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Dairy cows' responses to 2 separation methods after 3 months of cow-calf contact

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ABSTRACT

Weaning and separation remain among some of the biggest challenges for cow-calf contact systems, making the development of practical and low-stress separation methods mandatory for future success of these systems. This study aimed to compare behavioral and physiological responses of dairy cows to separation from their calves after 3 mo of full-time contact, with either the 2-step method (NF, 2 wks full-time contact while calves wore a nose flap, 1 wk fence-line contact before total separation, n = 18) or by gradual reduction of contact time between cow and calf (GR, 1 wk half day contact, 1 wk morning contact, 1 wk fence-line contact before total separation, n = 18). Vocalizations and searching behavior were observed on 4 d/wk from 1 wk before separation until 1 wk after total separation. During the same period, lying behavior and rumination time was automatically assessed via accelerometers and pressure sensors. Fecal and blood samples were collected twice per week from day −1 until +23 relative to separation start for analysis of fecal cortisol metabolites and the immune response. Milk yield in the parlor was continuously recorded. Statistical analysis was conducted using linear mixed effects models. We found no difference between the 2 separation methods in any of the examined behavioral and physiological response variables. However, a significant increase in vocalizations and searching behavior compared with baseline was present with both methods. Furthermore, there was a transient increase in physiological distress markers and a short-lived retention of milk yield at initiation of treatments, indicating that both methods induced distress for the cows. Descriptively, there were large interindividual differences between cows as well as a different temporal distribution in occurrence of behavioral responses, as GR cows vocalized most frequently during the week with fence-line contact and after total separation from the calf, while NF cows reacted strongest during the 2 wk while calves wore the nose flap. Milk yield was higher in NF than GR cows during the 2 wk while GR calves had time-restricted access to their dams and NF calves were prevented from suckling, but showed no difference afterward. However, similar evening milk yields of GR cows in the weeks with half-day and morning contact, indicated that the weekly reductions in contact time worked in a rather stepwise than gradual manner, which warrants further improvement of the GR method. Taken together, results showed that cows experienced distress during separation with the GR method, when implemented over 3 wk in 3 steps, as well as with the NF method, but differences between individuals were considerable.

Key words: cow-calf contact systems, gradual separation, stress, cows

INTRODUCTION

Dairy cow-calf contact (**CCC**) systems, which allow contact between calves and their dam or a foster cow for an extended time period (Sirovnik et al., 2020), have received increasing interest and demand from society (Placzek et al., 2021; Sirovica et al., 2022), scientists (Nielsen et al., 2023; Cook and von Keyserlingk, 2024), and farmers (Bertelsen and Vaarst, 2023; Hansen et al., 2023). Dairy cows are highly motivated for contact with their calf (Wenker et al., 2020) and form strong maternal bonds irrespective of whether they are kept with full-time or part-time contact to their calf (Jensen et al., 2024). The need for breaking this cow-calf bond later during weaning and separation is one of the biggest challenges for CCC systems from a welfare perspective (Johnsen et al., 2016) and was also reported as a main reason to discontinue this practice by Norwegian farmers (Hansen et al., 2023). Even at organic farms practicing CCC, wean-

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The list of standard abbreviations for JDS is available at adsa.org/jds-abbreviations-24. Nonstandard abbreviations are available in the Notes.

ing and separation is typically done earlier, on average when calves are 17 wk old (for Europe; Eriksson et al., 2022), than under natural conditions when the termination of suckling takes place at ~8 to 12 mo (Reinhardt and Reinhardt, 1981a). In addition, weaning is naturally not accompanied by separation, as dams form grazing and licking associations with their offspring that remain constant for several years (Reinhardt and Reinhardt, 1981b). It is therefore crucial to find practical and low-stress solutions for the weaning and separation process in CCC systems that consider the welfare of both the calves and their dams equally, to ensure high levels of welfare for the animals.

A promising separation method could be to gradually reduce the contact time between cow and calf. More so than other methods implemented in practice, this resembles the gradual decline in suckling bouts with increasing age of the calf during natural weaning (Reinhardt and Reinhardt, 1981a) and has additionally the potential that the pairs can habituate to periods of separation (Weary et al., 2008). In our previous publication we have compared the stress responses of 3-mo-old dairy calves during such a gradual weaning and separation process, where the contact time between cow and calf was reduced in 3 steps over 3 weeks, to a 2-step separation method with nose flaps that prevented the calves from suckling milk from the cow before they were separated (Vogt et al., 2024b). This study revealed that the employed gradual weaning and separation method was overall less stressful for the calves compared with the 2-step separation method with a nose flap, foremost allowing a more gentle physiological adaptation to the dietary change (Vogt et al., 2024b). However, it is unknown, if a gradual method is equally favorable for the dams, when compared with 2-step separation. Although a gradual reduction in contact time is more similar to the aforementioned naturally occurring slow reduction in nursing frequency of the calf (Reinhardt and Reinhardt, 1981a), it is also possible that the externally induced, repeated changes in opportunity for CCC could cause considerable distress for the cows due to multiple disruptions of established routines. At least in dairy cows tested at 1 mo after calving, it has been shown that cows, which could only reunite with their calves between morning and afternoon milkings, were in a more negative emotional state during a judgment bias test, compared with cows that had full-time contact to their calves (Neave et al., 2024b).

On the contrary, a 2-step separation process allows dams to keep largely unchanged contact to their calf during the first step while the calf is wearing the nose flap, at least theoretically, as this may depend whether it is cow- or calf-driven contact (defined in Sirovnik et al., 2020). In either case, the absence of nursing might reduce their motivation to reunite with the calf (Wenker et al., 2020, 2021) during the second step of complete separation. In support of this, studies in beef cows showed that 2-step separation with nose flaps leads to decreased vocalizations, pacing and walking behavior, but to increased grazing duration, after complete separation from calves compared with abrupt separation (Haley, 2006a; Ungerfeld et al., 2015), even when the beef suckler calves were weaned as early as 60 d of age (Ungerfeld et al., 2016). Similarly, dairy cows separated from their 5-wk-old calves (Haley, 2006b) or 12-wk-old foster calves (Loberg et al., 2007) show reduced behavioral responses to complete separation from their calves with 2-step weaning compared with abrupt weaning. Nonetheless, these cows show a slight behavioral response to prevention of nursing by their calves through nose flaps, but this to a lesser extent than at the time point of complete separation (Haley, 2006a; Ungerfeld et al., 2016). Additionally, there are reports that nonsuckled dairy cows that had only partial contact, meaning restricted contact during which nursing and some affiliative interactions are hindered (defined in Sirovnik et al., 2020), to their calves with udder nets show reduced motivation to reunite with their calf (Wenker et al., 2020) as well as reduced vocalizations during fence-line separation from their calves (Johnsen et al., 2018) compared with suckled cows. Taken together, these studies suggests that dams could benefit from the impeded nursing possibility during a separation process before total removal of their calves.

That said, results of the calves from the dams of this study and studies by other authors clearly show a negative effect of 2-step separation with nose flaps on weight gains (Boland et al., 2008; Enríquez et al., 2010; Wenker et al., 2022), nasal tissue integrity (8 mo old calves, Valente et al., 2022; 2 mo old calves, Wenker et al., 2022; 7 mo old calves of different weights, Kirk and Tucker, 2023; 3 mo old calves, Vogt et al., 2024b), and adaptation of the gastro-intestinal tract (Vogt et al., 2024b), as well as the emotional state (i.e., frustration, Vogt et al., 2024b) of the calves. Thus, even if we acknowledge the high practicability of 2-step separation with nose flaps especially for pasture-based systems, we now generally do not encourage this method as a welfare-friendly alternative for weaning and separation in dairy CCC systems with regard to calf welfare (Vogt et al., 2024a,b). Nonetheless, a detailed comparison of dairy cows' reactions to gradual separation from their calf to 2-step separation with nose flaps is still lacking and can enhance our understanding of the particular parts of the separation process that are afflicted with a high stress load for the dam, so that these can be avoided.

The aim of our study was therefore to compare behavioral and physiological responses of dairy cows indicative of distress (as defined by Moberg, 2000) during the process of separation from their calves either via gradual reduction of contact time between the cow and her calf or via 2-step separation using a nose flap for the calves. A secondary objective was to examine the effect of these separation methods on changes in social (i.e., affiliative and maternal) behaviors between cow and calf, as well as on milk yield of the cows over the course of the process. Based on previous results outlined above, we hypothesized that cows might show an overall reduced distress response when separated from their calves via the 2-step method compared with a gradual reduction in contact time to the calf.

MATERIALS AND METHODS

The study took place at the Thünen Institute of Organic Farming, Germany, from November 2019 till March 2020. All experiments were performed in accordance with the German Animal Welfare Act (Federal Republic of Germany, 2020; animal experiment number V244-51520/2019, MELUND Schleswig-Holstein).

Animals, Housing, and Feeding

Thirty-six dairy cows (mean age ± SD: 4.5 ± 2.0 years, range 2.2–9.4 years at start of the separation process; see Supplemental Table S1 for more details; see Notes) that nursed their own calf were included in the study. The required sample size was calculated a priori using G*Power 3.1 (Faul et al., 2007) for an assumed large effect ($f = 0.5$) with an acceptable α-error of 5% and β-error of 10%.

All cows were of the German Holstein breed, but lived separately in 2 different herds according to their horn status: 19 cows were from the horned herd and 17 cows belonged to the hornless (polled) herd. From the latter, only 12 cows were truly hornless, because the polled herd was in a transition phase from formerly horned into a completely genetically polled herd and the 5 animals that had horns were remaining older cows. The size of the cow herd varied from 45 to 49 cows (horned herd) and 43 to 48 cows (polled herd) throughout the experiment, due to integration of primiparous animals or culling and selling of cows.

The 2 herds were housed in identically mirrored parts of the same open-sided freestall barn (see Wagner et al., 2012 for a scheme of the barn). Each herd had a lying area that was separated into 50 straw-bedded cubicles (128.0 m^2 plus additional 64.0 m^2 headspace), a feeding area with a feeding rack (237.5 m^2), as well as a rubber-coated walking area (298.0 m^2) with one cow brush. The feeding area of each herd was divided into 2 different partitions, one for the early-lactating animals and one for the mid- to late-lactating animals. Access to the feeding partitions was automatically controlled via transponders in the cows' collars. Cows were fed with a TMR consisting of grass silage, corn silage, concentrate feed in the form of coarse grain, cattle salt, and mineral feed. The TMR was freshly provided twice per day after milking, which lasted approximately from 05:15 to 08:30 a.m. and 3:30 to 6:45 p.m. Water was provided ad libitum.

Cows calved in individual calving pens, where cow and calf stayed together for approximately 5 d to allow the cow-calf bond to establish. Afterward, cows were returned to the herd (or in case of primiparous cows introduced into the herd) and the calves were placed in a calf area that was directly connected to the cow area of the respective herd via an automatic selection gate. Until the weaning and separation process started, calves could enter the cow area at any time with exception of the milking hours, thus the pairs were kept with calf-driven, full, whole-day CCC (after Sirovnik et al., 2020). Within the cow area calves could share the lying and walking space of cows, but could not enter the cows' feeding area. Because cows were integrated into the herd ~5 d after calving, all experimental cows had been part of their herd for ~3 mo when the separation treatments began.

Treatment Groups

The separation process started when the related calf of a cow reached the age of 3 mo (mean ± SD: 93 ± 6.9 d at treatment start). Half of the cow-calf pairs was assigned to the 2-step weaning and separation method using a nose flap for the calves (**NF**, n = 18 cow-calf pairs), the other half of the cow-calf pairs was separated via gradual reduction of daily contact time between the pair (**GR**, n = 18 cow-calf pairs). Both separation treatments took place over a 3 week period (see Figure 1) before cow and calf were totally separated by moving the calves to the youngstock herd in a different barn located ~30 m away.

In the NF treatment, calves were equipped with the nose flap (Quiet wean, JDA Livestock Innovations, Canada) for 2 wk, allowing the cow theoretically whole-day contact to her calf, whenever it entered the cow area (except milking hours), without the possibility of nursing. After these 2 wk, the nose flap was removed and calves remained in the calf section for one more week, that is, the cows had one more week with only fence-line contact to their calves via the selection gate and the pen boundaries. This still allowed visual, auditory, olfactory, and limited tactile contact between the cow-calf pairs without the possibility for nursing. After these 3 wk of partial separation, cow and calf were totally separated by moving the calves to the youngstock barn. Once calves were in the youngstock barn, cows could still have some very limited auditory contact to their calf, but no further types of contact were possible.

In the gradual reduction treatment, the CCC time was reduced from full-time contact preweaning to half-day

Day relative to treatment start	-7	-6	-5	-4	-3	-2	-1	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
								BEGINNING OF PARTIAL SEPARATION ↓																					TOTAL SEPARATION ↓						
NF Treatment	Full time cow-calf contact							Full-time contact to calf with nose flap														Fence-line contact							Calf moved to Youngstock barn						
GR Treatment	Full time cow-calf contact							Half-day contact (8:30 a.m. - 3:30 p.m.)							Morning contact (08:30 a.m. - 12:00 p.m.)							Fence-line contact							Calf moved to Youngstock barn						
Fecal samples							X			X				X			X				X			X				X			X				
Blood samples							X			X				X			X				X			X				X							
Extra health check			X				X			X				X			X				X			X				X			X				
Behavioral observation	X	X	X	X				X	X	X	X				X	X	X	X				X	X	X	X				X	X	X	X			
Lying & rumination time measurement, milk yield	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X

Figure 1. Sequence of the separation process within the 2 separation methods and an overview of sampling time points for all assessed indicators. Treatments: Cows were separated from their calves via the 2-step method using a nose flap for the calves (NF; n = 18). Cow-calf pairs were separated by gradual reduction of cow-calf-contact time (GR; n = 18). Fence-line contact = limited contact of cow and calf through a selection gate and the pen boundaries without the possibility for nursing. Total separation = Calves were moved to the youngstock herd in another barn. Extra health check = detailed health check done by the experimenter in addition to the routine health check that was done daily by the barn personnel.

contact in the first week of the separation process. During the week of half-day contact, calves could enter the cow area between milkings during the day (~7 h, ~8:30 a.m.–3:30 p.m.). During the evening and night the calves had no access to the cow herd. After this week of half-day contact, the CCC time was further reduced to ~3.5 h per day, from the end of morning milking until noon. Following this week of morning contact, cows had one more week of fence-line contact to their calf through the pen boundaries as in the NF treatment. After these 3 wk of partial separation, the pair was totally separated by moving the calf to the youngstock barn. Access of calves to the cow section was automatically controlled via transponders in the calves' collars during the whole process.

Allocation of cow-calf pairs to the 2 treatments was balanced for sex of the calf (male/female) and herd affiliation of the cow-calf pair (horned/polled). An overview of attributes of cows and calves assigned to the 2 treatments can be found in Supplemental Table S1. The weaning age of the calves was staggered by 2 wk in both treatments to counterbalance the effect of the later milk loss in the GR treatment (milk loss at wk 3) compared with the NF treatment (milk loss at wk 1, described in detail in Vogt et al., 2024b).

Data Collection

A combination of behavioral and physiological indicators was used to evaluate the distress response of cows to the 2 separation methods. In addition, social behaviors between cow and calf were recorded to assess changes during the separation process that could be indicative of a reduced intensity of the cow-calf bond. Data from the milking parlor were complementarily collected to evaluate the effect of the 2 separation methods on changes in milk yield.

Data collection began in the week before the first calf reached the required weaning age (November 2019) and was terminated one week after total separation of the last cow-calf pair (March 2020), totaling 18 wk. Within this study period, each cow was sampled for a duration of 5 wk, including 1 wk before separation, the 3-wk partial separation phase, and the first week after total separation from the calf. An overview of all assessed indicators with the specific collection time points can be found in Figure 1.

Direct Behavioral Observation

Cows' behavioral responses (see Table 1 for definitions) to the separation procedure were observed directly between days −7 until +24 relative to treatment start. Details on the execution of behavioral observations can be found in Vogt et al. (2024b), because cows were observed simultaneously with their calves. In brief, observations were conducted always by the same observer on 4 consecutive days per week at (1) 2 h following morning milking, (2) 1 h before evening milking and (3) 2 h following evening milking. The observer switched between the 2 herds for observation every 30 min, because only one side of the barn (i.e., one herd), was visible to the observer at a time. This resulted in a total observation time of 2.5 h per day per cow. For behaviors observed with the scan sampling method (see Table 1), the number of scans in which the specific behavior was seen was calculated as percentage from all scans where the animal was visible to the observer, because sometimes experimental cows were hidden by other animals of the herd.

Because calves reached the required weaning age of 3 mo at different weeks within the study period, there was a maximum of 6 cow-calf pairs plus 3 cows whose calves had just been moved to the youngstock barn

Table 1. Ethogram of assessed behaviors

Behavior	Definition	Indicator classification	Mode[1]	Recording rule
Vocalization	Cow produces a clearly audible sound through the mouth (Ungerfeld et al., 2016), except coughing.	Separation distress	Direct	Continuous (frequency)
Searching behavior	Cow is moving parallel to and within 1 m of the pen partition up and down, or is standing at the selection gate or beside the pen partition (radius 2.5 m) with head elevated and eyes and ears focused in the direction of the calf's section and scanning (adapted from Enríquez et al., 2010; Ungerfeld et al., 2016).	Separation distress	Direct	Interval
Lying behavior	Recorded with IceTag leg-mounted sensors (validated for use in dairy cows by Trénel et al., 2009; Mattachini et al., 2013) containing a tri-axial accelerometer. Lying behavior (i.e., when the sensor is horizontal) is automatically calculated by the sensor's algorithm from a specific combination of acceleration forces in the 3 different planes (Trénel et al., 2009; Högberg et al., 2020).	Separation distress	Auto	Logging rate: 8 Hz
Lying bouts	Recorded with IceTag leg-mounted sensors (validated for use in dairy cows by Trénel et al., 2009; Mattachini et al., 2013) containing a tri-axial accelerometer. Lying bouts (i.e., the period between when the sensor is changing from vertical to horizontal and then back to vertical) is automatically calculated by the sensor's algorithm from a specific combination of acceleration forces in the 3 different planes (Trénel et al., 2009; Högberg et al., 2020).	Separation distress	Auto	Logging rate: 8 Hz
Rumination activity	Recorded with RumiWatch noseband pressure sensors (validated for use in dairy cows by Ruuska et al., 2016; Zehner et al., 2017) as >30 jaw movements/min with a minimum of 3 min duration as defined by the sensor's algorithm (Zehner et al., 2017).	Separation distress	Auto	Logging rate: 10 Hz
Nursing of the own calf	The cow's own calf has a teat of the dam in the mouth and makes sucking movements with the mouth or calf is butting the udder with the muzzle (adapted from de Passillé et al., 1992; Ungerfeld et al., 2016).	Maternal behavior	Direct	Interval
Cow-calf interaction	Cow is licking any body part of the calf or calf is licking any body part of the cow (tongue touches cow/calf), nose-calf-contact by the dam or nose-cow-contact by the calf (nose touches any body part of cow or calf), calf rubs its head against any body part of the cow (adapted from Jensen, 2011; Geburt et al., 2015; Johnsen et al., 2015).	Affiliative behavior	Direct	Interval
Social play of cow and calf	Cow and calf stand facing each other, pushing, rubbing or butting head against head/neck without force, often including rotating head movements (adapted from Jensen et al., 1998).	Affiliative behavior	Direct	One-zero

[1]Direct = direct observation, Auto = automatic recording with the use of sensors, Continuous = continuous behavior sampling, Interval = scan sampling every 3 min, One-zero = one-zero sampling every 3 min (i.e., presence [1] or absence [0] of the behavior within a 3 min sampling interval).

(n = 15 animals) in the horned herd and a maximum of 7 cow-calf pairs plus one cow whose calf had just been moved to the youngstock herd (n = 15 animals) in the polled herd that were simultaneously at any stage of the separation process within a week and needed thus to be observed simultaneously within an observation period.

Lying Times and Lying Bouts

Daily lying times and number of lying bouts of 26 of the 36 cows (13 per treatment, due to sensor availability) were automatically recorded with Ice Tag leg-mounted sensors (Ice Robotics Ltd., Edinburgh, UK; for validation studies and logging rate see Table 1). The IceTag sensors were attached on the lateral side, just above the fetlock, of the left hind leg of the cow from day −14 until +27 relative to the start of the separation process, but days −14 to −8 were taken as a habituation period for the cows and thus not analyzed. Also, the day of removal of the sensor (day +27) was omitted from the data set due to incomplete data for this day. The data were downloaded with IceManager 2010 software (Version 1.006, Ice Robotics Ltd., Edinburgh, UK) and then exported as summary per day into Microsoft Excel (Microsoft Corporation). From the total lying time per day in hours, the percentage of lying time per day was calculated. Lying bouts shorter than 4 min were considered as erroneous readings and deleted with an event filter from the raw data recorded by the IceTag sensor according to the threshold determined by Tolkamp et al. (2010).

Rumination Time

For assessment of the daily rumination time 30 of the 36 cows (NF = 14, GR = 16 due to sensor availability) were fitted with a RumiWatch noseband pressure sensor (Itin+ Hoch GmbH, Liestal, Switzerland; for validation studies and logging rate see Table 1) from day −14 until +27 relative to the start of the separation process. Again, days −14 to −8 were taken as a habituation period for the cows and not analyzed. Day of removal (day +27) or days of necessary replacement of the sensors were also omitted from the data set due to incomplete data for those respective days. Detailed information on the RumiWatch system can be found elsewhere (Ruuska et al., 2016; Zehner et al., 2017), but in brief, the sensor collects data of accelerations of the pressure tube located within the noseband, that is, pressure changes. The recorded pressure changes were processed with RumiWatch Manager 2 (Version 2.1.0.0.) and then converted into daily rumination time with RumiWatch Converter (Version 0.7.4.13, Itin+ Hoch GmbH, Liestal, Switzerland; output as summary per day). In a last step, values were manually converted from minutes to hours per day.

Fecal Cortisol Metabolites (FGCM)

Fecal samples of the cows were collected twice per week between days −1 and +23 relative to the start of the separation process. Sample collection always took place at ~07:30 a.m. (±60 min) in the morning, when cows returned from the milking parlor and were restrained in the feeding rack. Samples were collected directly from the cow's rectum with a gloved hand, homogenized in the hand, and transferred into a commercial fecal sampling tube. In case that the minimum amount of 5 g of feces could not be collected at first attempt, the experimenter returned to the cow at the end of sample collection before cows were released from the feeding rack for a second attempt. Fecal samples were extracted and analyzed with an 11-oxoetiocholanolone enzyme immunoassay (EIA, lab code 72a) as described previously (Palme and Möstl, 1997). This EIA measures 11,17-dioxoandrostanes, a group of fecal cortisol metabolites, and has been successfully validated for use in cows (Palme et al., 1999).

Hematologic Variables

For analysis of the total neutrophil (N) and lymphocyte (L) number and calculation of the N:L ratio, blood samples of cows were obtained twice per week between days −1 and +20 relative to start of the separation process, before collection of fecal samples. A qualified experimenter collected 2 mL of blood via puncture of the coccygeal (tail) vein into EDTA-coated (K EDTA) sampling tubes (S-Monovette by Sarstedt). Details on further handling and processing of blood samples can be found in Vogt et al. (2024b). Blood sampling was not possible in 2 cows (NF = 1; GR = 1).

Milk Yield

Cows were milked twice per day (5:15–8:30 a.m. and 3:30–6:45 p.m.) in a 2 × 4 tandem parlor (GEA Farm Technologies GmbH, Bönen, Germany). For technical characteristics of the milking parlor see Barth (2020). Milking routine started with manual stripping of the udder followed by cleaning of the teats with a fabric towel and attachment of the cluster. Milk yield was recorded per milking event using the herd management software DairyPlan C 21 (GEA Farm Technologies GmbH, Bönen, Germany) and then exported into Microsoft Excel (Microsoft Cooperation).

Data Analysis

Exclusion Criteria. Data points from sampling days at which cows showed clinical signs of severe sickness, or when cows were observed to be in estrus or when calves of the cows erroneously gained access to the cow area during periods in which they were supposed to be separated from the cows (hereafter called "illegitimate contact") were excluded from analysis. Examination of the health condition of cows was done twice per week at days of blood sampling. Assessment included scoring of lameness, nasal and ocular discharge, coughing, fecal consistency, and lesions and abrasions, as well as swelling of the joints. Additionally, information on udder infections was added based on reports from the milking personnel. Health scoring was done with a 0-1-2 system, in which 0 equaled a normal condition and a 2 equaled a severe symptom, for example, severe diarrhea, severe lameness, or infected lesions. In case that a cow was identified with a severe symptom, that is, scored a 2 in any of the health indicators on a specific day, or was treated with medications by the barn personnel or needed veterinary care that day, the cow was completely excluded from analysis for this day as well as the preceding and subsequent day.

Additionally, data points from days on which cows were identified to be in proestrus or estrus were deleted from the data because this can considerably affect their activity and behavior. For example, it has been reported that during estrus rumination time is reduced by 17% (Reith and Hoy, 2012) and cows may not lie down for 6 to 17 h (Brehme et al., 2008). Classification of proestrus or estrus state were done by observations of "(attempting to) mount another cow" and "standing to be mounted by another cow" (reviewed in Reith and Hoy, 2018) through the experimenter or the barn personnel in addition to automatic detection of increased activity via pedometers (GEA Farm Technologies GmbH, Bönen, Germany, described by Roelofs et al., 2005).

Details on determination of days with illegitimate contact between cows and calves can be found in Vogt et al. (2024b). In brief, illegitimate entrances of the calves into the cow area were monitored with video cameras and from these videos the percentage of time a calf spent illegitimately within the cow area (from the total time a calf had no entitlement for access to the cow area) was calculated. All collection days (for physiological indicators and automatically assessed behavioral indicators) and behavioral observation intervals (for the directly observed behavioral indicators) at which calves spent >2% of time illegitimately within the cow area were excluded from analysis. In case of the directly observed behavioral indicators, data for the subsequent observation interval after an illegitimate entry (e.g., evening observation after an illegitimate entry during the afternoon observation) was additionally deleted from the dataset because we expected dams that had just nursed their calves would have a lower motivation to reunite with their calf.

In summary, the final dataset contained only data points for observations or collection days on which cows had no severe health problem, were not in (pro)estrous, and cows and calves had spent ≤2% of time illegitimately together.

In case of FGCM concentrations, the data on health condition, estrus, and illegitimate contact from the day before collection of the fecal sample, instead of the sampling day, was used for correction of data points. This was necessary, because the peak in FGCM concentrations of cows occurs with a delay of 6 to 18 h after the stressor, depending of the type of stressor and the individual animal (Palme et al., 1999, 2000; Morrow et al., 2002). Thus, the FGCM samples collected at 07:30 in the morning reflect approximately the stress the cow experienced between noon and midnight of the preceding day. An overview of the remaining data points for each assessed parameter can be found in Supplemental Table S2 (see Notes).

Statistical Analysis. All statistical analyses were done using SAS Version 9.4 (SAS Institute Inc., Cary, NC). The individual cow (for indicators of distress and milk yield) or the cow-calf pair (for social behaviors) were treated as the experimental unit.

Indicators of Distress: Main Model. Analyses of cows' distress indicators (vocalizations [frequency/30 min], amount of searching behavior [percent of scans/30min], lying time [percent/24 h] and lying bouts [number/24 h], rumination time [hr/24 h]), FGCM concentration (ng/g), neutrophil number [cells/µL], lymphocyte number [cells/µL], and neutrophil:lymphocyte ratio) were conducted in line with analysis of distress responses of the calves (Vogt et al., 2024b). Before analysis, weekly means for each cow were calculated for frequency of vocalizations and percentage of scans with searching behavior, because these parameters were observed at several intervals per day. Data were then analyzed using a linear mixed effects model with repeated measures and the following fixed effects: treatment (GR or NF), phase (baseline, partial separation, total separation), the interaction between treatment and phase, lactation group of the cow (first lactation, second to third lactation, or fourth to seventh lactation), sex of the calf (male or female), as well as month of data collection (Nov., Dec., Jan., Feb., or Mar.). Weaning weight of the calf at the start of weaning (in kilograms) was included as continuous variable. The cow ID nested within herd (horned/polled) was included as a random effect. Additionally, the calendar week merged with herd was included as cross-classified random effect to account for the changing group compositions in the cow herds throughout the study (Cafri et al., 2015). The covariance structure was set to autoregressive. The degrees of freedom were corrected with the Kenward-

Roger adjustment. Weaning age of the calf at weaning start was not included in the model, because there was a correlation between weaning weight and age of the calf ($r_\rho = 0.7$, $P < 0.001$), and weaning weight proved to have the stronger effect on our used indicators.

To test for model assumptions, residuals of all models were graphically inspected for normal distribution and homoscedasticity. Data of frequency of vocalizations and amount of searching behavior were however considerably skewed toward the smaller values (Supplemental Figures S1 and S2; see Notes) returning funnel-shaped residuals. Because elimination of zeros (as, for example, implemented by Neave et al., 2024a for the same issue) could not significantly improve model fit due to a high occurrence of values between 1 and 10, data were square root-transformed before analysis and afterward back-transformed using the delta method for presentation of results. Square root transformation enhanced model fit; however, homoscedasticity could still not be fully achieved for these variables and thus it has to be acknowledged that our reported LSM of the main model underrepresent strong responses (but see the Indicators of Distress: Strong Behavioral Responses section).

Post hoc pairwise comparisons were calculated using a Tukey-Kramer test. Because there were 9 indicators for the distress response of the cows, significance levels needed to be corrected for multiple testing. This was done according to the Benjamini-Hochberg false discovery rate correction (Glickman et al., 2014) for results of the main effects of treatment and the phase (Supplemental Tables S3 and S4; see Notes), because there was a lack of significance of the treatment × phase interaction in all indicators. Results are presented as (back-transformed) LSM ± SE.

Indicators of Distress: Strong Behavioral Responses. As described in the previous section, there was a considerable range of frequency of vocalizations and amount of searching behavior by the individual cows, which is rather poorly reflected by the LSM and especially the SE of the main model. Therefore an additional analysis was run, for which responses of cows were classed as either a weak or a strong response. This analysis was based on the full data set, corrected for the exclusion criteria described in the Exclusion Criteria section, which means that it included the 5 observation time points of 30 min duration per cow per day. A response was classed as a strong response for the specific observation time point if the cow vocalized >10 times during the 30 min observation or showed searching behavior in >20% of observational scans (during the 30 min direct observation with scan sampling every 3 min, equaling approximately >6 min of searching behavior in 30 min). These selected thresholds for a strong response reflect a response that was greater than the median frequency of vocalizations or percentage of scans with searching behavior, respectively, over the whole 3-wk partial separation phase by all 36 cows after all zeros were excluded (otherwise even the 75% quartile was zero). The probability for occurrence of a strong vocal response (yes = 1; no = 0) or high amount of searching behavior (yes = 1; no = 0) was analyzed using a generalized linear mixed effects model with the same fixed and random effects as for the model described in the Indicators of Distress: Main Model section and with a binary distribution (logit-link). Distribution of strong responses in the 2 treatments over the different weeks of the separation process is given descriptively.

Indicators of Distress: Physiological Responses. For the hematologic indicators (neutrophil number [cells/μL], lymphocyte number [cells/μL], and neutrophil:lymphocyte ratio) and FGCM concentrations (ng/g), the main model described in the Indicators of Distress: Main Model section was additionally run with the exact sampling day instead of only the phase as sampling time point, because previous studies showed that physiological stress responses in cows are transient and can return to preweaning values within 48 h after start of the separation process (Whisnant et al., 1985; Acevedo et al., 2005).

Effect of the Separation Methods on Social Behaviors Between Cows and Calves. Analysis of changes in social behaviors between cow and calf comprised the amount of nursing behavior (percent of scans/30 min), the amount of cow-calf interactions (percent of scans/30 min), and occurrences of social play between cow and calf (occurrence: yes or no within a scan sampling interval).

For analysis of cow-calf interactions only the morning observation (2 × 30 min in the 2 h following morning milking) was included in the data set because this was the only observation time point at which cow-calf pairs of both treatments had an equal possibility for contact to each other. Cow-calf interactions were analyzed with the same linear mixed effects model with repeated measures as described in the Indicators of Distress: Main Model section, with the exception that it included the exact week (wk 1, wk 2, wk 3) of the partial separation phase instead of the pooled 3-wk partial separation phase as the sampling time point and consequently also the treatment × week interaction. As with vocalizations, data of cow-calf interactions had to be square root-transformed for analysis and was back-transformed using the delta method afterward.

For analysis of changes in nursing behavior only the GR treatment was included in the analysis because suckling was no longer possible after insertion of the nose flap in the NF treatment. Also, the afternoon observation time point (i.e., 1 h before evening milking) was not considered for analysis because this time point was not comparable between the weeks with half-day and morning contact within the GR treatment. However, nursing behavior of GR cows was only observed 3 times during the afternoon observation by all GR cows in the week with half-day contact and only 2 times during the baseline week anyway.

Taken together, the final data set for nursing behavior included only data for GR cows during the morning observation for the baseline week and the first 2 wk of the partial separation phase. Nursing behavior was analyzed with the same linear mixed effects model as cow-calf interactions, with the exception that treatment and treatment × week interaction was omitted from the model because only GR cows were considered.

Statistical analysis of social play between cow and calf was not possible due to a too low occurrence of this behavior during our observation time points and consequently a too low variance in data. Therefore, the results for social play are only presented descriptively.

Effect of the Separation Method on Milk Yield of the Cows. For analysis of the effect of the separation method on changes in milk yield (kg/day per cow), the same linear mixed effects model was run as for cow-calf interactions with the exact week of the partial separation phase as the sampling time point. For analysis of changes in yield at the specific milking times (kilograms per milking per cow), data were split into subsets separated by milking time before running the analysis.

RESULTS

Distress Responses of Cows to the 2 Separation Methods

There was no treatment × phase interaction for any of our behavioral or physiological indicators of distress (Table 2), hence there were no differences in distress responses of cows between the 2 separation methods over the 3-wk partial separation phase as well as in the week of total separation. However, there was a main effect of the phase for frequency of vocalizations and amount of searching behavior (Table 2), because cows in both treatments vocalized more frequently (post hoc: $t_{122} = -3.3$ [the subscript number indicates the degrees of freedom], $P = 0.003$) and showed more searching be-

Table 2. Model output for behavioral and physiological indicators of distress for cows that were separated from their calves via the 2-step weaning method using a nose flap for the calves (NF, n = 18) or by gradual reduction of contact time between cow and calf (GR, n = 18)[1]

			Phase			*P*-value (F-value$_{\text{Num DF,Den DF}}$)[2]		
Variable	T	N	Baseline	Partial separation	Total separation	T	P	T × P
Vocalizations[3,4] (frequency per 30 min)	NF	18	0.40 ± 0.14[A]	1.88 ± 0.39[B]	0.21 ± 0.09[AB]	0.03	**0.003**	0.30
	GR	18	0.46 ± 0.17[A]	3.72 ± 0.93[B]	2.71 ± 1.03[AB]	($F_{1,43} = 5.2$)	($F_{2,121} = 6.2$)	($F_{2,112} = 1.2$)
Searching behavior[3,4] (% of scans in 30 min)	NF	18	0.13 ± 0.04[A]	1.22 ± 0.25[B]	0.05 ± 0.02[A]	0.007	**0.0002**	0.33
	GR	18	0.30 ± 0.10[A]	3.91 ± 0.96[B]	1.88 ± 0.68[A]	($F_{1,39} = 8.1$)	($F_{2,121} = 9.1$)	($F_{2,110} = 1.1$)
Lying times (% of 24 h)	NF	13	42.66 ± 1.70	43.14 ± 1.55	42.24 ± 1.69	0.49	0.68	0.10
	GR	13	43.72 ± 1.71	43.05 ± 1.59	45.41 ± 1.64	($F_{1,20} = 0.5$)	($F_{2,196} = 0.38$)	($F_{2,167} = 2.4$)
No. of lying bouts (per 24 h)	NF	13	11.74 ± 1.74	11.98 ± 1.73	12.24 ± 1.75	0.94	0.06	0.57
	GR	13	11.22 ± 1.61	11.94 ± 1.61	12.25 ± 1.62	($F_{1,19} = 0.0$)	($F_{2,199} = 2.8$)	($F_{2,223} = 0.6$)
Rumination time (hr/24 h)	NF	14	9.44 ± 0.30	9.31 ± 0.27	9.10 ± 0.32	0.40	0.38	0.10
	GR	16	9.46 ± 0.28	9.29 ± 0.29	9.98 ± 0.39	($F_{1,20} = 0.7$)	($F_{2,127} = 1.0$)	($F_{2,130} = 2.3$)
FGCM (ng/g)	NF	18	19.17 ± 2.68	21.37 ± 2.43	19.37 ± 2.68	0.35	0.04	0.68
	GR	17	21.40 ± 2.87	24.34 ± 2.64	23.70 ± 2.77	($F_{1,31} = 0.9$)	($F_{2,172} = 3.3$)	($F_{2,155} = 0.4$)
Neutrophils (×1,000 cells/µL)	NF	17	2.01 ± 0.28	2.43 ± 0.19		0.14	0.08	0.62
	GR	16	2.47 ± 0.30	2.70 ± 0.24		($F_{1,37} = 2.2$)	($F_{1,137} = 3.1$)	($F_{1,130} = 0.2$)
Lymphocytes (×1,000 cells/µL)	NF	17	3.11 ± 0.22	3.18 ± 0.21		0.77	0.61	0.79
	GR	16	3.05 ± 0.24	3.07 ± 0.22		($F_{1,28} = 0.1$)	($F_{1,140} = 0.3$)	($F_{1,126} = 0.1$)
Neutrophil:lymphocyte ratio	NF	17	0.71 ± 0.08	0.79 ± 0.06		0.19	0.11	0.94
	GR	16	0.81 ± 0.09	0.89 ± 0.08		($F_{1,31} = 1.8$)	($F_{1,131} = 2.6$)	($F_{1,123} = 0.0$)

[A,B]Capital superscript letters indicate significant differences between phases independent of treatment with $P < 0.05$ (for those variables with a significant main effect of the phase).

[1]A Benjamini-Hochberg false discovery rate calculation confirmed the *P*-values in bold for rejection of the null-hypothesis with an $\alpha \leq 0.05$. The Benjamini-Hochberg false discovery rate calculation was done for the main effects of treatment (T) and phase (P) because there was no significant treatment × phase (T × P) interaction for any of the examined indicators. Baseline phase = the week before weaning start, partial separation = the 3-wk phase of partial separation from their calves through either the nose flap, limited daily contact times or through a fence-line; total separation = the first week after calves were moved to the youngstock barn. FGCM = fecal cortisol metabolites. Values are presented as (back-transformed) LSM ± SE.

[2]Num DF = numerator degrees of freedom, Den DF = denominator degrees of freedom.

[3]Means per week per cow calculated before analysis.

[4]LSM and SE back-transformed from square root transformation.

havior (t_{120} = −3.9, P = 0.0004) during the 3-wk partial separation phase than during the baseline phase (Table 2). Once the calves were moved to the youngstock barn (in the week of total separation), there was no significant difference in frequency of vocalizations (t_{124} = −1.1, P = 0.54) or amount of searching behavior (t_{126} = −1.0, P = 0.59) compared with baseline anymore (Table 2). An overview of results for the remaining main effects included in the models can be found in Supplementary Table S5.

Strong Behavioral Responses. In general, variability in frequency of vocal responses (range: 0–288, median: 0 vocalizations per 30 min) and amount of searching behavior (range: 0%–100%, median: 0% of scans) during the 4-wk separation phase was considerable, with a large proportion of weak behavioral responses and some very strong responses by cows. There was also a high interanimal variability in the maximum frequency of vocalizations (NF: range: 1–194, median: 59.5; GR range: 3–288; median: 126 vocalizations recorded at maximum per 30 min by a single cow) and maximum percentage of scans with searching behavior (NF: range: 0%–100%, median: 49.5%; GR range: 22.2%–100%; median: 81.8% of scans with searching behavior recorded at maximum per 30 min observation by a single cow) in both treatments. In sum, 14 NF cows and 16 GR cows (out of 18 cows per treatment) showed a strong vocal response, and 12 NF cows and 17 GR cows showed a high amount of searching behavior at least once during the separation process (Table 3). However, the statistical probability for cows to show a strong vocal response during the 3-wk partial separation phase, as well as during the total separation phase, was similar in both treatments (treatment × phase interaction: F-test statistic$_{\text{numerator degrees of freedom, denominator degrees of freedom}}$; $F_{2,774}$ = 1.8, P = 0.2). This was equally the case for the probability to show a high amount of searching behavior (treatment × phase interaction: $F_{2,858}$ = 0.04, P = 0.96).

Regarding the temporal distribution of responses, the largest proportion of strong vocal responses and largest proportion of high amounts of searching behavior within the GR treatment was recorded in the third week with fence-line contact, followed by the week of total separation (Table 3). Within the NF treatment, the largest proportion of strong responses for both indicators was recorded in wk 1 after the calves got the nose flap inserted, followed by the second week while calves were still wearing the nose flap (Table 3). Distribution of strong responses over the individual observation days is displayed in Supplemental Figures S3 and S4 (see Notes).

Physiological Distress Responses. Looking at the individual sampling days for the physiological indicators, there was a main effect of the sampling day on neutrophil numbers and FGCM concentrations (Table 4). Cows of

Table 3. Weekly distribution of strong vocal responses and high amounts of searching behavior by cows in the 2 treatments during the baseline, the 3-week partial separation phase, and the first week when calves were moved to the youngstock barn (i.e., total separation)[1]

				Sampling week				
Variable	Treatment	Unit	Baseline	Week 1	Week 2	Week 3	Total separation	Sum
Vocalization	NF	No. of SR	3	37	17	9	5	68
		% of all SR	—	54.4	25.0	13.2	7.4	100%
		No. per mor./noon/eve obs.	2/0/1	11/14/12	7/5/5	3/5/1	1/3/1	22/27/19
		No. of cows	3	10	7	5	2	14[3]
	GR	No. of SR	7[2]	21	8	54	28	111
		% of all SR	—	18.9	7.2	48.7	25.2	100%
		No. per mor./noon/eve obs.	2/1/4	2/0/19	2/2/4	29/15/10	16/10/2	49/27/35
		No. of cows	3	11	3	15	9	16[3]
Searching behavior	NF	No. of SR	0	23	10	6	3	42
		% of all SR	—	54.8	23.8	14.3	7.1	100%
		No. per mor./noon/eve obs.	0/0/0	7/9/7	4/4/2	2/3/1	1/2/0	14/18/10
		No. of cows	0	9	4	3	2	12[3]
	GR	No. of SR	3[2]	17	5	36	19	77
		% of all SR	—	22.0	6.5	46.8	24.7	100%
		No. per mor./noon/eve obs.	1/0/2	0/0/17	1/1/3	18/10/8	8/9/2	27/20/30
		No. of cows	1	10	3	13	7	17[3]

[1]Values are given as absolute numbers of strong responses (No. of SR), percentage of strong responses in the particular week of all strong responses recorded during the separation process (i.e., excluding the baseline; percent of all SR), absolute numbers of strong responses recorded during the morning (mor), afternoon or evening (eve) observation (No. per mor./noon/eve obs.) and the absolute number of cows that showed a strong response at least once during the respective week (No. of cows). A frequency of >10 vocalizations per 30 min observation was classed as a strong vocal response and a proportion of >20% of behavioral scans in which cows showed searching behavior (observed during 30 min direct observation with scan sampling in intervals of 3 min) were classed as a strong response for the amount of searching behavior. GR = Cow-calf pairs separated by gradual reduction of CCC time (n = 18). NF = Cows were separated from their calves with the 2-step weaning method using a nose flap for the calves (n = 18). Observations were conducted on 4 d per week at the following time points: 2 × 30min during the 2 h following morning milking (morning), 1 × 30 min during the 1 h before the start of evening milking (afternoon) and 2 × 30min during the 2 h following evening milking (evening).

[2]One particular cow was responsible for 5 of the 7 strong vocal responses and all 3 observations with a high amount of searching behavior during the baseline week.

[3]Cows that showed a strong vocal response or high amounts of searching behavior in more than one week were only counted once for the sum of high responding cows per treatment.

Table 4. Effect of the separation methods on total neutrophil and lymphocyte number, neutrophil:lymphocyte ratio, and fecal cortisol metabolite concentrations (FGCM) of dairy cows that were separated from their calves via 2-step weaning and separation using a nose flap (NF, n = 18) or gradual reduction of contact time to the calf (GR, n = 18)[1]

Variable	T	N	Baseline: −1	Week 1: 2	Week 1: 6	Week 2: 9	Week 2: 13	Week 3: 16	Week 3: 20	Total separation: 23	*P*-value (*F*-value$_{\text{Num DF,Den DF}}$): T	D	T × D
Neutrophils	NF	17	2.15 ± 0.28^{A}	3.12 ± 0.28^{B}	2.25 ± 0.27^{AB}	2.69 ± 0.28^{AB}	2.47 ± 0.29^{AB}	2.07 ± 0.30^{AB}	2.09 ± 0.28^{AB}		0.13	0.03	0.96
(×1,000 cells/μL)	GR	17	2.61 ± 0.30^{A}	3.20 ± 0.37^{B}	2.74 ± 0.35^{AB}	2.92 ± 0.34^{AB}	2.69 ± 0.37^{AB}	2.68 ± 0.42^{AB}	2.48 ± 0.45^{AB}		($F_{1,24}$ = 2.5)	($F_{6,108}$ = 2.5)	($F_{6,105}$ = 0.2)
Lymphocytes	NF	17	3.04 ± 0.23	2.94 ± 0.23	3.17 ± 0.22	3.19 ± 0.22	3.34 ± 0.23	3.33 ± 0.23	3.14 ± 0.23		0.69	0.13	0.23
(×1,000 cells/μL)	GR	17	3.00 ± 0.24	3.03 ± 0.26	3.16 ± 0.25	3.00 ± 0.25	2.83 ± 0.26	3.35 ± 0.27	2.99 ± 0.28		($F_{1,28}$ = 0.2)	($F_{6,104}$ = 1.7)	($F_{6,100}$ = 1.4)
Neutrophil:	NF	17	0.74 ± 0.09	0.92 ± 0.09	0.78 ± 0.08	0.88 ± 0.09	0.75 ± 0.09	0.65 ± 0.09	0.75 ± 0.09		0.14	0.07[2]	0.87
lymphocyte ratio	GR	17	0.83 ± 0.09	0.92 ± 0.11	0.84 ± 0.11	1.02 ± 0.10	0.95 ± 0.11	0.86 ± 0.12	0.89 ± 0.14		($F_{1,23}$ = 2.4)	($F_{6,101}$ = 2.0)	($F_{6,97}$ = 0.4)
FGCM (ng/g)	NF	18	20.06 ± 2.63^{A}	25.97 ± 2.67^{C}	19.54 ± 2.62^{A}	18.75 ± 2.61^{A}	23.83 ± 2.61^{AC}	24.53 ± 2.62^{BC}	18.16 ± 2.62^{AB}	19.21 ± 2.61^{A}	0.25	<0.001	0.62
	GR	18	22.25 ± 2.81^{A}	28.80 ± 3.15^{C}	22.79 ± 2.90^{A}	23.67 ± 2.85^{A}	26.62 ± 3.07^{AC}	27.67 ± 3.14^{BC}	25.51 ± 3.16^{AB}	24.20 ± 2.71^{A}	($F_{1,30}$ = 1.4)	($F_{7,132}$ = 6.0)	($F_{7,128}$ = 0.8)

$^{A–C}$Superscript letters indicate significant differences between sampling days independent of treatment with $P < 0.05$ (for those variables with a significant main effect of sampling day).

[1]Values are expressed as LSM ± SE. T = treatment, N = number of cows, D = sampling day, Num DF = numerator degrees of freedom, Den DF = denominator degrees of freedom.

[2]Individual pairwise comparisons were no longer significant after Tukey-Kramer adjustment.

both treatments showed neutrophilia (post hoc: t_{103} = 3.2, P = 0.03) and increased FGCM concentrations at the second day of the separation process (t_{133} = 4.5, P = 0.0004) compared with baseline values, but this was only short-lived in both indicators (Table 4). FGCM concentrations showed a second peak compared with baseline on d 16 (t_{156} = −3.3, P = 0.03, Table 4), that is, the first sample after introduction of fence-line contact, which was however not evident in neutrophil numbers.

Changes in Social Behaviors of Cow and Calf During the Separation Process

Changes in nursing behavior were only considered for the GR cows because NF calves could not suckle once the nose flap was inserted. Within the GR treatment, there was a main effect of the treatment week on the amount of scans with nursing behavior, as significantly more nursing behavior was observed after morning milking in the first week (i.e., half-day contact during the day, t_{75} = 3.0, P = 0.01) and second week (i.e., morning contact, t_{68} = 2.8, P = 0.02) of the separation process compared with the baseline observations (Table 5). However, there was no further increase in the amount of scans with nursing behavior from wk 1 with half-day contact to the second week with only morning contact (t_{68} = 0.4, P = 0.91, Table 5).

The percentage of scans with cow-calf interactions showed no differences between the 2 separation methods depending on the phase (Table 5). However, there was a main effect of treatment, because cow-calf interactions by GR pairs were more frequently observed than by pairs in the NF treatment (t_{27} = 2.9, P = 0.01), but this difference was already evident in the baseline (Table 5). Treatment week did not affect the amount of scans with cow-calf interactions (Table 5). Thus, partial separation of pairs neither led to significant adjustments in the amount of observed cow-calf interactions after morning milking compared with baseline in either treatment, nor when contact time was reduced from half-day to morning contact in the GR treatment (Table 5). In general, there was a high variability in the amount of cow-calf interactions between the individual pairs in the baseline (range 0%–36.4% of scans during morning observation, median: 0.0%) as well as during the 2 wk of partial separation (0%–31.8% of scans, median: 0.0%). Results for the remaining main effects on the amount of nursing behavior or cow-calf interactions can be found in Supplemental Table S6 (see Notes).

Social play between cow and calf was only observed in 1.1% of observations (observed 38 times in 20 different cow-calf pairs during 340 observations of 30 min during which several pairs were observed simultaneously, totaling 3,518 observations).

Table 5. Changes in social (i.e., maternal and affiliative) behaviors of cows in the course of the separation process[1]

Variable	T	N	Week			*P*-value (F-value$_{\text{Num DF;Den DF}}$)		
			Baseline	Week 1	Week 2	T	W	T × W
Nursing own calf (% of scans per 30 min)	GR	18	9.39 ± 1.94[A]	16.36 ± 2.55[B]	15.37 ± 2.20[B]		0.004 ($F_{2,70}$ = 6.2)	
Cow-calf Interactions (% of scans per 30 min)[2]	NF	18	2.14 ± 0.44	1.09 ± 0.22	1.06 ± 0.22	0.01 ($F_{1,27}$ = 8.5)	0.26 ($F_{2,141}$ = 1.3)	0.34 ($F_{2,151}$ = 1.0)
	GR	18	3.29 ± 0.69	2.56 ± 0.71	3.64 ± 0.93			

[A,B]Within rows, superscript letters indicate significant differences between weeks at $P \leq 0.05$.

[1]Cows were either separated from their calves via 2-step weaning using a nose flap for the calves (NF, n = 18) or by gradual reduction of contact time between cow and calf (GR, n = 18). Only morning observations (2 × 30 min in the 2 h following morning milking) were included for analysis of both indicators because this was the only observation time point that was comparable between treatments or between weeks within the GR treatment. T = treatment, W = sampling time point (week), T × W = interaction of treatment × week. DF = degrees of freedom, Num DF = numerator degrees of freedom, Den DF = denominator degrees of freedom. Baseline = the week before start of the separation process. Values are presented as LSM ± SE.

[2]The LSM and SE values were back-transformed from square root transformation.

Changes in Milk Yields of the Cows During the Separation Process

Daily milk yield (in kilograms) differed between treatments depending on the week (interaction treatment × week, Table 6). The NF cows reached a higher daily milk yield than GR cows during the first (t_{52} = −4.5, P = 0.0004) and second week (t_{53} = −4.1, P = 0.002) of the partial separation phase, but treatments did not differ during the third week with fence-line separation (t_{66} = 0.2, P = 1.0) or in the week of total separation (t_{48} = 0.3, P = 1.0, Table 6). Both treatments showed an increase in milk yield from baseline to the first week (GR: t_{398} = −5.2, P < 0.001; NF: t_{320} = −18.6, P < 0.001) and again from first to the second week of the partial separation phase (GR: t_{398} = −5.3, P < 0.001; NF: t_{364} = −6.1, P < 0.001, Table 6). Within the GR treatment there was a further increase in milk yield after implementation of fence-line contact in the third week as compared with the second week with morning contact (t_{400} = −6.4, P < 0.001). Hence, the milk yield of GR cows did not differ anymore from the yield of NF cows in the third week with fence-line contact (t_{66} = 0.2, P = 1.0). Also, yields in the week of complete cessation of nursing by the calves was not significantly different between the 2 treatments (NF wk 1 vs. GR wk 3, t_{64} = 3.0, P = 0.1, Table 6). Descriptively, milk yield of GR cows was reduced by ~4.3 L compared with cows separated with the NF method (mean ± SD: GR, 18.49 ± 6.20; NF, 22.82 ± 6.38 kg/day) when considered as average over the whole 3-wk partial separation phase.

Looking at the separate milking times, there was a main effect of the treatment week on morning milk yields (Table 6), as these were lower during the baseline week compared with all weeks of the partial separation process and the week of total separation (all P < 0.001) in both treatments. Also, an increase in morning milk yield from the first week to the second week (t_{390} = −6.4, P < 0.001) and to the third week (t_{331} = −4.4, P < 0.001) of the partial separation process was evident in both treatments (Table 6). However, there was no further increase in morning milk yield in the week of total separation compared with the third week with fence-line contact in either of the 2 treatments (t_{364} = 2.1, P = 0.24, Table 6). In this regard, descriptive analysis of the individual sampling days revealed that morning milk yield of cows slowly increased after introduction of the separation process and reached a quite constant level starting from about the third milking after treatment start (Figure 2).

In contrast to the aforementioned, evening milk yields of cows differed between the 2 separation methods depending on the week of the separation process (Table 6). Evening milk yield of GR cows was significantly lower than of NF cows in the first (t_{65} = −9.8, P < 0.001) and second week (t_{68} = −11.2, P < 0.001) of the partial sepa-

Table 6. Changes in milk yield of cows in the course of the separation process[1]

Variable	T	N	Week: Baseline	Week 1	Week 2	Week 3	Total separation	*P*-value (F-value$_{\text{Num DF,Den DF}}$): T	W	T × W
Total milk yield (kg/d)	NF	18	8.98 ± 0.91^{a}	20.14 ± 0.90^{cd}	23.77 ± 0.89^{e}	23.88 ± 0.89^{e}	23.13 ± 0.88^{e}	0.14	<0.001	<0.001
	GR	18	10.79 ± 0.94^{a}	14.48 ± 0.98^{b}	18.60 ± 0.99^{c}	24.16 ± 1.07^{de}	23.52 ± 0.92^{de}	($F_{1,30} = 2.3$)	($F_{4,337} = 190.5$)	($F_{4,314} = 25.9$)
Morning milk yield (kg)	NF	18	5.97 ± 0.69^{A}	11.58 ± 0.68^{B}	13.51 ± 0.67^{C}	13.62 ± 0.68^{CD}	12.89 ± 0.67^{BD}	0.23	<0.001	0.18
	GR	18	7.20 ± 0.72^{A}	12.07 ± 0.76^{B}	15.65 ± 0.77^{C}	14.35 ± 0.85^{CD}	13.16 ± 0.70^{BD}	($F_{1,27} = 1.5$)	($F_{4,331} = 96.1$)	($F_{4,326} = 1.6$)
Evening milk yield (kg)	NF	18	2.99 ± 0.47^{a}	8.73 ± 0.46^{b}	10.27 ± 0.46^{c}	10.42 ± 0.46^{c}	10.23 ± 0.45^{c}	<0.001	<0.001	<0.001
	GR	18	3.57 ± 0.49^{a}	2.37 ± 0.53^{a}	2.93 ± 0.53^{a}	10.01 ± 0.60^{bc}	10.25 ± 0.48^{bc}	($F_{1,27} = 27.4$)	($F_{4,348} = 127.3$)	($F_{4,344} = 76.5$)

[a–e]Lowercase superscript letters indicate statistically significant differences at $P \leq 0.05$ (all post-hoc pairwise comparisons between treatment × week combinations considered, for those variables with a significant T × W interaction).

[A–D]Capital superscript letters indicate significant differences between weeks independent of treatment with $P < 0.05$ (for those variables with a significant main effect of the week).

[1]Cows were either separated from their calves via 2-step weaning using a nose flap for the calves (NF, n = 18) or by gradual reduction of contact time between cow and calf (GR, n = 18). Morning milking lasted approximately from 5:15 to 8:30 a.m. and evening milking from 3:30 to 6:45 p.m. T = treatment, W = sampling time point (week), T × W = interaction of treatment × week. DF = degrees of freedom, Num DF = numerator degrees of freedom, Den DF = denominator degrees of freedom. Baseline = the week before start of the separation process, Total separation = the first week after calves were moved to the youngstock barn. Values are presented as LSM ± SE.

ration phase, but not during the third week ($t_{94} = -0.6$, $P = 0.99$) or the week of total separation ($t_{58} = 0.0$, $P = 1.0$, Table 6). Within the NF treatment, milk yields were lower during the first week than during the second ($t_{363} = -4.1$, $P = 0.002$) or third week ($t_{328} = -4.2$, $P = 0.001$) of the separation process, as well as compared with the week of total separation ($t_{327} = -3.5$, $P = 0.02$, Table 6). This was not the case for GR cows because evening milk yield of GR cows during the first week (with half-day contact, $t_{415} = 2.6$, $P = 0.2$) and the second week (with morning contact, $t_{354} = 1.3$, $P = 0.94$, Table 6) was as low as during the baseline week (with full-contact), which also did not differ from one another (GR wk 1 vs. wk 2: $t_{418} = -1.1$, $P = 0.98$). Only when fence-line contact was introduced in the third week, did the evening milk yield of GR cows considerably increase compared with baseline ($t_{390} = -11.5$, $P < 0.001$) and the other 2 weeks of the weaning and separation process (wk 1: $t_{375} = -13.1$, $P < 0.001$, wk 2: $t_{409} = -12.5$, $P < 0.001$, Table 6). Descriptive analysis of the individual sampling days showed a sharp increase in milk yield after introduction of fence-line contact (Figure 2), which is contrary to the slow adaptation in morning milk yield.

DISCUSSION

With this study, we aimed to compare behavioral and physiological responses of dairy cows to separation from their calf, after 3 mo of full-time contact, either via 2-step separation or via gradual reduction of contact time to the calf. A secondary objective was to investigate changes in social behaviors between cow-calf pairs as well as in milk yield of the cows in the course of the separation process. Based on the used indicators, the NF and GR treatment resulted in a similar stress load for the dams. In both treatments, cows reacted with a significant increase in vocalizations and searching behavior as well as with a transient increase in some physiological distress markers compared with the baseline, indicating that separating dams from their 3-mo-old calves with these methods is still a welfare concern. Descriptive analysis additionally revealed large interindividual differences between cows as well as a different temporal distribution in occurrence of strong responses in the 2 treatments. As expected, milk yield was higher in NF compared with GR cows during the 2 wk while GR calves had time-restricted access to their dams but NF calves were prevented from suckling by the nose flaps. However, once GR calves were completely prevented from suckling as well, milk yield of cows in the 2 treatments did not differ anymore. Interestingly, there was no difference in evening milk yield of GR cows, and neither in percentage of observations of nursing in the morning, between weeks with half-day and morning

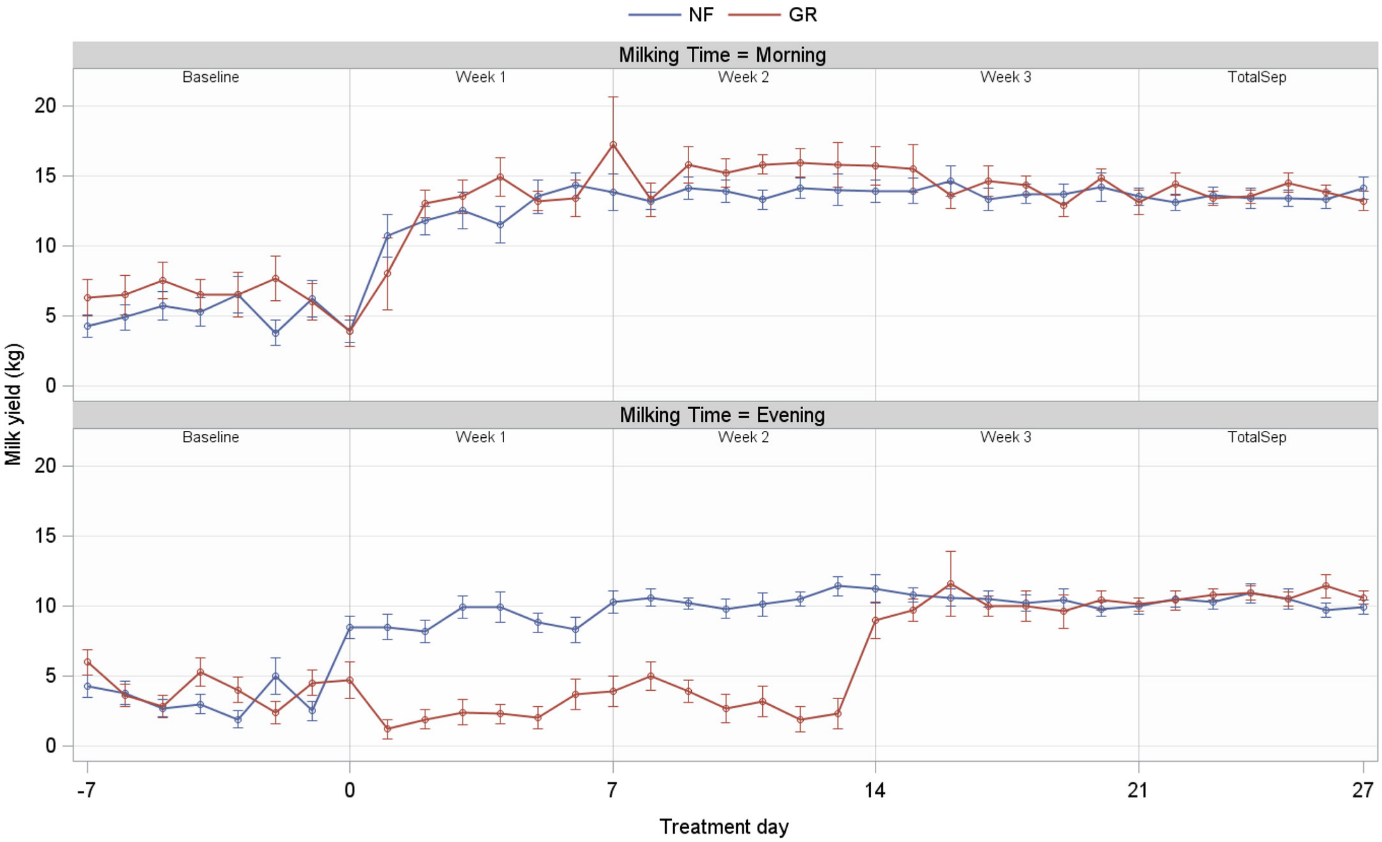

Figure 2. Milk yield of cows (mean ± SE, in kilograms) during morning (approximately 5:15–8:30 a.m.) and evening (approximately 3:30–6:45 p.m.) milking in the course of the 3 wk of partial separation (starting at day 0) and the first week after calves were moved to the youngstock barn (at day 21 (TotalSep = total separation). Cows were either separated from their calves via 2-step separation using a nose flap for their calves (NF, blue lines, n = 18) or by gradual reduction of contact time between cow and calf (GR, red lines, n = 18).

contact, indicating that our gradual separation method worked not as gradually as intended.

Distress Responses to the 2 Separation Methods Did Not Differ

Results did not confirm any significant differences in behavioral or physiological distress responses of cows separated from their calves with either the 2-step method or a gradual reduction of contact time to the calf. This implies that a partial contact to the calf for only a certain amount of hours of the day is overall a comparable stressor for the dams as a full-time contact to the calf with a limited possibility of physical interaction, namely nursing. Hence, the missing differences might be caused by the fact that both methods limited the physical contact to the calf, only in different manners. In line with this, Wenker et al. (2022) reported no differences in behavioral reactions to weaning of their calves in dairy cows that had limited physical contact to their 2-mo-old calves either due to fence-line separation or due to prevention of nursing by calves with nose flaps. Importantly, video monitoring confirmed that our NF calves significantly reduced the time they spent in the cow herd, compared with preweaning times, after 4 d of wearing the nose flap to ~3.5 h/d (Vogt et al., 2024a). This declined numerically even further to ~2.5 h/d at the fifth day after nose flap insertion (Vogt et al., 2024a) and is thus similar or even less than the minimum allowed morning contact duration of 3.5 h/d in the GR treatment. Consequently, both methods eventually reduced daily dam-calf contact, which might partly explain the overall similar results in cows. Despite the fact that our results confirmed no method as superior for stress reduction of dams during separation, there are still some major deductions we can infer from our findings, as described in the following sections.

Expectations Matter

Within both separation treatments, some of the dams reacted with a strong vocal response, as well as a high amount of searching behavior, which was not only transitory but present in several weeks of the process. In the NF treatment, cows showed descriptively the strongest reaction in wk 1, followed by wk 2 of the procedure (while calves wore the nose flaps), whereas GR cows showed the strongest response after fence-line - and total separation from their calves (Table 3). This different temporal distribution of strong responses by dams parallels the behavioral response we found in their calves (see Vogt et al., 2024b) and coincides with the time point of milk loss for both treatments as well as separation from each other in the GR treatment. A parallel pattern of cow and calf vocalizations during gradual separation in a CCC system has equally been reported by Johnsen et al. (2024) and a high correlation of calls by cow and calf were also observed under extensive conditions (Watts, 2001). This is plausible because both partners preferentially responded to calls from their own dam or calf (Marchant et al., 2002; Padilla de la Torre et al., 2016). Interestingly, the stronger behavioral reaction of our NF cows to prevention of nursing rather than to separation from the calf is the reverse of responses in beef cows, which reacted with more vocalizations and pacing behavior after final separation from their calf compared with prevention of nursing through nose flaps in several studies (Haley, 2006a; Ungerfeld et al., 2015, 2016). This is likely due to different housing conditions, because cows and calves in the latter studies were housed together in the same pen or paddock until separation, allowing unhindered access to calves by the cows, whereas in our study the calves had a separate calf area behind the selection gate to which cows had no access. Because NF calves significantly reduced the time they spent in the cow area after cessation of milk supply through the nose flaps (Vogt et al., 2024a), our NF cows already experienced separation from their calves during the 2 wk with intended partial contact. Consequently, the proportion of strong behavioral responses of NF cows in the second week of the separation process likely reflects the cows' motivation to regain contact to their calf (Watts, 2001), which remained in the calf area.

For the GR treatment, our results are largely in line with findings by Neave et al. (2024a), who used a very similar experimental design to us: dairy cows in a CCC system were gradually separated from their calves over 2 wk through reduction of contact time by 50% in the first and 25% in the second week, before total separation from the calf when it was 10 weeks old. Similar to our study, there was a high number of nonresponding animals, but those who vocalized did so when contact time was reduced to 50% or when pairs were totally separated, or both (Neave et al., 2024a). Importantly, this vocal reaction by gradually separated cows was cumulatively not significantly different from the response of the abruptly separated control treatment at the point of separation, but only differed in temporal distribution (Neave et al., 2024a). Furthermore, Jensen (2024a) reported that the gradually reduced daily contact times did not consistently reduce the measured suckling time of the calves used in the study by Neave et al. (2024a). Their findings and the descriptively strong vocal response to fence-line separation of GR cows in our study, both point toward the problem that the weekly reductions in contact time did not achieve the intended concurrent gradual increase in calves' nutritional independence from the dam. This is strongly supported by the similar probability to observe nursing after morning milking, as well as the comparable evening milk yields of GR cows in weeks with half-day and morning contact in our study. In general, an increase in nursing behavior during the mornings after temporary separation compared with baseline was expected, because this likely reflects the higher motivation (or necessity) to reunite sooner after the end of morning milking due to increased hunger of calves after the long hours of separation. In line with this, other studies reported that calves suckled 2 to 3 times as long at the next nursing when made more hungry by altering the milk availability in the cow's udder (de Passillé, 2001) or when separated from their dams for 9 h of the day (Roadknight et al., 2022). Also, Bertelsen and Jensen (2023) reported that half-day contact calves that were separated from their dam overnight, suckled in >90% of cases within 30 min after reunion with the dam, whereas this was only the case in ~50% of occasions for calves kept with whole-day contact to their dam, equally indicating that temporary separations increase the motivation to reunite soon. Interestingly, the probability to observe nursing was not further increased, but showed no difference, during the week with morning contact in our study compared with the week with half-day contact despite an even longer separation time. Furthermore, the similar evening milk yields of GR cows suggest that the amount of milk ingested by the calves over the day likely stayed the same in these 2 wk and did not decrease with the reduced contact time. This finding matches results of other studies about similar weight gains of dairy calves kept with half-day or full-time contact to their dams (Zipp, 2018; Roadknight et al., 2022). In our study, the missing difference in milk yield between the week with half-day and morning contact could either be due to a compensatory reaction of the calves, meaning that they drank higher amounts of milk during the shortened access times. Alternatively, this might also be because pairs generally did not nurse very frequently during the afternoon, as indicated by the very low number of nursing bouts we observed before start of evening milking. Hence, the missing 3.5 h afternoon contact during the second week of the separation process might not have significantly altered nursing times because it did not interfere with established nursing routines. This would also explain why we did not observe an increase in nursing behavior in the second week with morning contact compared with the week with half-day contact.

Generally, the 2 milking times created a routine for the animals in our barn, because calves were always separated into the calf area during milking and then pairs were able to reunite after cows came back from milking. Consequently, milking was a time point with high activity by all animals and all pairs established a pattern to reunite for nursing after the morning as well as evening milking. This is in line with observations of cattle under semi-natural conditions, which also showed the vast majority of suckling bouts reliably during the early morning and late afternoon, but only irregularly during the other times of the day (Reinhardt and Reinhardt, 1981a), which was recently also confirmed in a dairy CCC system (Johnsen et al., 2021a). This established routine would also explain the strong behavioral reaction in the first and third week, but the comparably weaker reaction in the second week of the separation process in GR cows. It seems likely, that the cows showed a strong behavioral response to unfulfilled expectation to reunite after evening milking during the first week, whereas the animals reacted to the unfulfilled expectation to reunite after morning milking in the weeks of fence-line and total separation. This theory is supported by the higher occurrence of behavioral reactions during evening observation in wk 1, while in wk 3 the behavioral reactions were more frequent during the morning and afternoon observations (see Table 3 and Supplemental Figures S3 and S4; see Notes). On the other hand, there was a relatively weaker behavioral reaction of GR cows in the second week with morning contact, in which no significant changes to these routines happened. In various studies it has been shown that a negative discrepancy from expectations can lead to a negative emotional response in cattle and other ruminants, described as frustration (e.g., Greiveldinger et al., 2011; Lambert and Carder, 2019), which was likely the case in our study. In further support of this, Nicolao et al. (2022) reported increased vocalizations for 3 d after separation in dairy cows that were used to very limited daily contact to their calf for only 20 min of nursing before morning milking, but had likely also formed an expectation to reunite at this time. This points toward the fact that disruption of established nursing patterns is a relevant stressor, irrespective of previously shortened contact times and further underlines the importance of taking prior expectations of cow and calf into account as important mediating factors of weaning and separation stress for evaluation and refinement of separation protocols, as already outlined by Neave et al. (2024a). Given that in consequence, our GR treatment was basically only comprised of 3 steps (prevention of evening nursing, prevention of morning nursing and removal of the calf), whereas the NF treatment had 2 steps (prevention of both nursings, removal of the calf), this could additionally explain why we did not see the previously hypothesized stronger stress response in GR cows, compared with NF, due to the repeated changes in routines.

Taken all together, this leads to the conclusion, that the reduction in milk consumption by GR calves only truly happened by prevention of suckling during the night in the first week, but not any further when afternoon contact was reduced in the second week, since this step led to no relevant disruptions of the nursing pattern. In consequence, removal of the nursing opportunity in the morning of the week of fence-line separation was rather abrupt, and not a subsequent gradual step as planned. Because results of a recent maximum price paid study by Jensen (2024b) suggest that a reduction of daily contact to the calf rather increases the cows' motivation to nurse their calves, rather than decreased it, this together might explain the strong behavioral reaction we observed in response to the step of fence-line separation, as already discussed for our calves (Vogt et al., 2024b) and by Neave et al. (2024a) for the missing difference to abrupt separation.

Toward a More Gradual Reduction in Milk Intake

As elaborated above, results of our GR cows suggest that the employed weekly reductions in CCC time did not achieve the intended parallel reduction in suckled milk amounts and thus in nutritional dependence of the calves. A possible option for refinement of the GR method could be a fence-line separation method where the calf can suckle through the fence and the cow or calf is driven away from the fence after a designated time of nursing. Contrary to our design, this could ensure a gradual reduction in actual nursing duration with each week and might therefore achieve better results. However, a disruption of the nursing act while suckling motivation is still high bears the risk that calves start nonnutritive sucking (de Passillé and Rushen, 1997; de Passillé, 2001), so that methods to partly empty the udder before cow and calf can reunite might be the better option to investigate in the future. Partial emptying of the udder could be achieved either through machine milking before reunion (given an acceptable milk ejection) or, in foster cow systems, through suckling by younger calves before reunion with the weaner calves.

Alternatively, providing calves with supplemental milk during a gradual separation process seems to truly reduce the amount of suckled milk from the dam as shown by steadily increasing milk yields (Sørby et al., 2024a). This practice led to a comparably weak vocal response of cows and calves when access to each other was finally terminated (Johnsen et al., 2024 on the same animals as Sørby et al., 2024a), which is contrary to the strong vocal response of cows in response to fence-line and total separation in our study and seems thus to be a promising refinement option for barns in which such procedures can be implemented.

Last, a gradual separation with smaller reductions in contact time over a longer time period could be examined in accordance with positive results in sheep, which showed little response at the point of total separation after gradual separation over 12 weeks (Orgeur et al., 1998). In this context, a recent study that compared a gradual decrease in dairy CCC contact time over a short (10 d) or long (4 wk) period, found only slight differences in behavioral responses of the cows between the 2 durations (Johnsen et al., 2024). At least for the calves though, the longer separation period showed some benefits (Johnsen et al., 2024; Sørby et al., 2024b), so that a longer separation process with smaller steps might be worth examining.

The Individual Matters

A prominent finding of our study were the great individual differences in the behavioral responses to separation (i.e., vocalizations and searching behavior) by cows in the NF as well as the GR treatment. These individual differences were greater than differences between the 2 separation methods, which is in line with recent reports of other separation studies in dairy CCC systems. For example, Johnsen et al. (2024) described that individual variability in vocal responses of cows to separation was greater than the treatment effect of a short or long separation period. In a previous study, the same authors also described that many cows showed no vocal response or spent little time close to the separation barrier during partial separation from their calves, which were tested for different levels of nutritional dependence from the dam (Johnsen et al., 2018). Also Neave et al. (2024a) reported a high amount of cows and calves with a lack of vocalizations, as well as various extremely marked behavioral reactions to gradual or abrupt separation of cow and calf. Analogous to this, there was a high variability in the amount of cow-calf interactions between individual pairs already in the preseparation period of our study, which is in line with results by Wenker et al. (2021) for dairy cows in a CCC system. These authors discussed that the large interindividual variability in calf-directed affiliative behaviors of cows in their study might be caused by the fact that certain cows have a greater interest for contact and interaction with their calf than others (Wenker et al., 2021). It is likely that these cows will also be the more emotionally negatively affected individuals with a consequently stronger behavioral response to separation. In sum, there is thus accumulating evidence that individual differences between cows might have a greater effect on stress experiences during separation from the calf than alternative separation methods per se, which strongly supports the idea to develop separation strategies that can be adapted to individual cow-calf pairs like proposed by Wenker et al. (2022) beforehand.

Toward More Individualized Separation Protocols

Selection criteria for more individualized separation protocols might not be that straightforward and further research is strongly encouraged here. In our study, calf sex and cow parity had no effect on the vocal response of cows to separation (see Supplemental Table S5), which is in line with results in beef (Stěhulová et al., 2017) and dairy cows (Neave et al., 2024a). However, in other studies multiparous cows reacted with a higher physiological (zebu cows; de Paula et al., 2023) and behavioral (beef cows; Ungerfeld et al., 2011) stress response to abrupt weaning compared with primiparous cows. These authors discussed that the cow-calf bond might be more intense in cows of higher parity (Ungerfeld et al., 2011).

A further possibility might be to wean calves according to their weight gain, as a proxy for physical development. However, in our study (see Supplemental Table S5) and that of Stěhulová et al. (2017), cows with heavier calves reacted with a stronger vocal response to separation. This might be due to a parallel response of cows to the calves' vocalizations, because in the study by Stěhulová et al. (2017) calves that grew faster before weaning also reacted with a stronger behavioral response to it. As discussed by Stěhulová et al. (2017), it is likely that calves that receive higher amounts of milk from their dam before weaning, either due to a high yield of the cow or simply due to more frequent nursing events, also lose access to higher amounts of milk during weaning and are therefore more stressed by the event. Partly in line with this, beef calves of high yielding dams, with higher growth rates, tended to show more fence-line pacing after weaning than calves from low yielding dams, but the frequency of vocalizations was similar in both groups (Ungerfeld et al., 2009). An individual weaning method based on weight gain of the calves might therefore not be straightforward. Where the facilities are available, intake of roughage, concentrate, or amounts of supplemental milk by the calves during the separation process is likely a more promising measure to tailor a suitable time point for total separation of the pairs, because a higher level of nutritional independence of the calf showed positive effects on cows' and calves' stress responses during weaning and separation in the past (Roth et al., 2008; de Passillé and Rushen, 2016; Johnsen et al., 2018, 2024). Such measurements could be done automatically on farm, for example with rumination collars or by the automatic milk feeder, but because intake of solid feed (Fröberg et al., 2008; Borderas et al., 2009; Sweeney et al., 2010) and often also of supplementary milk (Johnsen et al., 2015) is usually small during the preweaning period while calves can still consume large amounts of milk from the cow, this attempt will be primarily relevant for cow-calf pairs that are gradually separated over individualized long time periods.

Finally, the considerable differences in cows' responses to separation from the calf likely also reflect different personality traits of the cows, which have been shown to influence adult dairy cows behavioral and physiological reactions to stressful or novel situations in the past (Müller and Schrader, 2005; Kovács et al., 2015; Foris et al., 2018). It is noteworthy that in previous studies the frequency of vocalizations was associated with the personality trait of sociability, whereas the amount of locomotion in stressful situations was more related to the coping style of the individual and the personality trait of activity in cattle (cows, Müller and Schrader, 2005; calves, van Reenen et al., 2005). Future research might therefore attempt to quantify the effect of the most known personality traits that modify dairy cows' reactions during challenging situations (as summarized in Koolhaas and van Reenen, 2016) on the stress responses of cows to separation from their calf. Particularly, this should also take into account more passive coping, that is, less vocal individuals. As reviewed and discussed by Woodrum Setser et al. (2023) there is potential to develop automatic techniques to measure personality traits based on existing precision livestock technology already used in the barn, because specific behavioral patterns of dairy cows and calves in the home pen are associated with personality traits (Neave et al., 2022). Potentially, such systems could assist in identification of likely stronger affected cows in the future to support a move toward more individualized separation protocols.

Inhibition of Milk Ejection and Physiological Stress Responses After Separation Are Short-Lived

Machine milk yield of cows was reduced during the baseline period with full-time CCC, but increased rapidly within the week that calves were prevented from suckling in both treatments, which has repeatedly been described for CCC systems with varying levels of contact before (e.g., de Passillé et al., 2008; Johnsen et al., 2021b; Nicolao et al., 2022). As also frequently discussed beforehand (e.g., in Nicolao et al., 2022), this reduction can be explained by milk ingestion of the calves in combination with an impaired alveolar milk ejection of suckled cows in response to stimulation by machine milking (Lupoli et al., 2001; de Passillé et al., 2008). During the first 2 wk of the separation process, calves of our GR treatment could still suckle for part of the day, while NF calves were prevented from suckling immediately with treatment start, so the reduced (evening and total) milk yields of GR cows in the parlor compared with NF cows during these weeks are plausible.

Within the GR treatment, cows showed a weekly increase in total milk yield during the 3-wk partial separation phase (Table 6), which is in accordance with other studies on gradual separation that reported a steady increase of machine milk yield with each step that the contact time to the calf was reduced (Johnsen et al., 2021b; Sørby et al., 2024a). Interestingly, however, this weekly increase in total milk yield of GR cows was evident despite the fact, that evening milk yield during the first and second week of the separation process was comparable (as discussed previously) and suckling by calves was not able to influence morning milk yield. Descriptive examination of the individual milking times showed in this regard that the reduced total daily milk yield by ~4 L in wk 1 with half-day contact compared with wk 2 with morning contact (Table 6) was mainly caused by a lower morning milk yield during the first ~2 d after treatment start within wk 1 (Figure 2). Similarly, NF cows showed descriptively a decreased morning as well as evening milk yield in the first week of the separation process compared with the second week. This indicates that cows needed on average ~2 d to habituate to the change in contact allowance to their calves. A possible explanation for these reduced yields in the first days after start of the treatments might potentially be an inhibition of milk secretion caused by considerable amounts of milk remaining in the udder after the sudden reduction (GR) or complete discontinuation (NF) of suckling by calves compared with the frequent milk removal in the preseparation period. Although we did not directly test this, it has often been described in the literature that incomplete milk removal during milking or reduced milking frequency of dairy cows causes a decline in milk production (Bar-Peled et al., 1995; Penry et al., 2017; Albaaj et al., 2018). The exact mechanisms of regulation of milk synthesis and secretion are still quite unknown, but there are some suggestions of a decrease in mammary epithelial cells' secretory activity and cell apoptosis in conjunction with an altered integrity of tight junctions in the mammary epithelium (Deacon et al., 2023), modification of the responsiveness of the mammary gland to prolactin (Toledo et al., 2020), as well as diverse negative feedback mechanisms of different proteins, hormones, and growth factors contained in the milk itself (Weaver and Hernandez, 2016). Therefore, the reduced suckling of calves in combination with the likely impaired milk ejection at machine milking (Lupoli et al., 2001; de Passillé et al., 2008) probably led to an accumulation of milk in the mammary gland that negatively affected the milk synthesis, as already discussed by others for yields of CCC cows (e.g., Barth, 2020; Churakov et al., 2023). The fact that milk ejection was only transitorily impaired for ~2 d after start of our separation treatments aligns with findings by Albaaj et al. (2018), who reported that a high degree of milk remaining in the udder at milking had only short-term negative carry-over effects for 2 of the 7 following milkings. Once the cows in our study habituated to the (half-day) prevention of nursing after ~2 d, milk ejection in response to stimulation in the milking parlor probably normalized and thus milk yield increased, which is in line with suggestions by Bar-Peled et al. (1995).

As an alternative or complementary explanation, it is possible that the elevated stress levels of the dams caused by increased udder pressure as well as increased calf vocalizations, missing nursing by the calf (NF) or missing overnight contact to the calf (GR), reduced milk yields at the parlor during the first days after initiation of the separation process. In support of this, Pomiès et al. (2007) reported restlessness and vocalizations for 2 d in dairy cows in midlactation after a change of the milking routine from twice daily to only once-a-day milking, equally pointing toward stress in cows through reduction of milking (or in our case nursing) frequency. A reduced milk yield in reaction to stressful conditions or application of exogenous glucocorticoids and ACTH is a known phenomenon in dairy cows (Tančin and Bruckmaier, 2001; Bobić et al., 2011; Ponchon et al., 2017). The exact mechanism behind this is also still very little understood (discussed in Tančin and Bruckmaier, 2001; Bobić et al., 2011), but there is some evidence for inhibition of oxytocin at the central (brain; Bruckmaier et al., 1992, 1993) and peripheral level (mammary gland; Bruckmaier et al., 1997; Bruckmaier and Blum, 1998) in response to stress. Because it is likely that stress for our GR cows was highest in the first night without contact to the calf, it is plausible that we found the lowest yield at the first morning milking after start of separation, after which the yield gradually increased again.

An influence of stress on milk yields in our study is supported by the increase in FGCM concentrations and immune markers in both of our treatments at the second day of the separation process, which was the first sampling time point after treatment start. Although it has been shown that increased plasma cortisol levels are not directly related to a decrease in oxytocin levels, they often occur simultaneously in stressful situations (Sutherland and Tops, 2014). In general, the literature on physiological responses of cows to separation from their calves is comparatively sparse and especially results on cortisol responses of cows to separation are mixed depending, among other things, on calf age (Whisnant et al., 1985; Acevedo et al., 2005; Pérez-Torres et al., 2016) or time point of sampling (Loberg et al., 2007). However, an increased neutrophil:basophil ratio (Ungerfeld et al., 2011) and increased neutrophil numbers, paralleled by an elevated neutrophil:lymphocyte ratio (Lynch et al., 2010) were reported in beef cows following abrupt separation from their calves. Also, increased serum cortisol concentrations have been found in beef (Whisnant et al., 1985; de Paula et al., 2023) and zebu cows (Acevedo et al., 2005; Pérez-Torres et al., 2016) following temporary or total separation from their calves as well. In all of the latter studies, the increase in cortisol concentrations was only short-lived and had disappeared at the next sampling point, which is in agreement with the transitory increase in our FGCM concentrations. One possible explanation for this finding is that cortisol concentrations, as well as immune markers, increased in response to elevated unpleasant udder pressure after sudden reduction or cessation of suckling by the calf with start of the separation treatments. Although we did not measure udder pressure, this is likely, given the above discussed inhibition of secretion and measured temporary low morning milk yields of cows in both treatments for the first days of the procedure. In support of this, Bertulat et al. (2013) measured the highest udder pressure in dairy cows at the second day after sudden dry off and the highest FGCM concentrations one day later, which is in line with the reported time-delay between stressor and FGCM concentrations in other studies (e.g., Palme et al., 1999; Morrow et al., 2002). Consequently, increased udder pressure could partly explain the transitory increase in cortisol levels in the first week of our study, and because increased glucocorticoid levels can lead to neutrophilia (Weber et al., 2001; Burton et al., 2005), might also have influenced the immune response. Taken together, elevated stress levels of the dams due to sudden loss of nursing and contact to the calves in combination with impaired milk ejection at the parlor by inhibition of oxytocin probably led to considerable amounts of milk remaining in the udder, which resulted in unpleasant udder pressure, as well as the short-lived depression of milk secretion. This in turn probably itself caused stress to the animals, further increasing FGCM concentrations. Therefore the measured transitory increase in physiological markers and reduced milk yields likely reflect an interplay of several physical and emotional stressors that mutually affected each other.

Nonetheless, it remains unclear why evening milk yield of GR cows showed, in contrast to the morning milk yields, a rather sharp increase after initiation of fence-line separation in the third week. The high levels of vocalizations and searching behavior as well as increased cortisol levels in this week equally point toward considerable stress of cows after loss of suckling and contact to the calf at this time point. Therefore, an impeded milk ejection of GR cows at evening milking due to stress of the dams and a negative feedback inhibition through the sudden loss of suckling between milkings could be expected for the first days of the week with fence-line contact as well. The only difference might be that GR cows were already used to the missing suckling by calves after evening milking and during the night and therefore machine milking might have somehow better stimulated milk ejection at evening milking compared with conditions at morning milking in the first week. Future research is needed here to replicate (or reject) this finding and enhance our understanding of the underlying mechanisms.

Limitations

To judge which of our 2 separation methods with different steps at different time points was collectively the more gentle method for the cows, we chose to compare the cows' distress responses pooled over the whole 3-week partial separation phase. However, this statistical approach did not allow us to detect potential short-lived changes in the behavioral distress indicators and is a limitation of the study. This is especially relevant for lying behavior and rumination time, because previous studies showed reduced lying times in dairy cows within the first 8 to 72 h after separation from their calves (Loberg et al., 2007; Rhim, 2013; Neave et al., 2024a) as well as reduced lying and rumination behavior in beef cows during the first 72 h after separation from their calves (Ungerfeld et al., 2011). Given this evidence and the short-lived changes in physiological indicators found in our study, it is likely that our cows might have also reacted with reduced lying behavior or rumination time, or both, within the first hours after initiation of a new step of the separation process. However, Wenker et al. (2022) reported no difference in rumination time or time spent inactive in dairy cows that were separated via fence-line separation or 2-step separation with nose flaps from their calves. These cows were sampled for the first 4 d each after initiation of a new step within the separation processes and the findings are in accordance with our results.

A further major limitation of our study was that the chosen observation time points for assessment of stress responses did only allow for a very limited evaluation of changes in social behaviors within pairs, which was a secondary aim of our study. Foremost, we observed hardly any social play between cows and calves, which was likely because our observations covered mainly the times when cows came back from milking and thus were mainly occupied with feeding and nursing and there was in general a lot of agitation in the barn. We suspect that we would have observed more social play during later times of the day or if our afternoon observation would have lasted longer than 30 min, because there were fewer distractions and more space on the aisles at this time point. Furthermore, observations of nursing behavior were also not optimal. Although we observed nursing frequently, observation was hampered due to the necessity that the observer had to switch between the herds every 30 min. In consequence, we faced the problem that cows were often still at the feeding rack in the beginning of the observation (especially those that were last in milking order) and had only started nursing when the observer had to switch over to the other herd, where other cows had just finished nursing. Therefore we did not observe many complete nursing events and furthermore missed a relevant proportion of nursing events that happened in parallel at the other herd. Consequently, our data only reflect changes in probability to observe nursing at the observation time points, but do not allow for any further conclusions regarding the development of duration or frequency of nursing. The same limitations hold true for cow-calf interactions with the additional difficulties that we already found differences between treatments during the baseline and had a high amount of interindividual variability among cows including many observations with zero occurrences of cow-calf interactions. In addition, our observation mode was not optimal for assessment of all cow-calf interactions. Although recordings of affiliative behaviors between cow and calf generally show a good correlation between continuous and scan sampling, a sampling interval of 3 min is too long for reliable observation of shorter-lived affiliative behaviors (both in Manfrè et al., 2024). In our study this concerned the behaviors nose-to-cow or nose-to-calf contact, as well as the calf rubbing its head against the cow, which were likely often missed during scan sampling. The longer-lasting licking of the calf by the dam, which we considered as more important, could nonetheless be reliably assessed with our selected sampling interval (Manfrè et al., 2024). In summary, results of this study for changes in social behaviors between cow-calf pairs during the separation process are limited by the chosen observation time points and need confirmation by future studies specifically designed to measure these behaviors.

CONCLUSIONS

Dams reacted with a similar stress response to 2-step separation with nose flaps and a gradual reduction of contact time to their calf, potentially because both methods ultimately reduced the daily contact times and involved a rather abrupt termination of nursing. Regardless of the method, dams showed a longer-lasting behavioral stress response, a transitory increase in physiological stress markers, and an inhibition of milk ejection after initiation of the separation process, but interindividual differences were considerable. As shown by the similar milk yields in the week with half-day and morning contact, the weekly reductions of contact time in the gradual separation treatment worked in a rather stepwise than gradual manner, which needs future improvement. In addition, our descriptive results strongly support preceding demands to develop more individualized separation protocols and to consider previously formed expectations as important mediators of the distress response during development of future separation protocols.

NOTES

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Nonstandard abbreviations used: CCC = cow-calf contact; D = sampling day; Den DF = denominator degrees of freedom; DF = degrees of freedom; EIA = enzyme immunoassay; eve = evening; FGCM = fecal cortisol metabolites; GR = gradual separation method with weekly reduction of dam-calf contact time; mor = morning; NF = 2-step separation method with calves wearing a nose flap in the first step; Num DF = numerator degrees of freedom; P = phase; T = treatment; W = week.

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