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Welfare impacts during and after reproductive procedures for *in vivo* embryo production and transfer in Holstein dairy heifers

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ABSTRACT

Embryo technologies are routinely used in cattle, but the links between embryo technologies and cattle welfare have been poorly investigated. The aim of this study was to describe the behavioural, physiological and clinical responses of Holstein dairy heifers during and after five reproductive procedures: dominant follicle removal (**DFR**) by transvaginal follicular puncture, superovulation, double artificial insemination (**AI1** and **AI2**), embryo flushing (**EF**), and embryo transfer (**ET**). This longitudinal cross-over design used twelve nulliparous pubescent and cycled Holstein heifers, each acting as their own control. Behavioural recordings (video and continuous monitoring sensors), physiological and clinical examinations, and blood sampling were performed at regular intervals on each reproductive procedure from the start of contention up to 24 h after the end of the procedure. The heifers changed their behavioural and physiological responses during and after each of the five reproductive procedures. During the procedures, they displayed more abnormal postures of the body (e.g. arched back during all procedures; $P < 0.05$), hindlimbs (e.g. base-wide stance during all procedures except EF; $P < 0.05$), and head (e.g. lowered head during AI2, EF and ET; $P < 0.05$). They also displayed more signs of agitation, moving their body more (e.g. stepping aside during DFR, AI1 and ET; $P < 0.05$), their feet more (e.g. hoof lifting during DFR, EF and ET; $P < 0.05$) and their head more (e.g. neck stretching forward during AI2, EF, and ET; $P < 0.05$). They also showed increases in both plasma cortisol concentration (during AI2 and EF; $P < 0.05$) and heart rate (during DFR and AI2; $P < 0.05$). However, we did not observe any inflammatory response in plasma pro-inflammatory cytokines and haptoglobin or in macroscopic appearance of the vulvae 2 h after the procedures. During the 24 h after the procedures, the heifers spent less time ingesting and standing up after DFR ($P < 0.05$), less time ruminating after EF ($P < 0.05$), more time with activity after superovulation and more time without activity after EF ($P < 0.05$). Each reproductive procedure has specific responses in the heifers. Taken together, our findings suggest that heifer welfare was impacted both during and after the procedures involved in embryo production and *in vivo* transfer protocol. Acknowledging that some discomfort/pain may be present, it may be welfare-friendly to develop and apply refinement strategies during and after embryo technology procedures.

Implications

Embryo technologies are unquestionably efficient tools for improving cattle genetics and herd performances, but their welfare impact remains poorly understood. This study brings scientific insights on behavioural and physiological responses of dairy heifers during and after *in vivo* embryo production and transfer—including dominant follicle removal, superovulation, double artificial insemination, embryo flushing, and transfer. Observed changes in heifers suggest discomfort or pain related to these reproductive procedures. Ultimately, these findings will help refine assisted reproductive procedures, benefiting both animals and

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stakeholders through more ethical and effective cattle breeding practices.

Introduction

A wide variety of methods, tools and management practices have been developed over the past century to ensure or improve the fertility of individual dairy cows and the reproductive performances of cattle populations. Embryo production and transfer procedures are assisted reproductive technologies commonly used in the dairy industry to control venereal diseases (for a review, see Ritter et al., 2019) and increase genetic merit (Moore and Thatcher, 2006). There are currently two main ways in practice for producing embryos in cattle: *in vivo*-derived, or *in vitro*-produced (Ponsart et al., 2013). *In vitro*-produced procedures are the main approach used in America, whereas *in vivo*-derived procedures remain the most common approach in Europe (Viana, 2023). *In vivo*-derived embryo production and transfer encompasses five procedures: transvaginal follicular puncture (i.e. for dominant follicle removal; **DFR**), hormonal synchronisation protocols, trans-cervical artificial insemination (**AI**), trans-cervical embryo flushing (**EF**), and trans-cervical embryo transfer (**ET**). Although these procedures have increased genetic progress for a variety of productive and functional genetic traits, public attitudes toward reproduction practices based on such technologies are mainly negative because these invasive methods are perceived as unnatural (Pieper et al., 2016), and/or as violating the integrity of animals (Lund et al., 2023). Nevertheless, there has been little effort to investigate how the animals actually experience such procedures.

The animal's experience of a procedure can be explored through changes in physiological, behavioural indicators and behavioural tests. Physiological indicators include hormones like cortisol that are released by the hypothalamus–pituitary–adrenal axis, physiological changes due to activation of the autonomous nervous system (e.g. heart rate, respiratory rate, rumen contraction rate, body temperature (Prunier et al., 2013). If the procedure is associated with tissue damage, then in addition to changes in the above-mentioned biomarkers, there will also be a release of inflammatory markers such as cytokines and haptoglobin (Saco and Bassols, 2023; Ceciliani et al., 2012). Identifying the behavioural responses of the animal to a procedure is a fundamentally important concern in animal welfare research to explore and determine how animals experience the procedure and enable effective care actions to refine the procedure. When cattle experience discomfort or pain, they change their spontaneous behaviours both in their living area and when they are being handled (Herskin et al., 2018; Weary, 2014; Prunier et al., 2013). Behavioural tests can also be applied in order to explore the animal's emotional experience in relation with the procedure (Nielsen, 2020; Ede et al., 2019), e.g. motivation tests (Ledoux et al., 2023), or cognitive bias tests (Lagisz et al., 2020; Neave et al., 2013). Combining physiological and behavioural indicators, either spontaneous, or provoked in tests (Ede et al., 2019) gives a better characterisation of the emotions experienced by the animals (Veissier and Boissy, 2007; Désiré et al., 2002).

The impact of some reproductive procedures and technologies on cattle physiology and behaviour has been explored for uterus palpation per rectum (Giese et al., 2018; Kovács et al., 2014, 2016; Stojkov et al., 2015; Waiblinger et al., 2004; Nakao et al., 1994), vaginal examination (Pilz et al., 2012), AI (Koenneker et al., 2023; Waiblinger et al., 2004; Nakao et al., 1994), transvaginal follicular puncture (Chastant-Maillard et al., 2003) and embryo transfer (Koenneker et al., 2023) but not embryo flushing. Overall, these studies showed that most procedures induce agitation (Pilz et al., 2012; Waiblinger et al., 2004), arched back posture (Stojkov et al., 2015), cortisol release (Giese et al., 2018; Kovács et al., 2016; Nakao et al., 1994) and increased heart rate (Kovács et al., 2014, 2016) during the procedure. Another study in cows reported a cortisol response to handling but not to transvaginal follicular puncture (Chastant-Maillard et al., 2003). However, to our knowledge, no study has combined physiological, clinical and behavioural measurements to comprehensively analyse each of the reproductive procedures of the embryo production and *in vivo* transfer protocol in cattle, and these during and after each reproductive procedure.

The aim of this study was to describe the behavioural, physiological and clinical responses of Holstein dairy heifers to five reproductive procedures: DFR by transvaginal follicular puncture, superovulation, double AI, EF, and ET. We monitored that heifers' responses were during and 24 h after each procedure. Each animal was its own control.

Material and methods

Animals, housing and feeding

The study used 12 nulliparous, pubescent and cycled Holstein dairy heifers [age (mean ± SD) 15.8 ± 0.9 months; weight (mean ± SD) 441 ± 32 kg at intake to the experimental facilities]. We chose nulliparous heifers in order to standardise their reproductive history (i.e. with no previous experience of reproductive procedures).

The 12 heifers had been raised together in a commercial farm in the Maine-et-Loire department of France. The 12 heifers were purchased together and all brought together to the Eliance experimental facility at Nouzilly (France) on 02 March 2022 (i.e. at least 5 days before the start of the habituation phase of the study), after a 35-day quarantine period from 26 January 2022 (See Supplementary Table S1 for a detailed schedule). The Eliance experimental facility has a 9.26 × 45 m deep-bedded barn comprising several pens. The 12 heifers were loose-housed in three different pens. Each pen comprised four experimental heifers (one per batch) housed together with three contemporary heifers (i.e. not enrolled in the study). Each pen (11.76 × 5 m) had a 9.26 × 5 m area of deep-bedded straw and a 2.5 × 5 m concrete feeding alley covered with rubber mats, plus two automatic drinkers (La Buvette, France) and a fixed cow brush. Heifers were fed straw *ad libitum* and received 2 kg of concentrate twice daily (i.e. 4 kg per heifer) and had access to one salt lick. Uneaten ration was pushed back toward the heifers 4 times per day, at 0800, 1100, 1400 and 1700 h. Water was provided *ad libitum*.

Experimental design

This experimental study used a longitudinal cross-over design, with individual heifers serving as their own controls. Two situations were applied, i.e. an EXPERIMENTAL situation vs a CONTROL situation. In the EXPERIMENTAL situation, heifers were restrained and underwent a reproductive procedure (see below), while in the CONTROL situation, they were restrained but did not undergo any reproductive procedures. For practical reasons, it was only possible to perform the 'embryo flushing' (see below) reproductive procedure in up to 3 heifers at once (i.e. on the same morning). The experiment therefore comprised four batches of three heifers. Each heifer was allocated to a batch depending on her age. Finally, batch 1 had heifers numbered #1, #2 and #3, batch 2 had heifers #4, 5# and #6, batch 3 had heifers #7, #8 and #9, and batch 4 had heifers #10, #11 and #12. The three heifers of each batch were housed in three different pens. Each pen therefore comprised one heifer from each batch, and three contemporary (i.e. same-age, same-breed) heifers. The first pen comprised heifers #1, #4, #7

and #10, the second pen comprised heifers #2, #5, #8 and #11, and the third pen comprised heifers #3, #6, #9 and #12. The four batches were monitored from 7 March 2022 to 13 July 2022. We applied a Latin square design: Batch 2 (heifers #4, 5# and #6) and Batch 4 (heifers #10, #11 and #12) underwent the CONTROL situation then the EXPERIMENTAL situation; while Batch 1 (heifers #1, #2 and #3) and Batch 3 (heifers #7, #8 and #9) underwent the EXPERIMENTAL situation then the CONTROL situation (See Fig. 1, and Supplementary Table S1 for a detailed schedule).

During 2 weeks after their arrival at the experimental unit, the heifers underwent a daily training programme progressively including moving from the pen to the restraining cage or moving to the headlocks, restraint, simulation of puncture of the coccygeal vein (week 1), and effective puncture of the coccygeal vein (week 2). During the second week of training, each heifer was fitted with an abdominal belt equipped with a Polar Equine© recording system (Polar Oy Kempele, Finland) for 10 min per day to habituate them to the device.

Reproductive procedures for in vivo embryo production and transfer

After synchronisation of oestrus, the heifers underwent several reproductive procedures: DFR, superovulation, double AI, EF, and ET (Fig. 1). Handling individual differences has a significant impact on animal perception. Therefore, the heifers were handled by one technician (L.L.) and reproductive procedures were always performed by the same reproductive biotechnology technician (S.L.).

Dominant follicle removal by transvaginal follicular puncture

Before DFR, the heifers were submitted to an oestrus synchronisation protocol from day minus 9 (D_{-9}) to D_{-2} in order to obtain a standard oestrus (D_0). The protocol used a vaginal device of 1.55 g progesterone (PRID® DELTA, CEVA Santé Animale, Libourne, France) for 7 days, i.e. D_{-9} up to D_{-2}. At D_{-3}, the heifer received an intramuscular injection of 0.5 mg cloprostenol (ESTRUMATE, MSD, Beaucouzé, France). At D_{-2}, the vaginal device was removed. Standard oestrus was observed 42–48 h after device removal (D_0).

At D_{10}, the heifer was moved from the pen to the restraining cage (Mazeron, France) at 0900 h by a Eliance technician (L.L.). The Eliance reproductive biotechnology technician (S.L.) washed the heifer's sacrococcygeal area with iodised polyvinylpyrrolidone (Vétédine savon®, Vétoquinol, France). The technician (S.L.) then performed epidural anaesthesia in the sacrococcygeal space by slowly injecting 100–200 mg of procaine hydrochloride (Procamidor®, Richter Pharma, Austria) and then immediately emptied the heifer's rectum. The technician (S.L.) then cleaned and disinfected the anogenital area with water and an antiseptic soap (Vétédine solution®, Vétoquinol, France), and verified that the anaesthesia was effective by checking that the tail did not react to or resist handling. If the anaesthesia has not been totally effective (tail movement and/or resistance), an additional small dose of epidural anaesthesia (50% of the initial dose) was performed, and the technician tied the tail to the right side of the cage with a rope. The technician (S.L.) then inserted a guide containing the ultrasound probe (EXAPAD®, IMV technologies, France) per vagina and transrectally positioned the heifer's first ovary against the ultrasound probe. Once the dominant follicle (i.e. diameter larger than 8 mm) was visible and stabilised on the ultrasound scanner, the technician (S.L.) inserted a needle-holder fitted with a single-use 18G needle and suction system into the guide to transvaginally puncture the follicles. Following puncture of the dominant follicle, the technician cleaned and disinfected the anogenital area with water and antiseptic soap (Vétédine solution®, Vétoquinol, France), then applied a 1.55 g progesterone device (PRID® DELTA, CEVA Santé Animale, Libourne, France).

Superovulation

Superovulation treatment on donors lasted 4 days, from D_{12} to D_{15}, with the heifer headlocked. Each heifer received eight intramuscular injections of pFSH plus pLH (Stimufol®; Reprobiol, Belgium) in decreasing doses approximatively 12 h apart, once in the morning (0800 h) and once in the evening (1900 h), at 1.2 mL per injection on D_{12}, 1 mL per injection on D_{13}, 0.8 mL per injection on D_{14}, and 0.5 mL per injection on D_{15}. On D_{14} in

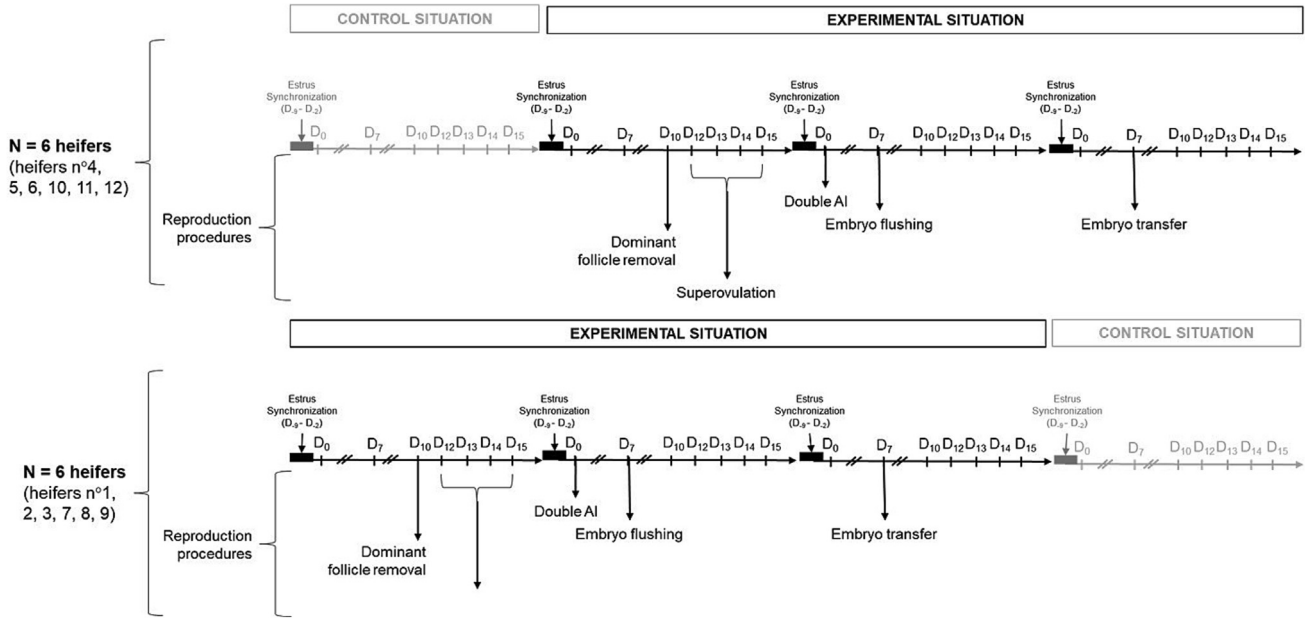

Fig. 1. Longitudinal cross-over experimental design* used to investigate the effects of reproductive procedures (dominant follicle removal, superovulation, double artificial inseminations, embryo flushing, embryo transfer) in 12 Holstein heifers. Abbreviations: D = Day; AI = Artificial insemination. *Two situations were applied, i.e. an EXPERIMENTAL situation vs a CONTROL situation. In the EXPERIMENTAL situation, heifers were restrained and underwent a reproductive procedure (dominant follicle removal, superovulation, double AI, embryo flushing, embryo transfer), while in the CONTROL situation, they were restrained but did not undergo any reproductive procedures. Each heifer underwent the control situation, then the experimental situation, or vice versa. Individual heifers served as their own control.

the morning, each heifer also received an intramuscular injection of 0.5 mg cloprostenol (ESTRUMATE, MSD, Beaucouzé, France). On D_{14} in the evening, the Eliance reproductive biotechnology technician (S.L.) removed the 1.55 g progesterone vaginal device (PRID® DELTA, CEVA Santé Animale, Libourne, France).

Double artificial inseminations

The heifer was headlocked by a Eliance technician (L.L.) and artificially inseminated twice, at 24 h (**AI1**) and 36 h (**AI2**) after the end of superovulation treatment, i.e. D_0 of a new oestrus cycle. AI1 and AI2 followed the same recto-vaginal-method procedure. The Eliance reproductive biotechnology technician (S.L.) emptied the heifer's rectum, then cleaned the anogenital area with a wet paper towel and dried it with a dry paper towel. The technician (S.L.) then placed a gloved hand in the heifer's rectum, held the cervix and guided the insemination gun (Kombicolor gun reference 018396 surrounded by a single-use alpha sheath reference 024485 and protection sheath reference 005563; IMV Technologies, France) through the cervix to deposit semen in the body of the uterus.

Embryo flushing

Embryos were collected 7 days after AI1, i.e. at D_7. The heifer was moved by a Eliance technician (L.L.) from the pen to the restraining cage at 0900 h. The Eliance reproductive biotechnology technician (S.L.) washed the heifer's sacrococcygeal area with iodised polyvinylpyrrolidone (Vétédine savon®, Vétoquinol, France), then performed epidural anaesthesia in the sacrococcygeal space by slowly injecting 100–200 mg of procaine hydrochloride (Procamidor®, Richter Pharma, Austria) and immediately emptied the heifer's rectum. The technician (S.L.) then cleaned and disinfected the anogenital area with water and an antiseptic soap (Vétédine solution®, Vétoquinol, France) and verified that the anaesthesia was effective by checking that the tail did not react or resist when handled. If the anaesthesia has not been totally effective (tail movement and/or resistance), an additional small dose of epidural anaesthesia (50% of the initial dose) was performed. The technician (S.L.) then tied the tail to the right side of the cage with a rope. The technician (S.L.) carried out embryo collection by applying the trans-cervical method. In a first step, she placed a gloved hand in the heifer's rectum and then introduced a cervical dilator (reference 007245, IMV Technologies, France) into the cervix for a few minutes. The technician (S.L.) then introduced the two-way collection catheter (Woerlein CH18 reference 005649, IMV Technologies, France) through the cervix into the first horn 10 cm beyond the uterine bifurcation, and inflated the cuff with 10–12 mL of air. The uterine horn was flushed using 500 mL flushing solution (Euroflush®, IMV Technologies, France) heated at 38 °C and collected back into a single-use embryo filter (Miniflush Minitübe, Germany). A second Eliance technician (L.L) injected the flushing solution by repeated injections from 10 to 60 mL of solution. The three-way collection catheter was then withdrawn back, and the same procedure was repeated for the second uterine horn. After each procedure, an intramuscular injection of 0.5 mg cloprostenol (Estrumate®, MSD, France) was given to flushed females to avoid any pregnancies and return to oestrus. After microscopic evaluation, embryos were classified for quality and staging and then cryopreserved using a slow freezing procedure as described in Janati Idrissi et al. (2021).

Embryo transfer

Before ET, the heifers were submitted to an oestrus synchronisation protocol to obtain a new standard oestrus (D_0) and allow ET at D_7. The synchronisation protocol started 9 days after the end of EF protocol. The protocol used a vaginal 1.55 g progesterone device (PRID® DELTA, CEVA Santé Animale, Libourne, France) for 7 days, from D_{-9} up to D_{-2}. At D_{-3}, the heifer received an intramuscular injection of 0.5 mg cloprostenol (ESTRUMATE, MSD, Beaucouzé, France). At D_{-2}, the vaginal device was removed. Standard oestrus was observed 42–48 h after device removal (D_0).

On D_7, the heifers were headlocked at 1500 h. The Eliance reproductive biotechnology technician (S.L.) emptied the heifer's rectum and then located the corpus luteum on one of the two ovaries, indicating the horn where the embryo would be deposited. The technician (S.L.) then washed the heifer's sacrococcygeal area with iodised polyvinylpyrrolidone (Vétédine savon®, Vétoquinol, France) and performed an epidural anaesthesia in the sacrococcygeal space by slowly injecting 100–200 mg of procaine hydrochloride (Procamidor®, Richter Pharma, Austria). The technician (S.L.) cleaned the anogenital area with a wet paper towel and dried it with a dry paper towel and then carried out embryo transfer by the trans-rectal method. The technician (S.L.) placed a gloved hand in the heifer's rectum, held the cervix and guided the transfer gun (reference 007240 surrounded by single-use sanitary sheath reference 005540 and protection sheath reference 005563; IMV Technologies, France) through the cervix to the horn ipsilateral to corpus luteum. As the objective was not to get gestating heifers, no embryos were transferred *per se*: the technician (S.L.) mimicked embryo deposition in the horn.

Data collection

On entry into the experimental facility, the heifers were fitted with collar-attached tri-axial accelerometers (AXEL® sensor) commercialised by ITK (Chateaubourg, France).

Behavioural recordings, physiological and clinical examinations and blood sampling were performed at regular intervals for each reproductive procedure from the start of contention up to 2 h after the end of the procedure (Fig. 2) by two graduate veterinary students (C.R. and P.DR). The heifers' activities were recorded continuously by monitoring sensors and by position from the start of contention up to 24 h after the end of the procedure.

In the EXPERIMENTAL situation for DFR, double AI, EF and ET, the animals' responses to the reproductive procedure were monitored and recorded at the start of restraint (T_0), at the end of reproductive procedure (T_{EP}), at 15 min after the end of the reproductive procedure (T_{EP}+0.25), and at 2 h after the end of the reproductive procedure (T_{EP}+2). The heifers' activities were continuously monitored from 15 min after the end of the reproductive procedure (T_{EP}+0.25) up to 24 h after the end of the reproductive procedure (T_{EP}+24).

In the EXPERIMENTAL situation for superovulation, the animals' responses to the reproductive procedure were monitored and recorded on D12, D13, D14 and D15, at 15 min after the end of the morning reproductive procedure (T_{EP}+0.25). On D12, D13, D14 and D15, the heifers' activities were continuously monitored from the end of the morning reproductive procedure (T_{EP}) up to 24 h after the end of the reproductive procedure (T_{EP}+24).

In the CONTROL situation, the animals' responses were observed at the same timepoints as in the EXPERIMENTAL situation.

Behavioural measures and observations

Behavioural observations were performed in the same way in both the EXPERIMENTAL and CONTROL situations.

- Behaviour during five reproductive procedures (i.e. DFR, AI1, AI2, EF and ET) was recorded from restraint (T_0) up to the end of the reproductive procedure (T_{EP}) using camcorders (Panasonic HC-VX870 4 K, Japan). When the heifer was headlocked for double AI (AI1 and AI2) and ET, two camcorders were used: one recorded the front of the heifer while the second recorded

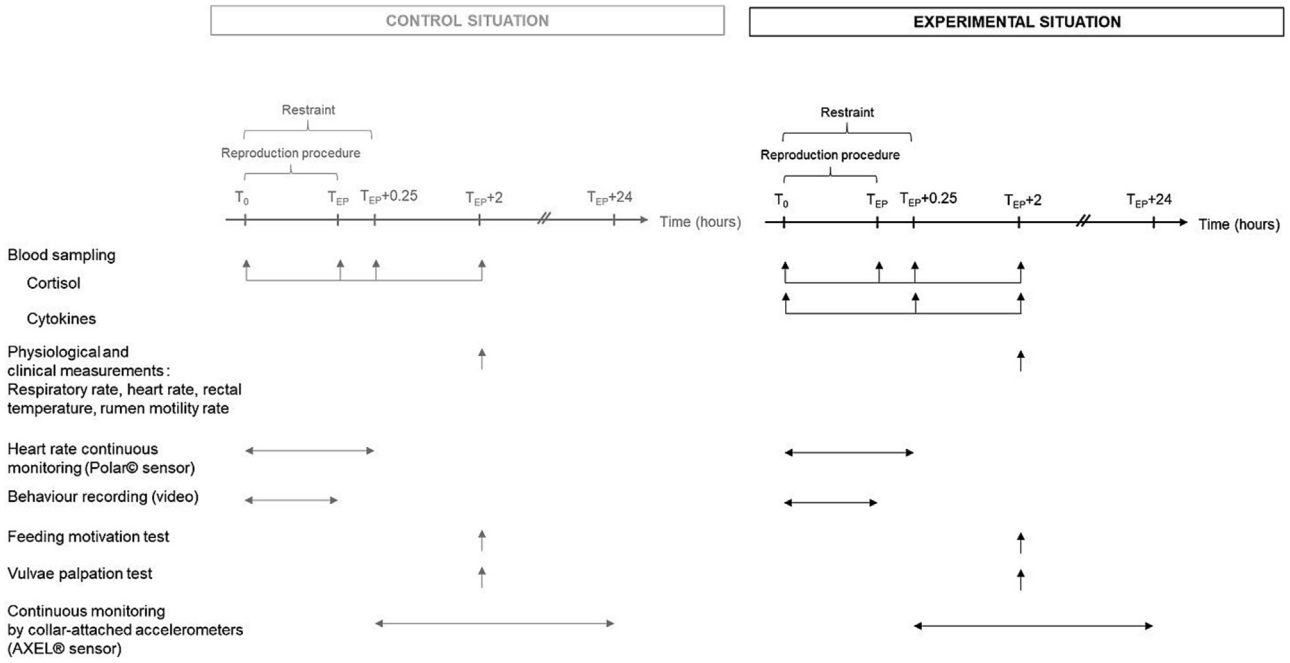

Fig. 2. Experimental protocol to investigate the effects of reproductive procedures (dominant follicle removal, double artificial inseminations, embryo flushing, embryo transfer) on physiological, clinical and blood (cortisol and cytokines) parameters and behavioural responses (behaviours during the procedure, feeding motivation test, and continuous activity monitoring using accelerometers) in 12 Holstein heifers. Measurements were taken at the start of restraint (T0), end of the procedure (T_{EP}), 15 min (T_{EP}+0.25), 2 h (T_{EP}+2), and 24 h (T_{EP}+24) postprocedure. Abbreviation: AI = Artificial insemination.

the rear part. When the heifer was restrained in a restraining cage for DFR and EF, three camcorders were used: one recorded the front of the heifer, the second recorded the rear part, and the third recorded the whole animal from above. A total of 288 videos were recorded: 72 for DFR, 48 for AI1, 48 for AI2, 72 for EF, and 48 for ET. An ethogram was developed to describe the heifers' behaviours during the procedures (Table 1). One technician (E.D.) in animal behavioural science with 23 years of experience in video-scoring and blinded to the procedure and to the situations scored the videos using The Observer XT 14 behaviour scoring software (Noldius, Wageningen, The Netherlands). The behaviours were scored either in seconds (i.e. time spent by the heifer in the item, i.e. 'state' behaviour) or in number (i.e. occurrence(s) within the duration of observation, i.e. 'event' behaviour) (Table 1). Behaviours in relation to tail posture were only taken into account for a vulvae palpation test (see below), as for reproductive procedures (excepted AI), the tail was either rope-tied (DFR, EF) and/or under anaesthesia (ET).

- A feeding motivation test was carried out 2 h after the end of each reproductive procedure (T_{EP}+2), i.e. DFR, D_{15} superovulation, double AI, EF and ET. The test protocol was similar to the protocol used in Ledoux et al. (2023). One graduate veterinary student (P.DR) placed a portion of the usual feed into the trough, as done every day, and then noted, for each heifer, whether or not it approached and whether or not it ate the feed within 3 min after distribution.
- Just after the feeding motivation test at T_{EP+2}, one graduate veterinary student (C.R.) performed a vulvae palpation test. The student placed himself behind the heifer, gently touched its vulvae and noted its behavioural reaction: no reaction, moving forward, moving back, arched back, stepping, lifting hoof, hoof to belly, kick, tail whipping, defecation (Table 1).
- Heifer activities and position were continuously monitored using collar-attached accelerometers. The data from the accelerometers were processed using FARMLIFE software (ITK, Châteaubourg, France) to provide information on animal activity and position. The accelerometer measures changes of inclination and lateral and vertical accelerations that ITK then translates into animal behaviour and posture. Every 5 min, the cow was classified as ingesting feed or ruminating or showing no motion (without activity) or in another activity according to the state in which it had spent most of its time over the previous 5 min. The position of the cow, i.e. standing up vs lying down, was also recorded. The sensors are able to record eating and rumination time with 89–90% accuracy (Delagarde and Lemonnier, 2015), position to 83% accuracy, and 'no activity' to 90% accuracy (Bouchon et al., 2019). This commercial device was recently used in bovine behaviour studies (Ledoux et al., 2023; Bacher et al., 2022):
  - o For DFR, AI2, EF and ET, heifers' activities were continuously monitored from 15 min after the end of the reproductive procedure (T_{EP}+0.25) up to 24 h after the end of the reproductive procedure (T_{EP}+24). Then, for each heifer, we calculated the estimated proportion of time spent in each activity and in each position per 24 h ("daily").
  - o For superovulation, heifers' activities were continuously monitored on D_{15} from the end of the morning reproductive procedure (T_{EP}) up to 24 h after the end of the reproductive procedure (T_{EP}+24). Then, for each heifer, calculated the estimated proportion of time spent in each activity and in each position per 24 h ("daily").

Physiological and clinical measurements, blood sampling and assays

For four reproductive procedures (i.e. DFR, double AI, EF and ET), at around 10 min before each procedure, each heifer was head-locked and fitted with an abdominal belt equipped with a Polar Equine© recording system (Polar Oy Kempele, Finland). A Wiko Power U10 was used as receptor. Heart rate was continuously recorded during the procedure from T_0 up to T_{EP}+0.25. (Fig. 2). During the procedure, a graduate veterinary student (P.DR.) collected blood samples into Na2-EDTA-coated vacutainer tubes by punc-

Table 1
Ethogram of dairy heifers' behaviours (behavioural states and events) observed when heifers were restrained at the headlock or in the restraining cage in CONTROL or EXPERIMENTAL situations during the reproductive procedures and the vulvae palpation test.

Behavioural states	Modalities	Description	Reference
Hind limb posture	Parallel	The heifer is standing with her hind legs parallel: the distance between her two ischium is equal to the distance between her two tarsal joints and between her two hooves	Nuss et al, 2020
	Tight	The heifer is standing with her hind legs tight: the distance between her ischium is wider than the distance between her tarsal joints and between her hooves	
	Base wide	The heifer is standing with her hind legs apart: the distance between her ischium is narrower than the distance between her tarsal joints and between her hooves	
Body posture and movements	Standing still	The heifer is standing still with her four hooves on the ground. She does not move.	
	Moving forward	The heifer moves forward/pushes on the barrier, with all feet on the ground or moving her feet	
	Moving back	The heifer moves back/pulls on the barrier, with all feet on the ground or moving her feet	
	Stepping	The heifer lifts her front and/or back feet, without going higher than the pastern joint, then lands them one after the other	Ginger et al., 2023
	Stepping aside	The heifer moves her hind feet from left to right and vice versa	Waiblinger et al., 2004
	Wriggling	The heifer is standing and moving her body without moving her feet: her four hooves are kept on the ground.	
	Unsteady	The heifer is standing unsteadily, sometimes with the body leaning against a wall, or stands and shifts her weight on her hindlegs at least once	
Back posture	Flat back	The heifer is standing with her back flat as described by Sprecher et al. (1997)	de Oliveira et al.,2014
	Arched back	The heifer is standing with her back arched as described by Sprecher et al. (1997)	de Oliveira et al.,2014
	Lordosis	The heifer is standing with her back in lordosis	
Head posture	Head horizontal	The heifer's poll is at 90° to the vertical post of the feed barrier/chute	Mialon et al 2012; de Oliveira et al., 2014
	Head diagonal	The heifer's poll is lowered and between 45° and 90° to the vertical post of the feed barrier/chute	Mialon et al 2012; de Oliveira et al., 2014
	Head downward	The heifer's poll is between 0° and 45° to the vertical post of the feed barrier/chute	Mialon et al 2012; de Oliveira et al., 2014
	Head up	The heifer's poll is beyond 90° to the vertical post of the feed barrier/chute	de Oliveira et al., 2014
Tail posture[1]	Tail whipping	The heifer is whipping her tail	
Behavioural events	Events	Illustration	Reference
Feet movements	Lifting hoof	The heifer lifts her hoof off the ground, higher than the pastern joint but lower than the tarsal joint	Ginger et al., 2023
	Hoof to belly	The heifer lifts her hoof off the ground, higher than the tarsal joint, but lower than the stifle joint	Ginger et al., 2023
	Kick	The heifer lifts her hoof off the ground higher than the hock, and thrown to the side or backward	Ginger et al., 2023
	Sliding	The heifer slides on the ground	
Vocalisation	Grunts: low monotone vocalisation	The heifer displays low monotone vocalisation	Mølgaard et al., 2012
	Mooing: low then high-pitched vocalisation	The heifer displays low and then high-pitched vocalisation	Mølgaard et al., 2012
Head events	Muzzle licking	The heifer licks her muzzle with her tongue	
	Sniffing or licking	The heifer sniffs or licks the environment	
	Head shaking	The heifer shakes her head up and down or from left to right	Waiblinger et al., 2004; Mølgaard et al., 2012
	Ear shaking	The heifer shakes her ears	
	Drooling	The heifer drools	
	Neck stretching forward	The heifer stretches her neck forward	
Elimination	Defecation	The heifer defecates	
	Miction	The heifer urinates	

[1] tail whipping was coded only during vulvae palpation test.

ture of the coccygeal vein at T_0, T_{EP}, $T_{EP+0.25}$ and T_{EP+2}. At T_{EP+2}, immediately after the feeding motivation test, a graduate veterinary student (C.R.) performed a clinical examination. The student (C.R.) performed a visual examination of the heifer's vulvae and noted its heat (normal vs hot), colour (white vs pinkish vs red), firmness (soft vs hard vs oedematous), whether there was vulvar discharge (present, absent), and if so, its colour (translucent vs opaque vs pink-coloured) and smell (normal vs foul).

The graduate veterinary student (C.R.) then recorded physiological parameters, i.e. rectal temperature (**RT**) with a thermometer, heart rate (**HR**) using a stethoscope to count heartbeats over a 20-s period, rumen motility rate (**RMR**) with a stethoscope for 2 min, and respiratory rate (**RR**) by counting the number of expansions of the thoracic wall during a 30-s period (Fig. 2). The student (C.R.) then sampled blood into Na2-EDTA-coated vacutainer tubes by puncture of the coccygeal vein and lastly removed the Polar Equine© belt.

For superovulation, the graduate student (C.R.) sampled blood into Na2-EDTA-coated vacutainer tubes by puncture of the coccygeal vein on D12, D13, D14 and D15 at $T_{EP+0.25}$. On D15, the student (C.R.) performed a clinical examination (see above) at $T_{EP+0.25}$ and then collected blood samples (see above) (Fig. 3).

The blood samples were centrifuged at 3 000 g for 20 min at 4 °C, and the plasma was collected and frozen at –80 °C until analysis to determine cortisol, pro-inflammatory cytokines IL-1β, IL-6, IL-8, and TNF-α, and inflammatory haptoglobin. Plasma cortisol concentration was determined by enzyme assay (Andanson et al., 2018). Concentrations of pro-inflammatory cytokines were deter-

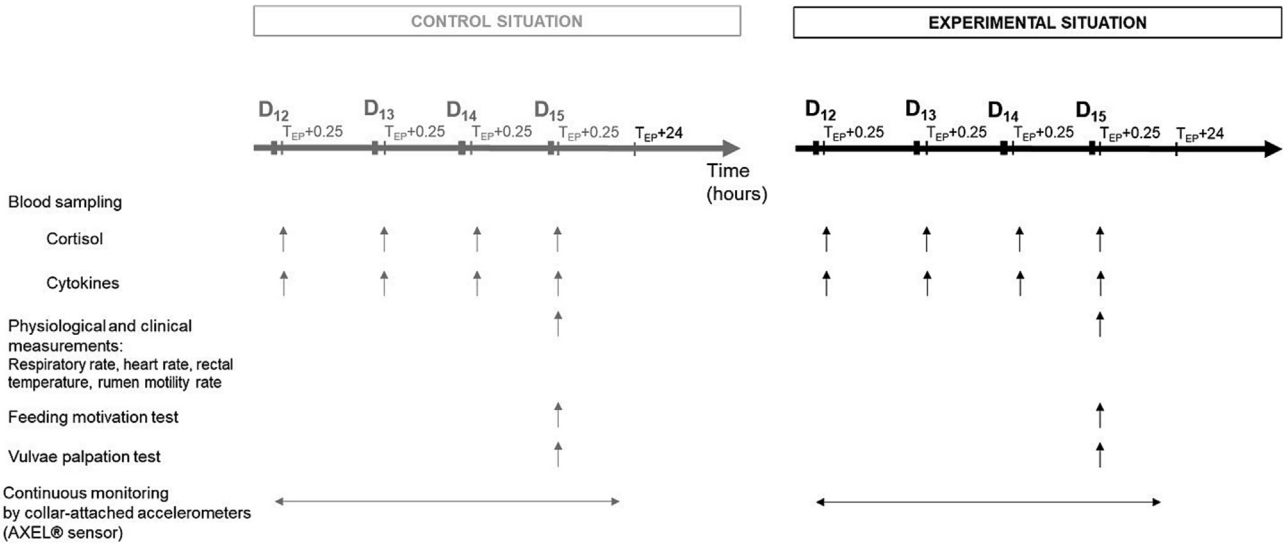

Fig. 3. Experimental protocol to investigate the effect of superovulation on physiological, clinical and blood parameters (cortisol and cytokines) and behavioural responses (feeding motivation test and continuous activity monitoring using accelerometers) in 12 Holstein heifers at 15 min after the end of the reproductive procedure (T_{EP}+0.25) on D12, D13, D14, and D15. Abbreviation: D = Day.

mined using a custom bovine MilliPlex xMAP cytokine assay (Merck Millipore, France; Lesueur et al., 2022). Data were recorded on a MagPix flow cytometer using Xponent software (Luminex, Austin, TX). Plasma haptoglobin concentration was determined by immunoprecipitation (Auboiron et al., 1990). All plasma determinations were performed at all the defined timepoints from T_0, T_{EP}, T_{EP}+0.25 to T_{EP}+2. Cortisol assays were performed in both CONTROL and EXPERIMENTAL situations at T_0, T_{EP}, T_{EP}+0.25, T_{EP}+2 for DFR, AI1, AI2, EF and ET (Fig. 2) and at T_{EP}+0.25 on D12, D13, D14 and D15 for superovulation (Fig. 3). Cytokine assays were performed in EXPERIMENTAL situations at T_0, T_{EP}+0.25 and T_{EP}+2 for DFR, AI1, AI2, EF and ET (Fig. 2). Cytokine assays were performed in both CONTROL and EXPERIMENTAL situations at T_{EP}+0.25 on D12, D13, D14 and D15 for superovulation (Fig. 3). Haptoglobin was only determined in EXPERIMENTAL situations at T_0 for DFR, at T_{EP}+0.25 on D12 for superovulation, at T_0 for AI1, and at T_{EP}+24 for ET.

Statistical analyses

Behaviour

None of the behavioural data followed a normal distribution. We therefore used non–parametric statistics. For behavioural data video-recorded during the procedure, we calculated the frequency of each behavioural event (expressed in occurrences per minute) and the duration of each behavioural state (expressed in % of time) for each reproductive procedure. We then ran CONTROL vs EXPERIMENTAL comparisons of data using pairwise Wilcoxon signed-rank tests (Siegel and Castellan, 1988).

The feeding motivation test and the heifer's behavioural reaction to the vulvae palpation test were analysed as qualitative paired series using McNemar tests (Siegel and Castellan, 1988), and we compared CONTROL vs EXPERIMENTAL situations for each reproductive procedure.

For time spent in each activity (ingesting, ruminating, without activity) and in the 'standing up' position recorded by collar-attached accelerometers, we conducted daily (24 h) analyses. For each reproductive procedure, we used a pairwise Wilcoxon signed-rank tests to compare CONTROL vs EXPERIMENTAL situations of each activity (ingesting, ruminating, without activity) and in the 'standing up' position.

Physiological, clinical, and blood parameters

To satisfy the assumptions of normality, plasma cortisols were log-transformed before analyses. For each reproductive procedure, we modelled the time-course changes in cortisol using linear mixed-effects models, with time (T_0, T_{EP}+0.25 and T_{EP}+2) and time × situation (EXPERIMENTAL vs CONTROL) interaction as fixed effects and group and heifer as nested random effects. To illustrate, the linear mixed model for plasma cortisol was lmer [log10 (plasma cortisol) ~time+time:situation + (1 | group/heifer)].

Plasma cytokines (IL-1β, IL-6, IL-8, and TNF-α) did not follow a normal distribution. In EXPERIMENTAL situation only, we compared plasma cytokines (IL-1β, IL-6, IL-8, and TNF-α) between the timepoints (T_0, T_{EP}+0.25 and T_{EP}+2) of the dominant follicle removal, AI1, AI2, embryo flushing and embryo transfer using Friedman tests, then Nemenyi posthoc tests for pairwise group comparisons. For superovulation, plasma cytokines were compared between each day (D12, D13, D14, D15) in CONTROL and in EXPERIMENTAL situations using Friedman tests, then Nemenyi posthoc tests for pairwise group comparisons. Then, we used a pairwise Wilcoxon signed-rank tests to compare CONTROL vs EXPERIMENTAL situations at a specific superovulation day.

In EXPERIMENTAL situation, we compared plasma haptoglobin between the timepoints (at T_0 for DFR, at T_{EP}+0.25 on D12 for superovulation, at T_0 for AI1, and at T_{EP}+24 for ET) using Friedman tests, then Nemenyi posthoc tests for pairwise group comparisons.

For RT, HR, RMR, RR, as these data did not follow a normal distribution, we used a pairwise Wilcoxon signed-rank tests to compare CONTROL vs EXPERIMENTAL situations for each reproductive procedure.

From continuous heart-rate measurements using the Polar Equine© recording system, we determined the mean heart rate (HRmean) between T_0 and T_{EP} and between T_{EP} and T_{EP}+0.25. For each reproductive procedure, we used a pairwise Wilcoxon signed-rank tests to compare CONTROL vs EXPERIMENTAL situations of HRmeans between T_0 and T_{EP} and between T_{EP} and T_{EP}+0.25.

Data recorded through visual examination of the vulvae (heat, colour, firmness, presence/absence of discharge and discharge characteristics) were analysed as qualitative paired series using McNemar tests. We compared the CONTROL vs EXPERIMENTAL situations for each reproductive procedure.

All analyses were performed using R software version 4.2.1. The lmer function in the lme4 package was used for the linear mixed-effect models, and the emmeans package was used to calculate least square means. The threshold for significance of a fixed effect was set at a *t*-value ≥ |2| (Bates et al., 2015) and a Tukey-adjusted *P*–value ≤ 0.05. Normality of the distribution and homogeneity of residuals were checked visually using plots of residuals and quantile–quantile plots of residuals and random effects. For the McNemar test, pairwise Wilcoxon signed-rank tests, Friedman tests and Nemenyi posthoc tests differences were considered significant at $P \leq 0.05$. Results from behavioural observations during the feeding motivation test and the reaction to the vulvae palpation are reported as the number of cows. Results from video-recorded behavioural data are reported as follows: the frequency of each behavioural event is reported as median [1st–3rd quartiles] number of occurrences per minute, and duration of each behavioural state is reported as median [1st–3rd quartiles] % of time. The results of clinical measurements, cytokines, haptoglobin, and continuous monitoring of heifer activities and position are reported as median [1st–3rd quartiles]. The results of cortisol are reported as least square means ± standard error (SE) in the text. The observed data are given in figures for plasma cortisol and for time spent in each activity (ingesting, ruminating, without activity) and in the 'standing up' position. Only significant results have been reported in the text. Non-significant results or trends are shown in the results tables.

Results

Heifers reaching endpoints and final dataset

No heifer reached endpoints as defined in the Ethics approval statement (see below). Due to a computer backup problem, the video from the camcorder recording the front of one heifer (cow 7, batch 3) during CONTROL Embryo flushing (24th June) was lost. The final set of video recordings therefore contained 287 videos (i.e. 72 videos for DFR, 48 videos for first artificial insemination (AI1), 48 videos for second artificial insemination (AI2), and 71 videos for EF, 48 videos for ET) instead of the 288 videos initially set.

One heifer (#8, batch 3) lost her collar with the attached accelerometer during the EXPERIMENTAL EF and during the EXPERIMENTAL ET procedures, and therefore, accelerometer data from only 11 heifers were used for this reproductive procedure.

We encountered problems with the Polar Equine© recording system i) during DFR for one heifer (#1, batch 1), and therefore, continuous heart rate data from only 11 heifers were used for this reproductive procedure; ii) during AI2 for one heifer (#7, batch 3), and therefore continuous heart rate data from only 11 heifers as used for this reproductive procedure; iii) during ET for two heifers (#1, batch 1; #7, batch 3), and therefore, continuous heart rate data from only 10 heifers were used for this reproductive procedure.

Duration of the reproductive procedures

DFR lasted (median, 1st-3rd quartiles) 903 [899–905] seconds in the CONTROL situation and 553 [497–614] seconds in the EXPERIMENTAL situation. AI1 lasted 242 [239–244] seconds in the CONTROL situation and 92 [89–103] seconds in the EXPERIMENTAL situation. AI2 lasted 240 [238–243] seconds in the CONTROL situation and 98 [88–106] seconds in the EXPERIMENTAL situation. EF lasted 2404 [2400–2406] seconds in the CONTROL situation and 2064 [1889–2279] seconds in the EXPERIMENTAL situation. ET lasted 602 [599–604] seconds in the CONTROL situation and 428 [384–485] seconds in the EXPERIMENTAL situation.

Parameters not significantly impacted by any of the reproductive procedures

Behaviour

The heifers never displayed kicks, low then high-pitched vocalisation, drooling, or defecation in any of the reproductive procedures, whatever the situation (Table 2).

During all the reproductive procedures, compared to CONTROL situation, there were no significant changes in the EXPERIMENTAL situation for two hind limb postures (frequency and duration of parallel hind limb, frequency of tight hind limbs), three body postures and movements (frequency of standing still, frequency and duration of moving back, frequency and duration of unsteady), one back posture (frequency and duration of lordosis), two head postures (frequency and duration of head up; duration of head downward), and for frequency of sliding the feet, frequency of muzzle licking, frequency of sniffing or licking, frequency of ear shaking, and frequency of miction (Table 2).

After all the reproductive procedures, for both 'motivation to feed' and 'reaction to vulvae palpation', there were no significantly difference between heifers in the EXPERIMENTAL and CONTROL situation (Supplementary Table S2).

Physiological, clinical, and blood parameters

In the EXPERIMENTAL situation, there were no significant changes in plasma haptoglobin (F = 3.43; *P* = 0.33) throughout the reproductive procedures (i.e. at T_0 for DFR (1.3 [0.86–1.72] μg/mL), at T_{EP}+0.25 on D12 for superovulation (1.6 [1.2–3.2] μg/mL), at T_0 for AI1 (2.2 [1.6–2.6] μg/mL), and at T_{EP}+24 for ET (1.9 [1.8–2.4] μg/mL). During all reproductive procedures, there were no significant changes in plasma IL-1β and IL-6 (Supplementary Tables S3 and S4).

After all reproductive procedures, at clinical examination (Table 3) at T_{EP}+2, compared to the CONTROL situation, there were no significant changes in the EXPERIMENTAL situation in rumen motility rate (RMR), heart rate (HR) and vulvae heat, colour, firmness and discharge (Supplementary Table S2).

Effect of dominant follicle removal on heifers' responses

Behaviour

During DFR, compared to the CONTROL situation, heifers in the EXPERIMENTAL situation spent less time stepping (*P* = 0.04), wriggled more frequently (*P* = 0.03), they had their back flat more often (*P* < 0.001) but for a shorter duration (*P* < 0.001), had their back arched for a longer duration (*P* < 0.001), stood in a base-wide hind limb posture more often (*P* = 0.02) and for a longer duration (*P* = 0.056), lifted a hoof more often (*P* = 0.03), and brought hoof to belly more often (*P* = 0.059) (Table 2).

Over the 24-h period studied (1 440 min) after DFR, heifers in the CONTROL situation spent 355.0 [330.0 – 411.2] min per day ingesting, 480.0 [425.0 – 530.0] min ruminating, 405.0 [348.8 – 426.2] min without activity, and the rest of the time in another activity. They spent 945.0 [913.8 – 1007.5] min standing up. In the EXPERIMENTAL situation, compared to the CONTROL situation, the heifers spent significantly less time ingesting (310.0 [270.0 – 356.2] min, *P* = 0.02) and standing up (800.0 [748.8 – 913.8] min, *P* = 0.003) (Fig. 4, Supplementary Table S5).

Physiological, clinical, and blood parameters

During DFR, in the EXPERIMENTAL situation, IL8 levels at T_0 (260.3 [168.6 – 460.6] $pg.mL^{-1}$) fell significantly to 216.0 [150.9 – 352.3] $pg.mL^{-1}$ at T_{EP}+0.25 (*P* < 0.05) (Supplementary Table S3). In the CONTROL situation, cortisol levels at T_0 (3.7 ± 1.2 $pg.mL^{-1}$) were significantly multiplied by 2.3 ± 1.3 (t value = 3.5) at T_{EP} and by 1.9 ± 1.3 (t value = 3.5) at T_{EP}+0.25 (Fig. 5, Supplementary

Table 2
Median [1st–3rd quartiles] and results of pairwise Wilcoxon signed-rank tests (V, *P*-value) for behavioural states (duration and frequency) and events observed in 12 Holstein heifers during CONTROL or EXPERIMENTAL reproductive procedures (dominant follicle removal, double artificial insemination, embryo flushing, and embryo transfer), under headlock or cage restraint.

	Dominant follicle removal				AI1				AI2				Embryo flushing				Embryo Transfer			
Item	CONT	EXP	V	*P*-value	CONT	EXP	V	*P*-value	CONT	EXP	V	*P*-value	CONT	EXP	V	*P*-value	CONT	EXP	V	*P*-value
Frequencies (No / min)																				
Hind limb posture																				
Parallel	0.17 [0.07–0.28]	0.34 [0.10–0.52]	15	0.12	1.00 [0.50–1.74]	1.35 [1.11–1.76]	28	0.42	0.89 [0.67–1.43]	0.99 [0.49–1.89]	36	0.85	0.46 [0.2–0.51]	0.36 [0.23–0.51]	42	0.85	1 [0.94–1.14]	1.45 [1.11–1.69]	21	0.18
Tight	0.2 [0.07–0.20]	0.12 [0–0.34]	25	0.5	1.00 [0.49–1.74]	0.68 [0.63–0.89]	55	0.23	0.99 [0.74–1.25]	0.53 [0–0.81]	64	0.052	0.3 [0.14–0.41]	0.24 [0.19–0.40]	28	0.42	0.9 [0.77–1.15]	0.91 [0.73–1.43]	29	0.47
Base wide	0.1 [0.05–0.20]	0.25 [0.10–0.39]	9	0.016	0.37 [0–0.80]	0.68 [0.55–1.02]	22	0.35	0.51 [0.37–0.81]	0.74 [0.63–1.30]	14	0.052	0.17 [0.11–0.29]	0.13 [0.08–0.16]	54	0.27	0.4 [0.20–0.80]	1.29 [0.93–1.44]	17	0.092
Body posture and movements																				
Standing still	0.63 [0.38–0.98]	0.76 [0.27–1.01]	27	0.38	1.90 [1.42–2.90]	1.39 [0.79–2.04]	60	0.11	1.95 [1.22–2.51]	1.72 [1.11–2.03]	47	0.57	0.91 [0.69–1.18]	0.72 [0.59–1.39]	41	0.91	1.94 [1.17–2.15]	2.52 [1.52–3.18]	24	0.27
Moving forward	0.07 [0–0.15]	0.05 [0–0.12]	25	0.85	0 [0–0]	0 [0–0]	0	NA	0 [0–0.06]	0 [0–0]	6	0.18	0.1 [0.02–0.20]	0.06 [0.05–0.10]	43	0.79	0.25 [0.10–0.53]	0 [0–0.03]	47	0.052
Moving back	0 [0–0.07]	0 [0–0.11]	14	0.62	0 [0–0]	0 [0–0]	3	0.37	0 [0–0.06]	0 [0–0]	6	0.18	0.06 [0–0.13]	0.05 [0.03–0.12]	34	0.73	0.05 [0–0.40]	0 [0–0.03]	24	0.44
Stepping	0.49 [0.17–0.56]	0.11 [0–0.40]	52	0.34	1.67 [0.93–2.14]	0.81 [0–1.17]	67	0.027	1.37 [0.70–1.94]	0.64 [0–1.39]	60	0.11	0.6 [0.42–0.84]	0.4 [0.28–0.66]	45	0.68	1.34 [0.68–1.63]	1.24 [0.94–1.57]	29	0.47
Stepping aside	0 [0–0]	0 [0–0]	0	NA	0 [0–0.06]	0 [0–0]	3	1	0 [0–0.06]	0 [0–0.12]	7	1	0 [0–0]	0 [0–0]	0	NA	0.05 [0–0.12]	0.19 [0.14–0.49]	11	0.027
Wriggling	0.07 [0–0.7]	0.09 [0–0.25]	6	0.032	0 [0–0]	0 [0–0]	1	1	0 [0–0]	0 [0–0]	1	1	0.17 [0.11–0.27]	0.23 [0.17–0.32]	36	0.85	0 [0–0]	0.33 [0–0.81]	0	0.014
Unsteady	0 [0–0]	0 [0–0]	0	0.37	0 [0–0]	0 [0–0]	0	NA	0 [0–0]	0 [0–0]	1	1	0 [0–0]	0 [0–0]	3	1	0 [0–0.12]	0 [0–0]	8	1
Back posture																				
Flat back	0.07 [0.07–0.08]	0.42 [0.27–0.49]	0	<0.001	0.25 [0.25–0.25]	1.32 [1.17–1.52]	0	<0.001	0.25 [0.25–0.25]	1.23 [0.96–1.36]	0	<0.001	0.06 [0.02–0.08]	0.14 [0.12–0.19]	5	<0.001	0.1 [0.10–0.10]	0.49 [0.46–0.63]	0	<0.001
Arched back	0 [0–0]	0.29 [0.24–0.39]	0	<0.001	0 [0–0]	0.66 [0.57–0.76]	0	<0.001	0 [0–0]	0.64 [0–57-0.68]	0	<0.001	0.01 [0–0.03]	0.11 [0.09–0.15]	7	0.009	0 [0–0]	0.37 [0.32–0.47]	0	<0.001
Lordosis	0 [0–0]	0 [0–0]	3	0.37	0 [0–0]	0 [0–0]	0	NA	0 [0–0]	0 [0–0]	0	NA	0 [0–0.02]	0 [0–0]	14	0.53	0 [0–0]	0 [0–0]	0	NA
Head Posture																				
Head horizontal	1.36 [0.87–1.67]	1.21 [1.01–1.92]	37	0.91	0.87 [0.44–1.48]	0.77 [0.64–1.31]	45	0.68	1 [0.44–1.80]	0.3 [0–1.20]	64	0.052	1.71 [1.22–1.82	1.4 [1.19–1.83]	50	0.15	0.25 [0.10–0.57]	0.62 [0.32–1.35]	10	0.045
Head diagonal	1.23 [1.11–1.80]	1.4 [1.27–1.62]	37	0.91	1.76 [1.50–2.05]	1.57 [1.27–2.46]	45	0.68	1.72 [1.42–2.56]	1.67 [1.35–1.9]	44	0.73	1.52 [1.26–2.46]	1.45 [1.33–1.56]	52	0.1	1.13 [0.90–1.52]	1.71 [1.59–2.35]	10	0.021
Head downward	0.33 [0.17–0.6]	0.42 [0.12–0.78]	40	0.97	1.13 [0.4–1.32]	0.73 [0–1.21]	52	0.1	1.23 [0.67–1.37]	1.29 [0–1.88]	40	0.97	0.42 [0.34–0.77]	0.3 [0.19–0.45]	58	0.024	0.8 [0.48–1.07]	1.49 [0.45–1.63]	21	0.18
Head up	0.3 [0.12–0.66]	0.26 [0.14–0.5]	46	0.62	0 [0–0.02]	0 [0–0]	6	0.18	0 [0–0.55]	0 [0–0]	15	0.059	0.48 [0.42–0.77]	0.36 [0.25–0.65]	52	0.1	0 [0–0]	0 [0–0.04]	1	0.2

	Dominant follicle removal				AI1				AI2				Embryo flushing				Embryo Transfer			
Item	CONT	EXP	V	*P*-value	CONT	EXP	V	*P*-value	CONT	EXP	V	*P*-value	CONT	EXP	V	*P*-value	CONT	EXP	V	*P*-value
Total duration of behavioural recording (s)	902.88 [899.50 –904.30]	552.98 [497.38 –613.48]			241.5 [239.13 –243.35]	91.42 [89.6 –102.36]			240.28 [238.57 –242.70]	97.64 [88.98 –105.72]			2403.86 [2400.39 –2405.58]	2063.82 [1889.19 –2278.91]			602.4 [599.28 –603.58]	427.64 [384.91 –484.05]		

(*continued on next page*)

Table 2 (*continued*)

Item	Dominant follicle removal				AI1				AI2				Embryo flushing				Embryo Transfer			
	CONT	EXP	V	*P*-value	CONT	EXP	V	*P*-value	CONT	EXP	V	*P*-value	CONT	EXP	V	*P*-value	CONT	EXP	V	*P*-value
Duration (% of time)																				
Hind limb posture																				
Parallel	33.27 [16.95–53.63]	32.63 [4.15–55.72]	38	0.69	25.78 [11.18–46.1]	27.62 [20.97–44.13]	32	0.62	33.35 [18.02–55.83]	19.43 [4.85–42.13]	53	0.3	49.77 [33.12–57.37]	49.37 [31.73–59.03]	39	1	39.72 [21.15–52.75]	35.58 [30.57–44]	47	0.57
Tight	34.68 [5.73–59.75]	13.7 [0–32.87]	53	0.083	52.77 [46.47–79.72]	11.57 [7.43–24.95]	75	0.002	45.33 [31.83–81.57]	3.6 [0–15.73]	68	0.021	29.63 [17.25–45.48]	33.58 [28.48–48.7]	36	0.85	45.05 [33.67–73.5]	17.18 [9.67–25.33]	70	0.012
Base wide	7.85 [4.73–33.95]	35.93 [9.82–89.17]	11	0.056	4.78 [0–7.47]	50.95 [27–61.12]	3	0.009	5.77 [1.95–15.65]	60.1 [26.8–82.4]	4	0.003	21.98 [9.27–24.83]	14.12 [9.02–17.42]	53	0.3	6.97 [2.47–14.47]	39.9 [31.47–60.05]	1	<0.001
Body posture and movements																				
Standing still	96.83 [95.42–98.88]	97.7 [95.65–99.52]	33	0.68	88.47 [84.95–92.52]	95.9 [92.8–100]	9	0.016	92.82 [89.7–95.1]	95.35 [92.83–99.02]	19	0.13	94.65 [93.68–96.72]	96.72 [93.95–97.77]	37	0.91	92.43 [87.88–94.85]	89.7 [84.95–93.13]	51	0.38
Moving forward	0.2 [0–0.38]	0 [0–0.43]	27	0.64	0 [0–0]	0 [0–0]	0	NA	0 [0–0.2]	0 [0–0]	6	0.18	0.33 [0.12–0.6]	0.2 [0.17–0.43]	40	0.97	0.82 [0.33–1.35]	0 [0–0.08]	51	0.019
Moving back	0 [0–0.25]	0 [0–0.55]	16	0.83	0 [0–0]	0 [0–0]	3	0.37	0 [0–0.22]	0 [0–0]	6	0.18	0.15 [0–0.55]	0.15 [0.08–0.52]	37	0.91	0.1 [0–1.42]	0 [0–0.1]	22	0.62
Stepping	1.98 [0.8–3.2]	0.25 [0–1.77]	65	0.042	10.43 [4.92–11.82]	3.85 [0–5.25]	70	0.012	6.18 [3.95–7.35]	2.48 [0–6]	57	0.18	3.3 [1.38–4.82]	1.38 [0.53–3.18]	53	0.3	4.93 [3.5–6.02]	4.72 [2.85–6.9]	44	0.73
Stepping aside	0 [0–0]	0 [0–0]	0	NA	0 [0–0.28]	0 [0–0]	3	1	0 [0–0.22]	0 [0–0.42]	8	1	0 [0–0]	0 [0–0]	0	NA	0.22 [0–0.65]	1.13 [0.43–3.57]	10	0.021
Wriggling	0.2 [0–0.42]	0.35 [0–0.75]	15	0.22	0 [0–0]	0 [0–0]	1	1	0 [0–0]	0 [0–0]	1	1	0.88 [0.55–1.9]	1.3 [0.85–1.87]	30	0.52	0 [0–0]	1.42 [0–3.3]	1	0.021
Unsteady	0 [0–0]	0 [0–0]	0	0.37	0 [0–0]	0 [0–0]	0	NA	0 [0–0]	0 [0–0]	1	1	0 [0–0]	0 [0–0]	4	0.79	0 [0–0.22]	0 [0–0]	8	1
Back posture																				
Flat back	100 [99.87–100]	86.93 [84.38–95.22]	78	<0.001	100 [100–100]	52.23 [42.7–55.48]	78	<0.001	100 [100–100]	34.53 [27.85–46.57]	78	<0.001	99.72 [98.5–100]	96.37 [95.23–97.27]	78	<0.001	100 [100–100]	77.93 [73.07–82.55]	78	<0.001
Arched back	0 [0–0]	13.07 [4.78–15.62]	0	<0.001	0 [0–0]	47.77 [44.52–57.3]	0	<0.001	0 [0–0]	65.47 [53.43–72.15]	0	<0.001	0.03 [0–1.28]	3.63 [2.73–4.77]	0	<0.001	0 [0–0]	22.07 [17.45–26.93]	0	<0.001
Lordosis	0 [0–0]	0 [0–0]	3	0.37	0 [0–0]	0 [0–0]	0	NA	0 [0–0]	0 [0–0]	0	NA	0 [0–0.22]	0 [0–0]	13	0.68	0 [0–0]	0 [0–0]	0	NA
Head Posture																				
Head horizontal	68.85 [49.18–81.27]	65.95 [35.72–79.27]	46	0.62	20.35 [11.55–24.7]	10.42 [4.38–27.63]	54	0.27	35.23 [18.15–46.58]	1.22 [0–17.62]	75	0.002	56.63 [54.83–74.13]	62.83 [56.03–81.12]	23	0.41	5.35 [1.05–15.18]	12.27 [1.45–26.6]	20	0.27
Head diagonal	28.12 [15.63–48.85]	28.1 [12.55–63.1]	32	0.62	32.55 [19.73–48.05]	67.03 [42.3–75.63]	17	0.092	18.05 [15.33–35.4]	72.15 [51.32–84.42]	5	0.005	33.68 [19.65–36.28]	30.27 [14.33–36.6]	35	0.9	14.88 [9.23–33.77]	40.57 [28.55–50.13]	21	0.18

Table 2 (*continued*)

Item	Dominant follicle removal				AI1				AI2				Embryo flushing				Embryo Transfer			
	CONT	EXP	V	*P*-value	CONT	EXP	V	*P*-value	CONT	EXP	V	*P*-value	CONT	EXP	V	*P*-value	CONT	EXP	V	*P*-value
Head downward	2.27 [0.88–2.57]	1.82 [1.15–4.08]	41	0.91	27.9 [6.88–55.8]	14.52 [0–31.23]	50	0.14	42.63 [12.72–48.05]	11.82 [0–48.68]	61	0.092	2.6 [1.62–5.28]	2.1 [1.33–2.73]	54	0.067	70.73 [35.08–87.32]	42.02 [17.8–59.27]	58	0.15
Head up	1.2 [0.8–2.2]	0.87 [0.55–2.45]	40	0.97	0 [0–0.32]	0 [0–0]	6	0.18	0 [0–3.65]	0 [0–0]	15	0.059	2.95 [2.52–4.7]	2.58 [1.07–3.15]	42	0.46	0 [0–0]	0 [0–0.12]	1	0.2
	Dominant follicle removal				AI1				AI2				Embryo flushing				Embryo Transfer			
Item	CONT	EXP	V	*P*-value	CONT	EXP	V	*P*-value	CONT	EXP	V	*P*-value	CONT	EXP	V	*P*-value	CONT	EXP	V	*P*-value
Frequencies (No / min)																				
Feet movements																				
Lift hoof	0 [0–0]	0.05 [0–0.25]	1	0.034	0 [0–0.06]	0 [0–0]	7	0.58	0 [0–0]	0 [0–0]	3	0.37	0.02 [0–0.05]	0.15 [0.1–0.29]	0	<0.001	0 [0–0.1]	0.18 [0–0.34]	4	0.033
Hoof to belly	0 [0–0]	0 [0–0.24]	0	0.059	0 [0–0.25]	0 [0–0]	15	0.059	0 [0–0.24]	0 [0–0]	10	0.1	0 [0–0]	0.11 [0.03–0.29]	0	0.004	0.1 [0.07–0.1]	0.15 [0.08–0.41]	9	0.016
Kick	0 [0–0]	0 [0–0]	0	NA	0 [0–0]	0 [0–0]	0	NA	0 [0–0]	0 [0–0]	1	1	0 [0–0]	0 [0–0]	0	1	0 [0–0]	0 [0–0]	0	0.37
Sliding	0 [0–0.07]	0 [0–0.12]	6	0.4	0 [0–0]	0 [0–0]	0	NA	0 [0–0]	0 [0–0]	1	1	0.04 [0.02–0.06]	0 [0–0.04]	32	0.68	0 [0–0.02]	0 [0–0]	6	0.86
Vocalisation																				
Low monotone	0.17 [0–0.36]	0 [0–0]	34	0.19	0 [0–0]	0 [0–0]	0	1	0 [0–0]	0 [0–0]	0	0.37	0.2 [0.1–0.67]	0.05 [0–0.11]	41	0.033	0 [0–0]	0 [0–0]	0	NA
Low then high- pitched	0 [0–0]	0 [0–0]	0	1	0 [0–0]	0 [0–0]	0	NA	0 [0–0]	0 [0–0]	0	NA	0 [0–0]	0 [0–0]	1	1	0 [0–0]	0 [0–0]	0	NA
Head events																				
Muzzle licking	0.1 [0–0.15]	0.1 [0–0.13]	35	0.48	0 [0–0.25]	0 [0–0]	10	0.1	0 [0–0.32]	0 [0–0]	10	0.59	0.15 [0.1–0.17]	0.1 [0.06–0.2]	44	0.36	0.1 [0–0.22]	0 [0–0.15]	24	0.91
Sniffing or licking	0.43 [0.2–0.97]	0.56 [0.25–0.74]	49	0.47	0 [0–0]	0 [0–0]	4	0.79	0 [0–0]	0 [0–0]	3	1	0.95 [0.65–1.29]	0.81 [0.57–0.97]	54	0.067	0 [0–0]	0 [0–0]	5	1
Head shaking	0.1 [0–0.35]	0.11 [0–0.2]	34	0.19	0 [0–0.25]	0 [0–0]	15	0.059	0.25 [0–0.62]	0 [0–0]	28	0.022	0.17 [0.02–0.52]	0.17 [0.04–0.2]	48	0.21	0.1 [0–0.22]	0.16 [0.09–0.51]	17	0.55
Ear shaking	0 [0–0]	0 [0–0]	0	NA	0 [0–1.77]	0 [0–0]	15	0.059	0 [0–1.07]	0 [0–0]	14	0.11	0 [0–0]	0 [0–0]	1	1	0.05 [0–0.33]	0.19 [0–0.33]	28	0.69
Drooling	0 [0–0]	0 [0–0]	0	NA	0 [0–0]	0 [0–0]	0	NA	0 [0–0]	0 [0–0]	0	NA	0 [0–0]	0 [0–0]	1	1	0 [0–0]	0 [0–0]	0	NA
Neck stretching forward	0.07 [0.05–0.15]	0.11 [0–0.47]	25	0.5	0 [0–0]	0.29 [0–0.66]	3	0.076	0 [0–0]	0.56 [0–0.62]	0	0.014	0.07 [0.05–0.11]	0.3 [0.15–0.4]	5	0.01	0 [0–0]	0.13 [0–0.27]	2	0.03
Elimination events																				
Defecation	0 [0–0]	0 [0–0]	3	0.37	0 [0–0]	0 [0–0]	0	NA	0 [0–0]	0 [0–0]	1	1	0 [0–0]	0 [0–0]	3	0.37	0 [0–0]	0 [0–0]	1	1
Miction	0 [0–0]	0 [0–0]	1	1	0 [0–0]	0 [0–0]	0	NA	0 [0–0]	0 [0–0]	0	NA	0 [0–0.02]	0 [0–0]	10	0.1	0 [0–0]	0 [0–0]	3	0.37

Abbreviations: AI1 = first artificial insemination; AI2 = second artificial insemination; CONT= Control situation; EXP = Experimental situation; NA indicates that no cows changing their behaviour between the two situations.
Abbreviations: AI1 = first artificial insemination; AI2 = second artificial insemination; CONT= Control situation; EXP = Experimental situation; NA indicates that no cows changed their behaviour between the two situations.

Table 3
Median [1st–3rd quartiles] and results of pairwise Wilcoxon signed-rank tests (V, *P*-value) for respiratory rate, heart rate, rectal temperature and rumen motility rate measured 2 h after the end of the reproductive procedures (T_{EP}+2) in 12 Holstein heifers under CONTROL or EXPERIMENTAL situations (dominant follicle removal, superovulation, double artificial inseminations, embryo flushing and embryo transfer).

Reproductive procedures	RR (breaths/min)				HR (beats/min)				RT (°C)				RMR (cycles/2 min)			
	CONT	EXP	V	*P*-value	CONT	EXP	V	*P*-value	CONT	EXP	V	*P*-value	CONT	EXP	V	*P*-value
Dominant follicle removal	24.0 [23.5–26.0]	29.0 [26.0–34.0]	0	0.004	66.0 [55.5–69.0]	70.5 [60.0–78.0]	24	0.25	38.3 [38.2–38.4]	38.3 [38.2–38.3]	43.5	0.37	7.5 [7.5–7.5]	7.5 [7.5–7.5]	2	0.77
Superovulation (D15)	28.0 [23.5–30.5]	30.0 [26.0–32.5]	27.5	0.7	63.0 [60.0–66.0]	69.0 [63.0–81.0]	11.5	0.06	38.1 [38.0–38.2]	38.3 [38.2–38.3]	6.5	0.04	7.5 [7.5–10.0]	7.5 [7.5–10.0]	20.0	0.79
AI1	32.0 [30.0–46.5]	30.0 [26.0–32.5]	61	0.01	84.0 [71.3–90.0]	82.5 [71.3–90.4]	29.5	0.88	38.5 [38.4–39.0]	38.7 [38.4–38.8]	37.5	0.72	7.5 [7.5–10.0]	10.0 [7.5–10.0]	15	0.35
AI2	28.0 [24.0–36.5]	29.0 [26.0–32.5]	35	0.47	73.5 [63.0–85.5]	68.5 [60.0–72.3]	46.5	0.25	38.3 [38.0–38.4]	38.1 [38.0–38.2]	53.5	0.27	7.5 [7.5–10.0]	7.5 [7.5–10.0]	7	0.48
Embryo flushing	32.0 [30.0–34.0]	30.0 [26.0–35.5]	31	0.89	63.0 [56.3–75.0]	63.0 [60.0–68.3]	25.5	0.88	38.4 [38.2–38.5]	38.5 [38.4–38.5]	14.5	0.20	7.5 [7.5–10.0]	7.5 [7.5–10.0]	10.5	1.00
Embryo transfer	33.0 [30.0–35.0]	38.0 [36.0–44.5]	5.5	0.01	81.0 [72.0–90.8]	70.5 [62.3–77.3]	61	0.09	38.6 [38.5–38.8]	38.6 [38.6–38.7]	14.5	0.20	10.0 [7.5–10.0]	7.5 [7.5–10.0]	14	1.00

Abbreviations: RR = respiratory rate; HR = heart rate; RT = rectal temperature; RMR = rumen motility rate; AI1 = first artificial insemination; AI2 = second artificial insemination; CONT= Control situation; EXP = Experimental situation

Table S6). Heifers in the CONTROL situation had a HRmean of 59.7 [56.4 – 63.2] beats/min between T_0 and T_{EP} and 55.3 [52.3 – 59.0] beats/min between T_{EP} and T_{EP}+0.25. Compared to heifers in the CONTROL situation, the HRmean of heifers in the EXPERIMENTAL situation increased significantly to reach 61.9 [59.7 – 71.2] beats/min between T_0 and T_{EP} (P = 0.01) (Table 4).

After DFR, at clinical examination (Table 3) at T_{EP}+2, compared to the CONTROL situation (24.0 [23.5 – 26.0] breaths.min^{-1}), the respiratory rate (RR) of heifers in the EXPERIMENTAL situation increased significantly to reach 29.0 [26.0 – 34.0] breaths.min^{-1} (P = 0.004).

Effects of superovulation on heifers' responses

Behaviour

Over the 24-h period studied (1 440 min) after superovulation, heifers in the CONTROL situation spent 272.5 [248.8 – 358.8] min per day ingesting, 582.5 [498.8 – 640.0] min ruminating, 365.0 [323.8 – 400.0] without activity, and the rest of the time in another activity. Heifers spent also 935.0 [876.2 – 963.8] min standing up. Compared to CONTROL situation, heifers in the EXPERIMENTAL situation spent significantly less time without activity (230.0 [157.5 – 325.0] min, P = 0.003) (Fig. 4, Supplementary Table S5).

Physiological, clinical, and blood parameters

During superovulation, in the CONTROL situation, IL8 levels of heifers at D_{12} (233.2 [157.7 – 359.7] pg.mL^{-1}) significantly increased to reach 249.9 [178.7 – 542.8] pg.mL $^{-1}$ at D_{14} (P < 0.05) (Supplementary Table S4). On D_{12}, compared to CONTROL situation (5.62 +/- 1.2 ng.mL^{-1}), cortisol levels of heifers in EXPERIMENTAL situation were significantly multiplied by 1.58 +/- 1.2 (t-value = 2.0) (Supplementary Table S6). At clinical examination, rectal temperature (RT) at D_{15} was significantly higher in heifers in the EXPERIMENTAL (38.3 [38.2 – 38.3] °C) vs CONTROL situation (38.1 [38.0 – 38.2] °C) (P = 0.04, Table 3).

Effects of double artificial inseminations on heifers' responses

Behaviour

During AI1, compared to the CONTROL situation, the heifers in the EXPERIMENTAL situation stood still for a longer duration (P = 0.02), stepped less often (P = 0.03) and for a shorter duration (P = 0.01), shook their head less often (P = 0.059) and ears less often (P = 0.059), they had their back flat more often (P < 0.001) but for a shorter duration (P < 0.001), arched their back more often (P < 0.001) and for a longer duration (P < 0.001), stood in tight hind limb posture for a shorter duration (P = 0.002), stood in a base-wide hind limb posture for a longer duration (P = 0.01), lifted their hoof to belly less often (P = 0.059) (P < 0.001; Table 2).

During AI2, compared to the CONTROL situation, the heifers in the EXPERIMENTAL situation had held their head horizontal less often (P = 0.05) and for a shorter duration (P = 0.02), held their head up less often (P = 0.059) and for a shorter duration (P = 0.059), held their head diagonal for a longer duration (P = 0.01), shook their head less often (P = 0.02), stretched their neck forward more often (P = 0.01), they had their back flat more often (P < 0.001) but for a shorter duration (P < 0.001), they had their back arched more often (P < 0.001) and for a longer duration (P < 0.001), stood in tight hind limb posture less often (P = 0.05) and for a shorter duration (P = 0.02), stood more often in a base-wide hind limb posture (P = 0.05) and for a longer time (P = 0.03) (Table 2).

Over the 24-h period studied (1 440 min) after AI2, heifers in the CONTROL situation spent 327.5 [277.5 – 360.0] min per day ingesting, 537.5 [498.8 – 565.0] min ruminating, 390.0 [308.8 – 441.2] min without activity, and the rest of the time in another activity. Heifers spent also 937.5 [902.2 – 956.2] min standing up (Fig. 4, Supplementary Table S5).

Physiological, clinical and blood parameters

After AI1, at clinical examination (Table 3) at T_{EP}+2, compared to the CONTROL situation (32.0 [30.0 – 46.5] breaths.min^{-1}), RR of heifers was significantly lower in the EXPERIMENTAL 30.0 [26.0 – 32.5] breaths.min^{-1} situation (P = 0.01).

During AI2, in the EXPERIMENTAL situation, TNFα levels at T_0 (284.8 [144.9 – 1061.6] pg.mL^{-1}) fell significantly to 253.9 [134.2 – 1123.3] pg.mL^{-1} at T_{EP}+ 0.25 (P = 0.03) (Supplementary Table S3). Cortisol levels at T_{EP}+0.25 were 6.46 ± 1.48 ng.mL^{-1} in the CONTROL situation. Cortisol levels at T_{EP}+0.25 were significantly multiplied by 1.78 ± 1.26 in the EXPERIMENTAL situation (t-value = 2.5) (Fig. 5, Supplementary Table S6). Heifers in the CONTROL situation had a HRmean of 63.0 [58.6 –72.5] beats/min between T_{EP} and T_{EP}+0.25. Compared to heifers in the CONTROL situation, the HRmean of heifers in the EXPERIMENTAL situation

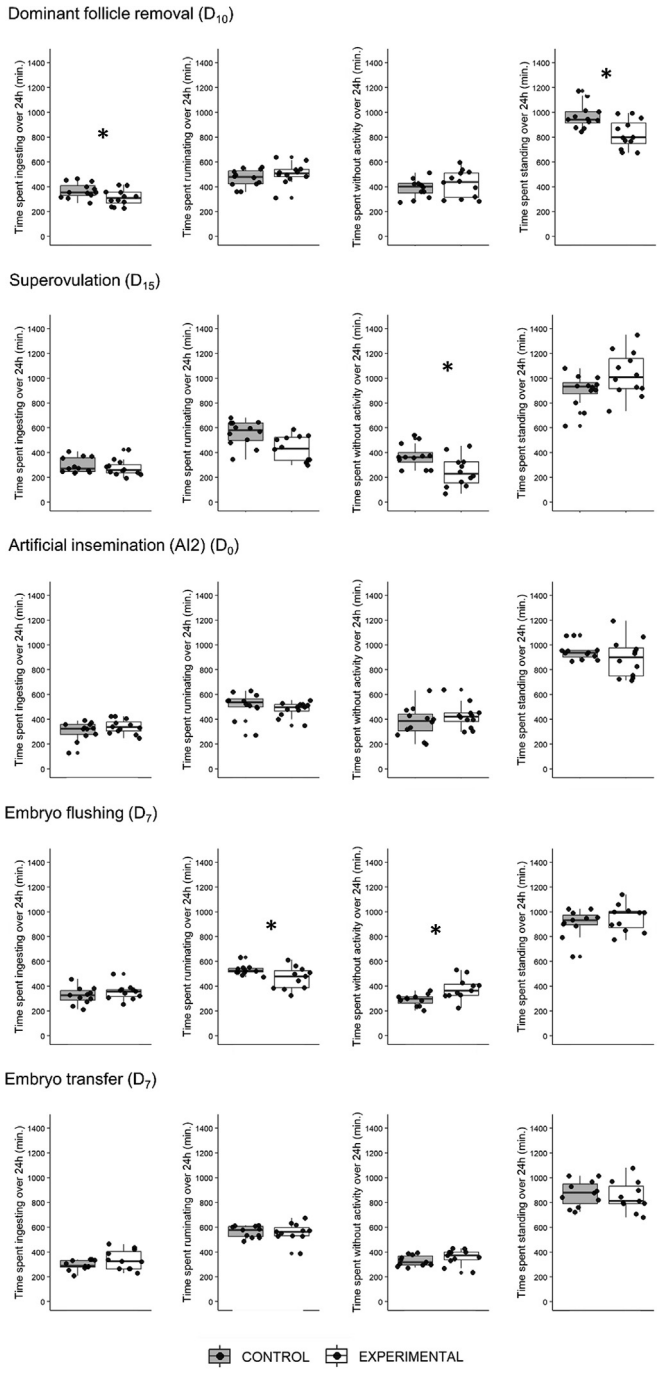

Fig. 4. Time spent ingesting, ruminating, without activity and standing over a 24-h period (1 440 min) following the end of reproductive procedures in 12 Holstein heifers under CONTROL or EXPERIMENTAL situations for dominant follicle removal on D15 of superovulation, second artificial insemination, embryo flushing, and embryo transfer. Grey boxplots represent heifers in CONTROL situation; white boxplots represent heifers in EXPERIMENTAL situation, black dots indicate individual values. Graphics flagged with an * indicate significant ($P \leq 0.05$) differences between CONTROL and EXPERIMENTAL situations. Abbreviation: AI = Artificial insemination.

increased significantly to reach 77.5 [65.3 – 84.6] beats/min between T_{EP} and T_{EP}+0.25 ($P = 0.01$) (Table 4).

Effects of embryo flushing on heifers' responses

Behaviour

During EF, compared to CONTROL situation, the heifers in EXPERIMENTAL situation had their head downward less often ($P = 0.02$), stretched their neck forward more often ($P = 0.01$), had their back flat more often ($P < 0.001$) and for a shorter duration ($P < 0.001$), had their back arched more often ($P = 0.01$) and for a longer duration ($P < 0.001$), lifted a hoof more often ($P < 0.001$), lifted hoof to belly more often ($P = 0.004$), and displayed less low monotone vocalisation ($P = 0.03$) (Table 2).

Over the 24-h period studied (1 440 min) after EF, heifers in the CONTROL situation spent 330.0 [287.5 – 365.0] min per day ingesting, 525.0 [515.0 – 545.0] min ruminating, 300.0 [262.5 – 290.0] min without activity, and the rest of the time in another activity. Heifers spent also 935.0 [897.5 – 972.5] min standing up. Compared to the CONTROL situation, heifers in the EXPERIMENTAL situation spent significantly less time ruminating (480.0 [387.5 – 525.0] min, $P = 0.05$) and significantly more time without activity (365.0 [327.5 – 415.0] min, $P = 0.01$) (Fig. 4, Supplementary Table S5).

Physiological, clinical and blood parameters

During EF, in the CONTROL situation, cortisol levels at T_0 (3.80 ± 1.17 ng.mL^{-1}) were significantly multiplied by 2.63 ± 1.26 at T_{EP} (t-value = 4.4) and by 1.78 ± 1.26 at T_{EP}+0.25 (t-value = 4.0). Cortisol levels at T_{EP}+0.25 were 6.76 ± 1.48 ng.mL^{-1} in the CONTROL situation. Cortisol levels at T_{EP}+0.25 were significantly multiplied by 2.63 ± 1.26 in the EXPERIMENTAL situation EXPERIMENTAL situation (t-value = 5.2).

After EF, cortisol levels at T_{EP}+2 were 3.72 ± 1.48 ng.mL^{-1} in the CONTROL situation. Cortisol levels at T_{EP}+2 were significantly multiplied by 2.88 ± 1.26 in the EXPERIMENTAL situation (t-value = 4.9) (Fig. 5, Supplementary Table S6).

Effects of embryo transfer on heifers' responses

Behaviour

During ET, compared to CONTROL situation, the heifers in EXPERIMENTAL situation moved forward less often ($P = 0.05$) and for a shorter duration ($P = 0.02$), stepped aside more often ($P = 0.03$) and for a longer duration ($P = 0.02$), wriggled more often ($P = 0.01$) and for a longer duration ($P = 0.02$), held their head horizontal more often ($P = 0.05$), held head diagonal more often ($P = 0.02$), stretched their neck forward more often ($P = 0.03$), had their back flat more often ($P < 0.001$) but for a shorter duration ($P < 0.001$), arched their back more often ($P < 0.001$) and for a longer duration ($P < 0.001$), lifted a hoof more often ($P = 0.03$), and lifted hoof to belly more often ($P = 0.02$) (Table 2).

Over the 24-h period studied (1 440 min) after ET, heifers in the CONTROL situation spent 290.0 [277.5 – 332.5] min per day ingesting, 580.0 [527.5 – 607.5] min ruminating, 320.0 [297.5 – 367.5] min without activity, and the rest of the time in another activity. They also spent 880.0 [792.5 – 950.0] min standing up (Fig. 4, Supplementary Table S5).

Physiological, clinical and blood parameters

During ET, cortisol levels at T_0 were 3.89 ± 1.23 ng.mL^{-1} in the CONTROL situation. Cortisol levels at T_0 were significantly multiplied by 2.19 ± 1.29 at T_{EP} (t-value = 3.0) and by 1.86 ± 1.29 at T_{EP}+0.25 (t-value = 2.4) in the EXPERIMENTAL vs in the CONTROL situation (Fig. 5, Supplementary Table S6).

After ET, at clinical examination (Table 3) at T_{EP}+2, compared to CONTROL situation (33.0 [30.0 – 35.0] breaths.min^{-1}), RR of heifers in EXPERIMENTAL situation was significantly higher (38.0 [36.0 – 44.5] breaths.min^{-1}) ($P = 0.01$).

Discussion

The heifers showed changes in their behavioural and physiological responses during and after each of the reproductive proce-

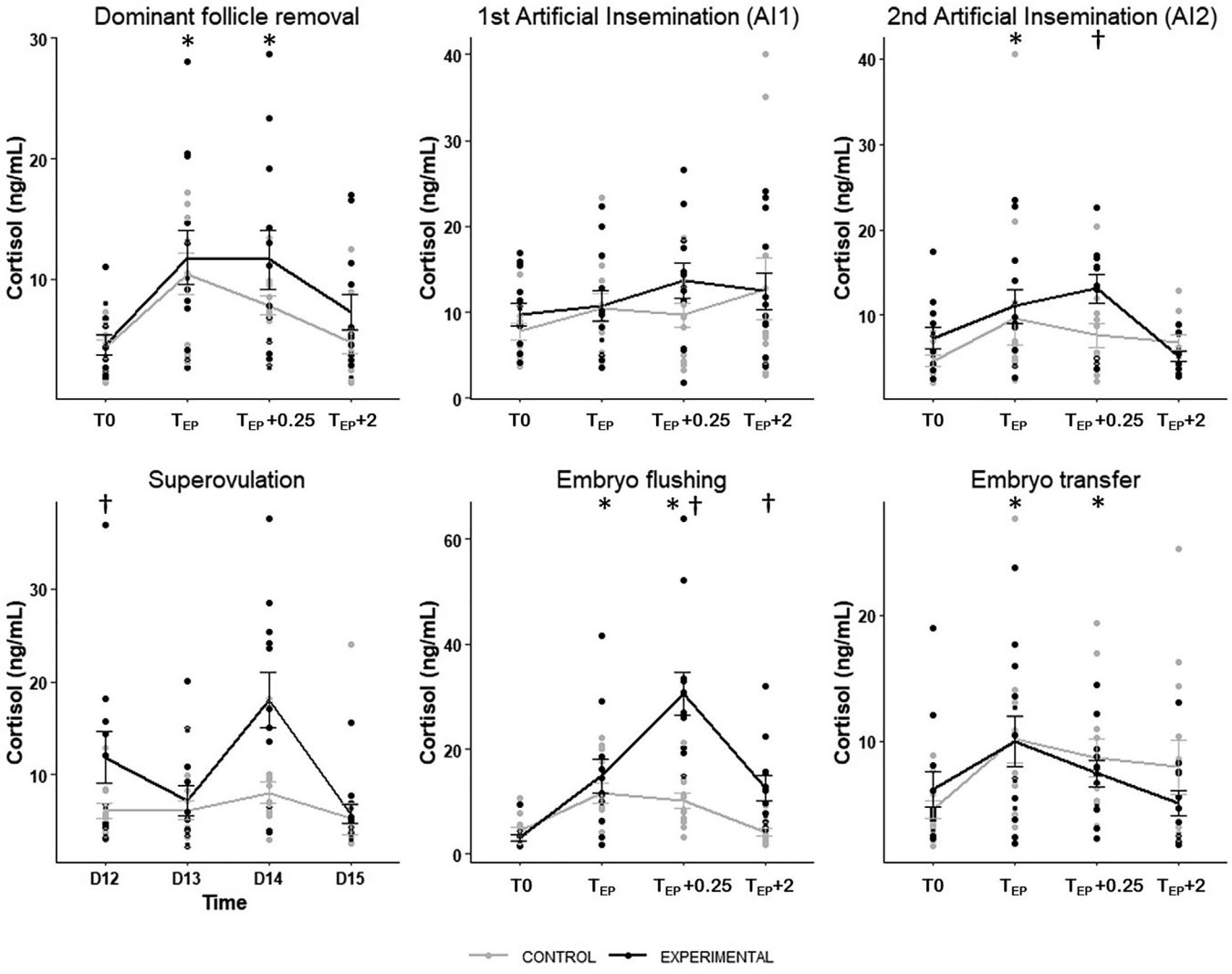

Fig. 5. Individual values (dots) and mean ± SE of plasma cortisol concentration (ng/mL) in 12 Holstein heifers under CONTROL or EXPERIMENTAL situations at the start of the reproductive procedure (T0), 15 min (T_{EP}+0.25) and 2 h (T_{EP}+2) after the end of the reproductive procedures (dominant follicle removal, D12-D15 of superovulation, double artificial insemination, embryo flushing and embryo transfer). Black dots and lines represent heifers in the EXPERIMENTAL situation and grey dots and lines represent heifers in the CONTROL situation. Means flagged with an * indicate significant differences between the values obtained at a given time-point and those obtained at T0 for the CONTROL situation ($P \leq 0.05$). Means flagged with a † indicate significant differences between CONTROL and EXPERIMENTAL situations at a specific time-point ($P \leq 0.05$). Abbreviations: D = Day; AI = Artificial insemination.

Table 4
Median [1st–3rd quartiles] and results of pairwise Wilcoxon signed-rank tests (V, *P*-value) for mean heart rate recorded using the Polar Equine© system from T0 to the end of the reproductive procedures (T_{EP}) and from T_{EP} to T_{EP}+0.25, in 12 Holstein heifers under CONTROL or EXPERIMENTAL situations (dominant follicle removal, double artificial inseminations, embryo flushing and embryo transfer).

Reproductive procedures	Heart Rate mean between T0 and T_{EP} (beats/min)				Heart Rate mean between T_{EP} and T_{EP}+0.25 (beats/min)			
	CONT	EXP	V	*P*-value	CONT	EXP	V	*P*-value
Dominant follicle removal	59.7 [56.4–63.2]	61.9 [59.7–71.3]	6	0.01	55.3 [52.3–59.0]	57.6 [53.5–63.9]	19	0.24
AI1	73.1 [61.7–82.9]	69.5 [65.3–75.3]	49	0.17	72.4 [70.1–76.8]	76.7 [67.6–85.4]	27	0.64
AI2	68.6 [64.3–73.2]	66.7 [60.8–71.7]	39	0.64	63.0 [58.6–72.5]	77.5 [65.3–84.6]	6	0.01
Embryo flushing	58.7 [55.9–63.3]	57.8 [54.0–61.3]	31	0.36	57.4 [52.0–62.4]	53.7 [53.1–58.7]	37	0.10
Embryo transfer	75.6 [67.1–78.8]	72.9 [65.2–82.1]	24	0.77	67.2 [64.7–69.4]	74.0 [61.0–75.2]	17	0.32

Abbreviations: CONT= Control situation; EXP = Experimental situation; AI1 = first artificial insemination; AI2 = second artificial insemination; T_{EP}+0.25 = 15 min after the end of the reproductive procedures.

dures. They displayed abnormal postures (body, hind limb, head) during all procedures and signs of agitation in Dominant Follicle Removal (DFR) and Embryo Transfer (ET). Their plasma cortisol concentration increased in superovulation, AI2 (second Artificial Insemination), and Embryo Flushing (EF). We did not observe any inflammatory response 2 h after the procedures. During the 24 h after some procedures, the heifers reorganised their activities (ingesting, ruminating, standing, without activity).

Although the heifers in the CONTROL situation were only handled and restrained, their plasma cortisol increased during sham DFR, sham AI2, sham EF, and sham ET. These findings demonstrated that handling *per se* induced stress in these animals, as already well described in the literature (Mialon et al., 2012; Grandin, 2010; Chastant-Maillard et al., 2003). Heifers displayed discomfort or pain, not only stress during and after the reproductive procedures. During the reproductive procedures, the heifers displayed postural changes, signs of agitation and defensive behaviours. This suggests that the procedures were associated with negative emotional experience. This is in line with the results of other studies performed on different reproductive procedures in dairy cows. Indeed, the majority of cows arched their back and stretched their neck and displayed agitation behaviours (moving sideways, tripping) during vaginal examination conducted with a Metricheck device (a metal rod with a cupule, inserted into the vagina), which can be compared to insertion of the insemination and transfer gun where the metal rod is inserted without catheterisation of the cervix (Pilz et al., 2012). Cows displayed behaviours reflecting restlessness (i.e. tail flicking, flinching, lifting a leg, stepping aside, head shaking) during transrectal palpation and sham AI (Waiblinger et al. 2004). These reactions have been widely described in animals experiencing pain and/or discomfort in various contexts (Weary et al., 2006; Prunier et al., 2013). However, some of these behaviours are observed in non-painful contexts. For instance, cows can be reactive at milking due to fear of humans (Rousing et al., 2006; Breuer et al., 2000). Our findings therefore suggest that the *in vivo* embryo production and transfer protocols induced discomfort and/or pain responses in the heifers during some procedures. This conclusion was expected and confirms the veterinarians' or technicians' experiences in the field.

The gold standard for determining whether pain is present is to compare the animal's responses during a procedure without anaesthetic against the same procedure with anaesthetic, as efficient locoregional anaesthesia suppresses the transduction and transmission of the nociceptive signal (Coetzee, 2013). Here, we did not compare the responses of heifers with or without anaesthetic for each procedure. However, the heifers underwent three procedures with anaesthetic (DFR, EF and ET) and two procedures without anaesthetic (superovulation and AIs). In the procedure where anaesthesia was not used, the heifers spent 48–65% (in AIs) with arched back, whereas in procedures with anaesthesia, they spent less time with arched back (13% in DFR, 4% in EF and 22% in ET). Thus, our study could suggest that increased time spent with arched back are signs of pronounced discomfort in heifers. Surprisingly, the magnitude of defensive behaviours (hoof lifting, hoof to belly) was higher during two procedures with anaesthesia (EF and ET) than during procedures without anaesthesia (AIs). Additional studies comparing each procedure with vs without anaesthesia or with different levels or protocols of anaesthesia and analgesia are needed to firmly conclude whether or not the procedures cause pain and, if so, to properly address its intensity and duration.

After some procedures, the heifers changed their activity patterns and their standing positions over 24 h. We expected to see behavioural change in the 24 h after superovulation treatment (increased time spent with activity) because the procedure is designed to induce oestrus behaviour, i.e. spending more time interacting with counterparts and less time resting (Röttgen et al., 2018). However, the activity pattern changes observed here following DFR (decreased ingestion and time spent standing) and EF (decreased rumination and increased time spent without activity) were unexpected. Our findings showed that the reproductive procedures have negative impacts on heifers long after the procedure ended.

The heifers' responses varied in terms of intensity and temporality depending on the reproductive procedure. This suggests that some reproductive procedures may have had a greater impact on the animals than others, which could be explained by several factors. First, all the reproductive procedures involved manipulation of the rectum alone, which is known to induce discomfort postures in cattle (e.g. arched back Stojkov et al., 2015). In addition, the heifers' reactions during the procedure may be due to different sensations – including discomfort – during the manipulation of the ovaries (DFR) or of the cervix and uterine horns (EF, ET, AI), as already reported or suspected in women (Buisman et al., 2022; Kwan et al., 2018; Ng et al., 1999) and in other mammals (Campbell and Sandøe, 2015; Berghold et al., 2007). Second, superovulation and AI lasted one to 2 min whereas DFR and ET lasted around 10 min and EF lasted 30 min. Third, AI was performed when the heifers had an open cervix whereas EF and ET were performed when they had a closed cervix. Fourth, the heifers were either isolated in another room and put in a restraining cage (i.e. for DFR and EF) or they were in a group headlocked in the feeding barrier (i.e. for AI, superovulation and ET) which may have induced lower stress (Boissy and Le Neindre, 1997). In addition, the heifers received local anaesthesia for DFR, EF and ET but not for AI or superovulation. However, as the experimental design stands, the procedures remained inter-dependent: the EF was performed after DFR, superovulation, and both AIs. The results presented here cannot identify whether one procedure impacted the heifers more or less than another, or which procedure had the most welfare impact. To know which of the five reproductive procedures had the greatest impact on welfare would enable us to target those that need to be refined in priority.

We had hypothesised that the series of reproductive procedures were associated with increased local and systemic inflammation. However, cytokine levels varied strongly between heifers and between procedures, thus preventing us from determining a clear pattern of response. In addition, there was no visible increase in haptoglobin levels. Our findings suggest that either the procedures did not induce systemic inflammation, or we were unable to characterise it because the sampling was not done at the appropriate time or on the appropriate matrix. We explored the inflammatory response at a systemic level through blood sampling, but not at local level using endocervical and endometrial cytological examination (Deguillaume et al., 2012), and so we were unable to reliably determine whether or not there was a local inflammatory response. We had chosen not to use a cytobrush here as we were concerned that it might induce local inflammation. Moreover, further studies examining lactate dehydrogenase (LDH) could better describe the extent of cell damages (Klein et al., 2020).

This study carries several limitations. First, our sample only included Holstein heifers naïve to all reproductive technologies. Our results can therefore only be extrapolated to Holstein heifers without any previous experience of reproduction. In practice, the high-genetic-value heifers usually undergo repeated embryo production procedures before embryo transfer, and so further studies are needed to address the impact of repetition of these procedures on female cow welfare. Second, the experiment was carried out over more than 4 months, resulting in inconsistent experimental conditions: the environmental parameters could not be controlled despite the cross-over design. The spring and early summer of 2022 were particularly hot periods in France (Météo France, https://meteo.data.gouv.fr/form), which likely (Gaughan et al.,

2000) increased respiratory rate and rectal temperature in heifers from batch 3 in CONTROL situation and heifers from batch 3 and 4 in EXPERIMENTAL situation. Third, assessment bias was only partly controlled: the technician who analysed the video recordings taken during the reproductive procedures was blind to the heifer's procedures and situations, while the clinical examinations were performed by one experimenter who knew the heifer's situation. However, the experimenter used well-defined clinical indicators (i.e. heartbeat counts, colour of the vulvae, etc) to not overestimate or underestimate each measure.

Conclusion

This study finds that the heifers had experienced some discomfort/pain during and after some procedures of the *in vivo* embryo production and transfer protocol. Acknowledging that some discomfort/pain is present, it is ethically essential to apply refinement strategies including a pharmacological approach and/or social, physical and cognitive enrichment, which should address both during and after the reproductive procedure.

Supplementary material

Supplementary Material for this article (https://doi.org/10.1016/j.animal.2025.101538) can be found at the foot of the online page, in the Appendix section.

Ethics approval

The study was conducted from February to July 2022 at the 'ELIANCE' experimental research platform (Agreement No. C371755), located at Nouzilly in France. All experimental protocols and procedures were carried out with the approval of the local institutional animal care and use committee (CEMEA Val-de-Loire No. 19; APAFIS agreement # APAFIS #34171-2021112610271360 v6) and conducted in full compliance with all applicable provisions established by European Directive 2010/63/EU on the protection of animals used for scientific purposes. All procedures were applied by trained staff members who performed the experiment in accordance with all relevant formally-named guidelines and regulations. The study was carried out in accordance with ARRIVE guidelines. All animals used in this study were handled in strict adherence to good clinical practices, and every effort was made to minimise suffering. Endpoints were defined before the start of the experiment: any heifers showing any signs of sickness or distress during the experiment were examined by a veterinarian and removed from the study. After the trial, the heifers returned to the herd at the experimental unit.

Data and model availability statement

The protocol was registered in the INRAE Quality Insurance system under number AQ938. Data supporting the findings of this study are available upon request from the authors, Dorothée Ledoux and Alice de Boyer des Roches.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) did not use any AI and AI-assisted technologies.

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Declaration of interest

None.

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