0	6 ± 0	-
	RTS	4.5 ± 0.5	4.2 ± 0.4	0.5[e]

BCS: body condition score; RTS: reproductive tract score; WW: weaning weight; [a] day; [b] kg; [c] cm^2; [d] Krustal-Wallis rank sum test; [e] Fisher's exact test; - Statistical test is not applicable.

RNA extraction, library preparation, and RNA sequencing

Total RNA was then isolated from PWBCs of 23 heifers using TRIzol™ reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. RNA yield was quantified using the Qubit™ RNA Broad Range Assay Kit (Eurogene, OR) on a Qubit® Fluorometer, and integrity was assessed on Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA) using an Agilent RNA 6000 Nano kit (Agilent, Santa Clara, CA). We obtained RNA integrity number (RIN) values ranging between 7.7 and 8.8. Furthermore, samples with rRNA ratios (28S:18S) greater than 1.5 were further processed for library construction (range 1.5-1.8). Libraries were prepared with the TruSeq Stranded mRNA Library Prep kit (Illumina, Inc., San Diego, CA) following manufacturer's instructions. Libraries were quantified with Qubit™ dsDNA High Sensitivity Assay Kit (Eurogene, OR) and quality was evaluated using the High Sensitivity DNA chip (Agilent, Santa

Clara, CA) on an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA). Libraries were sequenced in a HiSeq 2500 system at the Genomic Services Laboratory at HudsonAlpha, Huntsville, AL to generate 125 nucleotide long pair-end reads.

RNA sequencing data processing

Sequences were trimmed of their adapters and submitted to a custom build bioinformatics pipeline (Biase et al., 2016). Reads were aligned to the bovine genome (UMD3.1 (Zimin et al., 2009)), and sequences aligning to multiple places on the genome, with 5 or more mismatches were filtered out. The sequences were then marked for duplicates, and non-duplicated pairs of reads were used for gene expression study. The read-pairs were counted against the Ensembl gene annotation (Flicek et al., 2013) (version 1.87) using HTSeq (Anders et al., 2014). In order to remove quantification uncertainty associated with lowly expressed genes and erroneous identification of differentially expressed genes (Bullard et al., 2010; Robinson and Oshlack, 2010), we retained genes with more than one count per million (1 CPM) in 6 or more samples for downstream analyses, for each location independently.

Differentially expressed genes

Differences of transcript levels between samples at each research station were determined from fragment counts (Anders et al., 2013) using the Bioconductor packages "edgeR" (Robinson et al., 2010) and "DESeq2" (Love et al., 2014) in R software (Ihaka and Gentleman, 1996). Genes were considered detected if the counts per million was greater than one in 6 or more samples. For each experimental station, a gene was inferred as differentially expressed if the nominal P value was $\leq$ 0.01. This nominal P value corresponded to empirical false discovery rate (eFDR) of 0.02 for station A and 0.05 for

station B (Fig. 9), as calculated according to the procedure outlined elsewhere (Storey and Tibshirani, 2003), using 10000 randomizations of sample classification.

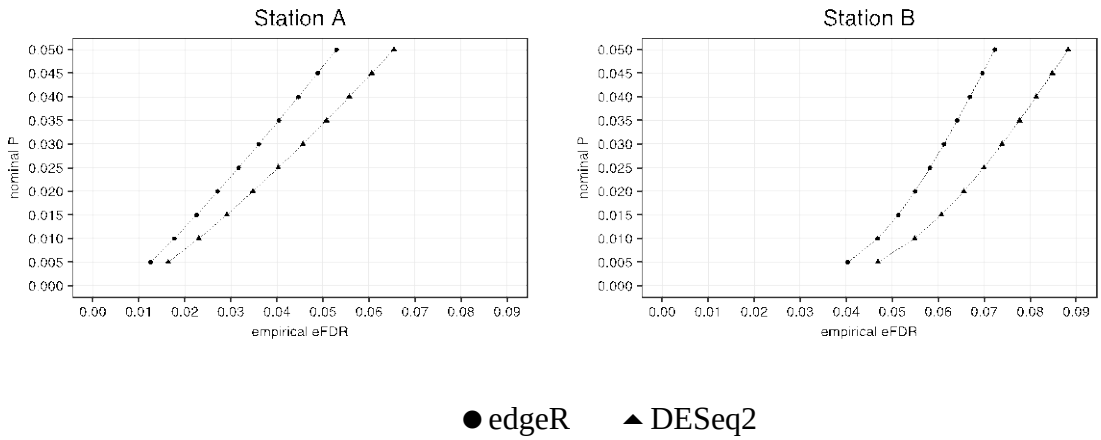


Fig. 9. Correspondence between nominal P values and empirical FDR. Nominal p value is designated on the y-axis, while calculated empirical eFDR is designated on the x-axis.

Validation of DEGs

We used RNA extracted from the PWBC of the 23 heifers from station A and B whose PWBC transcriptome was evaluated though RNA sequencing to confirm the DEGs by Real Time Quantitative Polymerase Chain Reaction (qPCR.) We synthesized complementary DNA from 500ng of total RNA and using oligodT_{15} (Promega, Madison, WI). Reverse transcription was carried out with SuperScriptII (Invitrogen, Carlsbad, CA) following manufacturer's recommendations. The final RT reaction was diluted 1:2 (v:v) and 1µl was used as template for each PCR reaction using Perfecta SYBR Green FastMix (Quanta Biosciences), and 100nM of each primer in a final volume of 10µl. Primers were designed using PrimerBlast application following the recommendations for obtaining target-specific primers for PCR (Ye et al., 2012). The reactions were assayed in a Roche Light Cycler 480 equipment (Roche) equipment with pre-incubation at 95°C for 1 min,

followed by 40 cycles at 95°C for 15 s and 60°C for 45 s. A melting curve was generated using the thermocycler's default parameters to validate the presence of a unique amplicon. The identification of unique amplicon is a proxy of primer specificity. Primer efficiency and cycle threshold (CT) was determined for all reactions using the LinRegPCR program (Ramakers et al., 2003).

We used *GAPDH* as a reference gene, which presented similar CT values across all samples (Fig. 10) and showed no difference of transcript abundance between the groups tested (P>0.9, t-test, Table 8). The ΔCT was calculated for each corresponding target gene relative to the reference gene, and the values of ΔCT were used as input for a t-test to assess the significance of differences between the two groups (Yuan et al., 2006). We inferred that the averages of gene expression levels were statistically different when $P \leq 0.1$. We adopted alpha = 0.1 for qPCR analysis because comparing normalized gene expression levels between groups with 6 samples in each group presents the power of 0.65 to detect an effect of one at the significance level of 0.1.

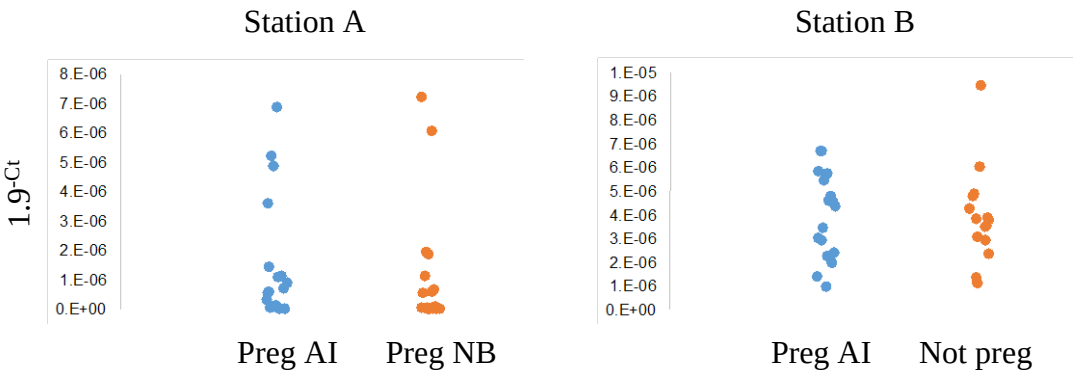


Fig. 10. Real time polymerase chain reaction data points for *GAPDH* transcripts. Please, see discussion in Livak and Schmittgen (Livak and Schmittgen, 2001) and in Schmittgen and Livak (Schmittgen and Livak, 2008) for the rationale on plotting the data as 1.9^{-Ct}. Y-axis denotes the data point for 1.9^{-Ct} and x-axis denotes heifer pregnancy outcome at Station A and Station B.

Table 8. Averages of real time polymerase chain reaction data points (1.9^{-Ct}) for *GAPDH* transcripts and summary of statistical tests to compare the averages.

Station A	Preg AI	Preg NB	F	$P_{(F\ test)}$	T	$P_{(t\ test)}$
	1.5E-06	1.6E-06	0.5950	0.1471	-0.0981	0.9224
Station B	Preg AI	Not preg	F	$P_{(F\ test)}$	T	$P_{(t\ test)}$
	3.9E-06	3.9E-06	0.8207	0.3492	0.0412	0.9674

AI: artificial insemination; NB: natural breeding.

Pairs of genes with expression ratios indicating fertility categorization

Fragments per kilobase per million reads (FPKM) were calculated using the function "rpkm()" from "edgeR". FPKM was used as the input for the calculation of top scoring pairs using the package "tspair" (Leek, 2009). The TSP approach (Geman et al., 2004) identifies genes whose transcript abundance ratios within each individual can classify subjects into binary categories. The ratios of the 20 TSP were used as input for hierarchical clustering of the samples, and the robustness of the clusters was calculated using 5000 randomizations with the R package "pvclust" (Suzuki and Shimodaira, 2006).

Results

Gene expression levels in PWBC associated with pregnancy outcome

We generated over 557.2 million pairs of reads, averaging 20.9 million pairs of reads uniquely aligned to the bovine genome UMD3.1 (Zimin et al., 2009) per sample (Table 9). We quantified expression levels of 12,538 genes in all samples. Of these genes, 10,422 were expressed in PWBC of heifers located at both experimental stations. Furthermore, 1,706 and 410 genes were exclusively expressed in PWBC of heifers located at experimental stations A or B, respectively (Fig. 11a). In order to strengthen the inferences of differentially expressed genes between heifers of differential pregnancy

classification, we analyzed the data from each station independently, and we adopted 2 algorithms implemented in the Bioconductor (Reimers and Carey, 2006) packages edgeR (McCarthy et al., 2012) and DESeq2 (Love et al., 2014). The fold changes estimated by both algorithms were very similar ($r>0.99$, $p<0.0001$) and we used the output from edgeR package to report the fold changes of differential gene expression.

The comparison of gene expression profiles in PWBC between AI-pregnant and NB-pregnant heifers resulted in 18 DEGs (Fig. 11b, eFDR≤0.02), of which *DMBT1*, *ADAM20*, *ALDH5A1*, *GSTM3*, *MNS1*, *P2RY12*, *TTLL1*, *UGT8* showed greater and *ANG*, *BOLA-DQB*, *FCER1A*, *KIR3DL1*, LOC107131247, LOC618633, *LYZ*, *PPP1R1B*, *SIGLEC14*, *TPPP* displayed lower expression levels in NB-pregnant compared to AI-pregnant heifers (Table 10, Fig. 11d). Despite the low number of DEGs, we identified significant enrichment (FDR≤0.002) for the GO biological process "metabolic process" (*ALDH5A1*, *GSTM3*, *LYZ*, *UGT8*).

The comparison of gene expression profiles in PWBC between AI-pregnant and non-pregnant heifers resulted in 6 DEGs (eFDR≤0.05, Fig. 9, Fig.11c). The genes *ALAS2*, *CNKSR3*, *LOC522763*, *TAC3*, *TFF2* presented greater transcript abundance in non-pregnant heifers, whereas transcripts for *SAXO2* were less abundant in PWBC of non-pregnant heifers compared to heifers that became pregnant to AI (Table 11, Fig. 11e). No significant GO term was identified when these 6 DEGs where tested for enrichment of biological processes or molecular functions.

Table 9. Number of read-pairs generated and aligned uniquely to the *Bos taurus* reference genome UMD 3.1.

Sample	N pairs sequenced	N pairs aligned	Alignment (%)
SL220764	36,128,269	30,457,166	84.3
SL220765	36,260,080	29,334,579	80.9
SL220766	19,966,449	17,252,337	86.4
SL220767	22,775,841	18,158,658	79.7
SL220768	14,805,290	12,403,405	83.8
SL220769	45,845,646	39,345,350	85.8
SL220771	16,970,202	13,927,564	82.1
SL220772	18,827,465	14,700,579	78.1
SL220773	18,309,120	15,031,613	82.1
SL220774	21,112,166	17,454,399	82.7
SL220775	32,485,522	27,287,566	84.0
SL253803	27,341,041	24,446,159	89.4
SL253804	25,477,964	22,971,157	90.2
SL253805	15,799,927	14,684,897	92.9
SL253806	18,093,354	16,667,914	92.1
SL253807	24,358,249	22,330,930	91.7
SL253808	18,861,053	16,636,677	88.2
SL253809	18,352,301	16,562,097	90.2
SL253810	21,052,090	18,776,453	89.2
SL253811	41,238,769	36,636,894	88.8
SL253812	18,757,099	16,756,241	89.3
SL253813	24,846,720	22,760,158	91.6
SL253814	19,575,486	16,879,869	86.2
Average	24,227,831	20,933,159	86.5

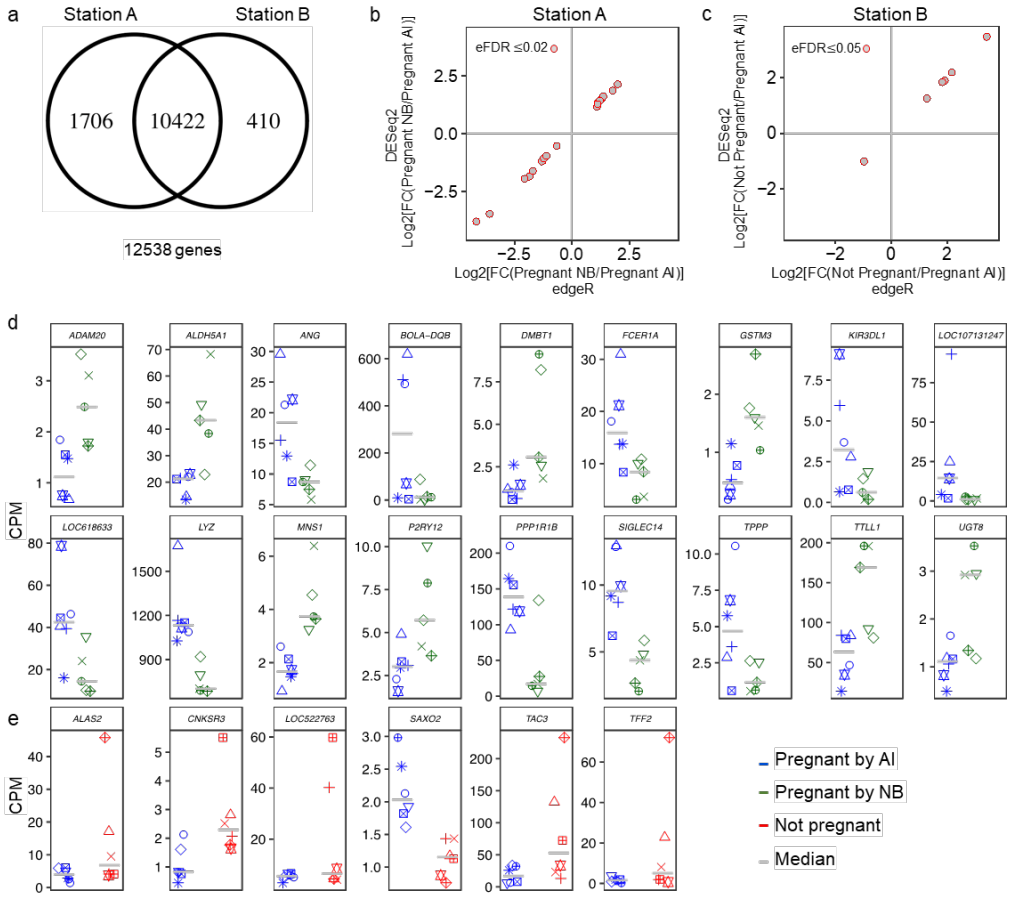


Fig. 11. Gene expression levels associated with pregnancy outcome. (a) Number of genes with expression estimated in PWBCs. (b,c) Fold change profiles obtained by 2 Bioconductor packages highlighting the genes inferred as differentially expressed between the 2 experimental groups. Y-axis denotes fold change ratio obtained from DESeq2; x-axis denotes fold change ratios obtained from edgeR. (d,e) Expression levels (counts per million, CPM; y-axis) for the DEGs between samples from NB-pregnant and AI-pregnant heifers obtained from Station A (d) and between samples from Not-pregnant and AI-pregnant heifers obtained from Station B (e). Within location, the shapes represent the same animals across gene charts.

We selected the genes *ALDH5A1*, *FCER1A*, LOC522763, *SIGLEC14*, *TAC3*, and *TTLL1* for independent assessment of differential gene expression by quantitative real-time polymerase chain reaction. The averages of fold change calculated from the PCR data were correlated to those obtained from RNA-seq (Spearman's correlation=0.94, P<0.02, Table 12). Therefore, we validated the results obtained by RNA-seq.

Table 10. Differentially expressed genes associated with pregnancy originated from artificial insemination or natural breeding.

Ensembl ID	Symbol	Description	LogFC (pregnantNB/pregnant AI)[#]
ENSBTAG00000022715	*DMBT1**	Deleted in Malignant Brain Tumors 1	1.98
ENSBTAG00000001842	*GSTM3*	glutathione S-transferase Mu 3	1.76
ENSBTAG00000012030	*TTLL1*	tubulin tyrosine ligase like 1	1.36
ENSBTAG00000000271	*MNS1*	meiosis specific nuclear structural 1	1.28
ENSBTAG00000021902	*ALDH5A1*	aldehyde dehydrogenase 5 family member A1	1.20
ENSBTAG00000004574	*UGT8*	UDP glycosyltransferase 8	1.13
ENSBTAG00000038377	*ADAM20*	ADAM metallopeptidase domain 20	1.11
ENSBTAG00000015837	*P2RY12*	purinergic receptor P2Y12	1.08
ENSBTAG00000026779	*LYZ*	Lysozyme C, non-stomach isozyme	-0.67
ENSBTAG00000045492	*ANG**	angiogenin, ribonuclease, RNase A family, 5	-1.11
ENSBTAG00000040580	LOC618633*	myeloid-associated differentiation marker-like	-1.22
ENSBTAG00000012887	*FCER1A*	Fc fragment of IgE receptor Ia	-1.28
ENSBTAG00000035868	*SIGLEC14**	Sialic Acid Binding Ig Like Lectin 14	-1.31
ENSBTAG00000047116	*TPPP*	tubulin polymerization promoting protein	-1.70
ENSBTAG00000006035	*PPP1R1B*	protein phosphatase 1 regulatory inhibitor subunit 1B	-1.85
ENSBTAG00000047971	*KIR3DL1*	killer cell immunoglobulin-like receptor, three domains, long cytoplasmic tail, 1 precursor	-2.05
ENSBTAG00000021077	*BOLA-DQB**	major histocompatibility complex, class II, DQ beta	-3.57
ENSBTAG00000047764	LOC107131247	multidrug resistance-associated protein 4-like	-4.15

* Genes annotated manually according to either KEGG pathways, Uniprot or NCBI Entrez databases. [#] LogFC: log fold change output by edgeR package.

Table 11. Differentially expressed genes associated with pregnancy originated from artificial insemination or failure to establish pregnancy.

Ensembl ID	Gene symbol	Description	LogFC(not pregnant/pregnant AI)[#]
ENSBTAG00000030814	*TFF2*	trefoil factor 2	3.42
ENSBTAG00000021807	*TAC3*	tachykinin 3	2.17
ENSBTAG00000001308	LOC522763*		1.92
ENSBTAG00000013178	*ALAS2*	5'-aminolevulinate synthase 2	1.82
ENSBTAG00000012674	*CNKSR3*	CNKSR family member 3	1.28
ENSBTAG00000003414	*SAXO2*	stabilizer of axonemal microtubules 2	-0.98

* Genes annotated manually according to NCBI Entrez databases. [#] LogFC: log fold change output by edgeR package.

Table 12. Validation of RNA-sequencing results contrasting the gene expression in PWBC between heifers with different pregnancy outcome.

Symbol	RNA-seq FC[a]	qPCR FC[a]	qPCR P	mRNA Accession #	Oligonucleotides	Amplicon (bp[b])	$\bar{x}$ PCR Efficiency
GAPDH	*	-	-	NM_001034034.2	TGGTGAAGGTCGGAGTGAAC ATGGCGACGATGTCCACTTT	91	1.9
Location A							
ALDH5A1	1.23	2.50	0.04	NM_001192735.1	CCCCAGCAAAAGAAAGGCG CTTCCGTGTGATCATGGCACT	99	1.9
FCER1A	-1.15	-1.74	0.02	NM_001100310.2	GTGGGCAGAATTCAGAGGCT GCCAGAAATAGTTGCTTTGAGGG	88	1.9
TTLL1	1.19	2.00	0.02	NM_001076171.1	AGAAGGACGAAAGCGGGAAG AACAGGTTGTAGTCGGCAGG	79	1.9
SIGLEC14	-1.22	-1.75	0.09	XM_015458276.1	TCCGGCTCAACGTCTCCTAT CTCCAGGATGGGCAGTGAC	98	1.9
Location B							
TAC3	2.17	3.04	0.05	NM_181017.2	GCACCTTCAAGTACCCTCCA TCTTCACGATGTAGCCCAGG	70	1.9
LOC522763	1.92	8.77	0.10	NM_001102069.1	GCACCGAGCTCTTGACTGAT GTGAAGGCTGAAGCTCAGGA	118	1.9

[a] FC: Fold change (pregnant NB/pregnant AI(Location A); non-pregnant/pregnant AI (Location B)), [b] bp: base pairs.
* *GAPDH* fold change in Station A: 0.7 and Station B: 0.9.

Detection of gene pairs to discriminate heifers pregnant by AI

Next, we used the top scoring pair approach (Geman et al., 2004) to test whether the ratio between transcript levels of 2 genes within samples discriminated heifers presenting distinct pregnancy outcomes. According to this approach, a gene's expression level is compared to the expression levels of all other genes. For instance, in station A, 12,128 genes formed 147,076,256 pairs, and 10,422 genes in station B formed 117,321,392 pairs.

The analysis of the transcriptome data from AI-pregnant and NB-pregnant heifers (station A) resulted in 1,520 pairs of genes that discriminate most of the heifers according to their pregnancy outcome (Overall score=1, P<0.0002, 5000 randomizations). The pair of genes with the greatest discriminatory score was *DTX4* and ENSBTAG00000038233, whereby the transcript levels of *DTX4* are greater than the transcript levels of ENSBTAG00000038233 in NB-pregnant in contrast with AI-pregnant heifers (Fig. 12a). Clustering of the samples using the ratios of transcript levels of the top 20 gene pairs (Fig. 13a) separated the heifers into 2 clusters that followed their pregnancy classification (Fig. 12b, P≤0.01, 5000 randomizations).

Analysis of the transcriptome data from 12 heifers sampled from station B, (AI-pregnant and non-pregnant) resulted in 88 gene pairs identified that separated most of the heifers in 2 groups (Overall score=1, P<0.0002, 5000 randomizations). The genes *U3* and *MMP19* formed the top scoring pair, in which the AI-pregnant heifers presented greater transcript abundance of *U3* compared to *MMP19*, and the opposite direction was observed for the non-pregnant heifers (Fig. 12c). Clustering of the samples using the ratios of transcript levels of the top 20 gene pairs (Fig. 13b) resulted in the formation of 2

clusters that separated the samples by pregnancy outcome (Fig. 12d, P<0.01, 5000 randomizations).

The TSP approach uses within subject transcript levels to calculate ratios between genes, and the analysis does not use variables that may create batch effects in animal experiments (i.e. time, genetic background, location of sampling). Thus, we interrogated the entire dataset (23 samples) under the binary classification of AI-pregnant (N=12) and AI-not-pregnant (N=11). There were 4 genes forming 2 pairs (C11orf54, *TAF1B*; *URB2*, ENSTAG00000039129) that discriminated 10 out of 12 heifers correctly (Fig. 12e, Overall score=0.83). The clustering of 10 out of 12 AI-pregnant heifers independently from NB-pregnant and non-pregnant heifers, showed non-trivial (P<0.003, hypergeometric test) patterns of ratios that identified heifers by pregnancy outcome, and clearly contrasted with ratio patterns obtained from random gene pairs (Fig. 12f).

Discussion

Our main goal was to identify differences in the transcriptome profile in PWBC at the time of AI in beef heifers with different pregnancy outcomes. In our experiment, we identified heifers that became pregnant to AI, to natural breeding, and heifers that failed to become pregnant during the breeding season. Sampling blood from heifers of similar age and other phenotypic parameters within herd was central for us to work with pubertal heifers of similar nutritional status, and thus focus on the differences associated with the physiology of reproduction driving the likelihood of pregnancy in beef heifers. Similar to other models of fertility and infertility in cattle (McMillan and Donnison, 1999; Peterson et al., 1999; Minten et al., 2013; Geary et al., 2016), the categorical pregnancy outcomes adopted in our study identify heifers with distinct fertility potential. In the current study,

we identified that variations in gene expression profiles of PWBC may be associated with the likelihood of a successful fertilization and pregnancy establishment.

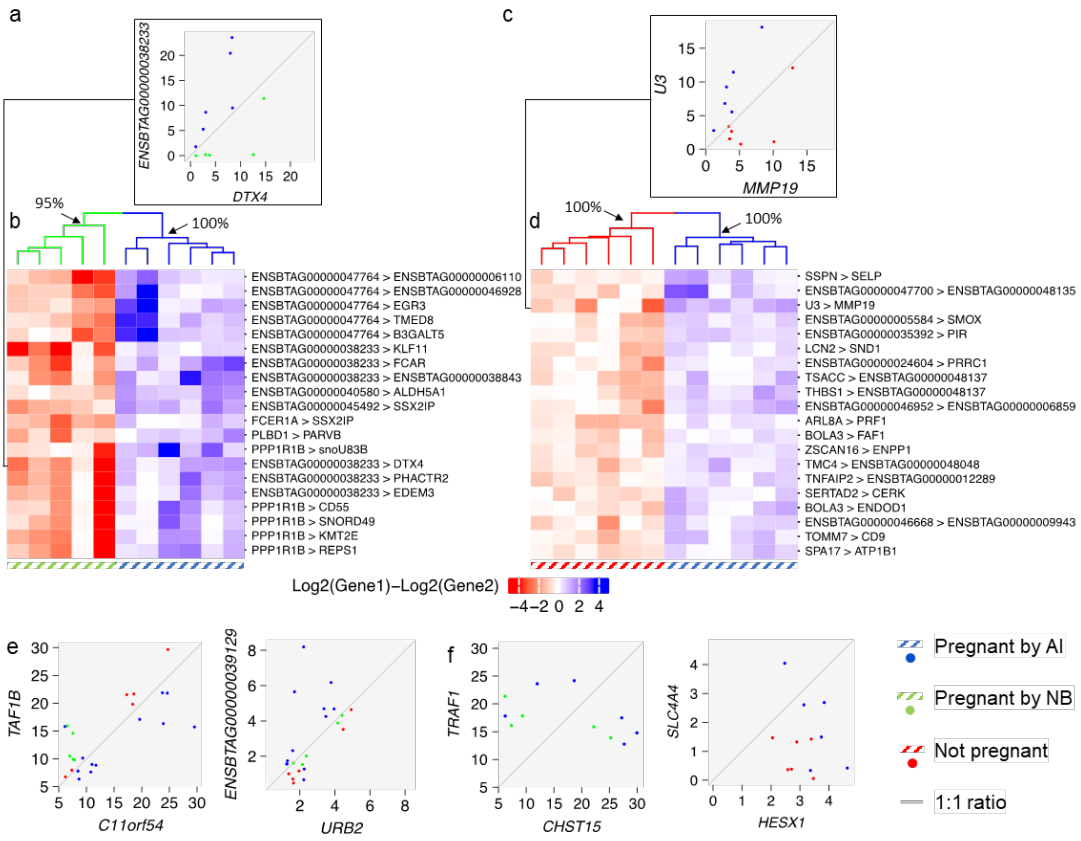
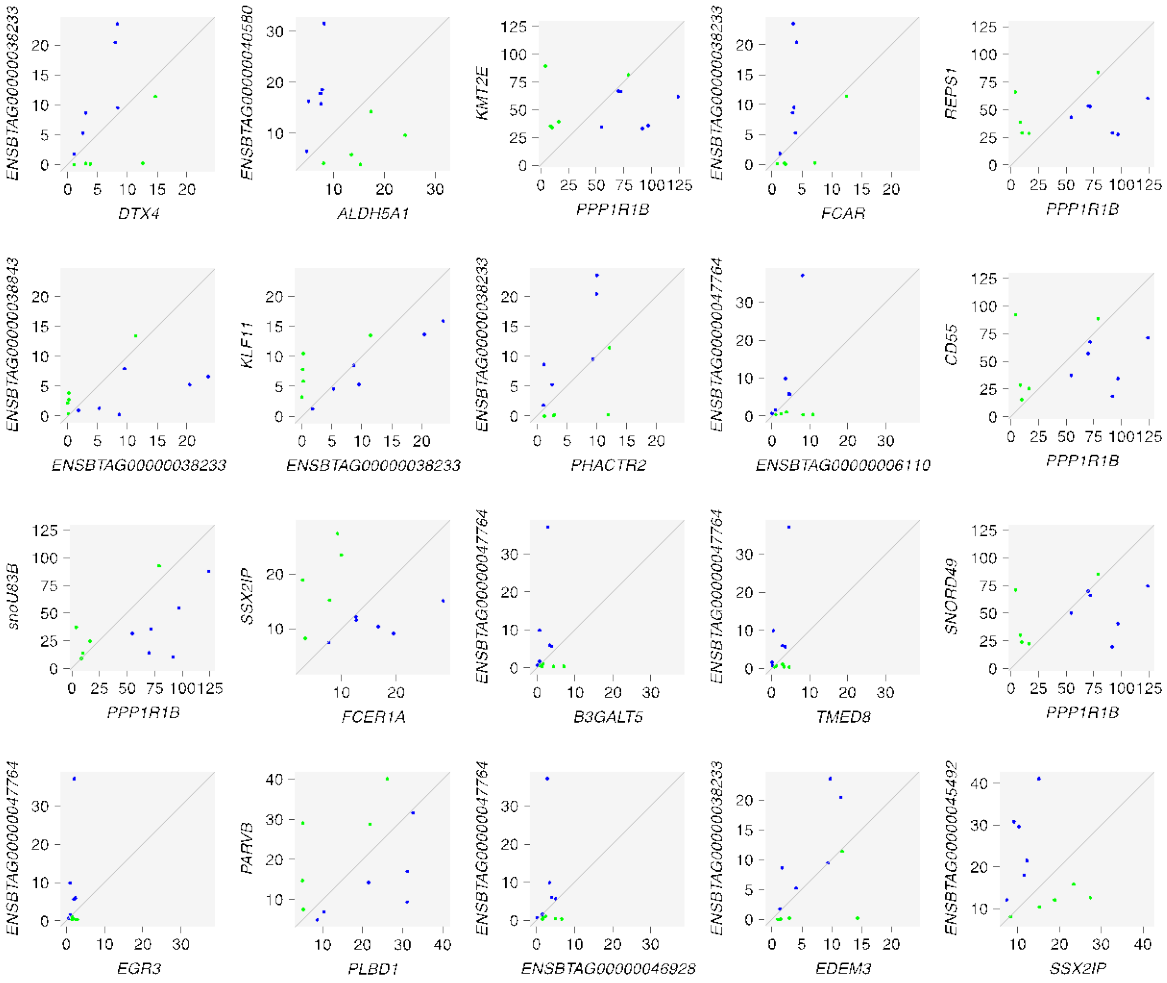


Fig. 12. Top scoring pairs for sample classification. (a,c) Most significant gene pair whose expression ratios discriminate (a) AI-pregnant from NB-pregnant heifers and (c) AI-pregnant from Not-pregnant heifers. Y-axis denotes FPKM expression levels of first gene and x-axis denotes FPKM expression levels of second gene in each pair. (b,d) Heatmap produced from the expression ratios of the top 20 gene pairs that discriminate (b) AI-pregnant from NB-pregnant heifers and (d) AI-pregnant from Not-pregnant heifers. Increasing intensity of blue coloring indicates increasingly positive ratio of gene 1:gene 2; increasing intensity of red coloring indicates an increasingly negative ratio of gene 1:gene 2. (e) Identification of two TSP with significant separation of AI-pregnant heifers from the others (NB-pregnant, non-pregnant); y-axis represents FPKM expression levels of one gene and x-axis represents FPKM expression levels of the 2nd gene in each pair. (f) Pairs of genes that demonstrate the null hypothesis of the top scoring pair approach. Y-axis represents FPKM expression levels of one gene and x-axis represents FPKM expression levels of the 2nd gene in each pair. Blue dots represent samples from AI-pregnant, green dots represent samples from NB-pregnant, and red dots represent samples from Not-pregnant heifers. Diagonal grey line represents a 1:1 ratio expression.

a



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b

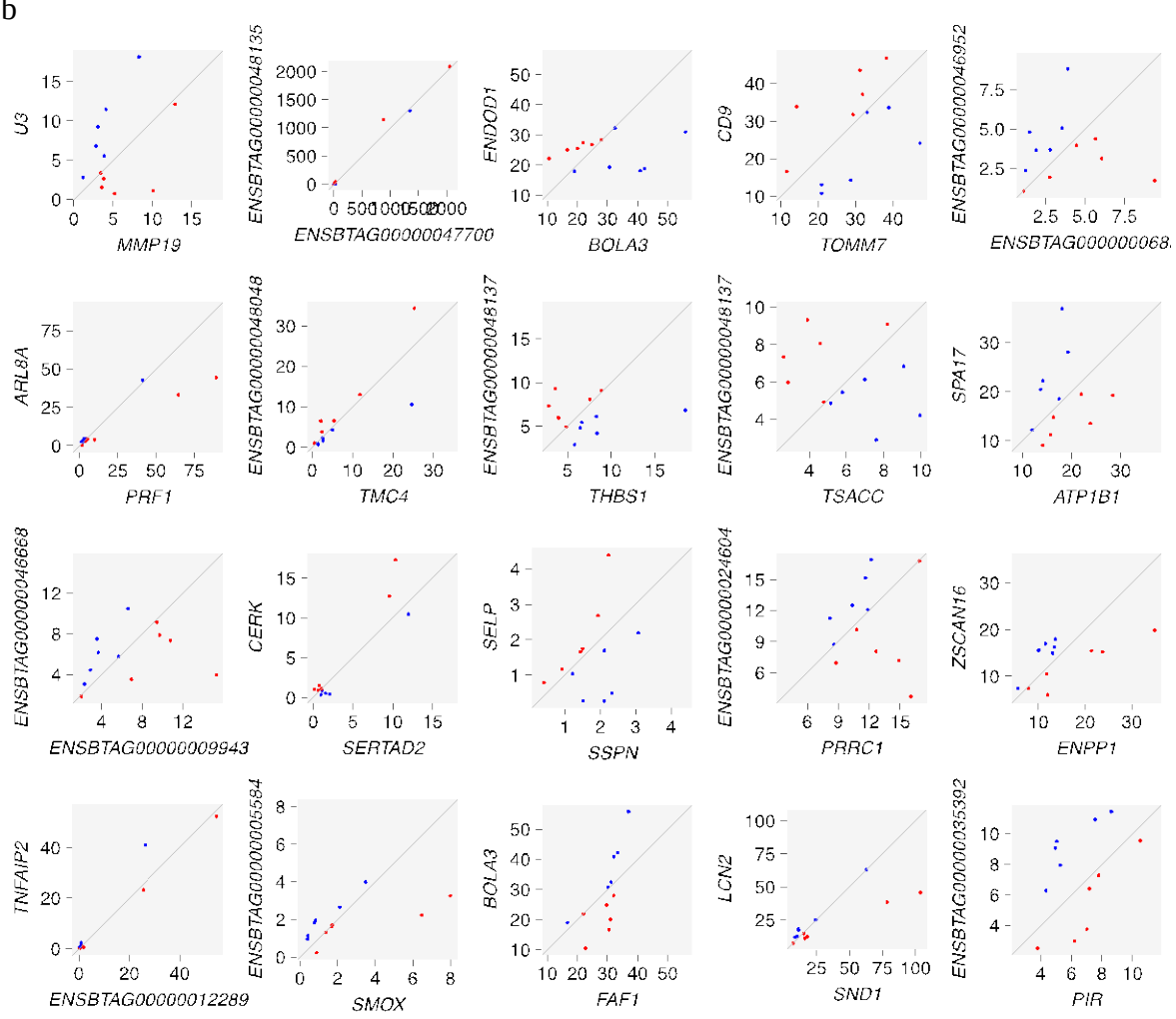

Fig. 13. Scatterplots of expression levels (FPKM) of the top scoring pairs that distinguish heifers according to pregnancy outcome. Top 20 TSPs for Station A (a) and B (b). Y-axis represents FPKM expression levels of one gene and x-axis represents FPKM expression levels of the 2nd gene in each pair. Blue dots represent samples from AI-pregnant heifers, green dots represent samples from NB-pregnant heifers, and red dots represent samples from Not-pregnant heifers. Diagonal grey line represents a 1:1 ratio of expression.

Considering the similarity of the heifers within location, as observed by age and phenotypic records (Table 7), genetic background, reproductive, health, and nutritional management, and other environmental effects within station, one could anticipate the low number of DEGs inferred in this study. Our very stringent approach for inferring DEGs

according to 2 independent algorithms was a reason for this observation. Nonetheless, this strategy (Suárez-Vega et al., 2015) greatly reduces the chance of inferring false positives by leveraging the strengths of both algorithms (Schurch et al., 2016). Other transcriptome investigations of endometrial tissues of beef heifers (Salilew-Wondim et al., 2010; Minten et al., 2013; Geary et al., 2016) or dairy cows (Moore et al., 2016) of different fertility potential yielded DEGs in the order of a few dozen. Of note, no previously identified DEGs have been found in more than one study. Furthermore, none of the DEGs identified in our study were observed in similar investigations focusing on women's fertility (Michou et al., 2003; Thum et al., 2004; Dons'koi et al., 2014). This observation is not surprising given the polygenic and complex physiology involving fertilization and pregnancy in females.

We identified 18 DEGs associated with heifer pregnancy to AI compared to pregnancy from natural breeding. Gene ontology analysis showed significant enrichment of the biological process "metabolic process", which included the genes "aldehyde dehydrogenase 5 family member A1" (*ALDH5A1*), "glutathione S-transferase Mu 3" (*GSTM3*), "UDP glycosyltransferase 8" (*UGT8*), and "Lysozyme C, non-stomach isozyme" (*LYZ*). The gene *ALDH5A1* is part of a family of aldehyde dehydrogenases that metabolizes aldehydes and reduces cellular toxicity. Additionally, there is evidence, in humans, that a functional ALDH5A1 is associated with the concentration of glutathione in the bloodstream (Niemi et al., 2014). Also in humans, it has been hypothesized that upregulation of *GSTM3* is a response to greater presence of cytotoxic products resultant of overabundance of reactive oxygen species (ROS)(Cortón et al., 2007) and the need for the conjugation of ROS to glutathione (Lamoureux and Rusness, 1992) to mitigate the

toxic effects of ROS. As evidence supports the link between oxidative stress and female infertility in humans (Lamoureux and Rusness, 1992; Ruder et al., 2009; Gupta et al., 2014), the upregulation of *ALDH5A1* and *GSTM3* in PWBC suggests that a greater presence of ROS species in the blood stream may reduce the likelihood of pregnancy success to AI in beef heifers, but do not prevent the heifers from becoming pregnant to a bull later in the breeding season.

Although no significant enrichment was observed, it was noteworthy that 4 out of 18 DEGs were related to "cytoskeleton organization" (*MNS1*, *TTLL1*, *TPPP*, *UGT8*). Interestingly, *UGT8* was down-regulated in the endometrium of women affected by implantation failure (Maekawa et al., 2017). The gene *FCER1A* is associated with "positive regulation of granulocyte macrophage colony-stimulating factor production" and was less expressed in NB-pregnant heifers. The down-regulation of the gene *FCER1A* in blood samples is associated with pre-term delivery in women (Enquobahrie et al., 2009). Another gene related to the immune system, namely *KIR3DL1*, showed the lowest transcript abundance (4-fold) in NB-pregnant compared to AI-pregnant heifers. Interestingly, recurrent miscarriage patients presented lower occurrence of *KIR3DL1* in their blood compared to healthy women (Faridi et al., 2009). When expressed in natural killer (NK) cells, KIR3DL1 inhibits (Rajagopalan and Long, 2005) the cytotoxic function or the adhesive capacity of NK cells [reviewed by (Pegram et al., 2011)].

We identified 6 DEGs in the PWBC of heifers associated with the pregnancy outcome of AI-pregnant or non-pregnant. It is critical to notice, however, that the inferences of *ALAS2*, *LOC52273*, *TAC3*, *TFF2* as DEGs, were mostly driven by some heifers that did not become pregnant, whereas others presented gene expression levels

equivalent to the heifers that became pregnant to AI. The gene *TAC3* encodes the protein neurokinin B, whose expression is negatively regulated by ovarian derived steroids (Rance and Bruce, 1994) and in turn stimulates the secretion of Gonadotropin-Releasing Hormone (GnRH) (Lehman et al., 2010), which has central function on the release of follicle-stimulating hormone and luteinizing hormone. On the same note, expression of the gene *CNKSR3* was upregulated by luteinizing hormone in women's endometrium (Humaidan et al., 2012) and follicular granulosa cells in buffalo cows (Rao et al., 2011). In specific heifers, the dysregulation of these 2 genes is suggestive of an alteration in the hormonal feedback between the ovary and the hypothalamic-pituitary axis in some of the heifers that did not become pregnant.

The TSP approach compares the levels of transcript abundance for each possible pair of genes expressed within a sample (Geman et al., 2004), and it has been used as a classification or prediction tool in biomedicine (Geman et al., 2004; Zhao et al., 2010; Czajkowski and Kretowski, 2011; Shi et al., 2011). We employed this approach to evaluate the usefulness of gene expression levels in PWBC at the time of AI for classification of heifers with different pregnancy outcome. For each experiment station, the use of transcript levels for the top 20 pairs of genes clustered AI-pregnant heifers separately from the others with 100% confidence of cluster formation. Because this approach is parameter free (Leek, 2009; Magis and Price, 2012) with the exception of the binary variable that separates subjects into 2 categories, we used the algorithm to identify TSPs in all 23 samples that could identity AI-pregnant heifers. The ratio between the expression levels for 4 gene pairs misclassified only 2 out of the 12 AI-pregnant heifers.

Our investigation focused on PWBC, which are mostly composed of circulating immune cells. The immune system and female fertility are connected at many levels with the reproductive function in cattle [reviewed by Fair (Fair, 2015)], and circulating cells of the immune system respond to reproductive hormones (Athreya et al., 1993; Giglio et al., 1994). Our results show that specific genes have transcript abundance correlated with whether a heifer became pregnant to AI, could become pregnant later by natural breeding, or failed to become pregnant. We hypothesize that PWBC change their transcriptome as the heifers undergo the follicular phase of their estrous cycle. These changes most likely reflect the heifer's readiness for fertilization.

The physiological relationship between the immune system of healthy heifers and their likelihood of becoming pregnant by AI is yet to be studied. In addition, further investigation is required to assess how our results may translate to other herds, especially when accounting for different management strategies, breeds, and genetic background. Although further work is needed to develop robust approaches to identify molecular markers in the transcriptome of PWBC, taken together, our results suggest a window of opportunity for the use of gene expression data as a source of prognostic molecular markers of pregnancy likelihood in beef heifers.

Summary and conclusions

At the time of AI, specific genes expressed in PWBC displayed differential transcript abundance in heifers classified according to their pregnancy outcome (AI-, NB-, non-pregnant). This variable expression is likely associated with the heifers' physiological condition that relates to their fertility at the time of AI. The data suggest that the heifer's metabolic status may be critical for the AI success, and impaired

hormonal regulation is among the multiple factors that may hinge the chances of pregnancy in beef heifers. Further investigation is needed to confirm these hypotheses. Using a parameter free approach, the transcript abundance of specific gene pairs distinguished most AI-pregnant, relative to NB- or non- pregnant heifers. This result showed that the transcriptome of PWBC has a promising potential to be used as a source of data to classify heifers of distinct potential to become pregnant.

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