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# Effect of using liquid semen on fertility in German Holstein Friesian dairy cattle: A randomized controlled clinical trial

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ABSTRACT

Low fertility rates in lactating dairy cows as well as restricted availability of semen doses of young bulls with high genetic merit are two major problems in the reproduction of dairy cows. By using liquid semen (LS), the number of doses per ejaculate can be increased. One of the challenges of optimizing the reproductive performance of dairy cows is the phenomenon of variable estrus lengths. The objective of this study was to determine whether the use of LS affects pregnancy outcome of dairy cows with delayed ovulation, when compared with frozen semen (FS). A randomized controlled clinical trial was implemented. In a split-sample procedure, 131 ejaculates were processed into LS (Caprogen, LIC, New Zealand) and FS (BioXcell, IMV, France). Both semen types of each ejaculate were inseminated under the same field conditions to cows showing natural or induced heat. Cows and semen type were allocated according to the last digit of the cows identification number (even = frozen semen, odd = liquid semen). Inseminations (n = 667) were conducted after localization of the pre-ovulatory follicle. Determination of ovulation was performed 24 h post AI per transrectal ultrasonographic examination. Ovulations were classified as delayed when the pre-ovulatory follicle was still present at ovulation control. The prevalence of delayed ovulations was 25.2%. Data of 667 inseminations were analyzed with a generalized linear mixed model including semen type ($P = 0.016$), parity ($P = 0.014$), backfat thickness ($P = 0.006$), estrus induction ($P = 0.010$), ovulation ($P = 0.265$) and the interaction term 'semen type by ovulation' ($P = 0.094$). Overall, a higher pregnancy per AI (P/AI) of LS (45.4%) than P/AI of FS (33.7%) was found. In cases of delayed ovulations, use of LS resulted in higher P/AI (46.8%) compared with FS (27.7%; $P = 0.017$). We concluded that the fertilizing capacity of LS in prolonged intervals from AI to ovulation might be greater when compared with FS and could be an efficient tool to improve fertility of lactating dairy cows with delayed ovulations.



## 1. Introduction

In the last decades, poor reproductive performance has been a limiting factor for fast genetic progress in the dairy cattle industry. Currently, there is great interest in using young breeding bulls of high genetic merit, which has led to the premature commencement of semen production. As a result, quantity and quality of suitable AI doses during the early production phase are usually unsatisfactory [1]. An alternative to overcome limitations of frozen semen is the use of liquid semen.

Furthermore, low fertility rates in dairy cows poses one of the greatest challenges in the dairy industry worldwide [2,3]. This is mirrored in the fact that poor reproductive performance is the main reason for involuntary culling of dairy cattle [3]. Besides the traditional visual observation of herds, there are numerous methods for heat detection that can be used including technical devices, all accompanied by their own advantages and disadvantages as reviewed by Roelofs et al. [4]. While heat detection is necessary to determine optimal timing for AI, if poorly executed, it can become one of the costliest factors on farms [5]. Inseminating cows too early leads to an insufficient number of fertile sperm once the oocyte reaches the oviduct. Inseminating cows too late, does not allow sperm the time to capacitate before the oocyte

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degenerates [6]. Several studies have investigated the optimal timing of AI in the past, forming the well-known a.m.-p.m. rule, where AI is implemented twice daily [7,8]. According to the a.m.-p.m. rule, cows seen in heat during the evening will be inseminated the next morning and cows seen in heat in the morning, will be inseminated in the evening. This ensures an interval of approximately 12 h from heat detection until AI. Contrary to this rule of thumb, it has been ascertained that AI once daily is just as effective as AI twice daily [9]. This finding is also supported by a recent study which used automated activity monitoring for estrus detection. It was observed that pregnancy outcome was greatest when AI happened once daily 7–24 h after onset of estrus, within 18 h after peak activity or 5 h before end of estrus [10]. However, even if estrus detection and AI are executed and timed correctly, the interval from onset of estrus until ovulation is extremely variable between individuals. Some researchers showed varying times from estrus to ovulation, e.g. 27.6 ± 5.4 h [11], 28.7 ± 8.1 h [12] or 30.0 ± 5.1 h [13]. Very long intervals, meaning ovulation has not occurred within 24 h after AI, we have termed as 'delayed ovulation'. In a field study, prevalence of delayed ovulations was 46% [14], which constitutes a serious impairment of herd fertility traits. Furthermore, if ovulation does not occur within 24 h after AI, the fertilization capacity of sperm rapidly declines [15] and a second AI would be strongly advisable. This is especially the case when frozen semen (FS) has been used.

Frozen semen is the most commonly used preservation method for bovine semen worldwide, in 1998 it accounted for 95% of all produced semen doses [16]. The other established, yet rarely used, semen type is liquid semen. When compared under various field conditions, LS and FS yielded similar pregnancy outcomes [17–20]. However, due to the avoidance of cryoinjury, LS maintains fertilizing capacity of spermatozoa for longer when compared to FS [15,21]. This discrepancy was reflected in the results of two recent investigations. Borchardt et al. [22] observed greater pregnancy per AI (P/AI) for LS compared to FS (27.5% and 20.0%; $P = 0.032$) after a CoSynch-56-protocol, a timed artificial protocol provoking a long interval from AI until ovulation. Tippenhauer et al. [10] used automated activity monitoring for estrus detection and observed a tendency for greater P/AI for LS compared to FS when cows were inseminated 5 h before the end of estrus ($P = 0.03$). In conclusion, both studies detected a tendency for superior pregnancy outcomes in cases of a prolonged interval between AI and ovulation when LS was used, compared with FS.

Nevertheless, previous investigations have not compared LS and FS under a strict randomized split-sample procedure in combination with determining the time interval between AI and ovulation. The aim of the present study was to determine the difference in P/AI when using LS or FS in dairy cows with a regular or prolonged interval between AI and ovulation. We hypothesized that animals with delayed ovulation would have better P/AI when using LS compared to FS.

## 2. Materials and methods

The study was designed as a randomized controlled clinical trial. A sample size of 967 was calculated for a different part of the study including biological samples of the animals. After validating the collected data, however, we conducted a post-hoc power analysis in G*Power (Version 3.1.9.6, University of Kiel, Germany) using a logistic regression procedure with large sample correction according to Demidenko [23] with variance correction (alpha = 0.05, $R^2$ within other X = 0.1, distribution of semen type = binomial, and $\pi$ for semen type = 0.49). All procedures were approved by the Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen (file number: 81-02.04.2019.A299).

### 2.1. Farms and animals

Lactating Holstein Friesian dairy cows housed on five commercial farms (1–5) in a western region in Germany were enrolled in this study. All cows were kept in free-stall barns, however, milking systems, feed, heat detection and management differed between farms. The herd size ranged from 163 to 743 animals and the milk yield (305 d) lay between 9,545 and 11,342 kg. Further information on basic farm characteristics is provided in Table 1.

### 2.2. AI center, bulls and semen processing

Bulls of high demand were selected according to pairing suggestions of the participating dairy farms. On each trial day, AI-technicians had AI doses of three Holstein-Friesian bulls and one Belgian Blue available. During the study period (June 2019 to April 2020), bulls aged between 1.5 and 3 years were used (4 Holstein Frisian, 3 Belgian Blue). All bulls were housed under the same conditions at the AI center of Rinder Union West eG in Borken, Germany. Their health status was regularly checked according to industry standards and statutory provisions.

Insemination doses for the trial were produced once weekly following standard operating procedures of the AI center. An overview of bull characteristics and average traits of native ejaculates is displayed in Table 2. Five to 40 ejaculates and 35 to 158 AI per bull were used for the trial. Ejaculates were collected using an artificial vagina. After passing quality check (>75% progressive motile sperm measured with Androvision v3.2, Minitüb, Germany; $>500 \times 10^6$ sperm/mL measured with SDM 5 Photometer, Minitüb, Germany) each ejaculate was split into two aliquots. One aliquot was processed using liquid semen diluent Caprogen ($9 \times 10^6$ sperm per straw; LIC, New Zealand). The other aliquot was diluted using BioXcell ($25 \times 10^6$ sperm per straw; IMV, France) and cryopreserved after 4 h of equilibration in 0.25 mL EcoStraws (Minitüb, Germany) at 4 °C. LS was filled into 0.25 mL 'MiniStraw for fresh semen' (Minitüb, Germany) and then stored in cooled water (13 ± 1 °C starting temperature) inside an insulated container, which was kept inside a Styrofoam box. Water temperature was checked at every removal of straws, but at the very least at the beginning and end of each work day. If water reached temperatures above 20 °C, straws contained therein were discarded. Frozen semen was stored in liquid nitrogen.

Quality of produced batches was controlled on the day of production by computer assisted sperm analysis (Androvision v3.2, Minitüb, Germany). For each batch, semen of three straws was pooled and incubated at 38 °C (thermo-resistance test). Both semen types had to maintain 45% total motile sperm after 120 min of incubation. Sperm were rated as motile when their head activity (HAC) was ≥0.087 rad. Liquid semen was also checked the following two days after production using straws that were exposed to similar temperature and transport stress as straws actually used for the trial.

### 2.3. Estrus induction, artificial insemination and ultrasound examinations

AI were performed the following two days after production of AI doses, mainly by the same two experienced AI-technicians. Farmers suggested animals for AI after spontaneous or hormonally induced estrus (Fig. 1). In case of hormonal estrus induction, two protocols were used, depending on the farm. The first protocol was a single application of prostaglandin F2α (PGF) after detection of a *corpus luteum* (PG, farms 2 and 3). The second protocol included an additional application of Gonadotropin releasing hormone (GnRH) 16 h prior to AI (PGG) (farms 1, 4 and 5). Whether heat was induced

**Table 1**
Descriptive characteristics of participating farms 1 to 5.

| Farm | Herd size, n [a] | AI included in data set, n [b] | 305 d Milk yield, kg [a] | Voluntary waiting period, d [a] | Calving interval, d [a] | Average DIM at AI, d [a] | Mean BFT 1d after AI ± SD, mm [b] |
|---|---|---|---|---|---|---|---|
| 1 | 668 | 332 | 9,545 | 60 | 408 | 124 | 9.2 ± 5.1 |
| 2 | 288 | 90 | 11,342 | 65 | 404 | 114 | 12.5 ± 7.3 |
| 3 | 163 | 28 | 10,158 | 45 | 378 | 95 | 9.1 ± 3.8 |
| 4 | 241 | 74 | 10,064 | 50 | 389 | 126 | 9.0 ± 4.6 |
| 5 | 742 | 143 | 9,606 | 60 | 388 | 117 | 13.6 ± 5.9 |

BFT = Backfat thickness, DIM = days in milk.
[a] Enquired through farm questionnaire.
[b] Derived from study animal measurements.

or which hormonal treatment was applied, was not part of our study design but choice of farmers or herdsmen. Cows in heat were identified by different heat detection systems, depending on the farm, but always amended by manual heat detection. Cows suggested for AI were pre-examined to assure correct timing for AI and to localize the pre-ovulatory follicle by rectal palpation as well as evaluation of vaginal discharge. Cows were excluded from the trial when vaginal discharge was doughy, purulent or already reddish in color. Other exclusion criteria were filling of the uterus, a palpable *corpus luteum* or a previous AI conducted less than 17 days before. If an animal was considered eligible, it was randomly allocated to receive either LS or FS based on the last digit of its unique animal identification number. Cows with even numbers were inseminated with FS and cows with odd numbers were inseminated with LS. Farmers, AI-technicians and other contributors were not blind to the allocated preservation method. Irrespective of the follicles location, the semen was always deposited in the body of the uterus. A transrectal ultrasound scan (the Easi-scan™ Ultrasound Scanner, IMV, France) 24 h after AI, conducted by the same veterinarian (MW), was used to determine if ovulation had occurred. Pregnancy diagnosis was conducted 32 ± 1 days after AI by using transrectal ultrasound scan performed by the same veterinarian (MW) or an experienced technician. Cows diagnosed not pregnant were reassigned to breeding after spontaneous or hormonal induction of estrus.

### 2.4. Assessment of backfat thickness

Backfat thickness (BFT) was measured by the same veterinarian (MW) 24 h post AI by ultrasound examination as described by Schroeder und Staufenbiel [24] using the Easi-scan™ (IMV, France) with a linear transducer in 'Detail' mode. The site of examination was located between the caudal quarter to one-fifth on a fictive line between *tuber coxae* and *tuber ischiadicum*. The unclipped hair was prepared with 80% diluted ethanol at the site of examination to ensure contact between transducer and skin. The linear transducer was placed vertically on the skin without pressure to avoid compression of the fat tissue. The backfat layer is limited by the skin and the *fascia trunci profunda,* which is located directly on top of the gluteal muscle. In an ultrasound image, the fat layer is displayed as a dark area between the brighter (base-) line representing the skin and the bright line representing the *fascia trunci profunda*. Often this dark area is divided by an additional thin white line representing the superficial fascia. After freezing the image, the distance from skin surface until *fascia trunci profunda* was measured to the nearest 0.1 cm.

### 2.5. Data collection and statistical analyses

The following information was recorded for each AI: farm, cow ID, parity, estrus induction, number of AI, days in milk, breeding date, semen type, sire, diagnosis of follicle control, diagnosis of ovulation control, backfat thickness and pregnancy diagnosis. Season of breeding was derived from breeding date (Winter: December, January, February; Spring: March, April, May; Summer: June, July, August; Autumn: September, October, November). Information was organized in Excel spreadsheets (Microsoft Office Professional Plus 2013, Microsoft Corp., Redmond, WA). Measurements of BFT were categorized into four groups (5 mm, 6–9 mm, 10–13 mm and >13 mm). Parity was categorized into three groups (1 gestation, 2 gestations and more than 2 gestations). Only complete data sets were used for statistical analyses with SPSS (version 25, SPSS Inc., Chicago, IL).

At first, we determined whether farm (1–5) or induction of estrus (spontaneous or induced; spontaneous, PG and PGG) was associated with prevalence of delayed ovulation by chi$^2$-tests.

Then, the GENLINMIXED procedure was used to create a multivariable mixed logistic regression model with pregnancy as a dependent variable (reference category = "not pregnant"). Cow within farm was considered the subject, number of AI was considered as repeated measurements. Univariable models were run for each relevant recorded parameter (parity, estrus induction, days in milk, breeding date [month and season respectively], semen type, sire, diagnosis of ovulation control, backfat thickness 1 day after AI) and biologically plausible interaction terms. In case these models yielded $P < 0.1$, parameters were tested as fixed effects in

**Table 2**
Overview of bull characteristics and average traits of raw ejaculates (± standard deviation).

| Bull | Breed | Age at enrollment, months | Ejaculates, n | AI with LS, n | AI with FS, n | Ejaculate volume, mL | Total sperm per ejaculate, sperm × $10^9$/mL |
|---|---|---|---|---|---|---|---|
| 1 | HF | 29 | 40 | 86 | 72 | 6.6 ± 1.3 | 1.392 ± 0.254 |
| 2 | HF | 27 | 11 | 31 | 26 | 5.8 ± 1.4 | 1.599 ± 0.213 |
| 3 | HF | 17 | 11 | 33 | 32 | 4.8 ± 1.7 | 1.590 ± 0.271 |
| 4 | HF | 20 | 29 | 67 | 81 | 6.2 ± 1.2 | 1.511 ± 0.235 |
| 5 | BB | 21 | 5 | 19 | 16 | 4.0 ± 0.7 | 1.270 ± 0.319 |
| 6 | BB | 20 | 19 | 48 | 71 | 4.3 ± 0.9 | 1.396 ± 0.326 |
| 7 | BB | 23 | 16 | 41 | 44 | 4.2 ± 1.3 | 1.548 ± 0.255 |

HF = Holstein-Frisian; BB = Belgian Blue; AI = Artificial insemination; LS = Liquid semen, FS = Frozen semen.

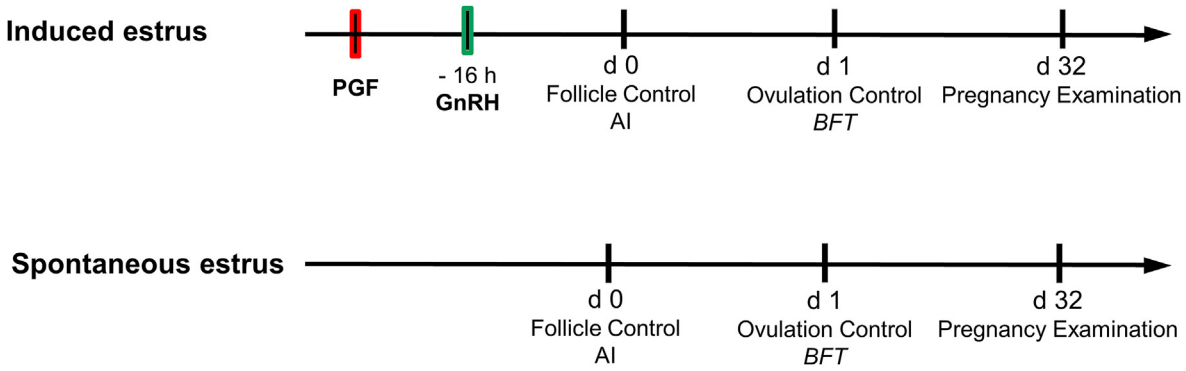


**Fig. 1.** Experimental design. Lactating dairy cows were suggested for AI after spontaneous or hormonal induction of estrus. Hormonal induction was accomplished either by single application of prostaglandin F2α (PGF) after detection of a corpus luteum (PG; marked red) or by application of PGF after detection of a corpus luteum with an additional application of gonadotropin releasing hormone (GnRH; marked green) 16 h prior to AI. Ovulation control and measurement of backfat thickness (BFT) were conducted 24 h after every AI. Pregnancy examination was performed after 32 ± 1 days. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

the final multivariable model. Variables were integrated stepwise starting with those with the lowest *P*-value. Only parameters with $P < 0.05$ were retained in the model. The interaction term 'semen type by ovulation' was retained in the model because it was the variable of interest. The final multivariable model contained semen type, parity, BFT, estrus induction, ovulation and the interaction term of semen type by ovulation as fixed effects. Post-hoc tests were conducted using Bonferroni correction. A *P*-value of <0.05 was considered significant.

## 3. Results

In total, 1,053 AI were carried out from June 2019 to April 2020. After exclusion of 386 AI due to; incomplete information, implausible diagnosis at time of ovulation control (e.g. pregnancy, potent *corpus luteum*, ovarian cyst, endometritis) or pregnancy examination (e.g. pregnancy too advanced), culling or selling before pregnancy examination, repeated AI within the first 17 days after AI for trial, more than 6 AI since last gestation and more than 250 days in milk at time of AI, a data set consisting of 667 (LS = 325; FS = 342) AI records was used for statistical analyses. In the final data set, five cows contributed four AI, 15 cows contributed three AI and 78 cows contributed two AI each, all the other data sets were contributed by single cows. The distribution of AI between farms can be seen in Table 3.

Overall, 25.2% of all ovulations were delayed. Pearson's chi$^2$-test showed an influence of farm on prevalence of delayed ovulations, but no influence of induction of estrus when comparing spontaneous and hormonally induced (PG and PGG) estruses ($P = 0.221$; Table 3). However, if spontaneous, PG induced and PGG induced estruses were distinguished, estrus induction indeed affected prevalence of delayed ovulations ($P = 0.006$). There were similar risks for delayed ovulation for spontaneous (23.1%) and PGG (24.3%) induced estruses, but a markedly higher risk for delayed ovulation after PG (44.0%).

Type of semen ($P = 0.016$), parity ($P = 0.014$), BFT ($P = 0.006$) and estrus induction ($P = 0.010$) had a significant influence on P/AI. Results of the multivariable analysis are illustrated in Table 4. In general, use of LS yielded higher pregnancy risk than use of FS (45.5%; 33.7%). Cows in their second lactation had a P/AI of 30.3%, which is lower than that of cows in their first or after their second lactation (44.7% or 44.0%, respectively). Cows with a BFT from 10 to 13 mm had highest P/AI (52.7%) compared to the other three groups (5 mm: 33.5%; 6–9 mm: 38.3%; ≥14 mm: 33.9%). Additionally, there was a higher likelihood of pregnancy for cows with spontaneous estrus (44.9%) when compared with hormonally induced estrus (34.2%).

There was a tendency ($P = 0.094$) towards reduced P/AI (27.7%) in cows inseminated with FS that had delayed ovulation compared with the other groups (AI with LS and delayed ovulation: 46.8%; AI with LS and regular ovulation: 44.0%; AI with FS and regular ovulation: 40.4%). After Bonferroni adjustment, the pairwise comparison of the interaction term of semen type by ovulation showed a significant difference in P/AI between usage of LS or FS ($P = 0.017$) in cows with delayed ovulations.

By conducting a post-hoc power analysis we determined the power to be 83.4% for the difference between P/AI in FS (33.7%) and LS (45.4%) for cows with complete records (n = 667).

**Table 3**
Results of three chi$^2$-tests concerning prevalence of delayed ovulations (ovulation >24 h after artificial insemination, measured by ultrasound examination) in Holstein dairy cows in different subgroups.

| Parameter | Category | Inseminations, n | Delayed ovulation, n | Prevalence, % | P($\chi^2$) |
|---|---|---|---|---|---|
| **Farm** | 1 | 332 | 63 | 19.0 | *0.002* |
| | 2 | 90 | 30 | 33.3 | |
| | 3 | 28 | 6 | 21.4 | |
| | 4 | 74 | 20 | 27.0 | |
| | 5 | 143 | 49 | 34.3 | |
| **Estrus (1)** | spontaneous | 329 | 76 | 23.1 | 0.221 |
| | induced | 338 | 92 | 27.2 | |
| **Estrus (2)** | spontaneous | 329 | 76 | 23.1 | *0.006* |
| | PG | 50 | 22 | 44.0 | |
| | PGG | 288 | 70 | 24.3 | |

PG = single application of prostaglandin F2α after detection of a *corpus luteum*; PGG = application of prostaglandin F2α after detection of a *corpus luteum* and application of Gonadotropin releasing hormone 16 h prior to AI.

**Table 4**
Multivariable mixed logistic regression model predicting pregnancy outcome after artificial inseminations (n = 667) with liquid or frozen semen in Holstein dairy cows with 95%-confidence intervals (CI) and significance values (P).

| Parameter | Category | N | P/AI, % | Lower CI | Upper CI | P-value |
|---|---|---|---|---|---|---|
| **Semen type** | LS | 325 | 45.4 | 31.8 | 59.8 | *0.016* |
| | FS | 342 | 33.7 | 21.9 | 48.0 | |
| **Parity** | 1 | 228 | 44.7[a] | 30.5 | 59.7 | *0.014* |
| | 2 | 177 | 30.3[b] | 18.8 | 45.0 | |
| | >2 | 262 | 44.0[a] | 30.4 | 58.6 | |
| **Backfat thickness** | 5 mm | 170 | 33.5[a] | 20.9 | 49.0 | *0.006* |
| | 6–9 mm | 208 | 38.3[a,b] | 25.4 | 53.2 | |
| | 10–13 mm | 136 | 52.7[b] | 37.3 | 67.7 | |
| | ≥14 mm | 153 | 33.9[a] | 21.2 | 49.4 | |
| **Estrus induction** | spontaneous | 329 | 44.9 | 31.4 | 59.1 | *0.010* |
| | induced | 338 | 34.2 | 22.5 | 48.2 | |
| **Semen type*ovulation** | LS*OV | 236 | 44.0[a] | 30.4 | 58.6 | 0.094 |
| | FS*OV | 263 | 40.4[a] | 27.4 | 54.9 | |
| | LS*DO | 89 | 46.8[a] | 30.8 | 63.5 | |
| | FS*DO | 79 | 27.7[b] | 15.4 | 44.6 | |
| **Ovulation** | OV | 499 | 42.2 | 29.5 | 56.0 | 0.265 |
| | DO | 168 | 36.7 | 23.8 | 51.9 | |

P/AI = Pregnancy per AI; CI = Confidence interval; LS = Liquid semen; FS = Frozen semen; OV = Ovulation within 24 h post AI; DO = Ovulation >24 h post AI (delayed ovulation).
[a, b] Different letters among categories of the same parameter indicate significant differences ($P < 0.05$).

## 4. Discussion

The present study compared the pregnancy outcome of LS and FS of split ejaculates in dairy cows housed on five different farms over the period of one year with special regard to delayed ovulations. We found that ovulations were delayed in more than a quarter of all cases. The group of cows with a BFT between 10 and 13 mm at time of insemination achieved the greatest P/AI. When comparing pregnancy outcomes of LS and FS, we not only demonstrated that LS yielded greater P/AI than FS in general, but also showed that performance of LS in cows with delayed ovulation was better when compared with FS.

The effect of LS and FS on semen quality and pregnancy outcome has been investigated in a large number of studies. While our findings agree with those of Crespilho et al. [25], Borges-Silva et al. [26] and Papa et al. [27], other studies have observed no association between pregnancy outcome and type of semen [17,19] or have even described advantages of FS under certain circumstances, for example; heat stress [28] or storage-time of LS [18]. Varying study designs compromise comparisons of investigations concerning semen preservation methods. One of the inconsistent factors in study designs is choice of semen diluents. Semen diluents, for both LS and FS, are available in great number and variation. While the present study applied Caprogen for dilution of LS, other studies have applied Botu-Bov [27,29] or even modified a standard TRIS-based diluent with plant-based additives [30]. Verberckmoes et al. [31] developed a LS diluent composed analogue to the plasma of the *cauda epididymis* with promising results. Murphy et al. [32] concluded that the milk-based diluent INRA96 could be a reliable alternative to Caprogen, with special regards to its preservative abilities during high fluctuations of ambient temperatures. Currently, however, Caprogen continues to be the gold standard for liquid preservation of semen [33]. In the future, it might be desirable to develop a long-term diluent (>3 days) without components of animal origin.

As mentioned before, one study found decreased calving rates in cows with short term heat stress when using LS [28]. The authors discussed high ambient temperatures and insufficient storage of LS-straws in regards to temperature as the main causes for the adverse effect on calving rates. Since our study was conducted through the hot summer season of 2019, an effect of season by semen type on P/AI was expected. The interaction of season and semen type, however, did not affect P/AI in our study. We assume that discarding LS straws when storage temperature reached over 20 °C prevented the use of impaired semen. According to Murphy [18], semen diluted with Caprogen yielded better progressive motility when stored at 15 °C compared to 5 °C, 22 °C and 32 °C. In the present study, only passive cooling (insulating) devices were used for storage of LS and therefore the critical threshold of 20 °C was chosen as a compromise between optimal sperm performance and practicality.

Pregnancy outcomes in cows with delayed ovulation were greater for LS compared to FS. This finding supports previous investigations suspecting superiority of LS when intervals of AI until insemination are prolonged [10,22]. It also underlines earlier conclusions regarding increased viability of LS when compared with FS [15,21]. The lack of freeze-thaw processes in LS might have contributed to superior viability when compared to FS due to the harmful effect of the freezing process on sperm membranes [34,35].

Delayed ovulation occurred in more than one quarter of all AI conducted during this trial, emphasizing the importance of this phenomenon. This is less than reported by Braun and Sarmento (46%) [14]. Nevertheless, it is important to mention that neither Braun and Sarmento [14] nor the present study distinguished between delayed ovulation and the failure of ovulation, which reportedly occurs with a prevalence of 6–7% in dairy cows [36,37]. Prevalence of delayed ovulation was also affected by type of estrus induction. Spontaneous estruses and PGG yielded similar risk of delayed ovulations. This might be caused by the additional GnRH administration before AI. Even though delayed ovulation risks were similar in spontaneous and induced estruses, P/AI was significantly greater for spontaneous estruses. Hormonal induction by single treatment with PGF resulted in a high prevalence of delayed ovulations. It is known, that the time needed until ovulation after a single treatment with PGF varies between individuals [38,39]. The main cause for this issue are follicular waves developing during the luteal phase [40]. Depending on the state of the follicular wave

when PGF is administered, ovulation will occur 3–5 days later [41]. This circumstance requires either a sufficient heat detection program or the confirmation of heat prior to AI to attain a reasonable P/AI [42]. Besides the aforementioned issue, the presence of additional *corpora lutea* at time of PGF treatment can reduce the estrus response and elongate the time needed until ovulation too [43]. Despite the thorough pre AI examination, we found that almost half of all cows in the PG group did not ovulate within 24 h after AI. Because of the described variability of the interval from onset of estrus until ovulation, implementing timed AI protocols is a reasonable option for farms to reduce the amount of work necessary for heat detection. Several researchers have shown that timed AI protocols are compliant with the use of LS [17,22,25,26] and would allow economical management of LS production as well.

Pregnancy outcome was also affected by parity, showing inferior results in the second lactation. Previous investigations came to inconsistent conclusions regarding influence of parity on reproductive performance. While most of the recent studies observed superior reproductive performance of primiparous cows [22,28,37,44], some did not detect a difference between primi- and multiparous cows at all [45,46].

In the present study, body condition was measured by BFT and yielded greatest P/AI when it was 10–13 mm. The substantial impact of body condition on reproductive performance is widely known in literature [47]. Body condition is most commonly assessed as body condition score (BCS). Various scales and methods for body condition scoring are described, but all are challenged by subjectivity when compared to the assessment of BFT [24]. According to Yukun et al. [48] who applied the BCS-scale described by Ferguson et al. [49], BFT from 10 to 13 mm roughly equates a BCS of 2.25–2.75. Although a BCS of 2.25–2.75 is considered low, this group yielded greatest P/AI. This finding is in contrast to other researchers who also assessed body condition at the time of insemination. Carvalho et al. [46] found that P/AI was greater with BCS >2.75 and lower with BCS <2.5. Another study observed greater likelihood for pregnancy establishment for BCS >3 compared with BCS <3 [44]. In the latter study, however, BCS was assessed 55–83 d after AI, which impedes a comparison of our results because most cows are likely to gain BCS points during lactation. Regardless, a possible bias in our data could be the high number of AI farm 1 contributed, as mean BFT was rather low at time of AI in that herd.

Another limitation of the present study was the unequal sperm count per straw for LS ($9 \times 10^6$ sperm) and FS ($25 \times 10^6$ sperm). Both sperm dosages clearly exceeded the level for compensation of bull related fertility differences [50]. When looking at New Zealand, where use of LS during breeding season is common, especially the total sperm numbers of LS doses used in the present study was high. In New Zealand, usually a tenth of the sperm count in FS is used in LS ($1–2 \times 10^6$ sperm) and still yields satisfactory results [21]. Regardless, the present study was designed to focus on the effect of a prolonged interval from AI until ovulation on the performance of LS and FS, which is similar to previous studies [10,22]. The aforementioned studies applied similar sperm count per straw for LS and FS ($10 \times 10^6$ and $20 \times 10^6$ sperm). Nevertheless, it also has been indicated that high numbers of sperm per straw might impair sperm quality with regard to oxidative stress and metabolic products [51].

## 5. Conclusions

Our findings indicate that LS retains its fertilizing capacity for longer periods when compared to FS in lactating dairy cows with delayed ovulations. Therefore, the use of liquid semen could be an effective tool to mitigate adverse effects of delayed ovulations on fertility in dairy cattle. Since LS bears the risk of wastage due to its short shelf-life, demand of insemination doses needs to be calculable. Therefore, synchronization protocols could be an effective tool in combination with the use of LS. When examining the interests of AI centers, the development of a LS diluent with extended shelf-life would be desirable to maximize yields of young bulls of high demand and minimize wastage of costly AI doses.

## CRediT authorship contribution statement

**Marie Wiebke:** Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Laura Pieper:** Validation, Formal analysis, Writing – review & editing. **Hakan Gürler:** Conceptualization, Methodology. **Ulrich Janowitz:** Resources, Supervision. **Markus Jung:** Conceptualization, Project administration. **Martin Schulze:** Writing – review & editing, Project administration, Supervision.

## Declaration of competing interest

The authors declare that there is no conflict of interest.

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