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Optimization of reproductive management of lactating dairy cows on farms using automated activity monitoring systems for estrus detection or protocols for timed artificial insemination

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ABBREVIATIONS

AAM	automated activity monitoring system	MLP	Milchleistungsprüfung
AI	artificial insemination	MUN	milk urea nitrogen
am	morning	n	number of samples
BCS	body condition score	NAHMS	National Animal Health Monitoring
BE	Besamungserfolg	ng	nanogram(s)
CI	confidence interval	no	number
CL	corpus luteum	NRC	national research council
°C	Celsius	OE	onset of estrus
d	day(s)	OR	odds ratio
DE; DU	duration of estrus	*P*	probability
d.h.	das heißt	P4	progesterone
DHIA	dairy herd improvement association	PA; PAE	peak activity of estrus, estrous intensity
DIM	days in milk	P/AI	pregnancy per artificial insemination
E2	estradiol	PGF	prostaglandin $F_{2\alpha}$
ECM	energy-corrected milk yield	pm	afternoon
EE	end of estrus	Q	quartile(s)
e.g.	exempli gratia	r	Pearson correlation coefficient
et al.	et alii/aliae/alia	s	second(s)
G1	first GnRH treatment	SCC	somatic cell count
G2	second GnRH treatment	SD	standard deviation
GDPG	Ovsynch with a double PGF dose d 7	SE	standard error of the estimate
GnRH	gonadotropin-releasing hormone	SEM	standard error of the mean
GPPG	Ovsynch with a second PGF dose d 8	TAI	timed artificial insemination
h	hour(s)	THI	temperature-humidity-index
ID	identification	TkB	terminorientierte künstliche Besamung
i.e.	id est	TMR	total mixed ration
i.m.	intramuscular	v.a.	vor allem
kB	künstliche Besamung	vs.	versus
kg	kilogram(s)	VWP	voluntary waiting period
LH	luteinizing hormone	wk	week(s)
m	meter(s)	µg	microgram(s)
mg	milligram(s)		
mL	milliliter		

1 INTRODUCTION

Reproductive performance of lactating dairy cows is of major importance to the profitability of dairy farms (Overton and Cabrera 2017). A strong association exists between timing of pregnancy during lactation and calving intervals, efficiency of milk production, and herd replacement dynamics (DeVries 2006; Giordano et al. 2012). Therefore, dairy farms implement intensive and complex reproductive programs to optimize insemination rate and overall fertility. Effective estrus detection programs as well as maximizing conception rates are essential contributors to achieve high reproductive performance.

Visual detection of estrus has been considered the gold standard management practice to facilitate artificial insemination (**AI**). Traditionally, this was based on monitoring cows for visual signs of estrus, primarily such as “standing to be mounted”. Estrus detection in dairy cows, however, not only requires time and expertise, but also became a great challenge in the dairy industry due to structural changes, such as more cows per employee, confined housing with footing surfaces less conducive to mounting behavior (Britt et al. 1986; Stevenson 2001), or increasing milk production (López-Gatius et al. 2005; Rivera et al. 2010). This raised concerns that mounting behavior may no longer be considered the ideal gold standard for estrus detection. Estrus detection rates in a recent survey of Canadian herds (Denis-Robichaud et al. 2016) were found to be below 50%. To reduce labor and improve estrus-detection efficiency, strategies were developed and have been implemented by the dairy industry to detect estrus in absence or in addition of visual signs of estrous behavior.

Automated activity monitoring (**AAM**) systems represent one strategy to improve efficiency and accuracy of estrus detection by providing continuous surveillance of behavioral and physiological parameters of dairy cows. Automated activity monitoring systems use the increase in activity to identify estrus events and precise timing of AI. Similar estrus-associated increases in number of steps (2 to 4 times) have been reported for a long time (Kiddy 1977; Redden et al. 1993; Roelofs et al. 2005). Pedometers attached to the cow’s leg and activity monitors available as neck-mounted or ear-attached devices were developed. These systems take advantage of the change in behavior to identify cows in estrus automatically. Whereas pedometers record changes in the number of steps, most of the modern AAM systems incorporate 3-dimensional accelerometers measuring accurate changes in physical activity in real time (Valenza et al. 2012; Schilkowsky et al. 2021).

Correct identification of true estrus events is crucial for AAM systems to achieve high performance. Aungier et al. (2015) reported 90% sensitivity but 17% false positive outcomes when comparing a neck-mounted AAM system (Heatime, SCR Engineers Ltd., Netanya,

Israel) with visual observation of estrous behavior. Similarly, another study (Schilkowsky et al. 2021) evaluating a modern ear-attached AAM system (Smartbow, Zoetis Inc., Parsippany, New Jersey, USA) reported a sensitivity of 93%. In fact, adoption rate of AAM systems on commercial dairy farms in recent years have increased. It was reported that by 2010 the bestselling system in the world was sold with approximately 1 million AAM tags (Stevenson and Britt 2017). In a survey conducted among German farm managers (Michaelis et al. 2013), the majority of farmers (94.1%) was satisfied after installation of the Heatime system and declared they would install the system again (94.5%).

Despite the increase in estrus-detection rates by using AAM systems there will be cows not detected in estrus and consequently not inseminated due to technical difficulties of the system (e.g., device failures, algorithm) or physiological limitations of cows to display estrous behavior (Stangaferro and Giordano 2019). Two recent studies evaluating AAM systems reported that 35 to more than 50% of cows not detected in estrus by the AAM system ovulated without showing signs of estrus, thus underwent silent ovulations (Valenza et al. 2012; Sauls et al. 2017). A further limitation of AAM systems is the management of the high prevalence of anovular cows near the end of the voluntary waiting period (**VWP**). Overall, the prevalence of anovular cows was 23.3% (ranging from 7.3% to 41.7%) at 50 to 65 days in milk (**DIM**) within eight US herds including approximately 6,000 cows (Bamber et al. 2009). This is a substantial proportion and demonstrates the great challenge for AAM systems regarding detection of estrus, as anovular cows do not show primary or secondary signs of estrus.

A second strategy to overcome the inefficiency of estrus detection is the use of protocols for synchronization of ovulation to facilitate timed AI (**TAI**). Hormone-based TAI breeding protocols have been incorporated widely into the reproductive management by dairy farmers since the late 1990s (Caraviello et al. 2006; NAHMS 2007). Such protocols aim to control first and subsequent AI services, but especially to identify nonpregnant cows (NAHMS 2007). Among several TAI protocols, the 7-d Ovsynch protocol is the one most commonly used (Pursley et al. 1995). Within the last years, a more advanced understanding of the physiology underlying the Ovsynch protocol has allowed to increase fertility to TAI. Certain requirements should be met to maximize its success: ovulation of a dominant follicle in response to the first GnRH treatment (**G1**), high progesterone (**P4**) concentration during the subsequent follicular development, complete regression of the corpus luteum (**CL**) at the time of the second GnRH treatment (**G2**), and timely ovulation after G2. It has been shown that cows that are properly synchronized achieve greater pregnancy per AI (**P/AI**) compared with cows that show spontaneous estrus (Santos et al. 2017). Therefore, modern TAI protocols (e.g. Double-Ovsynch) are considered “fertility programs” because they can achieve excellent reproductive performance if they are implemented correctly (Carvalho et al. 2018).

Initiating a new follicular wave by inducing ovulation of a dominant follicle to G1 was positively associated with P/AI (Bisinotto and Santos 2011). In the absence of a presynchronization protocol, however, ovulatory response to G1 occurs in only 45 to 50% of dairy cows (Pursley et al. 1995; Moreira et al. 2001). Ovulatory response is maximized if G1 is administered on d 5 to d 8 of the estrous cycle (Vasconcelos et al. 1999). Furthermore, increased P4 concentration at the initiation of the protocol was described to have a positive impact on the growth of the dominant follicle and on luteal regression, thereby improving fertility (Bisinotto and Santos 2011; Pursley and Martins 2011; Wiltbank et al. 2011; Denicol et al. 2012; Giordano et al. 2013). Another critical factor associated with P/AI is P4 concentration at G2. In a 7-d Ovsynch protocol, incomplete luteal regression has been observed in 12 to 21% of cows (Brusveen et al. 2009; Carvalho et al. 2015; Wiltbank et al. 2015). A marginal increase of P4 at G2 is associated with a strong negative impact on P/AI (Carvalho et al. 2018). The exact reason is unknown but gamete transport and endometrial thickness might be compromised in cows with incomplete luteal regression (Bennett et al. 1988; Souza et al. 2011). Elevated P4 concentration at the time of G2 are caused by the inability of a single prostaglandin $F_{2\alpha}$ (**PGF**) treatment to fully regress all luteal structures depending on the number and age of the CL (Stevenson 2016a). Modifications of the PGF treatment during an Ovsynch protocol, however, such as increased doses (Giordano et al. 2013; Barletta et al. 2018) or frequency of PGF treatments (Brusveen et al. 2009; Ribeiro et al. 2012; Wiltbank et al. 2015) have been developed. It has been shown that these strategies enhanced luteal regression and reduced P4 concentration at G2, leading to increased P/AI compared with a single standard dose of PGF in some of these studies (Ribeiro et al. 2012; Wiltbank et al. 2015). Another requirement is the timely ovulation after G2, which is not feasible in all of the cows (Pursley et al. 1995). Only an average of 85% of dairy cows were observed with synchronized ovulation after G2 (Santos et al. 2010). It has been shown that treatment of G2 at approximately 56 h after PGF and insemination occurring 12 to 16 h later would be optimal for pregnancy risk (Stevenson 2016b).

It is obvious that lack of complete and correct synchronization is a major factor involved in the success of TAI protocols. In this context, compliance of the farm personnel (i.e., administration of the correct dose and product at the correct time to the right cow) is another critical factor (Galvão et al. 2013). Furthermore, results from a survey of consumers in Germany indicated that most people (65%) generally perceived advanced reproductive technologies negatively despite lacking of basic knowledge about milk production (Pieper et al. 2016). Consumers would likely perceive hormone treatments to increase fertility to be unnatural and unwelcome, although negative pharmacological effects on animal or human health have not yet been described (Boogaard et al. 2011; Pieper et al. 2016).

Both strategies, the use of AAM systems as well as TAI protocols, implemented by the dairy industry to overcome some constraints in visual estrus detection are promising tools but need future research to further optimize reproductive performance.

For example, identification of the optimum timing of AI using AAM systems is challenging and there are only few studies available addressing this research question. Although similar intervals were observed between onset of increased activity and ovulation in studies evaluating AAM there was also a high variability among cows leading to potential errors in the timing of AI relative to ovulation (Roelofs et al. 2006; Valenza et al. 2012; Stevenson et al. 2014; Burnett et al. 2018). In addition, AAM systems do not only allow identification of a cow in estrus, but are also capable of evaluating specific estrous expression characteristics, which were reported to be strongly associated with the risk of pregnancy in lactating dairy cows (Madureira et al. 2015; Polsky et al. 2017; Madureira et al. 2019). This observation supports the assumption that AAM systems have more potential than simply detecting cows in estrus. Therefore, activity data might be useful in 3 different ways in herd management. They can be used 1) as a descriptive measure (i.e., how many cows are anestrous? Is there a change in the proportion of anestrous cows?), 2) as a predictive tool (i.e., based on the activity data at estrus, what is the chance of that cow to get pregnant?), and 3) in a prescriptive way, such that there are different reproductive management strategies for anestrous cows (e.g., enrolment in a TAI protocol such as a Double-Ovsynch protocol) and cows that showed estrous expression in early lactation (i.e., rely on estrus detection after VWP). Therefore, I wanted to evaluate a state of the art AAM system regarding its optimum timing of AI after detection of estrus as well as factors associated with estrous expression and fertility.

Future research on TAI protocols, on the other hand, should focus on optimization of the hormonal environment and ovarian dynamics during the protocol to achieve optimum P4 concentration at each treatment point of the protocol to optimize synchronization of cows, thereby increasing fertility. In this context, the two most successful strategies employed to TAI protocols are presynchronization before Ovsynch (e.g., Presynch-Ovsynch; G6G, Double-Ovsynch) in order to optimize P4 during follicular development by increasing the ovulatory response to G1 and/or presence of a CL at G1, and increasing the proportion of cows with complete luteal regression at the time of G2 (e.g., by adding an increased or a second dose of PGF). Therefore, I wanted to evaluate the effect of a dose frequency modification of the PGF treatment during an Ovsynch protocol. Results from this thesis can be used by farmers to make better use of AAM data as well as to provide a strategy to improve fertility using TAI protocols.

The overall objectives of this thesis were 1) to determine the association between the timing of AI and different characteristics of an estrus event (i.e., onset, peak, and end) using

an AAM system on P/AI in lactating Holstein cows inseminated with either fresh or frozen semen, 2) to identify plausible factors associated with estrous expression and subsequent P/AI in lactating Holstein cows using an AAM system, and 3) to evaluate two modifications (i.e., double dose, second administration 24 h apart) of the PGF treatment using cloprostenol in a 7-d Ovsynch protocol compared with a standard single PGF dose on P4 concentration at the time of G2 and P/AI in lactating Holstein cows.

Publication I and II were published in the Journal of Dairy Science (Impact Factor: 3.333). Publication III was published in Theriogenology (Impact Factor: 2.094).

2 PUBLICATION I

Timing of artificial insemination using fresh or frozen semen after automated activity monitoring of estrus in lactating dairy cows.

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2.1 Abstract

The objective of this observational experiment was to determine the association between the time of artificial insemination (AI) and pregnancy per AI (P/AI) in lactating Holstein cows inseminated either with fresh or frozen semen considering different characteristics of an estrus event (i.e., onset, peak, and end) using an automated activity monitoring system (AAM). A total of 3,607 AI services based on the alert of an AAM (Heatime; SCR Engineers Ltd., Netanya, Israel) were evaluated from 4 commercial dairy farms in Germany. Pregnancy diagnosis was performed by transrectal palpation 38 ± 3 d after AI or by transrectal ultrasonography 30 ± 3 d after AI. Estrus intensity was categorized based on peak activity of estrus (PAE) into low (35-89 index value), and high (90-100 index value) intensity. The mean (± standard deviation) duration of an estrus event (DE) was 14.3 ± 4.6 h. The mean (± standard deviation) interval from onset of estrus (OE; moment where index value was ≥35) to AI was 16.8 ± 8.0 h, from PAE to AI 11.9 ± 8.1 h and from end of estrus (EE; moment the index value returned to <35) to AI 2.5 ± 8.7 h. Primiparous cows had greater P/AI than multiparous, whereas first AI postpartum yielded greater P/AI compared with subsequent AI services. Type of semen was not associated with P/AI. Cows with heat stress 1 wk before AI had decreased P/AI. Cows with low estrus intensity (26.0%) were less fertile compared with cows showing high estrus intensity (32.8%). Cows with intermediate 100-d milk yield had decreased P/AI compared with cows with either low or high 100-d milk yield. There was a quadratic effect of the interval from OE to AI on P/AI. At 38 d after AI, P/AI was greatest for cows inseminated from 7 to 24 h after OE, within 18 h after PAE, or from 5 h before EE to 12 h after EE. There was no interaction between the interval from OE to AI and type of semen. There tended to be an interaction between the intervals from PAE to AI and type of semen and from EE to AI and type of semen. Cows inseminated with fresh semen within 5 h before EE had greater P/AI compared with frozen semen, whereas cows inseminated with frozen semen from 13 to 18 h after EE had greater P/AI compared with fresh semen. In conclusion, inseminating cows from 7 to 24 h after OE or 1 to 18 h after PAE yielded greatest P/AI irrespective of type of semen. In addition, high estrus intensity was positively associated with P/AI.

2.2 Key words

insemination time, activity monitor, pregnancy, cow

2.3 Introduction

Estrus detection in confined dairy cows is a great challenge as milk production increased (López-Gatius et al., 2005; Rivera et al., 2010) and cows are less likely to express estrous behavior on concrete surfaces (Britt et al., 1986). Estrus detection rate in a recent survey of Canadian herds was below 50% (Denis-Robichaud et al., 2016). The failure to submit cows for AI not only has a major impact on reproductive performance but also indicates unique potential to improve profitability (Overton and Cabrera, 2017). To improve efficiency and accuracy of estrus detection, automated activity monitoring systems (**AAM**) have been implemented on many farms. A new generation of AAM has been developed using accelerometers capable of detecting motion in all 3 spatial planes in real time. These systems continuously monitor physical activity in order to predict adequate timing of AI (Fricke et al., 2014). The time of ovulation relative to signs of estrus was similar among studies using AAM (Valenza et al., 2012: 28.7 ± 8.1 h; Stevenson et al., 2014: 25.7 ± 0.4 h; Burnett et al., 2018: 25.8 ± 10.2 h) but highly variable among cows leading to potential errors in the timing of AI in relation to ovulation (Roelofs et al., 2006; Valenza et al., 2012).

Precise identification of the optimum timing of AI relative to activity data from AAM, therefore, is challenging. There are only few studies available with this objective. In a large US field study, Stevenson et al. (2014) observed that the optimum interval from onset of estrus (**OE**) to AI differed between primiparous cows (13 to 16 h) and multiparous cows (0 to 12 h). In a field study from Canada (LeRoy et al., 2018), there was no difference in pregnancy per AI (**P/AI**) when multiparous cows were inseminated 0 to 24 h after onset of estrus. For primiparous cows, the odds of pregnancy were greatest if AI occurred 0 to 8 h after the activity alert.

While all of these studies used frozen semen for AI, a similar study evaluating optimum timing for AI with fresh semen after spontaneous estrus is missing. Results from a recent study (Borchardt et al., 2018) indicated that the optimum timing of AI might be different for fresh semen compared with frozen semen using 2 different timed AI (**TAI**) protocols. The authors speculated that viability of sperm in the female reproductive tract might be extended using fresh semen in a TAI protocol with a suboptimum timing of AI relative to ovulation (Cosynch-56). In fact, P/AI was greater for cows inseminated with fresh semen compared with frozen semen in a Cosynch protocol (27.5% vs. 20.0%, respectively, $P = 0.03$). Using an Ovsynch protocol with a more consistent timing of AI relative to ovulation, P/AI did not differ between fresh (32.2%) and frozen (28.6%) semen ($P = 0.33$).

Therefore, the objective of this study was to determine the association between the time of AI on P/AI in lactating Holstein cows inseminated either with fresh or frozen semen

considering different characteristics of an estrus event (i.e., onset, peak, and end) using an AAM. We tested whether cows inseminated closer to the onset of an estrus event would achieve greater P/AI with fresh semen compared with frozen semen. Furthermore, we tested whether P/AI would differ between fresh and frozen semen for cows inseminated closer to the end of an estrus event.

2.4 Materials and Methods

2.4.1 Study Design

This study was an observational cohort study including 6,603 AI services from 2,575 lactating Holstein cows (1,049 primiparous and 1,526 multiparous cows) from 4 commercial dairy farms in northeast Germany from July 2018 until May 2019. Inclusion criteria for herds were a herd size above 400 cows and the use of a neck-mounted AAM (Heatime; SCR Engineers Ltd., Netanya, Israel). Herd size ranged from approximately 430 to 1,200 cows per farm (Table 1). All cows were housed in freestall barns and milked twice daily. Milk yield ranged from 9,461 to 9,939 kg/305 d. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Freie Universität Berlin.

2.4.2 AAM

All cows were fitted with a neck-mounted AAM on the day of their first calving (herds A, B, D) or at approximately 40 DIM (herd C). The AAM was attached to the cows continuously (herds A, B, D) or was removed at 65 d after AI if a cow was diagnosed pregnant (herd C). Individual activity of each cow was recorded every 2 h by a wireless receiver box and transmitted to the accelerometer software (DataFlow II, SCR Engineers Ltd.) installed on the farm computer. The activity raw data from each cow was converted into an activity change index value using a proprietary algorithm (Figure 1). Index values for activity change ranged from 0 to 100 (0 = lowest [baseline], 100 = highest). Onset of estrus was defined as a cow exceeding an activity change index value of 35 (Madureira et al., 2015). End of estrus (**EE**) was defined by the first instance at which the index value fell below 35 again. The intensity of an estrus event (peak activity of estrus; **PAE**) was represented by the peak of the activity change index value during an estrus event. Estrus intensity was classified into 2 categories, low (35-89 index value) and high (90-100 index value) intensity. Duration of an estrus event (**DE**) was defined as the interval from onset to end of an estrus event. All characteristics were determined for each estrus event and plausible intervals from OE to AI (0 to 48 h), PAE to AI (-6 to 48 h) and EE to AI (-12 to 48 h) were calculated for each cow.

Files from the SCR system were exported using DataFlow II on a weekly basis for all cows that were inseminated within the last 7 d. These files were generated in the XLSX format (Microsoft Office 2013, Microsoft Corp., Redmond, WA). Each file contained the following information: cow ID, lactation number, last breeding date, AI number, DIM, days from last breeding, and last calving date. In addition, raw activity data, daily activity, and activity change were recorded for each cow within the past 7 d every 2 h. A software tool written in the open-source Python programming language (Python Software Foundation, Wilmington, DE) was used to process the AAM data from all XLSX files and generated a single result report XSLX file (J. L. Plenio, A. Bartel, Institute for Veterinary Epidemiology and Biostatistics, Freie Universitaet Berlin, Berlin, Germany; A. M. L. Madureira, R. L. A. Cerri, Applied Animal Biology, Faculty of Land and Food Systems, University of British Columbia, Vancouver, BC, Canada; W. Heuwieser, S. Borchardt, Clinic for Animal Reproduction, Freie Universitaet Berlin, Berlin, Germany; unpublished data). The report formatted in the wide (i.e., 1 line for each cow) and long (i.e., 1 line for each estrus event) format included the following information for each cow: cow ID, lactation number, last breeding date, AI number, DIM at AI, raw activity data (peak value and hour of raw activity peak), activity change (index value and hour for activity change start, peak, and end), and DE for each estrus event.

2.4.3 Reproductive Management

Lactating Holstein cows were inseminated based on the alert of the AAM, after visual estrus detection, or as part of a TAI program. Inclusion criteria for cows were: past the voluntary waiting period (Table 1) and eligible for breeding. Exclusion criteria after enrollment in the study were as follows: hormonal treatments before AI (n = 1,900) such as PGF (n = 268), GnRH (n = 978) or an Ovsynch protocol (n = 654); the use of sex-sorted semen (n = 9); and culling before a pregnancy diagnosis (n = 165). A list of cows eligible for breeding based on an activity alert was generated by the AAM. All farms had their threshold for an activity alert set at an index of 35. Based on these lists, estrus was verified by the AI technician via transrectal palpation of a highly contractile uterus and/or visualization of clear, stringy vaginal discharge. Eligible cows were inseminated at different intervals related to the AAM alert. In herds A and C, lists of cows eligible for breeding were printed at 0700 h and cows were inseminated once daily (herd A ~ at 1400 h, herd C ~ at 0800 h). In herds B and D, lists of cows eligible for breeding were printed twice daily (0300 and 1300 h). Cows in these herds were inseminated twice daily (in the early morning or in the afternoon). Cows were inseminated either with fresh or frozen semen based on farm-individual strategies. Cows were not randomly allocated to receive either fresh or frozen semen. Fresh semen was predominantly allocated to cows receiving TAI and therefore only a limited amount of straws was delivered to farms once a

week. Therefore, the use of fresh semen for cows inseminated based of the AAM alert in the study was dependent on the amount of straws left over from TAI on that specific day. Cows remained in the study until a confirmed pregnancy diagnosis (Table 1), which was performed on a weekly basis by transrectal palpation 38 ± 3 d after AI (herds A and C), by transrectal ultrasound beginning at 28 ± 3 d after AI (herd B) or at 32 ± 3 d after AI (herd D). For simplicity, the time at which pregnancy diagnosis was conducted will be referred to as 38 d after AI throughout this paper. In case of transrectal palpation, pregnancy was based on a verified pregnancy diagnosis defined by the presence of uterine fluid, asymmetry, and a positive fetal membrane slip. Non-pregnancy was based on absence of pregnancy at the day of examination or a rebreeding to an estrus before pregnancy diagnosis (Wiltbank et al., 2015). Positive pregnancy diagnosis performed by ultrasound was based on visualization of an embryo with a heartbeat. Cows diagnosed not pregnant were reassigned to breeding after spontaneous estrus or TAI.

Semen for this study was predominantly produced by genomically selected Holstein-Friesian bulls from two local AI centers (RinderAllianz GmbH, Woldegk, Germany and Rinderproduktion Berlin-Brandenburg GmbH, Groß Kreutz, Germany). Sires were not randomly allocated to the farms due to farm-specific breeding objectives. Ejaculates for producing fresh and frozen semen were collected using an artificial vagina per standard operating procedure twice a week (Monday and Wednesday) between 0600 and 0800 h. Ejaculates meeting the required quality standards (i.e., >70% progressive motility; >1.5 mL volume; >500 x 10^6 spermatozoa) were used for semen production. All ejaculates failing to meet these criteria were excluded from the study. The amount of fresh semen produced from each ejaculate was based on the requested number of straws for that day. The remaining semen was used for the production of frozen semen. Fresh semen was diluted using Caprogen liquid semen extender (Vishwanath and Shannon, 2000) to 10 x 10^6 spermatozoa/straw. After sealing, the 0.25-mL straws of fresh semen were dispatched to the farm technicians in the evening of the day of collection. Fresh semen was transported and stored in thermal insulated canisters with a temperature of 10 to 15°C until insemination, which happened within 3 d after delivery. Frozen semen was diluted using OptiXcel liquid semen extender (IMV Technologies, Normandy, France) to 20 x 10^6 spermatozoa/straw. Frozen semen was stored in 0.25-mL straws within a canister filled with liquid nitrogen at -196°C and allocated to the farms as required. For thawing, straws were placed in a water bath at 37°C for 15 s.

2.4.4 Data Collection and Statistical Analyses

Cow ID, parity, DIM, breeding date and time, number of AI, milk yield for the first 100 DIM, AI code (insemination according to AAM alert or hormone treatment) and type of semen

used for AI were obtained through the on-farm computer software (herdeW and herdeplus respectively; dsp agrosoft GmbH, Ketzin/Havel, Germany). If information about breeding time was not captured in the herd management software, it was documented on a list that was obtained on a monthly basis. All data were transferred to Microsoft Excel (Microsoft Corp.). The ambient temperature and relative humidity were recorded within each pen where eligible cows were housed using a Tinytag Plus II logger (Gemini Loggers Ltd, Chichester, UK). The logger was secured at a beam 3 m from the ground and hourly measured ambient temperature from -25 to 85°C with an accuracy of ± 0.3°C and a resolution of 0.01°C. Relative humidity was hourly measured from 0 to 100% with an accuracy of ±3.0% and a resolution of 0.3%. The temperature-humidity-index (THI) was calculated following the equation reported by the NRC guidelines (NRC, 1971): THI = (1.8 × T + 32) - ((0.55 - 0.0055 × RH) × (1.8 × T - 26)), where T = dry-bulb temperature and RH = relative humidity. Data from the logger were transferred to Microsoft Excel. The mean THI measured 1 wk before AI was used and classified into the categories low (<60), medium (60-68), and high (>68). For each of the 4 farms, cumulative milk yield within the first 100 DIM was classified into quartiles stratified by parity (Table 2).

All statistical analyses were performed using SPSS for Windows (version 22.0, SPSS Inc., Chicago, IL). To determine optimum timing of AI considering 3 intervals (from OE to AI, from PAE to AI, and from EE to AI), 3 different logistic regression models using the GENLINMIXED procedure of SPSS were built. Herd was considered a random effect. The unit of interest was the insemination and cow (nested within farm) was included as a random effect because some cows contributed more than 1 AI. A chi-square test was used to evaluate whether there was circadian dynamic of OE and if parity was associated with the frequency of high estrus intensity. Model building was conducted as recommended by Dohoo et al. (2009), where each parameter was first analyzed separately in a univariable model. Only parameters resulting in univariable models with $P \leq 0.10$ were included in the final mixed model. Because there was a strong correlation ($r = 0.66$; $P < 0.01$) between estrus intensity and estrus duration, we only considered estrus intensity in the statistical model. Selection of the model that best fit the data was performed by using a backward stepwise elimination procedure that removed all variables with $P > 0.10$ from the model. The initial model included the following explanatory variables as fixed effects: parity (primiparous vs. multiparous), type of semen (fresh vs. frozen semen), interval to AI (OE to AI vs. PAE to AI vs. EE to AI), estrus intensity (low vs. high), AI number (first AI vs. subsequent AI), THI (<60 vs. 60–68 vs. >68), and cumulative milk yield within 100 DIM [quartile (**Q**) 1 to Q4].

We tested all biologically plausible 2-way interactions. Because there were no interactions between type of semen and parity ($P = 0.42$), type of semen and THI ($P = 0.39$), type of semen and estrus intensity ($P = 0.35$), type of semen and 100-d milk yield ($P = 0.45$),

parity and the interval from OE to AI (P = 0.47), parity and the interval from PAE to AI (P = 0.77), parity and the interval from EE to AI (P = 0.30), or parity and estrus intensity (P = 0.40) on P/AI, these interactions were not included in the final statistical model. Regardless of the significance level, type of semen, interval (OE, PAE, or EE) to AI, and the interaction between interval (OE, PAE, or EE) to AI and type of semen were forced to remain in the final model. The final model therefore contained the following fixed effects: parity, interval (OE, PAE, or EE) to AI, estrus intensity, AI number, THI, cumulative 100-d milk yield, type of semen and the interaction between the type of semen and the interval (OE, PAE, or EE) to AI. A Bonferroni adjustment was used to account for multiple comparisons. Variables were declared to be significant when $P < 0.05$. A statistical tendency was declared when $P \geq 0.05$ and $P \leq 0.10$.

2.5 Results

Overall, 2,575 cows were enrolled in this experiment including activity information from 6,603 AI services. After exclusion of 2,996 AI services (45.4%) due to hormonal treatments before AI (n = 1,900), AI before an activity alert or services with incomplete activity data (n = 922), use of sex-sorted semen (n = 9) and culling before a pregnancy diagnosis (n = 165), a total of 3,607 AI services representing 1,979 cows (805 primiparous and 1,174 multiparous cows) were included in the final statistical analyses.

The mean (± SD) DE activity was 14.3 ± 4.6 h. The circadian distribution of cows reaching onset of estrus was similar during the day (P = 0.13). The mean interval from OE to AI was 16.8 ± 8.0 h, from PAE to AI 11.9 ± 8.1 h and from EE to AI 2.5 ± 8.7 h. The mean index value of PAE was 90.7 ± 14.6; 31.3% of cows had low estrus intensity and 68.7% had high estrus intensity. There was no association (P = 0.88) between parity and the frequency of high estrus intensity.

Overall P/AI was 29.3%. Primiparous cows (31.4%) had greater P/AI compared with multiparous cows (27.3%; P = 0.01). Number of AI was associated with P/AI (P = 0.01), as cows receiving first AI (31.5%) had greater P/AI compared with cows receiving second or greater AI (27.2%). Pregnancy per AI did not differ (P = 0.66) for cows inseminated with frozen semen (28.8%) or cows inseminated with fresh semen (29.8%). Intensity of estrus was associated with P/AI (P < 0.01) such that cows with low estrus intensity (26.0%) had decreased P/AI compared with cows with high estrus intensity (32.8%). Temperature-Humidity-Index measured 1 wk before AI had an effect on P/AI (P < 0.01). A low THI of <60 (36.1%) was associated with increased P/AI compared with a medium THI of 60 to 68 (28.6%; P < 0.01) and high THI of >68 (23.9%; P < 0.01), whereas P/AI did not differ (P = 0.14) between medium and high THI. There was an association between cumulative milk yield within 100 DIM and

P/AI (P = 0.03), as cows within Q2 100-d milk yield had decreased P/AI (25.7%) compared with cows within a Q1 (29.8%; P = 0.04), Q3 (30.3%; P = 0.03), or Q4 (31.6%; P = 0.01) 100-d milk yield.

2.5.1 Interval from OE to AI

There was an association between the interval from OE to AI and P/AI (P < 0.01; Figure 2A), as cows inseminated from 7 to 24 h after OE had greatest P/AI. Cows inseminated within 6 h after OE and 25 h or later after OE showed suboptimum P/AI (0-6 h: 20.8% vs. 7-12 h: 33.1% vs. 13-18 h: 34.9% vs. 19-24 h: 32.9% vs. 25-48 h: 26.2%). There was no interaction between the interval from OE to AI and type of semen (P = 0.56).

2.5.2 Interval from PAE to AI

There was an association between the interval from PAE to AI and P/AI (P < 0.01), as cows inseminated within 18 h after PAE had greatest P/AI. Cows inseminated before PAE and 19 h or later after PAE showed suboptimum P/AI (-6 to 0 h: 17.8% vs. 1-6 h: 33.0% vs. 7-12 h: 32.1% vs. 13-18 h: 36.4% vs. 19-24 h: 27.5% vs. 25-48 h: 21.2%). Overall, there tended to be an interaction between the interval from PAE to AI and type of semen (P = 0.09; Figure 2B). Cows inseminated within 6 h before PAE with frozen semen had greater P/AI compared with fresh semen (P = 0.01).

2.5.3 Interval from EE to AI

There was an association between the interval from EE to AI and P/AI (P < 0.01). Cows inseminated from 5 h before EE to 12 h after EE had greatest P/AI. Cows inseminated more than 5 h before EE and more than 13 h after EE showed suboptimum P/AI (-12 to -6 h: 27.2% vs. -5 to 0 h: 34.4% vs. 1-6 h: 32.5% vs. 7-12 h: 35.8% vs. 13-18 h: 21.6% vs. 19-24 h: 21.4% vs. 25-48 h: 15.8%). There tended (P = 0.07) to be an interaction between the interval from EE to AI and type of semen (Figure 2C). Cows inseminated with fresh semen within 5 h before EE had greater P/AI (P = 0.03) compared with cows inseminated with frozen semen. In addition, cows inseminated with fresh semen from 7 to 12 h after EE tended (P = 0.09) to have greater P/AI compared with cows inseminated with frozen semen. Cows inseminated from 13 to 18 h after EE showed greater P/AI (P = 0.03) when inseminated with frozen semen compared with fresh semen.

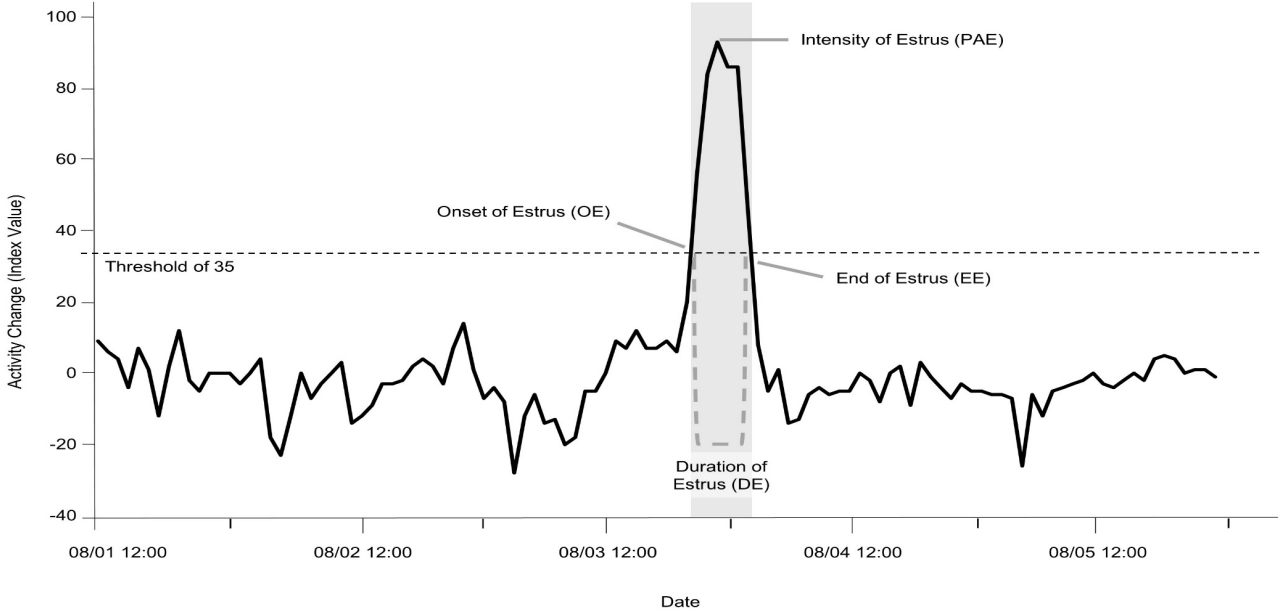
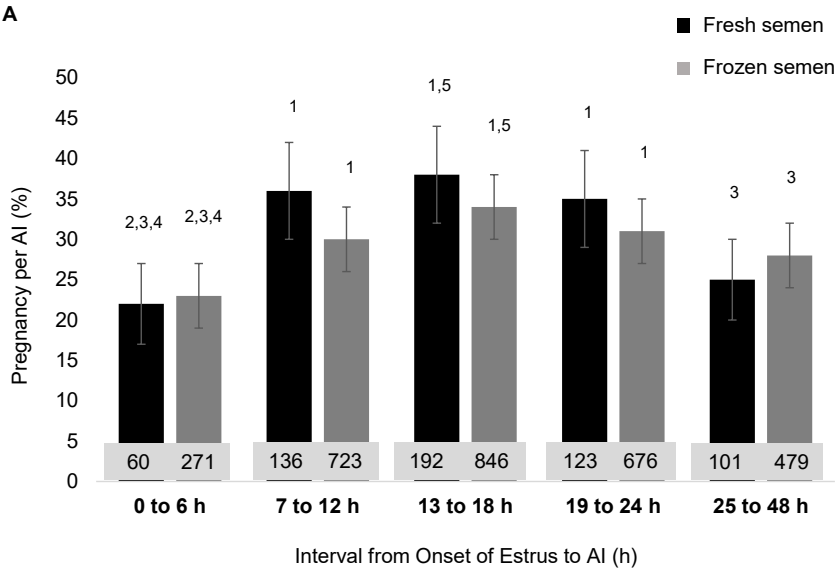
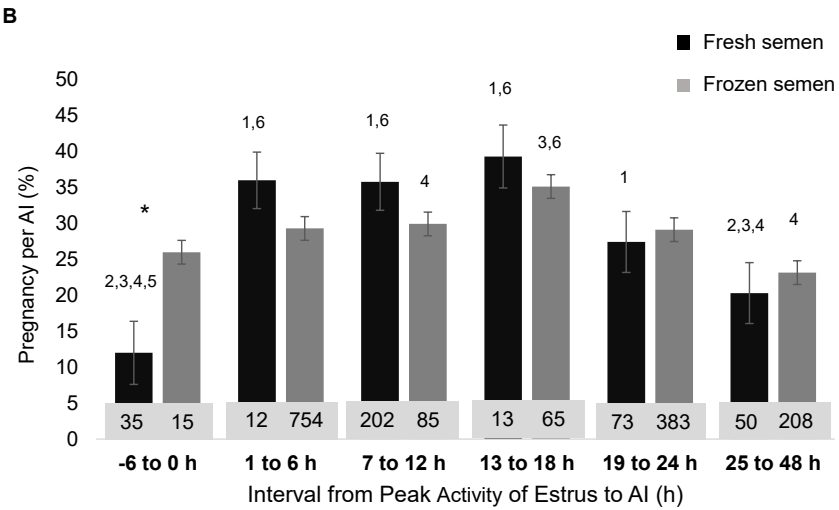


Figure 1. Schematic representation of a cow's activity change at an alert and estrus event characteristics used in the study generated by the DataFlow II software of Heatime (SCR Engineers Ltd, Netanya, Israel). Raw activity of each cow was continuously recorded in 2-h periods (dates are recorded as month/day) and converted into an activity change index value using a proprietary algorithm. Onset of estrus (OE) was defined as a cow exceeding an activity change index value of 35. End of estrus (EE) was defined by the first instance at which the index value fell below 35 again. Intensity of an estrus event (peak activity of estrus; PAE) was represented by the peak of the activity change index value during an estrus event. Duration of an estrus event (DE) was defined as the interval from onset to end of an estrus event.


A
Fresh semen
Frozen semen
Pregnancy per AI (%)
50
45
40
35
30
25
20
15
10
5
0
2,3,4
2,3,4
1
1
1,5
1,5
1
1
3
3
60
271
136
723
192
846
123
676
101
479
0 to 6 h
7 to 12 h
13 to 18 h
19 to 24 h
25 to 48 h
Interval from Onset of Estrus to AI (h)


B
Fresh semen
Frozen semen
Pregnancy per AI (%)
50
45
40
35
30
25
20
15
10
5
0
*
2,3,4,5
1,6
1,6
4
1,6
3,6
1
2,3,4
4
35
15
12
754
202
85
13
65
73
383
50
208
-6 to 0 h
1 to 6 h
7 to 12 h
13 to 18 h
19 to 24 h
25 to 48 h
Interval from Peak Activity of Estrus to AI (h)

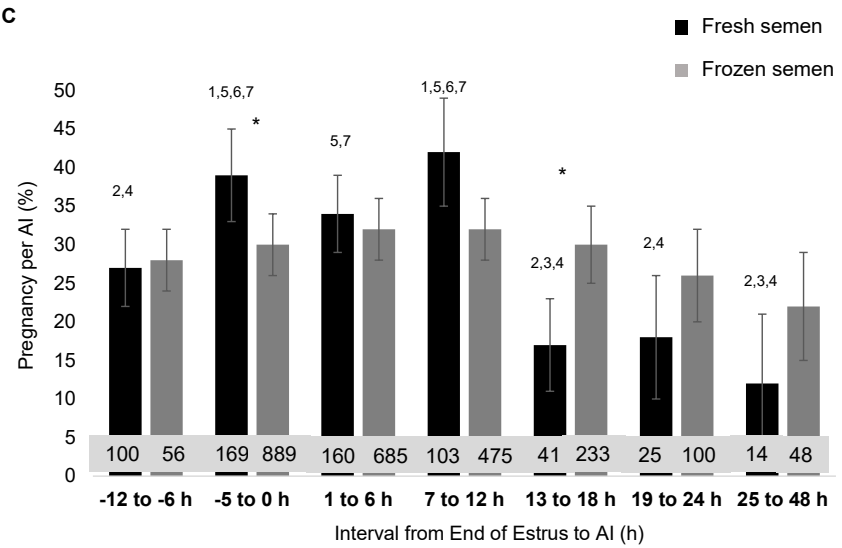


Figure 2. Pregnancy per artificial insemination (± SEM) for cows (n = 1,979) inseminated with either fresh semen (black bars) or frozen semen (grey bars) considering onset of estrus to AI (A), peak activity of estrus to AI (B), and end of estrus to AI (C) using a neck-mounted accelerometer system for estrus detection (Heatime; SCR Engineers Ltd., Netanya, Israel). Onset of estrus was defined as a cow exceeding an activity change index value of 35. Intensity of an estrus event was represented by the peak of the activity change index value during an estrus event. End of estrus was defined by the first instance at which the index value fell below 35 again. A Bonferroni adjustment was used to account for multiple comparisons. An asterisk indicates a significant difference ($P < 0.05$) between type of semen (i.e., fresh vs. frozen semen) within a time interval. Bars with different numbers indicate a significant difference ($P < 0.05$) among time intervals for either fresh or frozen semen. Therefore, time intervals were classified into numbers (1 to 5 for panel A, 1 to 6 for panel B, 1 to 7 for panel C) with number 1 beginning at the first time interval in ascending order. The number within each bar is the number of cows inseminated with this type of semen within that specific time interval.

Table 1. Descriptive information of the reproductive management from the 4 participating herds

Item	Herd A	Herd B	Herd C	Herd D
Average herd size (no. of cows)	1,224	716	427	558
Herd average 305-d milk yield (kg)	9,939	9,677	9,461	9,938
Voluntary waiting period (DIM)	42	40	45	42
Heat detection rate (%)	41	40	36	57
Conception rate (%)	28	28	38	28
21-d pregnancy rate (%)	12	11	14	16
Day of pregnancy diagnosis after AI	38 ± 3	28 ± 3	38 ± 3	32 ± 3
Method of pregnancy diagnosis	Transrectal palpation	Transrectal ultrasound	Transrectal palpation	Transrectal ultrasound

Table 2. Quartiles (Q1-Q4) of cumulative milk yield (kg) within the first 100 DIM from enrolled herds stratified by herd and parity

Quartile	Herd A	Herd B	Herd C	Herd D
Primiparous cows				
Q1	≤3,152	≤3,013	≤2,446	≤3,073
Q2	3,153–3,410	3,014–3,324	2,447–2,946	3,074–3,381
Q3	3,411–3,729	3,325–3,676	2,947–3,172	3,382–3,703
Q4	>3,729	>3,676	>3,172	>3,703
Multiparous cows				
Q1	≤4,050	≤4,018	≤3,438	≤3,416
Q2	4,051–4,483	4,019–4,472	3,439–3,698	3,417–3,822
Q3	4,484–4,899	4,473–4,881	3,699–4,261	3,823–4,322
Q4	>4,899	>4,881	>4,261	>4,322

2.6 Discussion

The objective of this study was to determine the association between the time of AI and P/AI in lactating Holstein cows inseminated either with fresh or frozen semen considering different characteristics of an estrus event (i.e., onset, peak, and end) using an AAM. We tested (1) whether cows inseminated close to the beginning of an estrus event would achieve greater P/AI with fresh semen compared with frozen semen and (2) whether P/AI would differ between fresh and frozen semen for cows inseminated closer to the end of an estrus event.

Overall, P/AI did not differ for cows inseminated with fresh or frozen semen. This is consistent with 2 previous studies that also compared pregnancy outcomes for AI with fresh or frozen semen (Bucher et al, 2009; Murphy et al., 2017). Similar P/AI for both types of semen was observed in beef cows allocated to TAI in a progesterone (**P4**)-supplemented 7-d Cosynch protocol (Bucher et al., 2009) and in grazing dairy cows in Ireland (Murphy et al., 2017). In both studies they used a reduced number of sperm per straw for fresh semen compared with frozen semen. For fresh semen, the authors therefore assumed an extended viability in the female reproductive tract and a higher sperm quality compared with frozen semen. A case report (Crespilho et al., 2012), however, showed increased P/AI for cooled semen (5°C) compared with frozen semen after TAI (61.5% vs. 45.7%, respectively). Collectively, studies evaluating reproductive performance of fresh and frozen semen are difficult to compare due to differences in sperm number per straw, semen diluents, storage time, temperature of semen, and type of AI (i.e., spontaneous estrus vs. TAI; Bucher et al., 2009; Crespilho et al., 2009; Murphy et al., 2017). Compromised sperm function after cryopreservation and its consequences for fertility has been widely studied (Watson, 1995; Gravance et al., 1998; Curry, 2000). Nevertheless, poor fertility resulting from compromised sperm viability in the female reproductive tract can be overcome by precise timing of insemination relative to OE and ovulation (Bombardelli et al., 2016; Borchardt et al., 2018). In a study by Borchardt et al. (2018), the optimum timing of AI was influenced by type of semen, as cows enrolled in a TAI protocol with a long interval from AI to ovulation (~ 24 h; Cosynch-56) achieved greater P/AI when inseminated with fresh compared with frozen semen (27.5% vs. 20.0%, respectively; $P = 0.03$). However, when cows were enrolled in a TAI protocol with a shorter interval from AI to expected ovulation (12–16 h; Ovsynch-56) no effect of type of semen (**P** = 0.33) was observed. These results further support the idea that viability of sperm in the female reproductive tract is extended using fresh semen compared with frozen semen. In our study, there was no interaction between the type of semen and the interval from OE to AI, but a tendency for an interaction between the type of semen and the intervals from PAE and EE to AI. Discrepancies to the latter study might be due to the

wide variation of ovulation timing among cows inseminated based on estrous behavior compared with cows receiving TAI (Roelofs et al., 2006; Valenza et al., 2012).

Similar intervals from onset of increased activity to ovulation were determined in 3 studies using neck-mounted AAM (Heatime), ranging from 25.7 ± 1.2 h to 28.7 ± 8.1 h (Valenza et al., 2012: 28.7 ± 8.1 h; Aungier et al. 2015: 27.0 ± 1.0 h; Bombardelli et al., 2016: 25.7 ± 1.2 h). These results are also consistent with the mean interval of 27.6 h from onset of standing estrus to ovulation determined with an electronic pressure-sensing system (Walker et al., 1996). All of these studies, however, reported a wide range of variation among cows observed for the interval from onset of activity to ovulation. In addition, one study reported 1.6 to 3.7 h longer intervals from OE, PAE, and EE to ovulation for multiparous cows compared with primiparous cows (Stevenson et al., 2014). Furthermore, variability in the DE and timing of AI relative to ovulation could contribute to poor fertility. In our experiment, the observed DE is in agreement with other studies (Dalton et al., 2001; Yoshioka et al., 2010; Burnett et al., 2018) and the circadian distribution of cows reaching OE was equal throughout the day. Whereas some studies support the latter finding (Dransfield et al., 1998; Xu et al., 1998; Bombardelli et al., 2016), others found estrous behavior to be more likely during the evening and nighttime (Hurnik et al., 1975; Esslemont and Bryant, 1976) or early morning and daylight hours (Van Vliet and Eerdenburg, 1996).

The results from the current study underline the importance of careful timing of AI relative to specific characteristics of an estrus event identified by an AAM as the intervals from OE to AI, PAE to AI and EE to AI were associated with pregnancy outcomes. The data suggests that adequate P/AI could be achieved when AI is performed only once daily, as cows showed greatest P/AI when inseminated from 7 to 24 h after onset of estrus. This corroborates older findings from Foote (1978) and Nebel et al. (1994) that demonstrated similar P/AI for once-daily versus twice-daily AI. Based on mounting behavior, however, greatest pregnancy outcomes resulted from AI between 4 and 12 h after the first standing event measured by an electronic pressure-sensing system (Dransfield et al., 1998). In other studies, the greatest P/AI was found when AI was performed between 9 and 15 h after OE (Aungier et al., 2015) or after the PAE (Bar, 2010), which agrees with our findings that showed increased P/AI for cows inseminated within 18 h after PAE.

Although we did not observe an interaction between parity and the intervals from OE, PAE, or EE to AI, 2 studies described differences in the optimum timing of AI for primiparous and multiparous cows using AAM (Stevenson et al., 2014; LeRoy et al., 2018). One study found greatest P/AI for primiparous cows inseminated from 13 to 16 h and for multiparous cows inseminated within 12 h after OE (Stevenson et al., 2014). Another study observed

greatest P/AI for primiparous cows inseminated within 8 h after OE, whereas for multiparous cows P/AI did not differ when inseminated up to 24 h (LeRoy et al., 2018). The relative discrepancy in the results of studies is likely caused by differences in experimental design, types of AAM, and different classification intervals (our experiment: mainly 5-h intervals; Stevenson et al., 2014: 3-h intervals; LeRoy et al., 2018: 8-h intervals), as the frequency of data transfer between AAM and on-farm computer differed.

Approximately two-thirds of the cows (68.7%) expressed high estrus intensity, whereas one-third (31.3%) showed low estrus intensity. This is noteworthy, as estrus intensity had a marked effect on P/AI. The positive association between high estrous expression and P/AI has been described earlier for lactating dairy cows and beef cows (Madureira et al., 2015a; Davoodi et al., 2016; Polsky et al., 2017). Cows with a greater PAE had an approximately 12 percentage units greater P/AI compared with those with lower PAE (Madureira et al., 2015). Estrous expression was reported to be influenced by various factors, such as milk yield, negative energy balance, stage of lactation, parity, and THI at time of estrus (Aungier et al., 2012; Madureira et al., 2015; Polsky et al., 2017). Furthermore, higher P4 concentrations on d 10 after AI were found in cows displaying high estrus intensity at AI (Madureira et al., 2019). Cows with low estrus intensity, on the other hand, had a higher risk of ovulation failure, abnormal timing of ovulation, and pregnancy losses (Pereira et al., 2016; Burnett et al., 2018; Madureira et al., 2019). Only few studies exist, however, that found no association between estrus intensity and pregnancy outcomes (Aungier et al., 2012; Bombardelli et al., 2016). Further research is warranted to better use data from AAM to support on-farm decision making, (e.g., by using PAE as a characteristic to select cows with superior estrus intensity to optimize pregnancy outcomes).

In the present study, P/AI decreased by 12.2 percentage units for cows exposed to a THI >68 1 wk before AI compared with cows exposed to a THI <60 1 wk before AI. Negative effects of heat stress include hormonal imbalances (Wilson et al., 1998), lower quality of the follicle and oocyte, and negative effects on embryo development and survival (Wolfenson et al., 1995; De Rensis and Scaramuzzi, 2003). In moderate climates heat stress decreased P/AI at a THI threshold of 73 (Schüller et al., 2014), with the greatest negative effect on P/AI observed 21 to 1 d before AI. Exposure to a mean THI of 73 or more during this period decreased P/AI by 19 percentage units, which is similar to our results. In contrast to other studies that found a reduced likelihood of pregnancy during periods of heat stress depending on the type of semen used (Schüller et al., 2016), we did not observe an interaction between type of semen and THI.

Interestingly, cows with intermediate (Q2) cumulative milk yield within 100 DIM had decreased P/AI compared with the other quartiles. The reason for this observation remains speculative. Previous research on the effect of milk yield on P/AI has been controversial, as some studies found no effect on P/AI (Dransfield et al., 1998; López-Gatius et al., 2005; Madureira et al., 2015), whereas others found a negative effect of milk yield on P/AI (Valenza et al., 2012; Bombardelli et al., 2016; Burnett et al., 2017). When evaluating an effect of milk yield on reproductive performance other variables closely associated with the probability of pregnancy, such as feeding management, housing, or skilled labor, are often ignored (LeBlanc, 2010). In addition, low-producing cows might be sick from diseases that will also affect the reproductive tract, whereas high-producing cows are more likely to be healthy and inseminated and less likely to be culled (Grohn and Rajala-Schultz, 2000; Santos et al., 2009).

One limitation of our study was that because we conducted this experiment as an observational cohort study, there was no option of randomizing type of semen and timing of AI. Yet our findings broaden the understanding about AAM-based estrus characteristics and subsequent timing of AI. External validity was improved by enrolling cows from 4 commercial farms in contrast to a more controlled study design on a single farm. Another limitation of our study was that cows were not systematically confirmed as being in estrus after the alert of the AAM and thus before AI by means of P4 concentration or transrectal ultrasonography of the ovaries. Studies measuring P4 concentrations of cows detected in estrus by an AAM, however, found only few cows to be false positive (Holman et al., 2011; LeRoy et al., 2018). In the study from Holman et al. (2011), prevalence of cows that were flagged by a neck-mounted AAM and inseminated having low serum P4 ranged between 86.3% and 97.6%."As our study was a field trial with 6,603 AI services, measurement of P4 concentrations was not feasible.

2.7 Conclusions

Using an AAM, inseminating cows from 7 to 24 h after OE or 1 to 18 h after PAE yielded greatest P/AI irrespective of the type of semen (fresh vs. frozen). Cows inseminated within 6 h before PAE with frozen semen had greater P/AI compared with those inseminated with fresh semen. Inseminating cows from 5 h before to 12 h after the EE yielded greatest P/AI. Cows inseminated with fresh semen within 5 h before EE had greater P/AI compared with those inseminated with frozen semen, whereas cows inseminated with frozen semen from 13 to 18 h after EE had greater P/AI compared with those inseminated with fresh semen. Our results confirm the well-known practice of once-daily AI. About two-thirds of cows expressed high estrus intensity, which yielded greatest P/AI. Further research is warranted to determine factors that affect estrus intensity.

2.8 Acknowledgments

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3 PUBLICATION II

Factors associated with estrous expression and subsequent fertility in lactating dairy cows using automated activity monitoring.

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3.1 Abstract

The objective of this observational study was to identify factors associated with estrous duration (DU) and intensity measured as the peak of activity (PA) change and subsequent fertility in lactating Holstein cows using a neck-mounted automated activity monitor (AAM; Heatime Pro; SCR Engineers Ltd., Netanya, Israel). Ambient temperature and relative humidity were recorded hourly to calculate the temperature-humidity-index (THI). A total of 5,933 estrus events from 3,132 cows located on 8 commercial dairy farms in Germany were used for this study. Farms participated in monthly DHIA testing. Pregnancy diagnosis was performed either by transrectal palpation (farm 1: 42 ± 3 d; farm 3: 40 ± 3 d; farms 4 and 8: 38 ± 3 d; farm 5: 43 ± 3 d after AI) or transrectal ultrasonography (farms 2, 6, and 7: 30 ± 3 d after AI). Estrous intensity was categorized based on peak activity of estrus into low (35 to 89 index value), and high (90 to 100 index value) PA. Overall, 73.5% of estrus events were of high PA. The mean (± standard error of the mean) DU was 14.94 ± 0.06 h. There was a strong correlation between DU and PA (r = 0.67). In the final statistical model, only PA was associated with pregnancy per artificial insemination (P/AI), with 1.35 greater odds of pregnancy for cows with high PA compared to cows with low PA. Increased THI 1 wk before AI was associated with shorter DU, lower PA and decreased P/AI. A small percentage of cows (4.7 %) showed short inter-estrus intervals (i.e., more than one activity peak within 7 d close to the event of estrus), resulting in reduced DU, PA and P/AI. The change of weighted rumination was associated with DU and PA, as a lower nadir was associated with a greater risk for high PA and long DU. There was no association, however, between the nadir of change of weighted rumination and P/AI. Whereas milk yield and somatic cell count from DHIA test date before AI was negatively associated with estrous expression, neither milk yield nor SCC were associated with P/AI. Surprisingly, multiparous cows expressed estrus with longer DU (13.15 ± 0.31 h) compared to primiparous cows (12.52 ± 0.32 h), whereas PA did not differ among parities. Pregnancy per AI was greater for primiparous (29.4%) than for multiparous cows (22.1%). An estrus event with long DU or high PA was more likely later in lactation. Milk fat, milk protein, milk urea nitrogen and lactose from the DHIA test date closest to AI had no association with estrous expression or P/AI. In conclusion, duration of estrus and PA were highly correlated, and particularly cows with high PA were associated with greater odds for pregnancy. A negative association between estrous expression and P/AI was identified for increased THI 1 wk before AI and cows with short inter-estrus intervals using AAM.

3.2 Key words

estrous expression, automated activity monitor, pregnancy per artificial insemination, dairy cow

3.3 Introduction

To reliably identify cows in estrus and to optimize timing of subsequent AI, automated activity monitoring (**AAM**) systems have been implemented on many farms (Roelofs et al., 2010; Stevenson et al., 2014; LeRoy et al., 2018). Besides mere identification of a cow in estrus, AAM systems also represent an accurate and useful tool for evaluation of a cow's individual estrous expression using real time data. Duration and intensity of expression of estrus were found to be strongly associated with the risk of pregnancy and embryonic development in lactating dairy cows (Madureira et al., 2015a; Pereira et al., 2016; Burnett et al., 2017). Cows having an estrus event of high intensity or long duration achieved greater pregnancy per artificial insemination (**P/AI**) compared to cows with an estrus event of low intensity or short duration (Madureira et al., 2015a; Polsky et al., 2017; Madureira et al., 2019).

Several factors affecting estrous expression in lactating dairy cows have been identified such as flooring (Britt et al., 1986), parity (López-Gatius et al., 2005; Madureira et al., 2015a; Burnett et al., 2017), body condition score (**BCS**; Aungier et al., 2012; Valenza et al., 2012), and milk yield (Rivera et al., 2010). Several studies observed a shorter and less intense expression of estrus in high-yielding dairy cows, most likely associated with an elevated metabolism of steroid hormones responsible for estrous behavior (Sangsritavong et al., 2002; López-Gatius et al., 2005; Rivera et al., 2010). Controversially, others reported a lack of an association between milk yield and estrous expression detected by AAM systems (Van Eerdenburg et al., 2002; Burnett et al., 2017). Furthermore, genomic merit for daughter pregnancy rate was associated with estrus characteristics, such as a greater peak activity and a trend for longer duration of estrus (Veronese et al., 2019; Chebel and Veronese, 2020).

Besides retrieving useful data from AAM systems, there might be other information available on farm that affect estrous expression and subsequent fertility in cows, such as climate data and DHIA test data. Climate loggers installed within the barns can continuously record ambient temperature and relative humidity for calculation of the temperature-humidity index (**THI**). With increasing THI at the day of estrus, the likelihood for low serum progesterone (**P4**) concentrations (<1 ng/mL) at the day of estrus and size of the pre-ovulatory follicle continuously decreased, probably thereby reducing estrous expression (Schüller et al., 2017). In addition, DHIA test data close to the AI, such as milk yield, milk fat, milk protein, lactose, milk urea nitrogen (**MUN**) or somatic cell count (**SCC**) might affect expression of estrus. It has been shown that mastitis and/or an elevated SCC around AI had a negative impact on fertility (Miller et al., 2001; Bijker et al., 2015; Fuenzalida et al., 2015). Controversial results exist regarding the association between fertility and MUN, which was suggested to be an adequate indicator of metabolic status (Eicher et al., 1999; Wattiaux and Karg, 2004). Several studies

observed a negative association between MUN and the risk of pregnancy (Hojman et al., 2004; König et al., 2008). Another study, however, showed that either very high or very low MUN concentrations were detrimental to fertility (Pehrson et al., 1992). In contrast to previous research having a similar scope, the objective of our study was to combine data from the AAM system (i.e., physical activity and rumination activity) and other data sources that are easily available on farm (i.e., climate and DHIA test data). One study (Tomic et al., 2015) found an effective integration of available on farm data from different data sources might improve on-farm decision making and lead to a more efficient herd management. Automated activity monitors create the opportunity for an objective and reliable submission of cows for AI based on the intensity of estrous expression. To take full advantage of this potential, the identification of risk factors for estrous expression and subsequent risk of pregnancy should be optimized by further investigation.

Therefore, the objective of this observational, retrospective study was to identify plausible factors associated with estrous expression and subsequent P/AI in lactating Holstein cows using an AAM system. Therefore, we evaluated the associations of parity, days in milk (**DIM**) at AI, THI 1 wk before AI, rumination activity around AI, and parameters from the DHIA test date closest to AI, such as milk yield (kg), SCC (x 10^3 cells/mL), fat (%), protein (%), lactose (%) and MUN (mg/L) with estrous expression (i.e., intensity measured as the peak of activity change and duration of estrus). In addition, risk factors associated with P/AI, particularly those related to estrous expression, were assessed. We hypothesized that factors associated with estrous expression will also have an impact on P/AI.

3.4 Materials and Methods

3.4.1 Study Design

This study was an observational, retrospective cohort study including 7,409 AI services from 3,870 lactating Holstein cows (1,374 primiparous and 2,496 multiparous cows) from 8 commercial dairy farms in northeast Germany from July 2018 until May 2019. Inclusion criteria for farms were a herd size above 400 cows, the use of a neck-mounted AAM (Heatime Pro, SCR Engineers Ltd., Netanya, Israel) and participation in monthly DHIA testing. Herd size ranged from approximately 400 to 1,200 cows per farm (Table 1). All cows were housed in freestall barns and milked twice or three times daily. Milk yield ranged from 9,105 to 11,900 kg per 305 d (Table 1). All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Freie Universität Berlin.

3.4.2 AAM

All cows were fitted with a neck-mounted AAM on the day of their first calving (farms 1, 2, 4, 6-8), at approximately 40 DIM (farm 3) or 14 d before their first calving (farm 5). The AAM was attached to the cows until culling (farms 1, 2, 4-8) or was removed at 65 d after AI if a cow was diagnosed pregnant (farm 3). Individual activity and rumination data of each cow were recorded in real time for 2 h periods by a wireless receiver box and transmitted to the accelerometer software (DataFlow II, SCR Engineers Ltd.) installed on the farm computer. The raw activity data from each cow was converted into an activity change index value using a proprietary algorithm. This algorithm calculates the difference of today's last hours of raw activity from the mean of last week's activity in the same period of the day weighted by the standard deviation of this specific cow. Index values for activity change ranged from 0 to 100 (0 = lowest, 100 = highest). The period where the cow's activity change index value was exceeding 35 was considered as an estrus event. For each estrus event, intensity and duration (**DU**) were determined. The intensity of an estrus event (**PA**) was represented by the peak value of the activity change index during an estrus event. Estrus intensity was categorized into low (35 to 89 index value) and high (90 to 100 index value) PA based on a previous study (Madureira et al., 2015a). Onset of estrus was defined as the time a cow exceeding an activity change index value of 35. End of estrus was defined by the first instance at which the index value fell below 35 again. Estrous duration was defined as the interval from onset to end of an estrus event. In addition, estrous behavior was classified into cows with a short inter-estrus interval and cows with no short inter-estrus interval. Cows with no short inter-estrus intervals were defined as cows with one period of increased activity within an observational period of 7 d around estrus (i.e., exceeding a threshold of 35, reaching an individual peak of activity and falling below 35 afterwards for one time). Cows with short inter-estrus intervals were defined as cows with more than one period of increased activity within an observational period of 7 d around of estrus (i.e., these cows had more than one activity peak as activity change had a drop of below 35 in between the observational period). Due to our SCR data report settings regarding the observational period, short inter-estrus intervals possibly ranged between a minimum of 4 h to a maximum of 7 d. In case of short inter-estrus intervals, number of activity peaks were recorded. The period with the higher peak of activity was considered to calculate duration, peak activity, and timing of AI in relation to an estrus event. Raw rumination data from each cow was converted internally into the change of weighted rumination using a proprietary algorithm. Change of weighted rumination is the difference of today's last hours of rumination from the mean of last week's rumination in the same period of the day weighted by the standard deviation of this specific cow. Index values for the change of weighted rumination ranged from + 100 to – 100.

Files from the SCR system were exported using DataFlow II on a weekly basis for all cows that were bred within the last 7 d on each farm. Each file contained the following information: cow ID, lactation number, last breeding date, AI number, DIM, days from last breeding, and last calving date. In addition, raw activity data, daily activity, activity change, rumination raw data, change of weighted rumination, and daily rumination data were recorded for each cow within the past 7 d every 2 hours. These files were generated in the XLSX format (Office 2013, Microsoft Deutschland Ltd.) using DataFlow II. A software tool, written in the open-source Python programming language (Python Software Foundation, Delaware, USA), was used to process all XLSX files and generated a single result report XLSX file (Plenio et al., 2020). The report formatted in the wide (i.e., one line for each cow) and long format (i.e., one line for each estrus event) included the following information for each cow: cow ID, lactation number, last breeding date, AI number, DIM at AI, raw activity data (peak value and hour of raw activity peak), activity change (index value and hour for activity change start, peak, and end), DU of estrus, and the nadir of change of weighted rumination (index value and hour) for each estrus episode.

3.4.3 Reproductive Management

Lactating Holstein cows were inseminated based on the alert of the AAM system, after visual estrus detection or receiving timed AI after hormonal intervention (i.e., 7-d Ovsynch protocol). Inclusion criteria for cows were: past the voluntary waiting period (Table 1), inseminated based on the alert of the AAM without any hormonal interventions, inseminated with frozen-thawed semen, and eligible for breeding. Exclusion criteria for cows were: inseminated with fresh or sexed semen or receiving a subsequent AI within 24 h. Cows were excluded after enrollment in the study when culled before a pregnancy diagnosis (n = 106). A list of cows eligible for breeding with an activity alert was generated daily by the AAM on each farm. Based on these lists, cows were inseminated at different intervals related to the AAM alert. Cows were inseminated once daily (farms 1, 3, 4, 8) or twice daily (farms 2, 5-7) following the am-pm rule with each cow receiving a single AI based on the AAM alert. Cows remained in the study until a confirmed pregnancy diagnosis (Table 1), which was performed on a weekly basis by transrectal palpation 38 ± 7 d after AI (farms 1, 3-5), by transrectal ultrasound beginning at 28 d after AI (farm 2) or at 32 d after AI (farms 6-8). For simplicity, the time at which pregnancy diagnosis was conducted will be referred to as 38 d after AI throughout the manuscript. In case of transrectal palpation, pregnancy was based on a verified pregnancy diagnosis defined by the presence of uterine fluid, asymmetry, and a positive fetal membrane slip. Non-pregnancy was based on absence of pregnancy at the day of examination or a rebreeding upon estrus before pregnancy diagnosis. Positive pregnancy diagnosis performed

by ultrasound was based on visualization of an embryo with a heartbeat. Cows diagnosed not pregnant were reassigned to breeding after spontaneous estrus or timed AI.

3.4.4 Data Collection and Statistical Analyses

Cow ID, parity, DIM, breeding date, and DHIA test data were obtained through the on-farm computer software (herdeW and herdeplus, respectively; dsp agrosoft GmbH, Ketzin/Havel, Germany).

The DHIA test data from 2018 were used to calculate average milk fat (%), milk protein (%), MUN (mg/L), and SCC (x 10^3 cells/mL) for each farm (Table 1). The DHIA data collected within 15 d after AI to 35 d before AI were used to evaluate associations between milk yield, milk fat, milk protein, lactose, MUN, and SCC and estrous expression and subsequent P/AI. If a cow had 2 DHIA test dates within the given range, we considered the DHIA test date closest to AI for analysis. Milk urea nitrogen was classified into 3 categories (i.e., < 150 mg/L, 150 – 300 mg/L, and > 300 mg/L). Somatic cell count was classified into 4 categories (< 100 x 10^3 cells/mL, 100 – 400 x 10^3 cells/mL, 401 – 1,000 x 10^3 cells/mL, and > 1,000 x 10^3 cells/mL).

Ambient temperature and relative humidity within the pen of cows eligible for breeding were recorded using a Tinytag Plus II logger (Gemini loggers Ltd, Chichester, UK). The logger was secured at a beam 3 m above the ground and measured ambient temperature hourly from -25 to +85°C with an accuracy of ± 0.3°C and a resolution of 0.01°C. Relative humidity was hourly measured from 0 to 100% with an accuracy of ±3.0% and a resolution of 0.3%. The THI was calculated following the equation reported by the NRC guidelines (NRC, 1971): THI = (1.8 × T + 32) - ((0.55 - 0.0055 × RH) × (1.8 × T - 26)), where T = temperature and RH = relative humidity. Data from the logger were imported into Microsoft Excel. The average of the THI within 1 wk before AI was used and classified into the categories < 50, 50 – 55, 56 – 60, 61 – 65, 66 – 70, and > 70. For simplicity, THI 1 wk before AI will be referred to as THI throughout the manuscript.

All statistical analyses were performed using SPSS for Windows (version 22.0, SPSS Inc., IBM, Ehningen, Germany). To identify important factors associated with estrous expression and subsequent P/AI, a linear regression model (DU) and 2 different logistic regression models (PA, P/AI) were built using the GENLINMIXED procedure of SPSS. Herd was considered a random effect. Cow was nested within farm. Number of AI was considered as a repeated measure because some cows had more than one AI within the observation time. Model building was conducted as recommended by Dohoo et al. (2009), where each parameter was first analyzed separately in a univariable model using the GENLINMIXED procedures as described above (Tables 2 and 3). Only parameters resulting in univariable

models with $P \leq 0.10$ were included in the final mixed models. Selection of the model that best fit the data was performed using a backward stepwise elimination procedure by removing all variables with $P > 0.10$ from the model. The initial models included the following explanatory variables as fixed effects: parity (primiparous vs. multiparous), DIM at AI, THI (< 50, 50 – 55, 56 – 60, 61 – 65, 66 – 70, and > 70), activity change at onset of estrus (35 to 100), change of weighted rumination (- 100 to + 100), estrous behavior (no short inter-estrus interval vs. short inter-estrus interval), and milk yield, milk fat, milk protein, lactose, MUN concentration (< 150 mg/mL, 150 – 300 mg/mL, and > 300 mg/mL), and SCC (< 100 x 10^3, 100 – 400 x 10^3, 401 – 1,000 x 10^3, and > 1,000 x 10^3) from the DHIA test date closest to AI. The initial model for P/AI additionally included DU and PA (low vs. high) as fixed effects. Furthermore, we tested whether timing of DHIA test date (before vs. after AI) was associated with the outcome variables. If the association was different when the DHIA test date was before an AI, we tested individual DHIA parameters separately in a univariable model.

The final model for DU therefore contained the following fixed effects: parity, DIM at AI, THI, change of weighted rumination, estrous behavior, milk yield and SCC. The final model for PA contained the following fixed effects: DIM at AI, THI, activity change at onset of estrus, change of weighted rumination, estrous behavior, and SCC. The final model for P/AI contained the following fixed effects: parity, DIM at AI, THI, estrous behavior, and PA. To account for multiple comparisons the *P* value was adjusted using a Bonferroni correction. Variables were declared to be significant when $P < 0.05$. A statistical tendency was declared when $P \geq 0.05$ and $P \leq 0.10$.

3.5 Results

Overall, 3,870 cows were enrolled in this experiment including activity information from 7,409 AI services. After exclusion of 1,476 AI services (19.9%) due to incomplete activity and rumination data from the AAM (n = 1,135), missing information regarding the DHIA test and/or THI data (n = 235), and culling before a pregnancy diagnosis (n = 106), 5,933 AI services representing 3,132 cows (1,094 primiparous and 2,038 multiparous cows) were included in the final statistical analyses.

There was a strong positive correlation (r = 0.67; $P < 0.01$; Figure 1) between DU and PA of an estrus event. Because of that, DU and PA were assessed independently.

3.5.1 Factors Associated with Duration of Estrus

The mean (± SEM) duration of estrous activity was 14.94 ± 0.06 h. Factors associated with DU are shown in Table 4. Duration of estrus was associated with parity ($P < 0.01$), as multiparous cows (13.15 ± 0.31 h) showed a longer estrous DU than primiparous cows (12.52 ± 0.32 h). There was a positive association between DIM at AI and DU ($P < 0.01$). The DU increased by 0.81 h when DIM increased by 100 days. An increase in THI ($P < 0.01$) had an impact on DU (THI < 50: 13.58 ± 0.32 h; THI 50 – 55: 13.42 ± 0.32 h; THI 56 – 60: 13.49 ± 0.33 h; THI 61 – 65: 12.90 ± 0.34 h; THI 66 – 70: 12.44 ± 0.34 h; THI >70: 11.18 ± 0.44 h; Figure 2A). When THI was < 60, cows had longer DU compared to a THI > 60 ($P < 0.01$). There was a tendency ($P = 0.05$) for cows having longer DU when THI was 61 – 65 compared to a THI of 66 – 70. A THI > 70 decreased DU ($P < 0.01$) compared to all the other THI categories below. The activity change at the onset of estrus was not associated with DU ($P = 0.30$). Overall, 4.7% (278/5,933) short inter-estrus intervals were recorded, resulting in shorter DU (11.37 ± 0.38 h) compared to estrus events with no short inter-estrus interval (14.30 ± 0.29 h; $P < 0.01$). The nadir of the change of weighted rumination was associated with DU ($P < 0.01$). If the nadir decreased by an index value of 10, DU increased by 0.78 h. Increasing SCC ($P < 0.01$) caused DU to decrease (< 100 x 10^3/mL: 13.15 ± 0.31 h; 100 – 400 x 10^3/mL: 12.93 ± 0.32 h; 401 – 1,000 x 10^3/mL: 13.06 ± 0.36 h; > 1,000 x 10^3/mL: 12.21 ± 0.37). Whereas DU was similar for cows with a SCC < 1,000 x 10^3/mL ($P > 0.11$), cows with a SCC > 1,000 x 10^3/mL had shortest DU ($P < 0.01$). Test day milk yield was associated with DU ($P = 0.01$). An increase in test day milk yield of 10 kg led to a decrease in DU of 0.20 h. There was no association between DU and milk fat ($P = 0.76$), milk protein ($P = 0.95$), lactose ($P = 0.67$), or MUN ($P = 0.29$).

In order to evaluate whether the association between the DHIA data and DU was dependent on the timing of the DHIA test relative to the estrus event we considered DHIA test data that occurred before an estrus event in a separate analysis including 3,307 AI services. Test day milk yield ($P < 0.01$), milk protein ($P < 0.01$), and SCC ($P < 0.01$) were associated with DU. An increase in milk yield of 10 kg led to a decrease in DU of 0.38 h. Duration of estrus increased with increasing milk protein, such that an increase of 1% in milk protein was associated with a 1.88 h longer DU. Increasing SCC resulted in shorter DU (< 100 x 10^3/mL: 15.15 ± 0.31 h; 100 – 400 x 10^3/mL: 14.62 ± 0.35 h; 401 – 1,000 x 10^3/mL: 15.09 ± 0.43 h; > 1,000 x 10^3/mL: 13.74 ± 0.44 h).

3.5.2 Factors Associated with Intensity of Estrus

Overall, 73.5% (4,362/5,933) of estrus events had high PA. Factors associated with PA are shown in Table 5. Intensity of estrus was not associated with parity ($P = 0.31$), with a similar percentage of primiparous cows (75.1%) reaching high PA compared to multiparous cows (73.8%). An estrus event with high PA was more likely later in lactation ($P < 0.01$). When DIM increased by 100 days, cows had 1.17 greater odds of an estrus event with high PA. Heat stress had an impact ($P < 0.01$) on the risk of an estrus event with high PA (THI < 50: 71.5%; THI 50 – 55: 68.3%; THI 56 – 60: 67.9%; THI 61 – 65: 62.2%; THI 66 – 70: 61.2%; THI >70: 52.5%; Figure 2B). The risk of an estrus event with high PA was greater when THI was < 60 ($P < 0.01$) compared to a THI > 60. Percentage of cows reaching high PA was similar ($P = 0.75$) for a THI from 61 to 70. A THI > 70 decreased the risk of high PA compared to a THI < 50 – 60 ($P < 0.01$) and 61 – 65 ($P = 0.04$) and tended ($P = 0.07$) to decrease the risk of high PA compared to a THI from 66 – 70. The activity change at onset of estrus was positively associated with the risk of an estrus event with high PA ($P < 0.01$). When activity change at onset of estrus increased by an index value of 10, cows had 1.38 greater odds of an estrus event with high PA. A greater percentage of cows with no short inter-estrus interval had high PA (76.1%) compared to cows with short inter-estrus intervals (50.2%; $P < 0.01$). The nadir of the change of weighted rumination was associated with PA ($P < 0.01$) such that an increase by 10 index values had 0.6 lesser odds of reaching an estrus event with high PA. Somatic cell count was associated with PA (< 100 x 10^3/mL: 65.8%; 100 – 400 x 10^3/mL: 64.3%; 401 – 1,000 x 10^3/mL: 69.0%; > 1,000 x 10^3/mL: 57.0%; $P = 0.01$). An estrus event with high PA was more likely for cows with a SCC < 1,000 compared to cows with a SCC > 1,000 ($P = 0.01$). Milk yield ($P = 0.51$), milk fat ($P = 0.11$), milk protein ($P = 0.25$), lactose ($P = 0.24$), and MUN ($P = 0.21$) had no impact on the percentage of cows reaching high PA.

In order to evaluate whether the association between the DHIA data and PA was dependent on the timing of the DHIA test relative to the estrus event we only considered DHIA test data that occurred before an estrus event in a separate analysis including 3,307 AI services. Test day milk yield was negatively associated with PA ($P = 0.02$). An increase of test day milk yield by 10 kg was associated with reduced odds for an estrus event with high PA (OR = 0.9). Milk protein ($P = 0.01$) and lactose ($P = 0.07$) were positively associated with PA, as a 1- percent increase of milk protein or lactose was associated with a 1.4 greater risk of high PA. A high SSC before an estrus event decreased the risk of an estrus event with high PA (< 100 x 10^3/mL: 76.4%; 100 – 400 x 10^3/mL: 72.0%; 401 – 1,000 x 10^3/mL: 75.4%; > 1,000 x 10^3/mL: 65.6%; $P < 0.01$). Milk urea nitrogen concentration before an estrus event tended to

be associated with PA (< 150 mg/L: 67.9%, 150 – 300 mg/L: 75.7%, > 300 mg/L: 72.9%; P = 0.06).

3.5.3 Risk Factors for P/AI

Overall P/AI was 25.6%. Factors associated with P/AI are shown in Table 6. Primiparous cows (29.4%) had greater P/AI compared to multiparous cows (22.1%; P < 0.01). Days in milk at AI were negatively associated with P/AI (P < 0.01). When DIM increased by 100 days, odds for pregnancy were 0.76. Temperature-Humidity-Index had an impact on P/AI (P < 0.01), as a low THI resulted in greater P/AI compared to a high THI (THI < 50: 32.6%; THI 50 – 55: 30.0%; THI 56 – 60: 32.2%; THI 61 – 65: 26.2%; THI 66 – 70: 25.2%; THI >70: 12.3%; Figure 2C). Pregnancy per AI was greater when THI was < 60 (P < 0.01) compared to a THI > 60. Cows tended (P = 0.07) to have similar P/AI when THI was 51 – 55 and 61 to 65. Similar P/AI (P = 0.65) was reached for a THI from 61 to 70. A THI > 70 decreased P/AI compared to all the other THI categories below. The activity change at onset of estrus was not associated with P/AI (P = 0.27). There was no association between the nadir of the change of weighted rumination and P/AI (P = 0.80). Whereas DU was not associated with P/AI (P = 0.27), we observed greater P/AI for cows with high PA (28.5%) than for cows with low PA (22.8%; P < 0.01). Pregnancy per AI was greater (P < 0.01) for cows with no short inter-estrus intervals (32.8%) than for cows with short inter-estrus intervals (19.5%). There was no association between P/AI and milk yield (P = 0.87), milk fat (P = 0.14), milk protein (P = 0.67), SCC (P = 0.82), lactose (P = 0.61), or MUN (P =0.84).

In order to evaluate whether the association between the DHIA data and P/AI was dependent on the timing of the DHIA test relative to the estrus event we only considered DHIA test data that occurred before an estrus event in a separate analysis including 3,307 AI services. Lactose was positively associated with P/AI (P < 0.01), such that a 1-percent increase in lactose was associated with 2.19 greater odds of pregnancy.

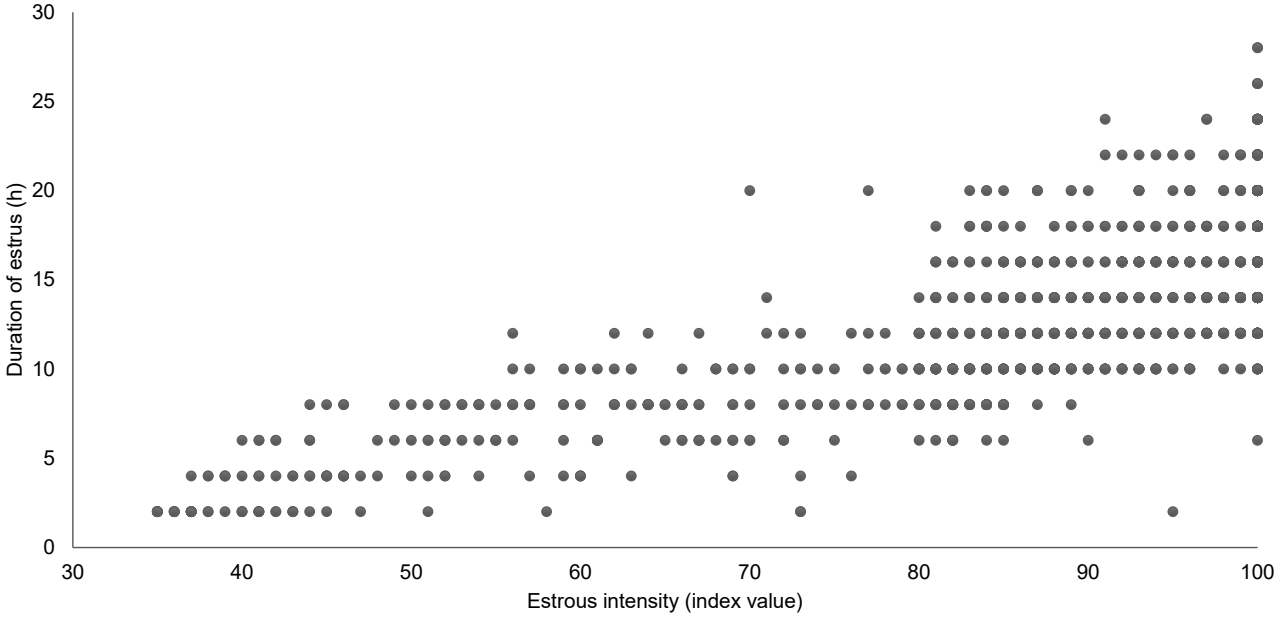
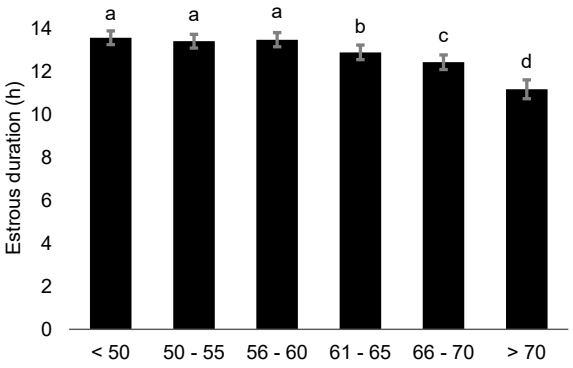
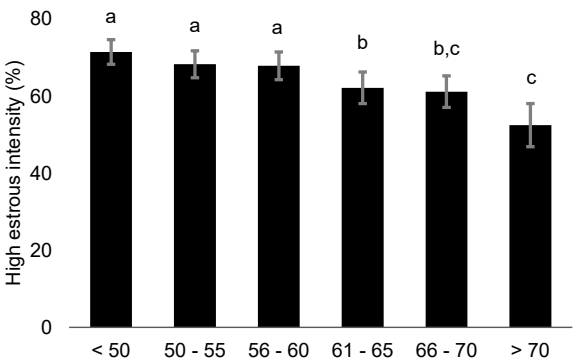


Figure 1. Association between peak of activity change and estrous duration (h) for 5,933 estrus events from 3,132 lactating dairy cows using a neck-mounted accelerometer system for heat detection (Heatime Pro; SCR Engineers Ltd., Netanya, Israel; r = 0.67; $P < 0.01$). Estrous intensity was represented by the peak index value of activity change during an estrus event. Estrous duration was defined as the interval from onset to end of an estrus event. Onset of estrus was defined as a cow exceeding an activity change index value of 35. End of estrus was defined by the first instance at which the index value fell below 35 again.

A


Estrous duration (h)
0
2
4
6
8
10
12
14
a
a
a
b
c
d
< 50
50 - 55
56 - 60
61 - 65
66 - 70
> 70

B


High estrous intensity (%)
0
20
40
60
80
a
a
a
b
b,c
c
< 50
50 - 55
56 - 60
61 - 65
66 - 70
> 70

C

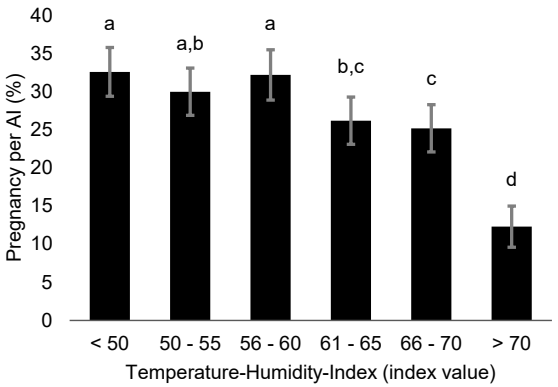

Pregnancy per AI (%)
0
5
10
15
20
25
30
35
40
a
a,b
a
b,c
c
d
< 50
50 - 55
56 - 60
61 - 65
66 - 70
> 70
Temperature-Humidity-Index (index value)

Figure 2. Association between average temperature-humidity-index (THI; index value) 1 wk before artificial insemination (AI) and estrous duration (h) (± SEM; Panel A), percentage of cows with high estrous intensity (%) (± SEM; Panel B), and pregnancy per AI (%) (± SEM; Panel C) using a neck-mounted accelerometer system for heat detection (Heatime Pro; SCR Engineers Ltd., Netanya, Israel). The THI was calculated as (1.8 × T + 32) - ((0.55 - 0.0055 × RH) × (1.8 × T - 26)), where T = temperature and RH = relative humidity and classified into the categories < 50 (2,131/5,933), 50 – 55 (1,322/5,933), 56 – 60 (993/5,933), 61 – 65 (680/5,933), 66 – 70 (633/5,933), and > 70 (174/5,933). Estrous intensity was represented by the peak index value of activity change during an estrus event. Estrous duration was defined as the interval from onset to end of an estrus event. Onset of estrus was defined as a cow exceeding an activity change index value of 35. End of estrus was defined by the first instance at which the index value fell below 35 again. Estrous intensity was categorized as low (35 to 89 index value) and high (90 to 100 index value). Bars with different letters denote statistical differences ($P < 0.05$).

Table 1. Descriptive information of enrolled dairy farms

	Farm							
Item	1	2	3	4	5	6	7	8
Average herd size	1,224	716	427	400	878	588	558	890
Average 305-d milk yield (kg)	9,939	9,677	9,461	11,900	11,428	9,105	9,938	10,520
Milking frequency per day	2	2	2	3	3	2	3	3
Average milk fat (%)	3.91	3.86	3.83	3.59	3.82	4.00	4.20	4.09
Average milk protein (%)	3.38	3.43	3.42	3.53	3.32	3.53	3.52	3.47
Average milk urea nitrogen (mg/L)	234	240	215	197	244	234	236	253
Average somatic cell count (x 10^3 cells/mL)	310	261	252	177	282	258	258	286
Voluntary waiting period (DIM)	42	40	42	70	60	40	42	40
Heat detection rate (%)	41	40	35	52	43	49	57	48
Conception rate (%)	28	28	38	42	33	36	28	35
21-d pregnancy rate (%)	12	11	13	22	14	18	16	17
Day of pregnancy diagnosis after AI	42 ± 3	30 ± 3	40 ± 3	38 ± 3	43 ± 3	30 ± 3	30 ± 3	38 ± 3
Method of pregnancy diagnosis	Transrectal palpation	Transrectal ultrasound	Transrectal palpation	Transrectal palpation	Transrectal palpation	Transrectal ultrasound	Transrectal ultrasound	Transrectal palpation

Table 2. Univariate associations between estrous duration (DU)[1], estrous intensity (PA)[2], or pregnancy per AI (P/AI[2]) and selected categorical risk factors of 8 commercial dairy farms (5,933 AI services from 3,132 cows)

Risk factors	N	Estrous duration		High estrous intensity		Pregnancy per AI	
		h	*P*-value	%	*P*-value	%	*P*-value
Parity			< 0.01		0.31		< 0.01
Primiparous	1,868	14.69 ± 0.33		75.1[a]		44.2[a]	
Multiparous	4,065	15.06 ± 0.32		73.8[a]		34.8[b]	
THI 1 wk before AI			< 0.01		< 0.01		< 0.01
< 50	2,131	15.32 ± 0.30[a]		77.7[ab]		40.9[a]	
50 – 55	1,322	15.08 ± 0.30[a]		74.9[bc]		37.8[a]	
56 – 60	993	15.19 ± 0.31[a]		74.8[abc]		40.4[a]	
61 – 65	680	14.61 ± 0.33[b]		70.9[cd]		32.9[b]	
66 – 70	633	13.99 ± 0.33[c]		68.1[d]		31.9[b]	
> 70	174	11.99 ± 0.44[d]		51.0[e]		16.3[c]	
Estrous Intensity[3]							< 0.01
Low	1,571					31.6[a]	
High	4,362					39.9[b]	
Short inter-estrus interval[4]			< 0.01		< 0.01		< 0.01
No	5,655	15.06 ± 0.31		75.3[a]		38.5[a]	
Yes	278	12.20 ± 0.41		52.4[b]		21.7[b]	
Milk urea nitrogen (mg/L)			0.10		0.21		0.84
< 150	343	14.44 ± 0.40[a]		70.3[a]		45,7[a]	
150 – 300	5,160	14.98 ± 0.32[b]		74.7[a]		42.3[a]	

> 300	430	15.09 ± 0.39[b]		73.5[a]	43.1[a]
Somatic cell count (x 10^3 cells/mL)			< 0.01	< 0.01	0.08
< 100	3,813	15.02 ± 0.31[a]		75.2[a]	38.9[a]
100 – 400	1,289	14.91 ± 0.33[a]		73.0[a]	35.6[b]
401 – 1,000	454	15.02 ± 0.37[a]		76.5[a]	37.0[ab]
> 1,000	377	14.03 ± 0.38[b]		65.0[b]	33.9[ab]

[a-d] Within a column with more than two categories, proportions with different superscripts differ ($P < 0.05$).

[1] LSM for estrous duration among levels of risk factors using linear regression after adjusting for farm as a random effect.

[2] LSM for probability of high intensity estrus and P/AI among risk factors using logistic regression after adjusting for farm as a random effect.

[3] Estrous intensity was categorized as low (35 to 89 index value) and high (90 to 100 index value).

[4] No short inter-estrus intervals were defined when a cow had one activity peak within an observational period of 7 d close to estrus (i.e., exceeding a threshold of 35, reaching an individual peak of activity and falling below 35 afterwards for one time within an observational period of 7 d). Short inter-estrus interval was defined when a cow had more than one activity peak within an observational period of 7 d close to estrus with a drop of activity change index below 35 between them.

Table 3. Univariate associations between estrous duration (DU)[1], estrous intensity (PA)[2,3], or pregnancy per AI (P/AI)[2] and selected continuous risk factors of 8 commercial dairy farms (5,933 AI services from 3,132 cows)

Risk factors	Estrous duration (h)		High estrous intensity (%)		Pregnancy per AI (%)	
	Estimate ± SE[4]	*P*-value	OR[5] (95% CI)	*P*-value	OR (95% CI)	*P*-value
DIM at AI / 100	0.80 ± 0.06	< 0.01	1.11 (1.03, 1.20)	0.01	0.75 (0.70, 0.81)	< 0.01
Onset of estrus (index value)[6] / 10	0.04 ± 0.04	0.30	1.40 (1.34, 1.45)	< 0.01	1.02 (0.99, 1.05)	0.27
Change of weighted rumination (index value)[7] / 10	- 0.80 ± 0.03	< 0.01	0.59 (0.57, 0.62)	< 0.01	0.96 (0.93, 0.99)	< 0.01
Milk yield (kg) / 10	- 0.36 ± 0.07	< 0.01	0.99 (0.98, 1.00)	< 0.01	1.00 (0.99, 1.01)	0.87
Milk fat (%)	0.28 ± 0.08	< 0.01	1.09 (1.01, 1.18)	0.02	0.95 (0.89, 1.02)	0.14
Milk protein (%)	1.56 ± 0.18	< 0.01	1.30 (1.09, 1.56)	< 0.01	0.79 (0.67, 0.93)	0.01
Milk lactose (%)	- 0.14 ± 0.32	0.67	1.49 (1.10, 2.03)	0.01	1.99 (1.48, 2.68)	< 0.01

[1] LSM for estrous duration among levels of risk factors using linear regression after adjusting for farm as a random effect.

[2] LSM for probability of high intensity estrus and P/AI among risk factors using logistic regression after adjusting for farm as a random effect.

[3] Estrous intensity was categorized as low (35 to 89 index value) and high (90 to 100 index value).

[4] SE = standard error of the estimate.

[5] OR = odds ratio

[6] Onset of estrus was defined a cow exceeding an activity change index value of 35.

[7] Raw rumination data from each cow was converted internally into the change of weighted rumination using a proprietary algorithm. Change of weighted rumination is the difference of today's last hours of rumination from the mean of last wk rumination in the same period of the day weighted by the standard deviation of this specific cow. Index values for the change of weighted rumination ranged from + 100 to – 100.

Table 4. Final linear regression model[1] for association between risk factors and estrous duration (DU) of 8 commercial dairy farms (5,933 AI services representing 3,132 cows)

Variable	Estimated estrous duration (h)	SE[2]	95% CI		*P* - value
			Lower CI	Upper CI	
Intercept	11.35	0.46	10.45	12.24	< 0.01
Parity					< 0.01
Primiparous	Referent				
Multiparous	0.64	0.13	0.38	0.89	
DIM at AI / 100	0.81	0.08	0.66	0.96	< 0.01
THI 1 wk before AI					< 0.01
< 50	Referent[a]				
50 – 55	- 0.16[a]	0.15	- 0.46	0.14	0.29
56 – 60	- 0.09[a]	0.17	- 0.42	0.23	0.57
61 – 65	- 0.68[b]	0.19	- 1.05	- 0.32	< 0.01
66 – 70	- 1.14[c]	0.19	- 1.52	- 0.76	< 0.01
> 70	- 2.40[d]	0.33	- 3.06	- 1.75	< 0.01
Change of weighted rumination (index value)[3] / 10	- 0.78	0.03	- 0.83	- 0.72	< 0.01
Short inter-estrus interval[4]					< 0.01
No	Referent				
Yes	- 2.93	0.26	- 3.43	- 2.42	
Milk yield (kg) / 10	- 0.20	0.08	- 0.36	- 0.05	0.01

Somatic cell count (x 10^3 cells/mL)					< 0.01
< 100	Referent[a]				
100 – 400	- 0.22[a]	0.14	- 0.49	0.05	0.11
401 – 1,000	- 0.08[a]	0.21	- 0.50	0.33	0.70
> 1,000	- 0.93[b]	0.23	- 1.38	- 0.48	< 0.01

[a-d] Within a column with more than two categories, proportions with different superscripts differ ($P < 0.05$).

[1] Model adjusted for the random effect of farm.

[2] SE = standard error of the estimate.

[3] Raw rumination data from each cow was converted internally into the change of weighted rumination using a proprietary algorithm. Change of weighted rumination is the difference of today's last hours of rumination from the mean of last wk rumination in the same period of the day weighted by the standard deviation of this specific cow. Index values for the change of weighted rumination ranged from + 100 to – 100.

[4] No short inter-estrus intervals were defined when a cow had one activity peak within an observational period of 7 d close to estrus (i.e., exceeding a threshold of 35, reaching an individual peak of activity and falling below 35 afterwards for one time within an observational period of 7 d). Short inter-estrus interval was defined when a cow had more than one activity peak within an observational period of 7 d close to estrus with a drop of activity change index below 35 between them.

Table 5. Final logistic regression model[1] for association between risk factors and estrous intensity (PA)[2] for 5,933 AI services representing 3,132 cows (1,094 primiparous and 2,038 multiparous cows) from 8 commercial dairy farms in Germany

			95% CI			
Variable	Estimated PA (%)	SE[3]	Lower CI	Upper CI	Odds Ratio	*P* - value
Intercept	- 2.74	0.21	- 3.15	- 2.32	0.07	< 0.01
DIM at AI / 100	0.16	0.04	0.07	0.24	1.17	< 0.01
THI 1 wk before AI						< 0.01
< 50	Referent[a]					
50 – 55	- 0.15[a]	0.09	- 0.33	0.03	0.86	0.10
56 – 60	- 0.17[a]	0.10	- 0.37	0.03	0.84	0.10
61 – 65	- 0.42[b]	0.11	- 0.64	- 0.21	0.66	< 0.01
66 – 70	- 0.47[b,c]	0.11	- 0.68	- 0.25	0.63	< 0.01
> 70	- 0.82[c]	0.18	- 1.17	- 0.47	0.44	< 0.01
Onset of estrus (index value)[4] / 10	0.32	0.02	0.28	0.37	1.38	< 0.01
Change of weighted rumination (index value)[5] / 10	- 0.51	0.02	- 0.56	- 0.47	0.60	< 0.01
Short inter-estrus interval[6]						< 0.01
No	Referent					
Yes	- 1.15	0.14	- 1.42	- 0.88	0.32	
Somatic cell count (x 10^3 cells/mL)						0.01
< 100	Referent[a]					

100 – 400	- 0.06[a]	0.08	- 0.22	0.10	0.94	0.43
401 – 1,000	0.15[a]	0.13	- 0.10	0.39	1.16	0.25
> 1,000	- 0.37[b]	0.13	- 0.62	- 0.12	0.69	< 0.01

[a-d] Within a column with more than two categories, proportions with different superscripts differ ($P < 0.05$).

[1] Model adjusted for the random effect of farm.

[2] Estrous intensity was categorized as low (35 to 89 index value) and high (90 to 100 index value).

[3] SE = standard error of the estimate.

[4] Onset of estrus was defined a cow exceeding an activity change index value of 35.

[5] Raw rumination data from each cow was converted internally into the change of weighted rumination using a proprietary algorithm. Change of weighted rumination is the difference of today's last hours of rumination from the mean of last wk rumination in the same period of the day weighted by the standard deviation of this specific cow. Index values for the change of weighted rumination ranged from + 100 to – 100.

[6] No short inter-estrus intervals were defined when a cow had one activity peak within an observational period of 7 d close to estrus (i.e., exceeding a threshold of 35, reaching an individual peak of activity and falling below 35 afterwards for one time within an observational period of 7 d). Short inter-estrus interval was defined when a cow had more than one activity peak within an observational period of 7 d close to estrus with a drop of activity change index below 35 between them.

Table 6. Final logistic regression model[1] for association between risk factors and pregnancy per AI (P/AI) for 5,933 AI services representing 3,132 cows (1,094 primiparous and 2,038 multiparous cows) from 8 commercial dairy farms in Germany

Variable	Estimated P/AI (%)	SE[2]	95% CI		Odds Ratio	*P* - value
			Lower CI	Upper CI		
Intercept	0.04	0.15	-0.25	0.33	1.04	0.78
Parity						< 0.01
Primiparous	Referent					
Multiparous	- 0.39	0.06	-0.50	-0.27	0.68	
DIM at AI / 100	- 0.28	0.04	- 0.35	- 0.20	0.76	< 0.01
THI 1 wk before AI						< 0.01
< 50	Referent[a]					
50 – 55	- 0.12[a,b]	0.08	- 0.27	0.03	0.89	0.12
56 – 60	- 0.02[a]	0.08	- 0.18	0.15	0.98	0.83
61 – 65	- 0.31[b,c]	0.10	- 0.50	- 0.12	0.74	< 0.01
66 – 70	- 0.36[c]	0.10	- 0.56	- 0.16	0.70	< 0.01
> 70	- 1.24[d]	0.21	- 1.66	- 0.82	0.29	< 0.01
Short inter-estrus interval[3]						< 0.01
No	Referent					
Yes	- 0.70	0.15	- 1.00	- 0.40	0.50	
Estrous Intensity[4]						< 0.01
Low	Referent					
High	0.30	0.07	0.17	0.43	1.35	

[a-d] Within a column with more than two categories, proportions with different superscripts differ ($P < 0.05$).

[1] Model adjusted for the random effect of farm.

[2] SE = standard error of the estimate.

[3] No short inter-estrus intervals were defined when a cow had one activity peak within an observational period of 7 d close to estrus (i.e., exceeding a threshold of 35, reaching an individual peak of activity and falling below 35 afterwards for one time within an observational period of 7 d). Short inter-estrus interval was defined when a cow had more than one activity peak within an observational period of 7 d close to estrus with a drop of activity change index below 35 between them.

[4] Estrous intensity was categorized as low (35 to 89 index value) and high (90 to 100 index value).

3.6 Discussion

The objective of this observational study was to identify risk factors associated with estrous expression and subsequent P/AI in dairy cows using AAM. Most of the risk factors associated with estrous intensity and duration had an equal impact on subsequent P/AI. Duration of estrus and PA were highly correlated, and particularly cows with high PA were associated with greater odds for pregnancy. Interestingly, not all the risk factors associated with estrous expression were necessarily associated with the subsequent risk of pregnancy.

Duration of estrus (14.94 ± 0.06 h) was similar to those reported by others using neck-mounted AAM for estrus detection (Aungier et al., 2015; Madureira et al., 2015a; Burnett et al., 2017). Few studies using AAM reported a high variability in DU (Valenza et al., 2012; Stevenson et al., 2014) that possibly contributes to incorrect timing of AI relative to ovulation leading to poor fertility in some cows. In contrast to other studies (Burnett et al., 2017; Burnett et al., 2018), DU was not associated with P/AI in the final statistical model after accounting for estrous intensity. This was due to the high correlation between DU and PA as already reported earlier by others (Madureira et al., 2015a). Intensity of estrus was found to be associated with P/AI, as cows with high PA had higher odds for pregnancy (OR = 1.35) compared to cows with low PA. In general, 73.5% of estrus events were of high PA, a percentage that appears above average compared to other studies evaluating PA using neck- or leg-mounted AAM (Madureira et al., 2015a; Polsky et al., 2017; Madureira et al., 2019). Direct comparisons among studies remain difficult, as studies differed in the type of AAM sensor used, in their experimental design (e.g., inclusion of timed AI), and their definition for low versus high PA. Substantial increases in P/AI for dairy cows showing a greater PA using neck-mounted AAM or a greater relative increase in activity using leg-mounted AAM have already been demonstrated earlier (Polsky et al., 2017; Burnett et al., 2018; Madureira et al., 2019). One study (Madureira et al., 2015a) noted an approximately 12 percentage units greater P/AI for cows receiving first postpartum AI with high PA compared to those with low PA. Some studies were not able to detect an association between estrous expression and subsequent P/AI (Yániz et al., 2006; Aungier et al., 2015; Burnett et al., 2017). These studies, however, are limited in their external validity and not comparable with previous research, as they were evaluating activity only during the first 50 DIM (Yániz et al., 2006), from seasonal calving pasture based cows (Aungier et al., 2012), or of insufficient sample size for showing an effect (Burnett et al., 2017). Lack of estrous expression in cows may be due to increased P4 concentrations around the time of estrus blocking the effects of estradiol (**E2**) in the region of the hypothalamus responsible for estrous behavior (Allrich, 1994; Woelders et al., 2014). Estradiol and expression of estrus near the time of AI were attributed a key role during fertilization, early embryonic development and

maintenance of pregnancy (Buhi et al., 2002; Galvão et al., 2004; Pereira et al., 2016). It was shown that cows with low PA had an increased risk of ovulation failure and a larger variation in ovulation timing (Madureira et al., 2019; Burnett et al., 2018). Moreover, cows with high PA were found to have a P4 profile on d 10 of the estrous cycle favorable for embryo development (Madureira et al., 2015b), endometrium receptivity and embryo survival (Davoodi et al., 2016).

Interestingly, in our study we observed about 5% of cows with short inter-estrus intervals. In a large sample of UK dairy herds including 42,252 cows from 159 herds (Remnant et al., 2015), the median interval between AI services was 22 d with a range of 18 to 28 d. They observed various factors to have an association with the inter-service interval and expected them to have a similar association with the inter-ovulatory interval, including parity, DIM, and AI service number. As we conducted a large observational, multi-site field study, assessment of information about ovarian profiles was not feasible and the reason for an association between short inter-estrus intervals and decreased DU, PA and P/AI remains speculative. Cows with short inter-estrus intervals as expressed here should therefore be examined more thoroughly in future studies to elucidate the physiological mechanisms (e.g., ovulation failure, delayed ovulation, hormonal profiles around estrus and after AI) behind their reduced fertility.

In the present study, THI 1 wk before AI had a severe impact on estrous expression and subsequent P/AI. Increasing THI decreased DU, PA, and P/AI, with the greatest drop if THI was above 70. Schüller et al. (2014) reported a negative effect of heat stress on P/AI both before and after the day of breeding. They found a THI ≥ 73 within 21 to 1 d before breeding had the greatest negative impact on P/AI, but also exposure to THI ≥ 73 for 1 h on the day of breeding was sufficient to decrease P/AI by 5%. Negative associations between heat stress and estrous expression or P/AI of dairy cows have also been demonstrated by others (López-Gatius et al., 2005; Peralta et al., 2005; Polsky et al., 2017). Heat stress was reported to reduce E2 and to increase P4 concentrations, which may lead to ovulation without expression of estrus (Roth et al., 2000; De Rensis and Scaramuzzi, 2003; Schüller et al., 2017). Reduced quality of the oocyte, as well as inhibited embryonic development and an increased risk of early embryonic death are associated with heat-induced changes in the uterine environment and luteal prostaglandin secretion (Roth et al., 2000; De Rensis and Scaramuzzi, 2003). Heat stress abatement strategies such as mechanical ventilation are pivotal to avoid poor reproductive performance during summer months (Mondaca, 2019). In addition, timed AI protocols (Hansen and Arechiga, 1999) or the combined use of different methods for estrus detection, e.g., AAM and visual observation, instead of using a single method (Peralta et al., 2005) can be used to improve reproductive performance during heat stress.

There was a strong association between rumination and estrous expression. A lower nadir of weighted rumination change resulted in longer DU and higher PA, but was not associated with P/AI. The decrease in rumination was reported to be between 17 to 19% during estrus or the day of AI (Reith and Hoy, 2012; Reith et al., 2014a; Pahl et al., 2015), but with high variations among cows and a longer average rumination time for multiparous cows (Reith and Hoy, 2012; Pahl et al., 2015). Changes in feeding behavior have been linked to restlessness and increasing physical activity of cows in estrus (Van Vliet and Van Eerdenburg, 1996). Our study is the first to evaluate associations between the change of weighted rumination during estrus and duration and intensity of estrus as well as subsequent P/AI. In the study from Reith et al. (2014a), clear estrus-related deviations from base levels were measured much earlier for rumination time than for activity. This finding again underlines the relevance of considering more than one behavioral trait for identifying cows in estrus, e.g., complementary incorporation of rumination time and changes when using AAM for estrus detection (Reith and Hoy, 2012; Reith et al., 2014b; Pahl et al., 2015).

Parity was associated with DU and P/AI, with multiparous cows (13.15 ± 0.31 h) having slightly longer DU than primiparous cows (12.52 ± 0.32 h), but a greater P/AI for primiparous (29.4%) than for multiparous cows (22.1%). Greater P/AI for primiparous cows is well known (Stevenson et al., 2014; Polsky et al., 2017). A possible reason may be the reduced risk of metabolic disorders for primiparous cows during early lactation (Tenhagen et al., 2004; Santos et al., 2010). Controversial results regarding the association between parity and physical activity exist and may in part be due to the use of (pre-) synchronization protocols, animals per pen, or insufficient sample size.

Interestingly, we found estrus events of long DU or high PA to be more likely at later DIM, whereas DIM were negatively associated with P/AI. There are only few studies also reporting shorter DU at earlier DIM (Burnett et al., 2018) and a lower relative increase in activity for cows at early DIM compared to cows at later DIM (Polsky et al., 2017). Authors from the latter study speculated that cows at later DIM could express more intense estrus, as they are more likely to have lower metabolic challenges. Furthermore, the absence of P4 during the first cycle postpartum may be responsible for decreased estrous intensity. The association between DIM and P/AI is controversial, as some authors reported no effect of DIM on P/AI (López-Gatius et al., 2005; Burnett et al., 2018) or greater P/AI for earlier DIM (Polsky et al., 2017). Most of the studies, however, observed greater P/AI for an AI occurring later in lactation (Tenhagen et al., 2003; Gábor et al., 2008; Friggens and Labouriau, 2010). In our study, the negative association between DIM and P/AI might be biased by selection of less infertile cows in later lactation, as cows with a negative pregnancy diagnosis remained in the study and received subsequent AI services. After all, it remains speculative whether these cows were

less fertile overall or if other factors, such as management, health issues or timing of AI also contributed to this interaction. Among others, BCS loss due to a negative energy balance is a major risk factor for estrous expression and fertility in lactating dairy cows (Aungier et al., 2012; Madureira et al., 2015a; Burnett et al., 2017). An extended delay of first AI was suggested in some studies for high yielding cows to enhance reproductive efficiency or profitability of cows (Tenhagen et al., 2003; Stangaferro et al., 2018; Niozas et al., 2019). High milk yield was associated with a decrease in walking activity (Adewuyi et al., 2006) and fertility (Butler, 2003; Wathes et al., 2007). In the present study, increasing milk yield marginally decreased DU, i.e., each 10 kg milk yield increase shortened DU by 0.2 h. Intensity of estrus was negatively associated with increasing milk yield from a DHIA test before an estrus event. It has been shown that high yielding cows have a higher feed intake and an increased metabolic clearance of E2 due to an increase in liver blood flow, making it more difficult to identify these cows in estrus (Sangsritavong et al., 2002; Wiltbank et al., 2006). In fact, some studies reported a negative association between estrous expression and increased milk yield (Lopez et al., 2004; Yániz et al., 2006; Madureira et al., 2015a), whereas others did not find an association (Van Eerdenburg, 2008; Valenza et al., 2012; Burnett et al., 2017). One study reported that each 1 kg increase in milk yield was associated with a 1.6% decrease in walking activity measured by a pedometer (López-Gatius et al., 2005). Consistent with our and numerous previous studies (Yániz et al., 2006; Polsky et al., 2017; Burnett et al., 2018) an association between milk yield and P/AI did not exist. In contrast, milk yield was found to affect P/AI in other studies (Royal et al., 2000; Valenza et al., 2012). A common perception is that high yielding cows have the greatest energy deficit, but cows with a higher milk yield were not necessarily those with a more severe or prolonged negative energy balance or with the greatest loss of BCS (Lucy, 2001). It should be questioned whether increasing milk yield inevitably causes decreased fertility or whether high production rather increases the demands on metabolism and management, which may not always be met (LeBlanc, 2010).

The association between elevated SCC and P/AI seems to be more consistent in the literature, as numerous studies reported reduced fertility for cows with both subclinical (Schrick et al., 2001; Lavon et al., 2011) and clinical (König et al., 2006; Hudson et al., 2012; Fuenzalida et al., 2015) mastitis. Comparison among studies remains difficult, however, because of their inconsistency on thresholds for defining elevated SCC and the breeding risk periods (SCC data from before and/or after DHIA test date). One study observed a 10 percentage units decrease in P/AI in cows with elevated SCC (> 200 x 10^3 cells/mL) 0 to 4 weeks before AI (Bijker et al., 2015). A possible explanation for the negative effects of mastitis occurring before AI could be a reduced pulsatile LH secretion, causing low E2 concentrations and a delayed ovulation in affected cows (Hockett et al., 2005; Lavon et al., 2010). One study found reduced

odds of pregnancy by 25% for cows with subclinical mastitis (> 150 x 10^3 cells/mL) within 3 d before to 32 d after timed AI (Fuenzalida et al., 2015). This was confirmed by another study evaluating data from more than 5,000,000 Holstein cows participating in the French Milk Control Program (Albaaj et al., 2017). They found a stronger negative association between an elevated SCC after AI compared to an elevated SCC before AI. Irrespective of timing of the DHIA test, we could not confirm results from previous studies, as P/AI was not influenced by elevated SCC in our study. Elevated SCC, on the other hand, decreased DU and PA, which in part is supported by a study (Morris et al., 2013) that found a tendency for lower PA in cows with elevated SCC (> 100 x 10^3 cells/mL) compared to cows with low SCC. Further research is warranted to elucidate the association between estrous expression and elevated SCC.

We found no association between estrous expression or P/AI and milk fat, milk protein, and MUN. We only observed a tendency for MUN from the DHIA test before an estrus event to be associated with PA. Milk urea nitrogen can be used for monitoring the protein-energy balance and for diagnosing feeding disorders (Eicher et al., 1999). It was suggested that high urea concentrations have detrimental effects on fertilization and embryonic development (Holtz et al., 1986; Broderick and Clayton, 1997). Studies evaluating associations between MUN and fertility, however, have been controversial. Whereas some support our finding that there is no association between MUN and the risk of pregnancy (Mucha and Strandberg, 2011; Löf et al., 2014), others found fertility to be negatively affected by high (Rajala-Schultz et al., 2001; Hojman et al., 2004; König et al., 2008) or either high or low (Pehrson et al., 1992) MUN concentrations. A study evaluating data from 60 commercial Ontario Holstein dairy herds (Godden et al., 2001) found the greatest odds of pregnancy for either low or high MUN concentrations within 45 d before AI, whereas MUN after AI did not affect P/AI. Equivocal results might be caused by factors involved, such as parity, lactation stage, health status, and season (Gooden et al., 2001; Wood et al., 2003; Hojman et al., 2004). Also DHIA test date, sampling time and feeding to sampling interval have been reported to highly influence MUN concentrations (Godden et al., 2001; Wood et al., 2003).

3.7 Study Limitations

As we conducted this experiment as a retrospective, observational study, there was no option of evaluating effects due to different farm management possibly associated with estrous expression and/or P/AI, such as pen stocking density, flooring, or heat abatement. Furthermore, we were not able to address associations between health issues and our outcome variables. As this was a multi-site study, an objective observation and consistent definition of cow's diseases was not feasible. External validity was improved by enrolling cows from 8 commercial farms in contrast to a more controlled study design on a single farm.

Another limitation of our study was that we did not include information about cow's breeding values. There are recent publications pointing out a genetic merit for fertility traits and its pronounced effects on estrous behavior (Veronese et al., 2019; Chebel and Veronese, 2020; Pinedo et al., 2020).

3.8 Conclusions

Results from the present study support the utilization of estrous expression measured by AAM for prediction of fertility and incorporation into management decisions to improve herd reproductive efficiency. Duration of estrus and PA were highly correlated, and particularly cows with high PA were associated with greater odds for pregnancy. However, risk factors, such as increased THI and short inter-estrus intervals were identified to be negatively associated with estrous expression and P/AI in dairy cows. For example, increasing THI decreased DU, PA, and subsequent P/AI, with the greatest decrease if THI was above 70. In addition, results from this study clearly show the negative impact of heat stress on fertility and emphasize the importance of heat abatement strategies in lactating dairy cows to improve reproductive performance. Furthermore, AAM systems have the potential to identify cows with short inter-estrus intervals. These cows should be the focus of future studies.

Interestingly, not all the risk factors associated with estrous expression (e.g., change of weighted rumination, milk yield, elevated SCC) were necessarily associated with the subsequent risk of pregnancy. This observational study highlights the potential of combining activity and rumination data from AAM systems with other information available on farm, such as THI data, to optimize estrus detection and pregnancy outcomes in dairy cows.

3.9 Acknowledgments

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4 PUBLICATION III

Effect of dose and timing of prostaglandin F2α treatments during a 7-d Ovsynch protocol on progesterone concentration at the end of the protocol and pregnancy outcomes in lactating Holstein cows.

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4.1 Abstract

The objective of this study was to evaluate the effect of two prostaglandin $F_{2\alpha}$ (**PGF**) treatments 24 h apart (500 µg of cloprostenol) and treatment with a double PGF dose on d 7 (1,000 µg of cloprostenol) during a 7-d Ovsynch protocol on progesterone (**P4**) concentration and pregnancy per artificial insemination (**P/AI**) in lactating Holstein cows. We hypothesized that treatment leads to a decreased P4 concentration at the second GnRH treatment (**G2**) and an increase in P/AI compared to the traditional 7-d Ovsynch protocol. A secondary hypothesis was that the treatment effect is influenced by the presence of a corpus luteum (**CL**) at the first GnRH treatment (**G1**). Two experiments were conducted on 8 commercial dairy farms in Germany. Once a week, cows from both experiments were assigned in a consecutive manner to receive: (1) Ovsynch (control: GnRH; 7 d, PGF; 9 d, GnRH), (2) Ovsynch with a double PGF dose (**GDPG**: GnRH; 7 d, 2xPGF; 9 d, GnRH), or (3) Ovsynch with a second PGF treatment 24 h later (**GPPG**: GnRH; 7 d, PGF; 8 d, PGF; 32 h, GnRH). All cows received timed AI (**TAI**) approximately 16 h after G2. Pregnancy diagnosis was performed by transrectal palpation (38 ± 3 d after TAI, experiment 1) or transrectal ultrasonography (35 ± 7 d after TAI, experiment 2). Whereas farms from experiment 1 used a Presynch-Ovsynch protocol (PGF, 14 d later PGF, 12 d later GnRH, 7 d later PGF, 2 d later GnRH, and 16 to 18 h later TAI) to facilitate first postpartum TAI, no presynchronization protocol was used on farms from experiment 2. In experiment 1, we enrolled 1,581 lactating dairy cows (60 experimental units) from 2 dairy farms. At G2, blood samples were collected from a subsample of cows (n = 491; 16 experimental units) to determine P4 concentration at G2. In experiment 2, we enrolled 1,979 lactating dairy cows (252 experimental units) from 6 dairy farms. Transrectal ultrasonography was performed to determine the presence or absence of a CL at G1. In experiment 1, treatment affected P/AI ($P = 0.01$) and P/AI was greater for GDPG (38.2%) and GPPG (38.9%) than for control cows (29.8%). Both, GDPG and GPPG cows had decreased P4 concentration at G2 compared with control cows ($P < 0.01$). Whereas both treatments increased the percentage of cows with very low P4 concentration (0.00 to 0.09 ng/mL) at G2, only the GPPG treatment decreased the percentage of cows with high P4 concentration (≥0.6 ng/mL) at G2 compared to the control group. In experiment 2, P/AI was greater for GPPG (37.4%) than for control cows (31.0%; $P = 0.03$) and tended to be greater than for GDPG cows (31.8%; $P = 0.05$). Cows from the GDPG group had similar ($P = 0.77$) P/AI compared to the control group. Pregnancy per AI did not differ between cows with a CL at G1 and cows without a CL at G1 (34.1% vs. 32.6%; $P = 0.50$). There was no interaction between treatment and presence of a CL at G1 on P/AI ($P = 0.61$). Combining data from the 2 experiments but excluding cows from experiment 1 receiving presynchronization before first TAI (n = 2,573; 312 experimental units), P/AI was greater for GPPG (40.3%;P < 0.01) than for control (31.8%) and GDPG cows (33.4%).

Between GDPG and control cows, P/AI did not differ (P = 0.46). We conclude that overall the addition of a second cloprostenol treatment on d 8 during a 7-d Ovsynch protocol increased P/AI compared to the traditional 7-d Ovsynch including a single cloprostenol dose on d 7 and to a double PGF dose on d 7. Doubling the cloprostenol dose on d 7 in a 7-d Ovsynch protocol did not affect P/AI. Future studies need to elucidate whether the treatment effect is modified by presynchronization of the first postpartum TAI.

4.2 Key words

prostaglandin, timed artificial insemination, fertility, dairy cow

4.3 Introduction

Protocols for synchronization of ovulation have been developed and widely adopted by the dairy industry to overcome the inefficiency of estrus detection and to allow timed artificial insemination (**TAI**) [1,2]. Presynchronization strategies, such as Presynch-Ovsynch [3,4] and Double-Ovsynch [5,6] were developed to improve fertility to TAI by increasing the percentage of cows at an optimal stage of the cycle at initiation of an Ovsynch protocol. This leads to a higher risk of ovulation after the first GnRH treatment (**G1**) resulting in a newly formed corpus luteum (**CL**) and a more optimum hormonal environment during the protocol [7-9]. The newly induced CL, however, does not regress completely in all cows treated with a single dose of prostaglandin $F_{2\alpha}$ (**PGF**) 7 d later. Cows bearing a mature CL at G1 were less affected by incomplete luteal regression after a single dose of PGF [10]. Although the exact mechanism needs to be determined, it was suggested that the mature CL induced luteal regression in the newly formed CL as well [11-13]. Incomplete luteal regression in a 7-d Ovsynch protocol has been observed in 12 to 21 % of cows treated with a single dose of PGF [13-15]. Incomplete luteal regression in these studies was defined as a progesterone (**P4**) concentration ≥0.4 ng/mL at the time of the second GnRH treatment (**G2**) [10]. Elevated P4 concentration at the end of the Ovsynch protocol may negatively affect oviductal and uterine motility thereby interfering with gamete transport [16]. Furthermore, elevated P4 at G2 was associated with decreased endometrial thickness, which led to compromised fertility after TAI [17].

Elevated P4 concentration at the time of G2 after a single PGF treatment is caused by incomplete luteal regression. The subsequent decrease in pregnancy per AI (**P/AI**) is particularly apparent in cows that started an Ovsynch protocol in a low P4 environment [11,18,19]. In addition, increased P4 at the start of the protocol has a positive impact on growth of the dominant follicle and on luteal regression thus improving fertility [20-22]. To overcome poor fertility caused by elevated P4 concentration because of incomplete luteal regression, two modifications in protocols for TAI have been tested: (1) an increased PGF dose on d 7, and (2) a second PGF treatment on d 8. Increasing the dose of PGF (500 to 750 µg of cloprostenol) in a 7-d protocol increased luteal regression in multiparous cows (+ 8.5%, $P = 0.03$) and tended to increase P/AI in all cows (+ 4.5%, $P = 0.05$) [8]. A recent meta-analysis [23] showed a clear benefit of a second PGF treatment on d 8 in a 7-d Ovsynch protocol regarding complete luteal regression (+11.6% units) and P/AI (+4.6% units).

Two recent studies have directly compared an increased dose (50 mg of dinoprost) and an additional treatment of PGF (25 mg of dinoprost) on d 8 in a 7-d Ovsynch protocol. One study [24] found that both modifications induced complete luteal regression in 96% (double dose of dinoprost) and 100% (second treatment with dinoprost 24 h later) of the cows,

respectively. Subsequent P/AI tended to be greater for cows treated with the double PGF dose (46.1%) than for cows treated with a second PGF dose 24 h later (36.7%). In contrast, Barletta et al. [25] observed that administering a second dose of dinoprost 24 h later improved the percentage of cows showing complete luteal regression (94%) compared with a double dose of dinoprost (89%). In this study, P/AI did not differ among treatments. A similar study comparing the effect of a double dose with a second administration of the PGF analogue cloprostenol in a 7-d Ovsynch protocol has not been reported.

The objective of this study was to compare the effect of two PGF treatments 24 h apart (500 µg of cloprostenol) with the treatment of a double PGF dose (1,000 µg of cloprostenol) during a 7-d Ovsynch protocol on P4 concentration at G2 and P/AI in lactating Holstein cows. Our primary hypotheses were that both treatments lead to a decrease in P4 concentration at G2 and an increase in P/AI compared with the traditional 7-d Ovsynch protocol. A secondary hypothesis was that the treatment effect is influenced by the presence of a CL at the beginning of the protocol.

4.4 Materials and Methods

All experimental procedures herein were conducted with the approval of the Institutional Animal Care and Use Committee of the Freie Universität Berlin.

4.4.1 Cows, Housing and Feeding

This study was conducted on 8 commercial dairy farms in northeast Germany from August 2018 through March 2019 (experiment 1, farms 7 and 8) and from February 2018 through June 2019 (experiment 2, farms 1 to 6), respectively. Average herd size was 674 and ranged from 325 to 1,380 lactating cows (Table 1). Cows were housed in free-stall barns equipped with either slatted (farms 1, 6, 8) or concrete floor (farms 2 to 5, 7). Cows had ad libitum access to feed and water and were fed twice daily a TMR diet consisting of corn silage and grass silage as forage with a corn- and soybean meal-based concentrate. The TMR was formulated to meet or exceed the dietary requirements for dairy cows [26]. Cows were milked twice daily at approximately 12-h intervals. The average milk production (305-d ECM, 4.0% fat, 3.4% protein) was 9,815 kg (range 8,362 to 10,592 kg).

4.4.2 Experiment 1: Treatments and Reproductive Management

Lactating Holstein cows (n = 1,581; 492 primiparous and 1,089 multiparous cows; 60 experimental units) from 2 commercial dairy farms (farms 7 and 8) were used in this experiment. First postpartum TAI was facilitated on both farms using a Presynch-Ovsynch

protocol (PGF, 14 d later PGF, 12 d later GnRH, 7 d later PGF, 2 d later GnRH, and 16 to 18 h later TAI). Cows received AI at detected estrus after the first PGF (farm 7: 80 ± 3 DIM and farm 8: 50 ± 3 DIM) or second PGF treatment (farm 7: 94 ± 3 DIM and farm 8: 64 ± 3 DIM). Cows not inseminated at estrus received TAI (farm 7: 116 ± 3 DIM and farm 8: 86 ± 3 DIM). For subsequent AI, nonpregnant cows received an Ovsynch protocol starting at the day of nonpregnancy diagnosis. All cows not inseminated at estrus were assigned in a consecutive manner to receive 1 of 3 synchronization protocols and TAI (Figure 1). In week 1, 2, and 3, all eligible cows were assigned to protocol 1, 2, and 3, respectively. In week 4, cows were assigned to protocol 1 again and so forth. Overall, each protocol was replicated 20 times resulting in 60 experimental units. Total number of replicates for each farm is shown in Table 1. Inclusion criteria for cows were: past the farm individual voluntary waiting period (**VWP**; Table 1) and eligible for breeding. Control cows (n = 524; 20 experimental units) received an Ovsynch protocol with a single PGF treatment (100 µg of GnRH; 7 d, 500 µg of cloprostenol; 9 d, 100 µg of GnRH). Cows in the second protocol (n = 514; 20 experimental units) received an Ovsynch protocol with a double dose of PGF (**GDPG**: 100 µg of GnRH; 7 d, 1,000 µg of cloprostenol; 9 d, 100 µg of GnRH). Cows in the third protocol (n = 545; 20 experimental units) received an Ovsynch protocol with a second PGF treatment administered 24 h later (**GPPG**: 100 µg of GnRH; 7 d, 500 µg of cloprostenol; 8 d 500 µg of cloprostenol; 9 d, 100 µg of GnRH). GnRH (gonadorelin acetate; Gonavet Veyx, Schwarzenborn, Germany) and the PGF analogue (cloprostenol; PGF Veyx Forte, Schwarzenborn, Germany) were from Veyx Pharma GmbH. All cows received TAI approximately 16 h after G2.

4.4.2.1 Experiment 1: Blood Sampling and Progesterone Assay

For a subsample of cows (n = 491; 16 experimental units; n (control) = 158 in 4 weeks, n (GDPG) = 180 in 6 weeks, n (GPPG) = 153 in 6 weeks), blood samples were collected at the time of G2 (i.e., 16 h before TAI) to analyze progesterone concentration. Blood samples were collected from September until November 2018 via venipuncture of the coccygeal vessel using an 18-gauge, 1.5-inch hypodermic needle (Vacuette, Greiner Bio-One GmbH, Kremsmünster, Austria). Blood was drawn into sterile, plastic, evacuated serum collection tubes without any anticoagulant (8 ml, Vacuette, Greiner Bio-One GmbH, Kremsmünster, Austria) and stored on ice for transport to the laboratory of the Freie Universität Berlin. After 4 to 6 h to allow clotting, samples were centrifuged at 4,500 x g for 5 min at approximately 20°C and serum was transferred into sterile vials (2 mL, Cryovial, Simport, Bernard-Pilon, Canada), frozen, and stored at -18°C until assayed for P4 concentrations. Serum P4 concentrations were determined in a single assay using a solid-phase, no-extraction RIA (MP Biomedicals, Solon, OH). Sensitivity was 0.05 ng/mL and the intra-assay CV was 12.2% based on a quality control

sample (2.50 ng/mL of P4) which was replicated within the assay. Pregnancy diagnosis was performed by the herd veterinarian via transrectal palpation at 38 ± 7 d after TAI hereafter referred to as 38 d after TAI (Table 1). Pregnancy was based on a verified pregnancy diagnosis defined by the presence of uterine fluid, asymmetry, and a positive fetal membrane slip. Nonpregnancy was based on absence of pregnancy at the day of examination or a rebreeding to an estrus before pregnancy diagnosis [14].

4.4.3 Experiment 2: Treatments and Reproductive Management

Lactating Holstein cows (n = 1,979; 553 primiparous and 1,426 multiparous cows; 252 experimental units) from 6 commercial dairy farms (farms 1 to 6) were used in this experiment. No presynchronization protocol was used to facilitate first postpartum TAI. Cows were enrolled to first postpartum TAI when they were not detected in estrus after a farm specific period after VWP (Table 1). Nonpregnant cows were enrolled at the day of nonpregnancy diagnosis. All cows were assigned in a consecutive manner to receive 1 of 3 synchronization protocols and TAI as described above (Figure 1). Inclusion criteria for cows were: past the farm individual VWP (Table 1) and eligible for breeding. Cows were excluded after enrollment when they had purulent vaginal discharge at the day of AI (n = 22) or signs of systemic illness such as lameness (n = 5) and mastitis (n = 27). Overall, each protocol was replicated 84 times resulting in 252 experimental units (n (control) = 674, n (second protocol, GDPG) = 641, n (third protocol, GPPG) = 664). Total number of replicates for each farm is shown in Table 1.

4.4.3.1 Experiment 2: Ovarian Ultrasonography

Transrectal ultrasonography of the ovaries was performed by the herd veterinarian to determine the presence or absence of a CL at d 0 (Draminski iscan with a 7.5-MHz linear-array transducer, Draminski S.A., 10-860, Olsztyn, Poland). Presence of a CL was based on a cutoff diameter of 15 mm [25, 27]. For each ovary, number of corpora lutea was counted and recorded. The diameter of the visualized luteal tissue was estimated by using an on-screen background grid of 10-mm squares. Luteal radius (r) was calculated as 0.5 x CL diameter and volume was calculated as $V = 4/3 \times \pi \times r^3$. For CL with a fluid-filled cavity, the cavity's diameter was measured, its volume calculated, and subsequently subtracted from the total CL volume. Pregnancy diagnosis was performed by the herd veterinarian via transrectal ultrasonography at 35 ± 5 d after TAI hereafter referred to as 38 d after TAI (Table 1). A positive pregnancy diagnosis was based on visualization of an embryo with a heartbeat. Nonpregnancy was based on absence of pregnancy at the day of examination or a rebreeding to an estrus before pregnancy diagnosis [13].

4.4.4 Statistical Analyses

Breeding information (i.e., cow identification, breeding date, calving date, lactation number) and results of pregnancy diagnosis were obtained from the on farm computer system, exported to Excel (Office 2010, Microsoft Deutschland Ltd., Munich, Germany) spreadsheets and analyzed using SPSS for Windows (version 22.0, SPSS Inc., IBM, Ehningen, Germany).

Based on a statistical power calculation using MedCalc (version 15.6.1, MedCalc Software, Mariakerke, Belgium), enrollment of 1,100 cows per treatment allowed for detection of a 5% point treatment difference in P/AI (95% confidence; 80% power) based on a 1-tailed test. The assumption of a 5% point treatment difference was based on a recent meta-analysis [23] that evaluated the effect of an additional PGF treatment on d 8 in a 7d Ovsynch protocol (+4.6% units P/AI for cows treated with a second PGF dose compared to cows treated with a single PGF dose).

For the analysis of P/AI after TAI, different logistic regression models using the GENLINMIXED procedure of SPSS were built for each experiment. Because both experiments were done with a similar experimental design, results were combined to more thoroughly test the effect of treatment in the Ovsynch protocol on P/AI. To avoid a possible effect of presynchronization on treatments, cows receiving first postpartum TAI from farms 7 and 8 (n = 572) were excluded from the combined analysis. Because of the experimental design, we considered week within farm as the experimental unit and cow as an observational unit. Farm and week within treatment and farm were included as random effects. Variables were declared to be significant when $P < 0.05$. A statistical tendency was declared when $P \geq 0.05$ and $P \leq 0.10$. Regardless of the significance level, treatment was forced to remain in the final model.

4.4.4.1 Experiment 1

The initial model for the analysis of P/AI after TAI included the following explanatory variables as fixed effects: treatment (control vs. GDPG vs. GPPG), parity (primiparous vs. multiparous), season of AI (winter from 1st of December to 28th of February, spring from 1st of March to 31rst of May, summer from 1st of June to 31rst of August, and autumn from 1st of September to 30th of November), and AI number (first TAI vs. subsequent TAI). Farm and week within treatment and farm were considered as random effects. We tested all biologically plausible 2-way interactions. Because there was no interaction between season and treatment ($P = 0.31$), this interaction was not included in the final statistical model. The final model contained the following fixed effects: treatment, parity, season of AI, AI number, interaction between AI number and treatment, and interaction between parity and treatment. To account for multiple comparisons the *P* value was adjusted using a Bonferroni correction.

The probability of a cow to become pregnant was modeled as:

$$\begin{aligned}\text{Logit}[\Pr(P/AI)] &= \beta_0 + \beta_1 \text{treatment}_{kj} + \beta_2 \text{parity}_{ki} + \beta_3 \text{season}_{kji} + \beta_4 \text{AInumber}_{kji} \\ &+ \beta_5 treatment_{kj} \times \text{AInumber}_{kji} + \beta_6 treatment_{kj} \times \text{Parity}_{ki} + farm_k \\ &+ week_{ktj} + \varepsilon_{kji}\end{aligned}$$

$$farm_k \sim N(0, \sigma^2_{farm})$$

$$week_{ktj} \sim N(0, \sigma^2_{week})$$

where k denotes farm, t denotes treatment, j denotes week, i denotes cow. Treatment, parity, season, AI number, the interaction between AI number and treatment, and the interaction between treatment and parity are included as fixed effects. Farm and week within treatment and farm are the random effects, and ε is the error term. β_1 to β_6 denote the estimated coefficients for each variable.

In a subset of animals (n = 491; 16 experimental units), a blood sample was collected at G2 in order to evaluate the effect of treatment on progesterone concentration. An ANOVA was performed using the GENLINMIXED procedure of SPSS. The outcome variable was progesterone at G2 (ng/mL). Because of the experimental design, we considered week within farm as the experimental unit and cow as an observational unit. Farm and week within treatment and farm were included as random effects. The initial model included the following explanatory variables as fixed effects: treatment (control vs. GDPG vs. GPPG), parity (primiparous vs. multiparous), and AI number (first TAI vs. subsequent TAI). We tested all biologically plausible 2-way interactions. Because there was no interaction between treatment and AI number (P = 0.51), or treatment and parity (P = 0.81), these interactions were not included in the final statistical model. The final model therefore contained the following fixed effects: treatment and parity. To account for multiple comparisons among treatments the P value was adjusted using a Bonferroni correction. Cows were stratified into 7 categories using P4 concentrations (from 0.00 to ≥0.60 ng/mL in 0.10 ng/mL increments) at G2. For cows in each category, P/AI was calculated to determine the threshold with the greatest decrease in P/AI (Figure 2). The threshold with the greatest decrease in P/AI was used to categorize low and high P4 concentration. Differences in the percentage of cows within each P4 category were analyzed by logistic regression. A Bonferroni adjustment was used to account for multiple comparisons.

4.4.4.2 Experiment 2

Model building was as described above. The initial model included the following explanatory variables as fixed effects: treatment (control vs. GDPG vs. GPPG), parity (primiparous vs. multiparous), season of AI (winter, spring, summer, and autumn), presence of a CL at G1 (yes vs. no), and AI number (first TAI vs. subsequent TAI). We tested all biologically plausible 2-way interactions. Farm and week within treatment and farm were considered as random effects. Because there was no interaction between parity and treatment (P = 0.21), AI number and treatment (P = 0.71), or season and treatment (P = 0.33), these interactions were not included in the final statistical model. Regardless of the significance level, treatment, presence of a CL at G1 and the interaction between treatment and presence of a CL at G1 were forced to remain in the final statistical model. The final model contained the following fixed effects: treatment, parity, season of AI, presence of a CL at G1, and interaction between treatment and presence of a CL at G1. To account for multiple comparisons the P value was adjusted using a Bonferroni correction.

$$
\begin{aligned}
&Logit[Pr(P/AI)] \\
&\quad = \beta_0 + \beta_1 \mathrm{treatment}_{kj} + \beta_2 parity_{\mathrm{ki}} + \beta_3 \mathrm{season}_{\mathrm{kji}} + \beta_4 CL/G1_{kji} \\
&\quad + \beta_5 treatment_{kj} \times CL/G1_{kji} + farm_k + week_{ktj} + \varepsilon_{kji} \\
&\qquad\qquad farm_k \sim N\left(0, \sigma^2_{farm}\right) \\
&\qquad\qquad week_{ktj} \sim N\left(0, \sigma^2_{week}\right)
\end{aligned}
$$

where k denotes farm, t denotes treatment, j denotes week, i denotes cow. Treatment, parity, season, presence of a CL at G1, and the interaction of CL at G1 and treatment are included as fixed effects. Farm and week within treatment and farm are the random effects, and ε is the error term. β_1 to β_5 denote the estimated coefficients for each variable.

4.4.4.3 Experiments 1 and 2

The initial model for the analysis of P/AI after TAI included the following explanatory variables as fixed effects: treatment (control vs. GDPG vs. GPPG), parity (primiparous vs. multiparous), season of AI (winter from 1st of December to 28th of February, spring from 1st of March to 31rst of May, summer from 1st of June to 31rst of August, and autumn from 1st of September to 30th of November), and AI number (first TAI from farms 1 to 6 vs. subsequent TAI). Farm and week within treatment and farm were considered as random effects. We tested all biologically plausible 2-way interactions. Because there was no interaction between parity and treatment (P = 0.80), AI number by treatment (P = 0.38), or season by treatment (P = 0.80), these interactions were not included in the final statistical model. The final model

therefore contained the following fixed effects: treatment, parity, and season of AI. To account for multiple comparisons the *P* value was adjusted using a Bonferroni correction.

The probability of a cow to become pregnant was modeled as:

$$\text{Logit}[\Pr(P/AI)] = \beta_0 + \beta_1 \text{treatment}_{\text{kj}} + \beta_2 \text{parity}_{\text{ki}} + \beta_3 \text{season}_{\text{kji}} + farm_k + week_{ktj} + \varepsilon_{kji}$$

$$farm_k \sim N(0, \sigma^2_{farm})$$

$$week_{ktj} \sim N(0, \sigma^2_{week})$$

where k denotes farm, t denotes treatment, j denotes week, i denotes cow. Treatment, parity, and season are included as fixed effects. Farm and week within treatment and farm are the random effects, and ε is the error term. β_1 to β_3 denote the estimated coefficients for each variable.

4.5 Results

Overall, 3,560 cows were enrolled in this experiment. After exclusion of 415 (11.7%) cows because of culling before pregnancy diagnosis (n = 102) or lack of protocol compliance (n = 313), 3,145 cows (312 experimental units) were included in the final statistical analyses.

4.5.1 Experiment 1

In experiment 1, overall P/AI at d 38 after TAI was 35.5%. Treatment affected P/AI (*P* = 0.01), as P/AI was greater for GDPG (38.2%; *P* = 0.01) and for GPPG cows (38.9%; *P* = 0.01) than for control cows (29.8%). Pregnancy per AI did not differ (*P* = 0.84) between GDPG and GPPG cows (Table 2). Primiparous cows tended (37.8%; *P* = 0.10) to have greater P/AI than multiparous cows (33.3%). There was an interaction between parity and treatment (*P* = 0.03), where primiparous cows treated with a double PGF dose (46.1%) showed greater P/AI than primiparous cows treated with a single PGF dose (28.8%; *P* < 0.01). Primiparous cows treated with a second PGF dose (39.4%) tended to have greater P/AI than primiparous cows from the control group (*P* = 0.05). Pregnancy per AI did not differ (*P* = 0.24) between primiparous cows treated with a second and a double PGF dose. For multiparous cows, treatment with a second PGF dose (38.3%) tended to improve P/AI compared to the GDPG (30.9%; *P* = 0.05) and the control group (30.9%, *P* = 0.05). Pregnancy per AI did not differ (*P* = 0.99) for multiparous cows from the GDPG and the control group. Season affected P/AI (*P* < 0.01). Pregnancy per AI was 51.2%, 13.3%, 42.3%, and 43.8% in spring, summer, autumn, and winter, respectively. There was no interaction between season and treatment (*P* = 0.31). Overall, P/AI for cows receiving first TAI did not differ (37.5%; *P* = 0.13) from cows receiving second or greater TAI (33.5%). There was an interaction between AI number and treatment (*P*

= 0.05). For cows receiving first TAI, P/AI was greater for GDPG (44.0%; $P = 0.02$) than for control cows (32.3%). There was no difference for GPPG cows receiving first TAI (36.7%) compared to control (32.2%; $P = 0.37$) or GDPG cows ($P = 0.15$). For cows receiving second or greater TAI, P/AI was greater for GPPG cows (41.1%; $P < 0.01$) than for control cows (27.5%) and tended to be greater than for GDPG cows (32.7%; $P = 0.05$). There was no difference ($P = 0.19$) in P/AI among GDPG or control cows receiving second or greater TAI.

Circulating mean P4 concentration differed ($P < 0.01$) among treatments at G2, with GPPG cows (0.49 ± 0.16 ng/mL; n = 153) having lower mean P4 concentration than control cows (1.22 ± 0.16 ng/mL; n = 158; $P < 0.01$). There was a tendency for GPPG cows to have lower mean P4 concentration than GDPG cows (0.73 ± 0.15 ng/mL; n = 180; $P = 0.08$). Furthermore, GDPG cows had lower mean P4 concentration at G2 than control cows ($P < 0.01$). To more closely evaluate the relationship between P4 concentration at G2 and the effect of a double or second PGF dose, cows were stratified into 7 classes based on P4 concentrations (Figure 2). Both treatments increased the percentage of cows with very low P4 concentration (0.00 to 0.09 ng/mL). Only the GPPG treatment decreased the percentage of cows with high P4 concentration (≥0.6 ng/mL) compared to the control group. There was no difference among percentage of cows with high P4 concentration for cows from the control group compared to the GDPG treatment. Based on the decrease in pregnancy outcomes, the threshold for P4 to define low versus high P4 concentration was set at <0.6 ng/mL and ≥0 .6 ng/mL, respectively. Overall, 62.9% of cows had low P4 concentration. Cows with low P4 concentration had greater P/AI than cows with high P4 concentration (36.9% vs. 17.4%, respectively). Treatment affected ($P = 0.01$) the percentage of cows with low or high P4 at G2. A greater percentage of GPPG cows (73.2%; $P < 0.01$) showed low P4 concentration than control cows (55.7%) and tended ($P = 0.06$) to have more cows with low P4 concentration than GDPG cows (63.3%). Percentage of cows with either low or high P4 concentration at G2 did not differ for control cows and GDPG cows did not differ ($P = 0.14$). Mean P4 concentration did not differ ($P = 0.90$) among parities (0.80 ± 0.16 ng/mL vs. 0.82 ± 0.14 ng/mL for primiparous and multiparous cows, respectively). Percentage of cows with low P4 concentration (<0.6 ng/mL) did not differ ($P = 0.16$) among parities (66.3% multiparous cows vs. 59.4% primiparous cows). Primiparous cows with low P4 concentration had greater P/AI (49.3%; $P < 0.01$) than primiparous cows with high P4 concentration at G2 (23.0%). Multiparous cows with low P4 concentration had greater P/AI (33.6%; $P < 0.01$) than multiparous cows with high P4 concentration at G2 (9.4%). There was no interaction between treatment and parity ($P = 0.81$). There was no difference ($P = 0.41$) in mean P4 concentration at G2 among cows receiving first TAI (0.88 ± 0.16 ng/mL) and cows receiving second or greater

TAI (0.78 ± 0.14 ng/mL). There was no interaction between treatment and AI number (P = 0.51).

4.5.2 Experiment 2

At d 38 after TAI, overall P/AI was 33.4%. Overall, primiparous cows (35.5%) tended (P = 0.10) to have greater P/AI than multiparous cows (31.3%). Number of AI had no effect on P/AI (first TAI 32.2% vs. ≥2 TAI 33.8%; P = 0.57). Season tended to affect P/AI (P = 0.06) which was 36.6%, 29.2%, 35.6%, and 32.2% in spring, summer, autumn, and winter, respectively. Overall, there tended to be an effect of treatment on P/AI (P = 0.05, Table 2). Control cows (31.0%) had lower P/AI than GPPG cows (37.4%; P = 0.03) and similar P/AI compared to GDPG cows (31.8%; P = 0.77). Cows from the GPPG group tended to have greater P/AI than cows from the GDPG group (P = 0.05).

At G1, the percentage of cows with a functional CL (luteal tissue ≥ 15mm) was 59.5% (989/1662) considering all 3 protocols. Presence of a CL at G1 did not affect P/AI (P = 0.50). Cows with a CL at G1 had similar P/AI (34.1%) to cows without a CL at G1 (32.6%). There was no interaction between treatment and presence of a CL at G1 (P = 0.61). Overall, there was no interaction between treatment and parity (P = 0.21), treatment and season of TAI (P = 0.33), or treatment and AI number (P = 0.71).

4.5.3 Combined Results of Experiments 1 and 2

Experiments 1 and 2 were conducted using a similar experimental design. Therefore, results were combined to achieve more statistical power to test the effect of treatment on P/AI. Cows receiving first postpartum TAI after presynchronization from experiment 1 (n = 572) were excluded from the combined analysis. At d 38 after TAI, overall P/AI was 35.1%. Overall, primiparous cows (37.4%) had greater (P = 0.03) P/AI than multiparous cows (32.9%). Number of AI had no effect on P/AI (first TAI 33.6% vs. ≥2 TAI 35.5%; P = 0.49). Season affected P/AI (P < 0.01), and P/AI was greater in spring (38.4%), autumn (39.5%), and winter (37.4%) than in summer (26.0%). Overall, there was a treatment effect on P/AI (P < 0.01). Cows receiving a second PGF dose on d 8 had greater P/AI (40.3%; P < 0.01) than cows receiving a single PGF dose (31.8%) and cows receiving a double PGF dose (33.4%). Cows receiving 1,000 µg of cloprostenol had similar P/AI to control cows (P = 0.46; Table 2). Overall, there was no interaction between treatment and parity (P = 0.82), treatment and season of TAI (P = 0.28), or treatment and AI number (P = 0.89).

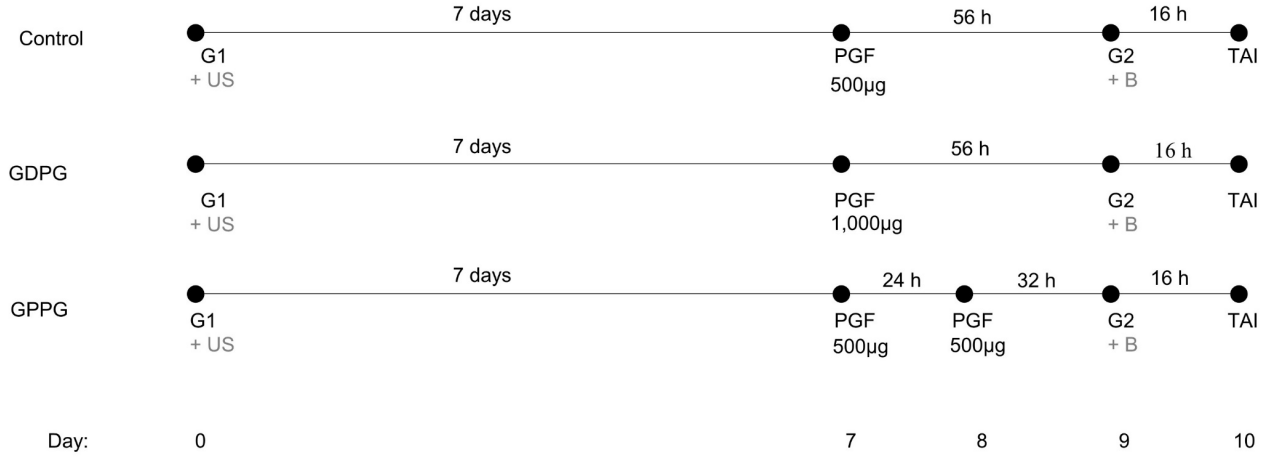

Figure 1. Schematic representation of the experimental study design used for experiments 1 and 2. Once a week, lactating Holstein cows were assigned in a consecutive manner to receive 1) Ovsynch (control: 100 µg of GnRH; 7 d, 500 µg of cloprostenol; 9 d, 100 µg of GnRH), 2) Ovsynch with a double dose of PGF (GDPG: 100 µg of GnRH; 7 d, 1,000 µg of cloprostenol; 9 d, 100 µg of GnRH), 3) Ovsynch with a second PGF treatment 24 h later (GPPG: 100 µg of GnRH; 7 d, 500 µg of cloprostenol; 8 d, 500 µg of cloprostenol; 9 d, 100 µg of GnRH). All cows received TAI approximately 16 h after G2. In experiment 1, blood samples were collected from a subsample of cows (n = 491; 16 experimental units; n (control) = 158, n (GDPG) = 180, n (GPPG) = 153) at the time of G2. In experiment 2, transrectal ultrasonography of the ovaries was performed in a subsample of cows (n = 1,979; 252 experimental units) at the time of G1. G1 = first GnRH treatment of the protocol; PGF = prostaglandin $F_{2\alpha}$; G2 = final GnRH treatment of the protocol; B = blood sample; US = ultrasonography; TAI = timed artificial insemination

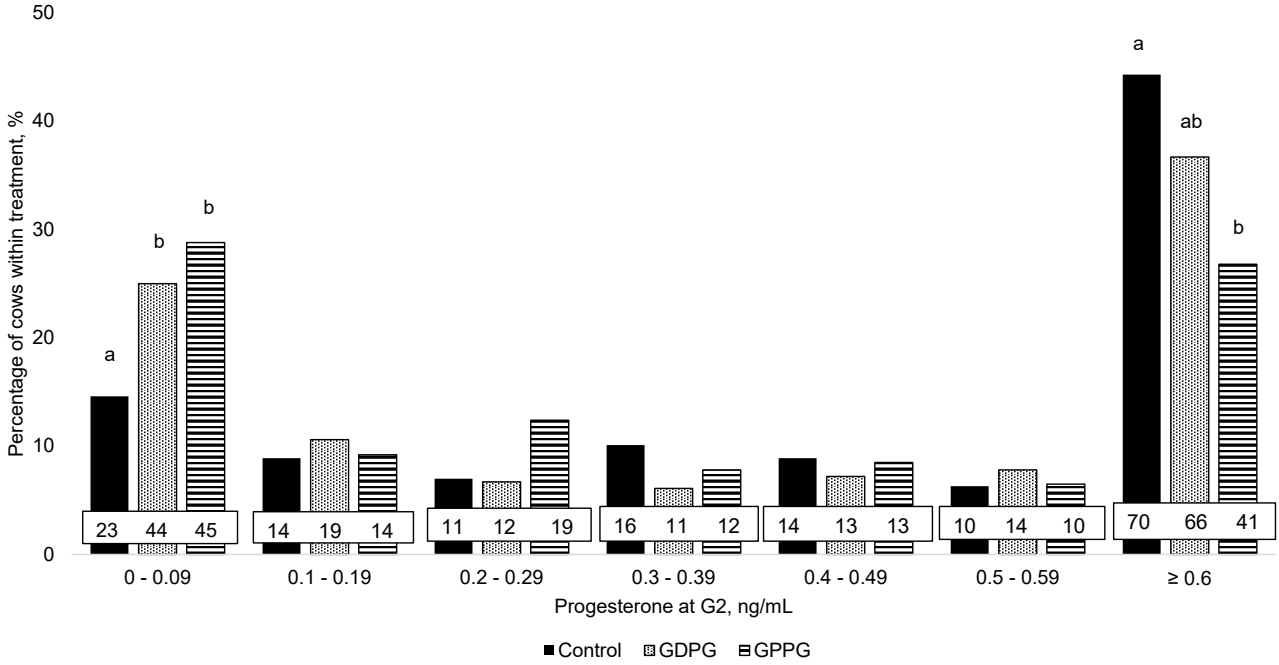

Figure 2. Treatment effect of one prostaglandin F_{2a} (PGF, control) dose vs. a double PGF dose (GDPG) vs. a second PGF dose 24 h later (GPPG) on the distribution of cows considering the concentration of progesterone (P4) at the time of the final GnRH treatment (G2) using data from cows that were synchronized and blood sampled in experiment 1 (n = 491; 16 experimental units). Once a week, lactating Holstein cows were assigned in a consecutive manner to receive 1 of 3 Ovsynch protocols: (1) Ovsynch (control: 100 µg of GnRH; 7 d, 500 µg of cloprostenol; 9 d, 100

μg of GnRH), (2) Ovsynch with a double dose of PGF (GDPG: 100 μg of GnRH; 7 d, 1,000 μg of cloprostenol; 9 d, 100 μg of GnRH), or (3) Ovsynch with a second PGF treatment 24 h later (GPPG: 100 μg of GnRH; 7 d, 500 μg of cloprostenol; 8 d, 500 μg of cloprostenol; 9 d 100 μg of GnRH). Within a progesterone category, bars with different letters denote statistical differences ($P < 0.05$). Numbers in the white boxes represent the number of cows within each category.

Table 1. Descriptive information for the 8 farms enrolled in the study (Experiments 1 and 2).

	Farm No.							
Item	1	2	3	4	5	6	7	8
Number of lactating cows	860	750	325	350	480	350	900	1,380
305-d ECM yield (kg)	9,685	10,551	10,102	8,362	10,299	9,518	10,592	9,408
Voluntary waiting period (DIM)	45	42	60	50	50	42	80	50
Median DIM at first TAI	90	70	120	130	100	95	115	85
Cows bred in estrus at first AI (%)	68	81	79	73	82	62	75	49
Cows bred in estrus at ≥2 AI (%)	61	63	72	55	80	68	71	50
Day of pregnancy diagnosis (days after AI)	35 ± 5	35 ± 5	35 ± 5	35 ± 5	35 ± 5	35 ± 5	38 ± 7	38 ± 7
21-d pregnancy rate (%)	22	15	13	15	14	14	19	17
Total number of replicates	66	75	30	21	36	24	30	30

Table 2. Comparison of the effect of treatment on pregnancies per AI [P/AI, % (number of cows)] 38 d after timed AI in experiments 1, 2 and combined results from experiments 1 and 2.

Parameter	Treatment[1]			*P*-value			
	Control	GDPG	GPPG	Treatment	C_1[2]	C_2[3]	C_3[4]
Experiment 1[5]	29.8 (493)	38.2 (475)	38.9 (515)	0.01	0.01	0.01	0.84
Experiment 2[6]	31.0 (572)	31.8 (528)	37.4 (562)	0.05	0.77	0.03	0.05
Combined results from experiments 1 and 2[7]	31.8 (888)	33.4 (814)	40.3 (871)	< 0.01	0.46	< 0.01	< 0.01

[1]Once a week, lactating Holstein cows were assigned in a consecutive manner to receive (1) Ovsynch (control: 100 µg of GnRH; 7 d, 500 µg of cloprostenol; 9 d, 100 µg of GnRH), (2) Ovsynch with a double dose of PGF (GDPG: 100 µg of GnRH; 7 d, 1,000 µg of cloprostenol; 9 d, 100 µg of GnRH), (3) Ovsynch with a second PGF treatment 24 h later (GPPG: 100 µg of GnRH; 7 d, 500 µg of cloprostenol; 8 d, 500 µg of cloprostenol; 9 d, 100 µg of GnRH). All cows received TAI approximately 16 h after G2.

[2]C1 = preplanned contrast comparing control and GDPG treatments.

[3]C2 = preplanned contrast comparing control and GPPG treatments.

[4]C3 = preplanned contrast comparing GDPG and GPPG treatments.

[5]Percentages for P/AI were derived from the GENLINMIXED model including treatment, parity, season of AI, AI number, the interaction between AI number and treatment, and the interaction between parity and treatment (60 experimental units). Farm and week within treatment and farm were considered as random effects.

[6]Percentages for P/AI were derived from the GENLINMIXED model including treatment, parity, season of AI, presence of a corpus luteum (CL) at the first GnRH (G1), and the interaction of treatment and presence of a CL at G1 (252 experimental units). Farm and week within treatment and farm were considered as random effects.

[7]Only non-presynchronized cows were included in this analysis (312 experimental units). Percentages for P/AI were derived from the GENLINMIXED model including treatment, parity, and season of AI. Farm and week within treatment and farm were considered as random effects.

4.6 Discussion

The objective of this study was to evaluate two modifications (i.e., double dose, double administration 24 h apart) of the PGF treatment using cloprostenol in a 7-d Ovsynch protocol on P4 concentration at the time of the second GnRH treatment and P/AI in lactating Holstein cows. Our primary hypotheses were that P4 concentration at G2 would decrease, whereas P/AI would be increased in the modified Ovsynch protocols (GPPG and GDPG) compared with the traditional 7-d Ovsynch protocol (control).

In experiment 1, we wanted to evaluate the effect of treatment on progesterone concentration at G2 and P/AI. In general, the intention of dose and frequency modifications of the PGF treatment is to decrease P4 concentration and increase the rate of luteal regression at the end of the TAI protocol, which is a critical factor for achieving high fertility [10]. There may be multiple mechanisms contributing to the decrease in fertility when P4 is elevated near AI, such as a negative influence on oviductal and uterine motility, affecting gamete transport [16]. It was shown that after adding P4 to in vitro fertilization media [28], blastocyst rate was reduced, suggesting that P4 might have direct effects on the subsequent embryo development. Furthermore, elevated P4 at G2 was found to decrease endometrial thickness, thus compromising fertility to TAI [17]. In our experiment, GPPG and GDPG cows had lower mean P4 concentration at G2 than control cows. Additionally, GPPG cows tended to have lower mean P4 at G2 than GDPG cows. Contrary to this, Barletta et al. [25] did not observe a difference in P4 concentration among treatments. In an orthogonal comparison, however, GPPG cows tended to have lower P4 concentration than control cows, whereas P4 concentration between GDPG and control cows did not differ. By stratifying P4 categories and calculating subsequent P/AI in our experiment, the greatest decrease in P/AI was found when P4 concentrations were greater or equal 0.6 ng/mL. Only the GPPG treatment decreased the percentage of cows with high P4 concentration (≥0.6 ng/mL) compared to the control group. As we have only taken one blood sample at the time of G2, it remains speculative whether cows with high P4 concentration were affected by incomplete luteal regression. We were not able to accurately assess luteal regression in our study, as its definition is mostly based on two blood samples taken at the time of the PGF treatment (high P4 indicating presence of a CL) and G2 (low P4 indicating complete regression of the CL) [8,29,30]. This method of evaluating luteal regression can be considered most accurate because cows without a CL at PGF will be excluded from the analysis. A lack of complete luteal regression was reported to occur in 12 to 21% of cows treated with a single PGF dose [14,15,31]. Especially cows ovulating after G1 had a greater risk of incomplete luteal regression, because they were bearing a younger CL at the PGF treatment, which did not completely regress after a single PGF dose [11]. This is

supported by a study, where a double dose of PGF (1,000 µg of cloprostenol) in a 5-d protocol had no beneficial effect on luteal regression and P/AI compared with a single PGF dose, whereas a second dose of PGF at d 6 improved luteal regression and P/AI [29]. Interestingly, Stevenson et al. [32] found a double PGF dose (50 mg of dinoprost) in a 5-d protocol effective to induce luteal regression using a threshold of 1 ng/mL for P4 concentration on d 8, but not when a more conservative threshold of 0.5 ng/mL was used. A greater percentage of cows treated with a second PGF dose (25 mg of dinoprost) on d 6 showed P4 concentration <0.5 ng/mL on d 8 resulting in greater P/AI. Their findings indicate that although treatment with a double PGF dose may decrease P4 concentrations with time, it may not achieve sufficiently basal concentrations in some cows to prevent reduced pregnancy outcomes [32]. Two studies [33,34], however, observed similar outcomes regarding luteal regression using a 5-d protocol with an increased PGF dose (50 mg of dinoprost and 375 µg of cloprostenol, respectively) versus an additional PGF treatment 24 h later (25 mg of dinoprost and 150 µg of cloprostenol, respectively). Stevenson et al. [24] found the GDPG treatment to be as effective as the GPPG treatment to induce complete luteal regression considering a P4 threshold of 0.4 ng/mL. These results, however, are based on an experimental design with a limited sample size (GPPG: 100 cows vs. GDPG: 99 cows) and several interactions among treatments. Literature seems more consistent with a clear benefit for GPPG cows, as shown in a meta-analysis including 6 manuscripts [23], where an additional PGF treatment in a 7-d Ovsynch protocol increased luteal regression by 11.6% units. Furthermore, a recent large dataset [10] including data from 14 different studies showed that P4 concentration at each treatment (i.e., G1; PGF; G2) during an Ovsynch protocol affected P/AI independently.

Mean progesterone concentration and percentage of cows with low P4 at G2 did not differ among parities. This finding is intriguing, because a greater percentage of primiparous cows was found to achieve low P4 concentration at G2 in some studies [8,30]. In other studies [13,25], however, the percentage of cows with low P4 concentration did not differ among parities.

We also assessed the effect of PGF modifications in a 7-d Ovsynch on P/AI in experiment 1. Both PGF modifications improved P/AI (GDPG: 38.2% and GPPG: 38.9%) compared with control cows (29.8%). Pregnancy per AI did not differ ($P = 0.84$) between GDPG and GPPG cows. One study [8] observed a tendency for greater P/AI for cows treated with an increased PGF dose (750 µg of cloprostenol) compared with a single PGF dose (500 µg of cloprostenol) synchronized with a Double-Ovsynch protocol. There are two other studies [24,25] with a similar experimental design comparing the effect of a double PGF dose (50 mg of dinoprost) and a second PGF dose on d 8 (25 mg of dinoprost) on P/AI in a 7-d Ovsynch protocol. One study [25] observed a tendency for greater P/AI 39 d after TAI for GPPG cows

compared with control cows (35% vs. 31%; *P* = 0.08). For GDPG cows, however, there was no effect on P/AI (32%; *P* = 0.34). The second study [24] did not include a control group, but contrary to the previous study, P/AI tended to be greater for GDPG than for GPPG cows (46.1% vs. 36.7%; *P* = 0.07). Divergent findings to our study might be caused by insufficient sample size (1,100 and 199, respectively), the use of a presynchronization protocol, or using dinoprost instead of cloprostenol as a PGF analogue. The latter assumption, however, seems to be unlikely, as similar results were found for both drugs regarding time to complete luteal regression, ovulation, and P/AI [35-37]. One study [38], nevertheless, found increased rates of estrus detection, conception and pregnancy for primiparous cows treated with cloprostenol compared to dinoprost. They speculated that cloprostenol might be able to faster decrease P4 concentrations, which increased estradiol production by dominant follicles. In contrast to other studies and to cows from our experiment 2, cows from experiment 1 were presynchronized (PGF, 14 d later PGF, 12d later G1 of the Ovsynch protocol) before receiving first TAI. Presynchronized cows showing estrous behavior received AI before the enrollment to 1 of the 3 treatments. Therefore, the remaining cows that were enrolled might represent a subpopulation of cows that are not fully representative as they are more prone to anovulatory conditions. In general, cows assigned to a 7-d Ovsynch at a random stage of the estrus cycle were found to have decreased chances of pregnancy compared to cows assigned to a presynchronization protocol [39,40]. Presynchronization leads to better fertility by inducing a greater proportion of cows in early diestrus at the initiation of an Ovsynch protocol, improving ovulatory response after G1, and preventing premature luteal regression [39-41]. Results from our first experiment suggest that the effect of a dose frequency modification in a 7-d Ovsynch protocol might be different among cows that were presynchronized for first postpartum TAI. Our experimental design has some limitations to fully address that question. Therefore, future studies need to elucidate whether the treatment effect is modified by presynchronization of the first postpartum TAI.

In experiment 2, we wanted to assess whether the effect of treatment was dependent on the presence of a CL at G1 as implicated by recent studies [12,19,25]. There was no significant interaction between treatment and presence of a CL at G1. There are several studies observing a beneficial effect of a second PGF dose on P/AI only in cows without a CL at G1 or with low P4 concentration at G1, respectively [12,19,25]. Unfortunately, we did not have information on P4 concentration at G1, which is considered as a gold standard to identify cows with a functional CL.

Another finding of interest was, that P/AI for cows with a CL at G1 did not differ from P/AI for cows without a CL at G1 (34.1% cows with a CL vs. 32.6% cows without a CL). This is inconsistent with other studies that have reported that cows with a functional CL or high P4

concentration at G1 have greater P/AI than cows lacking a CL or low P4 concentration at G1 [12,24,42]. Decreased P4 at G1 was associated with compromised embryo quality [43] and decreased fertility in lactating dairy cows [44]. The poor embryo development for these cows has been attributed to an overstimulation of the preovulatory follicle and the oocyte by a greater frequency of GnRH-induced LH pulses [44,45]. Particularly cows with low P4 concentration at G1 bear a young CL at the day of PGF treatment lacking full luteolytic response after a single PGF dose [10]. The reason why a younger CL lacks the ability to completely regress in response to a single treatment with exogenous PGF has stimulated discussions for a long time [46-48]. It is assumed that the number of PGF pulses rather than the magnitude of the pulses are critical for inducing complete luteal regression [48-50]. A study [50] simulating sequential PGF pulses at 12-h intervals in Holstein heifers found a continuous decrease in P4 after the fourth simulated pulse. A single PGF pulse, however, resulted in a 6-h decrease in P4 to a constant concentration for 3 d after treatment and a return to baseline concentration afterwards. Also the physiological uterine release of PGF was detected as rather a pulsatile than a consistent mechanism [51]. Endometrial and ovarian PGF pulses stimulate the release of oxytocin from the CL, which further stimulates uterine PGF secretion. If a larger and mature CL is present, PGF releases more luteal oxytocin that enhances uterine PGF secretion [47]. Intriguingly, one study [52] identified high affinity PGF receptors in CL of heifers on d 2, 4, 6, or 10 after ovulation, suggesting that the relative resistance to PGF in the young CL was not attributable to a deficiency of high affinity PGF receptors. Still, PGF-induced signaling pathways might be altered in young CL lacking luteolytic capacity [53]. The definitive lesion is still under investigation, but maintenance of high intraluteal P4 production may be central to the lack of luteal capacity in young CL [54]. This is supported by studies that observed cows with low circulating P4 concentration at the time of the PGF treatment have the highest risk for incomplete luteal regression [13,30]. These authors speculated that regression of the mature CL might be enhancing regression of the younger CL possibly because of a more pronounced decline in circulating P4. Cows with low P4 before the PGF treatment may not have as large of a decline in P4 concentration and consequently no increase of uterine PGF release [13,30].

The combined analysis of experiments 1 and 2 provided the most statistical power to evaluate the effect of PGF modifications in a 7-d Ovsynch on P/AI. Overall, proving an effect on P/AI is challenging to demonstrate because of the required sample size to detect a small effect size. Based on results from a recent meta-analysis [23] including 5,350 cows, incorporating a second PGF treatment on d 8 increased P/AI by 4.6% units. Therefore, sample size calculation in our study was based on a 5% unit difference in P/AI. In the combined results from our experiments, cows treated with a second PGF dose on d 8 had greater P/AI than control (+8.5% units) and GDPG cows (+6.9% units). Pregnancy per AI did not differ between

cows treated with a double PGF dose and control cows (+1.6% units). The different effect size of the two PGF modifications on P/AI might be explained by their difference in achieving low P4 concentration at G2 as we observed in experiment 1. In agreement with a previous study [14] we did not observe an interaction between treatment and parity. Contrary to this, Wiltbank et al. [13] only observed greater P/AI in multiparous cows when treated with a second PGF dose on d 8 compared with the control group (+4.1% units). The authors suggested that a second PGF dose might enhance fertility by effects other than decreasing percentage of cows with incomplete luteal regression at G2. As a possibility, a second PGF dose was found to achieve either low P4 concentrations or increased estradiol concentrations more quickly, thereby improving fertility [14,35].

4.6.1 Study Limitations

In contrast to our hypothesis, there was no significant interaction between treatment and presence of a CL at G1. Another surprising finding contrary to previous studies was that P/AI was not influenced by the presence of a CL at G1. One possible explanation for this disagreement might be the utilization of transrectal ultrasonography instead of P4 concentrations in blood samples to determine the presence or absence (P4 <1.0 ng/mL) of an active CL at G1. Sensitivity and specificity for transrectal ultrasonography to detect a functional CL at a random day of the estrus cycle were 89.4% to 91.2% and 39.8 to 45.7%, respectively [55,56]. In addition, the visual ability to detect a CL correctly by transrectal ultrasound can differ among technicians [56]. In that study, sensitivity of detecting a functional CL by 3 technicians ranged from 88.1% to 95.8% whereas specificity ranged from 20.5% to 70.2%. Utilization of ultrasonography instead of blood samples was based on the available resources for this study. Future studies should combine transrectal ultrasound and blood P4 concentration at G1 in order to evaluate the accuracy of ultrasound to detect a functional CL and whether the effect of PGF modifications is limited to cows without a functional CL at G1.

The study design was conducted by enrolling weekly cohorts of cows to 1 of the 3 protocols. In week 1, all cows were assigned to protocol 1 (control), in week 2 all cows were assigned to protocol 2 (GDPG), in week 3 all cows were assigned to protocol 3 (GPPG). In week 4, all cows were assigned to the control group and so forth. As it was a large field trial with 3 different protocols, this experimental design was chosen to simplify treatment administration and to assure a high level of compliance. The alternative experimental design in which cows were randomly assigned to one of the three protocols in each week was not practicable because of the logistical concerns that it created on the farms.

4.7 Conclusions

We conclude that overall, a second cloprostenol treatment on d 8 during a 7-d Ovsynch protocol decreased the proportion of dairy cows with high progesterone concentration at the time of G2 and increased P/AI compared to the traditional 7-d Ovsynch including a single cloprostenol dose on d 7. Treatment with a double dose of cloprostenol on d 7 during a 7-d Ovsynch only increased the proportion of cows with very low progesterone concentration at G2, but did not affect the proportion of cows with high P4 at the time of G2. In addition, doubling the cloprostenol dose on d 7 did not affect P/AI compared with a single dose of cloprostenol on d 7. The use of a presynchronization protocol, however, seems to influence the effect of a dose frequency modification of PGF treatment in an Ovsynch protocol. Presynchronized cows receiving first postpartum TAI had similarly increased P/AI treated with a double PGF dose compared to treatment with a second PGF dose. Using transrectal ultrasound, presence of a CL at G1 had no impact on the treatment effect. To evaluate the underlying physiological principles of achieving low progesterone concentration at the end of TAI protocols and to elucidate the possible effect of treatment by presynchronization of the first postpartum TAI, further research is warranted.

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5 DISCUSSION

The overall objectives of this thesis were 1) to determine the association between the timing of AI and different characteristics of an estrus event (i.e., onset, peak, and end) using an AAM system on P/AI in lactating Holstein cows inseminated with either fresh or frozen semen, 2) to identify plausible factors associated with estrous expression and subsequent P/AI in lactating Holstein cows using an AAM system, and 3) to evaluate two modifications (i.e., double dose, second administration 24 h apart) of the PGF treatment using cloprostenol in a 7-d Ovsynch protocol compared with a standard single PGF dose on P4 concentration at the time of G2 and P/AI in lactating Holstein cows.

Results from the first study underline the importance of careful timing of AI relative to specific characteristics of an estrus event (i.e., onset, peak, and end of estrus).

Using a modern, neck-mounted AAM system (Heatime), inseminating cows from 7 to 24 h after onset of estrus or 1 to 18 h after peak estrus activity yielded greatest P/AI irrespective of the type of semen (fresh versus frozen semen). Furthermore, cows inseminated with fresh semen from 5 h before to 12 h after end of estrus had greater P/AI compared with frozen semen, whereas cows inseminated with frozen semen from 13 to 18 h after end of estrus had greater P/AI compared with fresh semen. These results suggest that adequate fertility could be achieved by inseminating cows once daily, as already indicated by others (Foote 1978; Nebel et al. 1994). In contrast to these findings, Aungier et al. (2015) found greatest P/AI when cows were inseminated within a more limited period between 9 to 15 h after onset of estrus. Similar intervals between onset of estrus to ovulation were found in several studies, however, they also agreed on a wide range of variation among cows, which make precise timing of AI difficult and possibly contributes to poor fertility (Valenza et al. 2012; Aungier et al. 2015; Bombardelli et al. 2016). This fact might also in part explain our observation that we did not find significant interactions between the type of semen and the intervals from onset, peak, or end of estrus to AI. It was suggested by a recent study using TAI protocols (Borchardt et al. 2018) that P/AI might be affected by an interaction between the interval from AI to ovulation and the used type of semen. The authors observed that P/AI in a TAI protocol with suboptimum timing of AI relative to ovulation (Cosynch-56) was greater for fresh semen compared with frozen semen. Divergent findings to our study might also be due to my experimental design. As this was an observational study, there was no option of randomization of type of semen and timing of AI. Furthermore, cows were not systematically confirmed as being in estrus after the AAM alert. Nonetheless, our findings broaden the understanding about AAM-based estrous characteristics. External validity was not limited as we enrolled 4 commercial dairy farms.

Another finding of interest in this study was that only about two-thirds of cows expressed high estrous intensity (**PAE**), which was associated with increased P/AI. A positive association between intense estrous expression and P/AI has already been described earlier (Polsky et al. 2017; Burnett et al. 2018). While cows with low PAE showed a higher risk of ovulation failure or abnormal timing of ovulation (Pereira et al. 2016; Burnett et al. 2018), cows with high PAE were found to have a more optimum P4 profile after AI (Madureira et al. 2019) and a pattern of endometrial gene expression favouring establishment of a pregnancy (Davoodi et al. 2016). However, the exact biological mechanism is still under investigation.

In connection with the latter finding, the purpose of the second study was to identify factors associated with estrous expression and subsequent fertility in lactating dairy cows using AAM. We observed duration of estrus and PAE to be highly correlated and were able to confirm the finding from the latter study, as estrus events with high PAE were associated with greater odds of pregnancy.

Furthermore, there was a strong negative association between estrous expression and P/AI and an increased average temperature-humidity-index 1 wk before AI (**THI**). The greatest decrease for PAE and subsequent P/AI was observed when THI was above 70. Similarly, Schüller et al. (2014) reported the greatest negative impact on P/AI for a THI ≥ 73 within 3 weeks before breeding, but described decreasing P/AI already for an exposure to THI ≥ 73 for 1 h on the day of AI. Overall, this clearly shows the severe impact of heat stress on fertility and emphasizes the importance of heat abatement strategies (e.g., mechanical ventilation, TAI protocols, combined use of different methods for estrus detection) to improve reproductive performance.

In addition, AAM systems have the potential to identify cows with short inter-estrus intervals (5% of cows in our study). This is noteworthy, as these cows had dramatically decreased P/AI compared with cows with no short inter-estrus intervals. While various factors, such as parity, DIM, and the inter-ovulatory interval, were described to be associated with short inter-estrus intervals (Remnant et al. 2015), the reason for the observed negative association resulting in decreased estrous expression and P/AI in our study remains speculative. Because this was a large retrospective, observational study including 7 commercial dairy farms, assessment of information about ovarian profiles was not feasible. Therefore, these cows should be the focus of future studies to investigate the underlying mechanisms explaining their reduced fertility.

The aim of the third study was to evaluate the effect of dose and frequency modifications of the PGF treatment during a 7-d Ovsynch protocol on P4 concentration at the end of the protocol and on pregnancy outcomes.

Our results indicated that adding a second PGF treatment on d 8 decreased the proportion of cows with high P4 at G2 and increased P/AI compared with a single or a double PGF dose on d 7. A double PGF dose on d 7, however, was only able to increase the proportion of cows with very low P4 at G2 but did not affect overall P/AI. Previous literature reported controversial results about the effect of doubling the PGF dose during TAI protocols. Whereas similar results to our study regarding luteal regression and P/AI were found in a study using a 5-d TAI protocol (Ribeiro et al. 2012), another study (Stevenson et al. 2018) found a double PGF dose in a 7-d Ovsynch protocol as effective to induce luteal regression as a second PGF dose on d 8. In addition, the latter study interestingly found a tendency for a double PGF dose to increase P/AI compared with a second PGF dose. Overall, most of the literature seems more consistent with a clear benefit of a second PGF dose. A recent meta-analysis including 6 manuscripts (Borchardt et al. 2017) showed that a second PGF treatment on d 8 increased luteal regression by 11.6% units and P/AI by 4.6%. After all, comparison of studies is difficult, because of the divergent experimental designs including different PGF products, varying sample sizes, different P4 thresholds to define luteal regression, or the use of presynchronization protocols.

Presynchronization protocols seem to influence the effect of a dose frequency modification of PGF. At the initiation of an Ovsynch protocol, presynchronized cows were suggested to have a greater likelihood being in a stage of the estrous cycle (i.e., early diestrus) that favors ovulatory response to G1 and prevents premature luteal regression (Vasconcelos et al. 1999; El-Zarkouny et al. 2004). In fact, presynchronized cows from our experiment 1 had similarly increased P/AI when treated with a double PGF dose compared with a second PGF dose. However, our experimental design had some limitations to fully address this question. Therefore, future research is warranted to evaluate if the treatment effect of a dose frequency modification of PGF is influenced by presynchronization of the first postpartum TAI.

Another interesting finding in this study was that the presence of a CL had no impact on the PGF treatment effect. This contradicts other studies (Stevenson 2016a; Santos et al. 2016) observing a beneficial effect of a second PGF dose on P/AI only in cows without a CL at G1. After all, presence or absence of a CL in our study was assessed using transrectal ultrasonography instead of measuring P4 concentrations in blood samples which is considered the gold standard to identify cows with a functional CL. Sensitivity (88.1 to 95.8%) and specificity (20.5 to 70.2%) for transrectal ultrasonography differs among studies and may in part be influenced by different technicians (Bicalho et al. 2008; Sauls-Hiestermann et al. 2019).

Overall, future research on reproductive performance in dairy cows should focus on optimization of data generated by AAM systems, selective enrolment of cows to TAI protocols as well as custom tailored approaches to combine the use of AAM systems and TAI protocols.

Our studies again demonstrated the high potential of AAM systems as a useful tool for dairy farmers regarding decision-making aiming to improve reproductive management. Activity data can be used as a descriptive measure to identify and quantify cows being anestrous and to monitor the proportional dynamics of such cows. In addition, activity data may serve as a predictive tool as certain parameters (e.g., PAE) may be helpful to predict a cow's chance to become pregnant. Moreover, AAM data allow for prescription in order to customize reproductive management for individual subpopulations of cows, such that there are different strategies for anestrous cows (e.g., enrolment in a TAI protocol) and cows with estrous behavior in early lactation (i.e., rely on AI after spontaneous estrus detected after the end of VWP).

Another worthwhile approach for the dairy industry would be the development of adjusted algorithms or thresholds for AAM systems based on individual cows or at a farm level. As various factors (e.g., parity, THI, DIM, BCS) have been repeatedly demonstrated to interfere with estrous expression and therefore the ability to detect certain cows in estrus, it might be useful to take full advantage of the potential offered by AAM systems. Adjusted thresholds for increased activity could be explored, developed and adapted to individual dairy farms, e.g., applying thresholds according to season, parity, or farm level (Cerri et al. 2021).

Considering TAI protocols, future research is warranted to elucidate the possible interaction between modified PGF treatments and presynchronization of the first postpartum TAI. In order to evaluate the effect, future studies should be conducted as a 2 (effect of PGF modification) x 2 (effect of presynchronization) factorial design. As this was not the case in our study, we cannot draw any inference regarding this interaction from our results.

Combining AAM data and TAI protocols is another big topic further research should focus on. No difference was found for P/AI and days to pregnancy among cows receiving TAI or cows receiving AI after estrus detection using an AAM system (Neves et al. 2012). Similarly, other studies indicated that it is possible to achieve similar P/AI in intensive estrus detection programs compared with TAI programs (Fricke et al. 2014; Denis-Robichaud et al. 2016). There are comprehensive reproductive management strategies including both options (i.e., estrus detection aids and hormone treatments) with the aim to improve identification and treatment of cows that otherwise would not be inseminated.

One strategy would be to maximize the proportion of cows inseminated in estrus after the end of the VWP or after a previous AI service. Subsequent synchronization of ovulation and TAI should be used to ensure timely AI of cows not detected in estrus. For the success of this strategy, defining the period in which cows are expected to receive estrus detected AI is important.

Another strategy is synchronization of estrus using PGF treatments to inseminate all of the cows detected in estrus within a few days after the end of the VWP. Thereafter, cows not receiving estrus detected AI are enrolled to a TAI protocol.

There are other strategies available taking advantage of AAM data as a decision support tool to optimize reproductive management, such as the selective reproductive management. With this method, decisions can be made by evaluating estrous characteristics, such as the PAE, during the VWP. It has been shown recently (Borchardt et al. 2021) that cows with no estrus alert within the VWP expressed less intensive estrous behavior and had increased days to first postpartum AI and to pregnancy compared with cows with at least one estrus alert within the VWP. Therefore, this reproductive strategy aims to identify anestrous cows to subsequently enroll them to a fertility program, such as a Double-Ovsynch protocol, immediately after the end of the VWP. Cows showing at least one estrus alert within the VWP, on the other hand, could receive estrus-detected AI for first postpartum AI. Another approach for integration of AAM data to fine-tune reproductive management decisions was shown in a recent study (Burnett et al. 2019). Results indicated that treatment with GnRH at the time of AI for spontaneous estrus events improved P/AI, but interestingly only for cows with low PAE.

The appropriate reproductive strategy will be farm specific and might depend on available resources (e.g., labor to conduct estrus detection and/or hormone injections, funds to invest in AAM systems), facility design (e.g., availability of headlocks or a sort gate to improve compliance to a TAI protocol), environmental and physiological conditions favoring estrous expression, and overall management preferences. Overall, AAM systems allow a more targeted approach to hormone use contributing to higher sustainability of the dairy industry.

6 SUMMARY

Optimization of reproductive management of lactating dairy cows on farms using automated activity monitoring systems for estrus detection or protocols for timed artificial insemination

The overall objectives of this thesis were 1) to determine the association between the timing of artificial insemination (AI) and different characteristics of an estrus event (i.e., onset, peak, and end) using an automated activity monitoring (AAM) system on pregnancy per AI (P/AI) in lactating Holstein cows inseminated with either fresh or frozen semen, 2) to identify plausible factors associated with estrous expression and subsequent P/AI in lactating Holstein cows using an AAM system, and 3) to evaluate two modifications (i.e., double dose, second administration 24 h apart) of the prostaglandin $F_{2\alpha}$ (PGF) treatment using cloprostenol in a 7-d Ovsynch protocol compared with a standard single PGF dose on progesterone (P4) concentration at the time of the second GnRH treatment (G2) and P/AI in lactating Holstein cows.

To determine the association between the timing of AI with either fresh or frozen semen and different characteristics of an estrus event (i.e., onset, peak, and end) using an AAM system on P/AI, an observational study was conducted on 4 commercial dairy farms in Germany. The evaluation included a total of 3,607 AI services based on the alert of a neck-mounted AAM system (Heatime; SCR Engineers Ltd., Netanya, Israel). Pregnancy diagnosis was performed following farm individual strategies either by transrectal palpation 38 ± 3 d after AI or by transrectal ultrasonography 30 ± 3 d after AI. To evaluate whether cows were exposed to heat stress, loggers were installed within the barns that continuously recorded ambient temperature and relative humidity, thereby calculating the temperature-humidity-index (THI).

The software of the AAM system converted individual cow's raw activity data into an activity change index value using a proprietary algorithm. These index values ranged from 0 (lowest) to 100 (highest). Onset of estrus (OE) was defined as a cow exceeding an activity change index value of 35. End of estrus (EE) was defined by the first instance at which the index value fell below 35 again. The intensity of an estrus event was represented by the peak of the activity change index value (PAE) during an estrus event. Furthermore, PAE was classified into low intensity (35 to 89 index value) and high intensity (90 to 100 index value). Duration of an estrus event (DE) was defined as the interval from OE to EE.

The mean (± standard deviation) DE was 14.3 ± 4.6 h. The mean interval from OE to AI was 16.8 ± 8.0 h, from PAE to AI was 11.9 ± 8.1 h, and from EE to AI was 2.5 ± 8.7 h. Primiparous cows (31.4%) had greater P/AI than multiparous cows (27.3%) and first

postpartum AI (31.5%) yielded greater P/AI than subsequent AI services (27.2%). Heat stress decreased P/AI, such that exposure to a low THI of < 60 (36.1%) resulted in greater P/AI than exposure to a medium THI of 60 to 68 (28.6%) or a high THI of > 68 (23.9%) 1 wk before AI. For each farm, cumulative milk yield within the first 100 days in milk (DIM) was classified into quartiles. Cows with either low (29.8%) or high (31.6%) 100-d milk yield had greater P/AI than cows with intermediate (25.7%) 100-d milk yield. Estrous intensity was associated with P/AI. Cows with low PAE (26.0%) were less fertile compared with cows showing high PAE (32.8%). Type of semen was not associated with P/AI, as cows inseminated with frozen semen (28.8%) achieved similar P/AI compared with cows inseminated with fresh semen (29.8%). There was a quadratic effect of the interval from OE to AI on P/AI. Pregnancy per AI was greatest for cows inseminated from 7 to 24 h after OE. We did not observe an interaction between the interval from OE to AI and type of semen. Furthermore, greatest P/AI was achieved for cows inseminated within 18 h after PAE or from 5 h before EE to 12 h after EE. There tended to be an interaction between type of semen and the intervals from PAE to AI and from EE to AI. Cows inseminated with fresh semen within 5 h before EE had greater P/AI than cows inseminated with frozen semen. In addition, cows inseminated with frozen semen from 13 to 18 h after EE had greater P/AI than cows inseminated with fresh semen.

Results from this study allow one to draw the conclusion that inseminating cows from 7 to 24 h after OE or within 18 h after PAE yields greatest P/AI irrespective of type of semen. In addition, high PAE was favourable for P/AI. As only two-thirds of cows expressed high PAE, further research is warranted to assess factors associated with suboptimum estrous intensity and the underlying physiology.

The second study aimed to identify factors associated with estrous expression (i.e., DE and PAE) and subsequent fertility in lactating Holstein cows using the AAM system Heatime. Therefore, a total of 5,933 estrus events representing 3,132 lactating Holstein cows located on 8 commercial dairy farms in Germany were evaluated. All farms participated in monthly dairy herd improvement association's (DHIA) testing. To calculate the THI, climate loggers installed within the barns recorded ambient temperature and relative humidity hourly. Depending on individual farm management, pregnancy diagnosis was performed either by transrectal palpation ranging from 35 to 46 d after AI or transrectal ultrasonography at 30 ± 3 d after AI. In agreement with the first study, the same categories were used to categorize PAE (i.e., low = index values from 35 to 89; high = index values from 90 to 100).

Overall, the mean (± standard error of the mean) DE was 14.94 ± 0.06 h. More than two-thirds of estrus events (73.5%) were of high PAE. Because we found a strong correlation (r = 0.67) between DE and PAE, only PAE was used in the final statistical model as a fixed

effect. Cows with high PAE (28.5%) had 1.35 greater odds of pregnancy compared with cows with low PAE (22.8%). Heat stress indicated by an increased THI 1 wk before AI was associated with shorter DE, lower PAE and subsequently decreased P/AI. The change of weighted rumination measured by the AAM system was associated with estrous expression, as a lower nadir was associated with a greater risk for high PAE and long DE. There was no association, however, between the nadir of change of weighted rumination and P/AI. The AAM system detected a small percentage of cows (4.7%) with short inter-estrus intervals (i.e., more than one activity peak within 7 d close to the event of estrus). Cows with a short inter-estrus interval had reduced DE and PAE and showed decreased P/AI (19.5%) compared with cows with no short inter-estrus interval (32.8%). Furthermore, primiparous cows (29.4%) had greater P/AI than multiparous cows (22.1%). Interestingly, PAE did not differ among parities, but multiparous cows expressed estrus with longer DE (13.15 ± 0.31 h) than primiparous cows (12.52 ± 0.32 h). An estrus event with long DE or high PAE was more likely later in lactation.

Dairy herd improvement association's test data from the date closest to AI was associated with estrous expression or P/AI in different ways. We did not find an association between milk fat, milk urea nitrogen, or lactose with estrous expression or P/AI. There was an association, however, between milk protein, milk yield, and somatic cell count from DHIA test date before AI and estrous expression, but not P/AI. Increasing milk protein increased DE and the likelihood for an estrus event with high PAE, whereas an increase in milk yield and somatic cell count (especially > 1,000 x 10^3 cells/mL) was negatively associated with estrous expression.

In summary, DE and PAE were highly correlated, and particularly cows with high PAE were associated with greater odds for pregnancy. Whereas risk factors, such as DIM at AI, THI 1 wk before AI, and short inter-estrus intervals were associated with both estrous expression and P/AI, change of rumination, udder health, and milk yield were only associated with estrous expression but not with subsequent P/AI.

The objective of the third study was to evaluate two modifications (i.e., double dose, second administration 24 h apart) of the PGF treatment using cloprostenol in a 7-d Ovsynch protocol compared with a standard single PGF dose on P4 concentration at the time of G2 and P/AI in lactating Holstein cows. This study was subdivided into 2 experiments with a total of 8 participating commercial dairy farms in Germany. Cows from both experiments were assigned on a weekly basis in a consecutive manner to receive 1 of the 3 treatments: 1) a traditional Ovsynch including a standard single PGF dose of 500 µg cloprostenol (control: GnRH; 7 d, PGF; 9 d, GnRH), 2) an Ovsynch with a double PGF dose of 1,000 µg cloprostenol (GDPG: GnRH; 7 d, 2xPGF; 9 d, GnRH), or 3) an Ovsynch with a second PGF treatment of 500 µg

cloprostenol 24 h later (GPPG: GnRH; 7 d, PGF; 8 d, PGF; 32 h, GnRH). All cows received timed AI (TAI) approximately 16 h after G2. Pregnancy diagnosis was performed either by transrectal palpation at 38 ± 3 d after TAI (experiment 1) or transrectal ultrasonography at 35 ± 7 d after TAI (experiment 2). To facilitate first postpartum TAI, farms from experiment 1 used a Presynch-Ovsynch protocol (PGF, 14 d later PGF, 12 d later GnRH, 7 d later PGF, 2 d later GnRH, and 16 to 18 h later TAI), whereas no presynchronization protocol was used on farms from experiment 2. A total of 1,581 lactating dairy cows (60 experimental units) from 2 dairy farms were enrolled in experiment 1. Blood samples were collected from a subsample of these cows (n = 491; 16 experimental units) at the time of G2 to determine P4 concentration. In experiment 2, 1,979 lactating dairy cows (252 experimental units) from 6 dairy farms were enrolled. Transrectal ultrasonography was performed in all of the latter cows to determine the presence or absence of a corpus luteum (CL) at the time of the first GnRH treatment (G1).

In experiment 1, treatment affected P4 concentration at G2. The percentage of cows with very low P4 concentration (0.00 to 0.09 ng/mL) at G2 was increased by both treatments (n (GDPG) = 44/179, n (GPPG) = 45/154) compared with the control group (n = 23/158). Only the GPPG treatment, however, decreased the percentage of cows with high P4 concentration (0.6 ng/mL) at G2 compared with the control group (n (control) = 70/158, n (GDPG) = 66/179, n (GPPG) = 41/154). In addition, P/AI was greater for GDPG (38.2%) and GPPG (38.9%) than for control cows (29.8%). In experiment 2, GPPG cows (37.4%) had greater P/AI than control cows (31.0%) and tended to have greater P/AI than GDPG cows (31.8%). Pregnancy per AI did not differ for GDPG and control cows. Interestingly, cows with a CL at G1 (n = 989/1,662) had similar P/AI (34.1%) to cows without a CL at G1 (32.6%) and we found no interaction between treatment and presence of a CL at G1 on P/AI. Because both experiments were conducted using a similar experimental design, results were combined to achieve more statistical power to test the effect of treatment on P/AI. To avoid a possible effect of presynchronization on treatments, cows receiving first postpartum TAI from experiment 1 (n = 572) were excluded from the combined analysis (n = 2,573). Combined results indicated greater P/AI for GPPG (40.3%) than for control (31.8%) and GDPG cows (33.4%). There was no difference for P/AI between GDPG and control cows.

Results from this study demonstrate that compared with the traditional 7-d Ovsynch including a single standard PGF dose on d 7 adding a second PGF treatment on d 8 during a 7-d Ovsynch protocol decreased the proportion of cows with high P4 concentration at G2 and increased P/AI by 8.5 percentage units. Adding of a second PGF treatment on d 8 also seems to increase P/AI compared with a double PGF dose (+ 6.9 percentage units). Doubling the PGF dose on d 7 in a 7-d Ovsynch protocol only increased the proportion of cows with very low P4 concentration at G2, but did not affect P/AI compared with a single PGF dose (+ 1.6

percentage units). After all, it cannot be ruled out that the use of a presynchronization protocol influences the effect of a PGF dose frequency modification in an Ovsynch protocol. This was indicated by presynchronized cows receiving first postpartum TAI from experiment 1 having similarly increased P/AI treated with a double PGF dose compared with a second PGF dose. To confirm a possible interaction between the treatment effect and presynchronization, future research is warranted.

Overall, this thesis shows that 1) inseminating cows from 7 to 24 h after OE or within 18 h after PAE yields greatest P/AI irrespective of type of semen using a neck-mounted AAM system, 2) estrous expression is highly associated with greater odds for pregnancy, but the proportion of cows showing high estrous expression is limited as influenced by many risk factors, and 3) modification of the 7-d Ovsynch protocol using a second PGF treatment on d 8 is beneficial for P/AI compared with a single or a double PGF dose on d 7. Taken together, these results have the potential to improve reproductive performance on dairy farms using AAM systems or TAI protocols or a combination of both.

7 ZUSAMMENFASSUNG

Optimierung des Fruchtbarkeitsmanagements bei Milchkühen auf Betrieben mit Aktivitätssensoren zur automatischen Brunsterkennung oder Protokollen zur terminorientierten künstlichen Besamung

Die Ziele dieser Arbeit waren bei laktierenden Holstein-Kühen mit Aktivitätssensoren zur automatischen Brunsterkennung (AAM) 1) den Besamungszeitpunkt für Frischsperma und Tiefgefriersperma in Relation zu verschiedenen Brunstcharakteristika (d.h. Beginn, Maximum und Ende einer Brunst) hinsichtlich des Besamungserfolges (BE) zu ermitteln sowie 2) Risikofaktoren zu identifizieren, die die Brunstexpression und den darauffolgenden BE beeinträchtigen können. Ein weiteres Ziel war es 3) bei laktierenden Holstein-Kühen zwei Modifikationen der Prostaglandin $F_{2\alpha}$ (PGF) – Gabe (d.h. doppelte Dosis, zweite Gabe 24 h später) unter der Verwendung von Cloprostenol in einem 7-Tage (d) Ovsynch-Protokoll zu evaluieren hinsichtlich des Effekts auf die Progesteron-Konzentration (P4) zum Zeitpunkt der zweiten GnRH-Gabe (G2) und den BE verglichen mit einer einfachen Standarddosis PGF.

Um den Zusammenhang zwischen dem Besamungszeitpunkt mit entweder Frisch- oder Tiefgefriersperma und verschiedenen, mit AAM gemessenen Brunstcharakteristika (d.h. Beginn, Maximum und Ende einer Brunst) hinsichtlich des BE zu ermitteln, wurde eine Beobachtungsstudie auf 4 deutschen kommerziellen Milchviehbetrieben durchgeführt. Hierzu wurden insgesamt 3.607 künstliche Besamungen (kB), basierend auf dem Alarm des als Halsband angebrachten AAM (Heatime; SCR Engineers Ltd., Netanya, Israel), ausgewertet. Abhängig vom Betrieb wurde eine Trächtigkeitsuntersuchung entweder via transrektaler Palpation d 38 ± 3 nach kB oder via transrektaler Sonographie d 30 ± 3 nach kB durchgeführt. Um beurteilen zu können, ob Kühe Hitzestress ausgesetzt waren, wurden in den Ställen Logger angebracht, die kontinuierlich Temperatur und relative Luftfeuchtigkeit aufzeichneten und somit den Temperatur-Luftfeuchtigkeits-Index (THI) berechnen konnten.

Mittels eines geeigneten Algorithmus konvertiert die AAM-Software die individuelle Rohaktivität jeder Kuh in einen Aktivitätsänderungsindex. Dieser Index kann Werte annehmen von 0 (Minimum) bis 100 (Maximum). Als Brunstbeginn (OE) wurde das Überschreiten eines Aktivitätsänderungsindex‘ von 35 definiert. Das Brunstende (EE) wurde definiert als der erste Moment, in dem der Aktivitätsänderungsindex wieder unter 35 fiel. Die Brunstintensität wurde repräsentiert durch das Maximum des Aktivitätsänderungsindex (PAE) während einer Brunst. Des Weiteren wurde PAE unterteilt in geringe Intensität (Index von 35 bis 89) und hohe Intensität (Index von 90 bis 100). Als Brunstdauer (DE) wurde das Intervall zwischen OE und EE definiert.

Die mittlere (± Standardabweichung) DE betrug 14,3 ± 4,6 h. Das mittlere Intervall betrug 16,8 ± 8,0 h zwischen OE und kB, 11,9 ± 8,1 h zwischen PAE und kB und 2,5 ± 8,7 h zwischen EE und kB. Ein höherer BE wurde erlangt bei primiparen Kühen (31,4%) im Vergleich zu multiparen Kühe (27,3%) sowie bei Erstbesamungen (31,5%) im Vergleich zu Folgebesamungen (27,2%). Hitzestress eine Woche vor der kB minderte den BE, sodass ein niedriger THI von < 60 (36,1%) einen höheren BE zur Folge hatte als ein mittlerer THI von 60 bis 68 (28,6%) oder ein hoher THI von > 68 (23,9%). Für jeden Betrieb wurde die kumulative Milchleistung innerhalb der ersten 100 Tage in Milch (DIM) ermittelt sowie in Quartile unterteilt. Kühe mit entweder niedriger (29,8%) oder hoher (31,6%) 100-d Milchleistung hatten einen höheren BE als Kühe mit einer mittleren (25,7%) 100-d Milchleistung. Auch die Brunstintensität beeinflusste den BE. Kühe mit einer niedrigen PAE (26,0%) waren weniger fertil als Kühe mit einer hohen PAE (32,8%). Die Art des Spermas stand in keinem Zusammenhang mit dem BE, da mit Tiefgefriersperma besamte Kühe (28,8%) einen ähnlichen BE zeigten wie mit Frischsperma besamte Kühe (29,8%). Hinsichtlich des BE wurde ein quadratischer Effekt beobachtet für das Intervall von OE bis kB. Den höchsten BE hatten Kühe, die zwischen 7 und 24 h nach OE besamt wurden. Wir konnten keine Interaktion zwischen dem Intervall von OE bis kB und der Spermaart feststellen. Den höchsten BE erzielten Kühe, die innerhalb von 18 h nach PAE oder innerhalb von 5 h vor EE bis 12 h nach EE besamt worden waren. Für die Interaktion zwischen Spermaart und den Intervallen von PAE bis kB und EE bis kB wurde eine Tendenz beobachtet. Kühe, die innerhalb von 5 h vor EE mit Frischsperma besamt worden waren, hatten einen höheren BE als Kühe, die in diesem Zeitraum mit Tiefgefriersperma besamt wurden. Zusätzlich hatten Kühe, die innerhalb von 13 bis 18 h nach EE besamt worden waren einen höheren BE mit Tiefgefriersperma im Vergleich zu Frischsperma.

Diese Studienergebnisse erlauben den Schluss, dass eine kB innerhalb von 7 bis 24 h nach OE oder innerhalb von 18 h nach PAE den höchsten BE erzielt unabhängig von der verwendeten Spermaart. Eine hohe PAE begünstigte außerdem den BE. Da jedoch nur zwei Drittel der Kühe eine hohe PAE aufwiesen, sind weitere Forschungsarbeiten notwendig, um deren zugrundeliegende Physiologie zu ermitteln sowie Faktoren, die eine suboptimale PAE bedingen.

Das Ziel der zweiten Studie war es, Risikofaktoren zu identifizieren, die die Brunstexpression (d.h. DE und PAE) und den darauffolgenden BE bei laktierenden Holstein-Kühen beeinträchtigen auf Betrieben mit Heatime als AAM. Hierzu wurden insgesamt 5.933 Brunsten von 3.132 Kühen von 8 deutschen kommerziellen Milchviehbetrieben ausgewertet. Alle Betriebe nahmen an der monatlichen Milchleistungsprüfung (MLP) teil. Um den THI zu berechnen wurden Klimalogger zur stündlichen Messung von Umgebungstemperatur und relativer Luftfeuchtigkeit in den Ställen angebracht. Je nach Betriebsmanagement erfolgte eine

Trächtigkeitsuntersuchung entweder zwischen d 35 bis 46 nach kB via transrektaler Palpation oder zwischen d 30 ± 3 nach kB via transrektaler Sonographie. Wie bereits in der ersten Studie wurden die gleichen Kategorien gewählt zur Einteilung der PAE (d.h. niedrig = Index von 35 bis 89; hoch = Index von 90 bis 100).

Die mittlere (± Standardfehler) DE betrug 14,94 ± 0,06 h. Über zwei Drittel der Brunsten (73,5%) wiesen eine hohe PAE auf. Aufgrund der starken Korrelation (r = 0,67) zwischen DE und PAE wurde im finalen Statistikmodell lediglich PAE als fester Effekt verwendet. Kühe mit hoher PAE (28,5%) hatten eine 1,35 höhere Chance tragend zu werden als Kühe mit niedriger PAE (22,8%). Der durch einen erhöhten THI verifizierte Hitzestress eine Woche vor der kB bewirkte eine kürzere DE, niedrigere PAE und einen darauffolgenden schlechteren BE. Die vom AAM erfasste Änderung des gewichteten Wiederkäuens stand in Bezug zur Brunstexpression; ein niedriger Nadir war assoziiert mit einer höheren Wahrscheinlichkeit für eine Brunst mit hoher PAE und von langer DE. Zwischen dem BE und der Änderung des gewichteten Wiederkäuens war jedoch kein Zusammenhang erkennbar. Ein geringer Prozentsatz an Kühen (4,7%) wurde vom AAM mit einem kurzen Zwischenbrunstintervall (d.h. mehr als ein Aktivitätsmaximum innerhalb von 7 d zeitlich nah zur Brunst) detektiert. Kühe mit einem kurzen Zwischenbrunstintervall hatten eine verminderte DE, PAE und einen schlechteren BE (19,5%) verglichen mit Kühen, die kein verkürztes Zwischenbrunstintervall (32,8%) zeigten. Des Weiteren hatten primipare Kühe (29,4%) einen höheren BE als multipare Kühe (22,1%). Interessanterweise unterschied sich die PAE zwischen den Paritäten nicht, allerdings waren die Brunsten multiparer Kühe (13,15 ± 0,31 h) länger als die primiparer Kühe (12,52 ± 0,32 h). Lange oder intensive Brunsten traten außerdem mit einer höheren Wahrscheinlichkeit in einem fortgeschrittenen Laktationsstadium auf.

Die Daten der MLP mit zeitlich engstem Bezug zur kB waren auf unterschiedliche Art mit Brunstexpression oder BE assoziiert. Kein Zusammenhang bestand zum Gehalt an Fett, Harnstoff oder Laktose in der Milch. Einen Zusammenhang gab es jedoch zwischen Milchprotein, Milchleistung und somatischer Zellzahl der MLP vor der kB und der Brunstexpression, allerdings nicht dem BE. Ein Anstieg des Milchproteingehaltes erhöhte die DE und die Wahrscheinlichkeit für eine hohe PAE, wohingegen sich ein Anstieg der Milchleistung und der somatischen Zellzahl (v.a. > 1.000 x 10^3 Zellen/mL) negativ auf die Brunstexpression auswirkte.

Zusammenfassend waren DE und PAE eng korreliert und vor allem Kühen mit einer hohen PAE hatten bessere Chancen tragend zu werden. Wohingegen sich Risikofaktoren wie DIM zur kB, THI eine Woche vor kB und kurze Zwischenbrunstintervalle sowohl negativ auf die Brunstexpression als auch auf den BE auswirkten, waren die Änderung des gewichteten

Wiederkäuens, somatische Zellzahl und Milchleistung lediglich mit der Brunstexpression assoziiert und nicht mit dem darauffolgenden BE.

In der dritten Studie wurden bei laktierenden Holstein-Kühen zwei Modifikationen der PGF-Gabe (d.h. doppelte Dosis, zweite Gabe 24 h auseinander) unter der Verwendung von Cloprostenol in einem 7-d Ovsynch Protokoll untersucht. Beurteilt wurde der Effekt auf die P4-Konzentration zum Zeitpunkt von G2 und den BE verglichen mit einer einfachen Standarddosis PGF. Diese Studie wurde in zwei Experimente unterteilt, die insgesamt 8 aus Deutschland stammende, kommerzielle Milchviehbetriebe beinhalteten. Die Kühe beider Experimente wurden wöchentlich aufeinanderfolgend in eines der 3 Behandlungsprotokolle eingeteilt: 1) traditionelles Ovsynch mit standardmäßig einmaliger PGF-Gabe von 500 µg Cloprostenol (Kontrolle: GnRH; 7 d, PGF; 9 d, GnRH), 2) Ovsynch mit einer doppelten PGF-Dosis von 1.000 µg Cloprostenol (GDPG: GnRH; 7 d, 2xPGF; 9 d, GnRH), oder (3) Ovsynch mit einer zweiten PGF-Gabe von 500 µg Cloprostenol 24 h später (GPPG: GnRH; 7 d, PGF; 8 d, PGF; 32 h, GnRH). Alle Kühe erhielten eine terminorientierte künstliche Besamung (TkB) ungefähr 16 h nach G2. Eine Trächtigkeitsuntersuchung wurde entweder mit transrektaler Palpation d 38 ± 3 nach TkB durchgeführt (Experiment 1) oder mit transrektaler Sonographie d 35 ± 7 nach TkB (Experiment 2). Zur Erleichterung der ersten TkB postpartum nutzten die Betriebe von Experiment 1, nicht aber die von Experiment 2, ein Presynch-Ovsynch-Protokoll (PGF, 14 d später PGF, 12 d später GnRH, 7 d später PGF, 2 d später GnRH, und 16 bis 18 h später TkB). Insgesamt wurden 1.581 Kühe (60 experimentelle Einheiten) von 2 Betrieben in Experiment 1 aufgenommen. Von einem Teil dieser Kühe (n = 491; 16 experimentelle Einheiten) wurden Blutproben zu G2 entnommen um die P4-Konzentration bestimmen zu können. In Experiment 2 wurden 1.979 Kühe (252 experimentelle Einheiten) von 6 Betrieben aufgenommen. Bei all jenen Kühen wurde eine transrektale Sonographie durchgeführt, um Auskunft über das Vorhandensein eines Gelbkörpers (CL) zur Zeit der ersten GnRH-Injektion (G1) zu erhalten.

In Experiment 1 wurde die P4-Konzentration zu G2 vom Behandlungsprotokoll beeinflusst. Der prozentuale Anteil an Kühen mit sehr niedriger P4-Konzentration (0,00 bis 0,09 ng/mL) zu G2 wurde durch beide Behandlungsprotokolle erhöht (n (GDPG) = 44/179, n (GPPG) = 45/154) verglichen mit der Kontrollgruppe (n = 23/158). Allerdings war es nur mit dem GPPG-Protokoll möglich, den Prozentsatz an Kühen mit hoher P4-Konzentration (0,6 ng/mL) zu G2 im Vergleich mit der Kontrollgruppe zu verringern (n (Kontrolle) = 70/158), n (GDPG) = 66/179, n (GPPG) = 41/154). Zusätzlich erzielten Kühe der Protokolle GDPG (38,2%) und GPPG (38,9%) einen höheren BE als Kühe der Kontrollgruppe (29,8%). In Experiment 2 hatten Kühe des GPPG-Protokolls (37,4%) einen höheren BE als die Kontrollgruppe (31,0%) und tendierten zu einem höheren BE als Kühe des GDPG-Protokolls

(31,8%). Der BE zwischen dem GDPG-Protokoll und der Kontrollgruppe unterschied sich nicht. Interessanterweise hatten Kühe mit einem CL zu G1 (n = 989/1.662) einen ähnlichen BE (34,1%) wie Kühe ohne CL zu G1 (32,6%). Auch konnten wir keine Interaktion zwischen Behandlungsprotokoll und dem Vorhandensein eines CL zu G1 hinsichtlich des BE feststellen. Aufgrund des ähnlichen experimentellen Aufbaus beider Experimente wurden die Ergebnisse kombiniert, um einen Effekt der Behandlungsgruppe im Ovsynch-Protokoll auf den BE mit größerer statistischer Aussagekraft testen zu können. Um eine Beeinflussung der Ergebnisse durch eventuelle Effekte einer Vorsynchronisation auf die Behandlungsprotokolle auszuschließen, wurden die Kühe aus Experiment 1 (n = 572), die zur Erstbesamung anstanden, aus der kombinierten Analyse (n = 2,573) ausgeschlossen. Die Ergebnisse der kombinierten Analyse indizierten einen höheren BE für Kühe des GPPG-Protokolls (40,3%) als für Kühe der Kontrolle (31,8%) oder des GDPG-Protokolls (33,4%). Der BE für Kontroll- und GDPG-Kühe unterschied sich nicht.

Diese Studienergebnisse demonstrieren, dass eine zweite PGF-Gabe an d 8 in einem 7-d Ovsynch-Protokoll den Prozentsatz an Kühen mit hoher P4-Konzentration zu G2 senken und den BE erhöhen kann (+ 8,5%) im Vergleich mit einem traditionellen 7-d Ovsynch bestehend aus einer einfachen PGF-Dosis an d 7. Des Weiteren erhöht eine zweite PGF-Gabe an d 8 den BE (+ 6,9%) verglichen mit einer doppelten PGF-Dosis. Eine doppelte PGF-Dosis an d 7 in einem 7-d Ovsynch-Protokoll erhöhte lediglich den Prozentsatz an Kühen mit sehr niedriger P4-Konzentration, hatte mit der Kontrollgruppe verglichen jedoch keine Auswirkungen auf den BE (+ 1,6%). Allerdings kann nicht ausgeschlossen werden, dass der Einsatz von Protokollen zur Vorsynchronisation den Effekt einer Dosis-Frequenz-Modifikation der PGF-Gabe in einem Ovsynch-Protokoll beeinflusst. Dies indizierten die Ergebnisse aus Experiment 1, da hier zur Erstbesamung vorsynchronisierte Kühe einen ähnlichen Anstieg des BE aufwiesen sowohl nach Behandlung mit einer doppelten PGF-Dosis als auch mit einer zweiten PGF-Gabe. Um eine mögliche Interaktion zwischen dem Behandlungseffekt und einer Vorsynchronisation zu bestätigen, ist weitere Forschung notwendig.

Insgesamt zeigt meine Doktorarbeit, dass 1) unter der Nutzung von AAM die kB von Kühen innerhalb von 7 bis 24 h nach OE oder innerhalb von 18 h nach PAE den höchsten BE erzielt unabhängig von der verwendeten Spermaart, 2) Brunstexpression hochgradig mit den Chancen einer Trächtigkeit assoziiert ist, viele Risikofaktoren den Anteil an Kühen mit hoher PAE jedoch beeinflussen und einschränken, und 3) eine Modifikation des 7-d Ovsynch-Protokolls mit einer zweiten PGF-Gabe an d 8 sich positiv auf den BE auswirkt verglichen mit einer einfachen oder doppelten PGF-Dosis an d 7. Insgesamt haben diese Ergebnisse das Potenzial das Fruchtbarkeitsmanagement auf Milchviehbetrieben zu verbessern, die entweder AAM zur Brunsterkennung oder Protokolle zur TkB oder eine Kombination beider einsetzen.

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9 PUBLICATIONS

Research articles

Tippenhauer C M, Borchardt S (2019):
Optimierung des Besamungszeitpunktes: Wie nutze ich die Heatime-Daten bestmöglich?
Rind und Wir: Das Rinder-Fach-Journal, 25, S. 61.

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Oral presentations

Tippenhauer C M, Plenio J-L, Madureira A M L, Cerri R L A, Heuwieser W, Borchardt S (2019):
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Tippenhauer C M (2019, 2020):
Wie finde ich den optimalen Besamungszeitpunkt mit Hilfe von Heatime?
Rinder-Allianz-Brunch Sachsen-Anhalt. 15. / 16. / 23. / 24.10.2019.
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Tippenhauer C M (2020):
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53. Jahrestagung Physiologie und Pathologie der Fortpflanzung und gleichzeitig 45. Veterinär-Humanmedizinische Gemeinschaftstagung. Rostock-Warnemünde. 26.02. - 28.02.2020.
In: Reproduction in Domestic Animals, 55: 30. doi: 10.1111/rda.13591.

Poster presentations

Borchardt S, Tippenhauer C M, Steinmetz I, Heuwieser W (2019):
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11 DECLARATION OF INDEPENDECE

Hiermit erkläre ich, dass ich, Christie Marie Tippenhauer, alle Studien selbstständig durchgeführt und die vorliegende Arbeit selbstständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Tabelle 1. Eigener Anteil[1] an den Forschungsprojekten der vorliegenden Dissertation

	Studie 1[a]	Studie 2[b]	Studie 3[c]
Studienplanung	+++	+++	+++
Datenerhebung	+++	+++	++
Datenanalyse	+++	+++	++
Verfassen des Manuskripts	+++	+++	+++
Editieren des Manuskripts	+++	+++	++

[1]Legende: +++: > 70%
++: 50-70%
+: < 50%

[a]Timing of artificial insemination using fresh or frozen semen after automated activity monitoring of estrus in lactating dairy cows.

[b]Factors associated with estrous expression and subsequent fertility in lactating dairy cows using automated activity monitoring.

[c]Effect of dose and timing of prostaglandin F2a treatments during a 7-d Ovsynch protocol on progesterone concentration at the end of the protocol and pregnancy outcomes in lactating Holstein cows.

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