EVALUATION OF AN EAR-ATTACHED SENSOR FOR THE CHARACTERIZATION AND DETECTION OF ESTRUS EVENTS IN DAIRY CATTLE

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ABSTRACT

A study was conducted to evaluate the ability of a commercially available automated estrus detection (AED) system to detect cows in estrus. Estrus was synchronized in lactating dairy cows (n = 216) fitted with an ear-attached sensor that generated estrus alerts based on a combination of physical activity and rumination time data. For seven days after induction of luteolysis, cows were monitored for estrus behaviors using a combination of visual observation and automated monitoring of standing events. Ovulation time was determined via transrectal ultrasonography. The sensitivity, specificity, negative predictive value, positive predictive value and balanced accuracy of the AED system estrus alerts compared with the reference test were 92%, 69%, 53%, 96%, and 80%, respectively. Average alert duration was 13.5 h and interval from alert generation to ovulation was 23.8 h. Parity, milk production level, and circulating progesterone concentrations before estrus were all associated with significant changes in estrus expression.

BIOGRAPHICAL SKETCH

Ellen Schilkowsky was born on September 30, 1994 in Charlotte, North Carolina. As the daughter of a veterinarian, Ellen inherited a passion for animals and animal health and welfare. In May 2017 she graduated with a Bachelor's degree in Animal Science from North Carolina State University where she developed a specific interest in dairy cattle reproduction while volunteering in a bovine reproductive physiology research lab and working at the university teaching dairy. In August 2017 Ellen joined the Master's program at Cornell University in the Animal Science department under the supervision of Dr. Julio Giordano. Upon graduation, Ellen plans to pursue a career in veterinary medicine.

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CHAPTER I

LITERATURE REVIEW: DETECTION OF ESTROUS BEHAVIORS IN LACTATING DAIRY CATTLE

1. GENERAL INTRODUCTION

Today's competitive dairy economy has been increasingly encouraging farmers to optimize management practices in order to maximize profitability. Reproductive management has been no exception. However, as the average herd size continues to rise in the United States (NAHMS, 2018), implementation of traditional reproductive management practices becomes progressively more challenging due to the rise in labor demands. Reproductive performance is also compromised by factors such as selection pressure for higher milk yields, metabolic demands of increased milk production, nutrition, and other environmental and management factors (Ingraham et al., 1974; Roche et al., 2000; Royal et al., 2000). Thus, in order to combat these challenges, an abundance of new strategies and technologies for reproductive management have emerged over the last 20 to 30 years with the goal of improving and optimizing reproductive performance.

Artificial insemination (**AI**) has been largely responsible for the rapid genetic progress that has been made in the last century and currently, over 70% of pregnancies are achieved using AI (NAHMS, 2018). With so many farms using AI, it is critical that farmers have a way to accurately detect when cows are likely to ovulate so that they can time insemination such that the likelihood of conception is maximized. Moreover, since a high rate of submission to AI is necessary to achieve high pregnancy rates (Ferguson and Skidmore, 2013; Denis-Robichaud et al., 2016), the management approach must ensure that all eligible cows are inseminated in a timely manner following the end of the voluntary waiting period. The two main strategies for reproductive management on dairy farms are synchronization of estrus for timed artificial insemination (**TAI**) and estrus detection (**ED**) followed by artificial insemination (**EDAI**).

Timed AI protocols offer numerous benefits that ultimately lead to improved reproductive and economic performance of dairy herds (Giordano et al., 2012; Galvão et al., 2013; Fricke et al., 2014). For example, dairy farmers can increase the service rate by defining when cows will receive inseminations or increase pregnancy per AI using synchronization protocols designed to optimize fertility of TAI services. Thus, TAI has the potential to reduce time to pregnancy by affecting the two drivers of the pregnancy rate (Ferguson and Skidmore, 2013; Denis-Robichaud et al., 2016). In spite of the potential benefits of TAI, the majority of pregnancies in the United States are still generated using EDAI (NAHMS, 2018). Since EDAI programs rely on cows to spontaneously come into estrus, cows must be regularly monitored for expression of estrous behaviors, a labor demanding, and repetitive process. Detection of estrus is further complicated by the fact that that expression of estrus can also be inhibited by a multitude of biological, management, and environmental factors (Lopez et al., 2004; Palmer et al., 2010; Aungier et al., 2012; Schüller et al., 2017). Thus, methods to detect cows in estrus have evolved to address some of these shortcomings of traditional EDAI. Of particular interest is the development and performance of systems for automated estrus detection (**AED**). Chapter II describes a detailed analysis of the performance one such system and its potential to improve reproductive outcomes.

This chapter aims to describe the physiological and behavioral changes that occur during estrus, examine factors that can affect fertility and expression of estrus, and provide an overview of modern ED methods including AED systems.

2. THE BOVINE ESTROUS CYCLE

2.1. METESTRUS AND DIESTRUS

The estrous cycle of a lactating dairy cow is approximately 22 days long and cycles ranging from 13 to over 30 days have been reported (Remnant et al., 2015; Blavy et al., 2016). The cycle consists of four distinct phases characterized by specific patterns of ovarian and hormone dynamics: metestrus, diestrus, proestrus, and estrus. The central opposing hormones that are responsible for many of the changes observed during these phases are progesterone (**P_4**) and estradiol (**E_2**). Metestrus and diestrus together make up the luteal phase, a phase of the estrous cycle characterized by high P_4 levels and the presence of a corpus luteum (**CL**) on the ovary.

Metestrus begins around the time of an ovulation event. Stimulated by a surge of luteinizing hormone (**LH**) from the anterior pituitary gland prior to ovulation, the granulosa and theca interna cells of the ovulatory follicle undergo dramatic transformation during metestrus into the large and small steroidogenic cells of the CL, respectively (Alila and Hansel, 1984). The primary function of these cells is production of P_4 from cholesterol, which steadily rises throughout metestrus and into diestrus (Henricks et al., 1970; Alila and Hansel, 1984). Simultaneously, the developing CL is vascularized by an elaborate capillary system that will ultimately aid in P_4 transport to distal tissues including the uterus and hypothalamus (Fraser and Wulff, 2003). By the end of metestrus, on approximately day 3 to 5 of the estrous cycle, a functional, P_4-secreting CL is present at the location of the previously ovulated follicle.

Diestrus is the longest phase of the estrous cycle, typically lasting 10 to 12 days in cattle. This phase is characterized by high levels of P_4 production by the CL that peak around day 14 to 16 after ovulation (Henricks et al., 1970). The P_4 secreted by the CL enters circulation and prepares the reproductive tract for pregnancy, and acts directly on the uterine myometrium to reduce uterine contractility (Csapo, 1956). In the presence of sustained high concentrations of P_4, the P_4 receptor of the luminal epithelium of the uterine endometrium is eventually downregulated, resulting in the upregulation and/or downregulation of genes essential to uterine receptivity (Spencer et al., 2004, 2008). Progesterone also exerts a negative feedback mechanism on the release of gonadotropin-releasing hormone (**GnRH**) from the hypothalamus, keeping GnRH secretion at basal levels (Skinner et al., 1998). During estrus, a surge of GnRH results in the release of high amplitude, high-frequency pulses of LH from the anterior pituitary that induce ovulation. However, during the diestrus phase, high circulating concentrations of P_4 prevent the initiation of estrus and ovulation.

In most cows, cohorts of ~5 to 20 follicles are recruited from the resting pool 2 to 3 times during each estrous cycle, including during the luteal phase (Sunderland et al., 1994; Sartori et al., 2004). Typically a single dominant follicle develops from each recruited cohort; however, the high P_4 levels observed during the luteal phase exert negative feedback on the release of gonadotropins, thus preventing the dominant follicle from ovulating (Skinner et al., 1998). As a result, the follicle of the first or second follicular wave becomes atretic and a new follicular wave begins. In order for a dominant follicle to ovulate, P_4 concentrations must be low; thus, the CL must first undergo luteolysis. The process of luteolysis is as follows: during the late luteal phase, the sustained high concentrations of P_4 result in downregulation of the P_4 receptor and upregulation of E_2 receptors on the uterine epithelium. The binding of E_2 produced by a developing follicle to its receptor in the uterine epithelium then upregulates expression of the oxytocin receptor in epithelial cells of the endometrium (Silvia et al., 1991; Goff, 2004). In the absence of a conceptus, i.e. if the cow has not been inseminated or pregnancy was not established, the oxytocin receptors in the uterine epithelium are activated by oxytocin released by the pituitary first and then from the CL itself, resulting in pulsatile secretion of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) from the uterus (McCracken et al., 1984; Silvia et al., 1991). Thereafter, $PGF_{2\alpha}$ travels through the uterine vein and enters the ovarian artery through countercurrent exchange, where it can then act directly on the CL to halt P_4 production and induce regression of the luteal tissue (McCracken et al., 1999). Luteolysis marks the end of the diestrus phase and the beginning of the proestrus phase.

2.2. PROESTRUS AND ESTRUS

Proestrus and estrus are short phases of the estrous cycle characterized by reduced circulating concentrations of P_4 and the final growth and development of a preovulatory follicle. Proestrus begins at the onset of luteolysis and lasts approximately 2 to 5 days. During this time, P_4 levels fall rapidly and the CL begins to undergo structural disintegration. During proestrus, E_2 levels in circulation remain relatively low at ~<10 pg/mL until the onset of estrus, during which E_2 levels can rise to ~15 to 25 pg/mL (Henricks et al., 1971). In the absence of the negative feedback of P_4 during the proestrus phase, LH pulse frequency from the anterior pituitary increases. Simultaneously, expression of LH receptors on the theca interna of the developing follicle increase. Subsequently, the binding of LH to the LH receptors on the developing follicle stimulates E_2 production through the two-cell, two-gonadotropin model (Fortune and Armstrong, 1978). The process begins with LH binding to the LH receptor on the theca interna cells where is stimulates production of androgens from cholesterol. These androgens (primarily androstenedione) are then transported to the granulosa cells of the developing follicle where follicle-stimulating hormone (**FSH**) secreted by anterior pituitary gland binds to its receptor to stimulates production of E_2. In the absence of P_4, E_2 produced by the granulosa cells can then exert a positive feedback mechanism on the release of gonadotropins from the anterior pituitary, further increasing production of E_2 (Moenter et al., 2009). Eventually, the dominant follicle produces enough E_2 to induce behavioral changes, thus marking the end of proestrus and the beginning of estrus.

Estrus is the period of sexual receptivity in the cow and lasts anywhere from 6 to 24 h, with an average length of around 12 h (At-Taras and Spahr, 2001; Roelofs et al., 2005a;b; Stevenson et al., 2014). The behavioral changes observed during estrus are described in detail later in this chapter. In addition to behavioral changes, E_2 has a multitude of effects on the reproductive tract including increased blood flow to the reproductive tract and swelling of the genitals, myometrial tone, secretion of mucus, and leukocytosis in the uterus (Roelofs et al., 2010; Forde et al., 2011). These changes are all intended to prepare the reproductive tract for copulation, sperm transport, and fertilization. When E_2 reaches a threshold concentration in the blood, the GnRH surge center in the arcuate nucleus of the hypothalamus is activated, resulting in a surge of GnRH that travels through the hypothalamic-hypophyseal portal system to the anterior pituitary where it induces the release of large quantities of LH. This mass release of LH is referred to as the preovulatory LH surge and has several significant downstream effects. First, the LH surge is responsible for the removal of meiotic inhibition in the oocyte, allowing for the maturation process to be initiated (Channing et al., 1978). The LH surge also induces a shift from E_2 to P_4 production, a change that stimulates a cascade of events resulting in ovulation, including the production of collagenase by the theca interna cells to break down the follicle wall (Curry et al., 1986). On average, ovulation occurs between 24 to 30 hours after the onset of estrus behaviors (Walker et al., 1996; Roelofs et al., 2005b; Stevenson et al., 2014). After ovulation, a new CL will form on the ovary and a new estrous cycle begins.

2.3. ESTROUS BEHAVIOR

The characteristic behaviors displayed by cows in estrus are of critical importance for reproductive management, particularly at farms that incorporate AI at detected estrus as a substantial part of their reproductive management program. Estrus behaviors are caused by the high concentrations of E_2 that are produced by a preovulatory follicle. During estrus, E_2 binds to its receptor expressed in the mediobasal hypothalamus (**MBH**), a site identified as critical for induction of estrous behaviors (Blache et al., 1991). Activation of the E_2 receptor by E_2 induces a number of neurochemical changes, particularly the release of neurotransmitters from the MBH (Fabre-Nys et al., 1994). These neurotransmitters are received in the midbrain where they relay the signal and promote expression of estrous behaviors and sexual receptivity. Nevertheless, the manifestation of estrus and estrous behaviors can vary widely depending on physiological, environmental, and management factors that will be discussed in detail later in this chapter.

Typically, standing to be mounted is considered the primary sign of estrus and is often the best indicator of sexual receptivity in the cow. However, the prevalence of standing behavior is relatively low in dairy cattle. Indeed, a study by Van Vliet and Van Eerdenburg, (1996) et al. showed that only 37% of lactating dairy cows considered in estrus displayed standing behavior, although 50 to 60% is more typical (At-Taras and Spahr, 2001; Roelofs et al., 2005a). Furthermore, when present, standing estrus has an average duration of only ~6 hours (At-Taras and Spahr, 2001). In contrast, Roelofs et al. (2005a) showed that secondary signs of estrus began on average 30.6 hours before ovulation and remained present until 18.8 hours before ovulation for a total duration of 11.8 hours, or more than twice the average duration of standing activity alone. In addition, some secondary estrous behaviors were present in 90 to 100% of estrus events (Roelofs et al., 2005a). For these reasons, estrus is often detected based on secondary signs of estrus which may be subtler and sometimes more difficult to detect.

Often the most obvious secondary signs of estrus are those that involve interactions with other cows. Mounting activity, or mounting other cows, is indicative of estrus and was observed in 80% of cows in estrus according to a study by Van Vliet and Van Eerdenburg (1996). In addition to mounting activity, social and agonistic interactions between cows can become more frequent. One particular interaction that has been highly correlated with estrus is resting the chin on the rump of other cows. Similarly, cows in estrus will also often sniff or lick the vulvar region of other cows. Aggressive interactions are also observed more frequently during estrus than at other timepoints during the estrous cycle. Kerbrat and Disenhaus (2004) found that head-to-head butting was the most common agonistic behavior observed during estrus and was observed in 73.4% of cows. Oftentimes, vocalizations or "bellowing" observed in cows are also characteristic of an estrus event. Using a microphone attached to a neck collar, Schön et al. (2007) found that the vocalizations of heifers increased by 84% during estrus compared to heifers that were not in estrus.

Although difficult to identify and quantify without the assistance of activity monitoring systems, restlessness and overall activity levels increase predictably and dramatically around the time of estrus. Activity levels can be quantified in multiple ways depending on the system being used, but often they are reported as either number of steps, system-specific arbitrary activity units, or as a percentage increase above an established baseline level of activity. For example, Madureira et al. (2015) reported a 334% relative increase in activity during estrus when a leg-mounted pedometer was used. Also using pedometers, Roelofs et al. (2005b) demonstrated that increased activity was maintained for an average of ~10 hours, with a range of 6 to 18 hours. Since most studies report that a substantial increase in activity is observed in >85% of cows (Roelofs et al., 2005b; Dolecheck et al., 2015; Madureira et al., 2015) and activity levels can be easily quantified using automated sensors, the potential for success using AED systems that measure activity levels is promising. Furthermore, the onset of increased activity is closely associated with timing of ovulation, allowing for prediction of ovulation time and the potential to use this information to maximize fertility (Roelofs et al., 2005b; Stevenson et al., 2014).

Other secondary estrous behaviors that can be difficult to observe or quantify without the assistance of automated monitoring are the changes in rumination, dry matter intake (**DMI**), and water intake. On average, cows usually spend around a third of their day ruminating. Two previous studies showed the average daily rumination of non-estrual cows housed in freestall barns were 429 and 442 minutes, respectively, as measured by a microphone-based neck-mounted sensor (Reith and Hoy, 2012; Reith et al., 2014a). However, during estrus, rumination time often decreases. A previous study reported that 86.2% of cows experienced a decline in rumination time during estrus of 19.6% on average (Reith et al., 2014a). Another study using the same type of sensors showed that this apparent decline in rumination persists for 30 hours, on average (Pahl et al., 2015). On a related note, another study showed that 85.3% of cows had markedly reduced DMI on the day of estrus and 66.7% of cows had markedly reduced water intake observed primarily on the day prior to the onset of estrus (Reith et al., 2014b). Nevertheless, a reduction in rumination, DMI, and water intake is often also associated with adverse health events, necessitating the inclusion of either activity levels or the presence of other secondary signs of estrus in order to confirm an estrus event.

While behavioral changes are undoubtedly extremely valuable for ED, not all of the changes that can be used to detect estrus are behavioral. For instance, visual observation of a red, swollen vulva or the production of large quantities of clear vaginal discharge are indicative of an estrus event. In addition, body temperature can be an indicator of estrus. Using vaginal thermometry, Fisher et al. (2008) reported that body temperature rises by approximately 0.48° C within 4 hours of the ovulation-inducing LH surge. Lastly, as serum and milk concentrations of P_4 are lower in the days leading up to an estrus event, sensors that evaluate milk P_4 concentrations can be used to estimate when a cow is coming into estrus. However, because there is a large amount of variation in when cows will show estrus after luteolysis, alerts may not accurately estimate timing of estrus. Indeed, Roelofs et al. (2006b) reported that milk P_4 concentrations dropped below 5 ng/mL anywhere from 54 to 98 hours prior to ovulation, a range of nearly two days.

3. FACTORS THAT AFFECT EXPRESSION OF ESTRUS

3.1. INTRINSIC FACTORS

Estrus expression can vary widely between cows and even between estrus events for a single cow depending on a multitude of factors (Van Vliet and Van Eerdenburg, 1996; Lopez et al., 2004; Peralta et al., 2005; Madureira et al., 2015). This issue has important implications for reproductive management and fertility. First, cows that show less intense estrus events are less likely to be detected in estrus, potentially delaying insemination and prolonging the calving-to-conception interval. Secondly, there are a number of studies that have shown a direct relationship between estrus intensity and fertility. Gwazdauskas et al. (1981a) found approximately 10% lower pregnancy per AI (**P/AI**) for cows that failed to show standing estrus. Furthermore, Madureira et al. (2015) demonstrated that when using data from both a collar-mounted accelerometer and a leg-mounted pedometer, high levels of physical activity during estrus were associated with an approximately 12 percentage point increase in P/AI. Thus, a better understanding of the factors that affect expression of estrus in lactating dairy cows is critical to better understand the factors that might affect and drive dairy herd reproductive performance.

Milk production undoubtedly has a substantial impact on expression of estrus (Lopez et al., 2004; Rivera et al., 2010) and the steady decline in fertility in dairy cows (Dhaliwal et al., 1996; Lucy, 2001), and it is also directly related with a number of other factors that can affect reproduction including metabolism and nutrition (Ribeiro et al., 2013; Madureira et al., 2015). It has been well-demonstrated that high milk production is closely associated with decreased fertility (Dhaliwal et al., 1996; Lucy, 2001) as well as decreased intensity and duration of estrus (Lopez et al., 2004; Rivera et al., 2010). For example, one study reported that for every 1 kg increase in daily milk production, walking activity decreased by 1.6% (López-Gatius et al., 2005). One of the main proposed mechanisms for the effect of milk production on estrus expression is that due to greater dry mater intake to sustain milk output, high producing cows present greater metabolic rates than lower-producing cows (Moe, 1981; Sangsritavong et al., 2002). Thus, the metabolic clearance of steroid hormones, including E_2, is more rapid in high-producing cows (Sangsritavong et al., 2002). In this regard, a study by Lopez et al. (2004) showed that serum E_2 levels were significantly lower in lactating dairy cows that produced more or less than 39.5 kg of milk per day. Moreover, the duration and intensity of estrus were also compromised in the higher milk production group.

In dairy cows, milk production leads to a state of negative energy balance (**NEB**), whereby in early lactation, the cow is unable to consume the amount of nutrients necessary to meet the energy demands of lactation, thus leading to the mobilization of bodily fat stores (Bauman and Currie, 1980). When milk production is greater, this state of NEB may have adverse effects on reproductive physiological outcomes and the magnitude of the effect be more pronounced in cows in more severe NEB. For example, Markusfeld et al. (1997) showed that lower body condition score (**BCS**) (i.e., a parameter associated with energy balance) was associated with failure to express estrus and anovulation. Similarly, lower BCS was associated with decreased physical activity during estrus, decreased duration of estrus, and decreased P/AI (Madureira et al., 2015). Furthermore, as body lipid stores are mobilized, non-esterified fatty acids (**NEFA**) are produced. These NEFA, when present in excess in the body, have been shown to have detrimental effects (Adewuyi et al., 2005) such as reduced P/AI (Ribeiro et al., 2013). In addition, high serum NEFA concentration have been linked to reduced walking activity in postpartum dairy cows (Adewuyi et al., 2006). While it is difficult to tease out exactly which of these closely related factors has the greatest overall effect on estrus expression and fertility, it is possible to suggest that they may each contribute in part to reduced expression of estrus.

Interestingly, the reported effect of parity on expression of estrus in dairy cows has been conflicting. Although most studies reported reduced expression of estrus for multiparous cows (Peralta et al., 2005; Roelofs et al., 2005a; Madureira et al., 2015), others reported the opposite (Van Vliet and Van Eerdenburg, 1996). This may be due the variation between studies for the specific estrous behaviors used to measure estrus expression. For instance, Madureira et al. (2015) showed that multiparous cows in estrus had lowered levels of physical activity and shorter duration of increased activity associated with estrus compared with primiparous cows. However, multiparous cows in some studies displayed a higher frequency of standing events during estrus than primiparous cows (Van Vliet and Van Eerdenburg, 1996). It has been postulated that this increase in standing behavior is due to the greater amount of sexual experience that multiparous cows have compared with primiparous cows (Van Vliet and Van Eerdenburg, 1996).

Another factor that has the potential to affect expression of estrus is lameness. It logically follows that cows with reduced mobility would spend less time walking and expressing estrus than cows without such limitations. Indeed, Walker et al. (2008b) observed that lame cows spent more time lying down and less time walking, and expressed less estrous behaviors. Another study by the same group showed that when using a scoring system to quantify the intensity of estrous behaviors, lame cows had a 37% reduction in estrus intensity compared with non-lame cows. Although the most likely reason for reduced intensity of estrus in lame cows is simply impaired mobility, a few studies have explored a more advanced physiological mechanism. It has been shown that lame cows have lower maximum P_4 concentrations prior to the onset of estrus (Walker et al., 2008a). Thus, it has been hypothesized that this altered pattern of hormone secretion could in part contribute to the decreased intensity of estrus behaviors observed in lame cows.

Other adverse health events can also result in decreased fertility and estrus expression. Calving problems, metritis, clinical and subclinical endometritis, and digestive problems have all been associated with reduced P/AI on d 65 after AI (Ribeiro et al., 2013). The same study also showed that cows that were afflicted by more than one adverse health event had even greater reduced fertility than cows with only one adverse health event. Moreover, data from Aungier et al. (2012) showed that healthy cows were 3.6 times more likely to be detected in estrus by an neck-mounted accelerometer-based activity monitoring system than cows that had a uterine infection.

3.2. EXTRINSIC FACTORS

While intrinsic factors of individual cows may affect estrus expression, extrinsic factors associated with herd management and environmental conditions may also affect the ability of cows to express estrus and be detected in estrus. Some of these factors include housing type, herd size, and climatic conditions.

Dairy cattle housing systems vary considerably across the United States and globally, and there are certain aspects of many of the commonly used housing systems that have been associated with either improved or hindered estrus expression. The reported effect of freestall versus pasture-based systems has varied considerably. For instance, De Silva et al. (1981) showed that cows housed in tiestall barns had a greater number of standing events per hour during estrus (11.2 standing events/hour) compared with cows that were housed primarily in freestalls (6.5 standing events/hour) or pasture (5.4 standing events/hour). However, these results may have been influenced by the fact that the cows housed in tiestalls were limited to two 30-minute intervals per day during which they could interact with other cows. Interestingly, Palmer et al. (2010) observed the opposite. Cows housed in free-stall barns had fewer standing events and less intense estrous behaviors compared with cows in pasture-based systems. Other confounding environmental factors may affect estrus expression. For instance, within barn housing systems, flooring type can have a substantial effect on estrus expression. Concrete floors often used in confined housing systems may be problematic as there is evidence that cows housed on dirt floors display more frequent mounting behavior and a greater duration of mounting behavior than cows housed on concrete surfaces (Vailes and Britt, 1990). Furthermore, when a rubber coating was used to cover a concrete floor, the incidence of mounting activity was increased and the incidence of slipping or collapsing during mounting was decreased (Platz et al., 2008). Hence, slicker flooring types may discourage cows from the expressing estrus and reduce estrus intensity.

Herd size and stocking density can also affect estrus expression. When herds are larger, the likelihood that more cows will be in estrus at a given time increases, thus increasing the likelihood for the formation of sexually active groups (Roelofs et al., 2005a). Furthermore, when more cows are in estrus at the same time, estrus behaviors are intensified. For example, Yániz et al. (2006) showed that for each additional cow in estrus at the same time, walking activity of all of cows in estrus increased by an average of 6.1%. This increase in estrus behaviors can also improve estrus detection (**ED**) rates. Roelofs et al. (2005b) reported up to a 95% increase in ED rate by pedometer readings when more than one cow was in estrus at the same time. Similarly, stocking density can also affect estrus expression and ED. When stocking density is very low, the likelihood of cows in estrus meeting decreases, thus decreasing the likelihood for the formation of sexually active groups. Thus, as free stall stocking density increases, so does estrus expression and ED (Diskin and Sreenan, 2000) until excessive cow density begins to affect behavior.

Seasonal effects, primarily heat and humidity, are particularly detrimental to estrus expression and fertility (Senger, 1994). For example, a study by Schüller et al. (2016) observed that prolonged heat stress resulted in a 63 percentage point decline in pregnancy rate. Several studies have also demonstrated that heat stress reduces estrus intensity and duration and also alters hormone profiles around estrus (Gwazdauskas et al., 1981b; Younas et al., 1993; Schüller et al., 2017). A study by López-Gatius et al. (2005) found that overall walking activity was significantly decreased in the warm months (May to September) as compared to the cool months (October to April) and Yániz et al. (2006) found that walking activity at estrus was decreased when relative humidity was greater than 95%. Data from Reith et al. (2014a) demonstrated that cows are more likely to show estrus in the early morning hours from 0200 to 0800. One potential explanation for this observation is that cows may be more likely to demonstrate estrus behaviors during the cooler hours of the day.

4. METHODS AVAILABLE FOR DETECTION OF ESTRUS

4.1. OVERVIEW OF METHODS

As commercial dairy farms continue to increase in size and qualified labor becomes scarce, farms have increasing difficulties to dedicate personnel and time to detection of estrus. Furthermore, multiple biological, management and environmental factors reduce estrus expression in lactating dairy cows, making successful detection of estrous behaviors progressively more challenging. In order to address this concern, ED methods have evolved to help meet the needs of modern dairy farms. The traditional method of ED is visual observation of estrous behaviors such as standing to be mounted and other secondary estrus behaviors. However, visual observation requires substantial inputs of time and labor, which are often limited for some farms. Furthermore, not all cows display overt signs of estrus that can be easily detected by visual observation alone or the frequency of observation is insufficient to detect cows that display estrus during a short period of time. Thus, visual ED aids are often incorporated as a supplement to traditional methods. Common ED aids include the application of paint or pressure-sensitive patches to the tail head of cows, which can aid personnel by preserving evidence of recent standing behavior. While traditional ED and visual ED aids remain commonplace on dairy farms (NAHMS, 2018), some operations have transitioned to the use of fully automated ED systems. In the last 20 years, numerous commercially available AED systems have been developed and improved, giving farmers a variety of attractive alternatives over traditional monitoring methods.

Most AED systems consist of cow-attached automated sensors that monitor behavioral or physiological parameters known to change during estrus events such as standing activity, overall physical activity, rumination time, body temperature, milk P_4 concentrations, or combinations of these. Receivers or antennas capture and transfer data generated by the sensors to a computer with a specialized software that generates alerts when changes in the parameter(s) measured reach a certain threshold, indicating a high likelihood of an estrus event. Depending on the AED system, many other system-specific set of reports and graphs may be available. In some cases, the software may be integrated with the dairy herd management software, which allows exchange of individual cow and herd information, or can serve as the dairy herd management software itself. Although highly dependent on the ED program currently implemented at a farm, some of the AED systems may have numerous benefits over traditional methods of ED. Among the most common are: (1) the possibility of continuous rather than intermittent monitoring, which may increase the proportion of cows detected in estrus; (2) an objective evaluation of behavioral and/or physiological changes which may improve ED accuracy as compared to subjective evaluation by farm personnel, and (3) a reduction in labor demands as the AED system can eliminate the need to evaluate all cows or groups of cows once or more than once per day, and in cases that ED aids are used reduce or eliminate the use of such aids. Thus, these systems can complement or completely replace traditional methods for detection of estrus.

One of the earliest field applications of automated monitoring in dairy cattle was the use of pedometers by Kiddy (1977), who found that cows increased their number of steps by 275 to 393% during estrus events. These and other findings led to the development of one of the first commercially available pedometry systems marketed for use in dairy cattle, which quantified activity levels via an internal mercury switch (Arney et al., 1994). Another early type of AED system relied on monitoring standing estrus via a rump-mounted pressure sensor. In recent years, AED technology has advanced dramatically. Today, most modern AED systems monitor overall physical activity levels with the use of internal accelerometers that can track movement in three dimensions (Madureira et al., 2015; Dolecheck et al., 2016). Some of these systems also have the ability to monitor other parameters like rumination time, eating time and body temperature which may be used in algorithms to generate estrus alerts (Costa et al., 2016; Mayo et al., 2019). More recently, ear-attached accelerometers that monitor activity levels, rumination time, and other parameters have also become commercially available.

Since each AED system measures data for the parameters of interest differently and estrus alerts are created using advanced proprietary algorithms, the ability of these systems to detect cows in estrus can vary. The next section of this chapter will address the differences in performance between numerous commonly used AED systems and describe how the use of AED systems can help optimize fertility through optimal timing of insemination.

4.2. AUTOMATED ESTRUS DETECTION

4.2.1. AUTOMATED ESTRUS DETECTION SYSTEM PERFORMANCE METRICS

In order to determine the performance of an AED system, the alerts that are generated by the system must be compared with a reference test (**REF**) for detection of estrus. Examples of potential REF for estrus include visual observation of estrous behaviors, blood or milk P_4

dynamics to confirm low P_4 levels during estrus and ovulation, ovarian mapping by transrectal ultrasonography, or a combination of these. When an AED system estrus alert corresponds with estrus as determined by the REF it is considered a true positive (**TP**), failure to generate an alert for an estrus event detected by the REF results in a false negative (**FN**), alert generation without estrus detected by the REF is a false positive (**FP**), and lack of AED system alert and no estrus detected by the REF is a true negative (**TN**) (Firk et al., 2002). The most commonly reported performance metrics for AED systems in the literature are sensitivity (Se; i.e., the likelihood that an estrus alert is generated for an estrus event confirmed by the REF), and the positive predictive value (PPV; i.e., the likelihood that a cow with an AED system estrus alert is truly in estrus as determined by the REF). Sensitivity is calculated as TP / (TP + FN) × 100 and PPV is calculated as TP / (TP + FP) × 100 (Dohoo et al., 2014). Other metrics of interest that are less commonly reported are specificity (Sp; i.e., the likelihood that a cow that was not detected in estrus by the REF also did not have an alert from the AED system), and the negative predictive value (NPV; i.e., the likelihood that a cow for which no AED system alert was generated was truly not in estrus as determined by the REF). If sensitivity is too high, there is a high probability that there will be a relatively large percentage of false positives, thus decreasing the specificity and positive predictive value. If Sp is high then the opposite can be expected. Therefore, in order to achieve optimal performance, the thresholds to trigger estrus alerts must be set such that the occurrence of false positives and false negatives are balanced. A review by Firk et al. (2002) suggested that many AED systems on the market had a sensitivity in the range of 80 to 90%, but performance across systems varied. A summary of available performance metrics for numerous types of AED systems are presented in Table 1. At least in part, the variation in performance metrics observed, even for similar types of AED systems, can be explained by the choice of

REF, study design, management factors, sensor type and placement in the cow, and discrepancies between the alert-generating algorithms. Results from a number of studies will be explored to better understand the differences observed between AED system performance.

The ED method used as the REF can dramatically alter the perceived performance of an AED system. For instance, a recent study by Mayo et al. (2019) evaluated six AED systems using either blood P_4 dynamics or visual observation of standing estrus as the REF. The systems that were evaluated consisted of a pedometer, three leg-attached and one neck-attached accelerometer-based AED systems, and an ear-attached accelerometer-based AED system. When blood P_4 was used as the REF, the average sensitivity and specificity for all systems was 69.4% and 92.1%, respectively. On the other hand, when standing estrus was used as the REF, the average sensitivity and specificity for all systems was 80.2% and 56.5%, respectively. These results suggest that when using only blood P_4 as the REF, the FP rate may be low but the FN rate may be comparably higher. Conversely, for standing estrus as REF, the reverse is true. Interestingly, the system with the highest overall accuracy when blood P_4 was the REF was the ear-attached accelerometer-based AED system whereas the system with the highest overall accuracy when standing estrus was the REF was one of the leg-attached accelerometer-based AED systems. It is also interesting to note that although 4 of the 6 AED systems evaluated used physical activity measurements from one of the legs, the performance of these leg-attached systems varied as well, with Se ranging from 70.7 to 92.2% and PPV ranging from 60.3 to 70.6% when standing estrus was used as the REF. The greatest discrepancy was for Se for which a difference of up to 21.5% was observed between two systems. Collectively, these data indicated that the REF used in a particular study or for the same study, the sensor location on or in the cow, and sensor attributes given by the manufacturer can impact AED system performance

Table 1. Summary of the performance of various types of activity monitoring systems for automated detection of estrus in cattle.

Source	Sensor Type	Reference test for estrus	Se	Sp	PPV
Redden et al. (1993)	Leg-mounted pedometer	Milk P_4	80%	-	-
Roelofs et al. (2005b)	Leg-mounted pedometer	Visual Observation and Ovulation	83%	-	91%
Holman et al. (2011)	Leg-mounted pedometer	Milk P_4	59%	-	94%
	Neck-mounted accelerometer	Milk P_4	63%	-	74%
Aungier et al. (2012)	Neck-mounted accelerometer	Ovulation	-	-	79%
Chanvallon et al. (2014)	Leg-mounted pedometer	Ovulation	73%	-	72%
	Neck-mounted accelerometer	Ovulation	63%	-	84%
	Neck-mounted accelerometer	Ovulation	62%	-	66%
Dela Rue et al. (2014)	Leg-mounted accelerometer	Milk P_4 and insemination records	89%	-	99%
	Neck-mounted accelerometer	Milk P_4 and insemination records	81%	-	72%
Reith et al. (2014a)	Neck-mounted accelerometer	Conception	76%	-	-
Stevenson et al. (2014)	Neck-mounted accelerometer	Blood P_4 and follicle size	-	-	89%
Talukder et al. (2015)	Neck-mounted accelerometer	Mounting indicators	78%	57%	70%
Madureira et al. (2015)	Leg-mounted pedometer	Pedometer + Collar AED system	-	-	86%
	Neck-mounted accelerometer	Pedometer + Collar AED system	-	-	90%
Adriaens et al. (2019)	Neck-mounted accelerometer	Ovulation	83%	-	66%
Burnett et al. (2018)	Neck-mounted accelerometer	Ovulation	-	-	88%
Mayo et al. (2019)	Leg-mounted pedometer	Standing Estrus	92%	47%	60%
	Leg-mounted accelerometer	Standing Estrus	92%	55%	65%
	Leg-mounted accelerometer	Standing Estrus	71%	64%	62%
	Neck-mounted accelerometer	Standing Estrus	50%	77%	71%
	Ear-attached accelerometer	Standing Estrus	100%	40%	59%
	Leg-mounted accelerometer	Standing Estrus	76%	66%	61%
Schweinzer et al. (2019)	Ear-attached accelerometer	Standing Estrus + AED system	97%	98%	96%

metrics. An earlier study by Chanvallon et al. (2014) compared the performance of two estrus alert algorithms that generated estrus alerts using raw data from the same neck-mounted sensors, and found a difference of 1% in Se and 22% in PPV between algorithms. These results indicated that algorithm features can also substantially affect the performance of an AED system, even when using data from the same sensor.

Other studies suggested that performance of AED systems might improve when alerts are used in conjunction with other ED aids, or when data from multiple sensor types are combined. For example, Holman et al. (2011) compared the performance of a leg-mounted pedometer and a neck-mounted accelerometer-based AED system both individually and in conjunction with the use of rump-mounted scratch cards and KaMar patches (Kamar Inc., Steamboat Springs, Colorado, USA), or visual observation of estrous behaviors six times per day. The neck-mounted AED system and pedometers had baseline sensitivities of 58.9% and 63.3%, respectively. The inclusion of scratch card data along with AED system alerts improved sensitivity by 9.5% and 3.4% for the neck-mounted AED system and leg-mounted pedometer. Visual observation of estrous behaviors also improved sensitivity by 16.1% for the neck-mounted AED system and 11.1% for the pedometer. The use of KaMar patches resulted in the greatest increase of sensitivity, 16.7% and 12.6% for the neck-mounted AED system and pedometer, respectively. However, due to an increased in occurrences of FP alerts, the PPV decreased numerically when combinations of ED methods were used as compared to the AED systems individually. Thus, using ED aids as well as AED systems might improve ED sensitivity; however, the occurrence of FP alerts will likely increase. Furthermore, the combination of other behavioral or physiological parameters such as rumination time with physical activity also has the potential to improve the performance of AED systems (Reith et al., 2014a). This is because a majority of cows seem to have both an increase in activity and a decrease in rumination around estrus and a small proportion of cows seems to only have a decrease in rumination. The latter subgroup may be composed of cows with very low intensity of estrus or cows that do not express signs of estrus that can be measured by the sensors that monitor physical activity. Based on these observations, it was proposed that future efforts should focus on integration of rumination data into estrus alert-generating algorithms to aid in the identification of less intense or less evident estrus events.

Studies designed to evaluate the performance of recently developed ear-attached AED systems have been limited and had some experimental design limitations. In addition, the patterns of the parameters monitored or the factors that may affect the performance of these systems have not been described in detail. A recent study by Schweinzer et al. (2019) conducted an evaluation of an accelerometer-based ear-attached based AED system (Smartbow; Zoetis Inc., Parsippany, New Jersey, USA) using cows housed in a free-stall facility. Using an insemination that resulted in pregnancy as evidence of estrus (i.e., REF), sensitivity was calculated at 97%, which is in the upper range observed for most AED systems. A major caveat of the analysis including only pregnant cows after insemination as the REF was that only sensitivity could be calculated. In addition, evaluating an AED system using only estrus events form pregnant cows may not be ideal for several reasons. To name a few, the population of cows used for estimation of performance is biased and cows that become pregnant after insemination likely expressed more intense estrus events than cows that did not become pregnant. Thus, return to estrus from 18 to 25 d after a previous AI as was also used as REF for a separate analysis of performance. Estrus events were confirmed using a combination of visual observation of standing estrus and/or the presence of an estrus alert from another ear-attached AED system (CowManager Sensoor;

Agis Automatisering, Harmelen, the Netherlands). The reported overall accuracy of the AED system using return to estrus as REF was 96.3% with sensitivity of 96.6%, specificity of 97.7%, PPV of 95.8%, and NPV of 93.8%; all values in the upper range of performance for AED systems. Mayo et al. (2019) performed an evaluation of the CowManager ear-attached AED system (CowManager Sensoor; Agis Automatisering, Harmelen, the Netherlands) and also reported high values for accuracy, sensitivity, specificity, and PPV (90.8%, 89.5%, 100.0%, and 100.0% respectively) when blood P_4 was used as REF, although NPV was comparably low at 57.1%. However, as mentioned previously, performance metrics reported in the study varied considerably depending on the method used as REF. When standing estrus alone was used as REF, accuracy, sensitivity, specificity, PPV and NPV were 67.7%, 100.0%, 40.0%, 58.8%, and 100.0%. Therefore, while the ear-attached sensor evaluated was capable of detecting estrus events measured by multiple reference methods, it is difficult to discern the true performance of the system evaluated given the variability of the results. Taken together, these data suggested that a commercially available ear-attached accelerometer-based AED system may be an effective tool to detect cows in estrus. However, due to limited availability of studies and limitations of the only research studies that are available, further investigation of the performance of ear-attached accelerometer-based AED systems is needed.

4.2.2. TIMING OF INSEMINATION AND OVULATION RELATIVE TO AUTOMATED ESTRUS DETECTION SYSTEM ALERTS

In order to maximize the likelihood of conception, insemination time relative to the time of estrus and ovulation must be considered. While sperm can usually remain viable for at least 24 hours after insemination (Saacke et al., 2000), the oocyte has a shorter lifespan of approximately 8 to 10 h after ovulation, after which viability is greatly reduced (Hunter, 1985). Since sperm must also undergo capacitation before becoming capable of fertilization, insemination should be performed prior to ovulation in order to improve the chances of conception. Roelofs et al. (2006a) reported that fertilization rates and embryo quality were maximized when insemination was performed 24 to 12 hours prior to ovulation. Therefore, when using an AED system to detect estrus, it is beneficial to evaluate the expected window from alert generation to ovulation and to determine the optimal insemination time that will maximize the likelihood of conception.

Roelofs et al. (2005b) found that ovulation occurred on average 29.3 h after the onset of increased physical activity as measured by a pedometer. However, due to the nature of AED system alert-generating algorithms, alerts may not be generated until increased activity levels have been maintained for a certain period of time; thus, the onset of the alert does not necessarily correspond with the onset of estrous behaviors and should be considered separately. Indeed, Stevenson et al. (2014) found that while the interval from onset of standing behavior to ovulation was 26.4 h, the interval from the onset of an AED system alert to ovulation was 24.6 h, indicating that the alerts were generated on average 1.8 h after the onset of standing behavior. A study by Valenza et al. (2012) also demonstrated that physical activity as measured by a neck-mounted sensor began to increase approximately 28.7 h prior to ovulation and eventually peaked at an average of 20.4 h prior to ovulation. Stevenson et al. (2014) observed that P/AI was maximized for primiparous cows when insemination was performed between 6 and 13 h after the onset of AED system estrus alerts and for multiparous cows when insemination was performed from 0 to 12 h after the onset of an alert. Interestingly, LeRoy et al. (2018) found the opposite as there were no differences in P/AI for multiparous cows inseminated 0 to 8, 8 to 16, or 16 to 24 h after onset of an AED system alert. Primiparous cows on the other hand, benefitted from earlier insemination between 0 to 8 h after onset of alert and P/AI declined as insemination was delayed. Results from the latter study must be interpreted with caution since data from four different farms were included in the study and two different brands of neck-attached AED systems were used. Taking into account the discrepancies in the literature and the apparent detrimental effects of delaying insemination given the short viable lifespan of an ovulated oocyte, it seems reasonable to hypothesize that optimal P/AI might be achieved in both primiparous and multiparous cows if insemination is performed promptly after alert generation, particularly if insemination is performed only once daily. In support of this notion, Nebel et al. (1994) using 75 day nonreturn rates after insemination for 7,240 AI services found that P/AI was maximized when insemination was performed between 0 to 12 h after visual observation of estrous behaviors and was lowest when insemination was performed more than 24 h after observation of estrous behaviors.

5. SUMMARY

This literature review was intended to provide an overview of the physiology of estrus and estrous behaviors and the factors that can affect them. Furthermore, it summarizes available data on the performance of AED systems and explores how to use AED systems to optimize fertility.

During estrus, most cows display a multitude of characteristic behaviors including dramatically increased physical activity. Thus, AED systems can aid in ED by monitoring individual cow activity levels and generating alerts when activity levels reach a specific threshold, indicating a high likelihood of an estrus event. Other behavioral and physiological parameters that change during estrus such as rumination, eating, and body temperature may be used as an aid for creation of estrus alerts algorithms.

Although numerous studies have been conducted to evaluate the performance of various commercially available AED systems, variations in study design can present challenges for direct comparisons between systems. Thus, Chapter II is intended to provide a prospective evaluation of a commercially available recently developed ear-attached AED system for cattle, evaluate a series of factors that can affect its performance and the pattern of the parameters monitored around estrus and ovulation.

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CHAPTER II

EVALUATION OF AN EAR-ATTACHED SENSOR FOR THE CHARACTERIZATION AND DETECTION OF ESTRUS EVENTS IN DAIRY CATTLE

1. INTRODUCTION

Despite the advent of synchronization of ovulation protocols for timed AI (**TAI**) and their widespread adoption by dairy farms, submission of cows for insemination at detected estrus remains a significant aspect of dairy cattle reproductive management (Caraviello et al., 2006; Scott, 2016; NAHMS, 2018). Unfortunately, some dairy farms face challenges implementing effective programs for detection of estrus based on traditional methods such as visual observation (**VO**) of estrous behavior, tail-paint, or chalk removal, and activation of rump-mounted devices (Peralta et al., 2005; Holman et al., 2011). These methods are labor intensive and require consistent implementation, which is problematic for farms that lack or have limited availability of trained personnel to conduct ED. In addition, the performance of ED programs may be affected considerably by cow-to-cow variation because cow biological differences, management factors, and environmental conditions might limit expression of estrus or reduce estrus duration and intensity in lactating dairy cows (Lopez et al., 2004; Palmer et al., 2010; Aungier et al., 2012; Schüller et al., 2017). The effect of all these issues in isolation or in combination on traditional ED programs might reduce the overall reproductive performance of a dairy herd when all or most cows are expected to be submitted for AI after detected estrus.

An alternative approach to traditional methods for ED is the use of automated estrus detection (**AED**) systems. Most of the systems available today use sensors for continuous monitoring of behavioral, physiological, or performance parameters (Nebel et al., 2000; Dolecheck et al., 2015; Mayo et al., 2019) because of the evident changes in behavior and physiology that most bovine females experience during estrus (Kiddy, 1977; Van Vliet and Van Eerdenburg, 1996; Roelofs et al., 2005a). For example, monitoring physical activity (**PHA**) with pedometers or accelerometers attached to the leg or neck of cows is one of the most commonly used strategies to detect cows in estrus by AED systems (Madureira et al., 2015; Burnett et al., 2018; Mayo et al., 2019). In addition to changes in behavior that alter the level of overall PHA, cows also experience changes for other behavioral and physiological parameters such as rumination (Reith et al., 2014a), eating (Reith et al., 2014b), and body temperature (Fisher et al., 2008) that through recent development in technology can now be continuously monitored by sensors (Reith et al., 2014a; Dolecheck et al., 2015; Mayo et al., 2019). Due to technical or practical limitations, most sensors for monitoring behavioral and physiological parameters have traditionally been placed on the neck or leg of cows. Nevertheless, more recently, a novel type of AED system based on ear-attached sensors that monitor PHA and other behaviors such as rumination time (**RUM**) were developed for detection of estrus (Mayo et al., 2019; Schweinzer et al., 2019) as well as for health and feeding monitoring (Wolfger et al., 2015; Hill et al., 2017; Reiter et al., 2018). A few controlled studies reported that this type of AED system had reasonable performance for detection of estrus in lactating dairy cows (Mayo et al., 2019; Schweinzer et al., 2019); however, data available is still limited. In addition, the pattern and variation for the parameters monitored by AED systems based on ear-attached sensors around estrus and ovulation, and the effect of cow features and milk production on the overall performance of these systems have not been reported.

Thus, the objectives of this study were to: (1) determine the ability of an ear-attached accelerometer-based AED system (Smartbow; Zoetis Inc., Parsippany, New Jersey, USA) that monitored PHA and RUM to detect cows in estrus; (2) characterize the pattern of these parameters in relationship to estrus and ovulation; and (3) explore the effect of cow features and milk production on the ability of the AED system to detect cows in estrus and estrus parameters.

2. MATERIALS AND METHODS

All experimental procedures conducted with cows were approved by the Animal Care and Use Committee of Cornell University.

Farm and Cow Management

Lactating Holstein cows (n = 421; 120 primiparous and 301 multiparous) from the Dairy Unit of the Cornell University Ruminant Center (Hartford, NY) were enrolled into this observational prospective cohort study from December 2018 to April 2019. Cows were housed in a 6 row freestall barn with deep sand-bedded stalls, grooved concrete alleys, and headlocks on the feedline. Cows had ad libitum access to water and a once-daily fed total mixed ration diet was formulated to meet or exceed the nutrient requirements of lactating dairy cows producing 48 kg of milk per day with 3.8% fat and 3.1% true protein as determined by the Cornell Net Carbohydrate and Protein System (Higgs et al., 2015).

Cows were milked 3 times daily at approximately 8 hour intervals and milk weights were collected at each milking and stored on the dairy herd management software (DairyComp 305; Valley Agricultural Software, Tulare, CA). Average milk production for cows in the study during the study period was 43.5 kg per cow per day.

Synchronization of Estrus and Selection of Cows for Estrus and Ovulation Monitoring

Weekly, cohorts of 10 to 25 primiparous and multiparous cows were enrolled at 50 ± 3 DIM (n = 208) or at 18 ± 3 after a previous AI service (n = 213) and were subjected to an estrus synchronization protocol (Figure 1). Cows enrolled at 50 ± 3 DIM were not previously inseminated during the current lactation whereas cows enrolled 18 ± 3 after a previous AI service had already received at least one insemination and were past 65 DIM. At enrollment (d -14), all cows received an intramuscular injection of GnRH (100 μg Gonadorelin chloride, Factrel, Zoetis, Kalamazoo, MI), 7 d later cows received a second intramuscular injection of GnRH and a controlled internal drug release insert containing P_4 (1.38 g of P_4, CIDR, Zoetis, Kalamazoo, MI). On d 0, seven days after the second GnRH treatment and CIDR insertion, the P_4 insert was removed and cows received an intramuscular $PGF_{2\alpha}$ treatment (25 mg of Dinoprost tromethamine, HighCon Lutalyse, Zoetis, Kalamazoo, MI) to induce luteal regression. Twenty-four hours later cows completed the synchronization of estrus protocol with a second intramuscular $PGF_{2\alpha}$ treatment of the same dose. Cows detected in estrus by farm technicians before induction of luteolysis on d 0 were immediately inseminated and removed from the study.

Transrectal ultrasonography (**TUS**) of the reproductive tract and ovaries was performed using a portable ultrasound machine (Ibex Pro, E.I. Medical Imaging, Loveland CO) to measure and record in an ovarian map the location and size of ovarian structures present, and determine the pregnancy status of previously inseminated cows. For cows enrolled at 50 ± 3 DIM, TUS was conducted at the time of the second GnRH (d -7) and again one week later (d 0) to select cows to continue in the study. For cows enrolled at 18 ± 3 d after AI, TUS was first performed on d 0 of the experimental timeline to determine their pregnancy status and in nonpregnant cows the

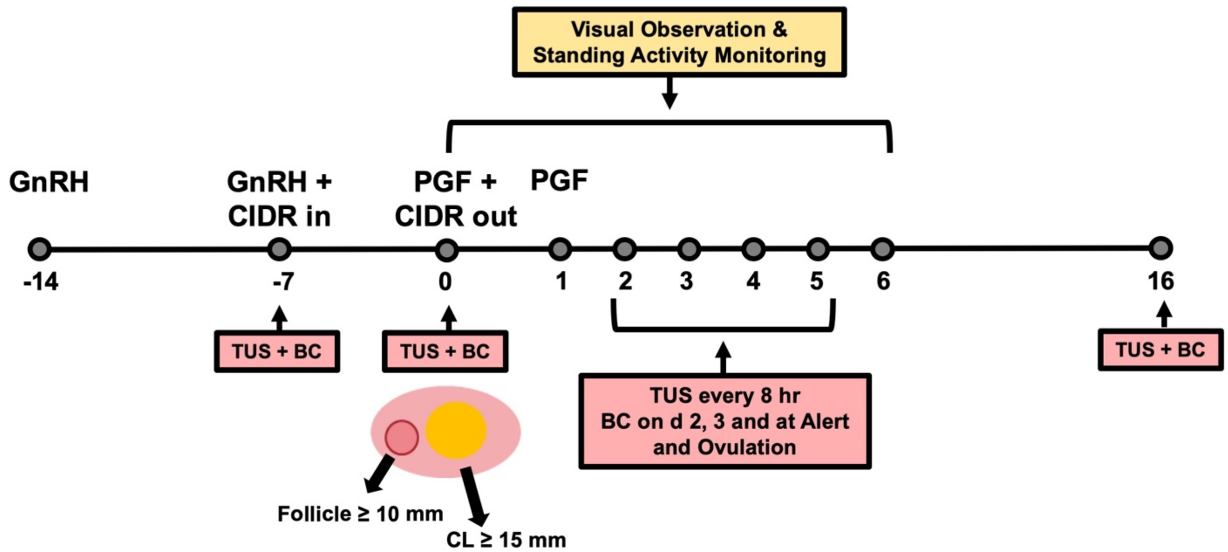

Figure 1. Graphical representation of experimental procedures. Every week, cows that had reached 50 ± 3 DIM or 18 ± 3 days after a previous insemination were enrolled in an estrus synchronization protocol. Two intramuscular GnRH treatments were given 7 d apart. On d 0, 7 d after the second GnRH, an intramuscular $PGF_{2\alpha}$ treatment was given to all cows that met the criteria for estrus detection. A second $PGF_{2\alpha}$ treatment was administered 24 h later. From d 0 to 6, cows were monitored for estrous behaviors by visual observation and a standing behavior monitoring system. Transrectal ultrasonography and ovarian mapping were performed on d -7 and 0 for cows enrolled at 50 ± 3 DIM and on d 0 for cows enrolled at 18 ± 3 days after a previous insemination to evaluate ovarian status. Transrectal ultrasonography was performed every 8 h from d 2 to 5 to determine timing of ovulation and on d 16 to confirm ovulation. Blood collection (**BC**) was performed on d -7, 0, 2, 3, at the time of automated estrus detection system alert and ovulation, and on d 16.

presence and size of ovarian structures. All measurements were conducted using the internal digital calipers of the ultrasound machine. Luteal volume was calculated as $V = 4/3 \times \pi \times r^3$, whereas the radius (r) was calculated as $0.5 \times$ CL diameter. For a CL with a cavity (anechoic area surrounded by echoic tissue), the volume of the cavity was calculated and subtracted from the total CL volume. All cows with at least one CL ≥15 mm in diameter and at least one follicle ≥10 mm in diameter on d 0 were eligible to continue in the study. Cows that did not meet these criteria were removed from the study.

Estrus and Ovulation Monitoring

Estrous behavior and ovulation were monitored for seven days (d 0 to 6) in all cows that remained in the study on d 0. Estrus was monitored through a combination of VO of behavioral signs of estrus by farm technicians and standing behavior recorded with a pressure-activated standing behavior monitoring system (HeatWatch, Cowchips LLC, Manalapan, NJ) attached to the tail head of the cow. Visual observation was conducted by farm technicians in charge of the farm reproductive management program (i.e., detection of estrus, insemination, hormonal treatments for synchronization of estrus and ovulation, pregnancy exams) a minimum of two times per day (once in the morning and once in the afternoon at approximately 12 h intervals) for at least 30 minutes during each session. Milking technicians and cow drivers assisted reproductive management technicians by recording estrous behavior observed during every milking shift. All primary (i.e., standing to be mounted) and secondary signs of estrus (i.e., mounting another cow, mounted but not standing, resting chin on the back of another cow, sniffing the vulva of another cow, or visibly increased restlessness or activity) observed were recorded in individual cow cards designed by the research team and provided to farm technicians. Pressure-activated transmitters from the HeatWatch system were placed inside a mesh pouch and affixed to the tail head using an adhesive. All cows were fitted with a transmitter by the time of first $PGF_{2\alpha}$ on d 0 and standing data was collected until d 6 or until the patch was detached by frequent standing events (n = 35), whichever occurred first.

A reference test for estrus (herein **RTE**) was created using a combination of data from VO and the standing behavior monitoring system. Cows with a record of VO of the primary sign of estrus, VO of a minimum of two secondary signs of estrus, or cows that met the definition of estrus based on standing behavior recorded by the pressure-activated tail head sensor were considered in estrus for the RTE. Estrus based on standing behavior was defined as a minimum of 2 standing events ≥ 1 second in duration within a window of 12 hours. Data from the pressure-activated tail head sensor was also used to gather information on the timing, duration, and intensity of estrus based on standing behavior. The definition of estrus based on standing behavior used in the current study was designed to remove potential false negative outcomes or artificially shorten the duration of standing behavior during an estrus event if the traditional definition of estrus (i.e., minimum of 3 standing events ≥ 2 seconds in duration within a window of 4 hours; Dransfield et al., 1998) based on the same monitoring method was used. Onset of standing estrus was defined as the timing of the first standing event detected by the standing behavior monitoring system and the duration of standing estrus was defined as the difference in hours between the first and the last standing event of the estrus event. Intensity of standing behavior was quantified by counting the total number of standing events per estrus.

On d 2, 48 hours after the first $PGF_{2\alpha}$ treatment, TUS of the ovaries was performed in all cows eligible for detection of estrus. The position and size of all follicles >10 mm present on the ovaries was recorded in an ovarian map. Thereafter, TUS was performed at approximately 8 h intervals until the end of d 5 or until two subsequent examinations following the first at which the largest follicle or two largest follicles were no longer visualized. Ovulation time was defined as the midpoint between the first examination at which the putative ovulatory follicle was no longer visualized and the prior examination. On d 16 of the study (i.e., 16 d after the first $PGF_{2\alpha}$ treatment for induction of luteal regression), all cows that ovulated during the period of estrus monitoring underwent TUS of the ovaries to confirm the presence of a CL on the same ovary where the ovulation was observed.

All cows detected in estrus during the experiment were inseminated. Cows not detected in estrus by VO, the pressure-activated tail head sensor, or both from d 0 to 6 were enrolled in a synchronization protocol to receive TAI according to farm management protocols. After this AI service cows were eligible for re-enrollment in the study at 18 ± 3 d after AI.

Data Collection from the Automated Estrus Detection System

At least three days before the first $PGF_{2\alpha}$ treatment for induction of luteal regression, all cows enrolled in the study were fitted with an ear-attached sensor that monitored PHA and RUM (Smartbow; Zoetis Inc., Parsippany, New Jersey, USA). A minimum of three days is required for the sensor software system to establish baseline activity levels for individual cows. From d 0 to 6, the software generated estrus alerts using an internal algorithm that combines data for PHA and RUM. The software also records and reports the number of minutes per hour that a cow spends ruminating and in three possible levels of PHA; i.e., inactive, active, and high active. The onset and end of estrus alerts is also reported and stored. After conclusion of the study, estrus alerts and raw PHA and RUM data were downloaded from the AED system web-cloud. The duration of estrus as measured by the AED system was defined as the difference in hours between the onset and end of the estrus alert. To calculate the intensity of the estrus alert, the amount of high activity in min/h was averaged for the hours during which the estrus alert was active.

Blood Sampling and Estimation of Progesterone Concentrations

Blood samples were collected by puncture of the coccygeal vein or artery using 8 mL heparinized evacuated tubes (BD Vacutainer, Franklin Lakes, NJ) to determine circulating concentrations of P_4. After collection, samples were immediately placed on ice for transport to the laboratory. Samples were centrifuged at 2000 x *g* for 20 minutes at 4°C. Plasma was collected and stored in duplicates in 2 mL plastic storage tubes at -20 °C until analyzed.

Samples were collected on d -7 for cows enrolled at 50 ± 3 DIM only. Thereafter, samples were collected in all cows on d 0, at 48 h and 72 h after the first $PGF_{2\alpha}$ treatment, and then at the time of TUS immediately after the first estrus alert and at the first time the putative ovulatory follicle was no longer visualized (Figure 1). Finally, a blood sample was collected on d 16 of the study from cows detected in estrus between d 0 and 6.

Plasma P_4 concentrations were estimated in duplicate samples using a commercial solid-phase, no-extraction radioimmunoassay (RIA, ImmuChem Coated Tube, MP Biomedicals, Costa Mesa, CA). In each assay (n = 12), samples with known concentrations of P_4 (5.0 ng/mL) were included at the beginning, middle, and end of each assay to assess reliability. Inter- and intra-assay coefficients of variation (CV) were 15.5% and 10.6%. The detection limit of the assay was 0.1 ng/mL and the minimum P_4 concentration required to consider a CL functional was 1.0 ng/mL.

Cow Features, Genomic and Milk Production Data Collection

At the time of the first TUS examination, the body condition of all cows was evaluated using a score (**BCS**) from 1 (emaciated) to 5 (obese) using 0.25 increments (Ferguson et al., 1994). For analysis, BCS was split into two levels; cows with BCS ≥ 3.0 were classified as being in high body condition and cows with BCS <3.0 were classified as being in low body condition. Body weight (**BW**) and locomotion score were recorded on d -3 of the study for cows enrolled at 50 ± 3 DIM and d 18 in cows enrolled 18 ± 3 d after AI. Locomotion scores ranged from 1 (sound) to 5 (severely lame) in one point increments as described in Sprecher et al. (1997). For analysis, all cows with locomotion scores ≥3 were considered lame and those with scores <3 were considered sound. Body weight was classified as high or low based on the median value within parity (i.e., primiparous or multiparous). Median body weight was 604 kg for primiparous and 691 kg for multiparous cows. Daily average milk production for the ten days preceding induction of luteolysis was classified as high or low based on the median value within parity. Median milk production for primiparous cows was 38 kg per day and for multiparous cows was 49 kg per day. Genomic merit for daughter pregnancy rate (**GDPR**) was collected from genomic reports (Clarifide Plus, Zoetis, Kalamazoo, MI) for animals that had been previously tested. Values for GDPR ranged from -3.5 to 4.2 with a median value 0.8 used to categorize cows in groups of high or low GDPR.

Statistical Analysis

Quantitative data from single data points and repeated measurements were analyzed by ANOVA with the MIXED procedure of SAS (Version 9.4, SAS Institute, Cary, NC). Models for outcomes of interest with single data points included selected explanatory variables depending on the outcome and comparison of interest. Models used for comparing size of ovarian structures for cows with high (>1 ng/mL) and low (<1 ng/mL) circulating concentrations of P_4 on d 0, included P_4 level group (i.e., high vs. low). Models for comparing timing and features of estrus events (i.e., timing from induction of luteolysis to estrus, duration of estrus, estrus intensity, and the estrus to ovulation interval) were offered the effect of P_4 level on d 0, parity, enrollment before or after first service (herein **service number**), and milk production, BCS, BW, locomotion score, and GDPR categorized in high and low groups for each variable as described above. In addition, the interaction between parity and service number and the interaction between parity and milk production were offered to the initial models.

Repeated measurements data for circulating concentrations of P_4 during the synchronization of estrus protocol and at 2 and 3 d after induction of luteolysis were compared for cows with high (>1 ng/mL) or low (<1 ng/mL) circulating concentrations of P_4 on d 0. Models included, P_4 group, time and their interaction as fixed effects whereas cow was included as a random effect. A spatial power covariance structure was used to account for the different timing of sampling. Data for P_4 concentrations were log-transformed because it was not normally distributed.

Time spent in each PHA level category (i.e., high active, active, and inactive) and RUM in minutes per hour were averaged for 8-h intervals to account for normal variation in raw hourly data reported by the AED system. For cows that failed to ovulate, the average ovulation time for cows with confirmed ovulation (i.e., 95 hours after induction of luteolysis) was used for analysis. Two different analyses were conducted. First, cows were grouped based on detection of estrus by the AED system and detection of ovulation as follows: estrus and ovulation, estrus and no ovulation, no estrus and ovulation, and no estrus and no ovulation. Due to the small number of cows in the estrus and no ovulation group (n = 2), this group was excluded from the analyses. In a second analysis, the same outcomes were compared but for groups of cows formed based on detection of estrus by the RTE and the AED system as follows: cows detected by both the RTE and AED system, cows detected only by the RTE, cows detected only by the AED system, and cows not detected in estrus by any method. Models for both analyses included group, time (i.e., hours before ovulation), the group by time interaction, and parity. Cow was included as a random effect. Data for time spent in the high active category was log-transformed because it was not normally distributed. For all analyses including repeated measurements data, the Least Significant Difference (**LSD**) post-hoc mean separation test was used for determination of differences between groups at specific time points.

Categorical data were analyzed by logistic regression using the GLIMMIX procedure of SAS. The proportion of cows detected in estrus, proportion of cows that ovulated, and proportion of cows in groups of cow features were compared for cows with high versus low P_4 on d 0. Models included P_4 level as a fixed effect. The proportion of cows detected in estrus and detected in estrus with confirmed ovulation were compared for the RTE and the AED system as well as for the RTE versus VO and the standing behavior system individually. Initial models were offered the method for detection of estrus and all other cow features and milk production groups as fixed categorical variables. The effect of cow features and milk production on detection of estrus and ovulation was analyzed using estrus event outcomes generated by the RTE and the AED system. Initial models were offered parity, service number, BCS, BW, locomotion score, milk production, GDPR, and d 0 P_4 group as well as the interaction between parity and service, and parity and milk production.

Model selection for all quantitative and binary outcomes evaluated was performed by backwards selection through stepwise removal of variables with $P > 0.10$. All comparisons with parameters from the standing behavior monitoring system contained only data from the 65% (141/216) of eligible cows that were detected in estrus by standing behavior.

Measures of test performance including Se, Sp, NPV and PPV were calculated using the FREQ procedure of SAS. Balanced accuracy was calculated using the average of Se and Sp. For initial calculations, all cows that completed the trial were included in the dataset and true positives (**TP**) and true negatives (**TN**) were determined using only the RTE. A secondary calculation of performance measures was performed which excluded cows for which RTE and ovulation data did not agree, i.e. cows that were detected by the RTE but for which ovulation was not observed or cows that not detected by the RTE but for which ovulation was observed. Therefore, only cows that were detected by RTE and for which ovulation was observed were considered TP and only cows that were not detected by the RTE and for which ovulation was not observed were considered TN. The FREQ procedure was also used to calculate the Cohen's Kappa statistic for interrater agreement between methods of detection of estrus and the REG and CORR procedures were used to analyze correlations between variables.

All values for quantitative outcomes and proportions are reported as Least Square Means and SEM unless otherwise stated. All explanatory variables were considered significant if $P < 0.05$ whereas $0.05 \leq P \leq 0.10$ was considered a tendency.

3. RESULTS

Cow Eligibility for Detection of Estrus

Of the 421 cows originally enrolled in the study, 216 were eligible to be detected in estrus from d 0 to 6. Cows were not eligible for various expected and unexpected reasons that are presented in Table 2. Of the final set of 216 cows used for analysis, 29% (62/216) were primiparous, 71% (154/216) were multiparous, and 73% (157/216) and 27% (59/216) had not or had received a previous insemination, respectively. In addition, 9% (19/216) of cows had a lameness score of ≥ 3 and 41% (88/216) had a BCS of < 3.0. Median BW and median milk production for the ten days preceding induction of luteolysis was 604 kg and 37.6 kg per day for primiparous cows and 691 kg and 49.1 kg per day for multiparous cows.

Of the cows with a CL ≥ 15 mm and at least one follicle ≥10 mm in diameter visualized by TUS on d 0, 11% (24/216) had circulating concentrations of P_4 <1 ng/mL, for which the mean P_4 concentration was 0.54 ng/mL. There was a group by time interaction ($P < 0.0001$) for circulating concentrations of P_4 for cows grouped based on P_4 levels on d 0. Cows with low P_4 (i.e., <1 ng/mL on d 0) had greater circulating P_4 on d -7, lesser on d 0, and similar at 48 and 72 h after induction of luteolysis than cows with high P_4 on d 0 (Figure 2). Thus, the circulating P_4 profile for cows with low P_4 on d 0 confirmed the presence of a functional CL at the time of the GnRH treatment on d -7 and luteal regression before the $PGF_{2\alpha}$ treatment on d 0 (Figure 2). The diameter of the largest follicle did not differ ($P = 0.40$) at the time of $PGF_{2\alpha}$ administration (16.2 ± 1.0 and 15.4 ± 0.3 mm for the low and high P_4 group) but it was greater ($P = 0.05$) for the low than the high P_4 group at 48 h after $PGF_{2\alpha}$ administration (18.2 ± 0.8 and 16.4 ± 0.3 mm for the low and high P_4 group). Total luteal volume differed ($P < 0.0001$) both at induction of luteolysis and at 48 h after induction of luteolysis, whereby cows in the low P_4 group (3.9 ± 0.9 and 0.7 ±

Table 2. Reasons for removal from study.

Reason	Number of Cows
Diagnosed pregnant on d 0	92
Inseminated during estrus synchronization	48
No CL $\geq$ 15 mm on d 0	45
Health issue, moved to hospital pen	5
Sold or died	5
Incomplete CL regression[1]	1
Insufficient data and/or lack of compliance	9
Total	**205**

[1]Plasma P_4 concentrations failed to reach <1 ng/mL by d 3; i.e. 72 h after $PGF_{2\alpha}$ treatment

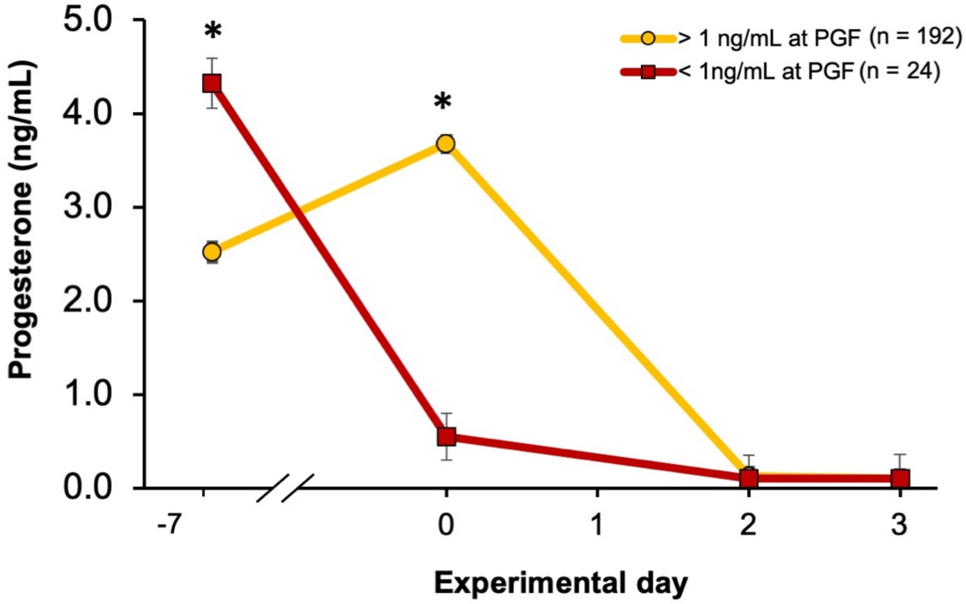

Figure 2. Circulating concentrations of progesterone on d -7, 0, 2, and 3 after induction of luteolysis with $PGF_{2\alpha}$ for cows with <1 ng/mL and >1 ng/mL on d 0. Within each time point, an asterisk (*) indicates differences ($P < 0.05$) for pairwise comparisons between groups.

0.2 cm^3) had less luteal tissue than cows in the high P_4 group (8.2 ± 0.3 and 1.2 ± 0.1 cm^3) on d 0 and d 2, respectively. In spite of the different ovarian dynamics during the synchronization of estrus protocol, cows with low P_4 on d 0 were included in the final analysis because the proportion detected in estrus by the RTE (83% vs 88% for low and high P_4), the proportion with detected ovulation (100% vs 92% for low and high P_4), the proportion of cows in BCS, BW, and locomotion score groups (i.e., high or low), and circulating P_4 concentrations following induction of luteolysis was similar ($P > 0.10$) compared with cows with high P_4 on d 0. We detected a tendency for the effect P_4 group on parity ($P = 0.06$) and service ($P = 0.08$) whereby a greater proportion of cows with low P_4 on d 0 were multiparous (88% vs. 69% for low and high P_4) and had not received a previous insemination (87% and 71% for low and high P_4). Progesterone level group on d 0 was; however, included as an explanatory variable for the evaluation of certain estrus and ovulation characteristics.

Average largest follicle diameter and CL volume for all cows was 15.5 mm and 7.7 cm^3 and 16.6 mm and 1.1 cm^3 on days 0 and 2, respectively. Average P_4 concentration was 3.3 ng/mL at induction of luteolysis and was 0.1 ng/mL at 48 h after induction of luteolysis. Out of all cows eligible for detection of estrus, 99% (214/216) and 100% (216/216) had circulating P_4 levels of <1 ng/mL by 48 and 72 h after induction of luteolysis, respectively.

Detection of Estrus and Ovulation

Of the cows eligible to be detected in estrus, 88% (190/216) had an estrus event recorded by the RTE as defined and conducted for this study. An ovulation was detected in 93% (201/216) of all cows eligible for detection of estrus and in 98% (187/190) and 54% (14/26) of the cows that were and were not detected in estrus by the RTE, respectively. Thus, out of the 216 cows eligible for detection of estrus and at risk of ovulating after d 0, 87% (187/216) were detected in estrus and ovulated, 1% (3/216) were detected in estrus and did not ovulate, 6% (14/216) were not detected in estrus and ovulated, and 6% (12/216) were not detected in estrus and did not ovulate within 7 d of induction of luteolysis.

A summary of estrus and ovulation outcomes for all methods of detection of estrus are presented in Table 3. The proportion of cows detected in estrus ($P = 0.23$) or detected in estrus and with confirmed ovulation did not differ ($P = 0.30$) between the RTE and the AED system. When the individual methods of detection of estrus used to create the RTE were compared with the RTE combining both methods, the proportion detected in estrus and detected in estrus with ovulation did not differ for VO ($P = 0.24$ for estrus, $P = 0.25$ for estrus with ovulation) but was less ($P < 0.0001$ for estrus, $P < 0.0001$ for estrus with ovulation) for the standing behavior sensor than the RTE (Table 3).

The Se, Sp, NPV, PPV, and balanced accuracy for detection of estrus based on the AED system alerts were 92 (95% CI 88 to 96%), 69 (95% CI 51 to 87%), 53 (95% CI 36 to 70%), 96 (95% CI 93 to 99%), and 80% (95% CI 70 to 92%), respectively. The Cohen's Kappa statistic for interrater agreement between the RTE and the AED system alerts was 0.54, indicating moderate agreement between the two methods (McHugh, 2012).

Additional evaluation of cows with presumptive false negative (**FN**) and false positive (**FP**) outcomes based on the AED system revealed that of 16 cows with a presumptive FN outcome, fourteen ovulated. Ovulation determined by the disappearance of the ovulatory follicle was confirmed by visualization of a CL and P_4 >1 ng/mL in all cows 16 d after induction of luteolysis. The remaining two cows, with no ovulation observed, were detected in estrus by both the standing behavior monitoring system and visual observation of increased activity and of

Table 3. Proportion of cows grouped based on detection of estrus by different methods and ovulation for 7 d after induction of luteolysis.

Item	RTE[1]	AED system[2]	VO[3]	Standing behavior[4]
Estrus	88.0%	84.3%	84.7%	65.3%*
Ovulation	86.6%	83.3%	83.3%	63.9%*
No Ovulation	1.4%	0.9%	1.4%	1.4%
No Estrus	12.0%	15.7%	15.3%	34.7%
Ovulation	6.5%	9.7%	9.7%	29.2%
No Ovulation	5.6%	6.0%	5.6%	5.6%

[1]RTE: combination of VO and standing behavior.
[2]Automated estrus detection system based on ear-attached sensor (Smartbow GmbH, Weibern, Austria).
[3]VO conducted a minimum of two times per day for at least 30 minutes during each session.
[4]Standing behavior monitored by sensor affixed to tail head (HeatWatch, CowChips LLC, Manalapan, NJ)
*Proportion of cows detected in estrus differed ($P < 0.0001$) from RTE.

mounting, resting chin on the back of, and sniffing the vulva of other cows. These data suggested that those were true FN for the AED system. On the other hand, of the 8 cows with a presumptive FP outcome based on the AED system, seven ovulated suggesting that the alerts from the system were appropriate and could not be considered FP outcomes. In these cows, circulating concentrations of P_4 at the time of the AED system alert and disappearance of the ovulatory follicle were 0.1 and 0.1 ng/mL and 100% (8/8) of the cows had P_4 <0.5 ng/mL at both time points. Ovulation could not be confirmed in these cows because they were immediately enrolled in a synchronization of ovulation protocol on d 6. After re-calculation of performance parameters based on assignment of cows into TP and TN groups based on RTE and ovulation, Se, Sp, NPV, PPV, and balanced accuracy for detection of estrus based on the AED system were 93 (95% CI 88 to 96%), 92 (95% CI 62 to 100%), 44 (95% CI 32 to 57%), 99 (95% CI 96 to 100%), and 93% (95% CI 75 to 98%), respectively.

Effect of Cow Features and Milk Production on Estrus Expression and Ovulation

For estrus outcomes generated by the RTE, parity affected estrus expression because fewer ($P = 0.02$) multiparous (91%) than primiparous (98%) cows were detected in estrus. In addition, we observed a tendency for cows enrolled after a previous AI ($P = 0.05$; 98% vs 92% for previous AI versus no previous AI) and cows in the high BW group ($P = 0.07$; 97% vs 94% for high and low) to be more likely to be detected in estrus by the referent test. No effect ($P > 0.10$) of the other factors evaluated (BCS, lameness score, milk production, and GDPR) was observed.

For estrus outcomes generated by the AED system, no effect ($P > 0.10$) of parity or any of the factors evaluated was observed on the proportion of cows detected in estrus.

The proportion of cows with a confirmed ovulation tended ($P = 0.09$) to be greater for primiparous (98%) than multiparous (91%) cows, but was not affected by any of the other factors evaluated ($P > 0.10$).

Estrus Event Features

A summary of estrus parameters measured by the AED system including the distributions of timing, duration, and intensity of estrus alerts as well as the interval from onset of estrus to ovulation are presented in Figure 3. For reference, standing estrus as measured by the pressure-activated sensor began at 68.2 ± 1.5 h after induction of luteolysis and continued for an average duration of 6.3 ± 0.4 h with average of 10.3 ± 0.8 standing events per estrus. The mean time from first standing event to ovulation was 27.2 ± 0.9 h.

Timing from induction of luteolysis to the onset of estrus as determined by both the AED system and the standing behavior monitoring system were affected ($P < 0.0001$) by P_4 concentration group. Onset of alerts from the AED system occurred earlier for low P_4 cows (52.6 ± 3.9 h) compared with high P_4 cows (73.8 ± 1.4 h) and the onset of standing estrus also occurred earlier for cows with low (46.4 ± 4.5 h) compared with high P_4 (69.2 ± 1.4 h) at $PGF_{2\alpha}$. Similarly, ovulation occurred earlier ($P < 0.0001$) for cows in the low (74.0 ± 3.5 h) versus high (97.8 ± 1.4 h) P_4 group. There was also an effect of GDPR group ($P = 0.02$), whereby cows with high GDPR (54.5 ± 2.9 h) displayed standing behavior earlier than cows with low GDPR (61.1 ± 2.6 h). There was a significant interaction between parity and milk group ($P = 0.02$) for onset of standing estrus, whereby primiparous high producing cows (49.1 ± 3.8 h) began standing behavior earlier than primiparous cows in the low (60.9 ± 3.9 h) production group and earlier

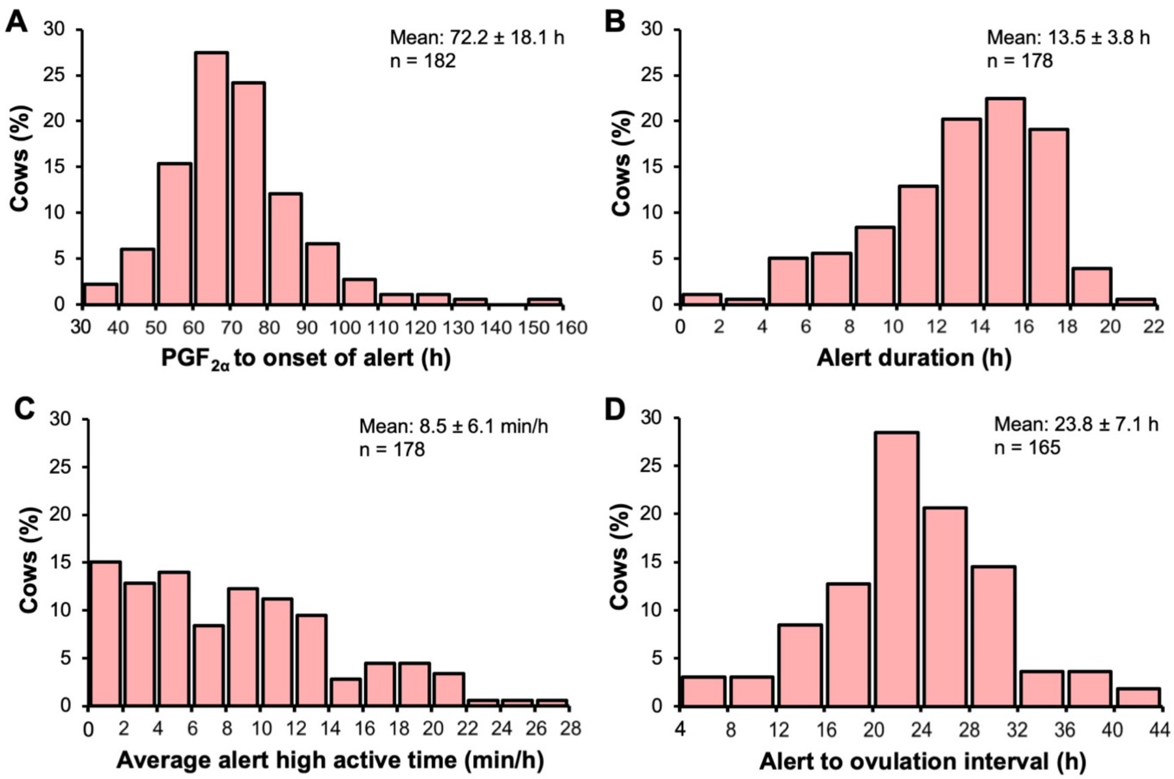

Figure 3. Frequency distributions of automated estrus detection system alert features. Means are reported as the arithmetic mean ± standard deviation.

than multiparous cows in all milk production groups (60.2 ± 2.9 and 61.2 ± 2.9 h for low and high).

Duration of estrus as measured by the AED system was affected by multiple factors. Estrus alert duration for cows in the low P_4 group (11.3 ± 0.9 h) was shorter (*P* = 0.02) than for cows in the high P_4 group (13.3 ± 0.3 h). There was a tendency (*P* = 0.08) for cows in the high milk production group (11.8 ± 0.9 h) to have shorter estrus alerts than cows in the low milk production group (12.8 ± 0.5 h). An interaction (*P* = 0.03) between parity and service number was also observed whereby primiparous cows that received a previous insemination (10.1 ± 0.9 h) had shorter estrus alerts than primiparous cows that did not receive a previous AI (12.7 ± 0.7 h) and all multiparous cows regardless of service number (13.1 ± 0.5 and 13.3 ± 0.8 h for first and second or greater service). In contrast, duration of standing estrus was only affected (*P* = 0.01) by service number because cows that received a previous insemination had shorter estrus (5.0 ± 0.7 h) based on standing behavior than cows that did not receive their first insemination (7.0 ± 0.4 h) before detection of estrus.

Estrus intensity as determined by the AED system was lesser (*P* = 0.002) for primiparous (6.5 ± 0.8 min per hr) than multiparous (9.5 ± 0.5 min per hr) cows. In addition, cows in the high milk production group (7.0 ± 0.6 min per hr) had less intense estrus events (*P* = 0.02) as measured by the AED system than cows in the low milk production group (9.0 ± 0.6 min per hr). For estrus intensity based on standing behavior, we observed an interaction (*P* = 0.01) between parity and service number because primiparous cows eligible for their first service had more recorded standing events (16.1 ± 1.6 standing events) than primiparous cows that received a previous insemination (9.6 ± 2.2 standing events) and both groups of multiparous cows (8.0 ± 1.0 and 10.2 ± 1.9 standing events) for multiparous cows without a previous AI and with a previous AI, respectively). In addition, cows with high GDPR tended ($P = 0.08$) to have more standing events (12.4 ± 1.2) than cows with low GDPR (9.5 ± 1.1).

The estrus to ovulation interval was also affected by a number of factors. Cows in the low P_4 group had a shorter estrus to ovulation interval as measured by the AED system ($P = 0.002$; 20.4 ± 1.8 vs 25.7 ± 1.1 h for low and high P_4) and standing behavior monitoring system ($P = 0.04$; 21.9 ± 2.9 vs 28.1 ± 0.9 h for low and high P_4). Cows in the high milk production group tended ($P = 0.07$) to have a shorter (22.1 ± 1.4 h) interval from onset of AED system alert to ovulation than cows in the low milk production group (24.0 ± 1.3 h), and cows with a lameness score of ≥ 3 (24.8 ± 2.0 h) tended ($P = 0.07$) to have a longer interval from onset of estrus alert to ovulation than cows with a lameness score of <3 (21.2 ± 0.9 h). We also observed an interaction ($P = 0.02$) between parity and service number such that primiparous cows eligible for their first service (21.6 ± 1.7 h) and multiparous cows eligible for their second or subsequent service (21.6 ± 1.6 h) had a shorter interval from onset of the AED system alert to ovulation than the other groups of cows (24.8 ± 2.0 and 24.1 ± 1.2h for primiparous cows with a previous AI and multiparous cows without a previous AI).

To further characterize the features of estrus events, correlations between various estrus parameters were explored. Significant correlations with an $R^2 > 0.3$ are displayed in Figure 4. We found that standing behavior usually began before the generation of an estrus alert, and the timing of the first standing event of estrus and the onset of the AED system alert were highly correlated ($P < 0.0001$, $r = 0.96$, $R^2 = 0.91$). In addition, the interval from AED system alert to ovulation was correlated ($P < 0.0001$, $r = 0.83$, $R^2 = 0.69$) with the interval from first standing event to ovulation and the duration of AED system alerts was weakly correlated ($P < 0.0001$, $r = 0.40$, $R^2 = 0.16$) with intensity of AED system alert. A tendency was found for the correlation

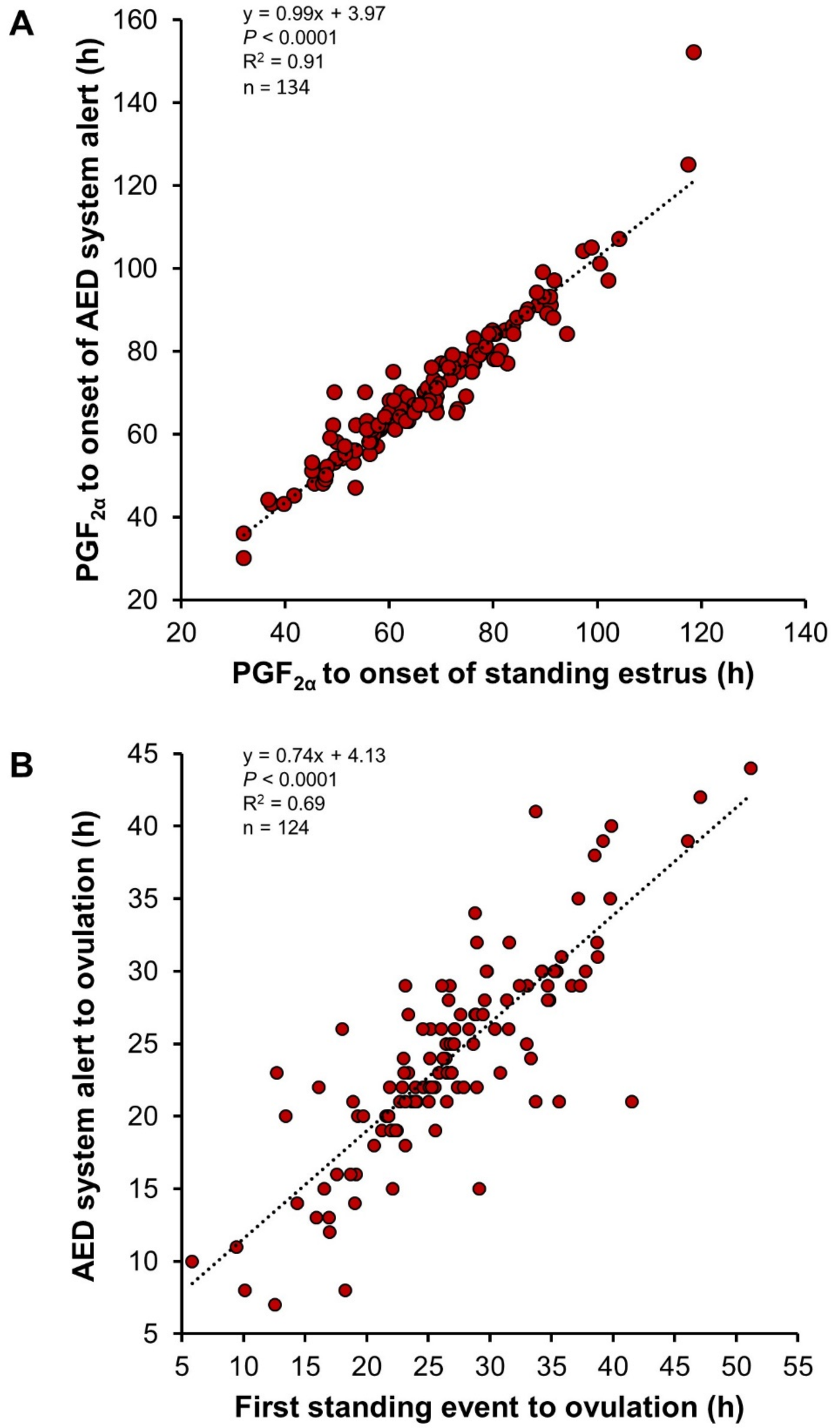

Figure 4. (A) Association between time (h) from administration of $PGF_{2\alpha}$ to onset of automated estrus detection system alert and time (h) from $PGF_{2\alpha}$ to onset of standing estrus. (B) Association between interval from onset of automated estrus detection system alert to ovulation (h) and interval from first standing event to ovulation.

between duration of AED system alerts and the interval from alert to ovulation (P = 0.05, r = 0.15, R^2 = 0.2). Conversely, no correlations were found between duration of the estrus alert and duration of standing behavior (P = 0.61, r = 0.05, R^2 = 0.00), intensity of alert and intensity of standing behavior (P = 0.99, r = 0.00, R^2 = 0.00), or alert intensity and alert to ovulation interval (P = 0.15, r= 0.11, R^2 = 0.01).

Patterns of Physical Activity and Rumination Time

Time spent on each of the three levels of PHA and RUM recorded by the AED system from 96 h before to the time of ovulation for groups of cows created based on the occurrence of estrus as determined by the AED system and ovulation are presented in Figure 5. Data for cows in the estrus and no ovulation group were not included in statistical analyses but are included in figures for descriptive purposes.

For minutes in the high active category, there was a group by time interaction (P < 0.0001) because cows detected in estrus by the AED system that subsequently ovulated had more minutes in high active from 32 to 16 h before ovulation than cows in the other two groups (Figure 5A). No group by time interaction (P = 0.22) or effect of group (P = 0.71) was observed for active time (Figure 5B). For inactive time there was an interaction between group and time (P < 0.0001), whereby minutes in the inactive category differed among groups from 56 to 48 h and 32 to 16 h before ovulation (Figure 5C). During the latter period, the estrus and ovulation group had the fewest inactive minutes, whereas the no estrus and ovulation group had intermediate levels of inactivity and the no estrus and no ovulation group had the most inactive time. Furthermore, we also observed an interaction (P < 0.0001) between group and time for RUM because cows in the estrus and ovulation group had reduced RUM from 32 to 24 h before

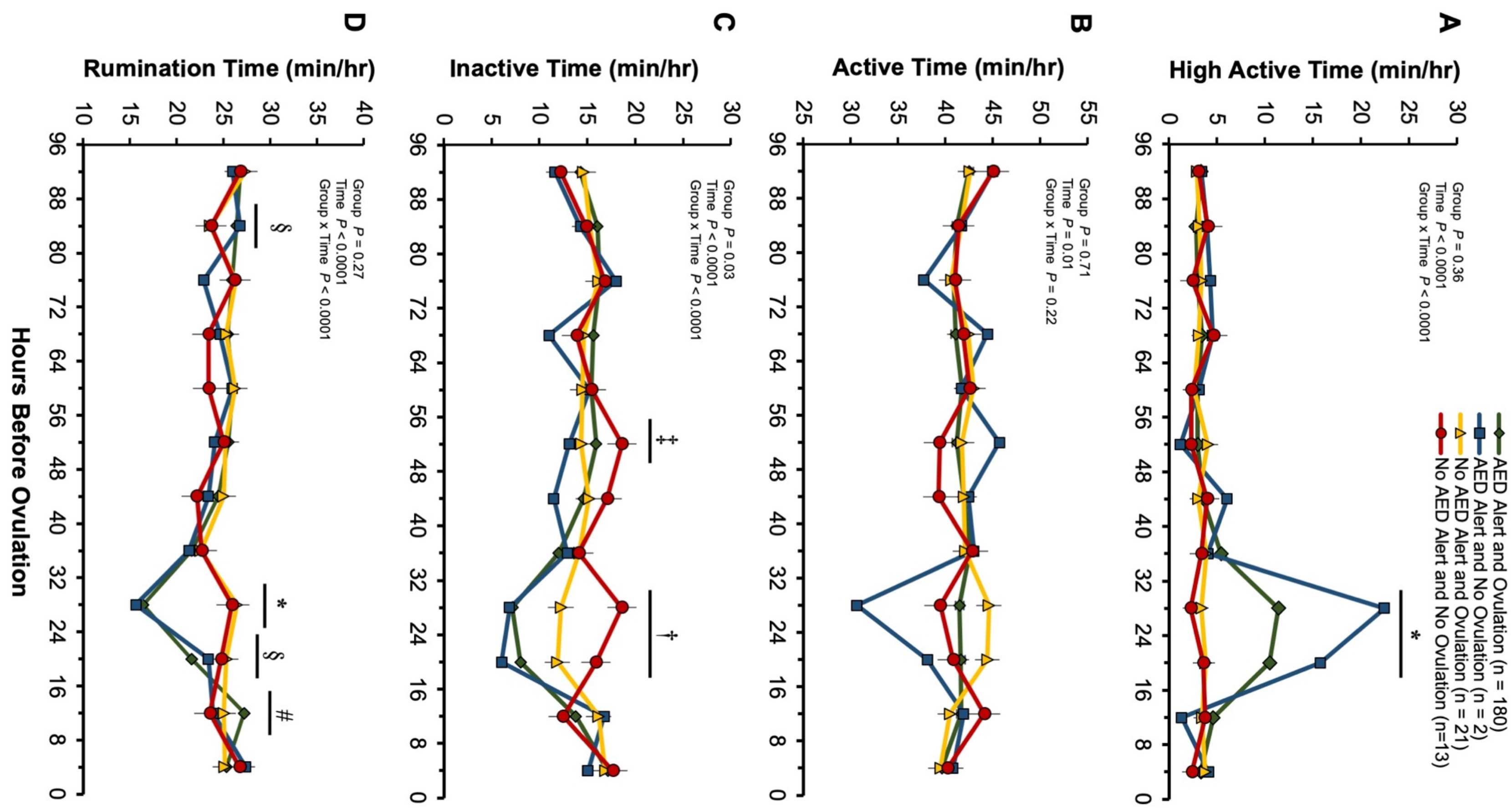

Figure 5. Comparison of time spent in each of three physical activity levels (high active, active, and inactive) and rumination time prior to ovulation time between cows detected or not detected in estrus by the automated estrus detection system and ovulating or failing to ovulate. Estrus and No Ovulation group was not included in statistical analysis and is shown here for illustrative purposes only. Raw means are displayed for high active time. For cows that failed to ovulate, the average ovulation time of 95 hours after induction of luteolysis was used. Within each time point, the following symbols are used to indicate differences for pairwise comparisons between groups ($P < 0.05$).

* Estrus and ovulation group different from all other groups

† All groups different from each other

‡ No estrus and ovulation group different from No estrus and no ovulation group

§ Estrus and ovulation group different from No estrus and ovulation group

Estrus and ovulation group different from No estrus and no ovulation group

ovulation compared with cows in the other two groups, and the no estrus and ovulation group from 24 to 16 h before ovulation (Figure 5D). Conversely, from 16 to 8 h before ovulation RUM for the estrus and ovulation group was greater than for cows in the no estrus and no ovulation group. There was no effect of parity on high active time ($P = 0.49$) or active time ($P = 0.94$); however there was a tendency ($P = 0.09$) for multiparous cows to display less inactive time on average (15.1 ± 0.4 vs. 14.4 ± 0.3 min/hr for primiparous and multiparous). Furthermore, overall RUM was lower ($P < 0.001$) in primiparous cows (24.1 ± 0.4 min/hr) as compared to multiparous cows (25.4 ± 0.3 min/hr).

To further explore reasons for some cows to be detected in estrus by the RTE but not by the AED system and vice versa, PHA and RUM patterns prior to ovulation were compared for cows detected in estrus by both the RTE and the AED system, only the RTE, only the AED system, or not detected in estrus by either method (Figure 6). From 32 to 24 h before ovulation, cows detected in estrus by both the RTE and the AED system had increased ($P < 0.05$) time in the high activity category compared with cows only detected by the RTE or not detected by either method (Figure 6A). High activity for cows detected by the RTE and AED system remained elevated from 24 to 16 h before ovulation, when it was greater ($P < 0.05$) than the other three groups. From 16 to 8 h before ovulation, time in high activity dropped for cows detected by the RTE and AED system but was greater ($P < 0.05$) than that for cows detected only by the AED system. No differences between groups of cows were detected for active time (Figure 6B). We observed a difference ($P < 0.05$) in inactive time from 88 to 80 h before ovulation for cows detected in estrus by the RTE and AED system, and cows that were not detected in estrus by either method compared with cows that were detected only by the AED system (Figure 6C). At 40 to 32 h before ovulation, cows that were detected by both methods had less ($P < 0.05$)

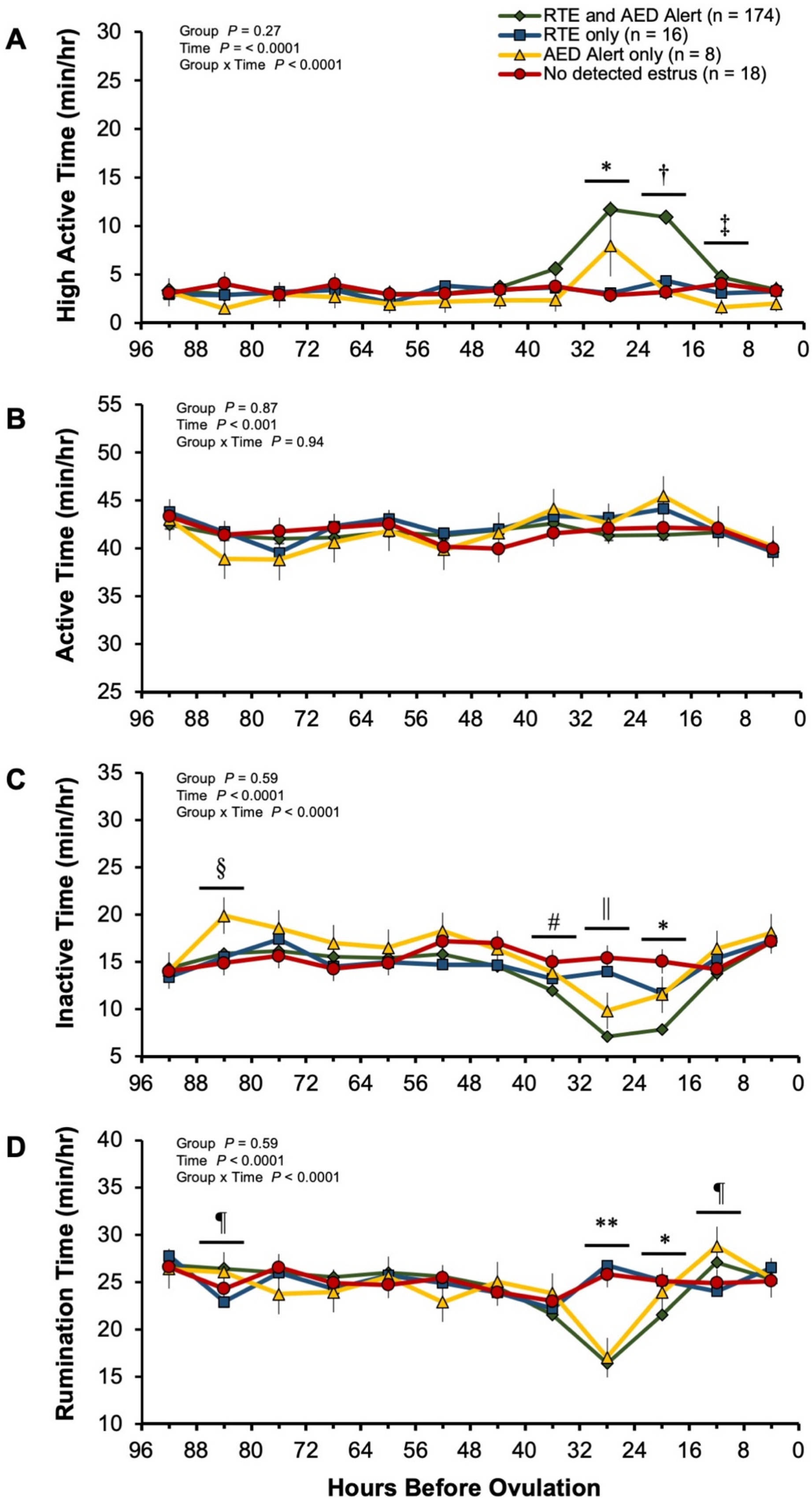

Figure 6. Comparison of time spent in each of three physical activity levels (high active, active, and inactive) and rumination time prior to ovulation between cows detected or not detected in estrus by the reference test for estrus (RTE) or the automated estrus detection (AED) system. For cows that failed to ovulate, the average ovulation time of 95 hours after induction of luteolysis was used. Within each time point, the following symbols are used to indicate differences for pairwise comparisons between groups ($P < 0.05$).

* RTE and AED system group different from RTE only and No detected estrus groups
† RTE and AED system group different from all other groups
‡ RTE and AED system group different from AED system only group
§ RTE and AED system and No detected estrus groups different from AED system group
\# RTE and AED system group different from No detected estrus group
|| RTE and AED system and AED system only groups different from No detected estrus group; RTE and AED system group different from RTE only
¶ RTE and AED system group different from RTE only group
** RTE and AED system and AED system only group different from RTE only and No detected estrus groups

inactive time compared with cows not detected in estrus. Furthermore, from 32 to 24 h before ovulation, inactive time was lower ($P < 0.05$) for cows detected by the RTE and AED system and cows detected only by the AED system compared with cows not detected in estrus, and was reduced ($P < 0.05$) for cows detected by the RTE and AED system compared with cows detected only by the RTE. Inactive time remained low ($P < 0.05$) for cows detected by the RTE and AED system compared with the RTE and cows not detected in estrus from 24 to 16 h before ovulation. Furthermore, RUM was reduced ($P < 0.05$) for cows detected in estrus by the RTE and AED system and cows detected only by the AED system from 32 to 24 h before ovulation compared with the other two groups (Figure 6D). From 24 to 16 h before ovulation, RUM remained lower ($P < 0.05$) for cows detected by the RTE and AED system than for cows detected by either the RTE only or not detected in estrus by any method. Finally, from 16 to 8 h before ovulation, RUM subsequently increased for cows detected in estrus by the RTE and AED system and was greater ($P < 0.05$) than for cows detected by only the RTE. Similar to the previous analysis, there was no effect of parity on high active time ($P = 0.41$) or active time ($P = 0.93$). Conversely, multiparous cows displayed less ($P < 0.001$) inactive time on average (15.3 ± 0.4 vs. 14.6 ± 0.3 min/hr for primiparous and multiparous) and RUM was lower ($P < 0.001$) in primiparous cows (24.0 ± 0.4 min/hr) as compared to multiparous cows (25.3 ± 0.3 min/hr).

4. DISCUSSION

In the current study, we evaluated the performance of an ear-attached accelerometer-based AED system for detection of estrus and characterized in detail estrus alerts features and the patterns of PHA and RUM as measured by the AED system to further understand the variation in these parameters around estrus and ovulation. We also investigated the effect of factors known to influence estrus and the associations between estrus features and time intervals for AED system-generated alerts.

Achieving maximal Se for detection of estrus events followed by ovulation is critical to the success of ED programs that use AED systems as the only or primary method to identify cows for insemination. Otherwise, cows that display estrus are not detected, extending the interval to first service or re-inseminations which reduces the service rate and thus, the pregnancy rate (Ferguson and Skidmore, 2013; Denis-Robichaud et al., 2016). The estimated Se and lack of difference in proportion of cows detected in estrus with ovulation between the AED system and the RTE suggested that the AED system was effective at detecting lactating Holstein cows in estrus. Compared with the RTE including VO of estrous behavior and continuous monitoring of standing behavior, only ~3% of estrus events followed by an ovulation were missed by the AED system. Estimating the implications of this difference in proportion of cows detected in estrus on herd reproductive performance was not possible in this study. Nonetheless, assuming that commercial farms have ED programs as effective as the RTE in our study (which may not always be the case), we speculate that the potential reduction in cows detected in estrus for the AED system would not be as detrimental as to preclude the use of the AED system as the primary method for detection of estrus. In addition, the potential negative implications of the lesser Se would be likely reduced for herds that submit cows for insemination using a combination of detection of estrus with the AED system and synchronization of ovulation for TAI (Giordano et al., 2012; Galvão et al., 2013; Fricke et al., 2014). Further research with more cows under commercial farm conditions is necessary to evaluate the implications on reproductive performance of conducting ED with the AED system used in the current study.

Comparing the performance of AED systems across studies is difficult because of the different types of alerts generated and parameters monitored by AED systems, different definitions for estrus events, and different REF used to detect estrus. Nevertheless, our observations for Se are in the range reported in previous studies that evaluated AED systems. For example, similar results (Se of 97%) were reported in a study using this particular AED system to detect lactating dairy cows in estrus under free-stall housing conditions (Schweinzer et al., 2019). In such study, an estrus event followed by pregnancy or estrus detected by a combination of another commercially available ear-attached sensor (CowManager Sensoor, Agis, Harmelen, Netherlands) and VO of estrus were used as REF (Schweinzer et al., 2019). Another recent study in which another commercially available ear-attached AED system (CowManager Sensoor, Agis, Harmelen, Netherlands) was evaluated, the observed Se was 89.5% when using circulating plasma P4 as an indication of ovulation as REF and 100% when standing estrus was used as REF (Mayo et al., 2019). In contrast to results for AED based on ear-attached accelerometers, the Se reported for detection of estrus in lactating dairy cows under confinement conditions for neck- (Chanvallon et al., 2014; Adriaens et al., 2019; Mayo et al., 2019) and leg-mounted sensors (Mayo et al., 2019) has varied dramatically (50 to 92%). Likely this was a reflection not only of the variation across multiple AED systems evaluated but also the fact that results were reported in a greater number of studies with more variable study conditions and differences for the REF used for detection of estrus (Chanvallon et al., 2014; Adriaens et al., 2019; Mayo et al., 2019; Schweinzer et al., 2019).

In attempt to better understand the reasons for the putative FN outcomes observed for the AED system (i.e. cows that were detected by the RTE but not by the AED system), we explored estrous behavior, ovulation, and AED system-recorded parameters in more detail. All except for two of the cows with a putative FN outcome ovulated and the two cows that failed to ovulate were detected in estrus by both methods used for the RTE. Thus, for these cows a FN was the correct outcome for the AED system. In spite of having an apparently fully functional sensor, cows with a FN outcome did not have a statistically significant or noticeable change in the pattern of PHA and RUM reported by the AED system during the peri-estrus and peri-ovulatory period. Unlike for cows that had an alert, the amount of high active and inactive time as well as the number of minutes spent ruminating per hour remained unchanged. This was surprising because all except for two cows had at least two secondary signs of estrus associated with increased PHA recorded by VO (i.e., active, mounted other cows, sniffing the vulva of other cows) and several cows, including those with no signs of estrus recorded by VO, had enough standing events recorded to be considered in estrus by the standing behavior monitoring system. Collectively, these data confirmed that the lack of an AED system alert was due to either no change or a change of insufficient magnitude for PHA and RUM around estrus and ovulation. We presume that there are some cows that, due to either biological reasons or environmental influence, show signs of estrus detectable by VO but do not increase their overall PHA levels or have reduced RUM as to be detected by the AED system. The lack of change in rumination is more difficult to explain but may be due to normal variation in response to endocrine changes associated with estrus (Lyimo et al., 2000). In agreement with our findings, Reith and Hoy (2012) reported that 6% of estrus events were associated with increased rather than decreased RUM and an additional ~20% of cows decreased their RUM around estrus by less than 10% of baseline levels. Further investigation is therefore required to determine the effect of variation in behavioral changes associated with estrus events on the ability of the AED system evaluated in this study and others to generate estrus alerts for cows that present less evident changes in behavior.

The value of an AED system for a dairy farm depends not only on the ability to detect cows in estrus (i.e., have high Se) but also avoiding an excessive number of FP alerts (i.e., have high Sp) (de Mol et al., 1997). Poor fertility of AI services due to insemination of cows not in estrus (Sturman et al., 2000; LeRoy et al., 2018) along with additional labor and cow disruption to either inseminate or confirm estrus are the main drawbacks of FP alerts. The Sp initially estimated was relatively low suggesting that the AED system generated a high proportion of FP outcomes. Nevertheless, the initial estimation did not account for possible FN outcomes generated by the RTE. As for all ED methods, both VO of behavioral signs of estrus and standing behavior monitoring have limitations that preclude identification of all estrus events followed by ovulation (Van Eerdenburg et al., 2002; Roelofs et al., 2005b; Stevenson et al., 2014). Insufficient observation frequency to identify cows with short estrus events and complete lack of or limited expression of certain behavioral signs of estrus are some of the main limitations for VO. No expression of standing behavior and fewer or shorter standing events than required to trigger an alert are major limitations of the standing behavior monitoring system (Dransfield et al., 1998; Lopez et al., 2004; Peralta et al., 2005). As the ultimate goal of all ED methods is to accurately identify cows that will ovulate rather than detect estrus alone, outcomes were re-classified as FP or TP based on the occurrence of both estrus and ovulation. When eliminating cows with inconsistent RTE and ovulation outcomes, the estimated Sp for the AED system increased by 23 percentage points and was within a more acceptable range. These data indicated than under similar conditions to those of our study, and when ovulation and estrus rather than estrus alone is the outcome of interest, a relatively small proportion of FP alerts should be expected (i.e., ~7%). The fact that all seven cows with an alert from the AED system that ovulated had either none or very few (i.e., two cows were observed mounting) estrous behaviors recorded by VO was surprising. In particular, because these cows had detectable changes in PHA before ovulation. Thus, it seems plausible that the AED system was capable of detecting changes in behavior that were not easily detected by VO because cows expressed estrus during a short period of time, had weak changes in behavior not easily detectable by VO, or both as it has been previously described for lactating dairy cows (At-Taras and Spahr, 2001; Roelofs et al., 2005b). It is also plausible that the AED system generated estrus alerts for these cows because of the substantial change in RUM. In contrast to PHA, which changed less around ovulation for cows detected only by the AED system, the change in RUM was of the same magnitude for cows detected only by the AED system and both the AED system and the RTE. Unlike for VO, the lack of standing behavior alerts in cows detected only by the AED system was not surprising because it is well known that not all cows that express secondary signs of estrus and ovulate express standing behavior (Van Vliet and Van Eerdenburg, 1996; Peralta et al., 2005; Roelofs et al., 2005b).

As the PPV and NPV are affected by the prevalence of the outcome of interest (Dohoo et al., 2014), the bias towards cows with a positive outcome in our study (88% estrus vs. 12% no estrus) favored the PPV. Thus, a cow with an estrus alert from the AED system had a high likelihood to be in estrus. Conversely, the low NPV indicated that a cow without an estrus alert had a relatively high likelihood of being in estrus reflecting the inability of the AED system to detect estrus events. Removing cows that ovulated but had a negative RTE outcome reduced the NPV even further in spite of the improvement in Sp because the number of cows with the outcome of interest (no AED system alert) was also substantially reduced. Balanced accuracy for the AED system was within a reasonable range, in particular when estimated after accounting for the occurrence of ovulation after estrus. Although not directly comparable given the different experimental conditions and differences between regular and balanced accuracy, the estimated balanced accuracy when not accounting for ovulation for the AED system evaluated in our study was greater (range of 9 to 22%) than for six other commercially available AED systems evaluated in a recent study (Mayo et al., 2019). As in our study, lactating dairy cows in the study by Mayo et al. (2019) were synchronized with a similar type of protocol and detection of estrus was conducted following a stringent estrous behavior scoring system. Taken together, the values for all performance parameters estimated for the AED system evaluated in our study suggested that under conditions similar to those of our study with lactating Holstein cows housed in free-stall barns and their estrous cycle synchronized, the AED system may be an effective method for detection of estrus. The system was successful at identifying cows that displayed estrous behavior and had a low FP rate if the occurrence of ovulation was accounted for classification of test outcomes. Detection of some cows in estrus by the AED system and not by the RTE showed that the AED system may generate estrus alerts for some cows not easily detected in estrus by VO or an automated standing behavior monitoring system. Nevertheless, such benefit may be totally or partially offset by the inability of the AED system to detect some cows in estrus that can be detected by other methods. Further investigation with more cows and at multiple farms is necessary to determine if the AED system would have the same performance when used under a wide range of commercial farm conditions.

We explored estrus alert features and the effect of multiple factors that may have affected these features to gain further insight into cow biology around estrus and ovulation, and for future consideration of these factors in the development and refinement of estrus alert algorithms. As expected, the mean and variation for time to onset of AED system alerts after induction of luteolysis with $PGF_{2\alpha}$ was within that reported in previous studies (Stevenson et al., 1999; Valenza et al., 2012) and suggested that alerts were associated with estrus rather than other non-estrus related events that may have affected PHA and RUM. A high correlation with the onset of standing behavior and the observed pattern of PHA and RUM in relationship to ovulation also indicated that the PHA clusters and reduction in RUM observed were associated with estrus. Although substantial variation was observed for duration of alerts (i.e., 1 to 21 h), on average cows had an active alert for at least half a day and a majority (66%) were longer than 12 h. From a practical perspective, a drawback of short alert duration could be that cows detected in estrus do not remain in the list for insemination for enough time. This may be of particular importance for farms that inseminate cows, and therefore check the list of cows to inseminate only once per day. This problem is avoided for the AED system evaluated in this study by maintaining cows in the software-generated alert list by a period of time set by the user. Indicators of estrus intensity as measured by AED systems may be used for decision-making as some recent evidence suggest that estrus intensity is associated with fertility of AI services (Garcia et al., 2011; Madureira et al., 2015, 2019; Burnett et al., 2018) and therapeutic strategies may be targeted to cows with different intensity of estrus (Madureira et al., 2019). As the AED system evaluated in this study lacked a parameter indicative of estrus intensity, we used as proxy the number of minutes in high active time. Interestingly, we observed substantial variation with some cows spending as little as two minutes per hour to as much as 28 min per hour in high active time. This large variation may facilitate sorting cows in groups of estrus intensity for implementation of targeted management strategies such as hormonal therapy, use of semen with different viability, modification of timing of insemination, or double inseminations. Further research with more cows should be conducted to determine if estrus intensity as measured by the AED system is actually associated with fertility of AI services or other biological outcomes of interest before this parameter is used as an aid for reproductive management decision-making. If an association between estrus intensity and fertility of AI services exist, it is unlikely to be mediated by factors related to intensity of standing behavior or timing of ovulation. In our study, the poor correlation between minutes in high active time (i.e., AED system alert intensity) and intensity of standing behavior suggested that time spent in high active was not a good indicator of the number of standing events during estrus. The poor correlation between alert intensity and alert to ovulation interval indicated that the intensity of the alert was not a good predictor of the timing of ovulation.

The interval from onset of estrus alerts to ovulation, and the association between timing of estrus alerts and the onset of standing behavior have implications for identifying an optimum timing of insemination for cows detected in estrus by AED systems. Although it may be challenging to adjust insemination time for individual cows or small subgroups of cows, a better understanding of the relationship between AED system-generated estrus alerts and timing of ovulation may be used for making adjustments to timing of AI that may improve fertility for a majority of cows. Of particular interest was the high correlation between the onset of estrus alerts from the AED system and the onset of standing estrus as measured by the rump-mounted pressure-activated sensor because of the known consistency for the interval from onset of standing behavior to ovulation (Walker et al., 1996; Stevenson et al., 2014). A consistent 4 h delay for the beginning of AED system alerts compared with the first standing event demonstrated that AED system alerts were triggered after the onset of standing estrus and most likely after the initial rise in PHA. At first this may seem counterintuitive because the onset of standing estrus usually occurs a few hours after changes in behavior associated with PHA are noticeable (Roelofs et al., 2005b; Stevenson et al., 2014). Nevertheless, this observation can be explained by the time lag between the initial rise in PHA and the time required by the AED system software to trigger an alert. Consistent with these observations was the 23.8 h interval from the onset of AED system alerts to ovulation which was shorter than the interval from first standing event to ovulation in this study, and ~5 h shorter than the interval from the onset of increased PHA to ovulation for a neck-mounted AED tag (Valenza et al., 2012). Interestingly, the timing from $PGF_{2\alpha}$ to onset of AED system alerts and standing estrus were highly correlated suggesting that the timing of an AED system alert may be predictive of the initiation of standing behavior, which is consistently associated with the timing of ovulation in dairy cows (Walker et al., 1996; Stevenson et al., 2014). Based on these findings, we estimated that insemination within 8 to 12 h of the onset of AED system alerts would have been optimal for >85% of the cows in our study. This timing of insemination would result in the presence of capacitated and viable sperm before or for as long as there is a viable oocyte present on the site of fertilization assuming that sperm capacitation requires approximately 6 h (Austin, 1974), sperm viability in the reproductive tract is reduced after ~24 h (Saacke et al., 2000), and oocyte viability is reduced after ~8 to 10 h of ovulation (Hunter, 1985). A graphical representation of these intervals in relationship to onset of an AED alert and timing of insemination is presented in Figure 7. Earlier insemination may compromise sperm viability for cows that ovulate later than 32 h of the AED system alert (9% of cows in our study). Conversely, later insemination may result in fertilization of a compromised oocyte for cows that ovulate less than 12 h after the onset of the AED system alert (5% of cows in our study). Interestingly, using the same standing behavior monitoring system than used in our study, Dransfield et al. (1998) showed that optimal pregnancy per AI was achieved when insemination occurred between 4 to 16 h after the onset of standing estrus.

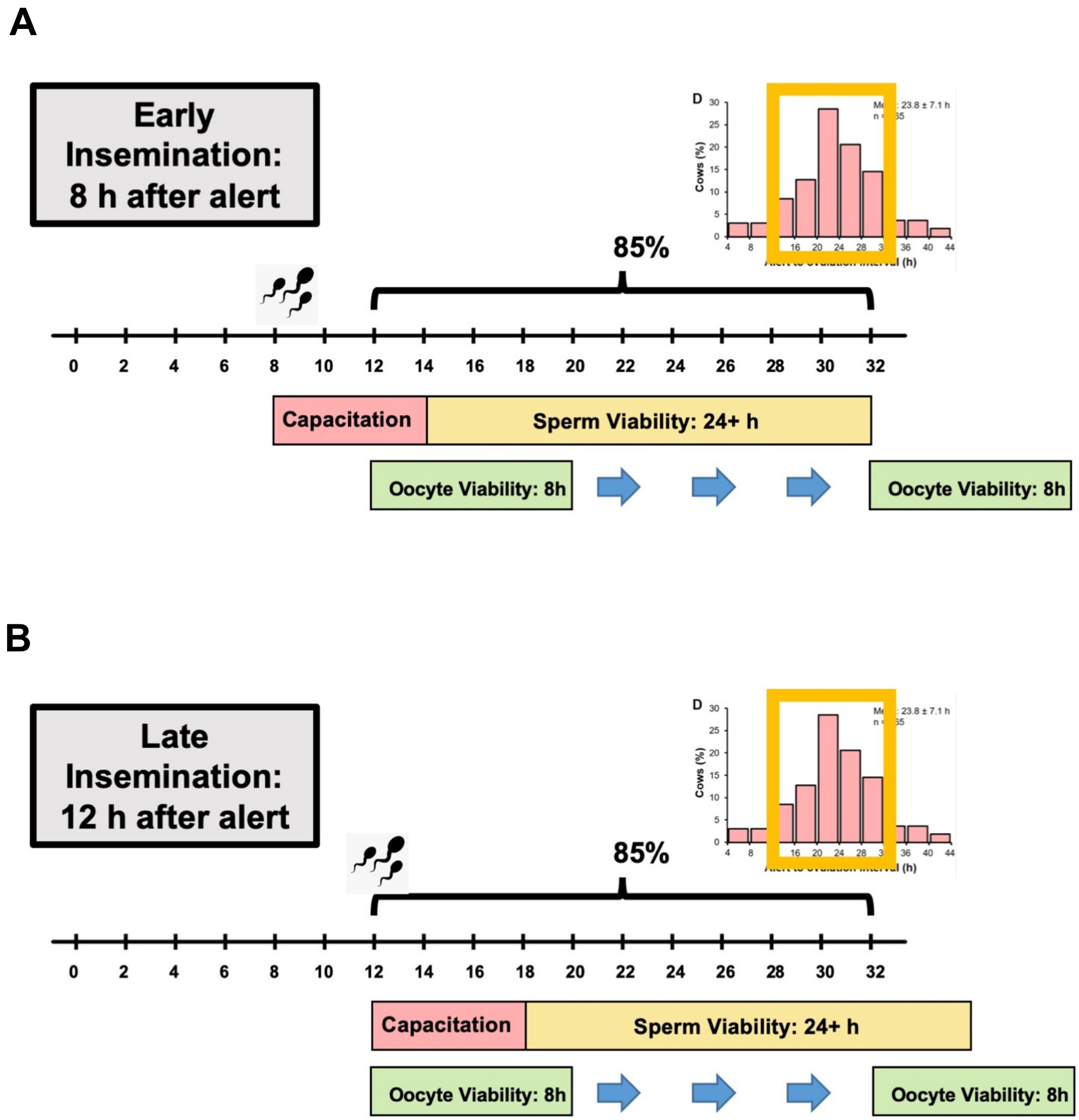

Figure 7. Graphical representation of gamete viability intervals in relationship to timing of automated estrus detection system estrus alerts and timing of insemination. Insemination can be performed as early as 8 h (A) after onset of automated estrus detection system alert or as late at 12 h (B) while maintaining overlap between oocyte viability and sperm viability for cows ovulating between 12 and 32 h after alert generation (85% of cows in the present study).

Such timing would correspond to 8 and 20 h after the onset of the alerts for the AED system evaluated in our study. A concern with the upper range of this interval would be that insemination beyond 12 h of the onset of AED system alerts may compromise fertility for several cows which may ovulate within 12 to 20 h after the onset of alerts (22% of cows in our study). Thus, additional research is necessary to determine the optimal timing of AI for cows detected in estrus by the AED system evaluated in our study.

A better understanding of the association between cow features and performance with expression of estrus, occurrence of ovulation, and estrus event features is valuable for development of predictive algorithms and for reproductive management decision-making. Both expected and unexpected results were observed and the effects of certain factors was mixed depending on the parameter evaluated. Parity was explored because of well-known associations with reproductive outcomes. For example, it is has been well documented that primiparous cows are more likely to show estrus than multiparous cows (Madureira et al., 2015; Burnett et al., 2018). In agreement, we observed an effect of parity on proportion of cows detected by the RTE but not by the AED system. Contrary to our expectations, we also observed more intense estrus alerts from the AED system for multiparous than primiparous cows. This is in contrast with a previous study which reported more intense estrus for primiparous than multiparous cows as determined by a visual observation scoring system (Roelofs et al. 2005). A plausible explanation for our observations is that certain behaviors that contribute to the estimation of PHA by the AED system may be more intensively expressed by multiparous than primiparous cows. This may be a particular feature of AED systems that use ear-attached accelerometers to monitor cow behavior. Service number was explored because cows that received a previous insemination are more likely to be cyclic and previously expressed estrus, which in turn, increases the likelihood of expressing estrus again (Thatcher and Wilcox, 1973; Chebel and Santos, 2010). The practical significance of the associations observed for estrus features as determined by the AED system remains uncertain due to lack of consistency of results and the effect of complex interactions. For example, primiparous cows that received a previous insemination had shorter duration of AED system alerts than all other cows, and the effect of service number on the interval from onset of AED system alert to ovulation was opposite for primiparous than for multiparous cows. The practical meaning of these associations is uncertain at the moment and requires further investigation if these parameters are to be considered for inclusion in predictive algorithms. In particular, because the lack of associations or the complex interactions observed in our study may be explained, at least in part, by the use of hormonal treatments to synchronize estrus.

Previous studies have demonstrated that high producing cows have shorter and less intense estrus than low producing cows (Lopez et al., 2004; Madureira et al., 2015) due, at least in part, to the effect of milk production on steroid hormone metabolism (Sangsritavong et al., 2002; Wiltbank et al., 2006). In agreement, we observed that cows in the greater milk production group had both shorter and less intense estrus events as measured by the AED system than cows in the group with lesser milk production. Cows in the greater milk production group also had a shorter interval (i.e., 2 h) from AED system alert to ovulation. A plausible explanation for the latter observation is that because high producing cows had less intense estrus events, more time was required to reach the threshold level for PHA and RUM that triggered the AED system alert. As milk yield of dairy cattle continues to increase (NAHMS, 2008) and daily milk production data becomes more readily available in commercial dairy farms, adjustments to estrus alerts algorithms based on level of milk production should be explored.

The effect of other cow features such as BW, BCS, lameness score, and genetic merit for fertility (i.e., GDPR) were either not associated with the outcomes of interest or the practical meaning of the associations observed would be likely minimal. Although it is possible that no true biological associations exist, it is also possible that our study design and sample size precluded detecting associations. Our study was underpowered to identify certain significant associations and the population of cows eligible for detection of estrus had lesser variation for the features of interest than an average population of dairy cows due the study eligibility criteria (i.e., removed cows that failed to respond to the synchronization protocol and cows with severe clinical disorders). Thus, the potential association between the cow features evaluated and estrus events should not be ruled out and investigated in a larger and more diverse population of dairy cows.

Finally, a factor that was consistently associated with several estrus features was the circulating P_4 concentration at the time of $PGF_{2\alpha}$ on d 0. The subgroup of cows assigned to the low P_4 group had circulating concentrations of P_4 indicative of the absence of a functional CL using the traditional definition of ≥1 ng/mL of P_4 most likely because of CL regression from d -7 to 0 and failure to ovulate in response to GnRH. The P_4 provided by the CIDR device prevented estrus expression prior to d 0, but once the CIDR was removed cows without a functional CL on d 0 experienced the endocrine and ovarian changes that lead to estrus and ovulation more rapidly than cows with a functional CL. Indeed, cows in the low P_4 group had larger follicles on d 2, and presented AED system alerts and ovulated nearly a full day earlier than cows in the high P_4 group. Similar to the effect of high milk production, shorter AED system alerts and a shorter interval from the beginning of the alert to ovulation were also observed for cows in the low P_4 group. In spite of these differences, the likelihood of expressing estrus was similar regardless of

P_4 concentration, thus cows in the low P_4 group remained in the study. Our observations were in agreement with previous studies for cows synchronized (Pursley et al., 1997; Stevenson and Lamb, 2016). Nevertheless, the value of this information for commercial farms that use AED systems may be limited until practical and cost-effective methods to estimate circulating P_4 concentrations become available and management strategies are developed to target cows with different P_4 levels.

5. CONCLUSION

We concluded that an automated estrus detection system that monitored physical activity and rumination behavior with an ear-attached sensor was effective at detecting cows in estrus when using a combination of visual observation of estrous behavior and continuous monitoring of standing behavior as reference test. Both physical activity and rumination time as recorded by the automated estrus detection system changed significantly around estrus and ovulation in most cows. After defining true positive and true negative outcomes based on detection of estrus by the reference test and the occurrence of ovulation, overall balanced accuracy as well as sensitivity and specificity were in excess of 90%. Differences in frequency and type of behaviors monitored by the automated estrus detection system and the reference test were the most likely reasons for lack of estrus alerts for some cows and for the generation of estrus alerts for some cows not detected in estrus by the reference test. We also concluded that cow features and milk production affected features of estrus alerts such as timing, duration, and intensity, and affected the interval from the onset of alerts to ovulation. Thus, some cow features and milk production level could be used to improve estrus alert algorithms and for the development of strategies to improve the fertility of AI services after detection of estrus with automated estrus detection systems using ear-attached sensors.

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CHAPTER III

OVERALL CONCLUSIONS AND FUTURE RESEARCH

1. OVERALL CONCLUSIONS

The purpose of the study presented in Chapter II of this thesis was to evaluate an ear-attached AED system to detect cows in estrus and characterize the patterns of activity and rumination during estrus and prior to ovulation. We were also interested in exploring some of the factors that have the potential to affect estrus expression as determined by the AED system. Taken together, this work may help elucidate new ways in which AED systems may be used to optimize reproductive performance in dairy cattle.

Our first aim was to evaluate the performance of the ear-attached AED system to detect cows in estrus. Other studies that have evaluated similar systems have shown large variation in performance metrics that are likely due to differences in study design and reference tests used to detect estrus, making it difficult to compare systems across studies. Of the limited available data, however, it seemed that ear-attached accelerometers tended to perform exceptionally well compared to leg- and neck-mounted systems, with sensitivity and specificity at or above 90%. In our study, we observed similar results. Using a reference test that combined visual observation with data from a pressure-activated standing behavior monitoring system, the AED system evaluated in this study had a sensitivity, specificity, negative predictive value, and positive predictive value of 92%, 69%, 53%, and 96% when only estrus was the outcome of interest. The low specificity and negative predictive values observed were possibly an indirect result of the study design, which was intended to maximize the percentage of enrolled cows that expressed estrus and ovulated. When a test to detect cows in estrus is evaluated using a limited number of true negative results, the specificity and negative predictive values are heavily impacted by misidentification of relatively few cows in estrus (i.e., putative false positive and false negative outcomes) by the AED system. A closer examination of the putative false positive and false negative outcomes revealed the likelihood that the reference test for estrus misidentified multiple cows as falsely being in estrus or falsely not in estrus. To address this concern, we removed cows from the dataset that had discrepancies between reference estrus and ovulation and recalculated the performance measures. The amended sensitivity, specificity, negative predictive value, and positive predictive value were 93%, 92%, 44%, and 99%, respectively.

In order to better understand why some cows were not detected by the AED system, we explored the activity patterns of cows that were detected and not detected by the AED system and ovulated or not after induction of luteolysis with $PGF_{2\alpha}$. As expected based on evidence from the literature, we observed that cows that were detected in estrus by the AED system and ovulated showed a very distinctive pattern of activity prior to ovulation, including increased time in high activity and decreased time in inactivity and ruminating compared with cows that were not detected in estrus and did not ovulate. Additionally, we observed that cows that ovulated but were not detected by the AED system did not appear to increase the amount of time they spent in high activity prior to ovulation or to decrease the amount of time spent ruminating. Active time appeared to increase slightly and inactive time appeared to decrease slightly prior to ovulation but these changes were apparently insufficient to trigger an alert.

In this study, we also measured timing of ovulation in order to suggest an optimal timing of insemination relative to the onset of AED alerts that might maximize P/AI. As an evaluation of the P/AI based on insemination time was outside of the scope of the study presented in this thesis, our preliminary conclusions are based on findings from previous studies and should be tested in a controlled trial. Based on the difference between onset of standing estrus and onset of AED system alert observed in our study (~4 hours), the interval from onset of alert to ovulation, and the optimal timing of insemination after onset of standing estrus reported in the literature (4 to 16 h after onset of standing estrus), we concluded that optimal P/AI could be achieved if insemination is performed between 8 to 12 hours after the generation of an estrus alert by the ear-attached AED system. As the mean ovulation time after alert generation observed in this study was 23.8 hours after alert generation, this insemination time would allow adequate time for sperm capacitation (~6 hours) but would not overextend the viable lifespan of sperm (~24 hours) or the ovulated oocyte (~8 to 10 hours) for a majority of cows.

Lastly, we explored numerous factors known or with the potential to impact estrus expression and/or estrus event attributes. Consistent with previous findings, we observed that high-producing cows had both shorter and less intense estrus events. Contrary to the literature, we found that multiparous cows had greater intensity of estrus than primiparous cows, and also we found that the variations in body weight, body condition score, lameness score, and genomic merit for daughter pregnancy rate on expression of estrus did not appear to be meaningfully associated with estrus expression. However, due to the nature of the study and the lack of extreme variation observed for body condition, lameness score, and genomic merit, these results may not be truly representative of the dairy cow population and further research using a larger and more diverse population of cows may be needed to appropriately evaluate these potential associations.

Overall, we believe that the data presented in Chapter II may help highlight some of the strengths and weaknesses of automated estrus detection in general and more specifically of ear-attached accelerometer-based AED systems. Our data suggested that the use of AED systems might be beneficial for detecting estrus in cows that may not show overt signs of estrus easily detectable by visual observation or other traditional methods despite increasing their overall activity levels. We observed that 3% (7/216) of cows in our study were detected in estrus by the AED system and ovulated but failed to be detected by the reference test, indicating that AED systems may be capable of detecting cows in estrus that would otherwise not be identified by most other conventional methods of estrus detection. Although some potential benefits of AED systems are evident, there may also be disadvantages to using AED systems as the only method of estrus detection. For example, sensor failure or dysfunction is possible, resulting in missed estrus events. Luckily, all of the sensors we used in this study remained functional, but all of the sensors used in this study were less than 6 months old. It is possible that if the sensors had been older, we could have encountered more challenges with sensor failure. Another disadvantage of AED systems is that cows that show less intense estrus events may be less likely to reach the threshold levels of the parameters measured to generate an alert. We observed this in the 7% (16/216) of the cows in our study that displayed a combination of primary and secondary signs of estrus but failed to be detected by the AED system. Although AED systems are unlikely to ever achieve 100% accuracy of estrus detection, we believe that future research and development of these systems may elicit methods to further improve their performance and optimize their potential as a reproductive management tool.

2. FUTURE RESEARCH

The study presented in Chapter II was somewhat limited in design such that the number of cows that truly did not express estrus was quite low, potentially contributing to the low observed specificity and negative predictive value. In order to more accurately determine these metrics, a study similar to the one presented in Chapter II could be performed but instead of including only cows that had their estrous cycles synchronized, a mix of cows with either high or low likelihood of being in estrus for an equal amount of time should be included. Synchronized, unsynchronized, and pregnant cows could be included. Collectively, the study should be aimed at improving the likelihood of having a more balanced number of positive and negative outcomes and thus a more robust calculation of specificity and negative predictive value.

The proprietary nature of the algorithms used in most AED systems creates challenges for their improvement, as the specific calculations used by the system are not disclosed and cannot be adjusted by persons outside the company that manufactures the system. Nevertheless, our findings showed that cows that failed to be detected by the AED system and either were detected by the reference test or went on to ovulate experienced a moderate decline in inactive time prior to the time of ovulation or expected ovulation. This suggests that detection of the characteristic increase in high active and/or active time for the system evaluated in this study may not be the only or the best method to detect estrus. Future research could further explore the patterns of decreased inactivity during estrus and leading up to ovulation and how that information could best be incorporated as part of an estrus alert generating algorithm. This could be particularly beneficial to aid in detection of cows that do not reach high enough levels of activity to generate an estrus alert.

Future research could also explore the potential value of adjusting algorithms based on cow-specific factors such as milk production data or parity. Consistent with previous findings, our data showed that high milk production resulted in a substantial decrease in both duration and intensity of estrus as measured by the ear-attached AED system. Thus, there is a possibility that high-producing cows in estrus may be less likely to reach the threshold levels of activity required to generate an estrus alert or they may be unable to maintain those levels of activity for long enough to generate an alert. Therefore, future studies could explore the possibility of integrating milk production data into estrus alert generating algorithms and either increasing the alert threshold for low producing cows or decreasing the alert threshold for low producing cows. In contrast to previous studies, our data also show that multiparous cows had more intense estrus events than primiparous cows, possibly due to the location at which activity data is collected (the ear). Further research could focus on validating this novel finding using the sensor from the present study and subsequently assessing various alert thresholds for primiparous and multiparous cows separately.

Lastly, one of the primary reasons for measuring timing of ovulation in our study was to suggest an optimal timing of insemination relative to the onset of AED alerts that might maximize P/AI. Based on the time intervals from estrus alerts to ovulation observed in this study and the optimal timing of insemination reported in the literature, we hypothesized that optimal P/AI would be achieved if insemination is performed between 8 to 12 hours after the generation of an estrus alert by the ear-attached AED system. Therefore, a future study could test the effect of insemination within windows of time after alert generation to either validate or refute our suggestion for timing of insemination after alert generation.