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Changes in social and feeding behaviors, activity, and salivary serum amyloid A in cows with subclinical mastitis

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ABSTRACT

The aim of this study was to identify detailed changes in behavior, and in salivary serum amyloid A (SAA), associated with subclinical mastitis. This included standard sickness behaviors, such as decreased activity, feeding and drinking (here labeled "core maintenance" behaviors), and less well-studied social, grooming, and exploratory behaviors (here labeled "luxury" behaviors). Luxury behaviors are biologically predicted to change at lower levels of mastitis infection and are, therefore, particularly relevant to detecting subclinical mastitis. Salivary serum amyloid A is a physiological marker of systemic inflammation, with levels in milk and serum already known to increase during subclinical mastitis. We investigated whether the same was true for SAA in cow saliva. Data were collected for 17 matched pairs of commercial barn-housed Holstein-Friesian cows. Each pair comprised a cow with subclinical mastitis (SCM) and a healthy control (CTRL), identified using somatic cell count (SCC; SCM: SCC >200 × 1,000 cells/mL; CTRL: SCC <100 × 1,000 cells/mL). SCM cows were selected for study ad hoc, at which point they were paired with a CTRL cow, based upon parity and calving date; consequently, the full data set was accrued over several months. Data were collected for each pair over 3 d: SCC (d 1), behavior (d 2), salivary SAA (d 3). All behaviors performed by the focal cows over a single 24-h period were coded retrospectively from video footage, and differences between the SCM and CTRL groups were investigated using the main data set and a subset of data corresponding to the hour immediately following morning food delivery. Saliva was collected using cotton swabs and analyzed for SAA using commercially available ELISA kits. We report, for the first time, that an increase in salivary SAA occurs during subclinical mastitis; SAA was higher in SCM cows and demonstrated a positive (weak) correlation with SCC. The behavioral comparisons revealed that SCM cows displayed reductions in activity (behavioral transitions and distance moved), social exploration, social reactivity (here: likelihood to be displaced following receipt of agonism), performance of social grooming and head butts, and the receipt of agonistic noncontact challenges. In addition, SCM cows received more head swipes, and spent a greater proportion of time lying with their head on their flank than CTRL cows. The SCM cows also displayed an altered feeding pattern; they spent a greater proportion of feeding time in direct contact with 2 conspecifics, and a lower proportion of feeding time at self-locking feed barriers, than CTRL cows. Behavioral measures were found to correlate, albeit loosely, with serum SAA in a direction consistent with predictions for sickness behavior. These included positive correlations with lying duration and the receipt of all agonistic behavior, and negative correlations with feeding, drinking, the performance of all social and all agonistic behavior, and social reactivity. We conclude that changes in salivary SAA, social behavior, and activity offer potential in the detection of subclinical mastitis and recommend further investigation to substantiate and refine our findings.

Key words: cows, salivary serum amyloid A, sickness, behavior, subclinical mastitis

INTRODUCTION

Clinical stages of infectious disease are typically easily identified by obvious physical symptoms and behavioral changes (so-called sickness behaviors; Hart, 1988). Subclinical infection, that is infection below the level of clinical detection, by definition, is more difficult to identify. However, based on the interactions between the immune and central nervous system that cause sickness behavior, predictions can also be made about behavioral changes during subclinical infection (Dantzer, 2004). These behavioral changes can be used as early warning signs of disease (Weary et al., 2009; von Keyserlingk et al., 2010) or to identify chronic subclinical infection levels. Mastitis remains a major concern in dairy cows with serious negative effects on welfare and productivity (Petersson-Wolfe et al., 2018). At subclinical levels,

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inflammation is present in response to the infection and milk production drops, but no abnormalities in the gland or milk are visible (Sordillo et al., 1997). It is therefore important for infection at any level to be identified and treated as soon as possible. Our main aim was to identify such changes in cows based on a detailed behavioral comparison between individuals with spontaneously occurring subclinical mastitis and healthy controls.

Behaviors in healthy animals can be divided, on the basis of immediate survival benefits, into "core maintenance" and "luxury" (e.g., Dawkins, 1990). Core behaviors (e.g., resting, feeding, and drinking) have immediate, short-term survival benefits. They, therefore, start to decline only during later disease (e.g., Littin et al., 2008) and have low sensitivity during early and low-level stages (e.g., Sepúlveda-Varas et al., 2014). In our comparison, we included standard core sickness behaviors, such as changes to feeding, previously shown to be associated with experimentally induced and spontaneously occurring clinical mastitis (Siivonen et al., 2011; Fogsgaard et al., 2012; Sepúlveda-Varas et al., 2016), in addition to so-called luxury behaviors, such as physical and social exploration, grooming, and various social interactions. These luxury behaviors have delayed longer-term benefits and are not essential for immediate survival, meaning that they are biologically predicted to change at lower levels of disease than other sickness behaviors due to a greater sensitivity to disease challenge (Littin et al., 2008; Weary et al., 2009), especially when energy resources are diverted to fighting infection (see also "low-resilience behaviors" in Littin et al., 2008).

In cows, effective health monitoring is hampered by logistical difficulties associated with direct animal observations within large open barns, reductions in human interaction linked with the substantial uptake in robotic milking units, and the tendency of cattle (for several biological reasons) to display only subtle indicators of pain or weakness (Gleerup et al., 2015). Advances in image analysis now allow automated recognition of individuals within a herd (Andrew et al., 2020), and accurate identification of health-related abnormal behaviors, such as foot disease (Gu et al., 2017). The identification of behaviors associated with subclinical disease (before the development of, or without, clinical symptoms) may therefore find application in future diagnostic software algorithms targeted at early disease monitoring in dairy cows (e.g., Wagner et al., 2020).

A second aim was to quantify levels of salivary serum amyloid A (**SAA**), a major acute phase protein in cows (Murata et al., 2004), in individuals with and without subclinical mastitis. Acute phase proteins are nonspecific inflammatory markers that fluctuate in response to infection. Increased SAA levels have been detected during subclinical mastitis within both serum and milk (Kováč et al., 2011; Kovačević-Filipović et al., 2012). Other studies have confirmed SAA presence in bovine saliva (Lecchi et al., 2012; Rahman et al., 2013), suggesting salivary SAA has the potential for use in noninvasive detection of infectious diseases, such as mastitis.

Raised systemic inflammation levels, whether during acute infection or as chronic inflammatory states, are predicted to be accompanied by a feeling of sickness or malaise in animals, as they are in humans (Dantzer et al., 2008; Weary et al., 2009). de Boyer des Roches et al. (2017) reported correlations between (serum) SAA and several pain indicators before and after experimentally induced intramammary challenge with *Escherichia coli*, including behavioral measures of attentiveness to surroundings. This suggested a direct link between serum SAA, infection levels and some sickness behavior in mastitic cows. A noninvasive means of monitoring systemic inflammation would also benefit future investigations into the effect of spontaneously occurring infections, such as mastitis, on cow welfare. In our study we tested for associations between salivary SAA levels and SCC (a standard measure of mastitis severity), and for associations between these physiological measures and behavior.

In summary, the purpose of this study was to identify differences in behavior and salivary SAA associated with spontaneous subclinical mastitis. To this end, we (1) compared the behavior and salivary SAA of cows with subclinical mastitis with that of matched healthy individuals, and (2) correlated behavioral variables with both SCC and salivary SAA. We predicted that salivary SAA would be higher in cows with subclinical mastitis than in matched healthy controls. We also predicted that luxury behaviors, here including a range of social behaviors, would decrease with subclinical mastitis, and that differences detected would correlate negatively with the physiological measures (SCC and salivary SAA).

MATERIALS AND METHODS

Ethics Statement

The study was conducted between October 2017 and February 2018 at the Bristol Veterinary School dairy farm. The experimental procedures were approved by the Animal Welfare and Ethical Review Board at the University of Bristol and conducted under University Investigation Number UB/17/061 "Behavioral markers of subclinical disease in dairy cows."

Animals

Focal cows (n = 34) were part of an indoor-housed commercial Holstein-Friesian dairy herd (n = 200) and resided within the low milk-yield group (approximately n = 80 cows) at the time of the study; having been part of the group for at least one month before data collection they were well-established within the social dominance hierarchy. Low-yield animals were studied as they were deemed to be of low risk for having, or developing, subclinical metritis or ketosis during the trial. Cows were housed within a freestall barn containing 93 lying cubicles (1.2 × 2.4 m) with sand bedding, 3 stainless steel tip-over drinking troughs, an automated swinging brush (DeLaval), and automatic floor scrapers. Figure 1 shows the layout and relative positioning of resources within the pen. Cows were milked 3 times daily (at 0600, 1400, and 2200 h) and fed a TMR once daily (0600 h).

Only clinically healthy nonlame cows (mobility score ≤1; AHDB, 2015) without physical symptoms of mastitis were selected for inclusion in the study. Clinical health was assessed using individual health records (to screen out individuals positive for Johne's disease or *Neospora*) and visual inspection of each cow (for skin lesions and lameness). All clinical health checks were performed by the lead author, in consultation with the herdsman, on the first day of data collection (d 1). Data were collected concurrently from cows in matched pairs, each comprising a cow with subclinical mastitis (**SCM**) and a healthy control (**CTRL**). Matching was performed on the basis of the following potential confounds: parity, pregnancy status (yes or no) and stage of pregnancy (days since insemination). Cows with a SCC >200 (×1,000 cells/mL) were classified as SCM (Madouasse et al., 2010), whereas cows with a SCC of <100 (×1,000 cells/mL) were classified as CTRL. The 34 focal cows comprised individuals that were pregnant (n = 24) and nonpregnant (n = 10), and individuals that were primiparous (SCM: n = 10; CTRL: n = 10) and multiparous. Multiparous cows included those that had calved twice (SCM: n = 1; CTRL: n = 1), 3 times (SCM: n = 1; CTRL = 1), 4 times (SCM: n = 2; CTRL: n = 3) and 5 times (SCM: n = 3; CTRL: n = 2). The time to expected calving date ranged from 55 to 236 d. Although data from the 2 cows in a matched pair were collected at the same time, the data for 17 pairs took several months to collect due to low mastitis incidence within the herd at the time of the study.

Somatic Cell Count

Composite quarter milk samples were collected between 1400 and 1500 h on d 1. Although pairs of cows had data collected at different dates, for the purpose of our experimental design, all cows had SCC data collected on d 1 and behavioral data on d 2. Day 1 of data collection was retrospectively assigned to cows based upon an individual meeting the criteria for SCM, and a CTRL cow was matched on the same day. Somatic cells were manually counted using a standard direct microscopic methodology (ISO, 2008) following staining with Newman-Lampert stain solution: Levowitz-Weber modification (Newman's Stain Solution: modified, 01375, Sigma-Aldrich).

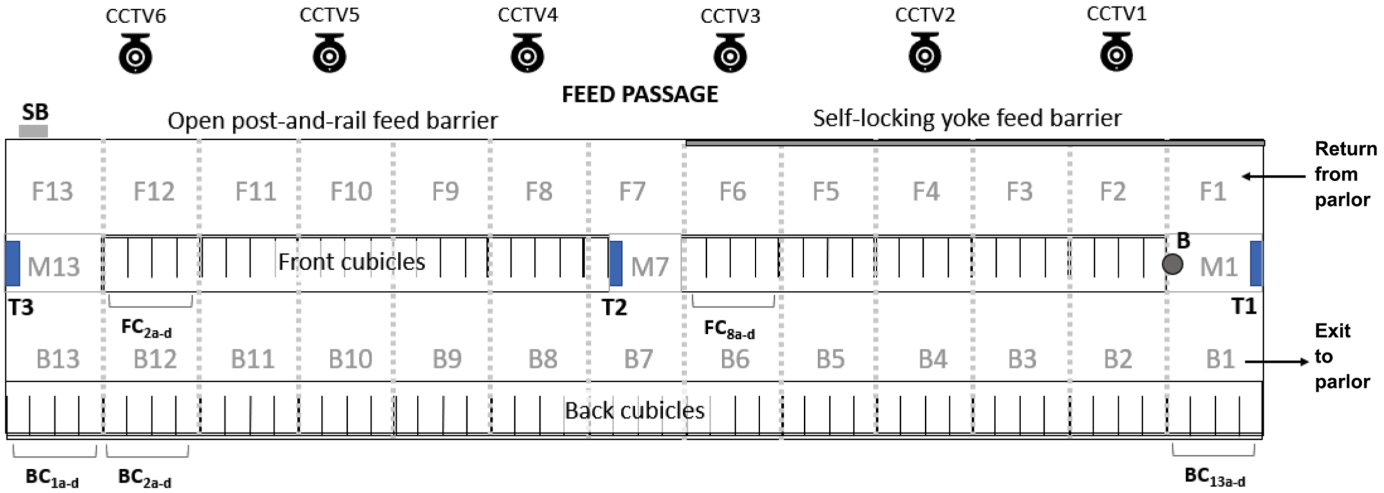

Figure 1. Plan of the home pen including closed-circuit television (CCTV) camera position and virtual division of floor space (F1–13, M1/7/13, B1–13) for logging cow position. SB = salt bin; T1–3 = water troughs; B = automatic rotating brush; FC = front cubicle; BC = back cubicle.

Behavioral Measures

Each focal cow was fitted with a colored collar to facilitate individual recognition on d 1. Two CCTV systems (N441L1T, Annke), including 6 cameras, recorded video footage from the entire low-yield pen. Continuous behavioral data were then coded retrospectively from video for each focal cow for 24 h starting from 00:00:01 h of d 2 by a single experienced coder who was unaware of the health status of the cows at the time of scoring; these data made up the 24-h data set. A subset of the behavioral data was also compiled for each cow using video recordings of the first 60 min following morning milking (**1hPostM1**) on d 2. This hour was chosen to coincide with the peak feeding time of the day (and, consequently, a period of predicted high social interactivity at the feed barrier) because fresh feed was delivered while the cows were in the parlor at morning milking. The start time of the 60-min observation period was specific to each focal cow, starting immediately following that cow's re-entrance into the home pen from the parlor exit.

All behavioral measures are described in Table 1. Three broad categories of behavior were of interest: social luxury behaviors, nonsocial luxury behaviors (such as self-grooming and exploring the physical environment), and core maintenance (including lying, feeding or drinking, and activity). The first category was the most detailed, comprising nonagonistic (e.g., allogrooming, social exploration) and agonistic (e.g., head swipes, head butts) behaviors, in addition to social reactivity (here defined as the likelihood of moving away or being displaced following the receipt of an agonistic behavior).

Total behavioral transitions, a measure of activity, was calculated using all behaviors, including those coded, but not analyzed individually (i.e., not listed in Table 1). These additional nonfocal behaviors included eat sand, lick salt, paw sand, run, shake head, stand, and walk. Proximity was investigated using nearest neighbor scores. When the focal cow was located at the feed barrier or resting in a cubicle, the number of other cows in immediate proximity were scored as 0, 1, or 2 near neighbors (Table 1). To enable an estimation of distance moved, the pen floorspace was hypothetically subdivided into 29 units (each 4.8 m wide; see Figure 1) and the location of each cow was noted every 5 min throughout the 24-h (and 1hPostM1) period. The number of floor units crossed was then used to estimate distance moved.

Data were not available for 0600, 1400, or 2200 h as the cows were in the milking parlor or collecting yard during these periods. To account for differences in total time visible (i.e., due to variations in time spent within the parlor) data in the 24-h data sets were standardized to either number per hour visible (behavioral events) or seconds per hour visible (behavioral states; e.g., to standardize data from a cow that was visible for 20 h, 42 min, and 12 s within a 24-h period, a division by 20.703 would be required).

Saliva Collection and SAA

Saliva was collected using a cotton swab (SalivaBio Children's Swab, Item No. 5001.06, Salimetrics) and then immediately stored at −80°C before analysis. This was performed on d 3 so as to prevent any potentially confounding effects of the saliva collection procedure on behavior (recorded on d 2). The SAA was measured in saliva from 31 cows (saliva volumes from 3 cows were too small to be analyzed), diluted 1:2, using a commercially available kit (Bovine Serum amyloid A protein ELISA Kit, EB0015, Finetest, Wuhan Fine Biotech Co. Ltd.). To assess the suitability of the kit for use with saliva an assay validation was performed. To determine parallelism (linearity) a displacement curve, produced by double-diluting a pooled saliva sample with assay buffer, was compared with a standard curve. Percentage binding (as a percentage of that recorded for the zero standard) was calculated, in addition to the log of the standard concentration (SAA standard) and the log of the inverse of the dilution factor (saliva sample), e.g., 1:4 was transformed to log(1/4). Parallelism was confirmed using a statistical test for the analysis of covariance (ANCOVA, SPSS Inc.). To measure assay accuracy the percentage recovery of exogenous SAA was calculated following the addition of 300 ng/mL SAA standard to a pooled saliva sample. Precision was assessed via intra- and interassay CV; the former was determined following the repeated measurement of aliquots of pooled saliva containing either high (quality control: $\mathbf{QC_{high}}$) or low ($\mathbf{QC_{low}}$) endogenous SAA within the same plate, whereas the latter was determined following the assay of QC_{high} and QC_{low} samples in different plates.

Statistics

Following tests for normality (Shapiro-Wilk analysis), all behavioral measures, SAA and SCC were compared between CTRL and SCM using paired samples t-test or Wilcoxon signed-rank tests (SPSS Statistics 24.0). Behavioral data from the continuous 24-h data set and 1hPostM1 data subset were separately analyzed. Because the experimental design required the performance of multiple comparisons between measures there was an increased associated risk of Type I errors. Use of Bonferroni correction procedures has been highlighted

Table 1. Cow behavioral measures used in the study

Measure (unit)	Definition
(1) Social (luxury) behavior	
(a) Agonistic interactions and tendency of being displaced (social reactivity)	
Body push: given[1] (n)	Performing a sideways shunt of the flank to make (often forceful) contact with the head or flank of a recipient; frequently results in displacement (e.g., when accessing a crowded feed barrier).
Body push: received[1] (n)	Receipt of a body push from another cow.
Body push: displacement[2] (%)	The percentage of all body pushes received by the focal cow that immediately (≤ 2 s) result in the focal cow being displaced.
Noncontact challenge: given[1] (n), received[1] (n), displacement[2] (%)	A threatening gesture aimed at displacing a conspecific (e.g., short-charging, determinedly approaching, or facing/staring at a conspecific with head lowered). Challenges can be given or received, and a percentage of those received will result in displacement.
Head butt: given[1] (n), received[1] (n), displacement[2] (%)	Using the front of the lowered head to strike the head/body of another cow with force. Often observed at the feed barrier as a single event where the rear or flank of a standing conspecific is targeted, or at the cubicles where lying conspecifics can be butted multiple times in quick succession. Head butts can be given or received, and a percentage of those received will result in displacement.
Head push: given[1] (n), received[1] (n), displacement[2] (%)	The use of prolonged/sustained contact of the forehead (occasionally the lateral aspect of the head) applied to a conspecifics head or body. Head pushes can be given or received, and a percentage of those received will result in displacement.
Head swipe: given[1] (n), received[1] (n), displacement[2] (%)	Quick sideways swipe of the head, whereby the lateral aspect of the head is used to make violent contact with the head of a conspecific. Usually observed at the feed barrier. May be performed singularly or multiple times in quick succession. Head swipes can be given or received, and a percentage of those received will result in displacement.
Mutual head butt[1] (n)	Mutual head-to-head butting between the focal cow and conspecific. Usually observed in prolonged bouts whereby the 2 cows stand head-down facing each other with or without sustained forehead contact between each butt.
Total agonistic: given[1] (n)	Body push: give; noncontact challenge: give; head butt: give; head push: give; head swipe: give.
Total agonistic: received[1] (n)	Body push: receive; noncontact challenge: receive; head butt: receive; head push: receive; head swipe: receive.
Total agonistic: displacement[2] (%)	The percentage of all agonistic interactions received by the focal cow that immediately (≤ 2 s) result in the focal cow being displaced. Here, percentage displacement following the receipt of agonistic behavior is equated with social reactivity.
(b) Nonagonistic interactions	
Allogroom give[1] (n,s)	Licking any body part of a conspecific (most frequently the head or main torso). Usually observed in prolonged bouts.
Allogroom receive[1] (n,s)	Receipt of licks from a conspecific. During licking the recipient usually stops all other activity and stands still.
Explore social[1] (s)	Explore cow (nose positioned close to another cow in the act of sniffing – no reciprocation); mutual sniff (reciprocal sniffing between a focal cow and conspecific, usually stood facing one-another with noses almost touching).
Mutual head rub[1] (n,s)	Mutual head-to-head rubbing performed by a focal cow and a conspecific; usually observed in bouts.
(c) Total social interactions	
Total social: given[1] (n)	Total agonistic: given; allogroom give; chin rest give (use of chin to exert pressure on the lateral posterior of a conspecific); head rub give (rubbing head on conspecific without reciprocation); mount give (bulling behavior where focal cow stands on back legs and rests her chest on the back/rump of a conspecific); mutual head butt; mutual head rub.
Total social: received[a] (n)	Total agonistic: received; allogroom receive; chin rest receive; head rub receive; mount receive; mutual head butt; Mutual head rub.
(2) Social proximity and feed barrier preference	
Feed barrier: no neighbors[2] (%)	Percentage of the total time spent with the head positioned over the feed barrier (open-rail or self-locking) during which the focal cow has no direct flank-to-flank contact with other cows.
Feed barrier: 2 neighbors[2] (%)	Percentage of the total time spent with the head positioned over the feed barrier (open-rail or self-locking) during which the focal cow has direct flank-to-flank contact with 2 other cows (one either side).
Lie: no neighbors[2] (%)	Percentage of the total time spent lying within a cubicle during which the focal cow is flanked by 2 unoccupied cubicles.
Lie: 2 neighbors[2] (%)	Percentage of the total time spent lying within a cubicle during which the focal cow is flanked by 2 occupied cubicles.
Feed barrier: open[2] (%)	Percentage of the total time spent with the head positioned over the feed barrier during which the focal cow was positioned at the open-rail section.
(3) Nonsocial (luxury) behavior	
Brush use[1] (s)	Proactive contact made between any body part and the mechanical brush.
Self-groom[1] (s)	Lick self; rub self (rub own body part against pen furniture); scratch self (using own foot to scratch own body part).

Continued

Table 1 (Continued). Cow behavioral measures used in the study

Measure (unit)	Definition
Explore environment[1] (s)	Explore food (holding nose close to food while sniffing, or moving or flicking nose around within food, without any obvious active ingestion); explore pen (licking or holding nose very close to any part of the barn structure or pen furniture); explore sand (holding nose close to sand bedding or flicking sand with nose).
(4) Maintenance (core) behavior	
Drink[1] (s)	Standing with muzzle placed within a water trough.
Feed[1] (s)	Ingesting or chewing food while standing with head traversing the feed barrier.
Lie[1] (s)	Horizontal recumbent resting position with abdomen in contact with the floor.
Head on flank[2] (s)	Lying with the head held in contact with the flank, pointing backward toward the rump. Associated with active sleep.
Activity: Transitions[1] (n)	Total number of behavioral changes performed during the observation period.
Activity: Distance moved[1] (units)	Number of units of floor space crossed during the observation period.

[1] Inclusion in 24-h data set and data subset of the first 60 min following morning milking on d 2.

[2] Inclusion in 24-h data set only.

as problematic (especially for animal behavioral studies, where sample sizes are often small) due to their tendency to increase Type II errors (Nakagawa, 2004). As an alternative to standard correction procedures we, therefore, calculated measures of observed (standardized) effect size in addition to *P*-values. Effect size measures the strength or magnitude of a relationship and, thereby, helps us to determine the strength of a statistical claim and whether a difference is real (i.e., it enables us to judge biological importance). Hedges' g-value (Equations 1 and 2), also termed Cohen's d-value for paired samples (Hedges, 1981; Cohen, 1988; Nakagawa and Cuthill, 2007), and 95% confidence intervals (**CI**) for effect size (Equations 3 and 4), were calculated for all measures that met the assumptions of normality:

$$g = \frac{\bar{x}_2 - \bar{x}_1}{\sigma_{\text{paired}}}, \quad [1]$$

where

$$\sigma_{\text{paired}} = \sqrt{\frac{(n_2 - 1)s_2^2 + (n_1 - 1)s_1^2}{n_1 + n_2 - 2}}. \quad [2]$$

$\bar{x}_1$ and $\bar{x}_2$ are the means of the 2 groups, σ_{paired} is the pooled standard deviation, n is the number of data points, and s^2 is the sample variance;

$$95\%\ \text{CI} = g - 1.96se_g \text{ to } g + 1.96se_g, \quad [3]$$

where

$$se_g = \sqrt{\frac{2(1 - r_{1,2})}{n} + \frac{g^2}{2(n-1)}}\ . \quad [4]$$

Here, se_g is the asymptotic standard error for the effect size, $n = n_1 = n_2$, and $r_{1,2}$ is the correlation coefficient between the 2 groups. For all behavioral measures that did not meet the assumptions of normality, bootstrap effect size values (Hedges' g-value with 95% CI, replicates = 2,000) were computed using the software package bootES (Gerlanc and Kirby, 2012; Kirby and Gerlanc, 2013) and R (version 3.2.2., www.r-project.org/). Effect size statistics were interpreted as follows: (1) the size of the effect (based upon the estimated g-values: ≤0.39 = small, 0.40–0.79 = medium, ≥0.80 = large); and (2) statistical significance (attributed to all measures where the associated 95% CI did not contain 0; Lee, 2016).

Interpretation of statistically nonsignificant *P*-values is possible using effect size confidence intervals in combination with the effect size (see Nakagawa and Foster, 2004). To identify those measures in the continuous 24-h data set that failed to reach statistical significance (between the SCM and CTRL cows), but could yet be biologically important, we used information from published studies to set accepted relative difference levels (**RDL%**, Table 2). Although these studies are not specific to subclinical mastitis, and include the effect of clinical (nonmastitic) infection and social status on behavior, it is assumed that they provide generous and relevant difference levels with which to compare our subclinical findings.

For those measures where no relevant literature was available (Other; Table 2), the average RDL was used (i.e., 20%; Table 2). For each measure, relative difference values (**RDV**) were calculated using the RDL% and the respective mean value from the CTRL group. The 95% CI_{RDV} were calculated using the confidence intervals from the effect size statistics and the (between-group) difference in means. In those cases where the 95% CI_{RDV} did not include the RDV, we conclude (with

Table 2. Summary of the relative difference levels (RDL%) and their sources, used in this study to ascertain the existence of a biologically meaningful difference in behavioral measures between cows, with and without subclinical mastitis, based on effect size

Behavioral measure	RDL%	Reference
Agonistic given: all categories	10	Sepúlveda-Varas et al., 2016
Agonistic received: all categories	10	Neave et al., 2018
Allogroom give	30	Galindo and Broom, 2002
Allogroom receive	80	Galindo and Broom, 2002; Hoonhout et al., 2017
Feed barrier: no neighbors	20	Manson and Appleby, 1990
Feed barrier: 2 neighbors		
Feed barrier: open	10	Huzzey et al., 2006
Brush use	30	Mandel et al., 2017
Self-groom	20	Fogsgaard et al., 2012
Activity: transitions, distance	10	Steensels et al., 2017; King et al., 2018
Feed	10	Dollinger and Kaufmann, 2013
Drink	20	Huzzey et al., 2007
Lie	10	Toaff Rosenstein et al., 2016
Other	20	

95% confidence) that the current study showed no important biological effect for that measure; we refer to these as biologically unimportant. In cases where the 95% CI_{RDV} did include the RDV, we conclude that a difference was inconclusive but plausible; we refer to these as biologically inconclusive. For example, if CTRL cows performed more body pushes than the SCM cows, yet this difference failed to reach statistical significance ($P \geq 0.10$), using the P-value alone we would dismiss this behavior as being unaffected by subclinical inflammation. However, if the corresponding RDV was within the 95% CI_{RDV} range (e.g., RDV = 0.08, 95% CI_{RDV} = −0.08 to 0.27) we would conclude that the effect is biologically inconclusive based upon our evidence (i.e., the difference may become significant given a larger sample size). Alternatively, if the RDV, in the above example, was 0.3, then we would conclude that the effect was biologically unimportant.

To test for correlations between physiological (SCC and SAA) and behavioral measures, we performed curve estimation regression statistics using the continuous 24-h data set (SPSS: ANOVA, coefficient of determination) following tests for normality. Due to the small sample size, standard deviations for the behavioral measures were already large, so outliers (±2 SD) deemed to be atypically/excessively low or high were considered and removed before data analysis. Such outliers comprised a maximum of a single data point (i.e., one matched pair) for any measure.

RESULTS

Behavioral Differences over 24 Hours and During 60 Minutes Immediately Following First Milking

Luxury Behavior: Social—Agonistic Interactions (and Tendency of Being Displaced). Over the 24-h period, cows with SCM gave significantly fewer head butts than healthy controls (Tables 3). Nonsignificant agonistic measures were classified, using RDV confidence intervals, as biologically inconclusive; this indicates that an effect may become evident given a larger sample size. These included the delivery of body pushes (RDV = 0.08, 95% CI_{RDV} = −0.08 to 0.27); noncontact challenges (RDV = 0.02, 95% CI_{RDV} = 0.00 to 0.15), head pushes (RDV = 0.03, 95% CI_{RDV} = −0.03 to 0.18), and head swipes (RDV = 0.17, 95% CI_{RDV} = −0.12 to 0.63), as well as total agonistic: given (RDV = 0.40, 95% CI_{RDV} = −0.31 to 0.73). The SCM cows also received fewer noncontact challenges than healthy cows over 24 h, but more head swipes (Table 3). With the exception of body push received, which was classified as biologically unimportant (RDV = 0.08, 95% CI_{RDV} = −0.01 to 0.02), all nonsignificant measures of agonistic interactions were classified as biologically inconclusive. This included the receipt of head butts (RDV = 0.07, 95% CI_{RDV} = −0.05 to 0.13), head pushes (RDV = 0.02, 95% CI_{RDV} = −0.00 to 0.14) and mutual head butting (RDV = 1.13 s, 95% CI_{RDV} = −0.19 to 8.03 s), as well as total agonistic: received (RDV = 0.34, 95% CI_{RDV} = −0.13 to 0.40). The SCM cows were significantly less socially reactive than healthy cows over 24 h (Table 3); for example, they were less likely to move away (displayed a lower percentage of displacement) following the receipt of agonistic interactions.

Cows with SCM gave significantly fewer head butts and head pushes during the 60 min following morning milking. The SCM cows also received significantly fewer total agonistic interactions than healthy controls, and specifically fewer body pushes and head butts during this same period (1hPostM1; Table 4).

Luxury Behavior: Social—Nonagonistic Social Interactions. Over the 24-h period, cows with SCM performed significantly less social exploration and al-

Table 3. Measures of social and other luxury behavior recorded from pair-matched cows with (SCM) or without (CTRL) subclinical mastitis over 24 h: parametric data (PD) analyzed using paired *t*-test, nonparametric data (NP) analyzed using Wilcoxon SR test.

		CTRL		SCM		Effect size statistics		
Measure (unit[1])	Test	Mean	SD	Mean	SD	Hedges' g	95% CI	Size of effect[2]
Body push: given[3] (n)	NP	0.8^{a}	0.8	0.5^{a}	0.3	0.47	−0.25 to 0.90	Medium, NS
Body push: receive[3] (n)	PD	0.8^{a}	0.3	0.8^{a}	0.4	0.11	−0.31 to 0.53	Small, NS
Noncontact challenge: given[3] (n)	PD	0.2^{a}	0.2	0.1^{b}	0.1	0.62	−0.01 to 1.25	Medium, NS
Noncontact challenge: received[3] (n)	PD	0.3^{a}	0.3	0.2^{c}	0.1	0.84	0.02 to 1.66	Large, Sig
Head butt: given[3] (n)	PD	1.1^{a}	0.9	0.6^{b}	0.4	0.65	0.10 to 1.20	Medium, Sig
Head butt: received[3] (n)	PD	0.7^{a}	0.4	0.6^{a}	0.3	0.37	−0.45 to 1.19	Small, NS
Head push: given[3] (n)	NP	0.3^{a}	0.4	0.1^{a}	0.1	0.52	−0.15 to 1.04	Medium, NS
Head push: received[3] (n)	PD	0.2^{a}	0.2	0.1^{b}	0.2	0.64	−0.01 to 1.29	Medium, NS
Head swipe: given[4] (n)	PD	1.7^{a}	0.8	2.3^{a}	1.4	−0.47	−0.23 to 1.18	Medium, NS
Head swipe: received[3] (n)	PD	1.4^{a}	0.9	2.0^{b}	1.5	−0.51	0.01 to 1.02	Medium, Sig
Mutual head butt[3] (s)	NP	11.3^{a}	11.9	5.3^{a}	4.0	0.66	−0.03 to 1.34	Medium, NS
Total agonistic: given[4] (n)	PD	4.0^{a}	2.6	3.3^{a}	1.5	0.32	−0.48 to 1.12	Small, NS
Total agonistic: received[3] (n)	PD	3.4^{a}	1.2	3.9^{a}	1.8	−0.30	−0.30 to 0.90	Small, NS
Body push: displacement[3] (%)	NP	51.5^{a}	14.0	53.7^{a}	17.9	−0.11	−0.53 to 0.85	Small, NS
Noncontact challenge: displacement[3] (%)	NP	84.4^{a}	13.4	76.9^{a}	23.3	0.39	−0.32 to 1.10	Small, NS
Head butt: displacement[4] (%)	PD	69.9^{a}	15.5	52.7^{d}	16.8	1.10	0.46 to 1.74	Large, Sig
Head push: displacement[5] (%)	NP	52.8^{a}	25.5	22.3^{c}	35.0	0.96	−0.02 to 2.23	Large, NS
Head swipe: displacement[3] (%)	PD	32.9^{a}	13.3	24.7^{c}	13.6	0.63	0.03 to 1.22	Medium, Sig
Total agonistic: displacement[4] (%)	NP	49.1^{a}	11.1	39.2^{d}	11.6	0.87	0.11 to 1.63	Large, Sig
Allogroom give[3] (s)	PD	18.2^{a}	14.0	10.1^{c}	9.7	0.70	0.05 to 1.35	Medium, Sig
Allogroom receive[3] (s)	NP	13.8^{a}	11.4	15.9^{a}	14.7	−0.16	−0.54 to 0.79	Small, NS
Explore social[3] (s)	PD	18.7^{a}	12.3	9.1^{d}	5.4	1.04	0.30 to 1.78	Large, Sig
Total social: given[3] (n)	NP	5.0^{a}	2.5	4.5^{a}	2.2	0.20	−0.53 to 0.87	Small, NS
Total social: received[3] (n)	PD	4.4^{a}	1.1	5.0^{a}	2.5	−0.34	−0.34 to 1.02	Small, NS
Feed barrier: no neighbors[4] (%)	PD	41.4^{a}	12.9	36.0^{b}	13.0	0.43	−0.02 to 0.88	Medium, NS
Feed barrier: 2 neighbors [3] (%)	PD	24.6^{a}	9.7	30.0^{c}	9.8	−0.56	0.11 to 1.01	Medium, Sig
Cubicle: no neighbors[4] (%)	PD	38.8^{a}	20.3	35.8^{a}	20.7	0.43	−0.02 to 0.88	Medium, NS
Cubicle: 2 neighbors[4] (%)	NP	21.9^{a}	19.6	18.6^{a}	20.6	0.08	−0.81 to 0.58	Small, NS
Feed barrier: open[4] (%)	PD	82.2^{a}	12.0	90.1^{c}	7.7	−0.81	0.10 to 1.53	Large, Sig
Self-groom[3] (s)	PD	21.2^{a}	7.9	26.0^{a}	10.8	−0.27	−0.58 to 1.12	Small, NS
Brush use[3] (s)	NP	29.0^{a}	43.9	11.9^{a}	9.0	0.53	−0.16 to 0.96	Medium, NS
Explore environment[3] (s)	PD	55.4^{a}	20.9	50.0^{a}	14.5	0.31	−0.24 to 0.86	Small, NS

[a–d] Mean values in the same row with different superscripts differ as follows: a = reference value; b = $P < 0.10$ (trend); c = $P \leq 0.05$; d = $P \leq 0.01$.

[1] Number and seconds transformed to "per hour visible."

[2] The size of the effect (based on estimated g-values): ≤0.39 = small, 0.40–0.79 = medium, ≥0.80 = large; significance (Sig) attributed only when 95% CI > 0, nonsignificance (NS) attributed when 95% CI contained 0.

[3] Number of data points used in statistical analysis: n = 32.

[4] Number of data points used in statistical analysis: n = 34.

[5] Number of data points used in statistical analysis: n = 22.

logrooming than healthy controls (Table 3). Receipt of allogrooming was not significantly different during 24 h, and this measure was classified as biologically unimportant (RDV = 11.02 s, 95% CI_{RDV} = −1.14 to 1.67 s).

In the 60 min following morning milking SCM cows, again, performed less social exploration than healthy cows but received less allogrooming than healthy controls (Table 4).

Luxury Behavior: Social—Total Social Interactions. Over the 24-h period, no significant differences were found in the total performance or receipt of social interactions (Table 3), and "total social: given" was classified as biologically unimportant in the context of this data set (RDV = 0.50, 95% CI_{RDV} = −0.26 to 0.43). "Total social: received," however, was classified as biologically inconclusive (RDV = 0.44, 95% CI_{RDV} = −0.21 to 0.64). This means that, given a larger sample size, it may have been possible to confirm that SCM cows did receive fewer social interactions over 24h. During the 60 min following morning milking, SCM cows did receive significantly fewer social interactions (Table 4).

Luxury Behavior: Social—Proximity and Feed Barrier Preference. Over the 24-h period, cows with subclinical mastitis spent a significantly greater proportion of their time at the feed barrier flanked by

Table 4. Differences in social and other luxury behavior identified between pair-matched cows with (SCM, n = 17) or without (CTRL, n = 17) subclinical mastitis during 60 min following morning milking: parametric data (PD) analyzed using paired *t*-test, nonparametric data (NP) analyzed using Wilcoxon SR test

		CTRL		SCM		Effect size statistics		
Measure (unit)	Test	Mean	SD	Mean	SD	Hedges' g	95% CI	Size of effect[1]
Body push: received (n)	NP	3.3^a	2.5	1.2^c	1.5	−0.98	0.29 to 1.67	Large, Sig
Head butt: given (n)	NP	1.7^a	2.4	0.5^b	0.6	−0.65	0.10 to 1.09	Medium, Sig
Head butt: received (n)	NP	1.1^a	1.4	0.3^b	0.6	−0.73	0.10 to 1.40	Medium, Sig
Head push: given (n)	NP	0.9^a	1.3	0.1^c	0.3	−0.84	0.24 to 1.38	Large, Sig
All agonistic: given (n)	PD	10.7^a	9.0	5.8^b	4.7	−0.70	−0.05 to 1.45	Medium, NS
All agonistic: received (n)	PD	4.7^a	2.6	2.5^c	1.9	−0.99	0.04 to 1.93	Large, Sig
Allogroom receive (s)	NP	37.6^a	66.1	1.9^c	3.2	−0.74	0.44 to 1.11	Medium, Sig
Explore social (s)	NP	21.2^a	28.8	6.5^c	7.6	−0.68	0.06 to 1.07	Medium, Sig
Total social: received (n)	NP	12.1^a	7.4	6.6^c	3.9	−0.91	0.17 to 1.51	Large, Sig
Self-groom (s)	PD	29.2^a	25.6	47.4^b	44.2	0.52	0.01 to 1.03	Medium, Sig
Explore environment (s)	PD	71.8^a	44.2	39.3^c	27.8	−0.91	0.11 to 1.71	Large, Sig

[a–c]Mean values in the same row with different superscripts differ as follows: a = reference value; b = $P < 0.10$ (trend); c = $P \leq 0.05$.

[1]The size of the effect (based upon estimated g-values): ≤0.39 = small, 0.40–0.79 = medium, ≥0.80 = large; significance (Sig) attributed only when 95% CI > 0, nonsignificance (NS) attributed when 95% CI contained 0.

2 neighbors than healthy controls (Table 3). Nonsignificant measures of social proximity were classified as biologically unimportant within the context of this study; this included the proportion of total time spent at the feed barrier without neighbors (RDV = 8.28%, 95% CI_{RDV} = −0.11 to 4.75%) and the proportion of total time lying within cubicles with either zero (RDV = 7.75%, 95% CI_{RDV} = −0.06 to 2.58%) or 2 (RDV: 4.38%, 95% CI_{RDV} = −2.69 to 1.93%) neighbors. The SCM cows also spent a significantly greater proportion of their time at the open section of the feed barrier over the 24 h (Table 3).

Luxury Behavior: Nonsocial—Self-Grooming and Brush Use. Over the 24-h period, differences in brush use and self-grooming were not significant but were classified as biologically inconclusive (brush use: RDV = 8.70 s, 95% CI_{RDV} = −2.74 to 16.45 s; self-groom: RDV = 4.23 s, 95% CI_{RDV} = −2.81 to 5.42 s). In the 60 min following morning milking, there was a tendency for cows with subclinical mastitis to perform more self-grooming than healthy controls (Table 4).

Luxury Behavior: Nonsocial—Environmental Exploration. Over the 24-h period, no differences were found in the amount of time spent exploring the environment (Table 3). "Environment explore" was also classified as biologically unimportant over 24 h in the context of this study (RDV = 11.08 s, 95% CI_{RDV} = −1.30 to 4.66 s). In the 60 min following morning milking cows with SCM explored the environment significantly less than healthy controls (Table 4).

Core Maintenance Behavior: Feeding and Drinking. For both the 24-h observations (Table 5) and the 60 min following morning milking (Table 6) no significant differences were evident for time spent feeding or drinking; both measures were classified as biologically unimportant over 24 h (feed: RDV = 82.39

Table 5. Measures of core maintenance behavior recorded from pair-matched cows with (SCM) or without (CTRL) subclinical mastitis over 24 h, analyzed using paired *t*-tests

	CTRL		SCM		Effect size statistics		
Measure (unit[1])	Mean	SD	Mean	SD	Hedges' g	95% CI	Size of effect[2]
Feed[3] (s)	823.9^a	167.5	823.8^a	281.2	0.00	−0.46 to 0.46	Small, NS
Drink[3] (s)	34.3^a	12.2	30.2^a	14.6	0.32	−0.32 to 0.96	Small, NS
Lie[3] (s)	$2{,}001.9^a$	211.6	$2{,}092.5^a$	367.6	−0.31	−0.26 to 0.88	Small, NS
Head on flank[4] (s)	143.5^a	49.7	178.7^c	74.8	−0.57	0.06 to 1.09	Medium, Sig
Transitions[4] (n)	55.6^a	8.2	48.6^c	7.8	0.91	0.08 to 1.74	Large, Sig
Distance[4] (unit)	8.2^a	1.9	6.8^c	1.5	0.88	0.11 to 1.65	Large, Sig

[a–c]Mean values in the same row with different superscripts differ as follows: a = reference value; b = $P < 0.10$ (trend) c = $P \leq 0.05$.

[1]All units transformed to "per hour visible."

[2]The size of the effect (based upon estimated g-values): ≤0.39 = small, 0.40–0.79 = medium, ≥0.80 = large; significance (Sig) attributed only when 95% CI > 0, nonsignificance (NS) attributed when 95% CI contained 0.

[3]Number of data points used in statistical analysis: n = 34.

[4]Number of data points used in statistical analysis: n = 32.

s, 95% CI_{RDV} = −0.03 to 0.03 s; drink: RDV = 6.86 s, 95% CI_{RDV} = −1.33 to 3.99 s).

Core Maintenance Behavior: Physical Activity and Lying Behavior. Over the 24-h period, SCM cows were significantly less active than healthy controls; they performed fewer behavioral transitions and moved over a smaller distance (Table 5). Total time spent lying did not differ significantly over the 24 h (Table 5) but time lying with their head on their flank did; SCM cows spent significantly more time in this position than healthy controls (Table 5). Total time spent lying was classified as biologically unimportant in the context of this study (RDV = 200.19 s, 95% CI_{RDV} = −23.54 to 79.67 s). In the 60 min following morning milking, again, cows with SCM were significantly less active (transitions and distance moved), but no differences in lying duration were found (Table 6).

Correlations Between Physiology and Behavior

Assay Validation. Parallelism ($F_{1,9} = 3.46$, $P > 0.05$) was confirmed between serial dilutions of saliva (range = 1:4 to 1:64) and SAA standards (range: 0, 9.38, 18.75, 37.5, 75, 150, 300 ng/mL), indicating that the ELISA kit was suitable for use with bovine saliva. Recovery of 300 ng/mL SAA from a spiked saliva sample was 93.76 ± 4.63% (n = 10). The intra-assay CV was 3.09% (250.87 ± 7.75 ng/mL, n = 10) for QC_{low} and 4.68% (1,360.33 ± 63.70 ng/mL, n = 10) for QC_{high}. The interassay CV was 2.77% (246.06 ± 6.81 ng/mL, n = 2) for QC_{low} and 3.89% (1,323.96 ± 51.43 ng/mL, n = 2) for QC_{high}.

SAA and SCC. The average SCC per group was the following: CTRL = 48.29 ± 28.33 (×1,000 cells/mL); SCM = 351.12 ± 176.73 (×1,000 cells/mL). A trend was found toward a significantly higher concentration of salivary SAA in the SCM cows (CTRL = 343.42 ± 269.60 ng/mL, SCM = 519.59 ± 315.43 ng/mL; $t_{1,12} = 1.93$, $P = 0.076$). A weak positive relationship was evident between SCC and salivary SAA levels ($F_{1,29} = 8.81$, $P = 0.006$, Figure 2).

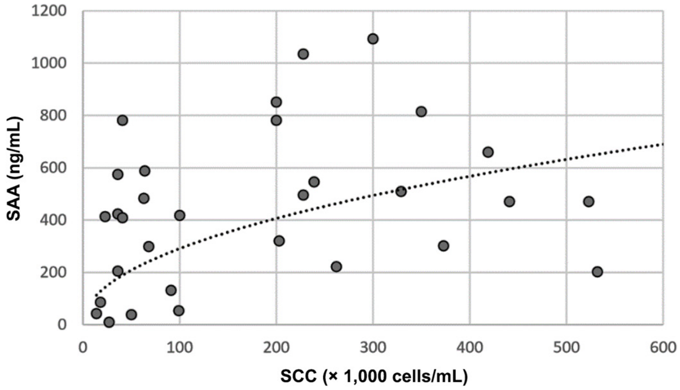

Figure 2. The significant positive relationship between SCC in milk and salivary serum amyloid A (SAA) in dairy cattle ($R^2 = 0.233$; $y = 31.715x^{0.4815}$).

SAA, SCC, and Behavior. All behavioral measures significantly correlated with SAA or SCC are presented in Table 7. The majority of these relationships are weak as indicated by R^2 values.

Luxury Behavior: Social. The significant relationship between SCC and "total social: received" was best modeled using a quadratic function. This described a negative association at low SCC levels and a positive association at higher levels, such that as cell counts rose in cows with low SCC (i.e., healthy CTRL cows) the tendency to receive social interactions would decrease, but in those cows with higher SCC (i.e., SCM cows) as cell counts rose further the tendency to receive social interactions increased. Significant positive relationships were found between salivary SAA and "total agonistic: received." Together these findings indicate that cows with high levels of salivary SAA received more social, including agonistic, interactions overall. Negative correlations were found between salivary SAA and the performance of several social measures, including head swipes, total social, and total agonistic, behavior. This indicates that cows with higher levels of salivary SAA initiated fewer social interactions overall.

Table 6. Differences in core maintenance behavior identified between cows with (SCM, n = 17) or without (CTRL, n = 17) subclinical mastitis during 60 min following morning milking: parametric data (PD) analyzed using paired *t*-test, nonparametric data (NP) analyzed using Wilcoxon SR test

		Control		SCM		Effect size statistics		
Measure (unit)	Unit	Mean	SD	Mean	SD	Hedge's g (bootstrap)	95% CI	Size of effect[1]
Transitions (n)	NP	99.1[a]	34.8	71.7[c]	24.6	−0.89	0.21 to 1.62	Large, Sig
Distance (unit)	PD	15.9[a]	6.6	12.5[b]	4.0	−0.63	−0.08 to 1.34	Med, NS

[a–c] Mean values in the same row with different superscripts differ as follows: a = reference value; b = $P < 0.10$ (trend); c = $P \leq 0.05$.

[1] The size of the effect (based upon estimated g-values): ≤ 0.39 = small, 0.40–0.79 = medium, ≥ 0.80 = large; significance (Sig) attributed only when 95% CI > 0, nonsignificance (NS) attributed when 95% CI contained 0.

Table 7. Significant correlations ($P < 0.05$; plus trends toward a correlation at $P < 0.10$) between behavioral measures (24-h data) and 2 markers of inflammation and mastitis infection[1]; given are curve estimation regression statistics (ANOVA, coefficient of determination, R^2) and the equation for the relationship (based upon the line of best fit)

Behavioral measure (unit)[2]	Correlation with salivary SAA and SCC
Noncontact challenge: received (n)	SAA: $F_{(1,29)} = 3.93$, $P = 0.057$; $R^2 = 0.119$; $y = 0.0003x + 0.165$
Head push: received (n)	SCC: $F_{(1,31)} = 5.87$, $P = 0.021$; $R^2 = 0.159$; $y = -1.990x + 5.520$
Head swipe: given (n)	SAA: $F_{(1,29)} = 6.89$, $P = 0.014$; $R^2 = 0.192$; $y = 2.3287e^{-9\text{E-}04x}$
Head swipe: received (n)	SCC: $F_{(2,30)} = 3.14$, $P = 0.058$; $R^2 = 0.173$; $y = 6\text{E-}06x^2 - 0.0018x + 1.621$
Total agonistic: given (n)	SAA: $F_{(1,29)} = 3.07$, $P = 0.090$; $R^2 = 0.096$; $y = 4.2715e^{-6\text{E-}04x}$
Total agonistic received (n)	SAA: $F_{(2,28)} = 4.23$, $P = 0.025$; $R^2 = 0.232$; $y = 6\text{E-}06x^2 - 0.004x + 3.817$
Head butt: displacement (%)	SAA: $F_{(1,29)} = 5.64$, $P = 0.024$; $R^2 = 0.163$; $y = 72.616e^{-5\text{E-}04x}$
	SCC: $F_{(1,31)} = 8.81$, $P = 0.006$; $R^2 = 0.221$; $y = -7.36\ln(x) + 95.594$
Head push: displacement (%)	SAA: $F_{(2,22)} = 4.14$, $P = 0.030$; $R^2 = 0.227$; $y = -0.0002x^2 + 0.1952x - 0.092$
Head swipe: displacement (%)	SAA: $F_{(1,29)} = 3.291$, $P = 0.080$; $R^2 = 0.102$; $y = -0.015x + 34.655$
Total agonistic: displacement (%)	SAA: $F_{(1,29)} = 4.63$, $P = 0.040$; $R^2 = 0.138$; $y = -0.0155x + 52.078$
	SCC: $F_{(1,32)} = 10.46$, $P = 0.003$; $R^2 = 0.246$; $y = 49.136e^{-7\text{E-}04x}$
Explore social (s)	SAA: $F_{(1,29)} = 4.34$, $P = 0.046$; $R^2 = 0.130$; $y = 0.0167x + 8.517$
	SCC: $F_{(1,31)} = 5.58$, $P = 0.025$; $R^2 = 0.153$; $y = 15.532e^{-0.002x}$
Total social: given (n)	SAA: $F_{(1,29)} = 6.42$, $P = 0.017$; $R^2 = 0.181$; $y = 6.0091e^{-7\text{E-}04x}$
Total social: received (n)	SAA: $F_{(2,28)} = 7.40$, $P = 0.003$; $R^2 = 0.346$; $y = 9\text{E-}06x^2 - 0.0061x + 5.057$
	SCC: $F_{(2,31)} = 4.90$, $P = 0.014$; $R^2 = 0.240$; $y = 2\text{E-}05x^2 - 0.0081x + 5.259$
Feed barrier: open (%)	SAA: $F_{(1,28)} = 4.08$, $P = 0.053$; $R^2 = 0.127$; $y = 3.244\ln(x) + 67.752$
	SCC: $F_{(1,32)} = 5.98$, $P = 0.020$; $R^2 = 0.158$; $y = 81.19e^{0.0003x}$
Explore environment (s)	SAA: $F_{(1,29)} = 5.96$, $P = 0.021$; $R^2 = 0.170$; $y = 8.690\ln(x) + 7.484$
Feed (s)	SAA: $F_{(1,29)} = 14.63$, $P = 0.001$; $R^2 = 0.335$; $y = 1{,}023.5e^{-6\text{E-}04x}$
Drink (s)	SAA: $F_{(1,29)} = 22.18$, $P < 0.001$; $R^2 = 0.433$; $y = -8.379\ln(x) + 79.816$
Lie (s)	SAA: $F_{(1,29)} = 9.10$, $P = 0.005$; $R^2 = 0.239$; $y = 0.5032x + 1{,}848.3$
	SCC: $F_{(2,31)} = 2.637$, $P = 0.088$; $R^2 = 0.145$; $y = -0.0019x^2 + 1.1955x + 1956.8$
Transitions (n)	SCC: $F_{(2,30)} = 5.50$, $P = 0.009$; $R^2 = 0.269$; $y = 0.002x^2 - 0.0934x + 59.272$
Distance (unit)	SCC: $F_{(2,30)} = 4.11$, $P = 0.026$; $R^2 = 0.215$; $y = 4\text{E-}05x^2 - 0.021x + 9.146$

[1] One from saliva (serum amyloid A, SAA), and one from milk (SCC).

[2] Number, seconds, and unit transformed to "per hour visible."

Significant negative relationships were found between measures of social reactivity, (i.e., the percentage of occasions when a cow was displaced following receipt of an agonistic interaction), and both inflammatory markers. This is indicative that cows with higher levels of salivary SAA or SCC demonstrate reduced social reactivity following the receipt of social stimuli, including cumulative agonism (total agonistic: displacement), and specifically in response to head butts (head butt: displacement) and head swipes (head swipe: displacement). Social exploration was positively associated with SAA and negatively associated with SCC, such that individuals with higher SAA levels explored other cows more, whereas individuals with higher cell counts explored other cows less.

Social Proximity and Feed Barrier Preferences. No significant correlations were evident for our measures of social proximity; however, salivary SAA and SCC were positively correlated with proportion of time spent at the open-rail feed barrier. This indicates that, with rising levels of either measure of inflammation, cows became less likely to feed at the self-locking barrier.

Luxury Behavior: Nonsocial. As with social exploration, environmental exploration was also positively associated with salivary SAA and negatively associated with SCC, such that cows with higher SAA levels performed more exploratory behavior overall, whereas cows with higher cell counts explored less. Self-grooming and brush use were not significantly correlated with either inflammatory measure.

Maintenance (Core) Behavior. Salivary SAA was negatively correlated with feeding and drinking, and positively correlated with time spent lying. This indicates that, within our study cohort, as SAA levels increased the cows spent less time feeding and drinking, and more time lying. Significant relationships between SCC and both the number of behavioral transitions and distance covered were best modeled using quadratic functions. These describe an initial decline in both measures of physical activity as SCC increased to approximately 300 (× 1,000 cells/mL), followed by an increase in both measures as SCC levels continued to rise (Figure 3).

DISCUSSION

The purpose of this study was to identify differences in salivary SAA and behavior in cows with spontaneously occurring subclinical mastitis, as compared with pair-matched healthy controls. Salivary SAA was found to be higher in SCM individuals, suggesting that it may have potential as a marker of low-level systemic inflammation in dairy cows. Higher salivary SAA levels were also correlated with several predicted behavioral changes. Furthermore, SCM cows displayed a reduction in the performance and receipt of various social behaviors (here, categorized as luxury behaviors), lower social reactivity (i.e., they were less likely to be displaced following the receipt of agonism), and reduced physical activity, but no difference in feeding, drinking or lying duration (maintenance behaviors).

Changes in Salivary SAA

We report here, for the first time, a positive (although weak) correlation between SCC and salivary SAA. Positive associations between SCC and nonsalivary SAA have previously been reported from cows with clinical mastitis and SCM (serum: de Boyer des Roches et al., 2017; milk: O'Mahony et al., 2006; Åkerstedt et al., 2007; Pyörälä et al., 2011). The SAA in saliva thus offers potential as a noninvasive means of detecting SCM, but further studies will be required to substantiate our preliminary findings.

Changes in Luxury Behaviors and Correlations with Salivary SAA

Luxury behaviors, as used here, include social and nonsocial behaviors that are deemed nonessential for survival within the short term; as such, we predicted that they would become downregulated during a subclinical infection.

Social Interactions. Over the 24-h period, total social behavior, given or received, remained unaltered between the 2 groups. The SCM cows did, however, receive fewer noncontact challenges and more head swipes. Head swipes are commonly observed at the feed barrier as a means of displacing competitors, and our result agrees with previous findings. Sick cows are typically reported to be displaced more frequently than healthy cows when feeding (Schirmann et al., 2016; Lomb et al., 2018; Neave et al., 2018;).

In the 60 min following morning milking (the period of peak feeding activity in the study herd) other differences emerged; cows with subclinical mastitis received fewer social interactions in total (specifically fewer head butts and body pushes) than the healthy controls. No corresponding reduction in the receipt of physical agonism was observed over the 24-h period. It is therefore possible that the SCM cows were avoiding peak feeding times, when aggression and feed competition are highest, as do low-ranking cows (Val-Laillet et al., 2008).

Interestingly, we also found positive correlations between salivary SAA and total social, and total agonistic, interactions received, indicating that those individuals with higher levels of systemic inflammation attracted more social attention. However, we cannot be sure whether SAA upregulation occurred in the cows with subclinical mastitis due to infection or following exposure to social stress. Upregulation of C-Reactive Protein (an acute phase protein known to increase during illness and stress) has, for example, been reported in zoo-housed gorillas following aggressive encounters (Fuller and Allard, 2018). In the current study, saliva samples were collected the day after behavioral video recordings. Therefore, an elevation in SAA could reflect agonistic encounters experienced during the previous day, independent of health status. Further study should address this potential confound.

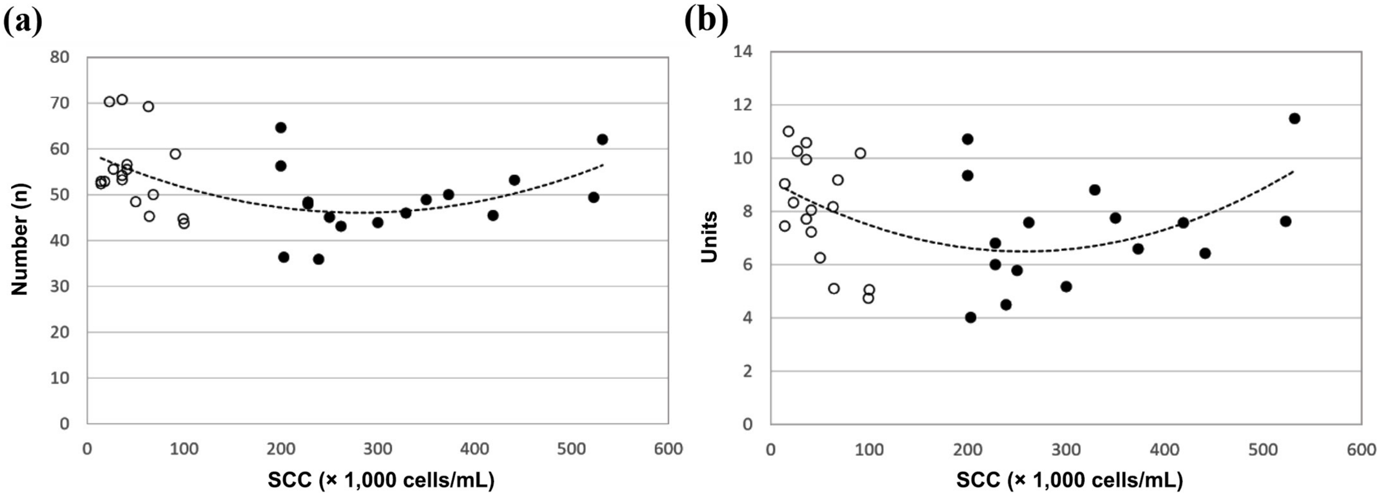

Figure 3. Quadratic relationship between SCC and 2 measures of activity in cows with (SCM = black), and without (CTRL = open circle) subclinical mastitis: (A) behavioral transitions; (B) distance covered.

In line with the prediction that social behaviors should decrease with subclinical mastitis, affected cows performed fewer head butts than healthy cows, and SAA was negatively (albeit loosely) correlated with the delivery of total social and agonistic interactions, including head swipes. Our results thus provide additional behavioral detail in support of existing literature which reports that cows with clinical (and subclinical) conditions often perform fewer agonistic interactions and competitive displacements than healthy individuals (Huzzey et al., 2007; Patbandha et al., 2012; Sepúlveda-Varas et al., 2014, 2016; Jensen and Proudfoot, 2017).

The relative social ranks of the focal cows and their interaction partners were not determined in the current study. It is possible, and an interesting focus for further study, that the relative social rank of an individual could be influenced by the effects of disease due to a loss of competitive vigor and motivation. High-ranking animals characteristically displace lower rankers from the feed barrier (DeVries et al., 2004; Huzzey et al., 2006), and lower rankers adjust their feeding patterns accordingly (DeVries et al., 2004).

Social Reactivity. Over the 24h period, cows with subclinical mastitis were less likely to be displaced than healthy cows following the receipt of agonism, described here as being less socially reactive. Correspondingly, reactivity declined with increasing inflammation levels, as demonstrated by negative correlations between the percentage displacement following the receipt of total agonistic interactions and both salivary SAA and SCC. These results appear, at first hand, to contradict previous findings of sick cows being less motivated to compete for access to feed based upon displacement (e.g., Huzzey et al., 2006, 2007; Goldhawk et al., 2009). However, where a cow positions herself at the feeder may be influenced by the social rank of others already feeding, as reported by Manson and Appleby (1990). They found that cows of the most dissimilar rank (low or high) maintained a greater distance from each other than cows of similar rank. It is thus conceivable that our SCM cows proactively avoided feeding positions next to individuals of higher competitive vigor, and preferentially selected the company of other sick or lower ranking cows. Subsequently, the receipt of moderate competitive aggression from an individual (with similarly moderate motivation to compete) may have been tolerable and thus, not provoke displacement. As others before us, we cannot rule out that this, and other findings on social behavioral differences, reflect underlying disparities in social status that have led to differences in health status. We here provide a first report of a reduction in social reactivity in cows with subclinical mastitis. By matching cows by parity, we sought some control over potentially confounding social rank biases. Further study is needed to substantiate differences in social interactions as markers of subclinical mastitis and other diseases.

Social Avoidance and Proximity. In the current study, cows with subclinical mastitis performed less social exploration than healthy controls, which indicates social avoidance or withdrawal. Furthermore, we observed a negative correlation between this measure and SCC, such that cows with higher cell counts spent less time exploring (sniffing) others. Sickness-driven social avoidance is well documented in laboratory-animals and humans and is predicted via the action of proinflammatory cytokines on the central nervous system (Kent et al., 1992; Bluthé et al., 1996; Dantzer and Kelley, 2007; Arakawa et al., 2010). Due to the limited opportunity for social avoidance in intensive systems, it has been relatively understudied in farm animals. We also found an unexpected, if weak, positive correlation between SAA and social exploration, which may, at least partially, be explained by the presence of both preclinical and postclinical individuals among our subclinical group. de Boyer des Roches et al. (2017, 2018) report behavioral changes including reduced environmental attentiveness during the preclinical phase of an experimentally induced mastitis model (i.e., before SCC and serum SAA upregulation), and during the acute phase (coinciding with raised levels of SCC and SAA), but not during the postclinical remission phase (high levels of SCC and SAA persist). This suggests that serum SAA may peak during postclinical remission, rather than during the acute phase of inflammation. Further study is therefore required to investigate when, and how, social exploration changes throughout the course of spontaneously occurring mastitis, and other diseases, to confirm its usefulness in the detection of subclinical disease states.

Over the 24-h period, cows with subclinical mastitis spent a greater proportion of their time feeding at the open-rail section of the feed barrier than healthy controls. A weak positive correlation was also found between this measure and SAA, which suggests that this preference increases with systemic inflammation. Barrier design is known to influence the incidence of agonistic behavior relating to feed access. Self-locking yokes have vertical bars which separate the necks of adjacent cows, and these are better at reducing competitive displacements compared with open post-and-rail barriers (Endres et al., 2005; Huzzey et al., 2006). Although our results appear to contradict previous findings it is possible that other factors contribute to the choice of feeding location, e.g., the open section provides better visibility and enables cows to quickly withdraw from potential agonistic interactions. Although our finding that the SCM cows spent a greater proportion of their time than healthy cows at the feed barrier in close contact with 2 (flanking) herd mates is at odds with the expectation of sickness-driven social avoidance, a perception of increased vulnerability may induce sick cows to feed in company. Future longitudinal studies could determine whether individuals change their feeding behavior in relation to health status, including whether they opt to feed among lower rankers when their own health is compromised.

Allogrooming. In agreement with our predictions relating to behavioral priorities during infection, cows with subclinical mastitis performed less allogrooming than healthy cows over the 24-h period and were allogroomed less during the 60 min following morning milking. Previous studies have reported similar findings in relation to social rank, with low-ranking cows performing and receiving less allogrooming than high rankers (Napolitano et al., 2009), and allogrooming decreasing more in low-ranking cows under conditions of increased competition (Val-Laillet et al., 2008). For health-related changes, Galindo and Broom (2002) found lame cows to be allogroomed more than nonlame cows, which is contrary to our result for SCM cows. Their finding was interpreted as a self-instigated coping strategy triggered by pain/discomfort associated with clinical lameness, which arguably applies less to subclinical mastitis.

Nonsocial Behavior. Against expectation, we did not observe a decline in brush use in cows with subclinical mastitis, nor a correlation between brush use and salivary SAA. Although grooming itself is a comfort activity that healthy cows are highly motivated to perform (McConnachie et al., 2018), brush use has been shown to decrease during nonmastitic disease (Toaff Rosenstein et al., 2016; Mandel et al., 2017; Weigele et al., 2018). The brush in our study was located central to many resources (feed barrier, water trough, and cubicles) and was readily accessible with minimal effort. A combination of small sample size and a trade-off between brush location and the sensitivity of brush use for detecting stress and morbidity (Mandel et al., 2013; 2017) may help to explain why no decline was detected in the current study.

We observed cows with subclinical mastitis to perform more self-grooming (including self-licking) than controls immediately following morning milking, presumably as a response to mild udder discomfort or as a substitute for allogrooming (see previously). However, this observation is at odds with existing literature that reports self-licking to remain unchanged (Siivonen et al., 2011), or decrease (Fogsgaard et al., 2012), during clinical mastitis. Based on our results the potential for the use of self-grooming as a marker of subclinical mastitis remains inconclusive and requires further investigation.

The weak positive correlation between SAA and environmental exploration described here was unexpected; a decline with increasing inflammation had been predicted. It is possible that our focal cows may have included individuals with early-stage, preclinical mastitis (associated with low SAA and sickness-driven reductions in environmental exploration), and individuals in postclinical remission (associated with high SAA and baseline exploration, as discussed previously; see "Social Avoidance and Proximity"). Ruminants generally display low levels of exploratory behavior in intensive housing, because it is a largely unstimulating environment (De Rosa et al., 2009). Although we did not find a reduction in environmental exploration in our SCM cows over the 24 h, they did explore less than controls during the 60 min following morning milking. This could reflect an avoidance of peak feeding because environmental exploration, as defined in our study, included sniffing feed.

Maintenance (Core) Behavior

Feeding and Drinking. Changes in feeding behavior have long been used to diagnose the onset of illness (Weary et al., 2009). We found a negative correlation between SAA and feeding duration, as would be predicted with sickness; however, the average inflammatory response within our SCM group was not sufficiently pronounced to trigger anorexia, as compared with CTRL cows. Sepúlveda-Varas et al. (2016) observed a decrease in feed intake (but not duration) before the diagnosis of clinical mastitis which may be attributed to underlying malaise. González et al. (2008) reported that some cows demonstrate a decrease in feeding duration with the onset of mastitis, whereas others show no change. Barn-housed cattle demonstrate highly synchronized feeding activity, with large peaks in both feeding and social competition coinciding with fresh food delivery, and smaller peaks following milking (DeVries and von Keyserlingk, 2005; Dollinger and Kaufmann, 2013). Mastitic cows, presumably to avoid adverse social interactions, have been shown to feed at less popular times such as early afternoon (Schirmann et al., 2016). Although we found no direct effect on feeding duration, differences in social interactions after morning milking, reported above, may be indicative of cows with subclinical mastitis avoiding peak feeding times.

Water and feed intake are positively related in cattle (Kume et al., 2010); however, drinking tends to be less affected by health than feeding (Hart, 1988). Water is more immediately vital for maintaining body function

(Kyriazakis and Tolkamp, 2011), and because drinking takes less time than feeding it is less prone to disruption from social competition (Huzzey et al., 2007). Although a reduction in water consumption has been reported in cows with mastitis (Lukas et al., 2008; Siivonen et al., 2011), and we observed a moderate negative correlation between SAA and drinking duration, the level of systemic inflammation within our subclinically sick cows may have been too low, or our sample size too small, for a difference to emerge. Overall, the persistence of feeding and drinking levels in our SCM cows fits the prediction that core maintenance behavior is conserved during early mastitic disease.

Lying. No difference in lying duration was found between our 2 groups. Lying is a highly prioritized behavior in cattle due to its importance in rumination (Jensen et al., 2005; Munksgaard et al., 2005) and dairy cows spend approximately 11 h/d recumbent (Ito et al., 2010). Increased lying duration, as a means of conserving energy and facilitating recovery, is a key adaptation for sickness, and we report a positive correlation between SAA and lying. Although extended lying duration has been frequently reported during nonmastitic clinical conditions (Toaff Rosenstein et al., 2016; Weigele et al., 2018; Barragan et al., 2018), lying may decrease during clinical mastitis (Fogsgaard et al., 2012, 2015; Medrano-Galarza et al., 2012; Yeiser et al., 2012), most likely due to udder pain (Cyples et al., 2012).

Our SCM cows lay with their heads held against their flank more than the healthy controls. This posture is primarily associated with rapid eye movement sleep, but cows are also known to display nonrapid eye movement (**NREM**) sleep and drowsing in this position (Ternman et al., 2012), and NREM (deep) sleep often increases during infection (Bryant et al., 2004; Opp, 2005). Crucially, the action of pro-inflammatory cytokines during the sickness response also predicts postural changes, such as curling up, that reduce surface area and associated loss of body heat as an adaptive behavioral change during sickness. On this basis, lying duration appears less promising for the detection of subclinical mastitis than our novel finding of a difference in lying posture.

Activity. Our finding that the SCM cows made fewer behavioral transitions and moved over a shorter distance than healthy cows agrees with our predictions, and with other studies that describe reduced activity before the clinical diagnosis of mastitis (Kester et al., 2015; Stangaferro et al., 2016; Veissier et al., 2017; King et al., 2018). The quadratic relationships between SCC and both behavioral transitions and distance moved described here are of interest because in cows with clinical mastitis, activity can increase (Siivonen et al., 2011; Medrano-Galarza et al., 2012), presumably due to udder discomfort and an associated reduction in lying time. Jadhav et al. (2018) argue that the threshold SCC value to delineate subclinical mastitis from normal should be 310, rather than 200 (×1,000 cells/mL), as conventionally judged (e.g., Madouasse et al., 2010). This higher value closely corresponds with the parabola vertex in both quadratic plots (Figure 3); that is, the point at which activity once again begins to rise.

Limitations

This study formed a preliminary investigation, to identify behaviors with potential for use as markers of subclinical mastitis and inform a wider schedule of focused research; longitudinal studies are now required to track changes in key behaviors with alterations in health status of individual cows. Due to an absence of pre-existing literature (luxury behavior, in this context, has been understudied), many behavioral measures were recorded over 24 h in the current study. Logistically this was time consuming and limited the amount of longitudinal data available per cow; priority was given to sample size. Because the analysis of a single 24-h period for any one cow provided only a snapshot it is not possible to conclude that the behaviors observed were typical for that cow at that level of SCC. Although focal cows were health-checked before inclusion in the study (to rule out clinical pathologies) we could not control for the presence of subclinical conditions other than mastitis, nor could we establish whether cows classified as having subclinical mastitis were in the preclinical or remission phase of disease. Finally, the social behavior of an individual cow fundamentally depends on social dynamics within the herd. Although this wider social context and, specifically, the rank relationships between study cows and their interaction partners, were beyond the scope of this investigation, we consider the initial identification of behavioral differences in a small group, within a complex and dynamic social environment, as encouraging. Further investigation is required into the interactions between social rank, health status and behavior in dairy cows.

CONCLUSIONS

Our detailed behavioral comparison between cows with subclinical mastitis and pair-matched healthy cows, conducted over 2 time periods (24 h and 60 min following morning milking), found that subclinical mastitis was associated with reduced activity, social exploration, receipt of nonagonistic social behavior, social reactivity (percentage displacement following receipt of agonism), and an increase in the receipt of head swipes.

Many social interactions can be considered luxury behaviors during sickness, and here we provide preliminary evidence that several social measures change during subclinical levels of mastitis, whereas core maintenance activities (including feed, drink, and lie) do not. Luxury behaviors therefore appear to offer greater potential for use in early disease detection. In addition, cows with subclinical mastitis altered their feeding behavior. They spent a greater proportion of their feeding time in direct contact with 2 neighbors, and a lower proportion of time feeding at the self-locking feed barriers, than the healthy cows. Although a positive relationship between SCC and salivary SAA was observed, and several correlations between SAA and behavioral measures occurred in a direction consistent with sickness behavior (including positive correlations with lying duration and the receipt of agonism, and negative correlations with feeding, drinking, the performance of total social and agonistic behavior, and social reactivity), the majority of associations were relatively weak. Following these initial steps to identify physiological and detailed behavioral changes associated with subclinical mastitis, we now recommend that observations be replicated in longitudinal studies, tracking individuals, with larger data sets to substantiate and refine our findings.

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