IMPACTS OF REPRODUCTIVE MANAGEMENT STRATEGIES AND GENETIC MERIT ON REPRODUCTIVE PARAMETERS OF DAIRY HEIFERS

By

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To my family and my fiancée, who supported my decisions, were comprehensive, and always there when I needed

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LIST OF ABBREVIATIONS

ACTB	Beta-actin
AED	Automated estrus detection monitoring device
AI	Artificial insemination
CCR	Cow conception rate
CL	Corpus luteum
CLO	Cloprostenol sodium
CM$	Cheese merit
DIN	Dinoprost tromethamine
DPR	Daughter pregnancy rate
ED	Early diestrus
ET	Embryo transfer
FM$	Fluid merit
GDPR	Genomic daughter pregnancy rate
GHCR	Genomic heifer conception rate
GM$	Grazing merit
HCR	Heifer conception rate
HH	High for GDPR class / High for GHCR class
HighGDPR	High class for GDPR
HighGHCR	High class for GHCR
HL	High for GDPR class / Low for GHCR class
IGF-1	Insulin like growth factor 1
IFN-τ	Interferon τ

IOFC	Income over feed cost
ISG15	Interferon stimulated gene 15
LH	Low for GDPR class /High for GHCR class
LL	Low for GDPR class/ Low for GHCR class
LowGDPR	Low class for GHCR
LowGHCR	Low class for GHCR
ME	Meteestrus
MID	Mid-diestrus
NM$	Lifetime net merit
PBL	Peripheral blood leucocytes
PE	Proestrus
PG	Prostaglandin
PIE	Prostaglandin induced estrus
Preg/Serv	Pregnancy per service
PSPB	Pregnancy specific protein B
Q1	Quartile 1
Q2	Quartile 2
Q3	Quartile 3
Q4	Quartile 4
RIA	Radioimmunoassay
RPL19	Ribosomal protein L 19
SEM	Standard error of the mean
SNPs	Single nucleotide polymorphisms

SPE	Spontaneous estrus
TAI	Timed artificial insemination
$T_{½}$	Half-life
THI	Temperature humidity index
TMR	Total mixed ration
VIS	Visual observation of estrus
21-d PregRate	21-d pregnancy rate
21-d ServRate	21-d service rate

Abstract of Thesis Presented to the Graduate School
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IMPACTS OF REPRODUCTIVE MANAGEMENT STRATEGIES AND GENETIC MERIT ON REPRODUCTIVE PARAMETERS OF DAIRY HEIFERS

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The objectives of this experiment was to evaluate the effects of $PGF_{2\alpha}$ formulations and methods of estrus detection on physiological parameters, estrous behavior, and reproductive performance of dairy heifers. Additionally, the association between fertility traits and physiological parameters, estrous behavior, and reproductive performance of dairy heifers are described. Holstein heifers (n = 1,019) were fitted with an automated estrus detection system (AED) and enrolled in the experiment around 11 months of age. Heifers were assigned to the $PGF_{2\alpha}$ (CLO: cloprostenol sodium or DIN: dinoprost thromethamine) and estrus detection (AED: automated estrus detection or VSI: visual detection of estrus) treatments in a 2 x 2 factorial design. At birth, heifers were genotyped and genomic daughter pregnancy rate (DPR) and heifer conception rate (HCR) were collected. Treatment with CLO increased percentage of heifers detected in estrus within 7 days after treatment and reduced progesterone concentrations at estrus but it had no effect on hazard of pregnancy. Automated estrus detection tended to improve hazard of pregnancy. Genomic daughter pregnancy rate was associated with greater ovulatory follicle size, estradiol concentrations, and estrus expression, whereas GHCR was negatively associated with estrous behavior. Selection of $PGF_{2\alpha}$ may be according to parameters other than

efficacy because reproductive performance was similar between CLO and DIN. Herds with inefficient visual estrus detection may benefit from AED. Selection of heifers for DPR is likely to improve signs of estrus and overall reproductive performance, but additional information is needed before HCR may be used extensively as a selection parameter.

CHAPTER 1
INTRODUCTION

Importance of Reproductive Performance for Dairy Production

The profitability of dairy herds is dependent on the efficiency of milk production, which may be simply evaluated as income over feed cost (**IOFC**). The IOFC is the difference between daily income from milk sales, which represents approximately 88% of the income of dairy operations, and daily cost of feeding lactating cows, which represents approximately 50% of the cost of dairy operations (Santos et al., 2010). Milk production of dairy cows is greatest during early lactation, with peak milk yield generally occurring around 5 to 8 weeks postpartum (Pollott, 2011). During early lactation, feed intake is insufficient to meet the energy and protein requirements of lactation and cows efficiently utilize body energy reserves for milk synthesis (Grummer et al., 2004), resulting in negative energy balance and maximum IOFC. The persistency of lactation, defined as the rate of decline in production after peak milk production (Cole and Null, 2009), is determined by genetics (Cole and Null, 2009), parity (Silvestre et al., 2009), use of recombinant bovine somatotropin (Van Amburgh et al., 1997), among other factors. Regardless of genetic composition of the herd, parity, or management strategies, the decline in milk yield is irreversible and IOFC declines sharply after approximately 100 d postpartum (Ribeiro et al., 2012).

Reproductive efficiency determines the percentage of time between two calvings that cows spend in the most profitable phase of their lactation. For example, if maximum IOFC is obtained in the first 60 d postpartum, cows in a herd with an average calving interval (interval between two consecutive calvings) of 16 months (485 d) would spend approximately 12% of this interval at maximum profitability. On the other hand, cows in a herd with an average calving interval of 12 months (364 d) would spend approximately 17% of this interval at maximum

profitability. Aside from decreasing average IOFC of the herd, reproductive inefficiency results in herds having cows with a wider distribution of days postpartum and a larger proportion of cows with extended lactation. Therefore, herds with inefficient reproductive management may require changes in nutritional management to prevent losses. Cabrera and Kalantari (2016) reviewed the literature and determined that having 3 different TMR instead of 2 different TMR would increase the IOFC because it would reduce waste from feeding low producing cows energy and protein rich diets. Other economic losses incurred from poor reproductive performance are increased culling because of reproductive failure (Machado et al., 2017), retention of larger number of replacement heifers (Kaniyamattam et al., 2016), reduced selection pressure on replacement heifers and, consequently, reduced genetic progress of the herd (Kaniyamattam et al., 2016). Thus, the objective of reproductive programs for lactating dairy cows is to increase 21-d pregnancy rates (**21-d PregRate**; percentage of eligible cows that become pregnant every 21 d after the end of the voluntary waiting period), through improvements in 21-d service rate (**21-d ServRate**; percentage of eligible cows that are serviced every 21 d after the end of the voluntary waiting period) and pregnancy per service (**Preg/Serv**; percentage of cows that conceive after a service), and maximize annuity value per cow per year (Neves and LeBlanc, 2015).

Reproductive Management of Dairy Heifers

For the reasons discussed previously, reproductive performance of lactating dairy cows is extremely important for financial success of dairy operations and is generally an area in which dairy owners, managers, and consultants spend significant time and resources on. Cost of rearing replacement heifers are lower than costs of feeding and managing the lactating herd, but still represents approximately 25% of the total cost of dairy operations (Santos et al., 2010) and is

second only to feeding the lactating herd (Gabler et al., 2000). Despite the importance of optimum replacement heifer rearing for the profitability and future of dairy operations, managers and consultants generally dispend less resources and time on reproductive management of heifers.

Inefficient reproductive management of heifers may result in a wide range of age at first calving (Ettema and Santos, 2004) and increased rearing costs of heifers (Stevenson et al., 2000). Aside from the direct impact of reproductive inefficiency on profitability of dairy herds by increasing age at first calving, inefficient reproductive management of heifers impacts milk production, reproductive performance, and health during the first lactation and productive life (Gabler et al., 2000; Ettema and Santos, 2004).

The goal of the reproductive management of Holstein heifers is to establish pregnancy at the appropriate size (60 to 65% of the mature body weight and 125 cm of wither height) at a reduced age (12 to 14 months of age) to shorten the interval from birth to the onset of the first lactation (Hoffman, 1997). Similarly to lactating cows, producers aim to increase 21-d PregRate of dairy heifers by increasing 21-d ServRate and Preg/Serv. In addition to improving heifer health and rate of growth, herds should adopt estrous synchronization or ovulation synchronization protocols to assure that heifers are serviced soon after achieving the desired weight and height (Penteado and Dias, 2013). Furthermore, genetic selection for reproduction traits associated with faster establishment of pregnancy (e.g. daughter pregnancy rate – **DPR**) should also be a part of the long term management of replacement heifers (Jonas and de Koning, 2015).

Manipulation of the Estrous Cycle of Dairy Heifers Using Reproductive Hormones

Reproductive management of heifers in US dairy herds is mainly based on visualization of spontaneous estrus (57.1%) and natural service (33.2%; NAHMS, 2007). Synchronization of estrous of dairy heifers with $PGF_{2\alpha}$ has the potential to increase 21-d ServRate compared with detection of spontaneous estrus without any detrimental effect to Preg/Serv (Stevenson et al., 2008). Prostaglandin $F_{2\alpha}$ treatment induces luteolysis of corpus luteum causing a decrease in progesterone concentration, growth of the dominant follicle, and synchronized estrous within 2 to 7 d after treatment (Martins et al., 2011a). Therefore, when $PGF_{2\alpha}$ treatment is combined with accurate detection of estrus, 21-d ServRate and 21-d PregRate should be greater compared with visualization of spontaneous estrus.

In dairies in which labor and systems for estrus detection are limiting factors, reproductive hormones (GnRH, $PGF_{2\alpha}$, progesterone inserts) for synchronization of ovulation and fixed time artificial insemination may be used. Ovulation synchronization protocols commonly used for lactating dairy cows (e.g. Ovsynch) tend to yield poor Preg/Serv in dairy heifers because while a large proportion of lactating dairy cows have 2 follicular waves, 44% of dairy heifers have three or more follicular waves (Sartori et al., 2004). New ovulation synchronization protocols with reduced interval from follicular wave recruitment to induction of ovulation, however, have yielded acceptable Preg/Serv in dairy heifers (Lima et al., 2013; Silva et al., 2015).

Tools for Estrus Detection in Dairy Heifers

The success of reproductive management of dairy heifers based on detected estrus is highly dependent on the efficiency and accuracy of estrus detection. The primary sign of estrus is an animal standing to be mounted (Forde et al., 2011). The duration of estrus was 14.0 ± 0.8 h and the number of times heifers were mounted when in estrus was 50.1 ± 6.4 events/heifer

among beef heifers (Stevenson et al., 1994) and the duration of estrus of dairy heifers was 9.7 ± 5.3 h (Yoshida et al., 2009). Therefore, unaided visualization of heifer standing to be mounted is quite difficult. Automated systems for detection of mounting activity (e.g. HeatWatch) are rarely used on commercial farms because they are cumbersome and expensive. An indirect estrus detection method commonly used by dairy farms is tail painting and mounting patches (e.g. Kamar), which are rubbed off or 'activated' when heifers are mounted (Kamphuis et al., 2012). These systems require daily monitoring of heifers to re-apply tail paint when necessary and to diagnose which heifer are rubbed off or activated, likely a consequence of mounting activity in the previous 12 or 24 h. If such systems are used but heifers are not monitored daily, inaccurate estrus detection and reduced Preg/Serv may result.

A multitude of automated estrus detection monitoring (**AED**) systems are available in the USA and each one has its nuances. In general, AED systems determine the occurrence of estrus according to changes in patterns of behaviors such as steps/walking, activity, and rumination (Chanvallon et al., 2014; Fricke et al., 2014b). Thus, most AED systems used in commercial dairy farms detect the occurrence of estrus based on secondary signs of estrus. Figure 1-1. depicts the activity and rumination graphs generated by the DataFlow2© software (SCR Inc., Netanya, Israel), one of the commercially available AED systems. The AED system in question records activity and rumination in 2-h intervals. Through a mathematical algorithm, the software calculates the momentary deviation of the activity/rumination from the average activity/rumination in the same time period during the previous 7 days. As seem in figure 1-1. deviations in activity/rumination from the animal's normal pattern are identified as estrus (depicted by the cow mounting symbol). Although differences among AED systems exist, they

generally utilize pedometers, 3D accelerometer, and microphones to record steps, activity, and rumination, respectively, and detect estrus through secondary signs.

Despite detecting estrus based on secondary signs of estrus, the sensitivity and specificity of AED detected estrus compared with ovulation determined by ultrasonography or visual observation of mounting activity are > 90% (Valenza et al., 2012; Dolecheck et al., 2015). Valenza et al. (2012) demonstrated a high level of agreement between an AED system based on changes in activity (SCR Engineers Ltd., Netanya, Israel) and a mounting detector (Kamar heatmount detector, Kamar Inc., Steamboat Springs, CO). Furthermore, standing to be mounted, the principal characteristic of cattle in estrus, was positively associated with duration of estrus and activity peak measured by an AED system (Silper et al., 2015b). In addition to providing continuous 24-h monitoring of individuals, AED systems remove human subjectivity from estrus detection (Reith and Hoy, 2017).

Strategies for Selection of Dairy Heifers with Improved Reproductive Performance

A large number of genetic traits that affect overall profitability of dairy operations are available for dairy producers to select animals (Calus et al., 2013). The most common strategy used to overcome questions regarding which traits to select for is to use an index, which is a composite of the most important traits the dairy desires to select for or against (Dekkers, 2007). The USDA Animal Improvement Programs Laboratory provides a few indexes for general use, such as the lifetime net merit (**NM$**), cheese merit (**CM$**), fluid merit (**FM$**), and grazing merit (**GM$**). All these traits include production related traits (e.g. yields of milk, fat, and protein), fertility related traits (e.g. daughter pregnancy rate – **DPR**, heifer conception rate – **HCR**, and cow conception rate – **CCR**), somatic cell score, productive life, functional type traits, and calving ability traits (Cole, 2017).

Decisions regarding which indexes to use and whether or not to create one's own index depend on several farm and market specific conditions. Nonetheless, the focus on selection for Holstein cattle for milk yield and type traits with disregard for functional traits such as reproduction traits resulted in a significant decrease in reproductive efficiency from the 50s to the early 2000s (Lucy, 2001). Thus, modern selection for Holstein cattle has been partly focused on improving fertility. Since the mid 2000's, NM$ has included reproduction traits such as DPR, introduced in 2004 (VanRaden et al., 2004), and CCR and HCR, introduced later (Kuhn et al., 2006). Daughter pregnancy rate is a measure of the hazard of pregnancy establishment of a bull's daughters compared with the population, whereas CCR and HCR are measures of the likelihood of pregnancy following a service of a bull's daughters compared with the population of lactating cows and heifers, respectively.

With advancements in technology, sequencing the genome of dairy cattle has become less expensive and readily available, allowing producers to genotype large populations of animals (García-Ruiz et al., 2016). The large-scale genotyping of cattle populations has produced high reliability genomic predicted transmitting ability (**GPTA**) values for several economically important traits (VanRaden et al., 2009). These traits started to be used first for the selection of sires, such that nearly all sires used for semen collection in the USA today are genomically tested, and are now commonly used for selection of female cattle (Wiggans et al., 2011). Genomic testing has had a great impact on genetic selection of dairy cattle because of the improved reliability, the reduced generation interval (faster selection of sires and dams with no need for progeny testing), and consequently faster genetic gain (García-Ruiz et al., 2016). Traits that have had historically low heritability, such as fertility traits, may benefit further from

genomic selection because of the increased accuracy of parental information and reliability of the test (García-Ruiz et al., 2016).

A

B

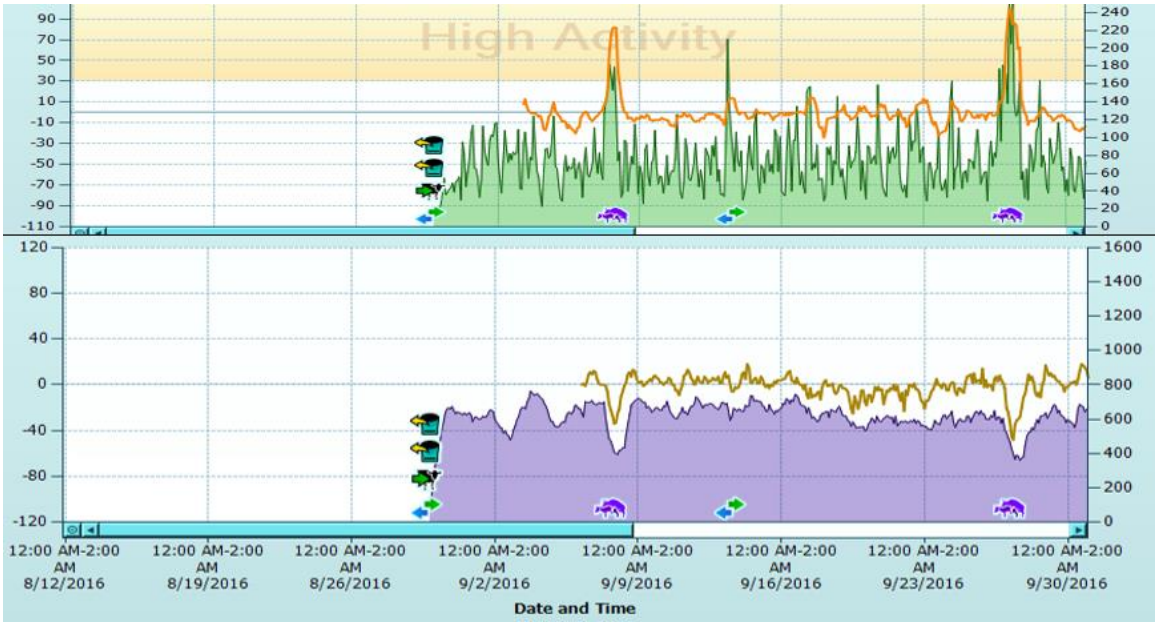

Figure 1-1. Activity data (green bars) and deviation (brown line; panel A) and rumination data (purple bars) and deviation (brown line; panel B). DataFlow2© (SCR Ltd., Netanya, Israel).

CHAPTER 2
EFFECTS OF TWO DIFFERENT PROSTAGLANDIN $F_{2\alpha}$ FORMULATIONS AND METHOD OF ESTRUS DETECTION ON ESTROUS CHARACTERISTICS AND REPRODUCTIVE PERFORMANCE OF DAIRY HEIFERS

Lifetime milk production and health of lactating cows are closely related to age and weight at first calving (Ettema and Santos, 2004). The objective of reproductive programs designed for dairy heifers is to have the majority of heifers calving in the stipulated time (≤ 24 months of age) and weight (560 Kg of live weight immediately after calving) to avoid large variations of age at first calving (Stevenson et al., 2008). Prostaglandin (**PG**) $F_{2\alpha}$ treatments fourteen days apart can be used to increase estrus rate, consequently increasing 21-d service rate (**21-d ServRate**) and improve overall success of reproductive programs for dairy heifers (Stevenson et al., 2000; Lopes et al., 2013). Currently available $PGF_{2\alpha}$ formulations include dinoprost tromethamine (DIN), a formulation composed of a molecule similar to endogenous $PGF_{2\alpha}$ that has a relatively short half-life (T½ ~ 9 min; Shrestha et al., 2012), and cloprostenol sodium (CLO), a formulation composed of a synthetic analogue of the $PGF_{2\alpha}$ molecule that has a relatively longer half-life (T½ ~ 3 h; Reeves, 1978).

Different authors (Martins et al., 2011a, 2011b; Pursley et al., 2012; Stevenson and Phatak, 2010) hypothesized that the cloprostenol sodium's longer half-life could induce faster and more thorough luteolysis and, consequently, increase 21-d ServRate and 21-pregnancy rate (**21-d PregRate**). Results from those studies, however, were not consistent. Pursley et al. (2012) and Martins et al. (2011b) showed that CLO treatment reduced progesterone concentrations faster, increased percentage of first lactation cows detected in estrus, increased pregnancy per service (**Preg/Serv**) in cows bred 3 and 4 d after the treatment, and increased 21-d PregRate, when compared with DIN treatment. Stevenson and Phatak (2010) showed that CLO treatment decreased percentage of cows with complete luteolysis compared with DIN treatment, but $PGF_{2\alpha}$

formulation did not affect Preg/Serv or 21-d PregRate. Methodologies used by Pursley et al. (2012) and Martins et al. (2011b) were different from methodologies used by Stevenson and Phatak (2010) and could make comparison of their results difficult. It is important to point out that both, Pursley et al. (2012) and Martins et al. (2011b), demonstrated that CLO treatment increased estrus detection and pregnancy rate among first lactation cows compared to DIN treatment. The authors speculated that reduced dry matter intake in primiparous cows compared with multiparous cows were the reasons of the different responses to CLO between primiparous and multiparous cows. According to the authors, lower dry matter intake in primiparous cows would result in lower hormonal clearance (Sangsritavong et al. 2002; Wiltbank et al., 2006) and longer $PGF_{2\alpha}$ half-life, greater luteolysis, and more intense behavioral estrous. Prostaglandin $F_{2\alpha}$ however, is metabolized and converted into a non-active molecule (13, 14-Dihydro $PGF_{1\alpha}$) mainly in the lungs (Shrestha et al., 2012). To the best of our knowledge, literature discusses possible association between high dry matter intake and high blood flow to the liver (Sangsritavong et al., 2002; Wiltbank et al., 2006), but whether the lungs would also have high blood flow due to high dry matter intake is uncertain. Furthermore, Pursley et al. (2012) and Martins et al. (2011b) did not measure dry matter intake of cows in their experiments. Thus, reasons for CLO to improve percentage of primiparous cows detected in estrus but not multiparous cows are still unknown.

Increasing estrus rate is important to improve reproductive performance (Lopes et al., 2013); however, increased estrus rate must be accompanied by accurate estrus detection, otherwise it can result in reduced Preg/Serv, compromising reproductive performance of dairy cows and heifers (Fricke et al., 2014b; Stevenson et al., 2014). More recently, automated estrus detection devices (**AED**), which determine estrus based on indirect signs (increased activity,

reduced rumination, etc.), have become more efficient, accurate, and affordable and their use by dairy operations has increased (Denis-Robichaud et al., 2016). Automated estrus detection devices have the ability to determine precisely the onset of estrus and the proper time of breeding, while minimizing human subjectivity during estrus detection on commercial farms (Fricke et al., 2017). Researchers have shown that AED can improve estrus detection rate and consequently 21-d Serv/Rate in dairy cows (Fricke et al., 2014b; Stevenson et al., 2014; Neves et al., 2015).

Therefore, we hypothesized that treatment of dairy heifers with CLO would reduce progesterone at estrus and improve estrus detection, estrous characteristics, service rate, Preg/Serv, and hazard of pregnancy compared with DIN treatment. Thus, our objectives were to evaluate progesterone and estradiol concentrations at estrus, percentage of heifers detected in estrus, estrous characteristics (e.g. duration, rumination nadir, and activity peak) measured by an AED, service rate, Preg/Serv, and hazard of pregnancy of heifers treated with CLO and DIN. Furthermore, we hypothesized that the use of an AED for estrus detection would improve estrus detection rate, service rate, Preg/Serv, and hazard of pregnancy of dairy heifers compared with detection of estrus by visual observation (**VIS**). Thus, our objectives were to evaluate service rate, Preg/Serv, and hazard of pregnancy in heifers detected in estrus by AED and VIS.

Materials and Methods

All procedures involving animals were approved by the animal care and use committee of the University of Florida (protocol #201609559).

Animals, Housing and Management

This study was conduct from March 2016 to December 2016 in a commercial dairy herd with approximately 4,200 replacement heifers, located in north central Florida. One thousand

and nineteen heifers between 10 and 11 months of age were enrolled in the study. All heifers were genotyped within 2 months of birth using a 50k single nucleotide peptide platform commercially available (Clarifide, Zoetis, Parsippany, NJ). Data referent to genomic breeding values for daughter pregnancy rate (**DPR**) and heifer conception rate (**HCR**) recorded within 2 months of birth were used. Starting at 12 months of age heifers were weighed weekly. Heifers with BW ≥ 340 kg were moved to a breeding pen and were treated with prostaglandin (PG) $F_{2\alpha}$ for synchronization of the estrous cycle. Heifers were housed in dry lots, with natural shade and no artificial cooling. The breeding pens had self-locking head stanchions on the feeding area. Heifers were fed twice daily (7:00 AM and 4:30 PM) a TMR formulated to meet or exceed the nutritional requirements of Holsteins heifers weighing ≥ 340 kg of live body weight and gaining 800 to 1,000 g of live body weight per day (NRC, 2001). Weather data (daily average temperature, humidity, and precipitation) from the Gainesville airport, located approximately 40 miles east of the dairy, were used to calculate daily temperature humidity index (THI) and precipitation. The percentages of days during the 30 d prior to and during the 30 d after the start of the reproductive program with THI ≥ 72 were recorded for each heifer. The cumulative precipitation during the 30 d prior to and during the 30 d after the start of the reproductive program were recorded for each heifer.

Automated Estrus Detection Device and Estrous Characteristics

At enrollment, an AED (Heat Rumination Long Distance, SCR Inc., Netanya, Israel) mounted on a collar was fitted on the left, cranial area of the neck of all heifers. The device determined activity through an accelerometer and rumination based on sounds of regurgitation and mastication through a microphone. Activity and rumination data were recorded for 2-h intervals. Estrus was determined according to changes in patterns of activity and rumination

within a 2-h interval compared with the average activity and rumination of the same period in the previous 5 and 7 d, respectively (DataFlow2©, SCR Inc, Netanya, Israel). An internal algorithm of the DataFlow2© software produced a heat index (0 = no estrus, 100 = maximum) according to the intensity of changes in activity and rumination. Daily, study personnel evaluated the activity and rumination patterns of heifers determined to be in estrus by the DataFlow2© software. On the day heifers were moved to the breeding pen, heifers with heat index < 50, duration of estrus < 6 h, and no change in rumination time were determined to have changes in activity pattern due to pen movement and not due to estrus and were, therefore, not inseminated. Heat index, activity peak (0 = no estrus, 100 = maximum activity), and rumination nadir (maximum difference in rumination time within a 2-h period during estrus compared with the average rumination of the same period in the previous 7 d) were recorded daily for all heifers in estrus. Study personnel evaluated each activity graph individually and determined the time of onset (2-h period when the activity threshold was surpassed), peak (2-h period when the activity change was maximum), and end (2-h period when the activity change was below the activity threshold) of estrus. Activity threshold was set at three fold above the average activity for the same period in the previous 5 d. Intervals from onset to peak of estrus and from onset to end of estrus were calculated. Characteristics of spontaneous estruses (**SPE**; estruses occurring before the start of the reproductive program) and $PGF_{2\alpha}$ induced estruses (**PIE**; estruses occurring after the start of the reproductive program) were recorded. Automated estrus detection monitor devices were removed from heifers at pregnancy diagnosis (28 d after service), when heifers received a second service, and when heifers were not detected in estrus within 28 d after the start of the reproductive program.

Study Design and Treatments

The study fallowed a completely randomized factorial design with 2 $PGF_{2\alpha}$ formulations (**PGFTRT**) x 2 estrus detection methods (**EDTRT**). Before the start of the reproductive program, heifers were randomly assigned to receive cloprostenol sodium (**CLO**, n = 505; Estrumate, Merck Animal Health, Summit, NJ) or dinoprost tromethamine (**DIN**, n = 490; Lutalyse, Zoetis, Parsippany, NJ) and for estrus detection by an automated estrus detection system (**AED**, n = 530; Heat Rumination Long Distance, SCR Inc., Netanya, Israel) or estrus detection by visualization of mounting activity or activation of a tail paint device (**VIS**, n = 465; Kamar, Kamar inc., Steamboat Springs, CO). When heifers were eligible to start the reproductive program (≥ 12 months of age and ≥ 340 kg of live body weight), a list containing animal's identification and respective treatments was available for study personnel at the dairy. Heifers were classified according to estrous cycle phase into metestrus (day 0 to 3), early diestrus (day 4 to 6), mid-diestrus (day 7 to 17), proestrus (day ≥ 18), and no estrus observed. Heifers in metestrus were treated with the assigned $PGF_{2\alpha}$ formulation 96 h later and heifers in early diestrus, mid-diestrus, and proestrus and heifers that had not had AED detected estrus were treated with the assigned $PGF_{2\alpha}$ formulation immediately. Heifers not serviced within 14 d of the first $PGF_{2\alpha}$ treatment received a second treatment with the same $PGF_{2\alpha}$ formulation. Heifers assigned to estrus detection method AED, did not receive a tail paint device at the beginning of the reproductive program, and were serviced at AED detected estrus informed by study personnel. Heifers assigned to estrus detection method VIS had a tail paint device placed by study personnel at the beginning of the reproductive program, and were serviced at estrus detected by farm personnel. According to the genetic selection program of the dairy, heifers were selected to receive artificial insemination (AI) or to receive embryo transfer (ET). Heifers detected in estrus were AI on the same morning or received an embryo 6 to 9 days after estrus

detection. As mentioned previously, all heifers had an AED fitted and estruses recorded, but estruses recorded by the AED system were reported to farm personnel only for heifers enrolled in the AED treatment.

Pregnancy Diagnoses and Reproductive Data

All heifers were examined for pregnancy by palpation per rectum of the uterine contents at 35 ± 3 d after the detected estrus that resulted in AI or ET. Pregnant heifers were re-examined by palpation per rectum of the uterine contents at 75 ± 3 d of gestation.

Pregnancy per service was calculated by dividing the number of heifers pregnant at 35 and 75 ± 3 d after estrus by the number of heifers serviced. Pregnancy loss was calculated by dividing the number of heifers not pregnant at 75 ± 3 d after service by the number of heifers pregnant 35 ± 3 d after service. Data regarding sire of insemination, sire and dam of embryo transfer, service technician, and reproductive outcomes were collected from an on-farm software (PCDART; Dairy records management system, Chapel Hill, NC).

Blood Sampling

In a subgroup of animals (n = 91), blood was sampled on the day of $PGF_{2\alpha}$ treatment and on the first morning after estrus was detected (1 to 24 h after onset of estrus). Blood was sampled by puncture of the coccygeal vein or artery into evacuated tubes containing K2 EDTA (Vacutainer, Becton Dickinson, Franklin Lakes, NJ). Immediately upon collection, tubes were placed in ice and kept refrigerated until transported to the laboratory for processing, within 2 to 3 h. Blood tubes were centrifuged at 1,500 × g for 15 min. Aliquots of plasma were frozen at −80 °C until assayed.

Analysis of Plasma Samples

Progesterone concentrations in plasma was determined by radioimmunoassay (**RIA**) using a commercial kit (Coat-a-Count, MP Biomedical LLC, Solon OH). Plasma harvested from heifers on days 4 (~1 ng/mL) and 10 (~ 4 ng/mL) of the estrous cycle were incorporated into each assay and used to calculate the CV. Intra and inter-assay CVs were 5.8 and 10.5 % respectively. Serum concentration of estradiol-17β were quantified by RIA as described previously by Jinks et al. (2013). Intra-assay coefficient of variance for estradiol assays was 2.73%.

Statistical Analysis

Data was analyzed using SAS version 9.3 (SAS Institute Inc., Raleigh, NC). Continuous variables were analyzed by ANOVA using the MIXED procedure. Data were evaluated for normality and homogeneity of residuals after fitting the model. Data violating the assumptions of normality were transformed before analysis. Rumination nadir values were multiplied by -1 and transformed to the natural log before analysis. Thus, positive rumination nadir values were excluded (n = 16). Outlier detection was performed, and rumination nadir transformed values < 2 were considered outliers and removed from the analysis (n = 4).

Likelihood of activity peak ≥ 80, heat index ≥ 80, pregnancy at 35 and 75 ± 3 d after service, and pregnancy loss between 35 and 75 ± 3 d after service were analyzed by logistic regression using the LOGISTIC procedure of SAS. Hazard of estrus, first service, second service and pregnancy were analyzed by the Cox proportional hazard ratio using the PHREG procedure of SAS. Interval from $PGF_{2\alpha}$ treatment that induced estrus to onset of estrus, interval from $PGF_{2\alpha}$ treatment to first service, interval from first service to second service, and interval from $PGF_{2\alpha}$ treatment to pregnancy were analyzed by the Wilcoxon test of equality using the LIFETEST

procedure of SAS. Concentrations of progesterone at $PGF_{2\alpha}$ treatment and at estrus were analyzed using a non-parametric procedure (Kurskal-Wallis; NPAR1WAY procedure).

Statistical models to evaluate characteristics of PIE included $PGF_{2\alpha}$ formulation, estrous cycle phase at $PGF_{2\alpha}$ treatment, number of $PGF_{2\alpha}$ treatments prior to the first AED detected estrus, and percentage of days with THI ≥ 72 and cumulative precipitation 30 days after the start of the reproductive program. Heifers that had been detected in estrus by the AED > 26 d prior to $PGF_{2\alpha}$ treatment (n = 10) and heifers detected in estrus > 168 h after the $PGF_{2\alpha}$ treatment (n = 106) were not included in the analysis of PIE estrous characteristics. Genomic breeding values for DPR and HCR were also included in the model to control for a possible influence of genotype on the outcomes.

For the analysis of the hazard of estrus, models included $PGF_{2\alpha}$ formulation, estrous cycle phase at the time of $PGF_{2\alpha}$ treatment, number of $PGF_{2\alpha}$ treatments prior to the first detected estrus, and percentage of days with THI ≥ 72 and cumulative precipitation 30 days after the start of the reproductive program. Genomic breeding values for DPR and HCR were also included in the model to control for a possible influence of genotype on the evaluated outcomes. When $PGF_{2\alpha}$ formulation and estrous cycle phase at $PGF_{2\alpha}$ treatment were associated with the hazard of estrus after $PGF_{2\alpha}$ treatment, the Wilcoxon test of equality (LIFETEST procedure) was used to characterize the association between $PGF_{2\alpha}$ formulation and estrous cycle phase at $PGF_{2\alpha}$ treatment and the interval from $PGF_{2\alpha}$ treatment that induced estrus to estrus.

Statistical models to evaluate pregnancy at 35 and 75 ± 3 d after service, pregnancy loss between 35 and 75 d for the first service included $PGF_{2\alpha}$ formulation, estrus detection method, the interaction between estrus detection method and $PGF_{2\alpha}$ formulation, estrous cycle phase at the $PGF_{2\alpha}$ treatment, the interaction between estrous cycle phase and $PGF_{2\alpha}$ formulation, the

interaction between estrus detection method and estrous cycle phase, technician, and percentage of days with THI ≥ 72 and cumulative precipitation within 30 days after the start of the reproductive program. Genomic breeding values for DPR and HCR were also included in the model to control for a possible influence of genotype on the evaluated outcomes. Statistical models to evaluate pregnancy at 35 and 75 ± 3 d after service and pregnancy loss after ET services also included embryo type (fresh in vivo produced embryo, frozen/thawed in vivo produced embryo, fresh in vitro fertilized embryo, and frozen/thawed in vitro fertilized embryo), embryo grade (excellent/good, fair, and poor), and days after estrus at embryo transfer (6 to 9 d).

For the analysis of the hazard of first service, models included $PGF_{2\alpha}$ formulation, estrus detection method, the interaction between estrus detection method and $PGF_{2\alpha}$ formulation, estrous cycle phase at the $PGF_{2\alpha}$ treatment, the interaction between estrous cycle phase and $PGF_{2\alpha}$ formulation, the interaction between estrus detection method and estrous cycle phase, and percentage of days with THI ≥ 72 and cumulative precipitation within 30 days after the start of the reproductive program. Genomic breeding values for DPR and HCR were also included in the model to control for a possible influence of genotype on the evaluated outcomes. When $PGF_{2\alpha}$ formulation and estrus detection method were not associated with the hazard of the first service, the Wilcoxon test of equality (LIFETEST procedure) was used to characterize the association between $PGF_{2\alpha}$ formulation on the interval from $PGF_{2\alpha}$ treatment to first service.

For the analysis of the hazard of second service, models included $PGF_{2\alpha}$ formulation, estrus detection method, the interaction between estrus detection method and $PGF_{2\alpha}$ formulation, and percentage of days with THI ≥ 72 and cumulative precipitation within 30 days after the start of the reproductive program. Genomic breeding values for DPR and HCR were also included in the model, to control for a possible influence of genotype on the evaluated outcomes. The

Wilcoxon test of equality (LIFETEST procedure) was used to characterize the association between $PGF_{2\alpha}$ formulation and the interval from first service to second service.

For the analysis of the hazard of pregnancy, models included $PGF_{2\alpha}$ formulation, estrus detection method, the interaction between estrus detection method and $PGF_{2\alpha}$ formulation, estrous cycle phase at the $PGF_{2\alpha}$ treatment, the interaction between estrous cycle phase and $PGF_{2\alpha}$ formulation, the interaction between estrus detection method and estrous cycle phase, and percentage of days with THI $\geq$ 72 and cumulative precipitation within 30 days after the start of the reproductive program. Genomic breeding values for DPR and HCR were also included in the model to control for a possible influence of genotype on the evaluated outcomes. When $PGF_{2\alpha}$ formulation and estrus detection method were not associated with the hazard of pregnancy, the Wilcoxon test of equality (LIFETEST procedure) was used to characterize the association between estrus detection method and the interval from $PGF_{2\alpha}$ treatment to pregnancy.

Models for estradiol concentrations after estrus was detected included $PGF_{2\alpha}$ formulation, estrous cycle phase at $PGF_{2\alpha}$ treatment, interval from onset of estrus to sample collection, and pregnancy at 35 ± 3 d. Models for progesterone concentrations after estrus was detected only included $PGF_{2\alpha}$ formulation.

For each of the statistical models collinearity was tested using the REG procedure of SAS with the "collin" and "VIF" functions. Variables with variance inflation factors $\geq$ 1.5 were considered collinear. In such cases, each variable was added to the model separately and the variable with the smallest *P* - value was retained. A backward stepwise elimination of variables with $P > 0.10$ until variables that remained in the model had $P < 0.10$. Statistical significance was considered at $P < 0.05$ and a tendency was consider when $0.05 < P \leq 0.10$.

Results

Age and Body Weight of Study Population

Mean age at $PGF_{2\alpha}$ treatment were CLO/AED = 376 ± 4 d, CLO/VIS = 377 ± 7 d, DIN/AED = 378 ± 6 d, and DIN/VIS = 378 ± 7 d. Mean (±SEM) weight at $PGF_{2\alpha}$ treatment were CLO/AED = 388.3 ± 26.3 Kg; CLO/VIS = 385.6 ± 27.5 Kg; DIN/AED = 383.7 ± 29.4 Kg; DIN/VIS = 380.6 ± 27.7 Kg.

Effects of $PGF_{2\alpha}$ Formulation on Detection and Characteristics of Estrous and Concentrations of Progesterone and Estradiol

The interaction between $PGF_{2\alpha}$ formulation and estrous cycle phase at treatment affected ($P = 0.02$) the percentage of heifers detected in estrus within 7 d of $PGF_{2\alpha}$ treatment, because a larger numerical difference between CLO and DIN was observed among heifers treated during early diestrus compared with heifers treated at mid-diestrus and proestrus, respectively (Figure 2-1.).

The interaction between $PGF_{2\alpha}$ formulation and estrous cycle phase at treatment affected ($P = 0.02$) the hazard of estrus. Treatment with CLO reduced ($P < 0.01$) the interval from $PGF_{2\alpha}$ treatment to estrus for mid-diestrus heifers (Figure 2-2.), but $PGF_{2\alpha}$ formulation did not affect the interval from $PGF_{2\alpha}$ treatment to estrus in early diestrus ($P = 0.95$) and proestrus ($P = 0.55$) heifers.

Prostaglandin $F_{2\alpha}$ formulation did not affect estrus duration ($P = 0.85$; Figure 2-3.) or rumination nadir ($P = 0.54$; Figure 2-4.). The interaction between $PGF_{2\alpha}$ formulation and estrous cycle phase affected ($P = 0.05$) the percentage of heifers with activity peak ≥ 80 because a greater percentage of heifers in early diestrus and proestrus treated with CLO had activity peak ≥ 80 than heifers treated with DIN, whereas a greater percentage of heifers in mid-diestrus treated

with DIN had activity peak ≥ 80 than heifers treated with CLO (Figure 2-5.). Similarly, the interaction between $PGF_{2\alpha}$ formulation and estrous cycle phase affected ($P < 0.01$) the percentage of heifers with heat index ≥ 80. A greater percentage of heifers treated with CLO in early diestrus and proestrus had heat index ≥ 80 than heifers treated with DIN, whereas CLO treatment of heifers in mid-diestrus resulted in slightly smaller percentage of heifers with heat index ≥ 80 than DIN treatment (Figure 2-6.).

At $PGF_{2\alpha}$ treatment, progesterone concentrations were not different ($P = 0.27$) between CLO and DIN treated heifers (Figure 2-7.). After detection of estrus, CLO treated heifers had ($P = 0.03$) lower progesterone concentrations than DIN treated heifers (Figure 2-8.). Estradiol concentrations after detection of estrus were not ($P = 0.49$) affected by $PGF_{2\alpha}$ formulation (Figure 2-9.).

Effects of $PGF_{2\alpha}$ Formulation and Estrus Detection Method on Reproductive Performance

Hazard of first service tended ($P = 0.06$) to be greater for CLO than DIN treated heifers (AHR = 1.14, 95% CI = 0.99 – 1.30). Estrus detection method did not ($P = 0.17$) affect the hazard of first service. The interaction between $PGF_{2\alpha}$ formulation and estrus detection method did not ($P = 0.65$) affect the hazard of first service. Interval from first $PGF_{2\alpha}$ treatment to first service was ($P = 0.04$) shorter in CLO than in DIN treated heifers (Figure 2-10.).

Prostaglandin $F_{2\alpha}$ formulation did not affect ($P = 0.87$) the hazard of second service. Automated estrus detection system tended ($P = 0.07$) to increase the hazard of second service compared with VIS (AHR = 1.19, 95% CI = 1.00 – 1.43). The interaction between $PGF_{2\alpha}$ formulation and estrus detection method did not affect ($P = 0.58$) the hazard of second service. Interval from first service to second service was ($P = 0.04$) shorter in heifers detected in estrus by the AED than in heifers detected in estrus by VIS (Figure 2-11.).

Pregnancy at 35 ± 3 d after first AI was not affected by $PGF_{2\alpha}$ formulation ($P = 0.39$), estrus detection method ($P = 0.95$), or the interaction between $PGF_{2\alpha}$ formulation and estrus detection method ($P = 0.47$; Table 2-1.). Pregnancy at 75 ± 3 d after first AI was not affected by $PGF_{2\alpha}$ formulation ($P = 0.29$), estrus detection method ($P = 0.77$), or the interaction between $PGF_{2\alpha}$ formulation and estrus detection method ($P = 0.91$; Table 2-1.). Pregnancy loss from 35 to 75 ± 3 d after first AI was not affected by $PGF_{2\alpha}$ formulation ($P = 0.43$), estrus detection method ($P = 0.39$), or the interaction between $PGF_{2\alpha}$ formulation and estrus detection method ($P = 0.15$; Table 2-1.).

Pregnancy at 35 ± 3 d after first ET was not affected by $PGF_{2\alpha}$ formulation ($P = 0.31$), estrus detection method ($P = 0.42$), or the interaction between $PGF_{2\alpha}$ formulation and estrus detection method ($P = 0.26$; Table 2-1.). Pregnancy at 75 ± 3 d after first ET was not affected by $PGF_{2\alpha}$ formulation ($P = 0.76$), estrus detection method ($P = 0.11$), or the interaction between $PGF_{2\alpha}$ formulation and estrus detection method ($P = 0.57$; Table 2-1.). Pregnancy loss from 35 to 75 ± 3 d after first ET was not affected by $PGF_{2\alpha}$ formulation ($P = 0.42$), estrus detection method ($P = 0.12$), or the interaction between $PGF_{2\alpha}$ formulation and estrus detection method ($P = 0.67$; Table 2-1.).

Pregnancy at 35 ± 3 d after the second AI was not affected by $PGF_{2\alpha}$ formulation ($P = 0.45$, estrus detection method ($P = 0.21$), or the interaction between $PGF_{2\alpha}$ formulation and estrus detection method ($P = 0.90$; Table 2-1.). Pregnancy at 75 ± 3 d after second AI was not affected by $PGF_{2\alpha}$ formulation ($P = 0.79$), estrus detection method ($P = 0.27$), or the interaction between $PGF_{2\alpha}$ formulation and estrus detection method ($P = 0.98$; Table 2-1.). Pregnancy loss from 35 to 75 ± 3 d after second AI was not affected by $PGF_{2\alpha}$ formulation ($P = 0.94$), estrus

detection method ($P = 0.78$), or the interaction between $PGF_{2\alpha}$ formulation and estrus detection method ($P > 0.99$; Table 2-1.).

Pregnancy at 35 ± 3 d after second ET was not affected by $PGF_{2\alpha}$ formulation ($P = 0.76$), estrus detection method ($P = 0.57$), or the interaction between $PGF_{2\alpha}$ formulation and estrus detection method ($P = 0.31$; Table 2-1.). Pregnancy at 75 ± 3 d after second ET was not affected by $PGF_{2\alpha}$ formulation ($P = 0.57$), estrus detection method ($P = 0.72$), or the interaction between $PGF_{2\alpha}$ formulation and estrus detection method ($P = 0.18$; Table 2-1.). Pregnancy loss from 35 to 75 ± 3 d after second ET was not affected by $PGF_{2\alpha}$ formulation ($P = 0.15$), estrus detection method ($P = 0.27$), or the interaction between $PGF_{2\alpha}$ formulation and estrus detection method ($P = 0.95$; Table 2-1.).

Prostaglandin $F_{2\alpha}$ formulation did not affect ($P = 0.59$) the hazard of pregnancy. Hazard of pregnancy tended ($P = 0.07$) to be greater for heifers detected in estrus by AED than in heifers detected in estrus by VIS (AHR = 1.17, 95% CI = 0.99 – 1.38). The interaction between $PGF_{2\alpha}$ formulation and estrus detection method ($P = 0.58$) did not affected the hazard of pregnancy. Interval from first $PGF_{2\alpha}$ treatment to pregnancy was ($P = 0.05$) shorter for heifers detected in estrus by AED than for heifers detected in estrus by VIS (Figure 2-12.).

Discussion

The interaction between $PGF_{2\alpha}$ formulation and estrous cycle phase at $PGF_{2\alpha}$ treatment affected the percentage of heifers detected in estrus within 7 d of treatment, because a larger numerical difference between CLO and DIN was observed among heifers in early diestrus than among heifers in mid-diestrus and proestrus. Furthermore, CLO shortened the interval from $PGF_{2\alpha}$ treatment to estrus among heifers in mid-diestrus, and reduced the progesterone concentration at estrus compared with DIN treatment. Prostaglandin $F_{2\alpha}$ formulation, however,

did not affect estradiol concentration at estrus or estrus duration and rumination nadir. The interaction between $PGF_{2\alpha}$ formulation and of estrous cycle phase at $PGF_{2\alpha}$ treatment was associated with the percentage of heifers with activity peak and heat index ≥ 80, because a larger numerical difference in the percentage of heifers with activity peak and heat index ≥ 80 between CLO and DIN was observed among heifers in early diestrus. Growth of a large follicle capable to produce enough estradiol to trigger estrus and ovulation is dependent on luteal regression (Goravanahally et al., 2009). After luteal regression occurs, interval from $PGF_{2\alpha}$ treatment to onset of estrus is dependent on age and maturity of the largest follicle at the time of treatment (Martins et al., 2011b). Prostaglandin $F_{2\alpha}$ luteolytic efficacy is highly dependent on the estrous cycle phase when the treatment is applied (Valldecabres-Torres et al., 2012; Ferraz Junior et al., 2016). Newly formed corpus luteum have concentrations of $PGF_{2\alpha}$ receptors similar to mature corpus luteum, but the ability of exogenous $PGF_{2\alpha}$ to induce luteolysis is reduced before day 5 or 6 of the estrous cycle (Wenzinger and Bleul, 2012). After day 16 of the estrous cycle, if maternal recognition of pregnancy is not established, oxytocin binds to its receptor in the uterus, which propagates secretion of endogenous $PGF_{2\alpha}$, and regression of the corpus luteum occurs spontaneously, with no need for exogenous $PGF_{2\alpha}$ treatment (Forde et al., 2011). In the current experiment, we hypothesized that dairy heifers would benefit from the longer half-life of CLO, which would increase the percentage of heifers detected in estrus and the hazard of estrus compared with CLO than DIN treatment. We used an AED to determine exact interval and characteristics of estrous to minimize human subjective during evaluation of estrous' characteristics. In the current experiment, the differences in percentage of heifers detected in estrus between CLO and DIN treatments was greatest among heifers treated at early diestrus, followed by heifers treated at mid-diestrus and proestrus, respectively. Since recently formed

corpus luteum are not fully responsive to $PGF_{2\alpha}$ treatments (Wenzinger and Bleul, 2012), we speculate that the longer half-life of CLO allowed a longer exposure of the newly formed corpus luteum to $PGF_{2\alpha}$, increasing the likelihood of luteolysis. On the other hand, heifers in proestrus benefited the least from CLO because they likely were undergoing or had undergone spontaneous luteolysis (Forde et al., 2011). The benefits of CLO to heifers in mid-diestrus was intermediary likely because at mid-diestrus a fully functional corpus luteum is present (Forde et al., 2011) and the half-life of the $PGF_{2\alpha}$ would not be as critical to induce complete luteolysis. Cloprostenol treatment reduced the interval from $PGF_{2\alpha}$ treatment to estrus, but only in mid-diestrus heifers. Since a greater proportion of heifers treated with CLO in early diestrus were detect in estrus, we expected CLO also to reduce the interval to estrus in early diestrus, not only in mid-diestrus heifers.

Estrous characteristics measured with an AED were previously associated with physiological signs of estrus such as clear vaginal mucus, uterine tone, visual mounting activity and standing to be mounted behavior (Silper et al., 2015). Because emergence of a dominant follicle capable of producing enough estradiol concentrations to trigger estrus expression should occur within 7 d of $PGF_{2\alpha}$ treatment (Forde et al., 2011), we only used heifers detected in estrus within 7 d of $PGF_{2\alpha}$ treatment in the analysis of estrous characteristics. Since CLO reduced progesterone concentrations at estrus, we expected it also to allow greater follicle growth and estradiol concentrations, and in turn produce more intense estrus compared with DIN treatment. Prostaglandin $F_{2\alpha}$ formulation, however, did not affect estradiol concentrations at estrus, estrus duration, and rumination nadir. Nonetheless, as discussed previously, treatment of heifers in early diestrus and proestrus with CLO resulted in greater percentage of heifers with activity peak and heat index ≥ 80. Thus, results from the current experiment suggest that although

progesterone concentrations at estrus were lower in CLO than in DIN treated heifers, reduction in progesterone concentrations in DIN treated heifers was likely enough to allow follicle growth and a rise in estradiol concentration to trigger estrus.

Treatment of dairy heifers with CLO reduced interval from $PGF_{2\alpha}$ treatment to first service. Reduced interval from $PGF_{2\alpha}$ treatment to first service most likely was due to the effects of CLO on percentage of heifers detected in estrus within 7 d of the first $PGF_{2\alpha}$ treatment. Pregnancy at 35 and 75 d after estrus and pregnancy loss from 35 to 75 d after estrus, for AI and ET services, were not affected by $PGF_{2\alpha}$ formulation. These results are in agreement with data by Stevenson and Phatak (2010), but are not in agreement with data by Pursley et al. (2012) and Martins et al. (2011b) who demonstrated that CLO treatment increased Preg/Serv in primiparous cows. Since progesterone concentrations at estrus were lower for CLO treated heifers, we expected it could improve Preg/Serv as previously reported by Colazo et al. (2017). Estradiol concentrations, however, were not affected by $PGF_{2\alpha}$ formulation and were enough to trigger estrus. Furthermore, mean progesterone concentrations at estrus among DIN treated heifers was only 0.11 ng/mL. Colazo et al. (2017) demonstrated that progesterone concentration > 0.5 ng/mL reduced Preg/Serv in cows. Thus, no practical benefit of the lower progesterone concentrations resulting from the CLO treatment was observed in the current experiment. Although CLO treated heifers had increased first service rate, hazard of pregnancy was not affected by $PGF_{2\alpha}$ formulation.

Estrus detection method did not affect the hazard of first service. Automated estrus detection monitoring system, however, increased the hazard of second service of non-pregnant heifers and tended to increase the hazard of pregnancy. Automated estrus detection systems allow for 24 h daily estrus detection (Fricke et al., 2014). Giordano et al. (2015) showed that

AED increased insemination of cows in estrus. Similarly, Fricke et al. (2014) showed that the interval to re-insemination of cows was shortened by the use of an AED. In the current experiment, we expected AED to increase hazard of first service and second service. The lack of effect of AED on hazard of first service may indicate that estrus detection by farm personnel was more intense for heifers that had not been serviced compared with heifers that had been serviced. Thus, heifers that did not conceive after the first service benefited the most from the AED in current experiment. Estrus detection using an AED did not improve Preg/Serv or pregnancy loss either on AI or ET services. Numerically, however, Preg/Serv was greater for heifers detected in estrus by the AED compared with VIS. Because AED increased hazard of second service and a numerical increase in Preg/Serv was noted, AED increased hazard of pregnancy in heifers in the current expeiment.

Treatment of dairy heifers with CLO treatment increased estrus detection within 7 d of treatment, tended to increase first service rate, and reduced progesterone concentrations at estrus compared with DIN treatment. These responses, however, are somewhat dependent on phase of the estrous cycle when heifers were treated with $PGF_{2\alpha}$ Estradiol concentrations and estrus characteristics, however, were minimally affect by $PGF_{2\alpha}$ formulation. Furthermore, $PGF_{2\alpha}$ did not affect Preg/Serv, pregnancy loss, or hazard of pregnancy. Results presented herein suggest that $PGF_{2\alpha}$ formulation may have a small or null impact on overall reproductive performance of dairy heifers and selection of $PGF_{2\alpha}$ formulation for dairy heifers should be according to others characteristics than efficacy.

Use of an AED for detection of estrus in dairy heifers tended to increase hazard of second service and pregnancy in a commercial dairy farm. Although improvements in reproductive performance observed herein can potentially increase profitability of heifer operations,

economical feasibility of the use of an AED for dairy heifers will vastly vary according to the type of reproductive program used the accuracy of estrus detection at the farm level.

Table 2-1. Effect of $PGF_{2\alpha}$ formulation and estrus detection method on pregnancy and pregnancy loss

	CLO		DIN		*P* - value		
	AED	VIS	AED	VIS	PGFTRT	EDTRT	PGFTRT x EDTRT
	First Service						
Pregnant							
Day 35 AI	49.6	47.1	51.6	54.2	0.39	0.95	0.47
Day 75 AI	45.9	43.3	50.0	50.0	0.29	0.77	0.91
Day 35 ET	34.0	26.9	37.8	36.5	0.31	0.42	0.26
Day 75 ET	29.3	21.3	33.3	27.0	0.76	0.11	0.57
Pregnancy loss							
AI	7.6	8.2	3.1	7.7	0.43	0.75	0.15
ET	14.0	20.7	11.9	26.2	0.42	0.12	0.67
	Second Service						
Pregnant							
Day 35 AI	37.0	26.7	36.2	25.0	0.45	0.21	0.90
Day 75 AI	33.3	22.2	36.2	25.0	0.79	0.27	0.98
Day 35 ET	31.5	39.5	31.6	26.7	0.76	0.57	0.31
Day 75 ET	29.6	37.2	29.0	20.0	0.57	0.72	0.18
Pregnancy loss							
AI	10.0	16.7	0.0	0.0	0.94	0.78	>0.99
ET	5.9	5.9	8.3	25.0	0.15	0.27	0.95

PGFTRT = $PGF_{2\alpha}$ formulation used; CLO = heifers were treated with $PGF_{2\alpha}$ formulation cloprostenol sodium (Estrumate, Merck Animal Health, Summit, NJ); DIN = heifers were treated with $PGF_{2\alpha}$ formulation dinoprost tromethamine (Lutalyse, Zoetis, Parsippany, NJ).
EDTRT = Estrus detection method used; AED = Automated estrus detection (Heattime, SCR Inc., Netanya, Israel); VIS = Estrus detection based on visual observation and mounting device activation (Kamar heatmount detector, Kamar Inc., Steamboat Springs, CO).
AI = Artificial Insemination
ET = Embryo Transfer

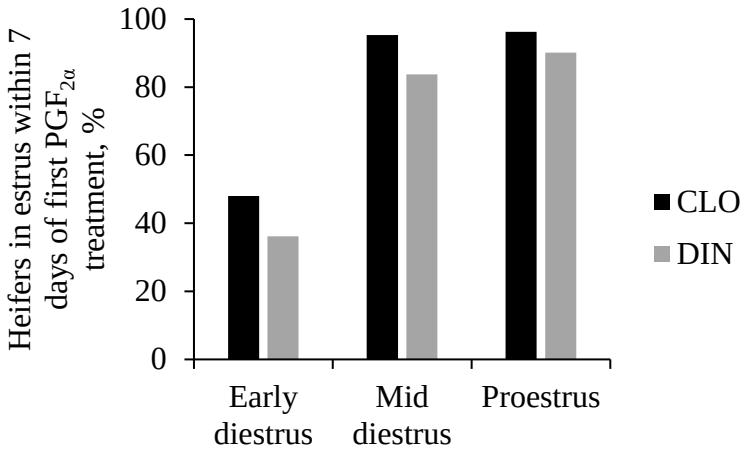

Figure 2-2. Effect of prostaglandin (**PG**) $F_{2\alpha}$ formulation on estrus detection by an automated estrus detection system (AED) within 7 days of first $PGF_{2\alpha}$ treatment according to the phase of the estrous cycle at $PGF_{2\alpha}$ treatment. CLO = heifers were treated with $PGF_{2\alpha}$ formulation cloprostenol sodium (Estrumate, Merck Animal Health, Summit, NJ); DIN = heifers were treated with $PGF_{2\alpha}$ formulation dinoprost tromethamine (Lutalyse, Zoetis, Parsippany, NJ). $PGF_{2\alpha}$ formulation - $P < 0.01$, estrous cycle phase at $PGF_{2\alpha}$ treatment - $P < 0.01$, $PGF_{2\alpha}$ formulation x estrous cycle phase at $PGF_{2\alpha}$ treatment - $P = 0.02$.

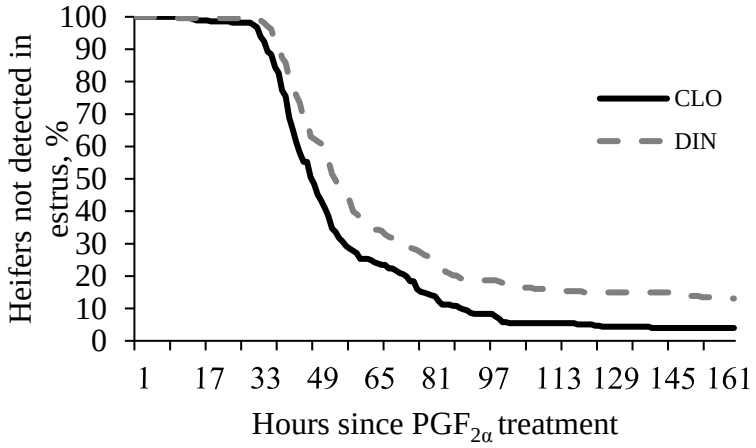

Figure 2-2. Effect of prostaglandin (PG) $F_{2\alpha}$ formulation on interval from $PGF_{2\alpha}$ treatment to onset of estrus only for mid-diestrus heifers. Mean ± SEM and median interval from PGF2α treatment to estrus: CLO = 58.3 ± 1.6 and 48.9 h, DIN = 72.8 ± 2.4 and 55.6 h. CLO = heifers were treated with $PGF_{2\alpha}$ formulation cloprostenol sodium (Estrumate, Merck Animal Health, Summit, NJ); DIN = heifers were treated with $PGF_{2\alpha}$ formulation dinoprost tromethamine (Lutalyse, Zoetis, Parsippany, NJ). $PGF_{2\alpha}$ treatment - $P < 0.01$.

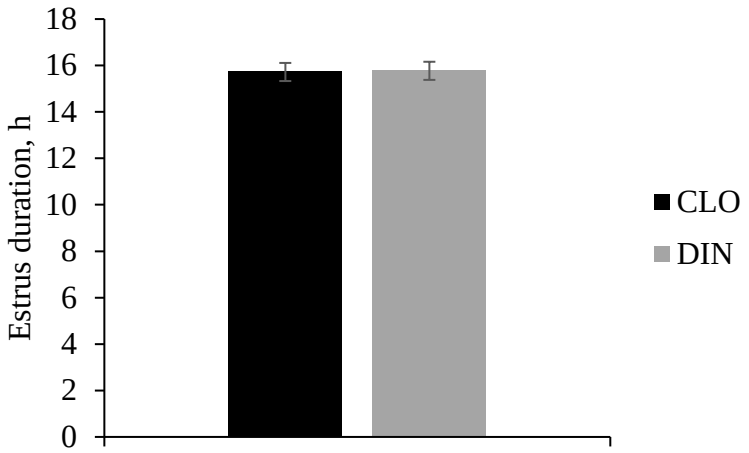

Figure 2-3. Effect of prostaglandin (PG) $F_{2\alpha}$ formulation on duration of estrus detected by an automated estrus detection system (AED) within 7 days of $PGF_{2\alpha}$ treatment. CLO = heifers were treated with $PGF_{2\alpha}$ formulation cloprostenol sodium (Estrumate, Merck Animal Health, Summit, NJ); DIN = heifers were treated with $PGF_{2\alpha}$ formulation dinoprost tromethamine (Lutalyse, Zoetis, Parsippany, NJ). $PGF_{2\alpha}$ treatment - P = 0.85.

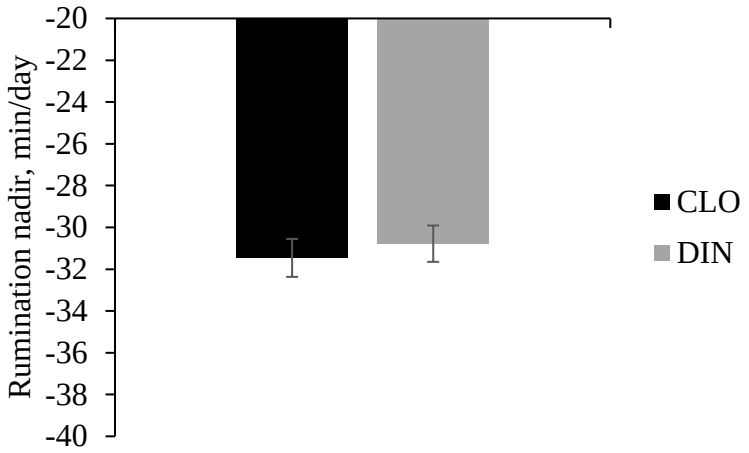

Figure 2-4. Effect of prostaglanin (PG) $F_{2\alpha}$ formulation on rumination nadir of estrus detected by an automated estrus detection system (AED) within 7 days of $PGF_{2\alpha}$ treatment. CLO = heifers were treated with $PGF_{2\alpha}$ formulation cloprostenol sodium (Estrumate, Merck Animal Health, Summit, NJ); DIN = heifers were treated with $PGF_{2\alpha}$ formulation dinoprost tromethamine (Lutalyse, Zoetis, Parsippany, NJ). $PGF_{2\alpha}$ formulation - P = 0.54.

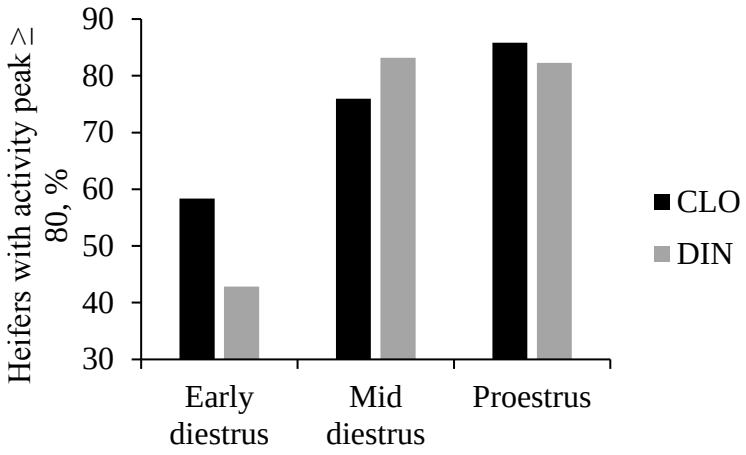

Figure 2-5. Effect of prostaglandin (PG) $F_{2\alpha}$ on percentage of heifers with activity peak ≥ 80 detected in estrus within 7 days of $PGF_{2\alpha}$ treatment according to the estrous cycle phase at $PGF_{2\alpha}$ treatment. CLO = heifers were treated with $PGF_{2\alpha}$ formulation cloprostenol sodium (Estrumate, Merck Animal Health, Summit, NJ); DIN = heifers were treated with $PGF_{2\alpha}$ formulation dinoprost tromethamine (Lutalyse, Zoetis, Parsippany, NJ). $PGF_{2\alpha}$ formulation - $P = 0.62$, estrous cycle phase at $PGF_{2\alpha}$ treatment - $P < 0.01$, $PGF_{2\alpha}$ formulation x estrous cycle phase at $PGF_{2\alpha}$ treatment $P = 0.05$.

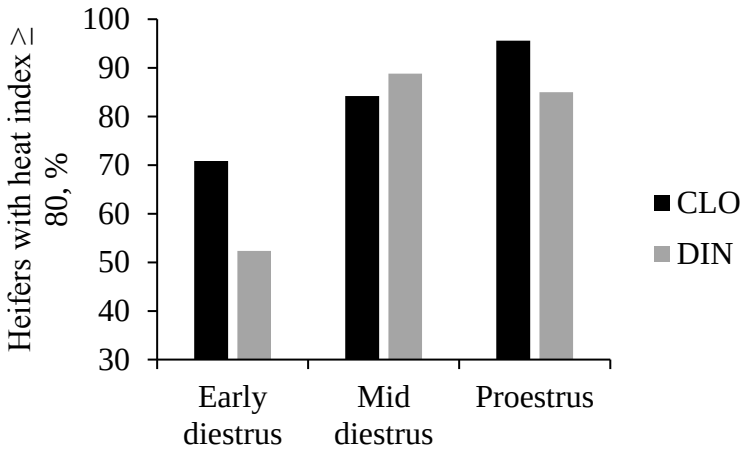

Figure 2-6. Effect of prostaglandin (PG) $F_{2\alpha}$ on percentage of heifers with heat index ≥ 80 detected in estrus within 7 days of $PGF_{2\alpha}$ treatment according to the estrous cycle phase at $PGF_{2\alpha}$ treatment. CLO = heifers were treated with $PGF_{2\alpha}$ formulation cloprostenol sodium (Estrumate, Merck Animal Health, Summit, NJ); DIN = heifers were treated with $PGF_{2\alpha}$ formulation dinoprost tromethamine (Lutalyse, Zoetis, Parsippany, NJ). $PGF_{2\alpha}$ formulation - $P = 0.02$, estrous cycle phase at $PGF_{2\alpha}$ treatment - $P < 0.01$, $PGF_{2\alpha}$ formulation x estrous cycle phase - $P < 0.01$.

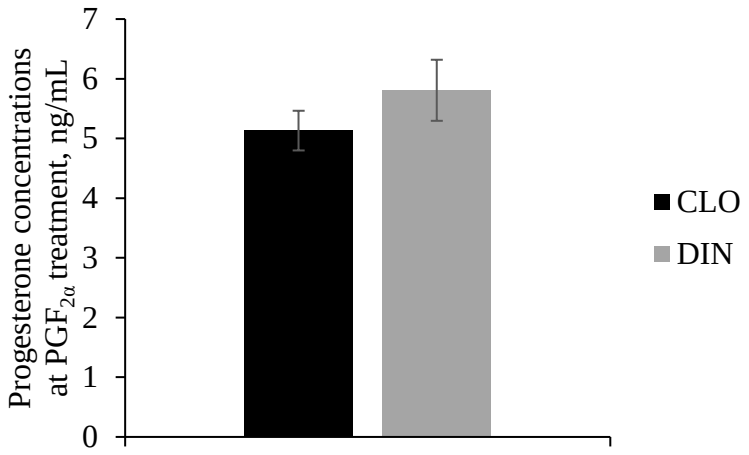

Figure 2-7. Progesterone concentrations at the day of prostaglandin (PG) $F_{2\alpha}$ treatment according to $PGF_{2\alpha}$ formulation. CLO = heifers were treated with $PGF_{2\alpha}$ formulation cloprostenol sodium (Estrumate, Merck Animal Health, Summit, NJ); DIN = heifers were treated with $PGF_{2\alpha}$ formulation dinoprost tromethamine (Lutalyse, Zoetis, Parsippany, NJ). $PGF_{2\alpha}$ formulation - $P = 0.27$.

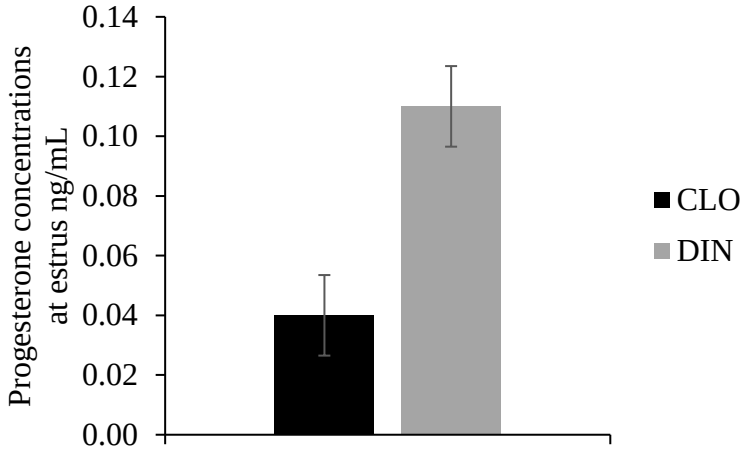

Figure 2-8. Effect of prostaglandin (PG) $F_{2\alpha}$ formulation on progesterone concentrations at estrus. CLO = heifers were treated with $PGF_{2\alpha}$ formulation cloprostenol sodium (Estrumate, Merck Animal Health, Summit, NJ); DIN = heifers were treated with $PGF_{2\alpha}$ formulation dinoprost tromethamine (Lutalyse, Zoetis, Parsippany, NJ). $PGF_{2\alpha}$ formulation - $P = 0.03$.

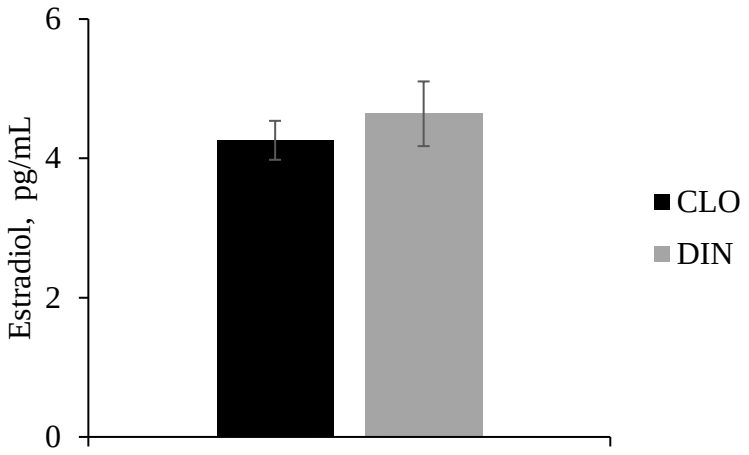

Figure 2-9. Effect of prostaglandin (PG) $F_{2\alpha}$ formulation on estradiol concentrations at estrus. CLO = heifers were treated with $PGF_{2\alpha}$ formulation cloprostenol sodium (Estrumate, Merck Animal Health, Summit, NJ); DIN = heifers were treated with $PGF_{2\alpha}$ formulation dinoprost tromethamine (Lutalyse, Zoetis, Parsippany, NJ). $PGF_{2\alpha}$ formulation - $P = 0.49$.

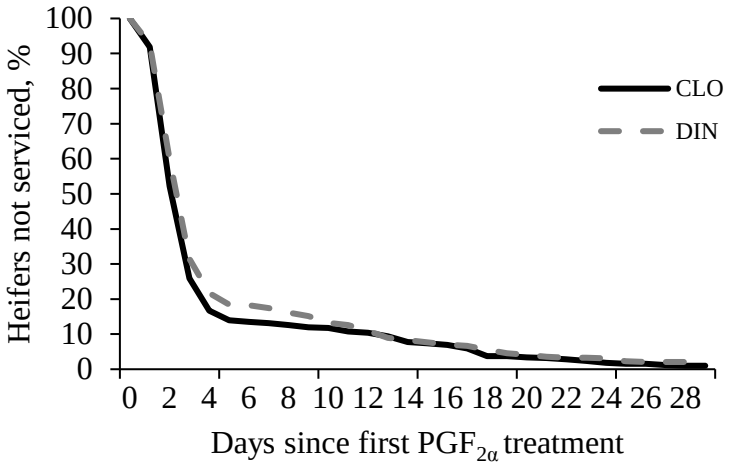

Figure 2-10. Effect of prostaglandin (PG) $F_{2\alpha}$ formulation on interval from $PGF_{2\alpha}$ to first service. Mean (±SEM) and median days to first service: CLO = 4.5 ± 0.2 and 3 d, DIN = 4.9 ± 0.3 and 3 d. Prostaglandin $F_{2\alpha}$ treatment: CLO = heifers were treated with $PGF_{2\alpha}$ formulation cloprostenol sodium (Estrumate, Merck Animal Health, Summit, NJ); DIN = heifers were treated with $PGF_{2\alpha}$ formulation dinoprost tromethamine (Lutalyse, Zoetis, Parsippany, NJ). $PGF_{2\alpha}$ formulation - $P = 0.07$.

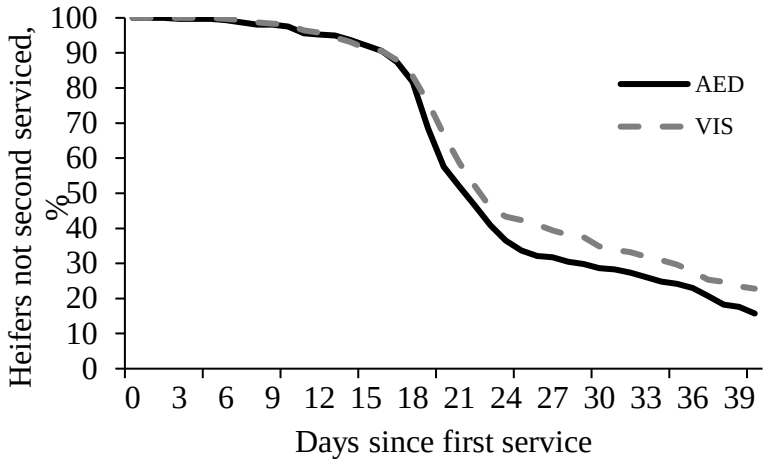

Figure 2-11. Effect of estrus detection method on interval from first to second service. Mean (±SEM) and median days to second service: AED = 22.5 ± 0.3 and 22 d, VIS = 23.3 ± 0.32 and 23 d. Estrus detection method: AED = Automated estrus detection (Heattime, SCR Inc., Netanya, Israel); VIS = Estrus detection based on visual observation and mounting device activation (Kamar heatmount detector, Kamar Inc., Steamboat Springs, CO). Estrus detection method - *P* = 0.04.

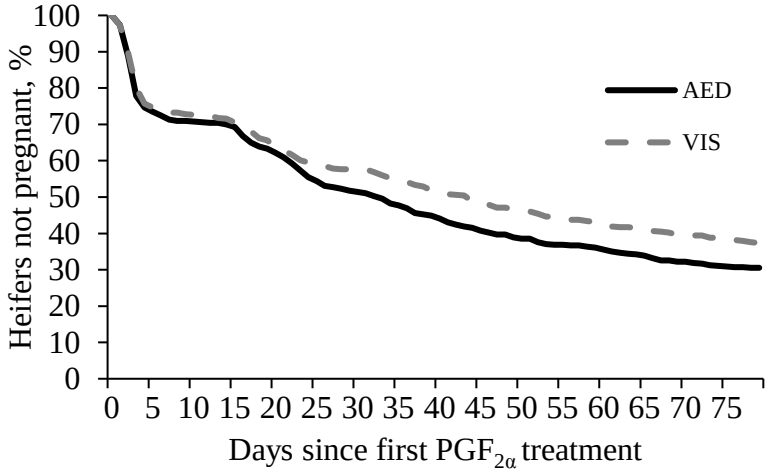

Figure 2-12. Effect of estrus detection method on interval from first prostaglandin (PG) $F_{2\alpha}$ to pregnancy. Mean (±SEM) and median to pregnancy: AED = 39.5 ± 1.4 and 33 d, VIS = 43.9 ± 1.5 and 44 d. Estrus detection method: AED = Automated estrus detection (Heattime, SCR Inc., Netanya, Israel); VIS = Estrus detection based on visual observation and mounting device activation (Kamar heatmount detector, Kamar Inc., Steamboat Springs, CO). Estrus detection method - *P* = 0.05.

CHAPTER 3
ASSOCIATION AMONG GENETIC MERIT FOR REPRODUCTION TRAITS AND ESTROUS CHARACTERISTICS AND FERTILITY OF HOLSTEIN HEIFERS

Reproductive performance is extremely important to maximize the profitability of dairy operations (Giordano et al., 2012). Factors such as reproductive management, nutrition, health, and genetics affect reproductive outcomes directly or indirectly. Genetic selection of dairy breeds until the early 2000s was mainly focused on production traits, while disregarding reproduction traits (Lucy, 2001). It is believed that such strategy contributed for the selection of cattle with reduced estrus expression and, consequently, reduced estrus detection and reproductive performance in modern dairy operations (Lopez et al., 2005). Although recent advancements in reproductive management has allowed for the insemination of cows and heifers following ovulation synchronization protocols, even animals subjected to such protocols have greater pregnancy per service (**Preg/Serv**) when they display estrus at the time of fixed time service. In a recent study, estrus expression was associated with increased fertility and decreased pregnancy losses following timed artificial insemination and fixed time embryo transfer (**TET**; Pereira et al., 2016).

Automated estrus detection monitoring devices (**AED**), based on changes in walking, activity and rumination patterns, have become more reliable for estrus detection and are being used in a growing number of dairies (Fricke et al., 2017). This technology has allowed the recording of estrus events and estrous characteristics (duration, intensity, etc.) from a large number of animals in a uniform manner. Burnett et al. (2017) demonstrated that estrous characteristics, such as duration, was positively associated with pregnancy per service (**Preg/Serv**) following artificial insemination (**AI**) in dairy cows. Studies that evaluate the associations among genetic merit, physiological parameters, and estrous characteristics present a

unique opportunity to understand how new strategies for genetic selection may affect estrus behavior and Preg/Serv.

In a series of experiments, Kommadath et al. (2011, 2013, 2017) and Woelders et al. (2014) recorded physiological estrous behavior signs visually and assigned an estrus score to dairy cows using a score previously described by Roelofs et al. (2005). Among the estrous behavior signs evaluated to assign estrus scores were mounting activity and standing to be mounted (Kommadath et al., 2011, 2013, 2017; Woelders et al. 2014), estrus signs that were positively associated with estrus duration and activity peak measured by an AED (Silper et al., 2015b). After recording estrus scores from several estrous cycles, cows were slaughter either at mid diestrus or at estrus and had brain collected for gene expression analyses. In these studies, estrus score was associated with a substantial number of genes expressed in different areas of the brain (Kommadath et al., 2011, 2013, 2017; Woelders et al., 2014), suggesting a possible genetic component driving estrous behavior in dairy cows.

Since the mid 2000's, genetic selection for dairy breeds has included reproduction traits such as daughter pregnancy rate (**DPR**), introduced in 2004 (VanRaden et al., 2004), and heifer conception rate (HCR), introduced in 2013. Daughter pregnancy rate is a measure of the hazard of pregnancy of a bull's daughters compared with the population, whereas HCR is a measure of the likelihood of pregnancy fallowing a service of a bull's daughter compared with the population (AIPL, 2013). With the advancement of genomic selection tools in recent years, genetic gains of selected traits in the US Holstein cattle population has been substantial (García-Ruiz et al., 2016). Despite improvements in the US Holstein population regarding reproduction traits such as interval from calving to first AI, 21-d pregnancy rate (**21-d PregRate**; percentage of eligible cows that become pregnant within a 21-d period), and calving interval, there is a lack

of information regarding the association among these genomic traits and estrus expression and estrous characteristics.

The hypothesis of the current study was that genomic merit values for DPR (**GDPR**) and for HCR (**GHCR**) are associated with estrous characteristics, hazard of service, Preg/Serv, pregnancy loss, and hazard of pregnancy in Holstein heifers. Therefore, the objectives of the current study were to evaluate the association among GDPR and GHCR and estrous characteristics, hazard of service, Preg/Serv, pregnancy loss, and hazard of pregnancy of Holstein heifers.

Materials and Methods

All procedures involving animals were approved by the animal care and use committee of the University of Florida (protocol #201609559).

Animal, Housing, and Management

This study was conduct from March 2016 to December 2016 in a commercial dairy herd with approximately 4,200 replacement heifers, located in north central Florida. One thousand and nineteen heifers, between 10 and 11 months of age, were enrolled in the study. All heifers were genotyped within 2 months of birth using a 50k single nucleotide peptide platform commercially available (Clarifide, Zoetis, Parsippany, NJ). Data referent to genomic breeding values for DPR and HCR recorded within 2 months of birth were used. Starting at 12 months of age heifers were weighed weekly. Heifers with BW $\geq$ 340 kg were moved to a breeding pen and were treated with prostaglandin (**PG**) $F_{2\alpha}$ for synchronization of estrous. Heifers were housed in dry lots, with natural shade and no artificial cooling. The breeding pens had self-locking head stanchions on the feeding area. Heifers were fed twice daily (7:00 AM and 4:30 PM) a TMR formulated to meet or exceed the nutritional requirements of Holsteins heifers weighing $\geq$ 340 kg

of live body weight and gaining 800 to 1,000 g of live body weight per day (NRC, 2001). Weather data (daily average temperature, humidity, and rain precipitation) from the Gainesville airport, located approximately 40 miles east of the dairy, were used to calculate daily temperature humidity index (**THI**). The percentages of days during the 30 d prior to and during the 30 d after the start of the reproductive program with THI $\geq$ 72 were recorded for each heifer. Cumulative precipitation during the 30 d prior to and during the 30 d after the start of the reproductive program were recorded for each heifer.

Automated Estrus Detection and Estrous Characteristics

At enrollment, an AED (Heat Rumination Long Distance, SCR Inc., Netanya, Israel) mounted on a collar was fitted on the left, cranial area of the neck of all heifers. The device determined activity through an accelerometer and rumination based on sounds of regurgitation and mastication through a microphone. Activity and rumination data were recorded in 2-h intervals. Estrus was determined according to changes in patterns of activity and rumination within a 2-h interval compared with the average activity and rumination of the same period in the previous 5 and 7 d, respectively (DataFlow2©, SCR Inc, Netanya, Israel). An internal algorithm of the DataFlow2© software produced a heat index (0 = no estrus, 100 = maximum) according to the intensity of changes in activity and rumination. Daily, study personnel evaluated the activity and rumination patterns of heifers determined to be in estrus by the DataFlow2© software. On the day heifers were moved to the breeding pen, heifers with heat index < 50, duration of estrus < 6 h, and no change in rumination time were determined to have changes in activity pattern due to pen movement and not due to estrus and were, therefore, not inseminated. Heat index, activity peak (0 = no estrus, 100 = maximum activity), and rumination nadir (maximum difference in rumination time within a 2-h period during estrus compared with the average rumination of the

same period in the previous 7 d) were recorded daily for all heifers in estrus. Study personnel evaluated each activity graph individually and determined the time of onset (2-h period when the activity threshold was surpassed), peak (2-h period when the activity change was maximum), and end (2-h period when the activity change was below the activity threshold) of estrus. Activity threshold was set at three folds above the average activity for the same period in the previous 5 d. Intervals from onset to peak of estrus and from onset to end of estrus were calculated. Characteristics of spontaneous estruses (**SPE**; estruses occurring before the start of the reproductive program) and $PGF_{2\alpha}$ induced estruses (**PIE**; estruses occurring after the start of the reproductive program) were recorded. Automated estrus detection monitors devices were removed from heifers at pregnancy diagnosis 28 d after service, when heifers received a second service, and when heifers were not detected in estrus within 28 d after the start of the reproductive program.

Reproductive Management

From enrollment to the start of the reproductive program all estruses were recorded. When heifer were eligible to start the reproductive program (≥ 12 months of age and ≥ 340 kg of live body weight) they were classified according to estrous cycle phase into metestrus (**ME**; day 0 to 3), early diestrus (**ED**; day 4 to 6), mid-diestrus (**MID**; day 7 to 17), and proestrus (**PE**; day ≥ 18), and no estrus observed. Heifers in metestrus were treated with $PGF_{2\alpha}$ 96 h later and heifers in early diestrus, mid-diestrus, and proestrus and heifers that had not had AED detected estrus were treated with $PGF_{2\alpha}$ immediately. Two $PGF_{2\alpha}$ formulations were used (cloprostenol sodium, Estrumate, Merck Animal Health, Summit, NJ; dinoprost tromethamine, Lutalyse, Zoetis, Parsippany, NJ). Fourteen days after the first $PGF_{2\alpha}$ treatment, heifers not detected in estrus received a second treatment with the same $PGF_{2\alpha}$ formulation. Despite all heifers being fitted

with the AED, 537 heifers were serviced at AED detected estrus, whereas 482 heifers were serviced at estrus detected by farm personnel based on visualization of mounting activity or activation of a tail paint device (Kamar, Kamar inc., Steamboat Springs, CO). According to the genetic selection program of the dairy, heifers were selected to receive artificial insemination (**AI**) or to receive embryo transfer (**ET**). Heifers detected in estrus were AI on the same morning or received an embryo 6 to 9 days after estrus detection.

Pregnancy Diagnoses and Reproductive Data

All heifers were examined for pregnancy by palpation per rectum of the uterine contents at 35 ± 3 d after the detected estrus that resulted in AI or ET. Pregnant heifers were re-examined by palpation per rectum of the uterine contents at 75 ± 3 d of gestation.

Pregnancy per service was calculated by dividing the number of heifers pregnant at 35 and 75 ± 3 d after estrus by the number of heifers serviced. Pregnancy loss was calculated by dividing the number of heifers not pregnant at 75 ± 3 d after service by the number of heifers pregnant 35 ± 3 d after service. Data regarding sire of insemination, sire and dam of embryo transfer, service technician, and reproductive outcomes were collected from an on-farm software (PCDART; Dairy records management system, Chapel Hill, NC).

Statistical Analysis

Data was analyzed using SAS version 9.3 (SAS Institute Inc., Raleigh, NC). Continuous variables were analyzed by ANOVA using the MIXED procedure. Data were evaluated for normality and homogeneity of residuals after fitting the model. Data violating the assumptions of normality were transformed before analysis. Rumination nadir values were multiplied by -1 and transformed to the natural log before analysis. Thus, positive rumination nadir values were

excluded (SPE = 6, PIE = 16). Outlier detection was performed, and rumination nadir transformed values < 2 for SPE (n = 4) and < 2.2 for PIE (n = 4) were considered outliers and removed from the analysis. Interval from onset of estrus to activity peak was square root transformed. Genetic merit for DPR and HCR are the predicted transmitting ability of a trait from the parent to its offspring. The GDPR and GHCR values used in this study were referent to the individuals used in the study; therefore, GDPR and GHCR values were multiplied by 2. Likelihood of activity peak ≥ 80, heat index ≥ 80, pregnancy at 35 and 75 ± 3 d after estrus, and pregnancy loss between 35 and 75 ± 3 d after estrus were analyzed by logistic regression using the LOGISTIC procedure. The hazard of estrus, of first service, and of pregnancy were analyzed by the Cox proportional hazard ratio using the PHREG procedure. Interval from the start of the reproductive program to the onset of first estrus and interval from the start of the reproductive program to establishment of pregnancy were analyzed by the Wilcoxon test of equality using the LIFETEST procedure.

Statistical models to evaluate SPE characteristics included GDPR (linear and quadratic), GHCR (linear and quadratic), the interaction between GDPR and GHCR, and percentage of days with THI ≥ 72 and cumulative precipitation 30 days before the start of the reproductive program. Statistical models to evaluate PIE characteristics included GDPR (linear and quadratic), GHCR (linear and quadratic), the interaction between GDPR and GHCR, percentage of days with THI ≥ 72 and cumulative precipitation 30 days after the start of the reproductive program, $PGF_{2\alpha}$ formulation, estrous cycle phase at $PGF_{2\alpha}$ treatment, and number of $PGF_{2\alpha}$ treatments prior to the first detected estrous. Heifers that had been detected in estrus by the AED > 26 d prior to the $PGF_{2\alpha}$ treatment (n = 10) and heifers detected in estrus > 168 h after the $PGF_{2\alpha}$ treatment (n = 106) were not included in the analysis of PIE characteristics. Nonetheless, heifers that displayed

estrus > 168 h after the $PGF_{2\alpha}$ and heifers that did not display estrus following $PGF_{2\alpha}$ treatment were censored for the purpose of the Cox proportional hazard ratio and Wilcoxon test of equality analyses.

For the analysis of the hazard of estrus after the start of the reproductive program, models included GDPR (linear and quadratic), GHCR (linear and quadratic), the interaction between GDPR and GHCR, percentage of days with THI ≥ 72 and cumulative precipitation 30 days after the start of the reproductive program, $PGF_{2\alpha}$ formulation, and estrous cycle phase at the time of the start of the reproductive program. When GDPR and GHCR were associated with the hazard of estrus after the start of the reproductive program, these variables were divided into quartile and the Wilcoxon test of equality (LIFETEST procedure) was used to characterize the association between GDPR and GHCR and the interval from the start of the reproductive program and first detected estrus.

Statistical models to evaluate the likelihood of pregnancy and pregnancy loss included GDPR (linear and quadratic), GHCR (linear and quadratic), the interaction between GDPR and GHCR, $PGF_{2\alpha}$ formulation, estrus detection method, the interaction between estrus detection method and $PGF_{2\alpha}$ formulation, estrous cycle phase at the start of the reproductive program, the interaction between estrous cycle phase and $PGF_{2\alpha}$ formulation, the interaction between estrus detection method and estrous cycle phase, technician, and percentage of days with THI ≥ 72 and cumulative precipitation within 30 days after the start of the reproductive program. Statistical models to evaluate the likelihood of pregnancy and pregnancy loss after ET also included embryo type (fresh in vivo produced embryo, frozen/thawed in vivo produced embryo, fresh in vitro fertilized embryo, and frozen/thawed in vitro fertilized embryo), embryo grade (excellent/good, fair, and poor), and days after estrus at embryo transfer (6 to 9 d).

For the analysis of the hazard of pregnancy after the start of the reproductive program, models included GDPR (linear and quadratic), GHCR (linear and quadratic), the interaction between GDPR and GHCR; $PGF_{2\alpha}$ formulation, the interactions between GDPR and $PGF_{2\alpha}$ formulation and between GHCR and $PGF_{2\alpha}$ formulation, the estrus detection method (AED vs. VIS) and the interactions between GDPR and estrus detection method, between GHCR and estrus detection method, and between $PGF_{2\alpha}$ formulation and estrus detection method; estrous cycle phase at the time of the start of the reproductive program and the interactions between GDPR and estrous cycle phase at the time of the start of the reproductive program, between GHCR and estrous cycle phase at the time of the start of the reproductive program, and between $PGF_{2\alpha}$ formulation and estrous cycle phase at the time of the start of the reproductive program; type of service (AI vs. ET) and the interactions between GDPR and type of service, between GHCR and type of service, between $PGF_{2\alpha}$ formulation and type of service, and between estrus detection method and type of service, and percentage of days with THI ≥ 72 and cumulative precipitation 30 days after the start of the reproductive program. When GDPR and GHCR were associated with the hazard of pregnancy, these variables were divided into quartile and the Wilcoxon test of equality (LIFETEST procedure) was used to characterize the association between GDPR and GHCR and the interval from the start of the reproductive program and first detected estrus.

For each of the statistical models, collinearity was tested using the REG procedure with the "collin" and "VIF" functions. Variables with variance inflation factors ≥ 1.5 were considered collinear. In such cases, each variable was added to the model separately and the variable with the smallest *P* – value was retained. In all models, a backward stepwise elimination procedure was adopted and variables with $P > 0.10$ were removed until all variables that remained in the

model had $P \leq 0.10$. Statistical significance was considered at $P \leq 0.05$ and a tendency was consider when $0.05 < P \leq 0.10$.

Results regarding the effects of $PGF_{2\alpha}$ formulation and phase of the estrous cycle at the start of the reproductive program on estrous characteristics and the effects of $PGF_{2\alpha}$ formulation, phase of the estrous cycle at the start of the reproductive program, and estrus detection method on reproductive responses are discussed in Chapter 2.

Results

Characteristics of the Study Population

Mean (±SEM) age and body weight at the start of the reproductive period were 377 ± 6 d and 384 ± 28 kg, respectively. Mean (±SEM) GDPR values in the study population were 1.65 ± 1.29 (range: -1.8 to 5.9; Figure 3-1.) and mean GHCR values for the study population were 1.34 ± 1.11 (range: -2.1 to 5.5; Figure 3-2.). Spearman's coefficient of rank correlation between GDPR and GHCR was 0.455 (95% CI = 0.405-0.503; $P < 0.01$; Figure 3-3.).

Association Among Genomic Daughter Pregnancy Rate and Heifer Conception Rate and Estrous Characteristics

Duration of the SPE tended ($P = 0.08$) to increase according to GDPR, but there was ($P < 0.01$) a negative association between GHCR and duration of SPE (Figure 3-4.). Percentage of days with THI ≥ 72 also was negatively associated with the duration of SPE (Table 3-1.). Interval from onset of estrus to activity peak tended ($P = 0.06$) to be negatively associated with GDPR and was ($P = 0.03$) positively associated with quadratic GDPR. Interval from onset of estrus to activity peak was negatively associated with percentage of days with THI ≥ 72 ($P < 0.01$) and positively associated with cumulative precipitation ($P = 0.03$) in the last 30 d prior to

the start of the reproductive program (Table 3-1.). Rumination nadir on the day of SPE was negatively associated with GDPR ($P = 0.03$) and positively associated with GHCR ($P = 0.05$; Figure 3-5), whereas cumulative precipitation in the last 30 days before the start of the reproductive program was ($P < 0.01$) negatively associated with rumination nadir (Table 3-1.). The likelihood of activity peak ≥ 80 tended ($P = 0.09$) to be positively associated with GDPR and was ($P = 0.04$) positively associated with cumulative precipitation in the last 30 days before the start of the reproductive program (Table 3-1.). There was a tendency ($P = 0.06$) for GDPR to be positively associated with the likelihood of heat index ≥ 80, but GHCR was ($P = 0.03$) negatively associated with the likelihood of heat index ≥ 80 (Table 3-1.; Figure 3-7.). Percentage of days with THI > 72 in the last 30 days before the start of the reproductive program was ($P = 0.01$) negatively associated with the likelihood of heat index ≥ 80 (Table 3-1.).

No association was observed among GDPR ($P = 0.24$) and GHCR ($P = 0.28$) and duration of PIE. Estrous cycle phase was associated ($P < 0.001$) with duration of PIE because ED heifers had shorter PIE, followed by MID and PE heifers, respectively. Duration of PIE was negatively associated with percentage of days with THI ≥ 72 ($P < 0.01$) and positively associated with cumulative precipitation ($P < 0.01$) in the first 30 days after the start of the reproductive program (Table 3-2.). Interval from onset of PIE to activity peak was positively associated with GDPR ($P < 0.01$) and GHCR quadratic ($P = 0.05$). Conversely, the interval from onset of PIE to activity peak was ($P = 0.02$) negatively associated with the interaction between GDPR and GHCR. Cumulative precipitation in the 30 days after the start of the reproductive program was ($P = 0.03$) positively associated with the interval from onset of PIE and activity peak. Estrous cycle phase on the day of $PGF_{2\alpha}$ treatment was ($P < 0.01$) associated with the interval from onset of PIE to peak activity because ED heifers had shorter interval from onset of PIE to activity peak

compared with MID ($P < 0.01$) and PE ($P < 0.01$) heifers. Rumination nadir on the day of PIE was ($P < 0.01$) negatively associated with GDPR (Table 3-2.). Cumulative precipitation in the 30 days after the start of the reproductive program was ($P < 0.01$) negatively associated with rumination nadir on the day of PIE (Table 3-2.). The estrous phase at $PGF_{2\alpha}$ treatment was ($P < 0.01$) associated with rumination nadir on the day of PIE because ED and MID heifers had greater rumination nadir at estrus than PE heifers (Table 3-2.). There was a tendency ($P = 0.06$) for GDPR to be positively associated with the likelihood of activity peak ≥ 80, whereas the interaction between GDPR and GHCR tended ($P = 0.10$) to be negatively associated with the likelihood of activity peak ≥ 80 (Table 3-2.). Estrous cycle phase was ($P < 0.01$) associated with likelihood of activity peak ≥ 80 (Table 3-2.), because ED heifers were less likely to have activity peak ≥ 80 than MID and PE heifers (Table 3-2.).There was no association between GDPR ($P = 0.74$) and GHCR ($P = 0.49$) and the likelihood of heat index ≥ 80 on the day of PIE (Table 3-2.). Estrous cycle phase was ($P < 0.01$) associated with the likelihood of heat index ≥ 80 on the day of PIE because ED heifers were less likely to have a heat index ≥ 80 than MID and PE heifers (Table 3-2.).

Genetic merit for DPR was ($P = 0.01$) positively associated with the hazard of estrus after the start of the reproductive program. Heifers in the 4th quartile for GDPR were detected in estrus in average 93.69 ± 6.20 h after the start of the reproductive program, followed by heifers in the 3rd quartile (109.02 h ± 6.66 h) and heifers in the 2nd (128.99 ± 7.35 h) and 1st (124.89 ± 7.19 h) quartiles, respectively (Figure 3-8.). There was no ($P = 0.93$) association between GHCR and hazard of estrus after the start of the reproductive program. Phase of the estrous cycle at the start of the reproductive program was ($P < 0.01$) associated with the hazard of estrus because heifers in PE (reference) at $PGF_{2\alpha}$ treatment were detected in estrus at faster rate, followed by heifers in

mid-diestrus (AHR = 0.760, 95% CI = 0.628, 0.920) and heifers in early dietrus (AHR = 0.143, 95% CI = 0.107) and metestrus (AHR = 0.139, 95% CI = 0.103, 0.187), respectively.

Association among Genomic Daughter Pregnancy Rate and Heifer Conception Rate and Pregnancy to First Service

Genetic merit for DPR was ($P < 0.01$) positively associated with the likelihood of pregnancy 35 ± 3 d after the first AI. Other factors associated with the likelihood of pregnancy 35 ± 3 d after the first AI were estrous cycle phase at $PGF_{2\alpha}$ treatment ($P = 0.01$) and service technician ($P = 0.04$). The interaction between GDPR and HCR was ($P = 0.03$) negatively associated with the likelihood of pregnancy 35 ± 3 d after the estrus resulting in the first ET. Other factors associated with the likelihood of pregnancy 35 ± 3 d after the estrus resulting in the first ET were the type of embryo ($P < 0.01$), phase of the estrous cycle at $PGF_{2\alpha}$ treatment ($P = 0.05$), and ET technician ($P < 0.01$). There was a tendency ($P = 0.09$) for the percentage of days with THI ≥ 72 in the first 30 days after the start of the reproductive program to be negatively associated with the likelihood of pregnancy 35 ± 3 d after the estrus resulting in the first ET.

The interaction between GDPR and GHCR tended ($P = 0.08$) to be positively associated with the likelihood of pregnancy 75 ± 3 d after the first AI (Table 3-3.). Phase of the estrous cycle at $PGF_{2\alpha}$ treatment was ($P = 0.05$) negatively associated with the likelihood of pregnancy 75 ± 3 d after the first AI, because ED and MID heifers were less likely to have pregnancy 75 ± 3 d after the first AI than PE heifers (Table 3-3.). Technician ($P = 0.02$) was associated with the likelihood of pregnancy 75 ± 3 d after the first AI (Table 3-3.). The likelihood of pregnancy 75 ± 3 d after the estrous resulting in the first ET was ($P = 0.01$) negatively associated with the interaction between GDPR and GHCR (Table 3-3.). Type of embryo ($P < 0.01$) and ET technician ($P = 0.02$) were associated with the likelihood of pregnancy 75 ± 3 d after the estrus

resulting in the first ET. Additionally, the interaction between $PGF_{2\alpha}$ formulation and phase of the estrous cycle at $PGF_{2\alpha}$ treatment ($P = 0.06$) and method of estrus detection ($P = 0.06$) tended to be associated with the likelihood of pregnancy 75 ± 3 d after the estrus resulting in the first ET (Table 3-3.).

There were no associations between GDPR ($P = 0.47$) and GHCR ($P = 0.84$) and the likelihood of pregnancy loss from 35 ± 3 to 75 ± 3 d after the first AI. Similarly, GDPR ($P = 0.80$) and GHCR ($P = 0.81$) were not associated with the likelihood of pregnancy loss from 35 ± 3 to 75 ± 3 d after the estrus resulting in the first ET. There was, however, a tendency for method of estrus detection ($P = 0.07$) and type of embryo ($P = 0.06$) to be associated with the likelihood of pregnancy loss from 35 ± 3 to 75 ± 3 d after the estrus resulting in the first ET.

Association among Genomic Daughter Pregnancy Rate and Heifer Conception Rate and Hazard of Pregnancy

The interaction between GDPR and estrus detection method tended ($P = 0.08$) to be and the interaction between GHCR and estrus detection method was ($P = 0.05$) associated with the hazard of pregnancy. Among heifers detected in estrus by the AED system, GDPR was ($P = 0.05$) associated with the interval from onset of the reproductive program to establishment of pregnancy (Figure 3-9.), but GHCR was not ($P = 0.26$) associated with the interval from onset of the reproductive program to establishment of pregnancy (Figure 3-10.). Among heifers detected in estrus visually by herd personnel, GDPR ($P = 0.97$; Figure 3-11) and GHCR ($P = 0.12$; Figure 3-12.) were not associated with the interval from onset of the reproductive program to establishment of pregnancy.

Discussion

In the current study, GDPR was positively associated with more intense characteristics of SPE such as duration and the likelihood of activity peak ≥ 80 and was negatively associated with rumination nadir on the day of SPE. Consequently, GDPR was positively associated with the likelihood of heifers having heat index ≥ 80 on the day of SPE. Conversely, GHCR was negatively associated with duration of SPE and the likelihood of activity peak ≥ 80 on the day of SPE and positively associated with rumination nadir on the day of SPE, resulting in a negative association between GHCR and the likelihood of heifers having heat index ≥ 80. The differences in characteristics of estrus according to GDPR and GHCR demonstrated herein are important because these characteristics are generally associated with mounting activity, vaginal mucus consistency, and uterine tone (Pahl et al., 2015; Silper et al., 2015) and may improve estrus detection efficiency and accuracy. Estrous behavior is the consequence of an orchestrated sequence of events, which lead to the acceptability of the male by the female, and are regulated by a network of genes that promote mating behavior (Woelders et al., 2014). During the growth phase, there is an increase in connectivity of hypothalamic neurons controlling behavior, followed by progesterone binding to its receptors amplifying estrogen-induced estrous behaviors (amplification phase), expression of sexual receptivity by the female (preparation phase females), expression of hypothalamic-driven mating behaviors (permission phase), and, finally, synchrony of mating and ovulation to elicit fertilization (synchronization phase; Kommadath et al., 2011, 2013). During these phases of sexual behavior, several genes are differentially expressed in the hypothalamus, amygdala, hippocampus, and pituitary of lactating dairy cows during estrus and mid-diestrus (Kommadath et al., 2011, 2013). Holmberg and Andersson-Eklund (2006) genotyped 427 Swedish Red and Swedish Holstein bulls to identify quantitative trait loci (QTL) contributing to the genetic variation in fertility, among which was heat intensity

score, a subjective assessment by dairy farmers of a cow's ability to display signs of estrus. In this study 5 QTL associated with heat intensity score were determined on *Bos taurus* autosomes 4, 7, 9, 13, and 25, of which QTL on Bos taurus autosomes 7 and 9 were significant at the genome level (Holmberg and Andersson-Eklund, 2006). There is, therefore, a clear aspect of genetic control of estrous behavior that could help explain the associations among GDPR, GHCR and estrous behavior. In a study conducted in Ireland, cows on the top quartile in genetic merit for milk yield and on the bottom 5% for calving interval had reduced duration of and activity during estrus compared with cows on the top quartile in genetic merit for milk yield and on the top 20% for calving interval (Cummins et al., 2012). Not surprisingly, cows on the top 20% for calving interval had shorter days open and increased Preg/Serv in the first two services postpartum compared with cows on the bottom 5% for calving interval. The positive associations between GDPR and estrous characteristics observed in the current study may reflect how genetic selection for this trait impacts reproductive performance of US dairy herds. Daughter pregnancy rate is a measure of the genetic merit associated with expected differences in 21-d PregRate when comparing animals or populations (VanRaden et al., 2004). The 21-d PregRate is highly dependent on 21-d service rate (**21-d ServRate**; percentage of eligible cows that are serviced every 21 d after the end of the voluntary waiting period or start of the reproductive program) and pregnancy per service (percentage of cows that conceive after a service). Since GDPR was marginally associated with the probability of pregnancy after AI and ET, it seems logical to speculate that the advancements in reproductive performance generally associated with the onset of genetic selection for DPR since the early 2000s may be a consequence of improved estrus expression by animals with greater GDPR and greater hazard of detection of estrus. To our surprise, GHCR was negatively associated with estrous characteristics evaluated in this study,

but was not associated with hazard of PIE or pregnancy. Furthermore, the interaction between GDPR and GHCR was associated with a decreased likelihood of heifers having activity peak ≥ 80. Genetic merit for HCR reflects the likelihood of a sire's daughters to conceive after a service compared with the population. The negative association between GHCR and estrous characteristics should be carefully studied in order to prevent a negative effect of selection for higher GHCR on estrus expression and detection in future generations of Holstein animals.

In a companion study, we evaluated the size of ovarian follicles and concentrations of estradiol, progesterone, insulin like growth factor 1 (**IGF-1**), pregnancy specific protein B (**PSPB**), and interferon stimulated gene 15 (**ISG15**) of heifers with high GDPR and high GHCR, high GDPR and low GHCR, low GDPR and high GHCR, and low GDPR and low GHCR. In that study, ovulatory follicle size and estradiol concentrations were greater for high GDPR animals and were not associated with GHCR. Since estradiol is secreted from follicles in the ovary (Jinks et al., 2013) and triggers estrous behavior (Reith and Hoy, 2017), these results shed light on why GDPR was associated with greater estrus duration and intensity in the current study. Together, these data provides evidence that GDPR drives physiological changes that alter estrous behavior and could have a major impact on estrous detection efficiency and accuracy by dairy herds.

In the current study, interaction of GDPR and GHCR tended to increase likelihood of pregnancy 75 ± 3 d after the first AI, and only GDPR increased likelihood of pregnancy 75 ± 3 d after second service. Surprisingly though, for ET services, the interaction of GDPR and GHCR decreased likelihood of pregnancy 75 ± 3 d after first service. In the companion study, the interaction between GDPR and GHCR was associated with ISG15 expression 19 ± 2 d after estrus. This interaction was because LH heifers had greater expression of ISG15 than LL heifers,

while HH heifers and HL heifers were not different from LH and or LL heifers. Although IGF-1 concentration was not statistically different according to GDPR and GHCR, IGF-1 concentration was numerically greater for LH heifers at 19 ± 2 d after estrus, which has been associated with expression of ISG15 and pregnancy establishment and maintenance (Ribeiro et al., 2014). Kuhn et al. (2006) demonstrated a significant positive association between parent average DPR and Preg/Serv of heifers of multiple breeds. Since HCR has been implemented recently in the genetic selection of dairy cattle, its true association with pregnancy per service is less understood. Ortega et al. (2016) evaluated 69 single nucleotide polymorphisms (SNPs) related to fertility trades in Holstein cattle and showed that a significant number of genes associated with DPR were associated with HCR. These results suggest that genes driving fertility outcomes in cows associated with DPR may be the same driving fertility in heifers associated with HCR. The remaining different genes that compose GDPR or GHCR but do not overlap, however, may be genes responsible for different functions that lead to improved reproductive performance but not necessarily by the same route.

In the current study, GDPR was associated with improved estrus expression, and faster onset of estrus after a $PGF_{2\alpha}$ treatment. These results indicate that genomic selection for DPR has the potential to select animals with improved estrus expression, duration, and intensity, which in turn could improve reproductive performance and profitability of dairy operations. Furthermore, due to increasing concern of costumers over use of hormones for milk production, selection for GDPR can be an alternative for farmers interested in reducing hormonal use for estrous cycle manipulation. Conversely, selection of dairy animals based on GHCR should be carefully evaluate because in the current study it was associated with reduced estrus duration and intensity. Reduction in estrus duration and intensity can be detrimental for reproductive performance, since

it can reduce estrus detection. Lastly, more studies are necessary to unravel how GDPR and GHCR drive pregnancy establishment and maintenance in dairy heifers.

Table 3-1. Final logistic regression model of factors associated with characteristics of spontaneous estrus

Variables	Estimates (±SE)	*P* – value
	Duration	
GDPR (linear)	0.118 ± 0.066	0.08
GHCR (linear)	-0.254 ± 0.008	<0.01
Pct THI ≥ 72[&]	-1.217 ± 0.450	<0.01
	Interval onset to peak of estrus	
GDPR (linear)	-0.0028 ± 0.0002	0.06
GDPR (quadratic)	0.0002 ± 0.0001	0.03
Pct THI ≥ 72[&]	-0.1098 ± 0.0176	<0.01
Precipitation[*]	0.0005 ± 0.0001	0.03
	Rumination Nadir	
GDPR (linear)	-0.515 ± 0.007	0.03
GHCR (linear)	0.515 ± 0.008	0.05
Precipitation[*]	-1.018 ± 0.001	0.01
	Activity Peak ≥ 80	
GDPR (linear)	0.058 ± 0.035	0.02
Precipitation[*]	0.073 ± 0.030	0.04
	Heat Index ≥ 80	
GDPR (linear)	0.075 ± 0.040	0.06
GHCR (linear)	-0.098 ± 0.047	0.03
Pct THI ≥ 72[&]	0.644 ± 0.260	0.01

[&]Pct THI ≥ 72: Percentage of days with Temperature Humidity Index (THI) above or at 72, in the 30 days prior to eligible for breeding

[*]Cum prec: Cumulative precipitation 30 days prior to reproductive period started

Table 3-2. Final logistic regression model of factors associated with characteristics of $PGF_{2\alpha}$ induced estrus

Variables	Estimates (±SE)	*P* – value
	Duration	
ECD[£] (ED vs. PE)	-3.641 ± 0.699	<0.01
ECD[£] (MID vs. PE)	-1.131 ± 0.491	0.02
Number of $PGF_{2\alpha}$ (1 vs 2)	2.040 ± 0.683	<0.01
Pct THI ≥ 72[&]	-1.272 ± 0.651	0.05
Precipitation[*]	0.215 ± 0.077	< 0.01
	Interval onset to peak of estrus	
GDPR (linear)	0.0045 ± 0.0003	< 0.01
GHCR (linear)	-0.0009 ± 0.0001	0.36
GDPR (quadratic)	0.0003 ± 0.0001	0.05
GDPR x GHCR	-0.0006 ± 0.0001	0.02
ECD[£] (ED vs. PE)	-0.1975 ± 0.0394	< 0.01
ECD[£] (MID vs. PE)	-0.0010 ± 0.0001	0.66
Number of $PGF_{2\alpha}$ (1 vs 2)	0.0314 ± 0.0044	0.07
Precipitation[*]	0.0003 ± 0.0001	0.03
	Rumination Nadir	
GDPR (linear)	-0.520 ± 0.005	< 0.01
ECD[£] (ED vs. PE)	1.336 ± 0.063	< 0.01
ECD[£] (MID vs. PE)	1.101 ± 0.033	< 0.01
Precipitation[*]	-1.014 ± 0.004	<0.01
	Activity Peak ≥ 80	
GDPR (linear)	0.096 ± 0.051	0.06
GHCR (linear)	0.064 ± 0.064	0.31
GDPR x GHCR	-0.021 ± 0.013	0.10
Number of $PGF_{2\alpha}$ (1 vs. 2)	0.747 ± 0.350	0.03
ECD (ED vs. PE)	-2.210 ± 0.447	< 0.01
ECD (MID vs. PE)	-0.293 ± 0.363	0.42
	Heat Index ≥ 80	
ECD[£] (ED vs. PE)	-1.625 ± 0.406	< 0.01
ECD[£] (MID vs. PE)	0.369 ± 0.338	0.28

[£]ECD: Estrous cycle day (ME: Meteestrus; ED: Early diestrus; MID: Mid-diestrus; PE: Proestrus)

[&]Pct THI ≥ 72: Percentage of days with temperature humidity index (THI) ≥ 72, in the 30 days after the start of the reproductive program

*Precipitation: Cumulative precipitation 30 days after the start of the reproductive program

Table 3-3. Final logistic regression model of factors associated with the likelihood of pregnancy after the first service (75 ± 3 d after service)

Variables	Estimates (±SE)	*P* – value
	First service	
Artificial Insemination		
GDPR (linear)	0.017 ± 0.065	0.77
GHCR (linear)	-0.069 ± 0.071	0.33
GDPR x GHCR	0.026 ± 0.015	0.08
ECD[£] (ED vs. PE)	-0.759 ± 0.319	0.02
ECD[£] (MID vs. PE)	-0.421 ± 0.248	0.09
Technician	-	0.02
Embryo Transfer		
GDPR (linear)	0.183 ± 0.075	0.01
GHCR (linear)	0.201 ± 0.097	0.04
GDPR x GHCR	-0.052 ± 0.022	0.02
ECD[£] (ED vs. PE)	-0.540 ± 0.653	0.41
ECD[£] (MID vs. PE)	0.589 ± 0.360	0.10
Technician	-	<0.01
Embryo type (1 vs. 4)[α]	1.932 ± 0.677	<0.01
Embryo type (2 vs. 4) [α]	-0.212 ± 0.941	0.82
Embryo type (3 vs. 4) [α]	1.201 ± 0.560	0.03

[£] ECD: Estrous cycle day (ED = Early diestrus; MID = Mid-diestrus; PE = Proestrus)

[α] Embryo type: 1 – fresh *in vivo* produced embryo, 2 – frozen/thawed *in vivo* produced embryo, 3 – fresh *in vitro* fertilized embryo, and 4 – frozen/thawed *in vitro* fertilized embryo.

Table 3-4. Final logistic regression model of factors associated with hazard of pregnancy

Variables	Estimates (±SE)	*P* – value
GDPR (linear)	-0.072 ± 0.057	0.21
GHCR (linear)	0.061 ± 0.088	0.49
GHCR (quadratic)	0.042 ± 0.022	0.06
Estrus detection method (AED vs. VIS)	0.176 ± 0.149	0.24
GDPR x estrus detection method	0.127 ± 0.073	0.08
GHCR x estrus detection method	-0.162 ± 0.083	0.05
ECD[£] (ED vs. PE)	-0.078 ± 0.149	0.60
ECD[£] (MID vs. PE)	-0.058 ± 0.120	0.63
ECD[£] (ME vs. PE)	-0.392 ± 0.162	0.02
Breeding code (AI vs ET)	0.438 ± 0.085	<0.01
Pct THI ≥ 72[&]	-0.207 ± 0.109	0.06

[£] ECD: Estrous cycle day (ME = Metaestrus; ED = Early diestrus; MID = Mid-diestrus; PE = Proestrus)

[&]Pct THI ≥ 72: Percentage of days with temperature humidity index (THI) ≥ 72, in the 30 days after the start of the reproductive program.

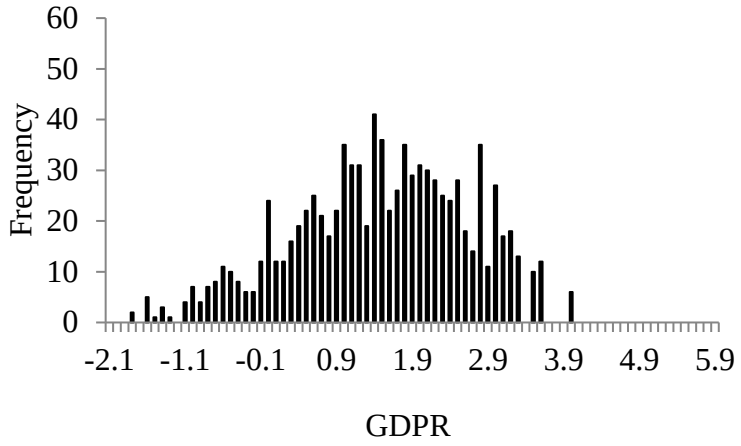

Figure 3-3. Distribution of genetic merit for daughter pregnancy rate (GDPR) values in the study population. Mean ± SD: GDPR = 1.65 ± 1.29 (range, -1.8 - 5.0).

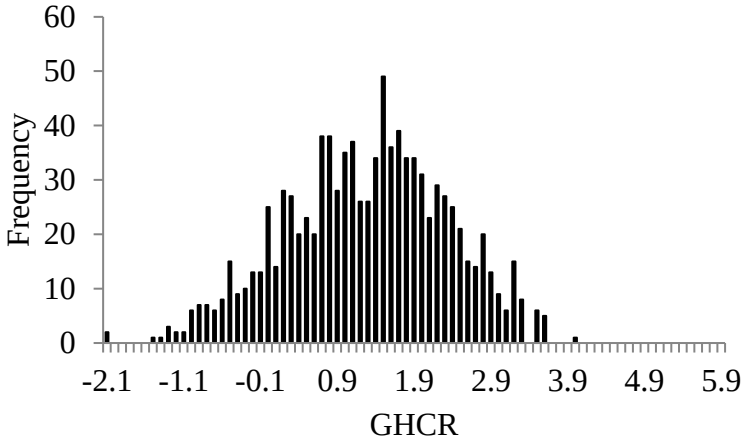

Figure 3-2. Distribution of genetic merit for heifer conception rate (GHCR) values in the study population. Mean ± SD: GHCR = 1.34 ± 1.1 (range, -2.1 - 5.5)

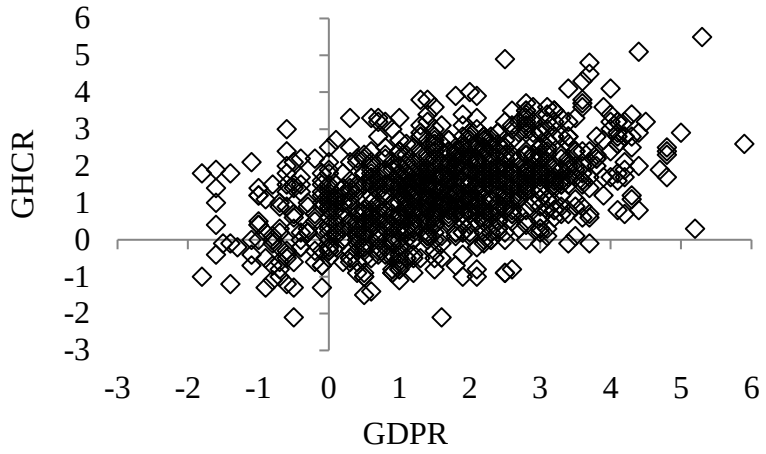

Figure 3-3. Correlation of genetic merit for daughter pregnancy rate (GDPR) and heifer conception rate (GHCR) (C). Spearman's coefficient of rank correlation = 0.455 (Confidence interval = 0.405-0.503; $P < 0.01$).

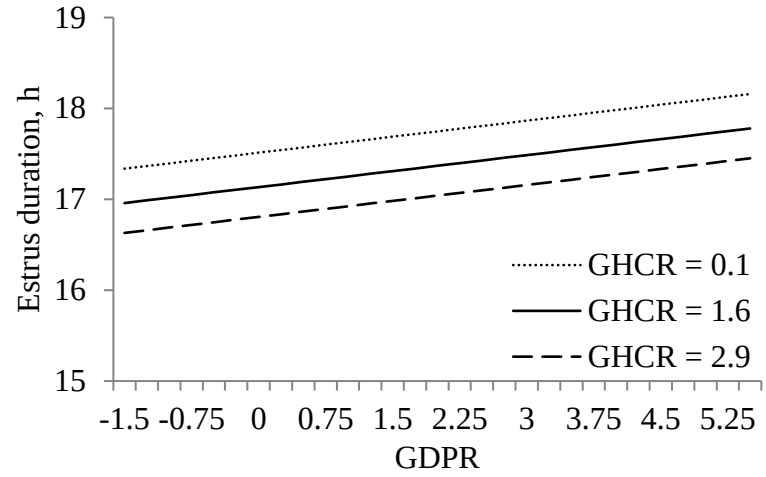

Figure 3-4. Duration of estrus according to genetic merit for daughter pregnancy rate (GDPR) and heifer conception rate (GHCR). GHCR = 0.1 (low), GHCR = 1.6 (intermediary), GHCR = 2.9 (high). GDPR - $P = 0.08$, GHCR - $P < 0.01$.

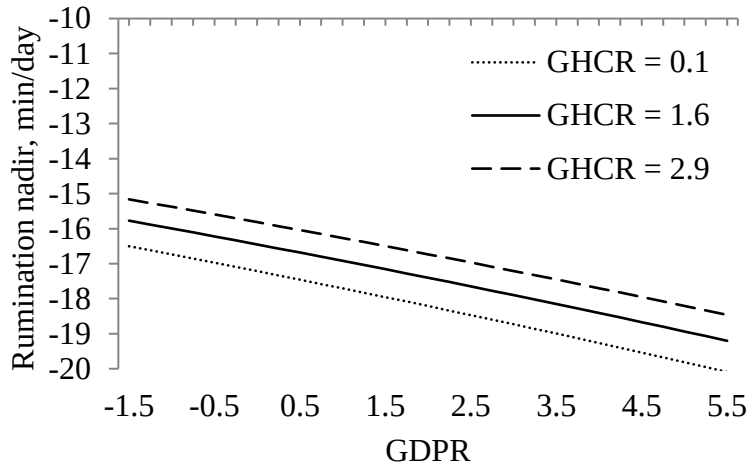

Figure 3-5. Rumination nadir according to genetic merit for daughter pregnancy rate (GDPR) and heifer conception rate (GHCR). GHCR = 0.1 (low), GHCR = 1.6 (intermediary), GHCR = 2.9 (high). GDPR - *P* = 0.03, GHCR - *P* = 0.05.

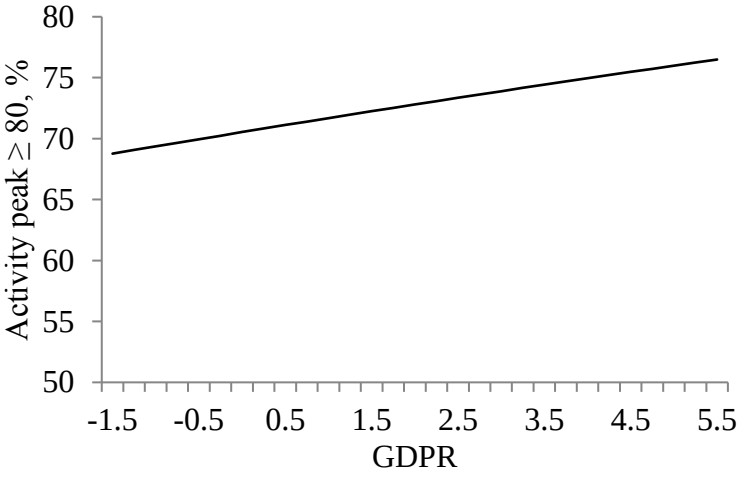

Figure 3-6. Activity peak according to genetic merit for daughter pregnancy rate (GDPR). GDPR - *P* = 0.02.

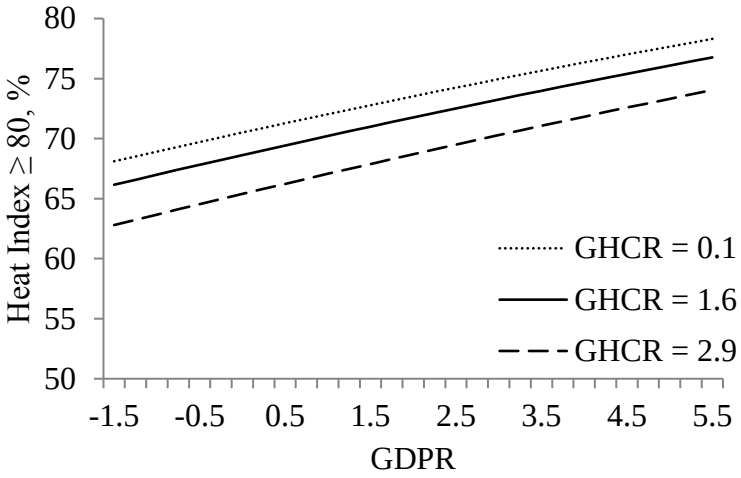

Figure 3-7. Heat index according to genetic merit for daughter pregnancy rate (GDPR) and heifer conception rate (GHCR). GHCR = 0.1 (low), GHCR = 1.6 (intermediary), GHCR = 2.9 (high). GDPR - $P = 0.03$, GHCR - $P = 0.05$. Heat index, percentage ≥ 80, according to GDPR ($P = 0.06$), when GHCR ($P = 0.03$) is low (0.1), intermediary (1.6), or high (2.9).

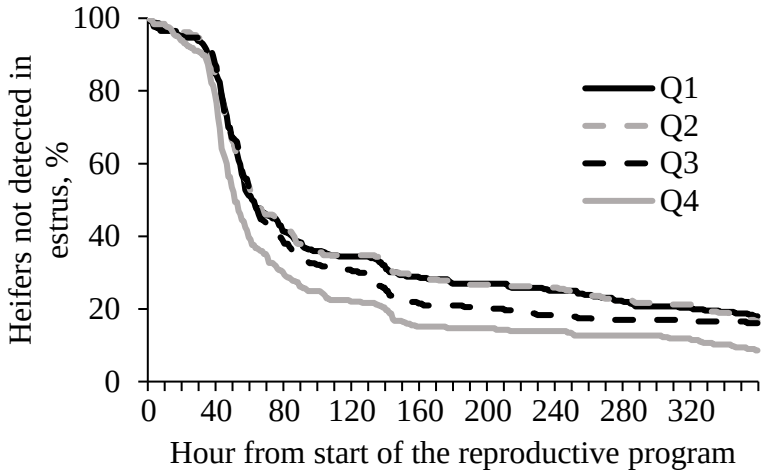

Figure 3-8. Interval from start of the reproductive program to first estrus detected by the AED according to GDPR quartile: Q1 = quartile 1 (GDPR = -1.8 to 0.8); Q2 = quartile 2 (GDPR = 0.9 to 1.7); Q3 = quartile 3 (GDPR = 1.8 to 2.5); Q4 = quartile 4 (GDPR = 2.6 to 5.9). Mean (±SEM) and median interval from the start of the reproductive program to first detected estrus: Q1 = 124.89 ± 7.19 and 58.5 h; Q2 = 128.99 ± 7.35 and 61 h; Q3 = 109.02 ± 6.66 and 60.2 h; and, Q4 = 93.69 ± 6.20 and 50.4 h. GDPR - $P < 0.01$.

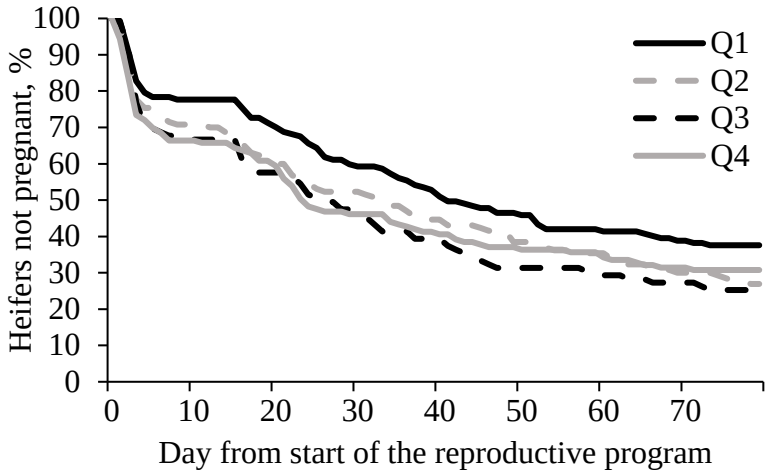

Figure 3-9. Interval from start of the reproductive program to pregnancy for heifers detected in estrus by an automated estrus detection device (AED) according to GDPR quartile: Q1 = quartile 1 (GDPR = -1.8 to 0.8); Q2 = quartile 2 (GDPR = 0.9 to 1.7); Q3 = quartile 3 (GDPR = 1.8 to 2.5); Q4 = quartile 4 (GDPR = 2.6 to 5.9). Mean (±SEM) and median interval from the start of the reproductive program to pregnancy: Q1 = 42.5 ± 2.3 and 41 d; Q2 = 38.4 ± 2.7 and 34 d; Q3 = 35.5 ± 3.1 and 26 d; and, Q4 = 37.2 ± 2.7 and 24 d. GDPR - *P* = 0.05.

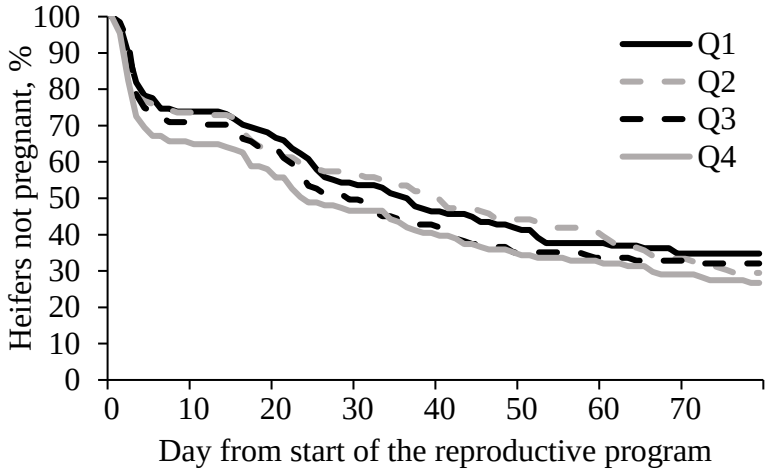

Figure 3-10. Interval from start of the reproductive program to pregnancy for heifers detected in estrus by an automated estrus detection device (AED) according to GHCR quartile: Q1 = quartile 1 (GHCR = -2.1 to 0.6); Q2 = quartile 2 (GHCR = 0.7 to 1.4); Q3 = quartile 3 (GHCR = 1.5 to 2.1); Q4 = quartile 4 (GHCR = 2.2 to 5.5). Mean (±SEM) and median interval from the start of the reproductive program to pregnancy: Q1 = 38.0 ± 2.3 and 37 d; Q2 = 42.0 ± 2.8 and 40 d; Q3 = 38.7 ± 2.8 and 29 d; and, Q4 = 35.5 ± 2.7 and 24 d. GHCR - *P* = 0.26).

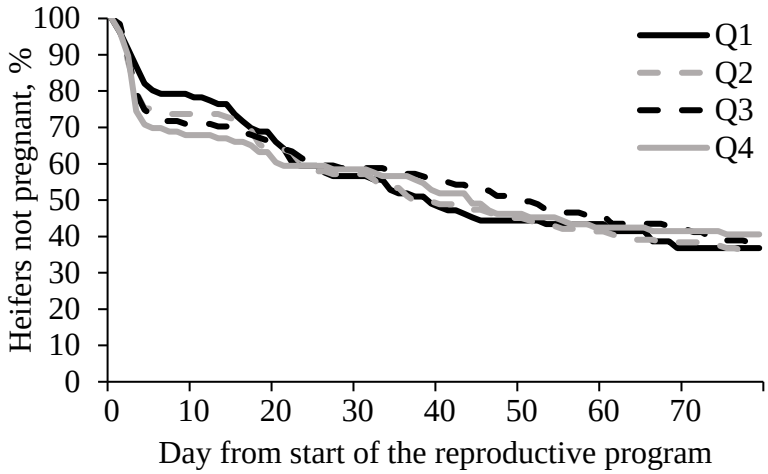

Figure 3-11. Interval from start of the reproductive period to pregnancy for heifers detected in estrus by visual observation (VIS) according to GDPR quartile: Q1 = quartile 1 (GDPR = -1.8 to 0.8); Q2 = quartile 2 (GDPR = 0.9 to 1.7); Q3 = quartile 3 (GDPR = 1.8 to 2.5); Q4 = quartile 4 (GDPR = 2.6 to 5.9). Mean (±SEM) and median interval from the start of the reproductive program to pregnancy: Q1 = 43.9 ± 3.1 and 39 d; Q2 = 42.8 ± 2.8 and 37 d; Q3 = 45.2 ± 2.9 and 50 d; and, Q4 = 43.6 ± 3.3 and 44 d. GDPR - $P = 0.97$.

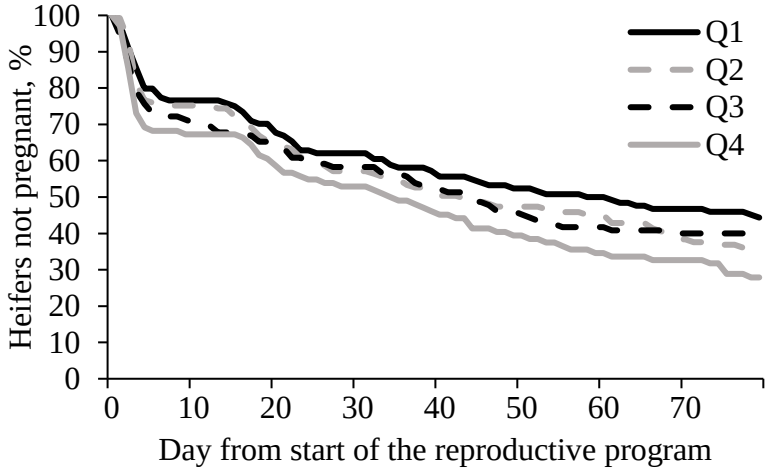

Figure 3-12. Interval from start of the reproductive period to pregnancy for heifers detected in estrus by visual observation (VIS) according to GHCR quartile: Q1 = quartile 1 (GHCR = -2.1 to 0.6); Q2 = quartile 2 (GHCR = 0.7 to 1.4); Q3 = quartile 3 (GHCR = 1.5 to 2.1); Q4 = quartile 4 (GHCR = 2.2 to 5.5). Mean (±SEM) and median interval from the start of the reproductive program to pregnancy: Q1 = 47.6 ± 2.9 and 60 d; Q2 = 44.4 ± 2.8 and 43 d; Q3 = 43.6 ± 3.1 and 44 d; and, Q4 = 39.0 ± 3.2 and 35 d. GHCR - $P = 0.12$.

CHAPTER 4
PHYSIOLOGICAL RESPONSES OF HOLSTEIN HEIFERS WITH HIGH AND LOW GENOMIC MERIT FOR FERTILITY TRAITS

Reproductive performance of Holstein cattle has declined over the past decades, with lowest breeding values for daughter pregnancy rate (**DPR**) recorded in the early 2000's (AIPL, 2005). One of the main factors negatively impacting reproductive performance is believed to be the intense genetic selection for milk yield with disregard for reproduction traits (Lucy, 2001; VanRaden et al., 2004). Between 1963 and 2003 an increment of 3,259 kg in breeding values for milk yield (AIPL, 2005) was observed; concurrently, breeding values for DPR decreased from approximately 16 in 1957 to negative values in the early 2000's (USDA, 2016). To halt the decline in reproductive performance associated with selection for productive traits alone, DPR was added to the genetic merit in 2003, allowing selection of Holstein animals with improved reproductive performance. Since the addition of DPR on genetic selection, breeding values for DPR have slightly increased, but still remain lower than the DPR values observed in the 1960's.

Because of its low heritability (Pryce et al., 2004), genetic progress for fertility traits such as DPR is low (García-Ruiz et al., 2016). With recent advances in genomic tools for prediction of breeding values and inclusion of genomic predicted transmitted ability (**GPTA**) values for DPR and other fertility traits, such as heifer conception rate (**HCR**), genetic progress for low heritable traits, such these fertility traits, significantly increased (García-Ruiz et al., 2016). Daughter pregnancy rate is a measure of the hazard of pregnancy of a bull's daughters compared with the population and genomic daughter pregnancy rate (**GDPR**) is a genomic predicted breeding value for DPR. Heifer conception rate is a measure of likelihood of pregnancy following a service for heifers of a bull's daughter compared with the population and genomic heifer conception rate (**GHCR**) is a genomic predicted breeding value for HCR.

Genomic fertility traits are associated with actual phenotypic values observed in the Holstein population (Mikshowsky et al., 2016; Ortega et al., 2016), but still little is known about how these genetic markers affect the phenotype. In recent experiments, researchers demonstrated that several genes represented by single nucleotide polymorphisms (**SNP**) known to be involved with endocrine system, cell signaling, immune function and inhibition of apoptosis, were also associated with fertility traits such GDPR and GHCR in Holstein cows (Cochran et al., 2013). Furthermore, many of the genes Cochran et al. (2013) demonstrated to be associated with fertility traits were previously shown to be associated with steroidogenesis in Holstein cows (Ortega et al., 2016). Although Cochran et al. (2013) and Ortega et al. (2016) provided valuable information about the possible functions of genes composing genomic predicted fertility traits (e.g. GDPR and GHCR), information about how genomic breeding values for fertility traits are associated with physiological responses in Holstein animals is not abundant.

The hypothesis of the current study was that Holstein heifers differing in GDPR and GHCR have significant differences regarding ovulatory follicle size, estradiol concentration at estrus, and progesterone, insulin like growth factor 1 (**IGF-1**), and pregnancy specific protein B (**PSPB**) concentrations after estrus, and expression of interferon stimulated gene 15 (**ISG15**) 19 d after estrus. Therefore, the objectives of the current study were to elucidate differences in ovulatory follicle size, estradiol concentration at estrus, and progesterone, IGF-1, and PSPB concentrations after estrus, and expression of ISG15 19 d after estrus of heifers in the extreme of GDPR and GHCR within a population of Holstein heifers.

Material and Methods

All procedures involving animals were approved by the animal care and use committee of the University of Florida (protocol #201609559).

Animals, Housing, and Management

The study was conducted from September to December 2016 in a commercial dairy herd with approximately 4,200 replacement heifers located in north central Florida. Ninety-nine Holstein heifers between 10 and 11 months of age were enrolled in the study. All heifers were genotyped within 2 months of birth using a 50k single nucleotide peptide platform commercially available (Clarifide, Zoetis, Parsippany, NJ). For the purpose of this study, data referent to genomic breeding values for DPR and HCR recorded within 2 months of birth were used.

Heifers selected for this experiment were in the top and bottom 50 percentile for GDPR or GHCR values in this population of 1,019 heifers. Heifers were classified as: high GDPR (range = 1.6 to 5.3), low GDPR (range = -1.8 to 1.0), high GHCR (range = 1.5 to 5.5), and low GHCR (range = -2.1 to 1.2). The resulting combinations of GDPR and GHCR class were, respectively: HH (n = 28), HL (n = 20), LH (n = 21), and LL (n = 30).

Starting at 12 months of age, heifers were weighed weekly and heifers with ≥ 340 kg of live body weigh were moved to a breeding pen and were treated with prostaglandin (**PG**) $F_{2\alpha}$ (cloprostenol sodium, Estrumate, Merck Animal Health, Summit, NJ) for synchronization of estrus. Heifers were housed in dry lots, with natural shade and no artificial cooling. The breeding pens had self-locking head stanchions on the feeding area. Heifers were fed twice daily (7:00 AM and 4:30 PM) a TMR formulated to meet or exceed the nutritional requirements of Holsteins heifers weighing 340 kg of live body weight and gaining 800 to 1,000 g of live body weight per day (NRC, 2001).

Automated Estrus Monitoring System

At enrollment, an automated estrus detection monitoring device (**AED**; Heat Rumination Long Distance, SCR Inc., Netanya, Israel) was fitted on the left, cranial area of the neck of all heifers. The AED determined activity through an accelerometer and rumination based on sounds of regurgitation and mastication through a microphone. Activity and rumination data were recorded for every 2 h periods. Estrus was determined according to changes in patterns of activity and rumination within a 2 h period compared with the average activity and rumination of the same period in the previous 5 and 7 d, respectively (DataFlow2©, SCR Inc, Netanya, Israel).

Reproductive Management

From enrollment to the start of the reproductive program, all estrus events were recorded. Heifer eligible to start the reproductive program (≥ 12 months of age and ≥ 340 kg of live body weight) were classified according to estrous cycle phase into early metestrus (estrous cycle day 0 to 3), early diestrus (estrous cycle day 4 to 6), mid-diestrus (estrous cycle day 7 to 17), proestrus (estrous cycle day ≥ 18), and no estrus observed. Heifers in early diestrus, mid-diestrus, proestrus and heifers that had no estrus observed were treated with $PGF_{2\alpha}$ immediately and heifers in metestrus were treated with $PGF_{2\alpha}$ 96 h later and heifers. According to the genetic selection program of the dairy, heifers were selected to be artificially inseminated (**AI**) or to receive an embryo transfer (**ET**). Heifers detected in estrus were artificially inseminated on the same morning or received an embryo 6 to 9 days after estrus detection.

Pregnancy Diagnoses and Reproductive Data

All heifers were examined for pregnancy by palpation per rectum of uterine contents at 35 ± 3 d after the detected estrus that resulted in AI or ET. Pregnant heifers were re-examined by palpation per rectum of the uterine contents at 75 ± 3 days of gestation.

Pregnancy per service was calculated by dividing the number of heifers pregnant at 35 and 75 ± 3 d after estrus by the number of heifers serviced. Pregnancy loss was calculated by dividing the number of heifers pregnant at 75 ± 3 d after estrus by the number of heifers pregnant 35 ± 3 d after estrus. Data regarding sire of insemination, sire and dam of embryo transfer, service technician, and reproductive outcomes were collected from farm records using dairy management software PCDART (Dairy records management system, Chapel Hill, North Carolina).

Blood Sampling and Ultrasonography of the Ovaries

Blood was sampled on the day of $PGF_{2\alpha}$ treatment, on the first morning after detected estrus (2 to 26 h after onset of estrus), and at 7, 14, 19 ± 2, 28, and 35 d after estrus. Samples were not collected when heifers returned to estrus and received a second service. Blood was sampled by puncture of the coccygeal vein or artery into evacuated tubes containing K2 EDTA (Vacutainer, Becton Dickinson, Franklin Lakes, NJ). Immediately upon collection, tubes were placed in ice and kept refrigerated until transported to the laboratory for processing, within 2 to 3 h of collection. Blood tubes were centrifuged at 1,500 × g for 15 min. Aliquots of plasma were frozen at -80 °C until assayed. Ovaries of heifers were evaluated by transrectal ultrasonography (MyLab^{TM}, Esaote North America, Inc., Fishers, IN) in the first morning after detected estrus (1 to 24 h after onset of estrus) and daily until ovulation was observed (disappearance of a follicle larger than 10 mm) or 96 h after onset of estrus.

Analysis of Plasma Samples

Progesterone concentrations was determined by radioimmunoassay (**RIA**) using a commercial kit (Coat-a-Count, MP Biomedical LLC, Solon OH). Plasma harvested from heifers

on days 4 (~1 ng/mL) and 10 (~ 4 ng/mL) of the estrous cycle were incorporated into each assay and used to calculate the CV. Intra and inter-assay CVs were 5.8 and 10.5 % respectively. Serum concentration of estradiol-17β were quantified by RIA as described by Jinks et al. (2013). Intra-assay coefficient of variance for estradiol assays was 2.73%. Concentrations of PSPB were analyzed using a commercially available quantitative ELISA assay (BioPRYN; BioTracking LLC, Moscow, ID) according to the method described by Humblot et al. (1988). Intra and inter-assay CVs were 3.5 and 8.2 % respectively. Concentrations of IGF-1 were determined by a commercial ELISA kit (Quantikine ELISA Human IGF-1 Immunoassay, R&D Systems) designed for human IGF-1, but with 100% cross-reactivity with bovine IGF-1, as described previously by Ribeiro et al. (2014). The intra-assay CV for IGF-1 was 8.0%.

Isolation of Peripheral Blood Leukocytes, mRNA Extraction, and Quantitative Real Time qPCR

Blood sampled 19 ± 2 d after estrus was used for isolation of peripheral blood leukocytes (**PBL**) according to Gifford et al. (2008). After centrifugation and harvest of plasma, buffy coat fractions were collected by pipetting and transferred to 15-mL conical tubes. A red cell lysis buffer was prepared (150 mM NH4Cl, 10 mM NaHCO3, and 1 mM EDTA; pH 7) and added to the buffy coat for a total volume of 15 mL. Tubes were inverted several times and incubated at room temperature for 5 min. Samples were then centrifuged at 300 × g for 10 min at 4°C and the supernatant was discarded. The PBL pellet was mixed with 5 mL of red cell lysis buffer, incubated at room temperature for 5 min, and centrifuged at 300 × g for 10 min at 4°C, and the supernatant was discarded. The PBL pellet was washed with ice-cold PBS and centrifuged at 300 × g for 10 min at 4 °C and the supernatant was discarded. The PBL pellet was re-suspended with 0.8 mL of Trizol (Molecular Research Center, Inc., Cincinnati, OH), transferred to 1.5-mL

microtubes, and stored at -80 °C. The time interval from blood collection and PBL sample storage at -80 °C was no longer than 6 h.

Extraction of mRNA was conducted according to the manufacturer's recommendations for the RNA-extraction kit (PureLink RNA Mini Kit; Invitrogen, Carlsbad, CA). The concentration of RNA was calculated by measuring absorbance at 260 nm, and 1 μg of total cellular mRNA was treated with DNase (RQ1 RNase-Free DNase; Promega, Madison, WI) and was used to synthesize complementary DNA using the DyNAmo cDNA Synthesis Kit (Thermo Scientific, Waltham, MA). Complementary DNA was then used for quantitative RT-PCR (ABI 7500 Sequence Detector; Applied Biosystems Inc., Foster City, CA). Three genes were investigated: ISG15 (target gene), beta-actin (**ACTB**; reference gene), and ribosomal protein L 19 (**RPL19**; reference gene). Primer reference and sequence are represented in Table 4-1. Each reaction mixture consisted of 3 μl of a 1:5 dilution of the cDNA, gene-specific forward and reverse primers, SYBR Green (Applied Biosystems Inc., Foster City, CA), and nuclease-free water in a total reaction volume of 20 μl. Reactions were run in duplicate and comprised 40 cycles of a three-step amplification protocol (30 sec at 95 °C followed by 45 sec at the optimized annealing temperature [57 °C-60 °C] and 1 min at 72 °C). Primer efficiency ranged from 81% to 85%. Melting curve analysis was also performed to ensure amplification of a single product.

Statistical Analysis

Data was analyzed using SAS version 9.3 (SAS Institute Inc., Raleigh, NC). Continues variables were analyzed by ANOVA using the MIXED procedure. Data were evaluated for normality and homogeneity of residuals after fitting the model. Data violating the assumptions of normality were transformed before analysis. Progesterone concentration values at estrus were transformed to the square root of the real value and ISG15 relative abundance values were

transformed to log natural of the real value to meet the assumption of normality of residuals. Data was back transformed for interpretation of the results.

All statistical models included GDPR class GHCR class and interaction of GDPR and GHCR class. Models for estradiol and progesterone at estrus also included pregnancy at 35 ± 3 d, interval from onset of estrus to blood sample collection (2 to 26 hours), linear and quadratic. For ISG15, models also included breeding code (AI vs. ET) and day after estrus when the sample was collected (19 ± 2 d). For the analysis of progesterone, PSPB, and IGF-1 concentrations after estrus, models also included sample, and the interactions between GDPR and sample, GHCR and sample, and GDPR, GHCR and sample, and breeding code (AI vs. ET).

For each of the statistical models collinearity was tested using the REG procedure of SAS with the "collin" and "VIF" functions. Variables with variance inflation factors ≥ 1.5 were considered collinear. In such cases, each variable was added to the model separately and the variable with the smallest *P*-value was retained. A backward stepwise elimination of variables with $P > 0.10$ until variables that remained in the model had $P \leq 0.10$ was performed. Statistical significance was considered at $P \leq 0.05$ and a tendency was consider when $0.05 < P \leq 0.10$.

Results

Descriptive data for GDPR and GHCR in the study population divided into classes are presented in Table 4-2 and distribution of GDPR and GHCR according to classes are presented in Figure 4-1. Descriptive data regarding number of heifers detected in estrus, number of heifers that ovulated according to ultrasound and according to progesterone concentrations, pregnant heifers at 35 ± 3 d after service, and pregnancy loss from 35 to 75 are described in Table 4-3.

Analysis of Physiological Differences Including All Heifers

Ovulatory follicle size was greater ($P < 0.01$) for High GDPR than Low GDPR heifers, but GHCR class was not ($P = 0.12$) associated with ovulatory follicle size. The interaction between GDPR and GHCR classes was not ($P = 0.82$) associated with ovulatory follicle size (Figure 4-2.). Estradiol concentrations after heifers were detected in estrus was greater ($P = 0.02$) for High GDPR than Low GDPR heifers, but GHCR class was not ($P = 0.21$) associated with estradiol concentrations after heifers were detected in estrus. The interaction between GDPR and GHCR class was not ($P = 0.60$) associated with estradiol concentrations after heifers were detected in estrus (Figure 4-3.).

Class of GDPR ($P = 0.88$) and GHCR ($P = 0.78$) and the interaction between GDPR and GHCR classes ($P = 0.56$) were not associated with progesterone concentration within 24 h after heifers were detected in estrus (Figure 4-4.). Class of GDPR ($P = 0.38$) and GHCR ($P = 0.38$) and the interaction between GDPR and GHCR classes ($P = 0.17$) were not associated with progesterone concentrations at 7 and 14 d after estrus (Figure 4-5.). Classes of GDPR ($P = 0.30$) and GHCR ($P = 0.71$) and the interaction between GDPR and GHCR classes were not ($P = 0.56$) associated with IGF-1 concentrations after heifers were detected in estrus.

Analysis of Physiological Differences Including Only Heifers Pregnant 35 ± 3 d After Estrus

Class of GDPR tended ($P = 0.08$) to be associated with greater progesterone concentrations at estrus (Figure 4-5.). Class of GHCR ($P = 0.43$) and the interaction between GDPR and GHCR classes ($P = 0.46$) were not associated with progesterone concentrations at estrus (Figure 4-5.). Class of GDPR ($P = 0.19$) and GHCR ($P = 0.98$) and the interaction between GDPR and GHCR classes ($P = 0.70$) were not associated with progesterone concentrations at 7, 14, 19 ± 2, 28, and 35 d after estrus (Figure 4-5.).

The interaction between GDPR and GHCR classes tended ($P = 0.08$) to be associated with relative expression of ISG15 19 ± 2 d after estrus, because LH heifers had greater expression of ISG15 than LL heifers, whereas the expression of ISG15 among HH heifers and HL heifers was intermediary (Figure 4-6.). Class of GDPR ($P = 0.87$) and GHCR ($P = 0.58$) and the interaction between GDPR and GHCR classes ($P = 0.41$) were not associated with PSPB concentrations 19 ± 2 d after estrus (Figure 4-7.). Concentrations of PSPB at 28 and 35 d after estrus were greater ($P = 0.03$) for High GDPR than Low GDPR heifers, but GHCR class ($P = 0.86$) and interaction between GDPR and GHCR classes ($P = 0.63$) were not associated with PSPB concentrations 28 and 35 d after estrus (Figure 4-7.).

Class of GDPR ($P = 0.50$) and GHCR ($P = 0.14$) and the interaction between GDPR and GHCR classes ($P = 0.48$) were not associated with IGF-1 concentrations within 24 h after estrus was detected and at 7, 14, 19 ± 2, 28, and 35 d after estrus (Figure 4-8.).

Discussion

In the current study, High GDPR heifers had greater ovulatory follicle size and estradiol concentrations, which may be explained by the fact that several SNPs associated with DPR are involved in steroidogenesis or are regulated by steroids (Ortega et al., 2016). Since an overlap of genes that compose GDPR and GHCR exists (Cochran et al., 2013), we expected GHCR also to be positively associated with greater ovulatory follicle size and estradiol concentrations. Proliferation of the pre-ovulatory dominant follicle drives estradiol concentrations (Vasconcelos et al., 2001; Forde et al., 2011). Estradiol triggers estrus expression, and is extremely important for accurate detection, and breeding of animals because it increases estrus intensity and duration, and facilitates estrus detection (Reith and Hoy, 2017). In a companion study, we evaluated the association among GDPR and GHCR and estrus duration and intensity (rumination nadir,

activity peak, and heat index) in dairy heifers. In the companion study, GDPR was positively associated with estrus duration and intensity, whereas GHCR was negatively associated with duration and intensity of estrus. Since in the current study, heifers with High GDPR had greater ovulatory follicle size, and estradiol concentrations, we speculate that one of the mechanisms by which heifers with high GDPR had longer and more intense estruses was due to greater estradiol concentrations. Genomic heifer conception rate was not associated with ovulatory follicle size or estradiol concentrations in the current study; however, numerically, smaller ovulatory follicles and estradiol concentrations were observed in LH heifers, which can potentially explain the negative association between GHCR and estrus duration and intensity observed in the companion study.

Class of GDPR and GHCR was not associated with progesterone concentrations at 7,and 14 d after estrus. Similarly, GDPR and GHCR classes were not associated with progesterone concentrations at 7, 14, 19 ± 2, 28, and 35 d after estrus, when only pregnant heifers 35 ± 3 d after estrus were included in the analysis. Progesterone is produced by luteinized granulosa and theca cells from the ovulated follicle (Forde et al., 2011), and has a crucial role on pregnancy maintenance (Stevenson and Lamb, 2016). Ortega et al. (2016) demonstrated that GDPR was associated with Preg/Serv and days open in a selected Holstein population. Genomic heifer conception rate is a measure of the likelihood of pregnancy after a service (Sun et al., 2014). Because GHCR is a newer trait, however, information about its association with actual Preg/Serv in heifers is limited. Cummins et al. (2012) performed a study to evaluate ovarian follicular dynamics, reproductive hormones and estrous behavior in lactating cows with high and low genetic merit for fertility traits. One of the main findings was that progesterone concentrations were greater in cows classified as high for fertility traits than in cows classified as low for

fertility traits. Therefore, Cummins et al. (2012) suggested that greater progesterone concentrations partially explained improved reproductive performance in cows classified as high for fertility traits. The lack of association among GDPR, GHCR, and progesterone concentrations in the current study, however, do not support the hypothesis by Cummins et al. (2012). We recognize that a small number of pregnant heifers was evaluated in the current study and additional studies are needed to confirm our findings. Nonetheless, when progesterone concentration at 7 and 14 d after estrus from all heifers was analyzed, GDPR and GHCR were not associated with progesterone concentrations, leading to the speculation that GDPR and GHCR indeed may not be associated with progesterone concentrations after estrus.

The interaction between GDPR and GHCR classes tended to be associated with ISG15 expression 19 ± 2 d after estrus because LH heifers had greater expression of ISG15 than LL heifers, whereas the expression of ISG15 among HH heifers and HL heifers was intermediary. Conceptus development and maintenance are highly dependent on a series of conceptus signaling that must be recognized by the dam (Ribeiro et al., 2014). In ruminants, IFN-τ is produced by the trophoblast and its responsible for the maternal recognition of pregnancy (Green et al., 2010). Interferon-τ stimulates a series of interferon stimulated genes that block the luteolytic cascade in endometrial cells and prevent regression of the corpus luteum (Ribeiro et al., 2014). Concentrations of IFN-τ in utero are dependent mainly of the size of the conceptus (Shirasuna et al., 2013). Interferon-τ also acts in peripheral cells (e.g. leukocytes), increasing expression of interferon-stimulated genes such as ISG15 (Ribeiro et al., 2014). Matsuyama et al. (2012) demonstrated that interferon stimulated genes responses in utero and in peripheral blood cells were similar, suggesting that ISG15 expression in peripheral blood leukocytes may be used as an indirect measure of early embryonic development. In a companion study, the interaction between

GDPR and GHCR classes were associated with Preg/Serv, and GDPR and GHCR were associated with the hazard of pregnancy. Therefore, these data combined suggest that GDPR and GHCR are associated with embryo development, maternal recognition of pregnancy, and maintenance of pregnancy.

Pregnancy specific protein B is secreted by binucleate trophoblastic cells and was previously described by Humblot et al. (1988) and Green et al. (2005) to be associated with conceptus development and pregnancy maintenance in heifers and cows. Ribeiro et al. (2014) demonstrated that cows with greater expression of ISG15 19 d after insemination also had greater PSPB concentrations 21 d after insemination. Since the interaction between GDPR and GHCR classes was associated with ISG15 expression 19 ± 2 d after estrus, we expected the interaction between GDPR and GHCR also to be associated with PSPB concentrations at 19 ± 2, 28, and 35 d after estrus. Class of GDPR and GHCR were not associated with PSPB concentrations at 19 ± 2 d after estrus. Class of GDPR, however, was associated with greater PSPB concentrations at 28 and 35 d after estrus, but GHCR was not associated with PSPB concentrations. Greater pre-ovulatory follicle size and estradiol concentrations are associated with improved endometrial environment, which favors pregnancy establishment (Madsen et al., 2015). The greater ovulatory follicle size and greater estradiol concentrations in high GDPR heifers could have led to improved uterine environment and hastened conceptus development, resulting in greater PSPB concentrations among high GDPR heifers at 28 and 35 d after estrus. Reasons for GHCR class to be associated with ISG15 at 19 ± 2 after estrus but not with PSPB concentrations on 28 and 35 d after estrus, however, are unknown and require further investigation.

There were no associations among GDPR and GHCR classes and IGF-1 concentrations at and after estrus. One of the possible mechanisms that would explain the upregulation of ISG15 expression and increased concentration of PSPB is IGF-1 induced conceptus growth, which could potentially lead to increased pregnancy maintenance and Preg/Serv (Ribeiro et al., 2014). Therefore, we hypothesized that GDPR and GHCR driven conceptus development, and consequently upregulations of ISG15 and greater concentration of PSPB, could result from differences in IGF-1 concentration. The lack of differences in IGF-1 concentration according to GDPR and GHCR classes could be the consequence of the small sample size and insufficient power of the current study because the IGF-1 concentration of LH heifers on day 19 ± 2 after estrus was approximately 22% greater than HH and HL heifers and approximately 43% greater than LL heifers. Genetic merit for DPR and GHCR are predictors of reproductive performance that share some genetic markers (Ortega et al., 2016) and the current study reinforces the hypothesis that both drive early conceptus development. Precise mechanisms by which GDPR and GHCR affect fertilization, embryo and conceptus development, and pregnancy maintenance, however, remain unknown.

The greater ovulatory follicle size and estradiol concentrations observed among high GDPR heifers in the current study may explain why high GDPR heifers have more evident sings of estrus and suggests that continued selection for GDPR could potentially improve estrous detection efficiency and accuracy on farm. The association of GDPR and GHCR with ISG15 expression by PBL and the association of GDPR with concentrations of PSPB after service suggest that both genetic markers are associated with embryo/conceptus development, but additional studies are necessary to further understand mechanisms by which GDPR and GHCR improve conceptus development.

Table 4-1. Primer reference and sequences for genes investigated by quantitative real-time PCR.

Target gene	Gene name	NCBI sequence	Primer	Primer sequence
ISG15	Interferon stimulated gene 15	NM_174366	Forward	5′-GGTATGAGCTGAAGCAGTT-3′
			Reverse	5′-ACCTCCCTGCTGTCAAGGT-3′
ACTB	β-actin	AY141970	Forward	5′-CTGGACTTCGAGCAGGAGAT-3′
			Reverse	5′-GGATGTCGACGTCACACTTC-3′
Reverse	Ribosomal protein L19	NM_001040516	Forward	5′-GCGTGCTTCCTTGGTCTTAG-3′
			Reverse	5′-ATCGATCGCCACATGTATCA-3′

Table 4-2. Descriptive GDPR and GHCR data for the study population.

*Class	N	Mean	SD	Min	Max
		GDPR			
HH	28	3.54	0.69	2.5	5.3
HL	20	2.87	0.69	1.6	4.2
LH	21	0.30	0.70	-1.8	1
LL	30	-0.50	0.60	-1.8	0.5
		GHCR			
HH	28	3.07	0.77	2.2	5.5
HL	20	0.57	0.39	-0.1	1.2
LH	21	2.33	0.53	1.5	3.3
LL	30	-0.28	0.61	-2.1	0.5

*Class (HH = High GDPR / High GHCR; HL = High GDPR / Low GHCR; LH = Low GDPR / High GHCR; LL = Low GDPR / Low GHCR)

Table 4-3. Descriptive data for the study population.

*Class	N	£Detected in Estrus	µOvulated according to US	¥Ovulated according to progesterone	Pregnant, 35 ± 3 d after estrus	Pregnancy loss from 35 to 75 d
HH	28	28	26	28	14	1
HL	20	15	13	15	6	0
LH	21	17	14	16	7	1
LL	30	24	16	24	6	2

*Class (HH = High GDPR/High GHCR; HL = High GDPR/Low GHCR; LH = Low GDPR/ High GHCR; LL = Low GDPR/Low GHCR)

µ Ovulated according to ultrasound (**US**) = Disappearance of a follicle ≥ 10 mm within 96 h after first ultrasound

¥ Ovulated Progesterone = Progesterone concentrations < 1 ng/mL on estrus day, and > 1 ng/mL 7 days after estrus

£ Detected in estrus = Detected in estrus by automated estrus detection device within 7 days of $PGF_{2\alpha}$ treatment

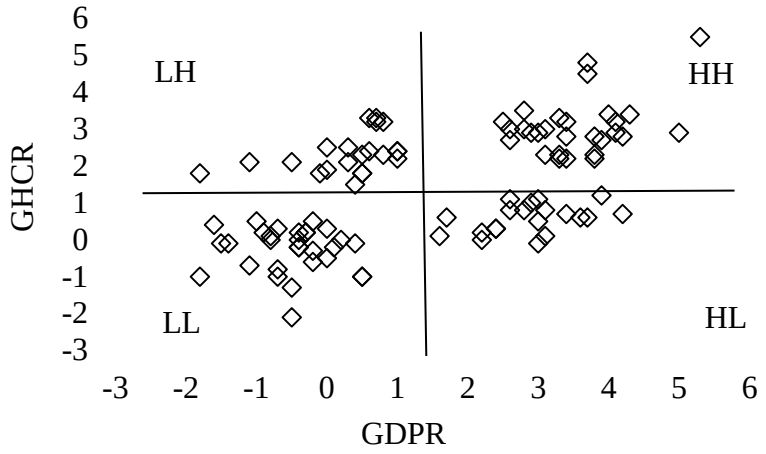

Figure 4-4. Genetic merit for daughter pregnancy arte (GDPR) and heifer conception rate (GHCR) breeding values in the study population. The bars represent the division of the population into classes used in the experiment: HH = High GDPR / High GHCR; HL = High GDPR / Low GHCR; LH = Low GDPR / High GHCR; LL = Low GDPR / Low GHCR.

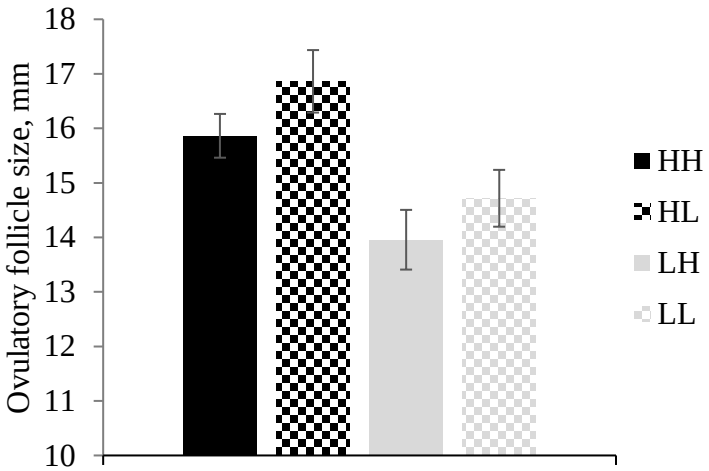

Figure 4-2. Ovulatory follicle size (all heifers) according to genetic merit for daughter pregnancy rate (GDPR) and heifer conception rate (GHCR) classes. HH = High GDPR / High GHCR; HL = High GDPR / Low GHCR; LH = Low GDPR / High GHCR; LL = Low GDPR / Low GHCR. GDPR - $P < 0.01$, GHCR - $P = 0.12$, GDPR x GHCR - $P = 0.82$.

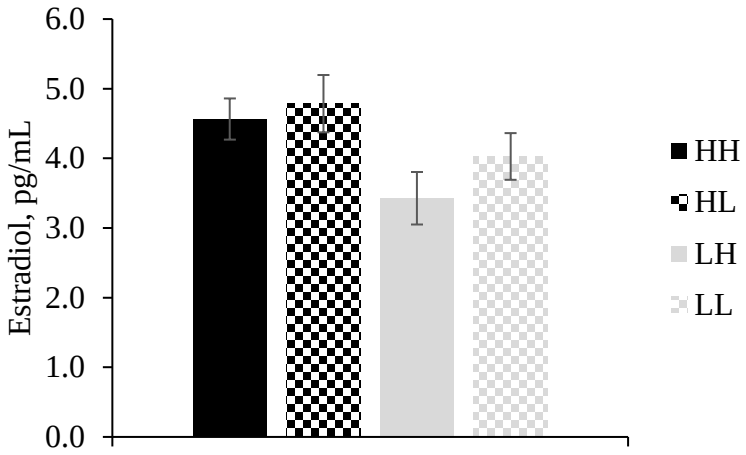

Figure 4-3. Estradiol concentrations at estrus (all heifers), according to genetic merit for daughter pregnancy rate (GDPR) and heifer conception rate (GHCR) classes. HH = High GDPR / High GHCR; HL = High GDPR / Low GHCR; LH = Low GDPR / High GHCR; LL = Low GDPR / Low GHCR. GDPR - *P* = 0.02, GHCR - *P* = 0.21, GDPR x GHCR - *P* = 0.60.

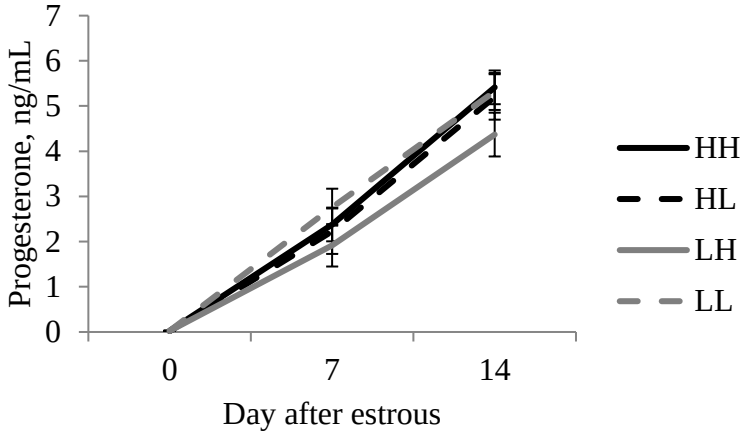

Figure 4-4. Progesterone concentrations at estrus, 7 and 14 days after estrus (all heifers), according to genetic merit for daughter pregnancy rate (GDPR) and heifer conception rate (GHCR) classes: HH = High GDPR / High GHCR; HL = High GDPR / Low GHCR; LH = Low GDPR / High GHCR; LL = Low GDPR / Low GHCR. Day 0: GDPR - *P* = 0.88, GHCR - *P* = 0.78, GDPR x GHCR - *P* = 0.56. Day 7 and 14: GDPR - *P* = 0.38, GHCR - *P* = 0.38, GDPR x GHCR - *P* = 0.17.

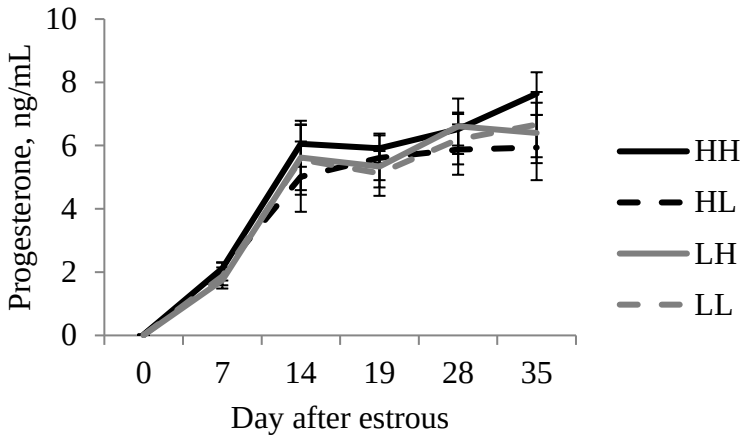

Figure 4-5. Progesterone concentrations at estrus, 7, 14, 19 ± 2, 28, and 35 days after estrus (only pregnant heifers 35 ± 3 d after service), according to genetic merit for daughter pregnancy rate (GDPR) and heifer conception rate (GHCR) classes: HH = High GDPR / High GHCR; HL = High GDPR / Low GHCR; LH = Low GDPR / High GHCR; LL = Low GDPR / Low GHCR. Day 0: GDPR - *P* = 0.08, GHCR - *P* = 0.43, GDPR x GHCR - *P* = 0.46. Day 7, 14, 19 ± 2, 28, and 35: GDPR - *P* = 0.19, GHCR - *P* = 0.98, GDPR x GHCR - *P* = 0.70.

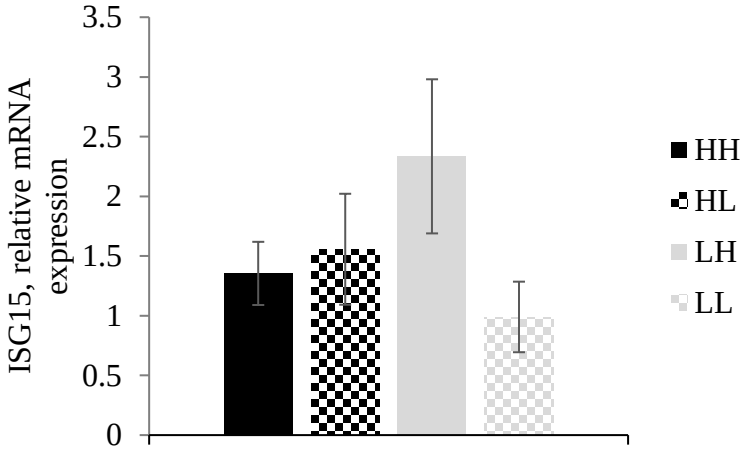

Figure 4-6. Interferon stimulated gene 15 (ISG15) 19 ± 2 days after estrus (only pregnant heifers 35 ± 3 d after service), according to genetic merit for daughter pregnancy rate (GDPR) and heifer conception rate (GHCR) classes: HH = High GDPR / High GHCR; HL = High GDPR / Low GHCR; LH = Low GDPR / High GHCR; LL = Low GDPR / Low GHCR. GDPR - *P* = 0.87, GHCR - *P* = 0.19, GDPR x GHCR - *P* = 0.07.

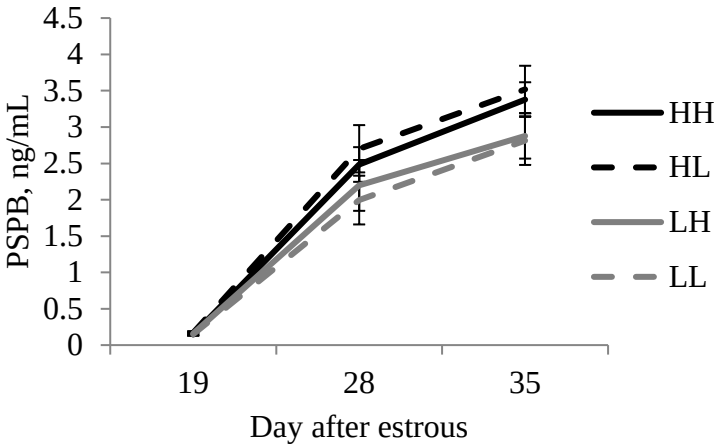

Figure 4-7. Pregnancy specific protein B (PSPB) concentrations 19 ± 2, 28, and 35 days after estrus (only pregnant heifers 35 ± 3 d after service), according to genetic merit for daughter pregnancy rate (GDPR) and heifer conception rate (GHCR) classes: HH = High GDPR / High GHCR; HL = High GDPR / Low GHCR; LH = Low GDPR / High GHCR; LL = Low GDPR / Low GHCR. Day 19 ± 2: GDPR - *P* = 0.87, GHCR - *P* = 0.58, GDPR x GHCR - *P* = 0.41. Day 28 and 35: GDPR - *P* = 0.03, GHCR - *P* = 0.86, GDPR x GHCR - *P* = 0.63.

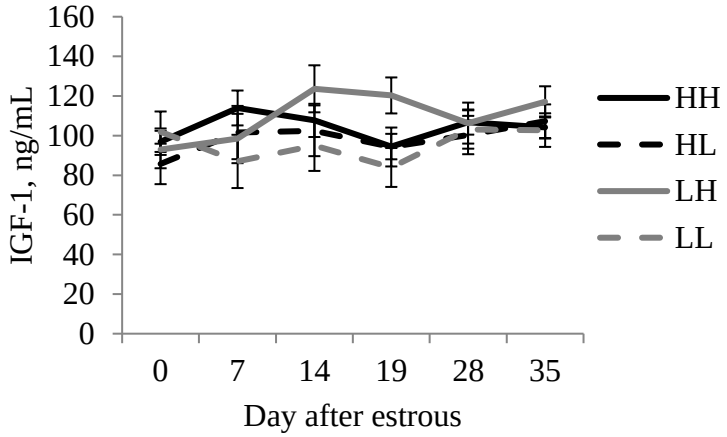

Figure 4-8. Insulin like growth factor 1 (IGF-1) concentrations at estrus, 7, 14, 19 ± 2, 28, and 35 days after estrus (only pregnant heifers 35 ± 3 days after service), according to genetic merit for daughter pregnancy rate (GDPR) and heifer conception rate (GHCR) classes: HH = High GDPR / High GHCR; HL = High GDPR / Low GHCR; LH = Low GDPR / High GHCR; LL = Low GDPR / Low GHCR. Day 0, 7, 14, 19 ± 2, 28, and 35: GDPR - *P* = 0.50, GHCR - *P* = 0.14, GDPR x GHCR - *P* = 0.48.

CHAPTER 5
CONCLUSION

Information about the efficacy and differences in response to $PGF_{2\alpha}$ formulations is not abundant for dairy heifers and data available from lactating dairy cows is controversial. Results presented herein provide new evidence about the differences in estrous behavior and hazard of estrus following $PGF_{2\alpha}$ treatments, suggesting that the heifers treated with cloprostenol sodium have lower progesterone concentration at estrus and are detected in estrus faster compared with dinoprost tromethamine. Despite the fact that cloprostenol sodium increased the proportion of heifers detected in estrus within 7 days of treatment and hazard of estrus, it did not affect Preg/Serv or hazard of pregnancy, the most important outcomes for dairy producers. Therefore, selection of $PGF_{2\alpha}$ formulation may be according to other parameters than efficacy.

Benefits of the use of an AED for detection of estrus of dairy heifers are not definite and may be a consequence of dairy heifers having greater duration and intensity of estrus compared with lactating dairy cows. In the experiment presented herein, however, AED improved the hazard of pregnancy likely because it improved the accuracy of estrus detection, observed as greater Preg/Serv. The feasibility of the use of AED for dairy heifers, however, remains uncertain and whether a farm will benefit from adopting the system will vastly vary according to the design of the reproductive program, and especially current efficiency and accuracy of estrus detection on each specific dairy.

Genomic fertility traits such as daughter pregnancy rate (GDPR) and heifer conception rate (GHCR), although vastly used in genomic selection for dairy cattle, lack information on their impact on physiological changes driving improvements in reproductive performance. Furthermore, the association among GDPR and GHCR and important phenotypes such as estrous behavior, have seldom been evaluated. The results from the current studies contribute to the

understanding on how GDPR and GHCR alter estrous behavior through physiological alterations, particularly of the ovulatory follicle and concentration of estradiol at estrus. Results presented herein reinforce the strategy of selecting heifers and cows for GDPR, which should lead to selection of animals with greater ovulatory follicle size, estradiol concentrations and improved estrus expression, duration, and intensity. On the other hand, the data from the current study suggest that GHCR could lead to reduction in estrous behavior and could potentially lead to reproductive losses in subsequent generations.

Together, these studies contribute with novel information that can be used by dairy farmers, researchers and other members of the dairy industry do advance and improve reproductive performance, improve genetic selection strategies, and profitability of dairy herds.

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BIOGRAPHICAL SKETCH

Anderson Veronese was born in Viadutos, a small, agriculture based, town in the southern state of Rio Grande do Sul, Brazil. He is the only child of Jaime Dionisio Veronese and Marines Bohm Veronese. His parents and grandparents, Felix and Leonora Bohm, owned a farm, where Anderson grew up. Since young, Anderson started working with his family on the farm, where they milked dairy cows, and finished swine for slaughter. At age of 15, Anderson did a training to learn how artificially inseminate cows. One year later, after having experience on breeding cows in his family dairy, he and an older cousin decided to partner and started a small business, providing artificial insemination service to dairy farmers in the town. After graduating on high school, Anderson decided to pursue a carrier in Veterinary Sciences, was approve on Federal Institute of Santa Catarina and started college in 2010. During college, Anderson started working closely with research under the supervision of Dr. Angela Veiga, an early mentor who develop his interest in science. During college breaks, Anderson did externships in a dairy production medicine and nutrition consulting in a company "Agropecuaria Dourado", where he develop several skills, had the chance to network with experienced professionals, and improved his knowledge about the dairy industry, as well as consulting and dealing with dairy farmers. In 2013, Anderson received a scholarship from the Brazilian academic mobility program, "Science without borders", funded by the federal government of Brazil, and came to US to spend one year as an exchange student at Maricopa Colleges, Phoenix-AZ. During this time, he improved his English skills, and did courses related to his field. Anderson returned to Brazil in July 2014, spend one year to finish his required classes, and returned to USA to do an externship under the supervision of Dr. Ricardo Chebel in July 2015. Following up the externship, Anderson was invite to stay at University of Florida to work with Dr. Chebel and pursue a Master of Science. He decided to accept the invitation, returned to Brazil for graduation in January 2016, and

immediately returned to Gainesville, where he has being working on his research and taking classes for his master degree program. He is expected to graduate in the fall 2017. Anderson's upcoming goals are to pursue a residency in production medicine and a doctoral degree at the same University.