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Salivary oxytocin response of dairy cows to nursing and permanent separation from their calves, and the influence of the cow-calf bond

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ABSTRACT

Oxytocin is a hormone involved in milk let-down, facilitating maternal behavior and parent-young bonding, and attenuating the stress response under challenge, but the release of oxytocin also appears to be dependent upon the social context. Dairy cows are commonly separated from their calves at birth, preventing maternal behavior and the establishment of a social bond. The growing interest in maintaining cow-calf contact provides a practically relevant context to study how oxytocin is affected by differing levels of cow-calf contact. Furthermore, the oxytocinergic system is likely affected by the stress of permanent cow-calf separation, depending on weaning method and strength of the cow-calf bond. Dairy cows were managed with full-time (23 h/d of calf contact), part-time (10 h/d of calf contact) or no calf contact (separated 48 h after birth), and then weaned by either: 50 % reduction in original calf contact time in wk 8 and 9 ('reduced-contact'), or calf contact time remained unchanged ('unchanged-contact'). Permanent separation from their calves occurred at wk 10 (n=14 for each treatment combination). Saliva was sampled in wk 8 before and after a nursing event over 3d, and in wk 10 before and after permanent separation (2 h after, and every 24 h thereafter for 3d), and analysed for oxytocin concentration. Cow-calf bond was measured as: motivation for cows to reunite with their calves (pressure cows were willing to exert on a weighted gate), and frequency and duration of social interactions between dam and calf. Cows with the most opportunity for calf contact (full-time; unchanged-contact) tended to have higher oxytocin concentrations around nursing, and oxytocin concentration around nursing tended to be positively related to proportion of total daily time together spent in physical contact. Over the 4-d post separation period, oxytocin response was generally stable for cows with male calves, but the pattern was variable for cows with female calves and in opposing directions for full- and part-time cows. Reduced-contact cows had greater oxytocin concentration over the separation period than unchanged-contact cows, but only if they had a male calf. In unchanged-contact cows, the oxytocin response to separation tended to increase if the cow-calf bond was stronger. These results highlight the complexity of the oxytocin response to different social situations, which depended on prior level of calf contact, calf sex, and strength of the cow-calf bond. Future research should explore how management practices influence social bonds and the oxytocinergic system, given their role in modulating stress resilience.

1. Introduction

Oxytocin is a neuropeptide hormone that plays a central role in maternal behavior, stress regulation, and lactation in mammals (e.g. see reviews Insel et al., 2001; Kendrick, 2000)). In livestock, oxytocin is known to facilitate maternal care, including grooming, nursing, and protection of young (Kendrick et al., 1987), and in social species, oxytocin helps maintain social bonds and interactions that are critical for social cohesion and group stability (Coulon et al., 2013; Rehn et al., 2014). Tactile contact also stimulates oxytocin release (Lürzel et al., 2020; Uvnäs Moberg and Petersson, 2022), an important component of social bonding in some species (Dunbar, 2010). Additionally, oxytocin release can attenuate physiological and behavioral responses to stress by downregulating the hypothalamic-pituitary-adrenal (HPA) axis (Amico et al., 2004; DeVries et al., 2003; Windle et al., 1997), which may motivate social reinstatement for group protection and social support

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(Bowen and McGregor, 2014; Grippo et al., 2009). Thus, oxytocin appears to be released during both socially positive (e.g. grooming and nursing) and stressful situations.

Dairy cows are often separated from their calves shortly after birth to maximize milk harvest, which has raised public concern and interest in managing cows and calves together (Placzek et al., 2020). However, farmers are often concerned about milk letdown at machine milking and milk loss to the suckling calf. Sustained oxytocin release is crucial for milk letdown and removal (see review by Bruckmeier and Blum (Bruckmaier and Blum, 1998)). In cows allowed to nurse, some studies report increased oxytocin (Lupoli et al., 2001) and no reduction in total milk yield after considering the amount the calf drank (de Passillé et al., 2008), while others indicate disturbed milk ejection, possibly due to lower oxytocin during machine milking (Akers and Lefcourt, 1982; Tancin et al., 2001). This conflicting evidence highlights the need to better understand the effects of cow-calf management on the oxytocinergic system.

Early cow-calf separation disrupts maternal behavior and bonding, potentially affecting the oxytocinergic system (Uvnäs-Moberg et al., 2001). The relationship between oxytocin concentration and expression of maternal behaviors, such as postnatal grooming, remains unclear (Geburt et al., 2015; Williams et al., 2001). Limited research has explored how prolonged interactions post-calving influence oxytocin concentrations. Management approaches, including varying daily contact duration, affect mother-offspring interactions (Bertelsen and Jensen, 2023a), possibly influencing bond strength and oxytocin secretion, but this remains to be investigated.

The physiological implications of separation after prolonged cow-calf contact are not well understood, but a negative behavioral response is suggestive of a strong mother-young bond (Meagher et al., 2019; Newberry and Swanson, 2008). One study reported reduced oxytocin and reduced milk yield after separation at 6 days of age (Tancin et al., 2001), possibly due to emotional distress inhibiting oxytocin release at milking (Bruckmaier et al., 1993). Conversely, oxytocin may increase to attenuate the cortisol response to stress, as seen in cows introduced to a new milking parlour (Sutherland and Tops, 2014) or fearful calves exposed to a novel environment (Yayou et al., 2014). However, exogenous oxytocin increased cortisol in isolated sheep, potentially reflecting the strength of the ewes' social bonds (Damián et al., 2021). Thus, the oxytocin response to calf separation may reflect maternal bond strength, which may be influenced by management practices.

Two management approaches that may ease the behavioural response to separation are restricted calf contact during rearing (Bertelsen and Jensen, 2023b; Wenker et al., 2022) and gradual reduction of contact before separation (Neave et al., 2024). These practices may reduce oxytocin due to decreased tactile stimulation, and weaken the maternal bond. For instance, rat mothers with limited physical contact with their pups had fewer oxytocin-containing brain cells compared to rats with total or no separation from their pups (Pedersen et al., 1995), suggesting that similar results may occur in cows.

This study aimed to investigate the salivary oxytocin response of dairy cows during two putatively contrasting social situations: a nursing event (positive) and permanent separation from the calf (negative). We examined the relationship between cows' oxytocin response in these social situations, management practices (initial calf contact duration and weaning method), and maternal bond strength. Nursing was considered a positive event based on evidence of cows' motivation to nurse (Jensen et al., 2024a; Wenker et al., 2020), and reduced behavioral and physiological indicators of stress upon reunion after separation (Boissy and Le Neindre, 1997; Schnaider et al., 2022). We predicted that cows with more daily calf contact and those abruptly separated would show a greater salivary oxytocin response to both nursing and permanent separation situations, indicating a stronger maternal bond and heightened stress response at separation.

2. Materials and methods

This study was conducted from September 2021 to August 2022 at the Danish Cattle Research Centre, Aarhus University (Tjele, Denmark). All animal procedures were approved by the Danish Animal Experiments Inspectorate (Permit No. 2021–15–0201–00989) in accordance with the Danish Ministry of Environment and Food Act No. 474 (May 15, 2014).

2.1. Animal management and treatment groups

Management of dairy cows and their assigned treatment groups are described in detail in (Neave et al., 2024). Briefly, a total of 84 Danish Holstein dairy cows and their calves (all from the same farm) were enrolled in 7 blocks of 12 cows each. Cows calved in an individual maternity pen (4 ×3.1 m) where they remained with their calf for approximately 48 h (range 42–66 h). For enrolment in the study, cow and calf needed to have no calving complication, no twin births, and the calf had to be able to suckle from the cow without assistance within 48 h. Cows and their calves were assigned to one of three calf-contact treatments, in blocks of 12: (1) full-time contact, apart from milking times (total 23 h/d of cow-calf contact); (2) part-time contact, between morning and afternoon milking at 0530 and 1530 h, and separation from the calf between afternoon and morning milking (total 10 h/d of cow-calf contact); (3) no-contact, where the cow was permanently separated from the calf after leaving the maternity pen. Assignment to calf-contact treatment occurred in pairs (i.e. two cow-calf pairs, to minimize stress of entering the experimental pen alone) until all 12 cows were assigned to a treatment group. Order of treatment assignment was pre-determined for each block on a rotational basis (e.g. in Block 1, first pair born was assigned to part-time, second pair to full-time, third pair to no-contact, and repeated for remaining three pairs). Once all 12 cows were enrolled in the block, the experimental period began (nominally referred to as wk 1) and cows were enrolled until wk 10 (end of experiment).

Each pen (treatment) within a block was balanced for two primiparous and two multiparous cows whenever possible (exceptions: one primiparous and three multiparous cows were in 3 of 7 full-time and no-contact pens, and 2 of 7 part-time pens). No cows previously experienced prolonged contact with their calves. Final distribution of primiparous and multiparous cows across full-time, part-time and no-contact treatments was similar (primiparous cows: n = 11, 12, 11, respectively; multiparous cows: n = 17, 16, 17, respectively). Average lactation of multiparous cows in full-time, part-time and no-contact treatments was 2.3 ± 0.6 (range 2–4), 2.7 ± 0.9 (range 2–5), and 2.9 ± 0.9 (range 2–4) lactations, respectively. The distribution of female and male calves in the full-time and part-time treatments was not randomized as part of the experimental design, but the final distribution was similar (17 females and 11 males in full-time; 14 females and 14 males in each of part-time and no-contact). At the start of the experiment (nominally wk 1), the age of suckling calves in the full-time and part-time treatments was on average 10 d (mean ± SD; full-time: 8.8 ± 6.9 d; part-time: 10.7 ± 5.9 d). The weights of female and male calves in week 7 of the experiment, when sampling commenced (see below), were (mean ± SD) 82.0 ± 12.8 kg and 88.8 ± 14.9 kg, respectively.

At wk 8 of the experimental period, cow-calf pairs in full-time and part-time treatments were pseudo-randomly assigned to one of two weaning treatments (in this study, weaning refers to the reduction of contact time between cow and calf): (1) gradual reduction in contact time between cow and calf until permanent separation from the calf at wk 10 ('reduced-contact'); (2) no change in contact time between cow and calf until wk 10 ('unchanged-contact'). Distribution of primiparous and multiparous cows across weaning treatments was balanced (reduced-contact: n = 12 primiparous, n = 17 multiparous; unchanged-contact: n =11 primiparous, n = 15 multiparous). No-contact cows did not experience any weaning treatment as they had no calf contact.

Overall, 28 cows were assigned to each of the three calf-contact treatments, within which 14 cow-calf pairs were assigned to each of reduced-contact and unchanged-contact treatments. This sample size was determined from a power analysis with α = 0.05 and power = 0.80, using the estimated mean and standard deviation of plasma oxytocin concentration of dairy cows during a nursing event reported in Lupoli et al. (2001), with a targeted 25 % difference in salivary oxytocin response between calf-contact treatments. This resulted in an estimated sample size of 31 cows per treatment. Due to logistical constraints at the research facility, it was not possible to enrol 8 blocks, so our sample size reached 28 cows per treatment over 7 blocks.

2.2. *Calf-contact treatment groups*

Full-time and part-time cows and their calves were housed in a dedicated barn in 7.5 m × 9 m pens bedded with straw (4 cows and 4 calves of the same treatment, per pen). Calves could move freely among cows in their pen, with exclusive access to two calf creep areas (3 × 3 m and 1.5 × 1.5 m) at the back corners of the pen containing *ad libitum* concentrate and hay. The larger calf creep area also offered water from a self-filling bowl. Cows had access to two rotating grooming brushes mounted to opposite sides of the pen, two self-filling water bowls, and two feed troughs (each 2 × 0.75 m) with *ad libitum* total mixed ration (approximately 50:50 concentrate to roughage ratio) replenished twice daily at 0800 and 2000 h; calves were also able to access this total mixed ration. Straw bedding was added daily and completely cleaned out every 4 wk.

No-contact cows were housed in a separate barn, in a pen (21.6 ×7.8 m) of 12 cows including 8 experimental cows (4 per block) and 4 non-experimental cows. These cows had no visual or auditory contact with their calves. The pen contained 12 lying stalls equipped with mattresses and topped with sawdust daily, 12 computerized feed bins (Insentec B.V., Marknesse, The Netherlands) in which cows were fed a total mixed ration twice daily at 1030 and 2000 h (the same ration provided to full- and part-time cows). The pen was also equipped with an automated rotating brush.

All cows were milked twice daily in a double 12 parallel milking parlour and individual milk yield volumes were recorded at each milking using in-parlour milk meters (SAC A/S, S. A. Christensen & Co.). Full-time and part-time cows were milked together at 0500 and 1530 h (first milking round), and no-contact cows immediately followed at 0530 and 1600 h (second milking round). Full-time and no-contact cows always returned to their home pen after each milking, while part-time cows were sorted from the herd after each afternoon milking and housed in a dedicated pen in the same barn as no-contact cows, without visual or auditory contact with their calves. This pen contained 14 lying stalls each equipped with a mattress and topped with sawdust daily, and fresh *ad libitum* total mixed ration delivered at 2000 h at a feed bunk with headlocks. After morning milking, part-time cows returned to their home pen with their calves.

2.3. *Weaning treatment groups*

Weaning treatments were initiated at wk 8. Two days prior, the pens were divided with 2 cow-calf pairs on either side of the fence; the reduced-contact treatment was always on the side with the large calf creep to accommodate restricting calves to this area. Cows in the reduced-contact treatment experienced reduced contact time with their calves (and thus also the available time for nursing) over a two-week period from wk 8 to wk 10. During wk 8, calf contact time was reduced to approximately 50 % of the original allotted time: full-time cows now had only 9.5 h/d with their calves, between 1100 and 2100 h, excluding afternoon milking; part-time cows now had only 4.5 h/d with their calves, between 1100 and 1530. During wk 9, calf contact time was further reduced to approximately 25 % of the original allotted time: full-time cows now had only 4.5 h/d with their calves, between 1100 and 1530; part-time cows now had only 2 h/d, between 1100 and 1300. Outside of the permitted contact times, research staff restricted reduced-contact calves to the large calf creep by blocking the exits with a temporary fence that allowed visual and limited physical contact with the dam, but not suckling. Cows in the unchanged-contact treatment experienced no change in their allotted calf contact time from wk 8 to wk 10.

2.4. *Permanent separation*

At wk 10, full- and part-time cows were permanently separated from their calves at 1100 h. Calves were removed from their home pens and placed in separation group pens (maintaining the same treatment groups) in the same barn as their dams. Cows still had auditory contact with their calves, but no visual contact (although some visual contact was possible for 3 pens that were positioned across the alleyway from the separation group pens). Cows and calves remained in their separate pens for 3 d after permanent separation, after which the experimental period ended with the final oxytocin sample (described below).

2.5. *Saliva sampling and oxytocin analysis*

We measured oxytocin in saliva; thus, future reference to oxytocin herein refers to salivary oxytocin. Oxytocin was measured from saliva at two key intervals: before and after a nursing event in wk 8 (for full-time and part-time cows only, since no-contact cows were not permitted to nurse), and before and after permanent separation in wk 10 (for full-time and part-time cows, plus no-contact cows as a negative control). While gently restrained in the home pen (for nursing samples) or in head locks (for separation samples), saliva was collected using a cotton swab (SalivaBio Children's Swab, Salimetrics LLC., Carlsbad, CA, USA) held by metal forceps and placed in the cow's lower cheek for 30 s. The swab was re-introduced if the cow rejected it. The swab was then placed in a labeled collection tube (Swab Storage Tube, Salimetrics LLC., Carlsbad, CA, USA) and immediately placed on ice and later stored at −20°C.

To assess the oxytocin response of dairy cows to a nursing event, saliva was sampled in wk 8 on 3 consecutive days before and 15-min after an observed nursing event, from full-time and part-time cows only. At the time of first sampling, reduced-contact cows had experienced a 50 % reduction in calf contact time for 5 days, and unchanged-contact cows had experienced no change in calf contact time. In a concurrent experiment, cows were tested for their motivation to nurse their calves (Jensen et al., 2024a); cows were presented the opportunity to nurse their calves after a period of restriction (approximately 1 h for unchanged-contact cows and 12 h for reduced-contact cows). Cows were walked from their home pen to the testing apparatus where they could push through a weighted gate to reunite with their calf and nurse for 10 mins (if they chose to do so). We aimed to collect saliva from all cows before and after this expected nursing event, which occurred at approximately the same time on consecutive days for each cow, and during 0800 to 1100 h; each day, each cow was sampled at approximately the same time within this time window. A saliva sample was collected just before the cow was taken for the motivation testing ('before' nursing sample), and another saliva sample was taken 15 mins after the cow finished nursing her calf in the testing apparatus ('after' nursing sample). There are only a few studies on the time lag from event to peak oxytocin concentration in saliva (e.g. Jong et al., 2015; MacLean et al., 2018) so we selected 15 min to be similar to a previous study on cattle (Lürzel et al., 2020). If the cow did not nurse in the testing apparatus, she was returned to her home pen and reunited with her calf, where the pair were observed for a nursing event and an 'after' sample was taken 15 mins after the calf stopped to nurse (n = 52 of 127 'after' samples obtained). If the calf stopped and restarted to nurse, the time was reset (only occurred once for 4 cows).

To assess the oxytocin response of dairy cows to permanent separation from their calves, saliva was sampled from cows of all treatments before and over the first 4 d after permanent separation in wk 10. As a baseline, a saliva sample was taken 3 d before permanent separation for unchanged-contact cows, and from no-contact cows as a baseline negative control. Since reduced-contact cows were already experiencing a reduction in calf contact just prior to permanent separation, a comparable baseline between the reduced- and unchanged-contact treatments was necessary; the baseline sample for reduced-contact cows was taken at the end of wk 7, the day before weaning was initiated. Permanent separation from the calf occurred at 1100 h in wk 10, corresponding to the time that reduced-contact cows expected to be reunited with their calves. Saliva was sampled 2 h after permanent separation (at 1300 h), and each 24 h thereafter for 3 d (26 h, 50 h and 74 h samples). No-contact cows were also sampled at these times as a negative control. To ensure samples were taken as close to 1300 h as possible, full- and part-time cows were briefly moved to a pen equipped with headlocks for a maximum of 15 min where two researchers simultaneously sampled all cows while in the headlocks. At the same time, no-contact cows were sampled by a third researcher while cows were in headlocks in their home pen. It typically took 1 min to sample the cow from the start of restraint to obtaining the sample, so any stress associated with this procedure was not expected to affect oxytocin concentration in the saliva.

On each day after saliva collection, samples were centrifuged for 20 min at 4°C at 1500 x g, and the supernatant was stored in 4 × 250 μL aliquots in Eppendorf tubes. Aliquots were immediately frozen at −20°C, then air-transported on dry ice overnight to University of Veterinary Medicine (Vienna, Austria) for laboratory analysis. The measurement of oxytocin in bovine saliva has been validated by Lürzel et al. (Lürzel et al., 2020) for detection, parallelism, accuracy and recovery. Oxytocin concentrations were analyzed by ELISA using the Cayman Chemical Oxytocin ELISA kit (No 500440, Cayman Chemical, Ann Arbor, MI, USA). Each sample was processed with a solid-phase extraction (see Lürzel et al. (Lürzel et al., 2020) for detailed methods), and analyzed in duplicate on the same plate to avoid inter-assay variation. Intra-assay CVs (calculated from the two duplicate samples) was (mean ± SD) 3.0 ± 2.5 %; samples were re-run if the CV between duplicates was over 8 %.

2.6. Measures of the cow-calf bond and nursing duration

We predicted that the oxytocin response of cows at nursing and/or permanent separation may also depend on the strength of the maternal bond. This was measured with two approaches. In the first, we used an experimental method wherein the strength of the maternal bond between cow and calf was measured by testing the motivation of the cow to push an increasingly weighted gate to reunite with her calf; see Jensen et al., (Jensen et al., 2024b) for details on methodology. This resulted in a maximum 'price' that the cow was willing to 'pay', where pushing higher bars of pressure was considered to indicate a stronger maternal bond. Two part-time cows failed to complete the required training to use the weighted gate, so these cows did not have this measure of the cow-calf bond. The second approach was an observational method wherein the bond between cow and calf was measured as (1) the amount of time spent touching (defined as any form of physical contact;dam's head or muzzle is in within 5 cm of her calf's body, including direct contact, grooming her calf, but excluding nursing), and (2) the amount of time spent in close proximity (defined as the distance between any part of the dam's head and any part of her own calf is equal to or less than the body length of the calf, including physical contact but excluding nursing). Behaviors were recorded continuously from video on an undisturbed day, 3 d before the first nursing sampling day, using cameras installed above each pen (HIKVISION DS-2CD2345FWD-I (2.8 mm)- Pro Series, Hangzhou Hikvision Digital Technology Co., Ltd, China). The entire period of the day during which the cow was permitted to be with her calf was observed (total 24 h excluding milking times for full-time + unchanged-contact cows; total 10 h for full-time + reduced-contact and part-time + unchanged-contact cows; total 4 h for part-time + reduced-contact cows). Cows from all blocks except 2 and 7 were observed (due to error in video coding for block 2; time contraints did not allow block 7 to be observed), resulting in 16 full-time cows (8 reduced-contact and 8 unchanged-contact) and 19 part-time cows (11 reduced-contact and 8 unchanged-contact), with 16 female calves and 19 male calves. Behaviors were recorded by two observers (interobserver relability = 0.73) using BORIS recording software (Friard and Gamba, 2016), and then total time spent engaged in each behavior was summarized for each cow and calculated as a proportion of time that the cow had access to her calf.

The duration of the nursing bout occurring between the 'before' and 'after' saliva samples at nursing was extracted from the recorded video, either from cameras installed above the testing apparatus (for cows that nursed there) or from cameras above the home pen (for cows that did not nurse in the testing apparatus, but subsequently nursed when returned to their home pen with their calf). If two nursing bouts for the same cow were observed, the duration of each were recorded separately. Nursing was defined as the calf with its head underneath the dam's belly near the udder area, which may be preceded by the calf moving towards the udder with a stretched neck and ears back (camera angle did not permit observation of actual mouth contact with the teat).

2.7. Final sample sizes

One multiparous part-time cow was excluded from the study in wk 3 because her calf was euthanized due to serious illness; thus, the remaining three cow-calf pairs in the pen were all assigned to the reduced-contact treatment in wk 8 (no pen division was performed to allow sufficient space). One primiparous no-contact cow was excluded from the study in wk 2 due to an injury unrelated to the experiment.

For the saliva samples at nursing, of the available 55 cows in full- and part-time treatments, samples were obtained from 36 cows for all three nursing events on the three consecutive days, from 6 cows for two nursing events, and from 7 cows for one nursing event. No samples could be obtained from 4 cows because they did not nurse in the available time frame (2 full-time and 2 part-time cows, all unchanged-contact), and 3 cows were excluded from sampling due to a safety issue with sampling (1 full-time + unchanged-contact cow) or were not tested in the testing apparatus (1 full-time + unchanged-contact cow did not meet learning criteria; 1 part-time cow was excluded from the study).

Saliva samples at permanent separation were obtained from all available 82 cows in full-time, part-time and no-contact treatments. However, due to the expense of analyzing oxytocin saliva samples, we first analyzed samples from Block 1–5; after examination of the data and emerging patterns, the remaining full-time and part-time treatment samples from Block 6 and 7 were analyzed, but no further no-contact treatment samples were analyzed (since these cows did not experience a weaning treatment). This resulted in a final (analyzed) sample size of 74 cows (14 full-time + reduced-contact cows; 14 full-time + unchanged-contact cows; 15 part-time reduced-contact cows; 12 part-time unchanged-contact cows; and 19 no-contact cows).

2.8. Statistical analysis

All statistical analyses were performed using SAS Studio (OnDemand for Academics, SAS Institute Inc.). The outcome variable oxytocin concentration in each of the nursing and separation data sets was assessed for approximation of a normal distribution using PROC UNIVARIATE and examining model residuals; a $\log_{10}$ transformation was required for oxytocin concentrations in response to the nursing event. For models described below, the fixed effects of calf-contact treatment, weaning treatment, sample day and sample period (in the case of nursing samples) were always retained; interaction terms and covariates were removed from the model with backwards elimination if $P > 0.1$ Statistical significance was declared at $P \leq 0.05$ and tendency at $0.05 < P \leq 0.1$.

Nursing. A linear mixed model (PROC MIXED) tested whether oxytocin changed in response to a nursing event, and if this depended on calf-contact and weaning treatments, calf sex and cow parity. The outcome variable was the $\log_{10}$-transformed oxytocin concentration, and the fixed effects were: calf-contact treatment (full-time; part-time), weaning treatment (reduced contact; no change in contact), sample period (before nursing; after nursing), day (1; 2; 3), cow parity (primiparous; multiparous), and calf sex (male; female). Relevant interactions tested were: the 2 and 3-way interactions of calf-contact treatment × weaning treatment × sample period, and the 2-, 3- and 4-way interactions of calf-contact treatment × weaning treatment × cow parity × calf sex. Interactions were removed from the base model using backwards elimination if $P > 0.1$ (all were removed according to this criteria). The following covariates were then tested in the base model one at a time and removed if $P > 0.1$: duration of nursing bout (four cows had two nursing bouts in the sampling interval; this was not a large enough sample size to statistically control for, so the total duration of nursing bouts was used), sample time, sample interval (between before and after samples), and milk yield on the sample day (daily yield, AM yield and PM yield were each tested separately). The interaction of milk yield × calf sex was also tested at this stage, but not retained ($P > 0.1$). The resulting final multivariate model included: calf-contact treatment, weaning treatment, sample period, day, cow parity and calf sex. Random effects of cow and block were included, accounting for the repeated observations of sample period and day, modelled with a compound symmetry covariance structure. Type III sum of squares and between-within calculation method for degrees of freedom were used.

Permanent separation. A linear mixed model (PROC MIXED) tested whether oxytocin changed in response to permanent separation, and if this depended on calf-contact and weaning treatments, calf sex and cow parity. Daily milk yield, AM milk yield and PM milk yield were tested separately in each model and removed if $P > 0.1$. First, we tested whether the baseline oxytocin concentration (before permanent separation) differed among treatments; calf-contact and weaning treatments were combined into 5 levels because no-contact cows did not experience any weaning treatment (full-time + unchanged-contact; part-time + unchanged-contact; full-time + reduced-contact; part-time + reduced-contact; no-contact). The final multivariate model contained the fixed effects of treatment, cow parity and calf sex, with block as a random effect. Next, we verified that oxytocin concentration did not change over the 5-d sampling period for no-contact cows (who never experienced permanent separation). The final multivariate model contained fixed effects of day, cow parity, calf sex and PM milk yield, with cow and block as random effects, accounting for the repeated observations of day. These first two analyses showed that baseline oxytocin concentration did not differ across treatments, and that oxytocin concentration of no-contact cows did not differ over the sampling period (see Results); thus, we could test the oxytocin response to permanent separation in cows housed with their calves and weaned by reducing calf contact or not. Fixed effects included were calf-contact treatment (full-time; part-time), weaning treatment (reduced-contact; unchanged-contact), day (before; +2 h; +26 h; +50 h; +74 h), cow parity (primiparous; multiparous), and calf sex (male; female). Relevant interactions tested were: the 2-, 3- and 4-way interactions of calf-contact treatment × weaning treatment × cow parity × day; the 2-, 3- and 4-way interactions of calf-contact treatment × weaning treatment × calf sex × day; and the 2-way interaction of calf sex × parity. Interactions were removed from the base model using backwards elimination if $P > 0.1$. Milk yield on the sample day, and its interaction with calf sex, were then tested in the base model and removed if $P > 0.1$ (daily yield, AM yield and PM yield were each tested separately). The resulting final multivariate model included: calf-contact treatment, weaning treatment, day, calf sex, cow parity, AM milk yield, weaning treatment × calf sex, AM milk yield × calf sex and the 3-way interaction of calf-contact treatment × calf sex × day (2-way combinations were retained). Random effects of cow and block were included, accounting for the repeated observations of day, modelled with a first-order autoregressive covariance structure. Type III sum of squares and between-within calculation method for degrees of freedom were used. Interactions were explored using the SLICE statement to partition effects by contact treatment and calf sex (comparing each day to the before baseline sample, adjusting for multiple comparisons using Dunnett's test), and partition effects by day (adjusting for multiple comparisons among contact treatment × calf sex combinations using Tukey-Kramer test).

Influence of cow-calf bond measured as maternal motivation. Maternal motivation to reunite with her calf as a measure of the cow-calf bond was tested for a possible effect on oxytocin concentration around nursing or at permanent separation. The maximum weight (bars of pressure) pushed by the cow to reunite with her calf ranged from 0 to 10 bars; however, the sample size became low (n = 2) for the lower and higher bars, so maximum weight pushed was categorized into 3 levels of maternal motivation: 'weaker' (0–2 bars; first quartile; n=12 cows), 'intermediate' (3–6 bars; second quartile; n=30 cows) and 'stronger' (7–10 bars; third quartile; n=13 cows). Separate linear mixed models (PROC MIXED) were conducted for each of nursing and permanent separation events, with the fixed effects of maternal motivation classification (weaker, intermediate, stronger), calf-contact treatment, weaning treatment and their 2 and 3-way interactions; interactions were removed if $P > 0.1$. Additional covariates tested in the model and removed if $P > 0.1$ were: sample period (before or after nursing, for the nursing model only) and day (1, 2 or 3 for the nursing model; before, +2 h, +26 h, +50 h or +74 h for the separation model). Random effects of cow and block were included, accounting for the repeated samples (sample period and/or day), modelled with either a compound symmetry (nursing model) or first-order autoregressive (separation model) covariance structure. Type III sum of squares and between-within calculation method for degrees of freedom were used.

Influence of cow-calf bond measured as social behaviors. Another measure of the cow-calf bond, social behaviors between the cow and her calf ('time spent touching' and 'time spent in close proximity'), were tested separately for their possible effects on oxytocin concentration around nursing (variable $\log_{10}$-transformed) or at permanent separation (variable in original scale). Separate linear mixed models (PROC MIXED) were conducted for each of nursing and permanent separation events, including the fixed effect of behavior (time spent in close proximity or touching, expressed as proportion of available time with calf; thus, calf-contact and weaning treatment were already accounted for in this variable). Model structure followed that described above for maternal motivation using backwards elimination if explanatory variables or interactions were $P > 0.1$.

We also tested whether calf sex and cow parity influenced the proportion of time spent in close proximity and time spent touching (PROC MIXED). Fixed effects included calf sex, cow parity and their interaction, with random effects of cow and block. After backwards elimination ($P > 0.1$), the final model for time spent in close proximity contained only calf sex; the final model for time spent touching (variable $\log_{10}$-transformed) contained calf sex and parity.

3. Results

3.1. Nursing

We did not observe an increase in oxytocin concentration after a nursing bout (Table 1), although there was large individual variation in how oxytocin changed after each of the three nursing events (see Supplementary Material). Oxytocin concentration tended to increase over the three day sampling period, and tended to depend on weaning treatment, calf-contact treatment and calf sex (Table 1). Overall, cows with the most opportunity for calf contact tended to have higher oxytocin concentrations: (1) cows with full-time calf contact tended to have a higher oxytocin concentration around nursing compared to cows with only part-time calf contact, and (2) cows that experienced no

Table 1
Back-transformed least squares means and 95 % confidence limits of salivary oxytocin concentration (pg/mL) of dairy cows around nursing, depending on sample period (before vs. after each nursing event), sampling day, calf-contact treatment, weaning treatment and calf sex. Saliva was sampled on three consecutive days, before and after a nursing event between 0900 and 1200 h during week 8 of the experimental period; during this time, half of the cows experienced a 50 % reduction in calf contact (reduced-contact) or did not (unchanged-contact). Cows were managed with their calves either full-time (24 h of calf contact, excluding milking time) or only part-time (10 h of calf contact, excluding milking time) where cows were separated from the calves at nighttime.

Variable	Estimate	Confidence limits	F-value (df)	P-value[a]
Sample period				
Before nursing	202.4	172.2 – 238.0	0.18 (1,48)	0.67
After nursing	209.5	178.2 – 246.3		
Sample day				
Day 1	180.2	150.2 – 216.2	2.9 (2,76)	***0.06***
Day 2	211.4	176.9 – 252.6		
Day 3	229.4	191.2 – 275.2		
Calf-contact treatment				
Full-time	232.4	190.7 – 283.3	3.3 (1,44)	***0.08***
Part-time	182.5	150.7 – 221.0		
Weaning treatment				
Unchanged-contact	235.8	188.9 – 294.3	3.9 (1,44)	***0.06***
Reduced-contact	179.8	151.8 – 213.1		
Calf sex				
Female	184.0	152.2 – 222.4	2.8 (1,44)	***0.10***
Male	230.5	188.7 – 281.6		

[a] Values in bold and italics indicates statistical tendencies between 0.05 and 0.1

change in calf contact time tended to have higher oxytocin concentration around nursing compared to cows that experienced reduced calf contact time. In addition, cows with male calves tended to have higher oxytocin concentration compared to cows with female calves.

Proportion of total daily time together spent touching (in contact, inclusive of sniffing and grooming) as a measure of the cow-calf bond tended to be positively associated with oxytocin concentration around nursing (back-transformed estimate and confidence limits: 0.016 pg/mL (0.0001 – 2.37 pg/mL); $F_{1,31} = 3.04$; $P = 0.09$), but this was not the case for our other measure of the cow-calf bond (maternal motivation), or its interaction with calf-contact or weaning treatments ($F_{2,42} < 2.0$; $P > 0.15$).

Cows with female calves tended to spend proportionally more time in close proximity (33.3 ± 5.0 % vs. 22.9 ± 4.7 % of total time, respectively; $F_{1,29} = 3.7$; $P = 0.06$) and spent more time touching their calves (backtransformed means and CL: 3.3 (1.8 – 6.3) % vs. 1.8 (1.0 – 3.3) % of total time, respectively; $F_{1,28} = 4.3$; $P = 0.05$) than cows with male calves. Multiparous cows also tended to spend proportionally more time touching their calves than primiparous cows (backtransformed means and CL: 3.2 (1.8 – 5.8)% vs. 1.9 (1.0 – 3.6)% of total time, respectively; $F_{1,28} = 3.1$; $P = 0.09$).

3.2. Permanent separation

Baseline oxytocin concentration (before permanent separation) did not differ among each combination of calf-contact and weaning treatments (no-contact: 480.0 ± 69.1 pg/mL; full-time + unchanged-contact: 471.4 ± 79.1 pg/mL; full-time + reduced-contact: 419.2 ± 79.1 pg/mL; part-time + unchanged-contact: 479.9 ± 84.5 pg/mL; part-time + reduced-contact: 488.9 ± 76.1 pg/mL; $F_{4,61} = 0.13$; $P = 0.97$). Furthermore, no-contact cows (who did not have any calf contact and thus experienced no permanent separation) did not differ in oxytocin concentration over the 5-d sampling period corresponding to the permanent separation period experienced by full- and part-time cows (before: 454.4 ± 71.6 pg/mL; 2 h: 382.4 ± 71.6 pg/mL; 26 h: 504.7 ± 71.6 pg/mL; 50 h: 557.1 ± 71.6 pg/mL; 74 h: 443.9 ± 71.6 pg/mL; $F_{4,72} = 0.88$; $P = 0.48$).

There was a 3-way interaction of contact treatment, calf sex and day relative to separation affecting oxytocin response of cows to permanent separation from their calf (Fig. 1; $F_{4,201} = 3.9$; $P < 0.001$). This relationship tended to be driven by cows that had female calves, but in opposite directions for full- and part-time treatments. For full-time cows that had female calves, oxytocin concentration increased with time since permanent separation ($F_{4,201} = 2.8$; $P = 0.02$); compared to before separation, oxytocin was greater at 26 h ($t_{1,63} = 2.5$; $P = 0.04$) and peaked 74 h after separation ($t_{1,63} = 3.1$; $P = 0.01$). In contrast, for part-time cows that had female calves, oxytocin concentration tended to be more variable over the separation period ($F_{4,201} = 2.4$; $P = 0.06$), but pair-wise comparisons of day before separation to each day after revealed no significant differences. For cows that had male calves (both full- and part-time treatments), oxytocin did not significantly change over the permanent separation sampling period ($F_{4,202} < 0.62$; $P > 0.65$).

When examining differences at specific timepoints since separation, oxytocin concentrations tended to differ before permanent separation ($F_{3,201} = 2.4$; $P = 0.07$), and 26 h after permanent separation ($F_{3,201} = 2.3$; $P = 0.08$), but there were no significant pairwise differences among contact treatment × calf sex combinations after Tukey adjustment. Oxytocin concentrations differed at 74 h after permanent separation ($F_{3,202} = 3.9$; $P = 0.01$), where greater oxytocin concentration was observed in full-time cows that had female calves (versus had male calves; $t_{1,44} = 3.5$; $P = 0.01$), and tended to be greater in part-time cows that had male calves (versus female calves; $t_{1,44} = 2.4$; $P = 0.08$). There were no differences among contact treatment × calf sex combinations for oxytocin concentration at 2 or 50 h after permanent separation ($F_{3,202} < 1.0$; $P > 0.41$).

There was also an interaction of weaning treatment and calf sex on the oxytocin response of cows to permanent separation (Fig. 2; $F_{1,48} = 4.7$; $P = 0.04$). For cows that had male calves, oxytocin concentration was greater if they experienced reduced-contact versus unchanged-contact ($t_{1,48} = 5.5$; $P = 0.02$), while cows that had female calves had similar oxytocin concentration regardless of weaning treatment ($t_{1,48} = 0.5$; $P = 0.50$). For unchanged-contact cows, those that had female calves had greater oxytocin concentration over the separation period than those that had male calves ($t_{1,48} = 8.7$; $P < 0.01$).

Pre-separation maternal motivation as a measure of the cow-calf bond tended to affect oxytocin concentration at permanent separation

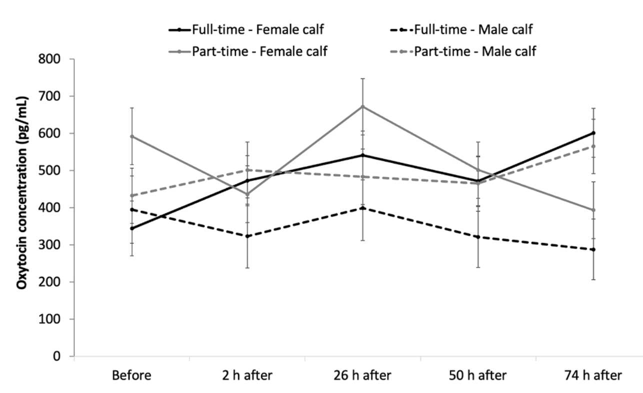

Fig. 1. Salivary oxytocin response (least squares means ± SE) of dairy cows to permanent separation from their calves at week 10 of the experimental period, depending on day relative to separation, calf-contact treatment and calf sex. Cows were managed with their calves either full-time (24 h of calf contact, excluding milking time) or only part-time (10 h of calf contact, excluding milking time) where cows were separated from the calves at nighttime. Color (black or gray) refers to calf-contact treatment, and line (solid or dashed) refers to calf sex.

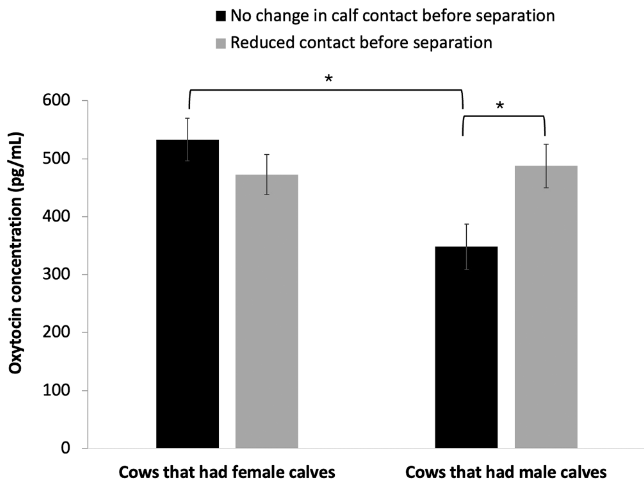

Fig. 2. Salivary oxytocin response (least squares means ± SE) of dairy cows to permanent separation from their calves at week 10 of the experimental period, depending on weaning treatment and calf sex. Values shown are the mean oxytocin concentration over the 5-d sampling period. Cows were weaned from their calves by either reducing contact time with the calf over a 2-wk period before permanently removing the calf (reduced-contact treatment) or by abruptly removing the calf (unchanged-contact treatment). Before separation, cows were managed either full-time (24 h of calf contact, excluding milking time) or only part-time (10 h of calf contact, excluding milking time) where cows were separated from the calves at nighttime. Asterisk indicates significant ($P < 0.05$) pair-wise differences.

depending on weaning treatment (Fig. 3; $F_{2,46} = 2.6$; $P = 0.08$). For unchanged-contact cows (i.e. did not experience reduced calf contact time prior to permanent separation) oxytocin concentration tended to increase with a stronger pre-separation maternal motivation (i.e. motivation to push increasing weights to reunite with the calf) ($F_{2,46} = 2.9$; $P = 0.07$). Time spent in close proximity or time spent touching as a measure of the pre-separation cow-calf bond (expressed as proportion of available time with calf) were not associated with oxytocin concentration in response to permanent separation ($F_{1,33} > 0.05$; $P > 0.59$).

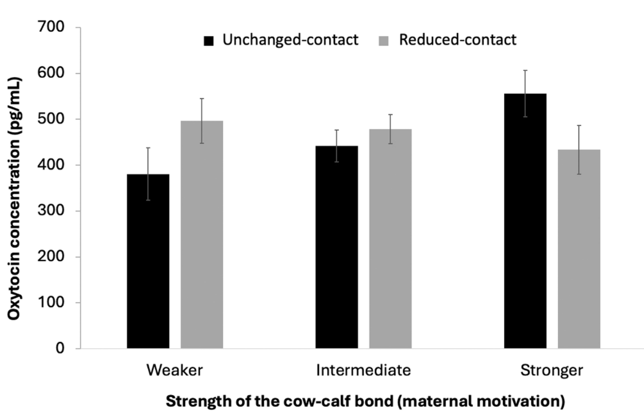

Fig. 3. The influence of the strength of the cow-calf bond on salivary oxytocin concentration (least squares means ± SE) in dairy cows during permanent separation from their calves at week 10 of the experimental period, depending on weaning treatment. Cows either experienced reduced calf contact time before permanent separation from the calf (reduced-contact) or did not (unchanged-contact). Cow-calf bond was measured as the maternal motivation of cows to reunite with their calf by pressing on an increasingly weighted gate, resulting in the maximum weight pushed (in bars of pressure) (see Jensen et al., 2024a). This was classified into 3 levels of motivation: weaker (pushed 0–2 bars), intermediate (3–6 bars) and stronger (7–10 bars).

4. Discussion

We examined changes in oxytocin concentration of dairy cows experiencing putatively positive (nursing of their calves) and negative (permanent separation from their calves) social situations, and how management practices and the cow-calf bond may affect these responses. As predicted, at nursing, oxytocin concentration generally tended to be greater in cows that had the most daily calf contact (full-time versus part-time; unchanged calf contact versus gradually reduced calf contact in the two weeks prior to separation). However, during permanent separation, changes in oxytocin concentration were influenced by factors including day relative to separation, daily calf contact, calf sex, and whether cows experienced reduced calf contact or not prior to separation. The cow-calf bond moderated the oxytocin response to both nursing and separation. These results highlight how management practices can affect the oxytocinergic system of dairy cows.

4.1. Nursing

Oxytocin plays a critical role in milk let-down (Bruckmaier and Blum, 1998), and its release is typically stronger during suckling than machine milking (de Passillé et al., 2008; Lupoli et al., 2001; Tancin et al., 2001). Although we expected oxytocin concentration to increase after a nursing bout, this was not observed, possibly due to our sampling time (15 mins post-nursing) missing the oxytocin peak. In plasma, peak oxytocin occurs shortly after nursing begins (Lupoli et al., 2001), but it is unclear when this peak occurs in saliva. However, cows with the most amount of calf contact tended to (full-time calf contact) or had significantly (unchanged contact) greater oxytocin concentration. This could be due to greater opportunity for nursing, leading to more teat stimulation (Uvnäs Moberg and Petersson, 2022). It also may reflect a stronger cow-calf bond, since oxytocin is thought to be responsible for eliciting and maintaining maternal behaviors between mother and young (Lee et al., 2009). Indeed, we found a tendency for a positive association between time spent touching (any physical contact between cow and calf) and oxytocin concentration around nursing. However, other studies in cattle (Geburt et al., 2015) and pigs (Valros et al., 2004) reported no association between physical contact and oxytocin concentration, and our other measure of bond strength, maternal motivation (measured as the willingness of cows to reunite with their calves), was not associated with oxytocin concentration around nursing. We also found that cows with male calves tended to have higher oxytocin concentrations, possibly because male offspring of ungulates tend to be larger (in the current study, males weighed about 7 kg more than females) and drink more milk, leading to more nursing time (Daleszczyk, 2004; Stěhulová et al., 2013; White et al., 2007); however, it was female calves that spent more time in close proximity and touching their dams, indicating a complex relationship between oxytocin, calf sex and maternal bonding behavior. It is likely that a combination of nursing time, social contact with offspring (apart from nursing) and lactation contribute to elevated oxytocin concentrations in dairy cows.

An alternative interpretation is that calf-contact and weaning treatment differences may be driven by *reduced* oxytocin in part-time and reduced-contact cows. Studies in rodents have explored the effects of repeated maternal separation on offspring, but rarely how this affects the dams (reviewed by Baracz et al., 2020a). One study found that repeated maternal separation in rats (6 h per day) reduced oxytocin-immunoreactive (IR) cells in the hypothalamus compared to shorter separation bouts (15 min per day) (Baracz et al., 2020b). This reduction could be due to the stress of prolonged separation, reversing pregnancy- and lactation-induced increases in oxytocin-IR cells. A similar mechanism may have occurred in our dairy cows experiencing periods of separation from their calves, but this requires further investigation.

4.2. Permanent separation

Weaning and permanent separation of dairy cows from their calves involves both physical and psychological stress, as it includes the cessation of suckling and the social loss of offspring (Weary et al., 2008). These processes are expected to affect oxytocin, which are involved in both milk letdown and the maintenance of the mother-young bond (Kendrick, 2000). Since these changes occurred simultaneously in our study, we cannot isolate which factor influenced oxytocin levels. However, this combination reflects typical management practices.

We observed changes in oxytocin concentration on the day of and in the days after permanent separation, depending on prior level of calf contact and calf sex. Cows that had full-time contact with female calves exhibited an increase in oxytocin concentration post-separation (3-way interaction of contact treatment, calf sex and day relative to separation), peaking at 74 hours. This aligns with previous findings showing that oxytocin can act as a buffer to the hypothalamic-pituitary-adrenal (HPA) stress response by reducing cortisol concentrations (Sutherland et al., 2012; Sutherland and Tops, 2014). Increases in oxytocin may also serve to facilitate social reinstatement. In our companion study, full-time cows showed more activity and 'searching' behaviors over the separation period compared to part-time cows, which are interpreted as efforts to seek contact with their calves (Neave et al., 2024). This evidence supports the dual role of oxytocin as both a stress-buffering mechanism and as a driver of social proximity seeking, which makes the interpretation of oxytocin response in such social contexts complex (Rault et al., 2017). Additional indicators of stress, such as cortisol and behavioral observations, may be beneficial to help distinguish oxytocin's role in stress buffering versus social motivation.

In contrast, cows with part-time contact with their female calves showed variable oxytocin responses. Tancin et al. (2001) also reported a decrease in oxytocin concentration in dairy cows separated from their calves after 6 days of restricted nursing (three times daily). This decrease in oxytocin occurred during milking, 10 mins post-separation, likely due to less udder fill from earlier periods of nursing. In our study, oxytocin was not measured during milking, and separation occurred midday (6 h after morning milking and 3 h before evening milking). The initial suppression of oxytocin post-separation (though not significant) may reflect hormonal adaptation to repeated separations that the part-time cows had experienced over the previous 10 weeks. Thus, at only 2 h after permanent separation, part-time cows might not have perceived this separation event as different from a typical day. This is supported by a study showing no plasma oxytocin increase after rodents experienced chronic repeated stressors (Hashiguchi et al., 1997). Our companion study (Neave et al., 2024) found that part-time cows spent more time lying and less time 'searching' on the day of separation compared to full-time cows, suggesting both behavioral and hormonal adaptation to repeated daily separations. This could explain the numerical oxytocin decline after its peak at 26 h post-separation, as cows likely realized they would not be reunited with their calves. Frequency of vocalizations also peaked at 26 h post-separation (Neave et al., 2024), a sign of distress and attempts at social reinstatement (Newberry and Swanson, 2008). Notably, Lürzel et al. (2020) showed a positive association between oxytocin concentration and vocalization frequency during a putatively positive human-animal interaction, highlighting the importance of context in interpreting oxytocin increases under social situations. Taken together, these results suggest that oxytocin appears to increase after permanent separation from the calf, likely in response to distress, but the previous experience of the cow (i.e., whether the cow was already familiar with daily separations and her expectations regarding timing of separation and reunion) appears to play a role in when oxytocin increases and if it is sustained.

Interestingly, the oxytocin response to permanent separation described above was only the case for cows with female calves; cows with male calves generally had a stable oxytocin concentration post-separation. This suggests that cows rearing female calves may be more distressed by separation than cows rearing male calves. This difference may reflect stronger maternal bonds with female offspring, as supported by other studies showing more intense mother-daughter bonding behaviors (Lidfors and Jensen, 1988; Viktor Reinhardt and Reinhardt, 1981). In support of this, we found cows with female calves spent more time in close proximity and physical contact with their calves before separation, compared to cows with male calves. This evidence of the cow-calf bond could explain the heightened oxytocinergic response in dairy cows separated from female calves.

Regarding our weaning treatment, cows that experienced a gradual reduction in calf contact did not show reduce the oxytocin response during separation compared to cows that had unchanged calf contact. This was unexpected, as gradual weaning methods like reducing cow-calf contact time are often hypothesized to weaken the maternal bond and ease the stress of separation (Wenker et al., 2022). Our results suggest that the two-week reduction in calf-contact time did not adequately mitigate the stress of permanent separation; our companion study supports this, wherein gradually reducing calf contact time did not reduce behavioral or vocal responses of cows to separation (Neave et al., 2024). Alternative weaning approaches, such as a fenceline weaning that allows for limited visual and/or limited physical contact (Johnsen et al., 2015) may better weaken the mother-young bond and reduce the oxytocin response to permanent separation, but this remains to be explored.

In summary, to our knowledge this is the first study to positively associate oxytocin concentration with measures of the maternal bond in a farm animal species (such as time spent touching; strength of motivation to nurse). However, this relationship was only observed in cows that experienced abrupt separation from their calf (i.e. unchanged calf contact before separation). Furthermore, of the unchanged-contact cows, those with female calves had greater oxytocin concentration post-separation than those with male calves. Together these results point to a pattern that oxytocin seems to be most responsive to mother-young separation in cows that reared female calves, in cows that experienced no change in calf contact time prior to separation, and in cows that were more strongly bonded to their calves.

The implications of our study are two-fold. First, dairy cows do seem to experience disruption to their oxytocinergic system after calf separation, consistent with evidence from studies in rodents and humans showing behavioral and physiological consequences following sudden partner loss, including depressive- and grief-like symptoms (Bosch and Young, 2018; Pohl et al., 2019). Second, management practices that limit cow-calf contact, such as only part-time contact or reducing calf contact over two weeks before separation, appear to contribute to oxytocin disruptions. Although reduced contact was expected to weaken the cow-calf bond and alleviate distress responses, the age of separation was far earlier than the 'natural' weaning age of 10 months (V. Reinhardt and Reinhardt, 1981), which may explain the strong negative physiological and behavioral responses observed. Careful consideration of weaning and separation methods is crucial to minimizing both physiological and behavioral stress of dairy cows.

4.3. Study limitations and future directions

The paucity of research on oxytocin's role in social situations for dairy cows and the complexity of our findings makes interpretation of our results challenging. While the behavioral indicators from our companion study were helpful, additional physiological measures of stress, such as cortisol, could have aided interpretation of results. Most studies referenced herein measured oxytocin from plasma, with few studies measuring oxytocin in saliva, which offers a non-invasive alternative for assessing stress and welfare (Rault et al., 2017). Future studies should explore how different sources of oxytocin respond under different affective social situations.

High individual variation in oxytocin concentrations, combined with our limited sample size, may have led to fewer statistically significant results. Individual differences in how cows perceived and responded to our putatively positive (nursing) and negative (separation) social situations, or in nursing motivation after a short period of calf contact restriction may have affected oxytocin concentration and contributed to individual variability. We did not specifically design our study to assess the influence of calf sex on oxytocin, though it emerged as an important factor. Other variables, like genetics, salivary rate, and oxytocin secretion dynamics, could explain the observed variability. Future research should consider these factors in study design.

The timing and conditions of sampling likely influenced our findings. The timing of peak oxytocin concentration in saliva after an event, relative to its peak in blood, is necessary to understand as no studies have investigated the dynamics of oxytocin release in saliva following a stimulus. Unlike previous research where oxytocin was measured during milking or nursing (Lupoli et al., 2001), we sampled before and after these events to avoid variation associated with milk let-down. Furthermore, the degree of separation between cow and calf (e.g. auditory but not visual contact) may have amplified stress in our study (Marchant-Forde et al., 2002). Future studies should explore the effects of complete sensory separation, such as moving calves to a different barn, which is a more common practice (Stěhulová et al., 2008).

Finally, the role of oxytocin as an indicator of emotional state in animals remains uncertain. Although there tended to be higher salivary oxytocin concentration in cows with more calf contact, suggesting a positive experience, oxytocin may also help regulate physiological homeostasis (Quintana and Guastella, 2020; Rault et al., 2017). Emotions themselves are often considered integral to maintaining allostasis, coordinating both internal and external processes. Oxytocin may be involved in maintaining homeostasis in response to varying stimuli, including both socially positive and challenging situations that were tested in our study. This perspective may explain the variability in oxytocin responses observed in our study, where oxytocin release could be influenced by both emotional and homeostatic factors. Future research should aim to disentangle the affective and allostatic roles of oxytocin in dairy cattle to better understand the hormone's response to different social situations.

5. Conclusions

Cows with the most opportunity for calf contact (housed full-time with their calves, and with unchanged calf contact before permanent separation), and cows that reared male calves, tended to have higher salivary oxytocin concentrations around nursing, possibly owing to greater nursing frequency. Oxytocin response of cows experiencing permanent separation from their calves was complex and influenced by the level of cow-calf contact, calf sex and weaning method. Overall, it appears that management practices that limit cow-calf contact may disrupt the oxytocinergic system of cows, which may affect how animals cope with separation stress. Given oxytocin's role in facilitating social behaviors and modulating the stress response, further work is necessary to explore how management practices can be designed to ensure physiological and psychological well-being of dairy cows.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.applanim.2024.106429.

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