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ABSTRACT

This study aimed to determine the effect of lipopolysaccharide (LPS)-induced mastitis with or without nonsteroidal anti-inflammatory drug (NSAID) on dairy cows' clinical, physiological, and behavioral responses in the milking parlor and freestalls as well as the specificity (Sp) and sensitivity (Se) of behavioral responses in detecting cows with LPS-induced mastitis. Twenty-seven cows received an intramammary infusion of 25 μg of *Escherichia coli* LPS in 1 healthy quarter. Following LPS infusion, 14 cows received a placebo (LPS cows), and 13 cows received 3 mg/kg of body weight of ketoprofen i.m. (LPS+NSAID cows). Cow response to the challenge was monitored at regular intervals from 24 h before to 48 h postinfusion (hpi) through direct clinical observations, markers of inflammation in milk, and via point-in-time direct behavioral observations in the barn and at milking. In LPS cows, infusion induced a significant increase of plasma cortisol levels at 3 and 8 hpi, milk cortisol levels at 8 hpi, somatic cell counts from 8 to 48 hpi, IL-6 and IL-8 at 8 hpi, milk amyloid A (mAA) and haptoglobin at 8 and 24 hpi, rectal temperature at 8 hpi, and respiratory rate at 8 hpi. Their rumen motility rate decreased at 8 and 32 hpi. Compared with before the challenge, significantly more LPS cows stopped feeding/ruminating and pressed their tail between their legs at 3 and 5 hpi, increased feeding/ruminating at 24 hpi, and had the tendency to be less responsive, dropping their head, and dropping their ears at 5 hpi. At milking, compared with before challenge, significantly more LPS cows lifted their hooves at forestripping at 8 hpi. The 2 groups showed similar patterns of response for milk cortisol, somatic cell count, respiratory rate, mAA, haptoglobin, and IL-6, IL-1β, and IL-8. Compared with LPS cows, LPS+NSAID cows had significantly lower plasma cortisol levels at 3 hpi, their rectal temperature decreased at 8 hpi, their rumen motility rate increased at 8 and 32 hpi, and their heart rate increased at 32 hpi. Compared with LPS cows, a significantly larger proportion of LPS+NSAID cows were feeding/ruminating, a lower proportion had ears down at 5 hpi, and a larger proportion lied down at 24 hpi. At milking, whatever the phase of milking, for "hoof to belly," 9 out of 14 cows did not show this behavior before infusion (Sp = 64%) and 14/14 did not kick during pre-infusion milking (Sp = 100%). Regarding sensitivity, at maximum, 5 cows out of 14 (Se = 36%) displayed "hoof to belly" after infusion. For "lifting hoof," 14/14 did not show hoof-lifting before infusion (Sp = 100%) and 6/14 displayed it after infusion (Se = 43%) at forestripping only. In the freestall barn, 9 behaviors had a Sp >75% (at minimum, 10/14 did not show the behavior) whatever the time point but Se < 60% (at maximum, 8/14 displayed the behavior). Finally, "absence of feeding and ruminating" had Sp of 86% (12/14 ate/ruminated) and Se of 71% (10/14 did not eat/ruminate) at 5 hpi. This study shows that feeding/ruminating, tail position, and reactivity at forestripping could be used as behavioral indictors for early detection of mastitis-related pain in dairy cows.

Key words: cattle, udder inflammation, welfare, pathophysiology, pain assessment

INTRODUCTION

Pain is highly detrimental for dairy cow welfare. Cows may experience pain during daily events such as routine veterinary procedures and surgeries and during diseases, all of which may lead to production and reproduction losses (Gröhn et al., 2004; Ahmadzadeh et al., 2009). Pain management is, therefore, essential for

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cow health and welfare, good dairy farming practices, and production and economic reasons.

Pain management begins with a pain assessment, which is based on detecting changes in physiological and behavioral indicators. Physiological indicators include hormones like cortisol that are released by the hypothalamus-pituitary-adrenal axis (**HPA**), physiological changes due to activation of the autonomous nervous system (**ANS**; e.g., heart rate, respiratory rate, rumen contraction rate, body temperature), and release of inflammatory markers (e.g., serum amyloid A, haptoglobin, cytokines; Prunier et al., 2013; Faure et al., 2017). Identifying behavioral signs of pain is a fundamentally important concern in animal welfare research to explore and determine to what extent animals experience pain, and in veterinary practice to enable effective care actions to be taken to treat the animal and monitor the success of that treatment (Rutherford, 2002). When cattle experience pain, they change their spontaneous behavior in their living area (Prunier et al., 2013) and their response to palpation of painful areas (Fitzpatrick et al., 2013; Lomax and Windsor, 2013; Raundal et al., 2014). Combining physiological and behavioral indicators leads to a better characterization of the pain experienced by animals in different contexts (de Boyer des Roches et al., 2017; Durand et al., 2021; Ledoux et al., 2023). To date, the diagnostic sensitivity (**Se**) and specificity (**Sp**) of each pain indicator over time has been described for physiological indicators (e.g., plasma and salivary cortisol, Substance P) but only for 2 indicators based on the behavioral response of cattle (Martin et al., 2022). There is, therefore, a lack of knowledge for the diagnostic Se and Sp of other behavioral responses.

Mastitis is a widely used model for udder inflammation and the associated signs of pain in cattle as it can be experimentally induced with various degrees of intensity (Schukken et al., 2011; Zimov et al., 2011; Leslie and Petersson-Wolfe, 2012; Fitzpatrick et al., 2013). The early clinical phases of mastitis can be experimentally induced by infusing LPS, a pro-inflammatory immunogenic cell-wall component of gram-negative bacteria, into the mammary gland. This provokes a local inflammation and a systemic response. Depending on the strain from which LPS is derived and the LPS concentration used, the severity and kinetics of the responses vary (Giovannini et al., 2017; e.g., LPS derived from *Escherichia coli* provokes acute responses). The response can be also modulated by administration of nonsteroidal anti-inflammatory drugs (**NSAID**), which has analgesic and antipyretic effects (Zimov et al., 2011; Fitzpatrick et al., 2013).

To date, the behavioral response of dairy cows with LPS in the udder have only been described in tiestalls (Siivonen et al., 2011; Zimov et al., 2011; Fitzpatrick et al., 2013; Giovannini et al., 2017), which is a setting that limits possibilities for cows to display a wide panel of behaviors. Furthermore, cows' responses to palpation at milking indicating hyperalgesia, or maybe even translating allodynia, have only been explored in cows with naturally occurring bacterial mastitis (Medrano-Galarza et al., 2012; Fogsgaard et al., 2015). Several studies have addressed pain indicators in farm animals in various contexts (Weary et al., 2006; Prunier et al., 2013) but without exploring their Sp (ability to detect pain-free animals) and Se (ability to detect animals in pain).

The aim of this study was to determine (1) the effect of LPS-induced mastitis with or without a NSAID on clinical and physiological responses in dairy cows, (2) the effect of LPS-induced mastitis with or without an NSAID on a large panel of behaviors in the milking parlor and freestall, and (3) the Sp and Se of behavioral responses in detecting cows with LPS-induced mastitis.

We used an *E. coli* LPS mammary challenge model with or without NSAID pain relief rapidly injected after the LPS procedure. We monitored cows' responses from 24 h before through to 48 h after the challenge using an extensive panel of physiological, clinical, and behavioral parameters. We hypothesized that (1) infusion of LPS in the udder provoked an acute and short-term clinical and pathophysiological response, (2) cows displayed behavioral indicators of pain both in the freestall barn and at milking, (3) these behavioral indicators of pain would be modulated by NSAID, and (4) these behavioral indicators of pain would vary in their Sp and Se.

MATERIALS AND METHODS

This experiment was carried out at the Herbipôle multidisciplinary experimental research platform (Herbipôle, INRAE, 2018), an upland ruminant farming systems research facility (https://doi.org/10.15454/1.5572318050509348E12) in Marcenat, France. The protocol and procedures were approved by the local CEMEA Auvergne institutional animal care and use committee (CE-05092.01, APAFIS agreement #2015043014541577) and conducted in full compliance with all applicable provisions established by European Directive 2010/63/EU. All procedures were applied by trained staff members who performed the trial in accordance with all relevant named guidelines and regulations. The study was carried out in accordance with ARRIVE guidelines (Percie du Sert et al., 2020). All animals used in this study were handled in strict adherence to good clinical practices, and every effort was made to minimize suffering. Before the challenge,

experimental cows went through clinical examination performed by an experienced veterinarian. The clinical examination included observation of lameness, nasal discharge, and respiratory disorder, and measurement of rectal body temperature, heart rate, respiratory rate, and rumen contraction rate. Endpoints and exclusion criteria were defined as follows before the start of the experiment: any cows showing any signs of sickness or distress during the experiment were examined by a veterinarian and were removed from the study if they crossed the threshold limit of rectal temperature above 42.5°C for 4 consecutive hours, together with a score above 12 on the de Boyer des Roches et al. (2017) grid.

Animals, Housing, and Feeding

The study used 28 primiparous Holstein dairy cows [age (mean ± SD) 3.2 ± 0.2 yr] that were between 100 and 163 (128.9 ± 15.8) DIM to ensure positive energy balance, with a BCS of 2.5 to 3, and were either pregnant or in the luteal phase. Primiparous cows were chosen to standardize their health history (i.e., with no previous mastitis). Because the literature does not include information on changes of behaviors in dairy cows housed in freestalls after an infusion of 25 μg of ultra-pure LPS from *E. coli* in the udder, we decided to calculate the sample size using a power *t*-test based on serum cortisol concentration data from Zimov et al. (2011). A sample size of 14 cows per treatment was the minimum to reach a probability of 90% (power) to show a difference in plasma cortisol concentration above 5 μg/mL with an estimated standard deviation of 3.9 and α-risk of 5% [formula in R software version 3.4.3 (2017): power.t.test (n = null, delta = 5, SD = 3.9, sig.level = 0.05, power = 0.90)]. The 28 cows were housed together in a loose-housing cubicle barn (244 m^2; space allowance per cow: 8.77 m^2) with 28 cubicles, 28 self-locking barriers, a trough (length: 222 cm), and an automatic rotating brush (DeLaval Sweden). The building was lit from 0500 h to 2000 h and dimmed lights were left on at night. Cows were fed once per day (at 1000 h) with a TMR based on hay (11.3 kg of DM per cow), beet molasses (0.4 kg of DM per cow), wrapped hay (5.8 kg of DM per cow), concentrate (2.7 kg of DM per cow, with nitrogen corrector and energy corrector), and minerals designed to meet the dietary requirements for lactation, as well as access to 1 salt lick. The mixed ration was pushed back toward the cows 3 times a day (at 1300, 1600, and 2200 h). The cows were milked twice a day (at around 0730 and 1630 h) by 2 experienced stockpersons in a 2 × 14 milking parlor adjacent to the barn. During milking, forestripping and pre- and postdipping were implemented. The cows were individually weighted after each milking by an automatic livestock weighting scale (DeLaval AWS100, DeLaval Corporate) situated at the exit of the milking parlor.

Experimental Design

The experiment examined the effects of experimental *E. coli* LPS infusion in the mammary gland and the effects of intramuscular injection of a NSAID, ketoprofen (Ketofen 10%, 3 mg/kg of BW; CEVA Santé Animale), or placebo (0.9% NaCl, 3 mL/100 kg, Bioluz) on cows' pathophysiological and behavioral responses. The experimental unit was the cow. The 28 cows were assigned to the following treatments: intramammary LPS challenge followed by intramuscular saline injection (LPS group, n = 14 cows) and intramammary LPS challenge followed by intramuscular injection of ketoprofen (**LPS+NSAID** group, n = 14 cows; see below). The random allocation was balanced between the 2 treatments based on (1) mean milk production in the first 3 mo of lactation, (2) maximum milk production within the first 3 mo of lactation, and (3) the SCC per quarter (see below).

For practical reasons, it was only possible to simultaneously monitor up to 10 cows at once. Therefore, the experiment comprised 3 groups: 1 group of 8 cows (LPS, n = 4; LPS+NSAID, n = 4), and 2 groups of 10 cows (per group: LPS, n = 5; LPS+NSAID, n = 5). The 3 groups were monitored from February to March 2019 with a 2-wk interval between groups. Each cow was allocated to a group depending on its calving date.

Intramammary E. coli LPS Challenge and NSAID Treatment

On the day before challenge (see Table 1 for details), all the udder quarters were checked for absence of intramammary infection by performing a SCC measurement using a DeLaval DCC cell counter (DeLaval Corporate) and bacteriological analysis on milk samples. For bacteriological analysis, 30 μL of milk was plated on sheep blood agar plates and incubated for 24 h at 37°C. An absence of colony on these plates combined with a SCC below 50,000 cells/mL on the day of the experiment allowed us to select only healthy quarters.

At T0, the cows were challenged with LPS and then received a NSAID or placebo. For each cow, 1 quarter was selected for LPS challenge based on the following criteria: (1) priority was given to hindquarters because they were more easy to access in the herringbone milking parlor and allowed comparisons with Fitzpatrick et al. (2013), (2) with the lowest cell count [i.e., SCC <50,000 cells/mL in the quarter at the evening milking on the day before challenge, i.e., at 1630 h before LPS

Table 1. Milk samples collected before and after LPS challenge to quantify SCC, bacteria, cortisol, IL-6, IL-1β, IL-8, amyloid A (mAA), and haptoglobin parameters in milk

Parameter	Milk sampling	Sampling time point[1]	Assay method
SCC	Pooled milk[2]	1 wk before LPS challenge	LIAL laboratory, Massif Central, France
	Each quarter[3]	T-16	DeLaval DCC cell counter (DeLaval Corporate)
	Inoculated quarter[4]	T-1, T8, T24, T32, T48	Fluorescence (Fossmatic model 90 apparatus; Foss Food Technology; Herry et al., 2017)
Bacteria	Each quarter[3]	T-24	Macroscopic evaluation of 30 μL of milk plated on sheep blood agar plates and incubated for 24 h at 37°C
	Inoculated quarter[4]	T-1, T8	Macroscopic evaluation of 30 μL of milk plated on sheep blood agar plates and incubated for 24 h at 37°C
Cortisol	Pooled milk[2]	T-24, T-16, T1, T8, T24, T32, T48	Competitive ELISA (Andanson et al., 2018)
IL-6, IL-1β, IL-8, mAA	Inoculated quarter[4]	T-1, T8, T24, T32, T48	Tri-delta ELISA kit (Herry et al., 2017)
Haptoglobin	Inoculated quarter[4]	T-1, T8, T24, T32, T48	ELISA (Bio-X Diagnostics Rochefort; Herry et al., 2017)

[1]Sampling time points before LPS infusion: T-24 = 0830 h; T-16 = 1630 h; T-1 = 0830 h; after LPS infusion: T8 = 1630 h; T24 = 0830 h; T32 = 1630 h; T48 = 0830 h.

[2]Pooled milk from the 4 quarters: 1 milk sample containing the mixed milk from all 4 quarters.

[3]Milk from each quarter: 1 milk sample per quarter (i.e., 4 milk samples in total).

[4]Milk from the inoculated quarter: 1 milk sample containing the milk from the inoculated quarter.

infusion (**T-16**)], and (3) with a negative bacteriological result (Table 1; Figure 1). The chosen quarter was challenged by infusion with 25 μg of ultra-pure LPS from *E. coli* O111 (tlrl-3pelps, InVivogen) diluted in 2 mL of sterile solution of Dulbecco's PBS containing 0.5% (wt/vol) of sterile BSA (solution for cell-culture, Sigma). This dose was chosen because it induces a moderate inflammatory reaction not exceeding 24 h (Jackson et al., 1990) and elicits behavioral and physiological responses in cows (Zimov et al., 2011; Fitzpatrick et al., 2013). The 2-mL LPS suspension was infused using a sterile syringe fitted to a 32-mm length cannula.

Lipopolysaccharide infusion was performed after the morning milking on the day of challenge at T0 (minimum–maximum: 0821–0840 h; Figure 1) by a trained milker after complete milking of the gland and while the cows were still in the milking parlor. The same procedure was repeated on all cows as follows: the milker aseptically cleaned the teat orifice with 70% isopropyl alcohol, and then infused the solution in-teat using a sterile cannula and massaged the base of the teat. The cows then returned to their home-pen and were headlocked. There, the cows received an intramuscular injection of either saline solution (0.9% NaCl, 3 mL/100 kg, Bioluz; LPS group) or ketoprofen (Ketofen 10%, 3 mg/kg; CEVA Santé Animale; LPS+NSAID group), rapidly injected after the challenge procedure (i.e., at an average of 30 min; minimum–maximum: 26–31 min; Figure 1). Volumes of saline solution injected were equivalent to the volumes of ketoprofen injected. A veterinarian performed the injections blinded to cow treatment (i.e., placebo or NSAID) as the syringes looked the same.

Data Collection

Blood and milk sampling, clinical measurements, and behavioral observations were performed at regular intervals from 24 h prechallenge up to 48 h postchallenge (Figure 1). Two veterinarians and 1 technician performed blood samples, 1 experienced veterinarian blind to cow treatment performed the clinical measurements, and 2 trained observers blind to cow treatment performed the behavioral observations, of which 1 observer performed the in-freestall observations and the other performed the at-milking observations.

Blood Sampling, Milk Sampling, and Clinical Measurements

Blood samples were collected from the caudal vein into 5-mL Vacutainer tubes containing Na_2-EDTA, when the cows were blocked in headlocks, at 9 time points (Figure 1). Plasma cortisol concentrations were determined by a competition ELISA (Andanson et al., 2018).

Milk samples were collected at the beginning of milking. Before sampling, each teat was cleaned with an individual wipe and the end of the teat was disinfected with a sterile compress and 70% alcohol. Milk samples served for quantification of cortisol concentration (Andanson et al., 2018), SCC, interleukins (IL-6, IL-1β, IL-8), amyloid A (**mAA**; Herry et al., 2017), and haptoglobin (Dan et al., 2018). All sampling time points and assay methods are presented in Table 1 and Figure 1.

Clinical measurements [rectal body temperature (**RBT**), heart rate (**HR**), respiratory rate (**RR**), and

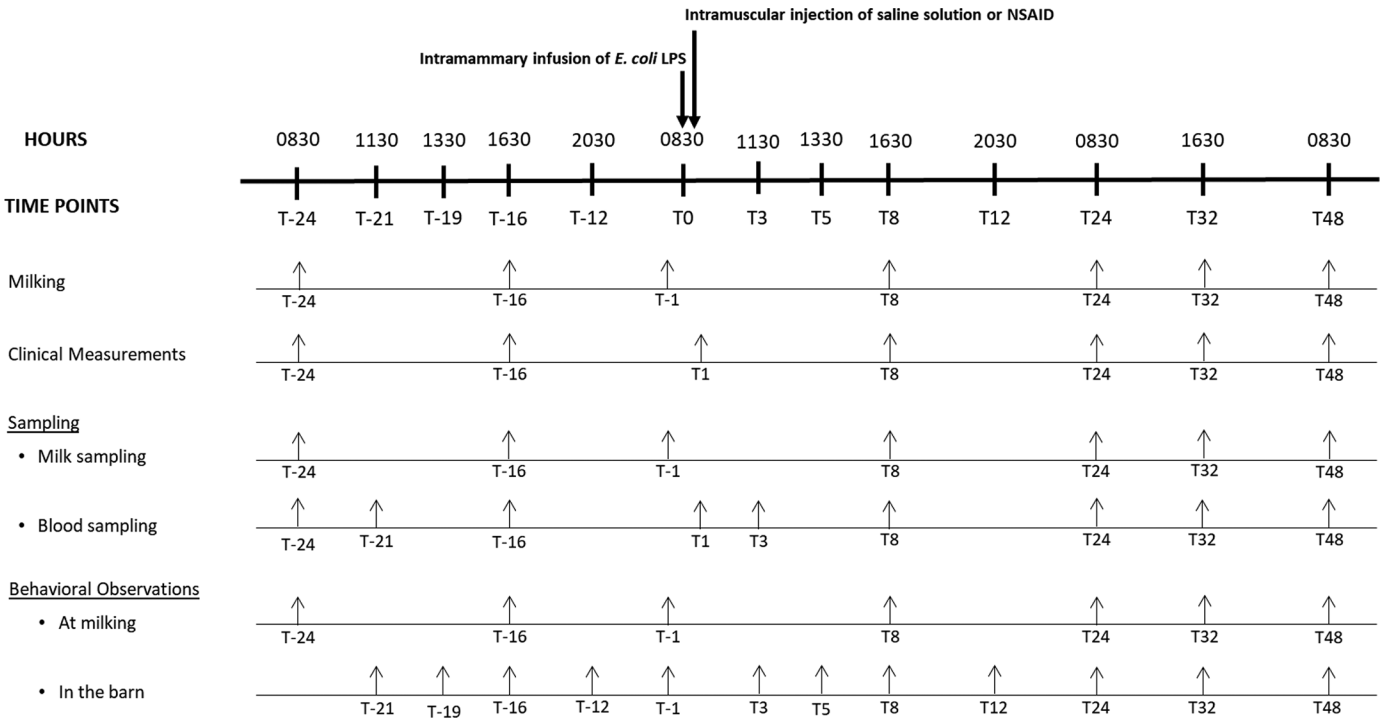

Figure 1. Experimental protocol for examining the effects of experimental 25 µg of *Escherichia coli* LPS infusion in the mammary gland and the effects of nonsteroidal anti-inflammatory drug (NSAID; ketoprofen, Ketofen 10%, 3 mg/kg BW; CEVA Santé Animale) or placebo (saline solution, 0.9% NaCl, 3 mL/100 kg, Bioluz) injection in 28 Holstein dairy cows. We observed milkings, clinical measurements, milk and blood sampling, and behaviors during the udder preparation for milking and in the barn. At T-1, data collection was performed before LPS infusion. At T+1, data collection was performed after LPS infusion and treatments (i.e., injection of saline solution or NSAID).

rumen contraction rate (**RCR**)] were performed when the cows were in headlocks, after the morning and afternoon milkings at 7 time points (Figure 1) by an experienced veterinarian blind to cows' treatments. The veterinarian used a veterinary thermometer (Veterinär Thermometer SC 12, SCALA Electronic GmbH) to record rectal temperature and a stethoscope (model Prof. Dr. Götzemit, Hauptner-Herberholz) to record heart, respiration, and rumen contractions.

Behavioral Observations

In case of estrus, cows were kept in the pen but excluded from behavioral observations as this physiological state generally induces an increase of activity, such as mounting other cows (Reith and Hoy, 2018).

Cows' reactions when the milker manipulated her udder were recorded by direct observations. From 24 h before infusion up to 48 h postinfusion at 7 time points (Figure 1), 1 trained observer blind to cow treatment recorded each cow's reactions when the milker manipulated her udder during the 3 phases of udder preparation for milking (**Phase**; i.e., teat cleaning, forestripping, and claw positioning) by all-occurrence sampling (Altmann, 1974; Bateson and Martin, 2021) using a dictaphone (Digital voice tracer, Philips LFH0622/00). The ethogram was adapted from Breuer et al. (2000) and Medrano-Galarza et al. (2012) and included the following 4 behaviors: stepping (**Step**, the hoof is lifted off the ground, without going higher than the pastern joint), lifting hoof (**Lift**, the hoof is lifted off the ground, higher than the pastern joint but lower than the tarsal joint), hoof to belly (**Belly**, hoof is lifted off the ground, higher than the tarsal joint, but lower than the stifle), kick (**Kick**, the hoof is lifted off the ground higher than the hock, and thrown to the side or backward), and the sum of the 4 behaviors (Step + Lift + Belly + Kick).

The cows' general behavior in the freestalls was recorded from 21 h before up to 48 h after the challenge, at 12 time points, by 1 trained observer blind to cow treatment using instantaneous focal-animal sampling (Altmann, 1974; Bateson and Martin, 2021; Figure 1). This method was chosen because it has already proven to be effective in detecting behavioral changes in relation to mastitis or following surgery in dairy cows (de Boyer des Roches et al., 2017; Durand et al., 2021), and it is in accordance with the way professionals (farmers, technicians, or veterinarians) punctually observe their animals in commercial farms. At each time point, the

observer quietly approached the barn, observed the cow for 10 to 20 s approximatively, and completed an individual assessment of the cow's behavior by standing at the boundary of the barn, 5 to 8 m from the focal cow so as not to disturb her. The behavior evaluation scheme was based on behaviors suggested to indicate pain described in the scientific literature on adult or juvenile cattle with pain due to tissue injury (e.g., castration, biopsy, surgery) or disease (e.g., mastitis). Each item was recorded either as "yes" (i.e., presence of behavior) or "no" (i.e., absence of behavior; Table 2).

Statistical Analyses

Clinical and Physiological Responses. To satisfy assumptions of normality, plasma cortisol, milk cortisol, and SCC were log-transformed before analyses. These parameters were then analyzed using linear mixed-effects models with time and the time × treatment interaction as fixed effects, and group and cow as nested random effects. To illustrate, the linear mixed model for plasma cortisol was lmer [$\log_{10}$ (plasma cortisol) ~time + time:treatment + (1 | group/cow)].

To account for the feed distribution-related circadian rhythm effect on clinical parameters (RBT, HR, RR, and RCR), the data were analyzed using the following 2 distinct linear mixed-effects models: a "morning" model that used data from morning time points [24 h before infusion (**T-24**), just before infusion (**T-1**), 24 after infusion (**T24**), and 48 h after infusion (**T48**)] and an "afternoon" model that used data from afternoon time points [16 h before infusion (**T-16**), 8 h after infusion (**T8**), and 32 h after infusion (**T32**)]. These 2 distinct linear mixed-effects models used time and the time × treatment interaction as fixed effects and group and cow as nested random effects. To illustrate, the "morning" linear mixed model for HR was lmer [HR ~time + time:treatment + (1 | group/cow)], where time as fixed effect included only data from time points T-24, T-1, T24, and T48.

Levels of IL-6, IL-1β, IL-8, mAA, and haptoglobin followed a nonnormal distribution due to the presence of numerous censored values (below the limit of detection of the analyzer) and were therefore investigated using nonparametric analyses (Siegel and Castellan, 1988). Interleukin levels at T-1, T32, and T48 did not reach the lower limit of quantification (**LLQ**; i.e., IL-6 = 468 pg/mL, IL-1β = 93 pg/mL, and IL-8 = 246 pg/mL) and so only data at T8 and T24 were used. Pairwise comparison between T8 and T24 for each group (LPS and LPS+NSAID) and between groups at T8 and T24 were performed using a Wilcoxon-Mann-Whitney test (respectively paired or not). Levels of mAA (LLQ = 1 ng/mL) and haptoglobin (lower limit of detection < 0.14 μg/mL) were compared between time points (T-16, T-1, T8, T24, T32, and T48) per group (LPS and LPS+NSAID) using a Friedman test and pairwise Wilcoxon rank sum test with Holm correction, and then at each time point (T-1, T8, T24, T32, and T48) between LPS cows versus LPS+NSAID cows using a Wilcoxon-Mann-Whitney test.

Behavioral Responses. Each behavioral response at milking (i.e., Step, Lift, Belly, Kick, and the sum of the 4 behaviors) was transformed into a binary variable. For each behavior, we defined the threshold as the occurrence at which at least half of the LPS-group cows expressed the behavior (i.e., 8 of 14 cows) at T-16 and at T-1 (Table 3). Thus, if the cow displayed an occurrence of the behavior above the threshold, a "yes" was assigned, and if she displayed an occurrence of the behavior equal to or below threshold, a "no" was assigned. Then, for each behavior, we compared the proportion of cows assigned to a "yes" modality at milking for each Phase and for the sum of the 3 milking phases (**SumPHASES**) before and after challenge, stratified by morning versus afternoon milking (i.e., T-16 vs. T8, T-1 vs. T24, T-16 vs. T32, T-1 vs. T48) using a McNemar change test (Siegel and Castellan, 1988).

To eliminate the circadian rhythm effect on cow's behavior in the barn (Veissier et al., 1989, 2017), we compared LPS cows' reactions at the same hour of the day before versus after infusion (i.e., T-21 vs. T3, T-19 vs. T5, T-16 vs. T8, T-1 vs. T24, T-16 vs. T31, T-1 vs. T48) using a McNemar change test (Siegel and Castellan, 1988).

We also compared cows' at-milking or in-freestall behaviors between LPS versus LPS+NSAID groups at each postchallenge time point (Figure 1) using Fisher's exact probability test or χ^2 tests (Siegel and Castellan, 1988).

Sp and Se of Behavioral Indicators. To define Sp and Se, we kept LPS cows but excluded LPS+NSAID cows because NSAID may have had an effect on inflammation. We considered that LPS cows were without udder inflammation before challenge and with udder inflammation after challenge as already demonstrated by Zimov et al. (2011) and Fitzpatrick et al. (2013). Specificity was calculated as the number of cows "detected without udder inflammation" out of the number of cows "without udder inflammation" (i.e., total number of LPS cows observed before challenge). Sensitivity was calculated as the number of cows "detected with udder inflammation" out of the number of cows "with udder inflammation" (i.e., total number of LPS cows observed after challenge). In detail, we considered that cows were "without udder inflammation" the day before the challenge (i.e., at T-21, T-19, and T-16) but "with udder inflammation" the day after the challenge (i.e., at T3, T5, and T8). For each at-milking behavior, a

Table 2. Ethogram of dairy cow behavior observed when cows were moving freely in their loose-housing freestall barn, at 12 time points from 21 h before up to 48 h after lipopolysaccharide infusion

Item	Modality	Behavior description
Resting	Yes	Cow is sleeping (lying or standing posture)
	No	Cow is not sleeping (lying or standing posture)
Lack of responsiveness (Banting et al., 2008; Hudson, 2008; de Oliveira et al., 2014; de Boyer des Roches et al., 2017; Durand et al., 2021)	Yes	Cow is not active, not sleeping and not ruminating, does not react to tactile, visual or audible environmental stimuli or does not react to all of these, or is oriented facing a wall
	No	Cow is active; does react to tactile, visual, or audible environmental, or all of these stimuli; when near other animals, can interact with or accompany conspecifics, or both; or is sleeping
Not attentive (Banting et al., 2008; Hudson, 2008; Gleerup et al., 2015; Durand et al., 2021)	Yes	Cow is standing or lying, but is neither active nor attentive to its environment
	No	Cow is standing or lying, and either active or attentive to its environment
No mammary compression of the inoculated quarter (Siivonen et al., 2011; Cyples et al., 2012)	Yes	Cow is in a posture (lying or standing) that does not put pressure on the challenged quarter
	No	Cow is lying and the lying posture puts pressure on the challenged quarter: the cow is lying on the side of the challenged quarter and 1 of its hindlegs is pressed against the udder
Lying (Molony et al., 1995; Ting et al., 2003; Zimov et al., 2011; Fogsgaard et al., 2012)	Yes	Cow is in sternal or lateral recumbency
	No	Cow is standing on her 4 feet
Unsteady (Ting et al., 2003; Siivonen et al., 2011; Chapinal et al., 2013; de Oliveira et al., 2014; de Boyer des Roches et al., 2017)	Yes	Cow is standing or lying unsteadily, sometimes with the body leaning against a wall; or standing with weight shifting on hindlegs at least once
	No	Cow is standing or lying steadily
Stretching forelegs, standing position (Robertson et al., 1994; Molony et al., 1995; Ting et al., 2003; de Oliveira et al., 2014)	Yes	Cow is standing with at least 1 foreleg partially or fully extended
	No	Cow is standing without forelegs partially or fully extended
Stretching forelegs, lying position (Robertson et al., 1994; Molony et al., 1995; de Oliveira et al., 2014)	Yes	Cow is lying with at least 1 foreleg partially or fully extended
	No	Cow is lying without forelegs partially or fully extended
Stretching hindlegs, standing position (Robertson et al., 1994; Molony et al., 1995; de Oliveira et al., 2014)	Yes	Cow is standing with at least 1 hindleg partially or fully extended caudally
	No	Cow is standing without hindlegs partially or fully extended
Stretching hindlegs, lying position (Robertson et al., 1994; Molony et al., 1995; de Oliveira et al., 2014)	Yes	Cow is lying with at least 1 hindleg partially or fully extended caudally
	No	Cow is lying without hindlegs partially or fully extended
Foot position on different floor (perched foot; Mølgaard et al., 2012)	Yes	Cow is standing with at least 2 feet on a different floor (cubicle, corridor)
	No	Cow is standing with all her feet on the same floor (cubicle, corridor)
Arched back (Ting et al., 2003; de Oliveira et al., 2014)	Yes	Cow is standing with its back arched as described by Sprecher et al. (1997)
	No	Cow is standing or lying in sternal or lateral recumbency, with the back not arched
Pressed tail (Mølgaard et al., 2012)	Yes	Cow's central part of tail pressed against vulva and udder, and distal part of tail pressed between hindlimbs
	No	Cow has a normal tail position: central part of tail slightly pressed against vulva
Tail whipping (Robertson et al., 1994; Molony et al., 1995; Ting et al., 2003; de Oliveira et al., 2014)	Yes	Cow is whipping its tail
	No	Cow is not whipping its tail
Head down (de Oliveira et al., 2014; Gleerup et al., 2015)	Yes	Cow's head is below the line of the spinal column
	No	Cow's head is at or above the line of the spinal column
Ears down (Gleerup et al., 2015; de Boyer des Roches et al., 2017)	Yes	Ears lower than spinal column, with increased gap between ears and the opening facing downwards
	No	Both ears forward or 1 ear forward or back and the other moving back and forth
No head or ear movement (Hudson, 2008; de Oliveira et al., 2014)	Yes	Cow does not react to environmental stimuli: cow displays neither ear nor head movement
	No	Cow reacts to environmental stimuli by displaying either ear or head movement, or both
No social contact (Ledoux et al., 2023)	Yes	Cow is not in contact with its nearest neighbor
	No	Cow is in contact with its nearest neighbor
No close proximity (Ledoux et al., 2023)	Yes	Cow's distance to her first neighbor is more than half a body length
	No	Cow's distance to her first neighbor is less than half a body length
Social isolation (Ledoux et al., 2023)	Yes	Cow's distance to her first neighbor is more than 1 body length
	No	Cow's distance to her first neighbor is less than 1 body length
No feeding or ruminating activity (Robertson et al., 1994; Molony et al., 1995; de Oliveira et al., 2014)	Yes	Cow is neither feeding nor ruminating
	No	Cow is feeding or ruminating

Table 3. Thresholds[1] for behavioral reaction[2] at each phase of udder preparation for milking (teat cleaning, forestripping, claw positioning) and for the total process of udder preparation for milking (SumPHASES) at the morning (a.m.) and afternoon (p.m.) milkings[3]

	Milking phase							
	Teat cleaning		Forestripping		Milking claw positioning		SumPHASES	
Item	a.m.	p.m.	a.m.	p.m.	a.m.	p.m.	a.m.	p.m.
Step	>1	>0	>0	>0	>0	>0	>1	>2
Lift	>0	>1	>0	>0	>0	>0	>1	>1
Belly	>0	>0	>0	>0	>0	>0	>0	>0
Kick	>0	>0	>0	>0	>0	>0	>0	>0
Sum	>2	>4	>1	>0	>0	>0	>3	>6

[1]Threshold defined as the occurrence at which at least half of the LPS group (n = 14) expressed the behavior (i.e., 8 of 14 cows) before LPS infusion (i.e., at T-16 for p.m. and at T-1 for a.m.).

[2]Step = stepping; lift = lifting hoof; belly = hoof to belly; kick = kick; SUM = step + lift + belly + kick.

[3]Thresholds were defined with the 14 Holstein-Friesian dairy cows inoculated with *Escherichia coli* LPS and injected with saline solution (LPS cows) half an hour after infusion; example: for "Step" during teat cleaning at the a.m. milking, "absence" is assigned if the cow displays 1 or no steps, and "presence" is assigned if the cow displays more than 1 step.

cow was considered to be "detected with udder inflammation" if that behavior was effectively observed based on the defined thresholds (see Table 3) and "detected without udder inflammation" otherwise. For each in-freestall behavior, a cow was considered to be "detected with udder inflammation" if that behavior was effectively observed and "detected without udder inflammation" otherwise (see Table 2).

All analyses were performed using R software version 3.6.2 (2019). Plasma cortisol, milk cortisol, SCC, RBT, HR, RR, and RCR were analyzed using linear mixed-effects models with the lmer function bundled with the lme4 package. As the lme4 package does not provide *P*-values, the fixed effect of a parameter was set as significant when the 95% confidence interval of that parameter did not contain 0 (Bates et al., 2014). For log-transformed parameters (i.e., plasma cortisol, milk cortisol, and SCC), confidence intervals at the true scale (i.e., not transformed) were calculated using the emmeans package. Normality of residuals and random effect distribution were visually verified using plots of residuals and quantile–quantile plots of residuals and random effects. At-milking and in-freestall behaviors were analyzed using McNemar, Fisher, or χ^2 tests with the threshold for statistical significance set at $P = 0.05$. For nonparametric analyses of IL-6, IL-1β, IL-8, mAA, and haptoglobin levels, the threshold for statistical significance set at $P = 0.05$.

RESULTS

Final Sample of Animals and Models Outputs

No experimental cow was in estrus at the moment of her monitoring. During clinical examination before the scheduled challenge, 1 cow showed signs of severe lameness (score 5 on Sprecher's scale; Sprecher et al., 1997) and was, therefore, excluded from the experiment before the challenge. No cows reached an endpoint. The final number of cows analyzed was, therefore, 27 (i.e., 14 LPS cows and 13 LPS+NSAID cows).

Outputs of all the linear mixed models are detailed in Supplemental Table S1 (https://doi.org/10.57745/LUFLAV).

Pathophysiological Responses to E. coli LPS Infusion in the Udder (LPS Cows)

Before *E. coli* LPS infusion (Figures 2 and 3; Table 4; Supplemental Table S1) at T-24, LPS cows had a basal $\log_{10}$ plasma cortisol level (expressed as estimated mean, 95% CI) of 1.42 [1.33 to 1.51; i.e., 26.2 (20.3 to 33.6) ng/mL], basal $\log_{10}$ milk cortisol level of −0.31 [−0.41 to 0.2; i.e., 0.49 (0.38 to 0.64) ng/mL], basal RBT of 38.3°C (38.2 to 38.5), basal HR of 80.2 (72.7 to 87.8) beats per minute (**bpm**), and basal RCR of 2.4 (1.9 to 2.8) contractions per 2 min. At T-16, LPS cows had a basal RBT of 38.5°C (38.4 to 38.7), basal HR of 82.3 (74.2 to 90.4) bpm, basal RR of 28.6 (23.9 to 33.4) cycles per minute, and basal RCR of 3.5 (2.7 to 4.2) contractions per 2 min. At T-1, LPS cows had a basal $\log_{10}$ SCC of 4.04 [3.90 to 4.18; i.e., 11,005 (7,879 to 15,373) cells/mL]. At T-1, IL-6, IL-1β, and IL-8 levels were below LLQ (i.e., IL-6 < 468 pg/mL, IL-1β < 93 pg/mL, and IL-8 < 246 pg/mL), mAA levels were equal to or below LLQ (i.e., 1 ng/mL), and haptoglobin level was below lower limit of detection (i.e., <0.14 μg/mL).

We detected a highly significant effect of *E. coli* LPS infusion on pathophysiological parameters (Figures 2 and 3; Table 4; Supplemental Table S1).

Compared with $\log_{10}$ plasma cortisol levels at T-24 [1.42 (1.33 to 1.51)], $\log_{10}$ plasma cortisol levels significantly increased by 0.42 (0.36 to 0.48) at T3 and by 0.07 (0.01 to 0.13) at T8 ng/mL (Figure 2A). Similarly, compared with $\log_{10}$ milk cortisol levels of −0.31 at T-24 (−0.41 to 0.2), $\log_{10}$ milk cortisol significantly increased by 0.54 (0.41 to 0.68) at T8 (Figure 2B).

Compared with $\log_{10}$ SCC at T-1 [4.04 (3.90 to 4.18)], SCC was significantly increased by 3.21 (3.01 to 3.4) at T8, 3.03 (2.84 to 3.23) at T24, 2.99 (2.79 to 3.18) at T32, and 2.81 (2.61 to 3.01) at T48 (Figure 2C). Compared with T24, IL-6 and IL-8 levels were significantly greater at T8 (respectively $P = 0.001$ and $P < 0.001$) and IL-1β levels tended to be greater at T8 ($P = 0.078$; Table 4). At T32 and T48, IL-6, IL-1β, and IL-8 levels in LPS cows were below LLQ. Compared with T-1, mAA levels were significantly increased by 180.1 μg/mL at T8 ($P < 0.001$), by 13.1 μg/mL at T24 ($P = 0.005$), by 23.5 μg/mL at T32 ($P = 0.001$), and by 73.4 μg/mL at T48 ($P < 0.001$). At T48, mAA levels were significantly greater than at T-1, T8, and T24 ($P < 0.05$ in all cases; Figure 3a). Compared with T-1, haptoglobin levels significantly increased by 23.9 μg/mL at T8 ($P < 0.001$), by 16.9 μg/mL at T24 ($P = 0.001$), by 14.9 μg/mL at T32 ($P < 0.001$), and by 22.9 μg/mL at T48 ($P = 0.001$; Figure 2b).

Compared with T-16, RBT significantly increased by 0.8°C at T8 (Figure 2D) and RR significantly increased by 6.9 cycles per minute at T8 (Figure 2F). Compared with T-24, HR significantly decreased by 9.4 bpm immediately after infusion (**T1**) and by 5.8 bpm at T24 (Figure 2E) and RCR significantly increased by 0.9 contractions per 2 min at T1 (Figure 2G). Compared with T-16, HR significantly increased by 8.8 bpm at T8 (Figure 2E) and RCR significantly decreased by 1.4 contractions per 2 min at T8 and by 1.4 contractions per 2 min at T31 (Figure 2G).

Pathophysiological Responses of LPS+NSAID Cows Compared with LPS Cows

Injection of ketoprofen significantly decreased (i.e., improved) endocrinal and clinical parameters in cows (Figure 2; Supplemental Table S1). At T1, LPS+NSAID cows had a significantly greater HR by 6.9 bmp than LPS cows (Figure 2E). At T3, LPS+NSAID cows had lower plasma cortisol levels than LPS cows by a factor of 0.7 (Figure 2A). At T8, compared with LPS cows, LPS+NSAID cows had a significantly lower RBT by 0.7°C (Figure 2D) and a significantly greater RCR by 1.0 contractions per 2 min (Figure 2G). At T32, compared with LPS cows, LPS+NSAID cows had a significantly greater HR by 8.4 bmp (Figure 2E) and a significantly greater RCR by 0.9 contractions per 2 min (Figure 2G). The LPS+NSAID and LPS cows showed similar response patterns for milk cortisol in milk, SCC, RR, mAA, haptoglobin, IL-6, IL-1β, and IL-8 (Figure 2B, 2C, 2F, and 3; Table 3; Supplemental Table S1).

Behavioral Responses of LPS Cows to E. coli LPS Infusion in the Udder.

Behavior During Udder Preparation for Milking. Following LPS infusion, cows were significantly more reactive during forestripping at T8 (Supplemental Table S2, https://doi.org/10.57745/LUFLAV).

At 1630 h on the day of challenge (T8), during forestripping, 6 out of 14 cows lifted their hoof, whereas they did not before (T-16), and 8 out of 14 cows did not lift their hoof at T-16 and at T8 (McNemar test, $P = 0.04$).

In addition, for SumPHASES, 5 out of 14 cows lifted their hoof, whereas they did not before (T-16), 3 out of 14 cows did not lift their hoof at T-16 and at T8, and 6 out of 14 cows lifted their hoof at both T-16 and T8 (McNemar test, $P = 0.07$). At 0830 h, 2 d after challenge (T48), for SumPHASES, 5 out of 14 cows lifted their hoof, whereas they did not before (T-1), 4 out of 14 cows did not lift their hoof at T-1 and T48, and 5 out of 14 cows lifted their hoof at both T-1 and T48 (McNemar test, $P = 0.07$).

Behavioral Responses in Freestalls. Following LPS infusion, LPS cows significantly changed their feeding or ruminating activity and tail posture at T3, T5, and T24 (Supplemental Table S3, https://doi.org/10.57745/LUFLAV).

At 1130 h on the day of challenge (T3), 6 out of 14 cows were no longer feeding or ruminating, whereas they did before (T-21), and 8 out of cows 14 fed or ruminated at both T-21 and T3 (McNemar test, $P = 0.04$).

At 1330 h on the day of challenge (T5), 2 behaviors significantly changed. First, 6 out of 14 cows pressed their tail, whereas they did not before (T-19), and 8 out of 14 cows did not press their tail at T-19 and at T5 (McNemar test, $P = 0.04$). Second, 9 out of 14 cows were no longer feeding or ruminating after challenge (T5), whereas they did at T-19, 1 out of 14 cows was not feeding and ruminating at T-19 and at T5, 1 out of 14 cows was feeding or ruminating at T5 but did not at T-19, and 3 out of 14 cows were feeding or ruminating at T-19 and at T5 (McNemar test, $P = 0.03$). In addition, 5 out of 14 cows were less responsive at T5, whereas they were not before (T-19), and 9 out of 14 cows were responsive at T-19 and at T5 (McNemar

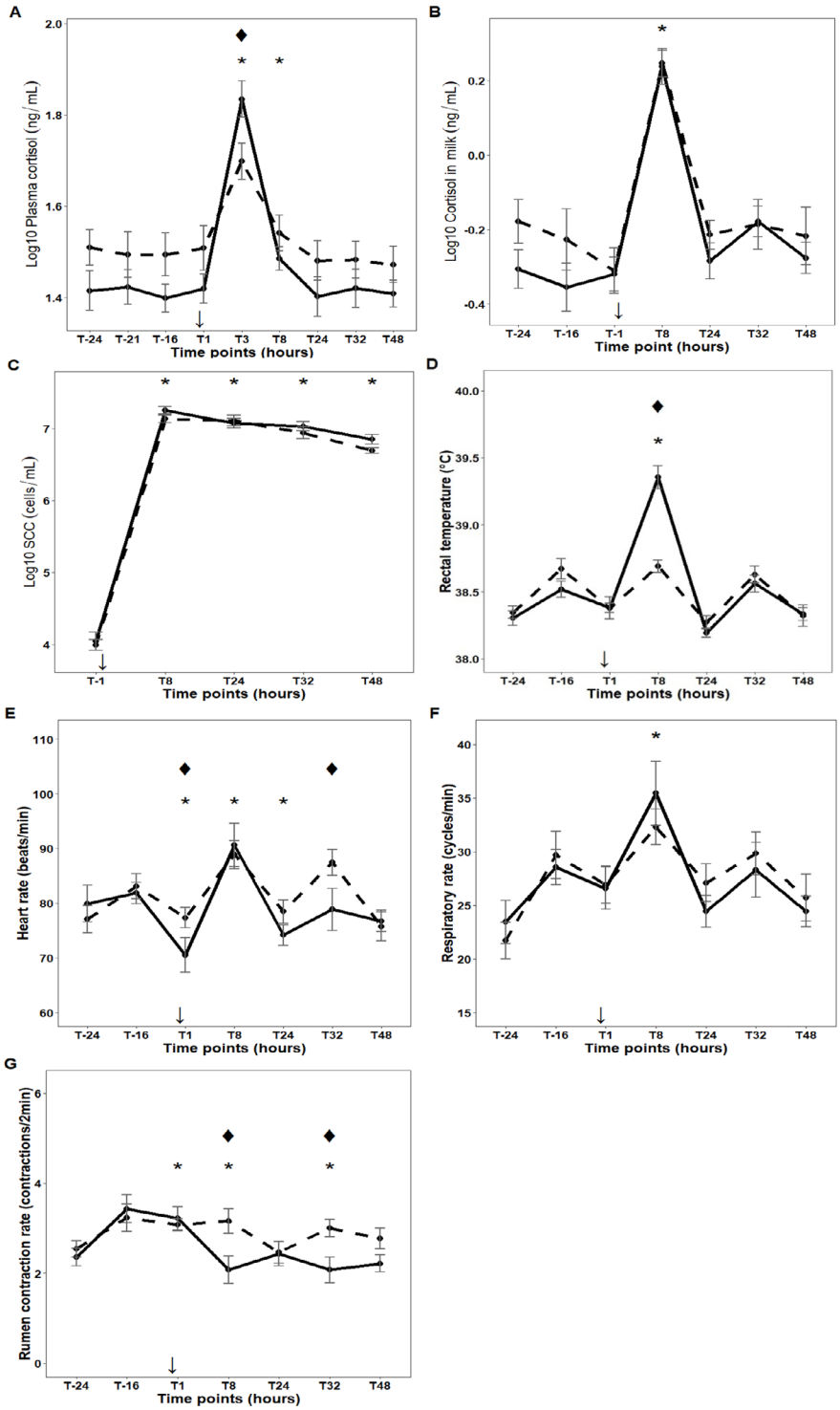

Figure 2. Time-course changes (mean ± SE) in physiological and clinical parameters: (A) $\log_{10}$ plasma cortisol, (B) $\log_{10}$ milk cortisol, (C) $\log_{10}$ milk SCC, (D) rectal temperature, (E) heart rate, (F) respiratory rate, and (G) rumen contraction rate in 27 Holstein-Friesian dairy cows inoculated with *Escherichia coli* LPS. Half of them were injected with saline solution (LPS cows, n = 14) and the other half injected with 3 mg/kg BW of ketoprofen (LPS+NSAID cows, n = 13) half an hour after infusion. Time-course changes were reported at a series of time points (i.e., 24, 21, 16 h, and just before infusion; T-24, T-21, T-16, and T-1, respectively), immediately after infusion (T1), and at 3, 8, 24, 32, and 48 h postinfusion (T3, T8, T24, T32, and T48, respectively). Solid lines represent LPS cows, and dashed lines represent LPS+NSAID cows, downward arrows indicate when LPS and treatments were performed (i.e., T0), an asterisk indicates significant differences between a specific time point and a reference time point (i.e., intercepts: T-24, T-16, or T-1) in LPS cows, and ◆ indicates significant differences between LPS and LPS+NSAID cows at a specific time point.

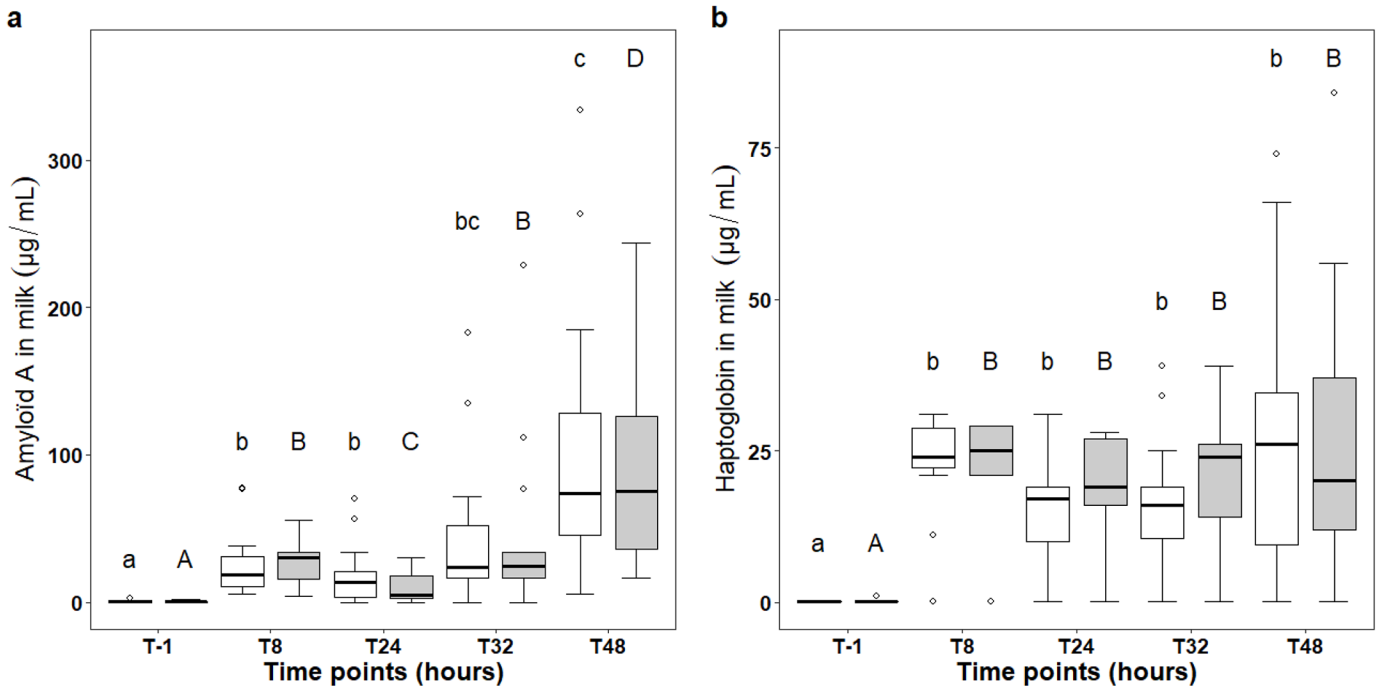

Figure 3. Time-course changes in (a) milk amyloid A and (b) milk haptoglobin in 27 Holstein-Friesian dairy cows inoculated with *Escherichia coli* LPS. Half of them were injected with saline solution (LPS cows, n = 14) and the other half injected with 3 mg/kg BW of ketoprofen [LPS + nonsteroidal anti-inflammatory drug (NSAID) cows, n = 13] half an hour after infusion. Time-course changes were reported just before infusion (T-1) and at 8, 24, 32, and 48 h postinfusion (T8, T24, T32, and T48, respectively). White boxes represent LPS cows and gray boxes represent LPS+NSAID cows. Lowercase letters (i.e., a–c) indicate significant differences between time points in LPS cows, capital letters (i.e., A–D) indicate significant differences between time points in LPS+NSAID cows, and ◊ indicates significant differences between LPS and LPS+NSAID cows at a specific time point. Within each boxplot, the central line represents the median (50% of data were greater than this value, which is the middle of data set), boxes represent the upper quartile (25% of data are higher than this value) and lower quartile (25% of data are lower than this value), whiskers represent the minimum (lowest value, excluding outliers) and maximum (highest value, excluding outliers), and circles represent outliers.

test, $P = 0.07$). Regarding head posture, 5 out of 14 cows were observed with a lowered head at T5, whereas they had not at T-19, 5 out of 14 cows were observed with a lowered head at both T-19 and T5, and 4 out of 14 cows did not have their head down at T-19 and T5 (McNemar test, $P = 0.07$). Regarding ear postures, 5 out of 14 cows had ears down at T5 but not at T-19, and 9 out of 14 cows did not have ears down at T-19 and T5 (McNemar test, $P = 0.07$).

At 0630 h on the day after challenge (T24), 7 out of 14 cows were feeding or ruminating, whereas they did not at T-1, 2 out of 14 cows were not feeding or not ruminating at both T-1 and T24, and 5 out of 14 cows were feeding or ruminating at both T-1 and T24 (McNemar test, $P = 0.02$).

Behavioral Responses of LPS+NSAID Cows Compared to LPS Cows

Behavioral Responses During Udder Preparation for Milking. During SumPHASES, 4/13 LPS+NSAID cows versus 10/14 LPS cows lifted their hoof (Fisher's exact probability test, $P = 0.06$; Supplemental Table S4, https://doi.org/10.57745/LUFLAV).

Behavioral Responses in Freestalls. Compared with LPS cows, LPS+NSAID cows recovered normal feeding/ruminating activity, ear posture, and body posture after challenge (Supplemental Table S5, https://doi.org/10.57745/LUFLAV).

At 1330 h on the day of challenge (T5), compared with LPS cows, significantly more LPS+NSAID cows fed or ruminated (LPS+NSAID cows, 10/13; LPS cows, 4/14; Fisher's exact probability test, $P = 0.02$), and significantly fewer LPS+NSAID cows had their ears down (LPS+NSAID, 0/13; LPS cows, 5/14; Fisher's exact probability test, $P = 0.04$).

At 1800 h on the day after challenge (T24), significantly more LPS+NSAID cows (10/13) were lying down compared with LPS cows (4/14; Fisher's exact probability test, $P = 0.02$).

On the day of challenge, at 1130 h (T3), 3/13 LPS+NSAID cows versus 0/14 LPS cows were in social

Table 4. Changes of IL-6, IL-1β, and IL-8 levels in 27 Holstein-Friesian dairy cows inoculated with *Escherichia coli* LPS, half of which were injected with saline solution (LPS cows, n = 14) and the other half with 3 mg/kg BW of ketoprofen (LPS+NSAID cows, n = 13) half an hour after infusion, at 8 h (i.e., T8 = 1630 h), and 24 h (i.e., T24 = 0830 h) after LPS infusion[1]

Item		T8		T24		Time effect: T8 vs. T24[2]				Treatment effect[3]					
						LPS		LPS+NSAID		T8		T24			
		LPS	LPS+NSAID	LPS	LPS+NSAID	V	*P*-value	V	*P*-value	W	*P*-value	W	*P*-value		
IL-6 (pg/mL)	Median (first quartile–third quartile)	1,942 (1,764–3,015)	2,352 (1,066–2,808)	468 (468–516)	468 (468–587)	105	0.001	91	<0.001	89	0.94	93.5	0.91		
IL-1β (pg/mL)	Median (first quartile–third quartile)	159 (70.3–228)	207 (154.8–318)	93.0 (93.0–106)	93.0 (88.5–109)	81	0.078	87	0.002	61.5	0.16	100.5	0.65		
IL-8 (pg/mL)	Median (first quartile–third quartile)	2,747 (1,687–7,025)	6,206 (4,918–11,856)	568 (511–688)	554 (528–636)	102	<0.001	90	<0.001	55.5	0.09	97.5	0.77		

[1]Wilcoxon signed rank test were used to test whether IL levels changed between T8 versus T24 in each group [LPS and LPS+ nonsteroidal anti-inflammatory drug (NSAID)]; example: for the LPS group, IL-6 levels were significantly higher at T8 than at T24 (Wilcoxon signed rank test: V = 105 and $P = 0.001$). Wilcoxon-Mann-Whitney tests were used to test whether IL levels changed between LPS versus LPS+NSAID groups at each time point (T8 and T24); example: at T8, IL 6 levels were not significantly different between the LPS and LPS+NSAID groups (Wilcoxon-Mann-Whitney test: W = 89 and $P = 0.94$).

[2]For the LPS and LPS+NSAID groups, Wilcoxon signed rank tests were used to test for time effect (comparison of T8 vs. T24; V = value of the test).

[3]Wilcoxon-Mann-Whitney tests were used to test for treatment effect (comparison of LPS vs. LPS+NSAID) at T8 and T24 (W = value of the test).

contact (Fisher's exact probability test, $P = 0.10$). At 1330 h (T5), 3/13 LPS+NSAID cows versus 0/14 LPS cows were socially isolated (Fisher's exact probability test, $P = 0.10$). At 2030 h (T12) at 6/13 LPS+NSAID cows versus 2/14 LPS cows were neither feeding nor ruminating (Fisher's exact probability test, $P = 0.10$). On the day after challenge at 0600 h (T24), 6/13 LPS+NSAID cows versus 2/14 LPS cows pressed their inoculated quarter (Fisher's exact probability test, $P = 0.10$).

Sp and Se Assessment of Behavioral Responses in LPS-Induced Mastitis

Behavioral Responses During Udder Preparation for Milking. Table 5 details Sp and Se for each behavioral response observed at milking for all milking phases.

During forestripping and milking claw positioning, Step, Lift, Belly, and Kick had Sp between 64% and 100%, where Sp = 64% signifies that 9 out of 14 cows did not display these behaviors before infusion (T-16). These behaviors had a Se below 43% after infusion (T8) as no more than 6 cows out of 14 displayed them. When taking into account all behaviors at the same time (the sum of the 4 behaviors), Sp and Se at forestripping and for SumPHASES were 64% (Table 5). Whatever the milking phase, Sp for Kick always reached 100% (Table 5).

Behavioral Responses in Freestalls. Table 6 details Sp and Se for each behavioral response observed in freestalls.

First, 12 behaviors ("lack of responsiveness," "unsteady balance," "stretching forelegs" in standing or lying position, "stretching hindlegs" in standing position, "foot position on different floors," "arched back," "pressed tail," "tail whipping," "ears down," "no head and ear movement," "no close proximity," and "social isolation") had an Sp >80% for at least 1 time point before LPS infusion, but also had an Se <33% at least once after LPS infusion. Second, the Sp and Se of 3 behaviors (i.e., "resting," "not attentive," and "lying") varied according to time points. For instance, resting had an Sp of 100% at T-21 and 71% at T-16 but only 50% at T-19. Third, 2 behaviors ("inoculated quarter not compressed" and "no social contact") had Sp <36% for all time points before infusion but had an Se ≥79% for all time points after infusion. Fourth, at 1330 h, Sp of "head down" reached 64% (T-19) and its Se reached 71% (T5). Finally, "no feeding or ruminating" had Sp above 64% at all time points (Sp = 100% at T-21, 86% at T-19 and 64% at T-16) but a Se of 71% only at T5.

Table 5. Behavioral reactions during udder preparation for milking in 14 Holstein-Friesian dairy cows inoculated with *Escherichia coli* LPS and injected with saline solution (LPS cows) half an hour after infusion[1]

Behavioral reaction at milking	Sp[2] T-16, 1630 h	Se[3] T8, 1630 h
Teat cleaning		
Step[4]	57 (8/14)	64 (9/14)
Lift	57 (8/14)	64 (9/14)
Belly	64 (9/14)	36 (5/14)
Kick	100 (14/14)	7 (1/14)
Sum	57 (8/14)	57 (8/14)
Forestripping		
Step	64 (9/14)	36 (5/14)
Lift	100 (14/14)	43 (6/14)
Belly	100 (14/14)	7 (1/14)
Kick	100 (14/14)	21 (3/14)
Sum	64 (9/14)	64 (9/14)
Milking claw positioning		
Step	79 (11/14)	29 (4/14)
Lift	71 (10/14)	29 (4/14)
Belly	100 (14/14)	14 (2/14)
Kick	100 (14/14)	0 (0/14)
Sum	71 (10/14)	43 (6/14)
SumPHASES		
Step	57 (8/14)	50 (7/14)
Lift	57 (8/14)	79 (11/14)
Belly	64 (9/14)	43 (6/14)
Kick	100 (14/14)	21 (3/14)
Sum	64 (9/14)	64 (9/14)

[1]Specificity (Sp) and proportion of cows not displaying reaction at 16 h before infusion, and sensitivity (Se) and proportion of cows that displayed reaction at 8 h postinfusion.

[2]Sp = number of cows displaying the modality "without udder inflammation" of each behavior at milking, out of the total number of cows observed at the specific time point.

[3]Se = number of cows displaying the "without udder inflammation" modality of each behavior at milking, out of the total number of cows observed at the specific time point.

[4]Example for "Step" during teat cleaning at 1630 h (i.e., Sp at T-16 and Se at T8): 8 cows out of 14 cows in total (57%) were identified "without udder inflammation" 16 h before LPS infusion (i.e., at T-16 = 1630 h) and 9 cows out of 14 cows in total (64%) were identified "with udder inflammation" at 8 h after infusion (i.e., at T8 = 1630 h).

DISCUSSION

Pain associated with mastitis—and the ways to alleviate it—has recently attracted attention, but most studies were on cows housed in tiestalls. To our knowledge, the present study is the first to give an overview over 48 h of the behavioral (in the barn, during udder preparation for milking), clinical, and physiological responses of primiparous dairy cows to udder infusion with LPS in cows observed both in the freestall barn and at the milking parlor. Physiological and clinical responses showed that cows displayed signs of acute mastitis of short duration with local and systemic signs modulated by i.m. administration of ketoprofen. We identified behavioral indicators associated with the LPS challenge and expressed by cows in freestalls (i.e., reduction of feeding/ruminating activity, tail pressed against udder) or during udder preparation for milking (i.e., hoof-lifting at forestripping). Nonsteroidal anti-inflammatory drugs administration counteracted the acute LPS-induced HPA and ANS responses, and cows were able to keep feeding/ruminating and to display baseline ear posture at 5 h postinfusion. Of all the behavioral changes observed, only 2 of them, (i.e., "no feeding/ruminating" in the freestall, and "hoof-lifting at forestripping") were sufficiently specific and sensitive to detect udder inflammation in cows under mild mastitic challenge.

Pathophysiological and Clinical Responses Associated with LPS-Induced Mastitis and NSAID

For this study, we used a mammary challenge that consisted in infusing 1 quarter of the udder with LPS from of *E. coli* O111. The LPS-induced mastitis model we used has been described in the literature to mimic the early steps of *E. coli* mastitis, in particular the onset of inflammation (Carroll et al., 1964; Blum et al., 2000). Yet, presumably because LPS is removed at the next milking and also detoxified by acyloxyacyl hydrolase, there is no prolonged exposure to the LPS pro-inflammatory signal, as is the case when inflammation is triggered by infection with live *E. coli* bacteria, resulting in a reduced inflammatory response of the host, both in intensity and duration (Blum et al., 2000; Mehrzad et al., 2007; Herry et al., 2017; Védrine et al., 2018). The model used here was, therefore, slightly different from bacteria-related mastitis in terms of duration and amplitude. It, however, produced clinical and pathophysiological responses that are very similar to those observed during the early phase of a moderate mastitis episode due to gram-negative bacteria.

In the present study, the challenge induced changes in all physiological and clinical parameters at 3 h postinfusion (HPA) and 8 h postinfusion (inflammation, ANS, and HPA). There was an increase of the inflammatory indicators classically monitored in cattle in blood or milk, such as cytokines (IL-1β and IL-6), serum mAA, mAA, haptoglobin, and SCC (Eckersall et al., 2001; Hisaeda et al., 2011; Zimov et al., 2011; Fitzpatrick et al., 2013; de Boyer des Roches et al., 2017, 2018). In this experiment, cytokines (IL-6, IL-1β, and IL-8) peaked in milk at 8 h postinfusion and fell back to basal values (i.e., below the quantification threshold of our method) at 32 h postinfusion. Concerning HPA indicators, cortisol concentrations peaked at 3 h postinfusion in plasma and at 8 h postinfusion in milk, and fell back to basal values at 24 h postinfusion. This pattern of response is consistent with previous data (Zimov et al., 2011; de Boyer des Roches et al., 2018). The plasma cortisol levels were multiplied by a factor of just 2.63 at T3, whereas other studies using experimen-

Table 6. Behavioral reactions in the barn in 14 Holstein-Friesian dairy cows inoculated with *Escherichia coli* LPS and injected with saline solution (LPS cows) half an hour after infusion[1]

	Sp[2]			Se[3]		
Behavioral reaction in the barn	T-21, 1130 h	T-19, 1330 h	T-16, 1630 h	T3, 1130 h	T5, 1330 h	T8, 1630 h
Resting	100 (14/14)	50 (7/14)	71 (10/14)	21 (3/14)	21 (3/14)	57 (8/14)
Lack of responsiveness	100 (14/14)	100 (14/14)	100 (14/14)	0 (0/14)	36 (5/14)	0 (0/14)
Not attentive	100 (14/14)	50 (7/14)	71 (10/14)	21 (3/14)	57 (8/14)	57 (8/14)
Inoculated quarter not compressed	7 (1/14)	14 (2/14)	21 (3/14)	79 (11/14)	86 (12/14)	93 (13/14)
Lying	79 (11/14)	43 (6/14)	57 (8/14)	36 (5/14)	36 (5/14)	43 (6/14)
Unsteady	93 (13/14)	93 (13/14)	100 (14/14)	7 (1/14)	7 (1/14)	0 (0/14)
Stretching forelegs, standing	100 (11/11)	100 (6/6)	100 (8/8)	0 (0/9)	0 (0/9)	0 (0/8)
Stretching hindlegs, standing	73 (8/11)	40 (2/5)	88 (7/8)	33 (3/9)	44 (4/9)	38 (3/8)
Stretching forelegs, lying	67 (2/3)	100 (8/8)	100 (6/6)	0 (0/5)	20 (1/5)	0 (0/6)
Stretching hindlegs, lying	0.00 (0/3)	50.0 (4/8)	50 (3/6)	60 (3/5)	60 (3/5)	33 (2/6)
Feet on different floors	91 (10/11)	100 (6/6)	88 (7/8)	11 (1/9)	56 (5/9)	25 (2/8)
Arched back	100 (14/14)	100 (14/14)	100 (14/14)	0 (0/14)	14 (2/14)	0 (0/14)
Pressed tail	93 (13/14)	100 (14/14)	100 (14/14)	29 (4/14)	43 (6/14)	0 (0/14)
Tail whipping	93 (13/14)	100 (14/14)	100 (14/14)	14 (2/14)	21 (3/14)	21 (3/14)
Head down	21 (3/14)	64 (9/14)	57 (8/14)	77 (10/13)	71 (10/14)	57 (8/14)
Ears down	86 (12/14)	100 (14/14)	93 (13/14)	0 (0/14)	36 (5/14)	7 (1/14)
No head and ear movement	93 (13/14)	79 (11/14)	79 (11/14)	0 (0/14)	36 (5/14)	43 (6/14)
No social contact	7 (1/14)	36 (5/14)	14 (2/14)	100 (14/14)	86 (12/14)	86 (12/14)
No close proximity	79 (11/14)	93 (13/14)	57 (8/14)	50 (7/14)	14 (2/14)	50 (7/14)
Social isolation	93 (13/14)	100 (14/14)	86 (12/14)	21 (3/14)	0 (0/14)	7 (1/14)
No feeding or ruminating activity[4]	100 (14/14)	86 (12/14)	64 (9/14)	43 (6/14)	71 (10/14)	36 (5/14)

[1]Specificity (Sp) and proportion of cows not displaying reaction at 21, 19, and 16 h before infusion, and sensitivity (Se) and proportion of cows that displayed reaction at 3, 5, and 8 h postinfusion.

[2]Sp = number of cows displaying the "without udder inflammation" modality of each behavior in the barn, out of the total number of cows observed at the specific time point. Time points before LPS infusion: T-21 = 1130 h; T-19 = 1330 h; T-16 = 1630 h.

[3]Se = number of cows displaying the "without udder inflammation" modality of each behavior in the barn, out of the total number of cows observed at the specific time point. Time points after LPS infusion: T3 = 1130 h; T5 = 1330 h; T8 = 1630 h.

[4]Example for "no feeding or ruminating activity" at 1330 h (i.e., Sp at T-19 and Se at T5): 12 cows out of 14 cows in total (86%) were identified as "without udder inflammation" at 19 h before LPS infusion (i.e., at T-19 = 1330 h) and 10 cows out of 14 cows in total (71%) were identified as "with udder inflammation" at 5 h after LPS infusion (i.e., at T5 = 1330 h).

tally induced *E. coli* or endotoxin-induced mastitis have reported 4- to 5-fold increases (Hopster et al., 1998) or even a 10-fold increase (de Boyer des Roches et al., 2018). This difference can be explained by the lower severity of the challenge because only the LPS fraction from *E. coli* bacteria was infused. The ANS indicators were affected by the infusion of LPS in the udder as follows: RBT, HR, and RR peaked at 8 h postinfusion before falling back to their initial value at 24 h postinfusion, and RCR decreased from 8 h postinfusion to 32 h postinfusion. The increase of RBT, HR, and RR and the decrease of RCT were consistent with previous observations (Lehtolainen et al., 2003; Banting et al., 2008; Zimov et al., 2011; Fitzpatrick et al., 2013). So, as expected (Prunier et al., 2013), activation of the sympathetic system and decrease in gastrointestinal motility due to a reduction in parasympathetic activity were identified as signs of pain. Thus, the infusion of 25 μg of ultra-pure LPS from *E. coli* O111 in the udder of primiparous dairy cows housed in freestalls was associated with significant inflammatory, ANS, and HPA responses that all peaked soon after the challenge. The period of 3 h postinfusion to 8 h postinfusion seemed to be the most critical time-window for the challenged cows in terms of pathophysiological response, supporting that the model used here was relevant for refining behavioral indicators of intramammary inflammation in dairy cows in freestalls and at the milking parlor.

In our experiment, NSAID treatment administered approximately 30 min after LPS infusion significantly counteracted the effect of LPS injection on the HPA axis (plasma cortisol), and on RBT, which confirm its antipyretic effect. Nevertheless, as in other studies (Anderson et al., 1986; Dascanio et al., 1995; Zimov et al., 2011), NSAID treatment of mastitis failed to influence the mammary gland response, specifically for local signs of inflammation such as SCC, haptoglobin, and amyloid A concentrations in milk.

Behavioral Changes Associated with LPS-Induced Mastitis and NSAID

Infusion of LPS in the udder and the associated acute ANS, HPA, and inflammatory responses induced

some behavioral changes in cows that were detectable by direct behavioral observations. In freestalls, cows decreased their feeding/ruminating activity, adopted antalgic postures, and were more reactive during udder preparation for milking. Nonsteroidal anti-inflammatory drugs administration counteracted some of these effects.

In the freestall barn, LPS cows stopped feeding/ruminating at 3 h postinfusion and 5 h postinfusion, increased feeding/ruminating at 24 h postinfusion, and pressed their tail between their legs at 5 h postinfusion. More of them also tended to be less responsive, dropping their head and dropping their ears at 5 h postinfusion. Such changes in posture, attention, and activity have been previously described as behavioral indicators of pain (Rutherford, 2002; Weary et al., 2006; Prunier et al., 2013; Steagall et al., 2021). Given this behavioral response, the infusion of LPS in the udder, therefore, seemed to be associated with pain.

The timeline of the in-freestall behavioral responses in the barn are in line with previous findings. The LPS cows decreased their feeding and ruminating activity in the first 9 h following 25 μg of *E. coli* LPS challenge in the udder, as previously reported (Zimov et al., 2011). Tail-pressing at 5 h postinfusion also corresponds to previous findings after percutaneous needle liver biopsy (i.e., at 4.5 h; Mølgaard et al., 2012). However, this change in tail posture manifested earlier here than after infusion with 1 mL of a bacterial suspension (1,000 cfu/mL) of *E. coli* in the udder, where tail-pressing characterized the phase of 12- to 24-h postinfusion phase (de Boyer des Roches et al., 2017). This could be explained by the LPS challenge used here. Lack of responsiveness and lowered head at 5 h postinfusion are partially consistent with the timeline observed after *E. coli* infusion in the udder, where this behavior was observed from 4 h postinfusion up to 24 h postinfusion (Fogsgaard et al., 2012; de Boyer des Roches et al., 2017).

Reactivity in LPS cows during udder preparation for milking increased during forestripping in the first milking after LPS challenge (i.e., at 8 h postinfusion), corresponding to the acute phase of the pathophysiological process, but not after nor in other stages of the milking process. Cows can show reactivity at milking due to several reasons, including fear of humans (Breuer et al., 2000; Rousing et al., 2006). Udder preparation for milking is known to induce more stepping/lifting/kicking responses than milking itself (Rushen et al., 1999), but to our knowledge, the cows' reactions at each phase (teat cleaning, forestripping, milking claw positioning) has never been described. Here, the increase in cows' reactions to forestripping between pre- and post-LPS challenge suggests that they experienced increased Se, potentially corresponding to allodynia while the milker pinched the teats. Several authors have reported that udder inflammation led to more stepping or kicking or both responses at milking than in healthy cows or than before the cows were infected. This was reported 6 h after infusion of 25 μg of *E. coli* LPS in the udder (Fitzpatrick et al., 2013) and for several days in cows with naturally occurring mastitis milked automatically (Fogsgaard et al., 2015) or in a parlor (Medrano-Galarza et al., 2012). Although cows in our study were more reactive after the LPS infusion than before, the absence of kicking response after challenge suggests that the cows experienced less pain here than in naturally occurring mastitis (Medrano-Galarza et al., 2012; Fogsgaard et al., 2015) or because they were more sick and, therefore, less responsive (Rasmussen et al., 2011).

Among the behavioral changes observed in the freestall or at the milking parlor after LPS infusion in the udder, only 3 were counteracted by the antipyretic or analgesic or both effects of ketoprofen. In the freestall, cows receiving ketoprofen kept feeding or ruminating and had normal ear posture at 5 h postinfusion and more of them were lying down at 24 h postinfusion. Similar findings were observed in cows receiving flunixin meglumine after 25 μg of *E. coli* LPS challenge in the udder (Zimov et al., 2011), which spent more time eating at 9 to 12 h postinfusion compared with cows with placebo. The 4-h delay to NSAID effect observed in Zimov et al. (2011) may be explained by the fact that flunixin meglumine was administered 4 h after versus immediately after the LPS infusion here. There were more LPS+NSAID cows lying down at 24 h postinfusion compared with LPS cows. Because lying is very important for cow welfare (European Food Safety Authority, 2009; Tucker et al., 2021), this result suggests that ketoprofen decreased discomfort in dairy cows with udder inflammation. To our knowledge, this is the first study to demonstrate an effect of NSAID on lying behavior in cows at 24 h postinfusion; for example, Zimov et al. (2011) only recorded lying behavior up to 12 h postinfusion, and Fitzpatrick et al. (2013) did not record lying behavior. Administration of NSAID did not significantly counteract the increase of reactivity during udder preparation for milking, whereas Fitzpatrick et al. (2013) found NSAID (meloxicam) effects. This result could be explained by our small sample size. Indeed, at 8 h postinfusion, the majority (11/13) of LPS+NSAID cows did not lift their hoof at forestripping, whereas 6/14 LPS cows did hoof-lift. In the present study, we calculated the sample size based on serum cortisol data from Zimov et al. (2011) study because, unfortunately, knowledge was missing about expected differences regarding cow's behavior in freestall barns and at milking. Another hypothesis is intramuscular injection of ketoprofen could not alleviate local pain,

contrary to meloxicam (Fitzpatrick et al., 2013). Therefore, ketoprofen improved cows' general state assessed through lying and eating behavior, probably through the combination of its antipyretic and analgesic effects. However, further studies are necessary to explore its analgesic effects on behavioral signs of pain at milking.

Taken together, the changes in behaviors suggest that pain was present in the acute phase (i.e., 3 h postinfusion to 8 h postinfusion) of the response following infusion of LPS in the udder, as already described in cows with naturally occurring mastitis (Fogsgaard et al., 2015), after an *E. coli* mastitis challenge (Fogsgaard et al., 2012; de Boyer des Roches et al., 2017), or a 25 μg of *E. coli* LPS challenge (Zimov et al., 2011; Fitzpatrick et al., 2013). However, the behavioral changes observed in freestalls and during udder preparation for milking (e.g., no kicking) here were less marked than in other situations such as naturally occurring mastitis (Medrano-Galarza et al., 2012; Fogsgaard et al., 2015) or mammary challenge with entire bacteria (Fogsgaard et al., 2012). As the magnitude of response gives some indication of the pain level experienced by animals (Rutherford, 2002), and as we did not record severe pain behaviors, we might suggest the level of pain experienced by cows here would seem to be moderate. In the present study, the behavioral changes observed in cows with LPS-induced mastitis suggest that the 3 dimensions of pain (sensory, affective, cognitive; Mogil, 2009; Moriarty et al., 2011; Sneddon et al., 2014) could have been affected. Indeed, after the challenge, cows changed their postures and reacted more during udder preparation for milking. These behavioral changes reflect the sensory dimension of pain, as previously described in various species (Mogil, 2009; Moriarty et al., 2011; Low, 2013; Sneddon et al., 2014). In addition, our study may also have allowed us to explore the 2 other components of pain (i.e., affective and cognitive). First, our results suggest that LPS cows' changes in activities after the challenge may reflect the affective dimension of pain; for example, the cows might have reoriented their activity (e.g., feeding) due to pain, as already described in rodents (Mogil, 2009). Second, the reduction of responsiveness observed in cows during acute udder inflammation may reflect the cognitive dimension of pain, as already described in humans in various pain contexts (Moriarty et al., 2011) or in cows with mastitis (de Boyer des Roches et al., 2018). Taken together, these preliminary results suggest that the intramammary challenge affected the 3 dimensions of pain in dairy cows. Further studies using other devices and more extensive affective and cognitive behavioral tests are nevertheless necessary to confirm our hypotheses.

Behavioral signs of sickness and pain may overlap (Weary et al., 2006, 2009). Here, we were not totally able to distinguish indicators of the cows' "feeling sick" or under "malaise" state with indicators of the cows' "being in pain." For example, the following responses described here corresponded to those described in cows with infectious mastitis [either experimentally induced (de Boyer des Roches et al., 2017) or naturally occurring (Fogsgaard et al., 2015)]: hyperthermia, lack of responsiveness (de Boyer des Roches et al., 2017), and decrease of feeding (Fogsgaard et al., 2015). Other reactions overlapped less, such as the reaction to palpation when the milker touches or manipulates the cow's udder at forestripping, which may indicate hyperalgesia or maybe even allodynia (Medrano-Galarza et al., 2012; Fogsgaard et al., 2015). Therefore, the present study allowed us to identify pain for some indicators. However, there was a risk of overlapping between "being in pain" and "feeling sick" indicators for the behavioral reactions detected in the barn. In addition, these behaviors may not be specific to pain in general, and when seeing in a nonexperimental herd as part of daily management, it may indicate other states (e.g., primiparous cows being more reactive to milking that multiparous ones).

Sp and Se of Behavioral Changes Associated with LPS-Induced Mastitis

This study identified the Sp and Se of behavioral changes associated with pain due to LPS-induced mastitis, because the behaviors displayed by cows have been previously characterized as behavioral indicators of pain in cattle (see above; Weary et al., 2006; Prunier et al., 2013). We chose to only calculate Se between 3 and 8 h postinfusion which corresponded to the moment when mastitis was the most severe. We hypothesized that during this time period, cows were certainly in pain. Conversely, we calculated Sp using observations before the LPS infusion to be sure that the cows were healthy and not in pain.

During udder preparation for milking, "hoof to belly" and "kicking" had a Sp between 64% and 100%, whatever the phase. This is in line with previous observations showing that cows with naturally occurring mastitis milked automatically (Fogsgaard et al., 2015) or in a milking parlor (Medrano-Galarza et al., 2012) display kicking, especially in the first stages of mastitis, and less after. However, these 2 behaviors ("hoof to belly" and "kicking") had a Se below 36% in our study. This could be due to a lower level of pain experienced by cows after the LPS challenge compared with under naturally occurring mastitis (Medrano-Galarza et al., 2012; Fogsgaard et al., 2015) or because they were sicker and, therefore, less responsive (Rasmussen et al.,

2011). Hoof-lifting behavior at forestripping also had a Sp of 100% and a Se of 43%. These results are concordant to the only study that monitored hoof-lifting in cows with naturally occurring mastitis milked in a parlor (Medrano-Galarza et al., 2012), which also found that mastitic cows lifted their hoof more frequently in the first 2 d of mastitis than after recovery, but did not find any difference between mastitic and healthy cows.

The following 9 in-freestall behavioral indicators had a Sp above 75% but a Se below 60% at all times of the day (1130, 1330, 1630 h): "lack of responsiveness," "unsteady balance," "stretching forelegs" while standing, "foot position on different floors," "arched back," "pressed tail," "tail whipping," "ears down," "no head and ear movement," and "social isolation." Therefore, if a cow displays 1 of these 9 behaviors, then it is confirmed as "with udder inflammation and in pain." However, in commercial farms, a large proportion of cows that are experiencing udder inflammation and pain will not display these behaviors and, therefore, will not be detected as "with udder inflammation and in pain." In this case, the cows that are "with udder inflammation and in pain" but not detected will be missed and therefore fail to get drug management; however, all cows detected "with udder inflammation and in pain" will benefit from drug management. One solution to detect more cows "with udder inflammation and in pain" could be to repeat behavioral observations of these highly specific behaviors.

We also identified some behaviors with Sp below 60% and Se above 64%. During udder preparation for milking, step and hoof-lifting had the highest Se (64%) and Sp of 57% but only during teat cleaning. Indeed, cows are known to display such behaviors without udder inflammation and associated pain (Rushen et al., 1999; Breuer et al., 2000; Rousing et al., 2006). The following 3 in-freestall behaviors had Se between 57% and 100% and Sp between 7% and 64%: inoculated quarter not compressed, head down, no social contact. Most cows "with udder inflammation and in pain" will display 1 of these behaviors, and thus get detected as "with udder inflammation and pain." However, many cows without udder inflammation will also display these behaviors. In this case, all the cows truly "with udder inflammation and in pain" will benefit from drug management, but some of the cows falsely detected as being "with udder inflammation and in pain" will also get drug management. These sensitive behaviors are, therefore, good warning indicators but warrant confirmation by further examination or additional behavioral observations of the cows.

Finally, 1 behavior (no feeding/ruminating) had a Sp of 86% and a Se of 71% for a specific time point (5 h postinfusion). This result is consistent with already known changes in feeding/ruminating activity in cows after infusion of LPS (Zimov et al., 2011; Fitzpatrick et al., 2013) or bacteria (Fogsgaard et al., 2012) in the udder. Therefore, to detect udder inflammation and associated pain, this indicator should only be used a few hours after feed distribution and during a quiet moment without stockmen in the barn. This moment corresponds to a period where cows normally eat or ruminate (Arnold, 1984; Veissier et al., 1989). Therefore, udder inflammation and pain should be considered in the case of absence of feeding and ruminating activity detected at a time when cows would normally typically be eating or ruminating given its good Se and Sp.

CONCLUSIONS

The pain associated with the challenge was concomitant with inflammatory, ANS, and HPA responses, and was detected via point-in-time observations of 2 behavioral responses ("absence of feeding/ruminating" in the freestall, "hoof-lifting" during udder preparation for milking) from 3 to 8 h after the challenge. The Se and Sp of the behavioral indicators suggest that trained professionals could identify cows based on "kick" and "lack of responsiveness" behaviors but would probably still miss some mastitic cows. Their identification could probably be improved by including observations of "hoof-lifting" or "absence of feeding/ruminating," or by repeating behavioral observations of these highly specific behaviors. Further studies are needed to examine Se and Sp of combined indicators as well as to examine whether these behavioral changes could also indicate pain from other clinical conditions typically encountered in a dairy cow herd.

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