

**From the Department of Animal Breeding and Genetics
Professorship of Animal Husbandry, Behaviour and Welfare
of the Justus Liebig University of Gießen**

HOW TO REDUCE WEANING AND SEPARATION STRESS IN DAIRY COW-CALF CONTACT SYSTEMS?

**A comparison of a gradual process with the two-step nose flap
method and an evaluation of different stress indicators**

DISSERTATION

for the award of the doctoral degree (Dr. agr.)

in the Faculty of Agricultural Sciences, Nutritional Sciences and Environmental
Management of the Justus Liebig University of Gießen

submitted by

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**Declaration in accordance with the Doctoral Regulations of the Faculty
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“I declare that the doctoral thesis here submitted is entirely my own work, written without any unauthorized help by a third party and solely with the assistance referred to in the thesis. I have indicated in the text those texts that have been quoted from already published sources, either verbatim or by analogy and all statements based on verbally conveyed information. During the research carried out by me and referred to in the doctoral thesis, I have at all times followed the principles of good scholarly practice as defined in the Statute of Justus Liebig University Gießen for Ensurance of Good Academic Practice.”

List of included papers

Paper I

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Paper V

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Author contributions

Funding acquisition for studies II & IV was done by me with the help of Dr. Kerstin Barth. Planning and design of studies I-IV was done by me under thorough supervision of Prof. Dr. Uta König von Borstel, Prof. Dr. Susanne Waiblinger and Dr. Kerstin Barth. Design of study I was further supervised by Prof. Dr. Rupert Palme, who was additionally consulted for queries regarding fecal samples during studies II and IV. Design of study V was done in collaboration by Dr. Janja Sirovnik, Rebecca Simon and me under supervision of Dr. Kerstin Barth, Dr. Steve Smith, Prof. Dr. Susanne Waiblinger, Prof. Dr. Gesine Lühken and Prof. Dr. Uta König von Borstel. My part in study V was the overall experimental design, while Janja Sirovnik and Rebecca Simon designed the exact sampling protocol for the barn and laboratory analysis under supervision of Gesine Lühken.

Management and execution of data collection in the barn for all experimental studies was done by me with assistance of technicians. Processing of data from automatically assessed behaviors was done by me. Video analysis for studies II- IV was done by me with assistance of students and technicians. Laboratory analysis of fecal samples for studies I, II & IV was done by me (sample preparation) and laboratory technicians. Laboratory analysis of blood samples for study II & IV was done by an external medical diagnostics provider. Laboratory analysis of blood samples for study V was done by Rebecca Simon and laboratory technicians.

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“You never change things by fighting the existing reality. To change something, build a new model that makes the existing model obsolete.”

Richard Buckminster Fuller

Summary

Early separation of cow and calf within 24 hours after birth is increasingly questioned by society and producers within the dairy industry. Cow-calf-contact (CCC) systems, which allow extended contact between cow and calf, are an alternative to this practice with many advantages. However, the weaning and separation process is one of the biggest challenges for these systems and thus, the development of practical, low stress separation methods for CCC systems is crucial. The present thesis aimed to compare behavioral and physiological responses of 36 dairy cows and their calves to two different weaning and separation methods that were hypothesized to have considerable potential to reduce stress of the pairs during the process: two-step weaning and separation with nose flaps (NF, 2 weeks access to dam with a nose flap, 1 week fence-line contact before total separation, n=18 pairs) and a gradual reduction of contact time between cow and calf before total separation (GR, 1 week half day contact, 1 week morning contact, 1 week fence-line contact before total separation, n= 18 pairs). Pairs were kept with full-time contact for the entire pre-weaning period and the weaning and separation process started when calves were 3 months old. As a secondary aim, this thesis examined the use of fecal cortisol metabolites (FGCM) for unweaned calves and changes in relative telomere length (RTL) in both cows and calves as indicators for stress during weaning and separation, since both of these indicators were hypothesized to better reflect, to a limited degree, the stress the animals experience over a longer time span, compared to the commonly used plasma or salivary cortisol measurements.

For validation of FGCM concentrations as a stress indicator in unweaned dairy calves (experiment I, **study I**) five German Holstein calves of 3 mo of age were exposed to a 45 min transport stressor once before and once after weaning. All voided fecal samples were collected for 24 h after termination of the transport. FGCM concentrations showed a clear peak after the stressor in dairy calves pre- and post-weaning, suggesting that FGCM concentrations are a valid stress indicator in younger calves, just as they are for older cattle. However, peak FGCM concentrations tended to be higher and the time lag of excretion of FGCMs after the stressor was numerically longer in calves on the pre-weaning compared to the post-weaning diet. Thus, it is concluded that FGCM concentrations are not a suitable indicator for comparison of pre- and post-weaning states in calves with a change in diet.

For examination of cows and calves' stress responses to the GR and NF weaning and separation method (experiment II, **studies II-V**) direct behavioral observations were conducted on 4 days per week from 1 week before weaning start and during the 3-week weaning and separation period. In the first week after total separation, only the cows but not the calves were observed, since calves were in a different building out of sight. Locomotor play levels and lying behavior of the calves and lying behavior and rumination time of the cows was automatically recorded with sensors. Blood and fecal samples, for analysis of FGCM concentrations, RTL and the immune response, were taken twice per week over the whole 3-week weaning and separation phase up from the day of treatment start (day 0, or for cows day -1). A further sample of the cows was taken in the first week after total separation from the calves. Milk yield in the parlor was continuously recorded and calves were routinely weighted once per week. Additionally, the selection gate which allowed calves to change sides from the calf area into the cow area was

video monitored for 24 hours per day during 1 week before nose flap insertion and during the 14 days in which calves had free access to the cow area while wearing the nose flap, in order to determine changes in time that calves still spend in the cow area after insertion of a nose flap. Statistical analysis was conducted using (generalized) linear mixed models.

Results for calves (**study II**) showed that NF calves had lower average daily weight gains, a stronger decrease in the number of lying bouts and reduced locomotor play levels compared to GR calves, indicating that the abrupt change in diet with the NF method apparently compromised adaption of the gastrointestinal tract (GIT) and led to a greater energy deficit in NF compared to GR calves. Furthermore, NF calves showed a high number of suckling attempts with the nose flap for at least 3 days, which together with the reduction in lying bouts and locomotor play behavior hints towards a negative affective state in these calves. The neutrophil:lymphocyte ratio of NF calves was elevated on day 3 after insertion of the nose flaps compared to baseline, but showed no change for GR calves on any sampling day. Total mixed ration feeding times were higher in NF than in GR calves, but this was not reflected in respective high weight gains, pointing towards a possible impairment of feed intake by the nose flap. Lastly, pressure marks (12 calves) and partly even injuries (6 calves) were present at the nasal septum of NF calves when nose flaps were removed after the 14 day period. Collectively, it is concluded that two-step weaning with nose flaps cannot be classified as a high-welfare weaning and separation method for dairy calves. In contrast, we saw higher weight gains during the weaning and separation period and a lower decrease in lying bouts and locomotor play levels compared to baseline in gradually weaned calves, indicating a better adaption of the GIT to the dietary change as well as a likely less compromised affective state. Nonetheless, GR calves showed a higher frequency of vocalizations and more searching behavior compared to the NF group. This was confounded by the different possibilities for cow access and associated differing necessity for vocal communication in the two treatments, but nevertheless demonstrated that the gradual weaning and separation method also provoked stress and frustration in the calves, which was especially evident in the third week with fence-line separation from the cows. It is concluded that both methods caused stress for the calves, but that overall the GR method was favorable for calves compared to two-step separation with a nose flap (**study II**).

Video observations of the calves' time spent in the cow herd (TIC) after insertion of the nose flap (**study III**) revealed that calves significantly reduced their TIC at the fourth day of wearing the nose flap compared to their individual baseline, after which times stayed at a lower level. This indicates that in 3-month-old dairy calves the nose flaps need to be worn for a minimum of 4 days in order to effectively reduce the calves' suckling motivation, while there seems to be no benefit of using the nose flap longer than this time. However, these times were subject to individual variability already during the baseline and need replication. It was not assessed whether injuries at the calves' nasal septum were already present in our NF calves after the 4 days of nose flap usage, but results in 8-month-old beef calves suggest that this is the case after 5 days of usage.

With regard to the cows (**study IV**) we found no difference between the two separation methods in any of the examined behavioral and physiological response variables. However, a significant increase in vocalizations and searching behavior compared to baseline was present with both methods. Further, 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 high inter-individual 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 weeks while calves wore the nose flap. Milk yield was higher in NF than GR cows during the 2 weeks while GR calves had time-restricted access to their dams and NF calves were prevented from suckling, but showed no difference afterwards anymore. However, similar evening milk yield 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. It is concluded that cows experienced stress during separation with the GR method, when implemented over three weeks in three steps as in this study, as well as with the NF method, but differences between individuals were considerable.

Analysis of RTL lengths of cows and calves (**study V**) showed no difference for calves and only a very weak tendency for shorter RTL in cows separated with the GR compared to the NF method. Furthermore, there was no correlation of RTL with health indicators (injuries at nasal septum of calves through the nose flap) or any of our other stress indicators (e.g. FGCM concentrations, frequency of vocalizations or searching behavior), which was potentially due to a too short sampling interval. Thus, it is concluded that RTL as sampled in the present thesis does not seem to be a valid indicator of weaning and separation stress in cattle.

Given the overall beneficial effect of the gradual weaning and separation method for the calves and the missing difference between the two methods for the cows, the general conclusion from this thesis is that a gradual method is overall favorable for reduction of weaning and separation stress of dairy cow-calf pairs compared to two-step separation with nose flaps. However, results indicated that the gradual separation protocol with three reductions in contact time over three weeks as used in the present thesis worked in a rather stepwise than gradual manner and also provoked considerable stress in cows and calves, which clearly demands for future refinement of the gradual weaning and separation method as compared to the protocol used for this thesis. Recommendations for possible refinement are given in the general discussion of this thesis.

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List of abbreviations

ACTH	= Adrenocorticotrophic hormone
ADG	= Average daily gains
AMF	= Automatic milk feeder
CCC	= Cow-calf contact
CPA	= Counts of peak acceleration measurements
EIA	= Enzyme immunoassay
FGCM	= Fecal (glucocorticoid) cortisol metabolites
GIT	= Gastrointestinal tract
GR	= Gradual reduction of contact time
L	= Lymphocytes
LSM	= Least square means
N	= Neutrophils
N:L ratio	= Neutrophil: lymphocyte ratio
NF	= Two-step weaning with a nose flap
RTL	= Relative telomere length
S.E.	= Standard error
TIC	= Time spent in cow herd
TMR	= Total mixed ration
TotalSep	= Total separation

General Introduction

Introduction

Two recently published perspective papers (Brombin et al., 2019; Cook and von Keyserlingk, 2024) provided a good summary of the necessary changes the modern dairy industry will need to face if it wants to maintain public trust in the future, including i.a. termination of tiestall housing, dealing with surplus calves, painful procedures and zero-grazing systems. Both have highlighted termination of early separation of cows and calves shortly after birth as a key step for future sustainability of the dairy industry, as this practice is rejected by consumers around the world (e.g. Boogaard et al., 2010; Ventura et al., 2013; Busch et al., 2017; Cardoso et al., 2017; Hötzel et al., 2017; Sirovica et al., 2022), mainly due to a perceived unnaturalness and animal welfare concerns of the consumers (reviewed in Placzek et al., 2021). At the same time, an increasing number of practitioners already started to raise their calves with contact to the dam or a foster cow, in a so called cow-calf contact (CCC) system (Sirovnik et al., 2020), for several years or are interested in implementation of such a system in the future (Eriksson et al., 2022; Neave et al., 2022; Bertelsen and Vaarst, 2023; Johanssen et al., 2023).

The commonly cited reasons for the routine practice of early separation of cow and calf include better options to monitor calf health, colostrum intake and milk ingestion, an increased amount of saleable milk, prevention of disease transmission from the cow to the calf and a prevention of stress at later separation (Flower and Weary, 2001; Sumner and von Keyserlingk, 2018; Beaver et al., 2019). However, there is little scientific evidence for a negative effect of prolonged contact on cow and calf health (Beaver et al., 2019) while morbidity and mortality of calves kept in the conventional calf rearing systems is also high in many cases (Santman-Berends et al., 2019, 2021; Roche et al., 2023), as already discussed by others (Wenker et al., 2022b; Cook and von Keyserlingk, 2024). Furthermore, conventional calf rearing practices, including early separation from the dam, individual housing in the first weeks of life and restrictive feeding, can compromise the physiological and social development of the calf as reviewed in detail elsewhere (Cantor et al., 2019; Costa et al., 2019; Lorenz, 2021). On the other hand, CCC systems have the potential to increase welfare of cows and calves by providing opportunities to fulfill highly motivated social behaviors. For example, dairy cows have been shown to push high weights in order to receive access to their calf (Wenker et al., 2020), while separation from the cow or the calf induces a negative judgement bias in both partners of the pair (Daros et al., 2014; Lecorps et al., 2021 as discussed by Cook and von Keyserlingk (2024)). Recent systematic literature reviews (Beaver et al., 2019; Meagher et al., 2019) showed a range of advantages of prolonged CCC, which include that calves which were raised with full contact to a cow show a consistent reduction in abnormal oral behaviors, such as cross-sucking, and increased weight gains during the suckling period (provided an adequate milk supply, see Nicolao et al., 2022). Also, contact to cows increases social competence of the calves when confronted with an unfamiliar cow (Buchli et al., 2017) and during integration into the cow herd as heifers (Wagner et al., 2012). Regarding health, evidence points towards beneficial or neutral effects on calf scours, as well as a reduced risk of mastitis in dairy cows that are suckled by their calves (Beaver et al., 2019). This is in line with reports by farmers already practicing CCC, which name improved health of the animals, improved labor satisfaction and less time consumption, as well as perception of this practice as a more natural system as their main reasons for implementing this practice (Vaarst et al., 2020; Eriksson et al., 2022). Recently, the

scientific panel on Animal Health and Animal Welfare of the European Food Safety Authority (EFSA) reviewed the most recent scientific knowledge on the welfare of calves in common husbandry systems under European legislation. They also came to the conclusion that contact between cow and calf for longer durations should be provided in the future, but that this implementation requires further research and scientific guidance (EFSA, 2023).

1.1 A need for practical low-stress weaning and separation methods for CCC systems

Among the most frequently cited barriers by farmers for implementation of prolonged cow-calf contact are an inappropriate barn design and required labor (Vaarst et al., 2020; Eriksson et al., 2022), as well as an impaired alveolar milk ejection of suckled cows in the parlor (Lupoli et al., 2001; de Passillé et al., 2008). The latter decreases the amount of salable milk in combination with the high amount of milk ingested by the calves (Meagher et al., 2019; Barth, 2020) and is thus financially challenging. Furthermore, the need for breaking the established 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). In a survey among European farmers already practicing a CCC system, 87% reported problems during weaning and separation (Eriksson et al., 2022). Also, separation stress was a major reason to discontinue this practice in a survey of Norwegian farmers (Hansen et al., 2023) and has been identified as a key barrier for adoption of CCC systems during interviews with farmers in New Zealand (Neave et al., 2022). In order to facilitate implementation of CCC systems in practice on a larger scale and enhance welfare of animals kept in these systems, there is thus a high necessity for development of practical and low-stress weaning and separation strategies for CCC systems.

1.2 Stress during weaning and separation

For the calf, the weaning and separation process entails loss of milk as main nutritional source, loss of access to the udder as suckling opportunity (which has been shown to be itself rewarding in artificially reared calves, see Jasper et al., 2008) and loss of maternal care and associated emotional comfort (Enriquez et al., 2011). For the dairy cow, the process entails mainly loss of the contact to the calf and associated pleasurable experiences, such as grooming and nursing (discussed in Newberry and Swanson, 2008). Under natural conditions, weaning is usually a gradual process during which the dams' milk availability gradually decreases and the calves concurrently increase the time they spend foraging for solid food and spending time with social peers apart from the dam (discussed in Newberry and Swanson, 2008; Lynch et al., 2019). In free-ranging beef cattle it was observed that cows initiate the majority of the nursing bouts when calves are very young, but that this changes with increasing age of the calf, and that older calves initiate nursing more often than their dams (Lidfors et al., 1994). With increasing age of the calf, the number of daily suckling bouts declines (Reinhardt and Reinhardt, 1981a; Das et al., 2000) until the cow completely rejects the calf's nursing attempts (discussed in Enriquez et al., 2011), which has been observed to happen at variable ages between 7-14 months of age (Reinhardt and Reinhardt, 1981a). According to parent-offspring conflict theory (Trivers, 1974), the dams will wean their young when their investment in the current offspring is greater than the benefit the young achieves from it (at a point when the calf does not depend on milk supply by the dam anymore), while the dam increasingly benefits from preserving energy and resources for investment in future offspring. Thus, the exact weaning age depends on several

factors, including i.a. the available food resources and time until the next offspring is born (discussed in Newberry and Swanson, 2008; Burns et al., 2010) as well as sex of the calf (females weaned with about 9 months, males with about 11 months of age, Reinhardt and Reinhardt, 1981a). Naturally, weaning is, however, not accompanied by separation, as preferential relationships between the dam and her offspring remain constant for several years (Reinhardt and Reinhardt, 1981b) and also after birth of the next calf (Veissier et al., 1990). Furthermore, Bertelsen (2023) and Hvidtfeldt Jensen (2024) already questioned, if the reported natural weaning age is representative for modern dairy cattle which are bred to produce extremely large amounts of milk and are usually kept indoors, sheltered from weather and predation, and with *ad libitum* feed. Thus, their ability to invest in future offspring is less compromised when continuing to nurse the current calf, which could theoretically delay the age that modern dairy cows would wean a calf compared to the reported 7-14 months in semi-wild zebu cattle, when left to decide the time point themselves (discussed in Hvidtfeldt Jensen, 2024).

In contrast to this, weaning and separation in European dairy CCC systems is typically done a lot earlier, when the calf is around 12.5 to 17 weeks old, in conventional or organic farms respectively, and on 85% of the farms also abruptly (Eriksson et al., 2022). As reviewed by Weary et al. (2008), pre-mature and abrupt weaning and separation leads to high levels of stress for the calf, which is further intensified, if the process is accompanied by additional stressors such as changes in spatial surroundings, loss of known peers, mixing with new conspecifics and altered handling and management routines. In several studies on beef and dairy calves, weaning and separation has been shown to result in reduced growth rates (Roth et al., 2009; Campistol et al., 2016), impaired immune function (Lynch et al., 2010a), changes in the leucocyte and erythrocyte population in blood (O'Loughlin et al., 2014), increases in plasma cortisol levels (Hickey et al., 2003) as well as strong behavioral responses such as increased activity levels and frequent vocalizations (Lidfors, 1996; Jasper et al., 2008), reduced play behavior (Enriquez et al., 2010) and lying time (Price et al., 2003; Haley et al., 2005). These changes are not only related to hunger (Thomas et al., 2001), but also to the loss of contact to the dam, since abrupt maternal separation has been shown to cause a pessimistic judgement bias even in nutritionally independent dairy calves (Daros et al., 2014). Thus, like already concluded by Weary et al. (2008) weaning and separation is of obvious welfare concern for these animals.

Regarding the dams, it has been shown that the cows are less affected by the separation process than their calves (Acevedo et al., 2005; Pérez-Torres et al., 2016; Stěhulová et al., 2017; Nicolao et al., 2022), which might be explained by the evolutionary greater risk of death for calves compared to their dams in case that the pair fails to reunite under natural conditions (discussed in Newberry and Swanson, 2008). Nonetheless, the cows likely experience discomfort due to high udder pressure as well as a negative affect when pleasurable experiences, such as grooming and nursing of the calf, are suddenly withdrawn (discussed in Newberry and Swanson, 2008). In beef and dairy foster cows it has been shown that abrupt removal of the calves caused increased walking and pacing behavior, a decrease in grazing and lying times as well as a high frequency of vocalizations (Loberg et al., 2007; Ungerfeld et al., 2011, 2015) which lasted about 2-4 days. Also dairy cows kept with full or part-time contact to their calf showed an increase in vocalizations when they were abruptly (or gradually) separated from their 10-week-old calf

(Neave et al., 2023). On the other hand, physiological responses of cows to separation are often rather small (Acevedo et al., 2005; Loberg et al., 2007; Orihuela and Hernandez, 2007; Lynch et al., 2010b; Pérez-Torres et al., 2016).

1.3. Alternative weaning and separation methods

To reduce the stress that cows and calves experience during the weaning and separation process, alternative strategies have been proposed which separate the stress of losing milk as the main nutritional source and the loss of social contact to the dam to two different points in time. These alternative strategies include the use of udder nets (Orihuela and Hernandez, 2007; Johnsen et al., 2015c), two-step separation with nose flaps (Haley et al., 2005; Wenker et al., 2022a) as well as fence-line separation (Price et al., 2003; Bertelsen and Jensen, 2023). A two-step separation of cow and calf entails that prior to permanent total separation of the pair, suckling by the calf is prevented with a so-called nose flap (as defined by Sirovnik et al., 2020, see Fig. 1), which prevents the calf from taking a teat into its mouth. This is also the way how udder nets work. During fence-line separation, partial physical contact between cow and calf (mostly visual, olfactory, auditory and some limited tactile contact) through a fence without a suckling opportunity is allowed for a specific time period before separation (Sirovnik et al., 2020). Vice versa, cow and calf can also be separated first before the calf is weaned off the milk, for example by providing the calf with the opportunity to continue suckling from a foster cow (known as 'combined rearing', Sirovnik et al., 2020) or from an artificial milk source (Johnsen et al., 2015a, 2018) after separation from its mother.

In addition to these methods, some studies investigated the effect of restricted suckling periods (of only 30min once or twice per day) and temporal weaning (complete separation of pairs for 3-4 days) before total separation (Acevedo et al., 2005; Pérez-Torres et al., 2016, 2021) in beef cattle on the stress response during final separation of the pair, which was favorable for the restricted suckling compared to the temporal weaning method (Acevedo et al., 2005). Similar approaches have recently been studied in dairy CCC systems, including responses to separation after short-time contact (20min - 2.5 hours once or twice daily, Nicolao et al., 2022), part-time/half-day contact (Nicolao et al., 2022; Bertelsen and Jensen, 2023; Neave et al., 2023) or partial contact (without a suckling opportunity, Wenker et al., 2022a) before separation. However, weaning and separation also induced stress in cows and calves that experienced short-time or half-day contact before separation (Nicolao et al., 2022), and the overall behavioral response to weaning did not differ in dairy cows or calves kept with half-day contact or whole-day contact beforehand (Bertelsen and Jensen, 2023; Neave et al., 2023). Only calves which were reared with partial contact to their dam, without the possibility of suckling, showed no stress response at the point of separation from their dam (Wenker et al., 2022a), which has been discussed to be related to an overall diminished cow-calf bond in these pairs through the complete prevention of nursing (Wenker et al., 2020, 2021, 2022a).

1.3.1 Fence-line separation

Compared to the above-mentioned alternative weaning and separation methods, the use of fence-line separation has been studied more intensively. Beef calves that have been weaned via fence-line separation show fewer vocalizations and less pacing behavior but higher cumulative post-weaning weight gains and spend more time eating and lying down than abruptly separated calves (Price et al., 2003). However, while Taylor et al. (2020) also reported higher weight gains in calves weaned via fence-line separation compared to the abrupt method, there was no effect on weight gains in the study by Burke et al. (2009) and even the opposite was the case in a study by Enriquez et al. (2010). The latter reported lower weight gains and more vocalizations in fence-line than abruptly separated beef calves. This is in line with results from zebu calves that showed more vocalizations and spend less time lying down when temporarily separated by a fence-line from their dam compared to calves that were (temporarily) abruptly separated with no contact to their dams anymore (Solano et al., 2007). Furthermore, studies showed that cows (Enriquez et al., 2010; Pérez-Torres et al., 2016) as well as calves (Price et al., 2003; Enriquez et al., 2010) spend a high amount of time near the fence-line barrier in the first days after fence-line introduction, suggesting a high motivation of the pair to reunite and potentially frustration (reviewed in Enriquez et al., 2011; Orihuela and Galina, 2019).

This is largely in line with findings for fence-line separation in dairy CCC systems. For example, one week of fence-line contact to their dam before total separation led to an overall reduced behavioral response in dairy calves compared to abrupt weaning and separation, and the response to introduction of the fence was stronger than to total separation afterwards (Bertelsen and Jensen, 2023). In line with this, Johnsen et al. (2015b) reported that the behavioral response of cows and calves was reduced when the pair was separated with a fence-line compared to a solid wall that allowed only auditory contact, but that a strong vocal response was nevertheless evident in the first days following introduction of the fence (Johnsen et al., 2015b). However, when cows and calves were prevented from suckling through udder nets and calves were provided with an alternative milk source for some time beforehand, introduction of fence-line separation caused only little stress to the animals (Johnsen et al., 2018). This finding seemed to be largely linked to the additional milk source than to the fence-line separation, since those cows and calves in this study which had the regular nursing contact to each other, also reacted with a moderate vocal response to introduction of fence-line separation (Johnsen et al., 2018).

1.3.2 Two-step separation with nose flaps

Like fence-line separation, two-step separation with nose flaps has the advantage that the stress of losing milk as main nutritional source and the loss of social contact to the dam is divided over two time points, which is why the usage of nose flaps is considered as a preferable alternative to abrupt weaning and separation for beef suckler systems (Haley et al., 2005; Ungerfeld et al., 2015, 2016). In beef cows whose calves had been wearing a nose flap beforehand, it has been shown that this method leads to positive effects on behavioral stress indicators after complete separation from calves, including reduced vocalizations, pacing and walking behavior, with a concurrent increase in grazing durations, compared to dams that were abruptly separated from their calves (Haley, 2006a; Ungerfeld et al., 2015). These positive

effects were also present when the calves were weaned with 2 month of age, which is much younger than the usual ~6 months for beef suckler calves (reported by Enriquez et al., 2011), and authors concluded that two-step weaning and separation is also beneficial for the cows when the nutritional dependence of the calf is still high (Ungerfeld et al., 2016). In line with this, dairy cows separated from their 5-week-old calves (Haley, 2006b) or 12-week-old foster calves (Loberg et al., 2007) showed reduced behavioral responses to complete separation from their calves when separated with the two-step method with nose flaps compared to abrupt separation. On the whole, the dams' stress responses seem to be reduced by prevention of nursing of their calves before total separation, which is supported by recent findings in dairy cows which suggest that the absence of nursing reduces the cows' motivation to reunite with the calf (Wenker et al., 2020, 2021).

With regard to the calves, two-step weaning with nose flaps has been shown to reduce beef and dairy calves' vocalizations and time spent walking (Haley et al., 2005; Loberg et al., 2008; Enriquez et al., 2010), increase time eating and lying down (Haley et al., 2005; Boland et al., 2008; Loberg et al., 2008), as well as reduce heart rates and cortisol levels (Loberg et al., 2008) compared to an abruptly weaned control group at the time of separation. On the other hand, there are consistent reports of low weight gains in calves during the time when they wear the nose flap compared to abruptly or fence-line weaned calves (Boland et al., 2008; Burke et al., 2009; Enriquez et al., 2010; Wenker et al., 2022a). Furthermore, calves show increased walking activity (Loberg et al., 2008; Hötzel et al., 2012; Lambertz et al., 2015; de la Cruz-Cruz et al., 2021) and several failed suckling attempts in the first days after fitting of the nose flap (Enriquez et al., 2010; Hötzel et al., 2012), which indicates that two-step separation with nose flaps likely leads to frustration in the calves (discussed in Enriquez et al., 2011). Additionally, nose flaps have been shown to cause nasal abrasions and open wounds with or without bleeding and secretion in 7-8 month old beef calves (Lambertz et al., 2015; Freeman et al., 2021; Valente et al., 2022), which might potentially even initiate pituitary abscesses (Fernandes et al., 2000), and leave nose flaps questionable with regard to calf welfare. However, at the time point of determination of the experimental design for this thesis (in 2019) there was no information available whether nose flaps also cause lesions to the nostrils of dairy calves, which are much narrower at the time point of weaning compared to those of beef suckler calves due to the typically much younger weaning age in dairy systems (in the meantime it has been reported that injuries at the calves' nostrils were also present in 2-month-old dairy calves after a 14 day usage of the nose flap, Wenker et al., 2022). Furthermore, studies in beef calves report no differences in stress indicators at separation when nose flaps were used for shorter periods of only 3 or 7 days (Haley et al., 2005; Alvez et al., 2016) compared to longer durations, which implies that shorter usage of nose flaps could be an option to prevent lesion development. However, to date there is no scientific guidance regarding the minimum duration that nose flaps need to be worn for an effective cessation of the suckling motivation of calves.

In general, a scientific evaluation of a two-step weaning and separation process with nose flaps in dairy CCC systems is missing, especially in the view of the fact that this method has repeatedly been discussed as a possible option for weaning and separation of dairy cows and their calves in the past (e.g. Sirovnik et al., 2020; Schneider and Ivemeyer, 2021; Barth et al., 2022; Eriksson et al., 2022). The major advantage of two-step weaning and separation with

nose flaps is the high practicability for farms, as in contrast to other alternative weaning and separation methods it requires no modifications of the barn (Barth et al., 2022) or adjustments of on-farm routines for animals or personnel. This is especially valuable for separation of cows and calves in pasture-based systems, where options for alternative weaning strategies are more limited. Farmers that currently run pasture-based dairy CCC systems mention two-step weaning and separation with a nose flap repeatedly as a practical approach to reduce the stress of cows and calves during the procedure (e.g. “De Öko Melkburen”, Germany or “The Ethical Dairy”, Scotland). Additionally, a recent survey among farmers running CCC systems and already using nose flaps, revealed that farmers value the lower number of vocalizations by cows and calves during weaning with nose flaps not only as a sign of reduced stress levels of the animals, but also because the frequent vocalizations during weaning are emotionally challenging for them and lead to additional worries that others may mistake these as an indication for mistreatment of the animals (Waiblinger and Hebesberger, 2023; personal communication S. Waiblinger, University of Veterinary Medicine, Vienna, Austria). Thus, an in-depth scientific evaluation of behavioral and physiological stress responses of younger dairy calves and their dams to a two-step weaning and separation procedure with nose flaps is warranted.

1.3.3 Fence-line vs. Two-step separation with nose flaps

As described in detail above, fence-line separation and two-step separation with nose flaps can both reduce the stress response of cows and calves compared an abrupt separation. Some studies in beef calves have also investigated these two methods in direct comparison to each other and showed that the calves react with more vocalizations, pacing, walking and seeking behavior to introduction of a fence-line than to insertion of the nose flap (Boland et al., 2008; Enriquez et al., 2010), while these responses were reversed, i.e. higher in nose-flap than in fence-line separated calves, at the time point of final separation from the dam in the study by Enriquez et al. (2010). In contrast, there were no differences between fence-line and nose flap separated calves in pacing behavior, eating or lying time after total separation in the study by Boland et al. (2008). In a further study, Haley (2006a) compared the response of calves to introduction of a fence-line to the response to final separation from the dam after 4 days of nose flap usage and reported a stronger behavioral reaction for the fence-line separated animals. Considering the beneficial effects of both methods at the different time points, Haley (2006b) hypothesized that potentially a combined weaning and separation strategy, comprised of prevention of nursing with nose flaps followed by fence-line contact before total separation, may offer the best weaning and separation method.

With regard to dairy CCC systems, a recent study (published after determination of the experimental design for this thesis) compared two-step separation with nose flaps to fence-line separation over 14 days each in 2-month-old dairy calves and their dams (Wenker et al., 2022a). Results showed no differences between the methods in activity patterns or time spent ruminating in the cows, and neither in physiological stress responses (plasma or hair cortisol levels and serum IgG concentrations), health status or behavioral responses in standard behavior tests in the calves (Wenker et al., 2022a). The missing differences in standard behavior tests were with the exception of higher levels of solitary play in the novel object test by calves weaned with the nose flap compared to those weaned with a fence line, which was discussed to

potentially reflect a rebound behavior due to the increased space availability in the test arena (Wenker et al., 2022a). Moreover, calves weaned and separated with the nose flap had lower weight gains during the weaning period and also showed higher activity levels in the post-weaning period compared to fence-line separated calves, so that the authors concluded that fence-line separation is the more effective method for reduction of weaning and separation stress for dam-reared dairy calves (Wenker et al., 2022a).

1.3.4 Gradual weaning and separation as a potential low-stress method

A shared problem of all commonly used weaning and separation methods (abrupt, fence-line or two-step weaning with nose flaps) is that the calves abruptly lose the milk, which has been proposed to contribute to weaning stress (Weary et al., 2008). An alternative approach is a gradual weaning and separation process, during which the contact time of the calves and their dams is gradually restricted, so that cow and calf can potentially habituate to being separated from each other. At the point of determination of the experimental design for this thesis, a gradual weaning and separation process had not yet been scientifically investigated in dairy cattle, but was considered to be a promising method by the author for several reasons. First, it resembles more the naturally occurring, slow reduction of milk intake during natural weaning, which is typically a gradual process during which the number of daily suckling bouts declines with age of the calf over the course of several months (Reinhardt and Reinhardt, 1981a; Vitale et al., 1986) as described in more detail above. Second, Weary et al. (2008) already suggested that during the increased times which cow and calf spend apart before total separation, the pairs are potentially able to habituate to the longer periods of separation from another, so that the final separation is less stressful. With regard to the calves, it is also likely that they will increase their intake of solid feed during the hours of separation from the dam, which might ease their transition from milk to solid feed as their main nutritional source during the weaning process (Weary et al., 2008). In this regard it has been shown in conventional calf rearing systems, that gradually weaned calves consume higher amounts of concentrates pre-weaning, so that they suffer less often from reduced post-weaning weight gains or even weight loss compared to abruptly weaned calves (Sweeney et al., 2010; Khan et al., 2011; Omidi-Mirzaei et al., 2015; Wolfe et al., 2023). Furthermore, calves that are gradually weaned from bucket feeding or an automatic milk feeder vocalized less (Jasper et al., 2008; Scoley et al., 2019; Bittar et al., 2020) and showed no decrease in lying times after cessation in milk supply (Scoley et al., 2019) compared to abruptly weaned calves. Due to these positive effects, a gradual reduction in milk supply, either by diluting the milk/milk replacer with water or by feeding the calves less often or with smaller amounts of milk, is also the recommended weaning method for artificially reared dairy calves compared to an abrupt weaning off milk (Jasper et al., 2008; Sweeney et al., 2010; Steele et al., 2017).

On the other hand, it is possible that the repeated reductions in contact time allowance and thus repeated forced temporary separations from the dam (or calf respectively) are perceived as stressful by the animals. At least in other species it has been shown that repeated maternal separations can cause i.a. hypersensitivity to pain or chronic stress in the young (discussed in Newberry and Swanson, 2008; Meagher et al., 2019). Also, it was recently reported that dairy cows with part-time contact to their one-month-old calves showed a more negative emotional

state during a judgment bias test, compared to cows that had full-time contact to their calves (Neave et al., 2024). The authors likewise discussed that this negative bias could potentially be related to the extended periods of daily separation from the calf (Neave et al., 2024), which could also lead to reduced welfare of cows during a gradual separation process. Taken together, a gradual weaning and separation method shows potential to facilitate the development of nutritional independence of the calf from the dam and to allow habituation of cow and calf to being separated from another, but might as well impose considerable stress on the animals.

Studies using gradual weaning and separation strategies in other species reported mixed results in this regard. In 28 day old piglets, it was shown that a gradual reduction in sow contact (5, 7, and then 9h separation per day) led to less maladaptive behavior, as well as little change in plasma cortisol concentrations post-weaning, but also reduced weight gains in comparison to control piglets that were abruptly weaned (Ruyter et al., 2017). Lambs that were gradually separated from the dams over 10 days (day 0-5: full-time contact, day 5-10: half day contact overnight, day 11: complete separation) showed a higher cortisol increase and a lower cell-mediated immune response, as well as reduced growth rates in comparison to abruptly separated lambs at 24-30h post-partum (Sevi et al., 2003). However, in this study the separation of the lambs took place directly after birth, when the lambs were still nutritionally fully dependent from the mother. In a study by Orgeur et al. (1998) during which lambs were fully separated with 3 months of age, but were temporary separated from the ewe for increasing durations from 3.5 weeks of age, lambs showed a strong vocal response during the beginning of the temporary separations, but became accustomed to the procedure with repeated separations and then showed little response during total separation at 3 months of age. In comparison, lambs in this study that had not experienced the temporary separations, reacted with significantly more vocalizations during total separation and also more vocalizations than the temporarily separated lambs had shown at any time point before. Given this mixed evidence and the above described potential of a gradual method, an in-depth scientific evaluation of behavioral and physiological stress responses to this method also in dairy cattle is warranted.

Recently (after determination of the experimental design for this thesis) some studies on gradual weaning and separation strategies in dairy cow-calf contact systems have been published. So far no positive effect of a gradual weaning and separation method on reduction of behavioral stress responses in dairy cows or calves compared to abrupt separation was reported, with the exception that gradually separated cows showed an earlier recovery of lying times after separation (Neave et al., 2023). A gradual decrease in contact time over 9 days prior to total separation in a cow-driven CCC system provoked a moderate vocal response during the first two days after pairs were totally separated, while vocalizations at initiation of the treatment were not recorded (Johnsen et al., 2021). Comparison of a short (10 day) or long (28 day) duration of reductions in contact time revealed that a longer process had beneficial effects on the calves' weight gains (Sørby et al., 2024) and behavioral response to separation (Johnsen et al., 2024), while there was no positive or negative effect on behavioral responses in the cows (Johnsen et al., 2024).

1.4 A demand for alternative stress indicators for gradual weaning and separation methods

Exposure of dairy and beef cattle to common stressors such as for example transport (Knowles et al., 1999; Gebresenbet et al., 2012) or isolation from peers (Boissy and Le Neindre, 1997; Hernandez et al., 2014) causes changes in physiological stress indicators that are evident from the beginning of the procedure. This is likely less so during induction of weaning and separation processes, especially if animals are not simultaneously regrouped and/or moved to unfamiliar surroundings. While the insertion of the nose flap itself can be expected to cause stress in calves, which likely increases with time as the calf gets hungrier, the time point when stress will occur in their dams is less obvious, especially when dams are continued to be machine milked as commonly done in CCC systems. Similarly, it can be expected that pairs which are weaned and separated by gradual reductions in contact time with the aid of smart gates, will often not notice that their contact allowance has been reduced until they try to pass the smart gate for the next time, which can take place after a variable amount of time. And even then, a denied access must not necessarily be immediately perceived as stressful by the animals, since cows and calves are often used to being restricted from entry into the meeting or feeding areas during specific time points of the day, e.g. during milking, and have learned that the gates will open at a later time point again. Therefore, determination of sampling time points for (especially gradual) weaning and separation protocols is not straightforward. While behavioral observations can be conducted over longer time periods, or even permanently with video observations, assessment of physiological stress responses is often limited to one or a few samples per day due to necessary restraint and handling of the animal that causes itself stress (Sheriff et al., 2011) and can bring nuisance to the actual treatment effect of interest. However, low sampling frequency has the risk of missing the crucial time points and subsequent false evaluation of the stress load of a treatment, which is especially so with saliva and blood cortisol samples that reflect the stress the animal perceives at the moment of sampling (measurable 3-5min after a stressor and reaching peak values with a delay of about 15-30 min (Lay et al., 1996; Sheriff et al., 2011) and an additional 10 min delay in saliva cortisol (Hernandez et al., 2014)). Furthermore, these sampling materials have the disadvantage that their results are influenced by the episodic pattern of cortisol secretion into the blood (Ladewig and Smidt, 1989). In this context, Loberg et al. (2007) reported no increase in salivary cortisol concentration in dairy cows after abrupt separation from their foster calves, and authors ascribed the missing increase to the fact that their sampling interval might have missed the peak in salivary cortisol concentrations. Thus, there is a demand for physiological stress indicators that are less dependent on the exact sampling time points and are able to integrate stressors which the animals experience over a longer time span.

1.4.1 Fecal cortisol metabolites and hair cortisol

One suitable indicator in this regard could be fecal cortisol metabolites, i.e. metabolites of circulating glucocorticoids, including cortisol, that are excreted via the bile into the gut, where they are mixed with the digesta passing through the intestine over several hours and are subsequently measurable in the feces of an animal (Palme, 2005). Since fecal cortisol metabolites are integrated into the feces over a longer time period, they are less affected by the episodic fluctuations of cortisol concentrations in the blood. Therefore they are considered to give, to a limited degree, a better representation of the cumulative secretion of hormones

compared to serum cortisol samples (discussed in Touma and Palme, 2005; Ashley et al., 2011; Palme, 2012). However, while measurement of fecal cortisol metabolites is a well-established method to assess stress in adult dairy cattle (e.g. transport: Palme et al., 2000; claw trimming: Pesenhofer et al., 2006; sudden dry off: Bertulat et al., 2013; heat stress: Rees et al., 2016), this method has never been used in pre-mature dairy cattle. Therefore, an investigation is needed to examine, if fecal cortisol metabolites reflect physiological stressors in young dairy calves the same way they do in older cattle. With regard to a potential evaluation of weaning and separation stress with fecal cortisol metabolites it is of additional interest to assess how the milk diet affects the composition, time lag and concentration of excreted fecal cortisol metabolites in comparison to a solid-based diet, since the diet changes substantially between the pre- and post-weaning state of calves.

A further useful indicator for the stress an animal experiences over longer time periods is analysis of cortisol concentrations in hair samples. Since systemic cortisol is integrated into the growing hair shaft, cortisol concentrations in hair samples might allow for a pooled representation of the stress an animal experienced during the growing period of the examined hair (discussed in Burnett et al., 2015; Heimbürge et al., 2019). It was considered to use hair cortisol concentrations as an indicator for separation stress for this thesis, but an analysis could unfortunately not be realized due to financial restrictions. Since this method is not well-established yet and there is still some uncertainty about the underlying mechanisms of cortisol incorporation into the hair shaft (Heimbürge et al., 2019) as well as evidence that local production of glucocorticoids in hair follicles (Keckeis et al., 2012) might confound an actual representation of systemic cortisol levels in hair samples (discussed in Palme, 2012), it was decided not to use this method. However, recently hair cortisol concentrations were used to compare different separation methods for dam-reared dairy calves in a study by Wenker et al. (2022), which reported no significant differences in hair cortisol concentrations between calves weaned with a nose flap or by fence-line separation or the control group (without dam-contact). This result was paralleled by the calves' plasma cortisol concentrations and authors discussed that the missing treatment differences were likely caused by the high individual variability in physiological stress responses of calves, which were evident in both, measured plasma and hair cortisol concentrations (Wenker et al., 2022a).

1.4.2 Relative telomere length

Finally, the shortening rate of telomeres has also recently been proposed to be able to integrate positive and negatives life experiences by animals over a longer time period and was therefore described as a potential indicator for cumulative welfare of an animal (Bateson, 2015). Since each step of a separation process (weaning of the milk/cessation of suckling by the calf, partial separation from the cow/calf, total separation from the cow/calf and change in housing conditions) will lead to peaks in acute stress reactions, the use of telomere shortening rates might be a useful indicator to integrate these several peaks over time into one measurements of the overall stress the animals experience during the whole weaning and separation treatment. Telomeres are repetitive DNA-sequences at the end of vertebrate chromosomes which protect the chromosomal coding regions from damage during cell division and DNA repair (Brown et al., 2012; Bateson, 2015). As elaborated in Bateson (2015), telomere shortening occurs with

each cell division, but the shortening (and repair) rate depends on several factors, including the life stage of an individual or the tissue type. Furthermore, telomere length is negatively affected by oxidative stress, inflammation and/or increased serum cortisol levels, which typically occur during stressful life events. In humans it has been shown in this regard that telomere shortening occurs at an accelerated rate during negative life experiences (Bateson, 2015; Bateson and Poirier, 2019), but that positive lifestyle interventions can prevent and possibly even reverse telomere attrition (Schutte et al., 2020). In cows, reduced telomere length has been shown in association with heat stress (Seeker et al., 2021), infections with bovine leukaemia virus (Szcotka et al., 2019) and stress during parturition (O'Daniel et al., 2023). However, it is still unknown yet if relative telomere shortening rates do also reflect social stressors like cow-calf separation in dairy cattle and thus if they can be used as a potential indicator for the overall stress the animals experience over the course of the whole weaning and separation process, which warrants research.

2. Aims, objectives and outline of the thesis

The overall aim of this thesis was to investigate separation methods that facilitate a low stress weaning and separation process of bonded cows-calf pairs kept in a dairy cow-calf contact system. Specifically, this thesis aimed to compare behavioral and physiological responses of dairy cows and calves to two-step weaning and separation with nose flaps to a gradual reduction in contact time between cow and calf (see Fig. 1). These methods were chosen, since review of the literature suggested that both have potential to reduce the stress that cows and calves experience during the weaning and separation process and the gradual weaning and separation method had not yet been scientifically assessed for dairy CCC systems at the time point of determination of the experimental design for this thesis.

In order to achieve a comprehensive comparison of these two separation methods, this thesis additionally examined the use of fecal cortisol metabolites for unweaned calves and changes in relative telomere length in both cows and calves as indicators for stress as a secondary aim, since these indicators were hypothesized to reflect, to a limited degree, the stress the animals experience over a longer time span, compared to the commonly used plasma or saliva cortisol measurements. Thus, this thesis builds on two separate experiments: a pilot study and the following main experiment (Fig. 2).

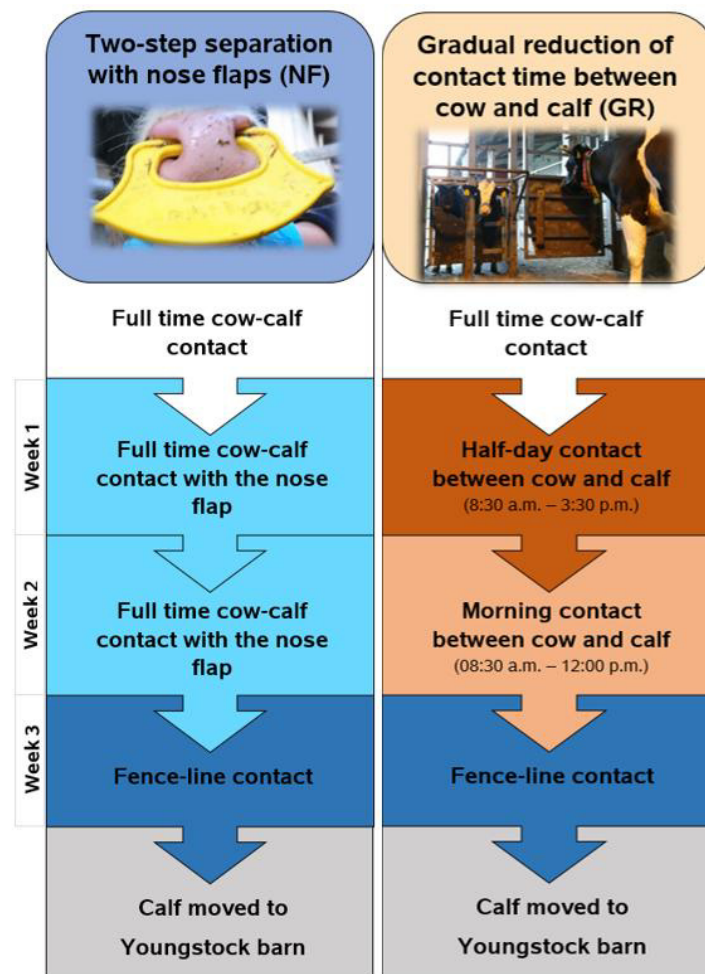


Fig 1.) Schematic overview of the two compared weaning and separation methods

The specific objectives of this thesis were to:

- assess, if fecal cortisol metabolite concentrations reliably reflect a (transport) stressor in unweaned dairy calves on a primarily milk-based diet in the same way they do this for older cattle on a solid-based diet (**study I**)
- determine which of the two separation methods leads to overall lower behavioral and physiological stress responses in dairy calves (**study II**)
- investigate how much time dairy calves still spend in the cow area, as a proxy for suckling motivation, after they are prevented from suckling their dam through a nose flap (**study III**)
- determine which of the two separation methods leads to overall lower behavioral and physiological stress responses in dairy cows (**study IV**)
- investigate, if the two separation methods cause changes in social (i.e. affiliative and maternal) behaviors of the cow-calf pairs (**study IV**)
- compare the effect of the two separation methods on changes in milk yield of the cows (**study IV**)
- assess, if changes in relative telomere length reflect the stress that cows and calves experienced during the two separation methods in order to evaluate if relative telomere length is a suitable indicator for social stressors in cattle (**study V**)

Experiment I
Validation of fecal cortisol metabolite concentrations as a stress indicator for dairy calves on a primarily milk-based diet (study I)
Experiment II
Behavioral and physiological stress responses of calves to gradual separation and two-step separation with a nose flap (study II)
Changes in dairy calves' time spent in the cow herd after insertion of a nose flap (study III)
Behavioral and physiological stress responses of cows to gradual separation and two-step separation with a nose flap & Effects of the two separation methods on changes in social behaviors between pairs and milk yield of the cows (study IV)
Effect of the two separation methods on relative telomere length of cows and calves (study V)

Fig. 2) Overview of studies enclosed in this thesis

Sample size calculation

The sample for experiment I (**study I**) included all calves that were available in the barn, which were born close enough to each other to allow for weaning at the same time point. This were 5 calves.

The required sample size for experiment II (**study II-V**) was calculated using G*Power 3.1 (Faul et al., 2007) for assessment of two independent groups with several dependent variables using repeated measures ANOVA and an assumed large effect ($f=0.5$). The alpha level was set to 5% and the beta error to 10%. The resulting sample size was 34 cow-calf pairs, which corresponds to a sample size of 17 cow-calf pairs per separation treatment. This was increased by one pair each to a final sample size of 18 cow-calf pairs per treatment, to allow for an unexpected drop-out of single individuals from the experiment.

Study I

Fecal cortisol metabolites reflect transport stress in 3-month-old dairy calves pre- and post-weaning: A pilot study

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Fecal cortisol metabolites reflect transport stress in three-month-old dairy calves pre- and post-weaning – a pilot study

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Abstract

Measurement of fecal cortisol metabolites (FGCMs) is a well-established, non-invasive method to assess stress in adult dairy cattle. However, for unweaned dairy calves this procedure has not yet been validated and it can be expected that the milk proportion of the diet may influence the resulting FGCM concentrations. The aim of this study was therefore to assess, if a peak in FGCM concentrations in response to a stressor can be measured in unweaned dairy calves on a largely milk-based diet. If so, further objectives were to examine whether maximum FGCM concentrations as well as the time lag until they are reached, are comparable to the values in the same calves on a solid-based diet after weaning. For this study, five German Holstein calves of about three months of age (93-102 days pre-weaning) were exposed to a 45 min transport stressor once before and once after weaning, which was three weeks apart. All voided fecal samples were collected for 24 hours after termination of the transport. FGCMs were analyzed with an 11-oxoetiocholanolone enzyme immunoassay and changes in FGCM concentrations relative to the individual baseline were calculated (FGCM_{rel}). Results showed a clear peak in FGCM concentrations on both diet types. The peak FGCM_{rel} concentrations tended to be higher when the calves were on the pre-weaning diet (at peak: +233±25% increase relative to baseline) in comparison to the post-weaning diet (+124±23%). Considering the whole 24 hour sampling period, the FGCM_{rel} concentrations were for all calves significantly higher on the pre-weaning diet than on the post-weaning diet. There was also a numerical difference in the delay between occurrence of the stressor and appearance of the peak FGCM_{rel} concentrations in feces, as the time lag was 1.5±1.2 hours longer when the calves were on the pre-weaning diet compared to the post-weaning diet. In conclusion, our results suggest that FGCM concentrations are a useful stress marker for unweaned dairy calves in the same way they are for older cattle, but that FGCM_{rel} concentrations tend to be higher in unweaned than in weaned calves and are thus not directly comparable.

Keywords: dairy calves, glucocorticoid metabolites, weaning stress, diet, transport

1. Introduction

Measurement of fecal cortisol metabolites (FGCMs) is a well-established, non-invasive method to assess stress in adult dairy cattle for evaluating challenging situations like transport (Palme et al., 2000), claw trimming (Pesenhofer et al., 2006), regrouping (Wagner et al., 2012; Mazer et al., 2020), heat stress (Rees et al., 2016; Veissier et al., 2018) or sudden dry off (Bertulat et al., 2013). For unweaned dairy calves, it is however unknown how the milk diet affects the composition, time lag and concentration of excreted FGCMs. This is of special interest for evaluating weaning distress in young calves, as the diet changes substantially between the pre- and post-weaning period.

In general, there is a considerable variation between species, sexes of the same species, as well as life-history stages with regard to the glucocorticoid metabolites formed in feces (Palme et al., 2005). Thus, a careful validation of the employed enzyme immunoassay (EIA) is needed in each new species under investigation (Palme et al., 2005; Palme, 2019). An EIA measuring 11,17-dioxoandrostanes (11,17-DOAs) proved to be well suited for evaluating adrenocortical activity in adult cows (Palme et al., 1999, 2000), but up to date it was unknown if this is also the case for young dairy calves.

Additionally, there is a species-specific time lag between the peak concentration of cortisol in the blood plasma and the peak FGCM concentration, which is closely related to the intestinal transit time of the animal (Palme et al., 2005). For example, the maximum FGCM concentrations were measured about 12 hours in sheep, 24 hours in ponies and 48 hours in pigs after the end of intravenous infusion of radiolabeled ^{14}C -steroid hormones (Palme et al., 1996). In cattle, peak concentrations occurred about 10 hours (range: 6 to 18.7 h) after respective plasma cortisol peaks following ACTH injection (Palme et al., 1999). With regard to unweaned dairy calves, it is uncertain whether such a peak in FGCM concentrations can equally be measured at all, and if so, if the time lag until FGCM concentrations peak is comparable to the lag times measured in older cattle.

Specifically, it is unknown how the milk content of the diet of unweaned calves affects the composition, time lag and concentration of excreted FGCMs in contrast to weaned calves or adult cattle on a solids diet. In different species it has been highlighted, that the diet of an animal can have a distinct effect on FGCM concentrations (e.g. Dantzer et al., 2011; von der Ohe et al., 2004; Wasser et al., 1993). Especially the dietary fiber content was reported to influence steroid concentrations, but results are contradictory depending on the studied species. In female baboons for example, increased dietary fiber was positively correlated with fecal cholestanone excretion (Wasser et al., 1993). Equally, enhancement of fiber in the diet of laying hens also led to an increase in FGCM concentrations and to an increased excretion rate (Alm et al., 2014). In contrast to that, experimental addition of dietary fiber decreased corticosterone metabolite concentrations in droppings of European stonechats (Goymann, 2005) and fecal cortisol metabolite concentrations in Meishan sows (Jiang et al., 2019). In dairy cattle, the effect of the dietary fiber content on FGCM concentrations has not yet been investigated. However, it was shown that changing the amount of dry matter in the diet of lactating dairy cows did not affect fecal progesterone metabolite concentrations (Rabiee et al., 2002).

Taken together, up until now it is unknown whether FGCMs can be used to assess stress levels in unweaned dairy calves the same way they can be used for weaned calves or adult cattle on a solid diet. Given that the diet has a significant effect on FGCM concentrations in other species, it can be assumed that results will differ when the dairy calves are fed primarily with milk (high fat, no fiber) pre-weaning or roughage and concentrate (low fat, high fiber) post-weaning.

The aim of this study was therefore to assess if a peak in FGCM concentrations in response to a stressor (transport) can also be measured in unweaned dairy calves on a largely milk-based diet. If so, further objectives were to examine whether maximum FGCM concentrations as well as the time lag until they are reached, are comparable to the values in the same calves on a solid-based diet after weaning.

2. Materials and Methods

2.1 Animals, housing and feeding

Experiments were performed in accordance with the German Animal Welfare Act (Federal Republic of Germany, 2020; animal experiment number V244-10476/2020, MELUND Schleswig-Holstein). The study was conducted in May and June 2021 with 5 (2 male and 3 female) dairy calves housed at the Thünen Institute of Organic Farming in Trenthorst, Germany. All calves were of the German Holstein breed and about three months old (mean \pm SD: 98 \pm 3.94 days of age) when the treatment started. These five study animals included all available calves of the barn that were born close enough to each other to allow for weaning at the same point of time. The calves were housed indoors in a pen of 68m², of which 12.6m² was straw bedded lying area. Next to the five study animals, there were eight other calves present, totaling 13 animals in the calf area. Before the second data collection started, one male calf was sold, leaving 12 animals in total in the calf area for the second data collection.

During the pre-weaning phase, the calves were provided with 12 liters of whole milk per day from an automatic milk feeder (Förster Technik) and had additional ad libitum access to hay and a total mixed ration (TMR, composed of about 69.3% grass silage, 27% corn silage, 3% concentrate feed in the form of coarse grain, 0.4% cattle salt and 0.3% mineral feed) in their home pen. Additionally, the calves had access to a concentrate feeder (Förster Technik) with an allowance of 1.5 kg concentrate per animal and day distributed in portions of 50 g over the day. The concentrate feed was composed of wheat, triticale, field bean and mineral feed. Thus, the calves were on a diet that consisted mainly of milk, but included also roughage and concentrate feed in the pre-weaning period. For weaning, milk allowance was reduced continuously over two weeks by \sim 0.9 liters per day, so that after two weeks the calves were weaned off milk completely. Calves were about 3.5 months (107-116 days) old when they were fully weaned off milk. During the post-weaning phase, the calves only had access to the same ad libitum hay, TMR and 1.5 kg concentrate feed in their home pen, but no milk allowance at the automatic milk feeder anymore. Ad libitum access to water was guaranteed at all times and all calves were accustomed to human handling.

2.2 Experimental design and procedures

A repeated measures design was used. Calves were subjected to a stress-eliciting situation once before weaning, i.e. on the 'milk' diet, and once again after weaning on the 'solid' diet. To provoke a stress response, the transport-naïve calves were loaded into a conventional animal trailer (Arne's Smedie Vognen Denmark) and were transported around the area of the home stable once before weaning and once again after weaning. The transport started at 10:00 a.m. at both occasions. The journey lasted 45 minutes and included short passages of cobblestone (about one-eighth of the total distance) and one sudden brake process. The trailer was 2.5 x 6 m in size and no bedding was provided for the 45 minutes drive, so that calves were transported on the anti-slip checkered plate of the trailer. Sample collection started immediately after unloading of the calves and lasted always 24 hours. Transport route and driver were the same for the journey before and after weaning.

Immediately after the first transport and the pre-weaning sample collection (age of calves: 93-102 days) was completed, the two week weaning process started. Once they were fully weaned off milk, the calves were given one week to allow for the gastrointestinal tract to adapt to the dietary changes. Then the second transport and sample collection for the post-weaning phase took place, which was respectively exactly three weeks after the first transport and sample collection (age: 114-123 days). All samples were collected in the home pen of the calves, where they stayed for the whole study period to prevent any stress caused by changes in housing conditions. Thus, the weaned calves were not moved to the youngstock barn until the study was terminated.

Feed and water intake by the calves was not measured. As hay, TMR and water were available ad libitum to the calves the whole time, the time points of the last feed and water intake before transport will have varied between the individuals.

Weather conditions at the transport day were about 14°C and rain showers mixed with sun in the pre-weaning phase and about 20°C and a mix of sun and clouds without precipitation for the post-weaning transport.

2.3 Sample collection

About 1.5 hours before the transport started a baseline fecal sample was obtained from each calf by rectal stimulation on a calf weighing scale during the weekly health check and all animals were marked with colored collars and animal marking spray for identification during the sample collection. None of our calves had scours during the sample collection period. Since birth, animals were weighted and the health assessment was done routinely every week. Thus, they were accustomed to being moved onto the weighing scale.

Further sample collection was done following Kleinsasser et al. (2010). Collection of fecal samples started at the moment the calves were unloaded from the trailer and a sample of every defecation of the five study calves was collected immediately after voidance for a period of 24 hours. As soon as one of the five study calves defecated, about 2-5 g of feces were collected from different areas of the pile of feces into a conventional fecal test tube. Care was taken to collect only fecal material without any contact to the ground or contamination of straw or other

pen material. Then the fecal sample was immediately placed into a cooling box and time of voidance was noted. The cooling box was changed every 30 minutes and all samples collected within the 30 minutes period were transferred to a freezer and kept at -20°C until further analysis.

2.4 Analysis of fecal cortisol metabolites

Extractions of the samples followed the established protocol (Palme et al., 2013). In brief, defrosted (60°C; 30 minutes) fecal samples were homogenized, weighed (0.5 g) and extracted with 5 ml of 80% methanol. FGCMs were analyzed in a portion of the supernatant with an 11-oxoetiocholanolone enzyme immunoassay (EIA), previously validated for cattle (Möstl et al., 2002). Intra- and interassay coefficients of variation were below 10% and 15%, respectively. This EIA measures FGCMs with a 5 β -3 α -ol-11-one structure (for details, including cross-reactions of the antibody see Möstl et al., 2002). With the EIA measuring 11,17-dioxoandrostanes (11,17-DOA), which is typically used for fecal samples of adult cows (Palme et al., 2000), the resulting FGCM values from the samples of some calves were too low, so that the 11-oxoetiocholanolone EIA was preferred.

2.5 Statistical analysis

Statistical analysis was performed with SAS Version 9.4. Baseline concentrations included only pre-transport values, namely the one sample taken per animal before a transport started. Differences of the baseline FGCM values between the pre- and post-weaning phase were analyzed using a paired t-test. Results are given in ng per gram wet feces. The difference of the defecation frequency between the two diets was analyzed using a Wilcoxon signed-rank test.

For further analysis, following Kleinsasser et al. (2010), the fecal samples collected in the 24 hours sampling period after the transport were grouped into three-hour intervals. If more than one sample was present in a specific time interval, the mean value for the individual calf in this interval was calculated. Note that not for every calf a sample was available in each interval as defecation was irregular. This way the mean FGCM value in ng per gram wet feces per interval for each calf was obtained. Based on this mean value in ng per gram, the change in FGCM concentrations (increase or decrease, respectively) in percent relative to the individual baseline was calculated according the formula described in Bertulat et al. (2013): $FGCM_{rel} = [(FGCM - \text{baseline value}) : \text{baseline value}] \times 100$. This relative change was computed for each calf for each interval, so that the influence of sex, weather conditions and individual personality as well as individual physiology and daily rhythms was reduced, because each animal acted as its own control.

A linear mixed effects model with repeated measures was run in SAS for analysis of the $FGCM_{rel}$ values with phase (pre-weaning/post-weaning), interval, as well as the interaction between phase and interval as fixed effects. The calf number was included as random effect. Sex of the calf and weaning age (the latter as continuous variable) was dropped from the model as they were not significant. Model requirements (normal distribution, homoscedasticity) were

checked graphically. Pairwise differences between specific phase and interval interactions were calculated using a Tukey-Kramer posthoc test. The FGCM_{rel} results are presented as least square means \pm S.E.

The differences between the time lags until occurrence of the peak FGCM_{rel} concentrations in feces during the pre- and post-weaning phase were analyzed using a paired t-test. When calf 2 was dropped from the model as an outlier in an additional analysis, a Wilcoxon signed-rank test had to be used for calculation of the time lags, as data were not normally distributed anymore.

3. Results

3.1 Total number of defecations and individual progression over time per calf

The total number of defecations of the calves differed between the two types of diet, as all calves tended to defecate less often when they were on the pre-weaning ‘milk’ diet (mean \pm S.E. 9.2 ± 0.58 defecations per 24 h collection period), than when they were on the post-weaning ‘solids’ diet (16.4 ± 2.25 , signed rank statistic (S)= 7.5, $p= 0.06$, see Table 1).

Table 1.) Number (No.) of defecations per 24 h of each calf when the calves were on the pre-weaning diet (milk + roughage + concentrates) or on the post-weaning diet (only roughage + concentrates).

Calf No.	No. of defecations <u>pre-weaning</u> per 24 h collection	No. of defecations <u>post-weaning</u> per 24 h collection
1	7	12
2	10	14
3	10	15
4	10	25
5	9	16

FGCM baseline concentrations tended to differ between the two diet types (mean \pm S.E. pre-weaning: 180 ± 28 ng/g; post-weaning: 109 ± 10 ng/g, $t_4= 2.5$, $p= 0.07$). Four calves showed higher baseline concentrations during the pre-weaning phase than during the post-weaning phase. Only one individual (calf 2) showed similar baseline concentrations in the two phases (see Fig. 1).

A clear peak in FGCM concentrations was detectable in all animals for the pre-weaning as well as the post-weaning period, except for calf 2 during the pre-weaning phase (see Fig. 1). The variation between individual calves regarding the time lag of the peak concentration after occurrence of the stressor was greater in the post-weaning phase than during the pre-weaning phase (see Fig. 1).

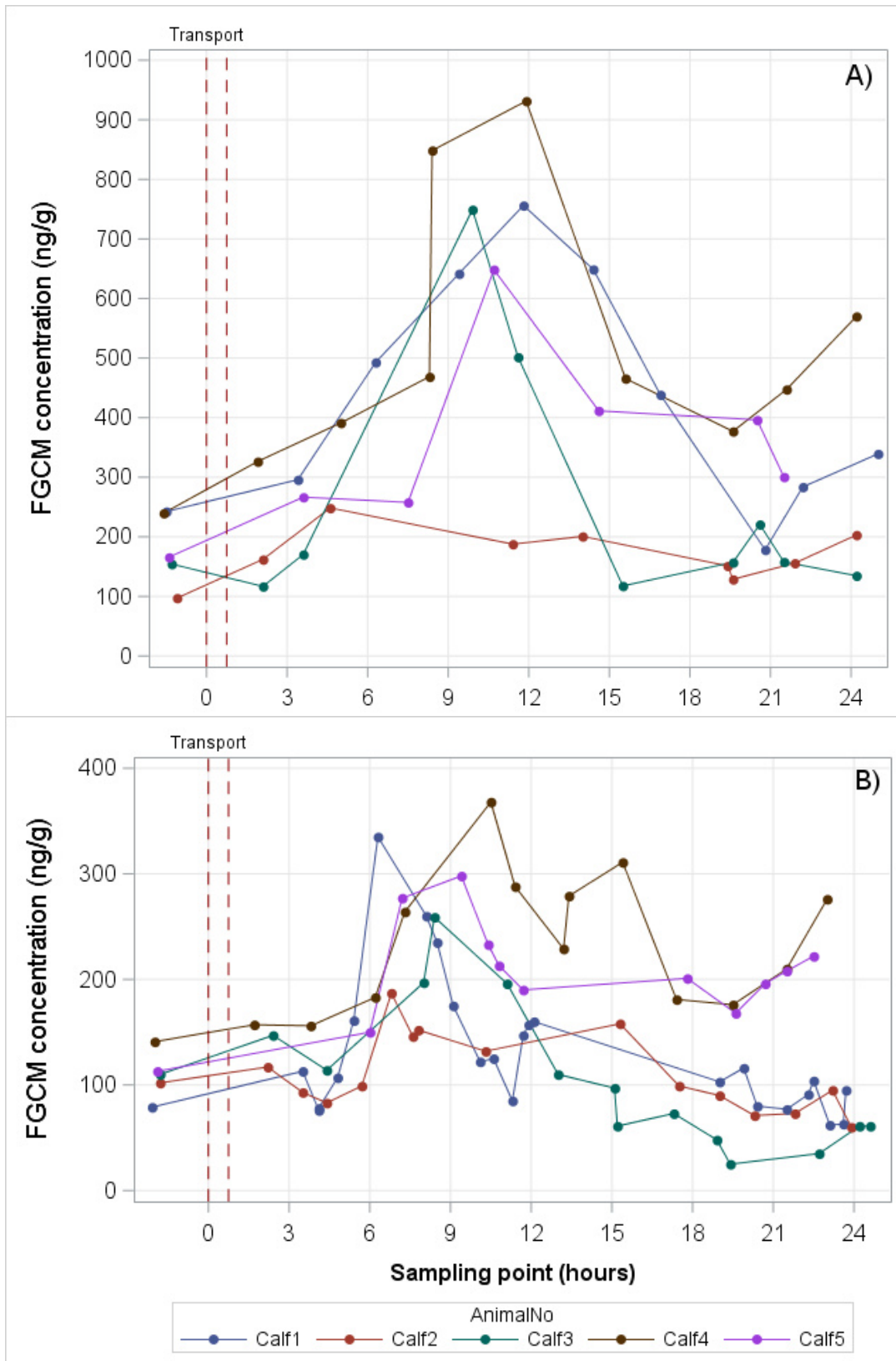


Fig 1.) Individual fecal cortisol metabolite (FGCM) concentrations (ng/g wet feces) of five dairy calves over a 24 hour sampling period following a 45 min transport stressor on (A) the pre-weaning diet (milk + roughage + concentrate) and (B) the post-weaning diet (only roughage + concentrate). Sampling point 0 represents 10:00 a.m. in the morning and each sampling point refers to the hours after start (not end) of the transport stress. Please note the different scales of the y-axes.

3.2 Percentage increase in FGCM concentrations relative to the individual baseline

Results showed that the phase had a significant effect on the mean increase in FGCM concentrations relative to individual baseline values (FGCM_{rel}), as the mean FGCM_{rel} concentration changes over the whole 24 hour sampling period were significantly higher in the pre-weaning phase (LSM±S.E.: +90.6±14.5%) than in the post-weaning phase (+46.9±14.2%, $F_{1,47}= 11.6$, $p=0.001$, Fig. 2).

During the pre-weaning ‘milk’ phase, the FGCM_{rel} concentration was highest when the samples were obtained between 9-12 hours after transportation (+233.2±25.3% increase relative to baseline, Fig. 2A). This was significantly higher than all other intervals in the pre-weaning phase (all $p<0.05$) except for the adjacent intervals 6-9, as well as 12-15 hours after the transport stressor (see Fig. 2A).

The highest increase in FGCM_{rel} concentrations on the post-weaning ‘solid’ feed diet was found at 6-9 hours after transportation (+124.0±23.1% increase relative to baseline). In comparison to the other intervals, this did not differ significantly from the intervals between 9 until 18 hours after transportation (all $p>0.05$, see Fig. 2B). In general, the differences between the intervals were less pronounced on the post-weaning ‘solids’ diet than on the pre-weaning ‘milk’ diet.

However, although the peak increase was more pronounced and occurred one interval later when calves were on the pre-weaning diet compared to the post-weaning diet, the FGCM_{rel} concentrations of the respective peak intervals of the two diets (pre-weaning 9-12 hours: 233.2±25.3% vs. post-weaning 6-9 hours: 124.0±23.1%) only tended to differ significantly from each other (adjusted $p=0.09$).

The time lag between occurrence of the stressor and appearance of the peak increase in FGCM_{rel} concentrations in feces was 1.5±1.2 hours (mean ± S.E.) longer when the calves were on the pre-weaning diet (peak after 9.8±1.4 hours) than when they were on the post-weaning diet (peak after 8.3±0.8 hours), but this difference was not significant ($t_4= 1.2$, $p= 0.29$). In four calves the peak occurred earlier in the post-weaning phase than in the pre-weaning phase; only in calf 2, where a clear peak was lacking, this was reversed and the peak occurred later in the post-weaning phase (see also Fig. 1). When calf 2 was dropped from the model, the time lag between the transport stress and the FGCM_{rel} peak concentration was 2.4±1.0 hours (mean ± S.E.) longer when the calves were on the pre-weaning diet than when they were on the post-weaning diet ($S= -5$, $p = 0.13$).

For better comparability the absolute FGCM concentrations for the peak intervals are given as well, which were in the mean (±S.E.) 618±121 ng/g on the pre-weaning diet at 9-12 hours after transport and 233±21 ng/g on the post-weaning diet at 6-9 hours after the transport stressor.

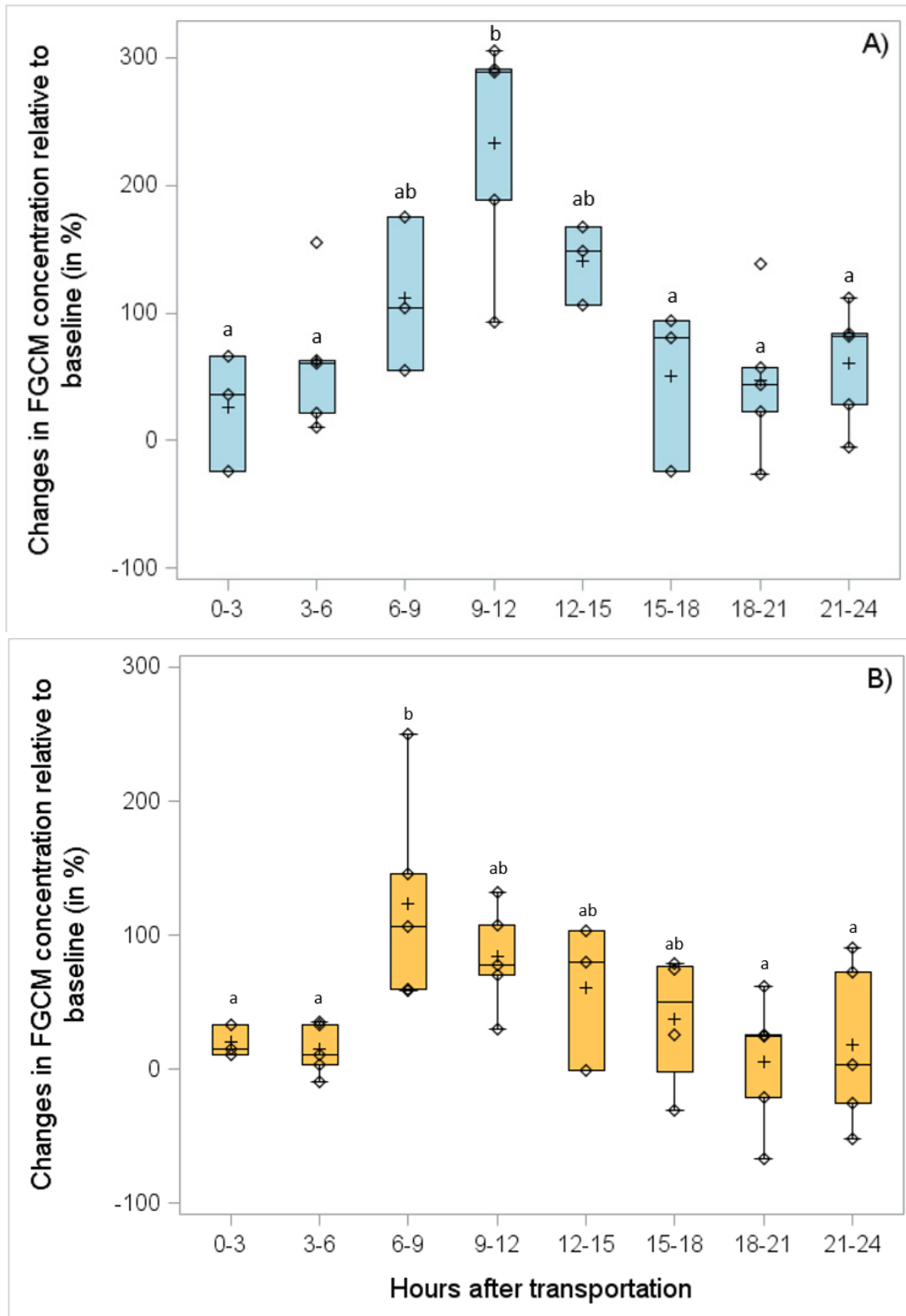


Fig 2.) Boxplot graphs of changes in fecal cortisol metabolites concentrations (in %) relative to the individual baseline ($FGCM_{rel}$) per three hour sampling interval for the five dairy calves on (A) the pre-weaning diet (milk + roughage + concentrate) and (B) the post-weaning diet (only roughage + concentrate). Baseline values comprise only one sample taken from each calf before the transport stressor started. The diamonds display the values of individual calves in each interval. Mean values are displayed by the plus symbol. Note the small sample size of only five calves and that not for every interval a value was present for each calf as defecation was irregular. Different letters (a-b) indicate statistically significant differences at $p < 0.05$.

4. Discussion

In this study, the stress response of young dairy calves to a transport stressor was evaluated before and after weaning by measuring FGCMs. Results revealed that FGCM concentrations on the pre-weaning diet increased significantly and showed a peak in response to the transportation stress, which indicates that FGCM concentrations can be used to assess stress in unweaned calves the same way as for weaned dairy calves. The peak increase in FGCM_{rel} concentrations tended to be more pronounced when the calves were on the pre-weaning 'milk' diet in comparison to the post-weaning diet based on solid feed. However, this was only a tendency and not significantly different between the two phases, which was probably caused by the small sample size of only five animals and the high individual variability. Considering the whole 24 hour sampling period though, the FGCM_{rel} concentrations were for all calves significantly higher on the pre-weaning diet than on the post-weaning diet. The time lag between occurrence of the stressor and the reflecting peak FGCM_{rel} concentrations in feces was numerically, but not significantly, longer when the calves were on the pre-weaning diet (9.8 ± 1.4 hours) than when they were on the post-weaning diet (8.3 ± 0.8 hours). In a study by Palme et al. (2000) eight transport-experienced, lactating dairy cows, were transported for two hours on country roads and samples were taken from every spontaneous defecation over a period of 48 hours after the transport. Peak concentrations occurred 12 hours after the transport and FGCM concentrations were significantly higher from 8-16 hours after the start of the transport, compared to all other sampling intervals (Palme et al., 2000). Thus, the measured time lags in our study were shorter than the peak measured in adult cattle after transportation, but fall within the given range of 8-16 hours. Also, the measured times in our study are comparable to the time lag observed in adult cattle after the fixation for claw trimming where FGCM concentrations peaked nine hours after the stressor (Pesenhofer et al., 2006).

Although a different EIA was used in our study, the increase in FGCM_{rel} concentrations of 124% relative to the individual baseline during the post-weaning phase is comparable to results of other studies in adult cattle that were exposed to different stressors. For example, an analysis of the stress reaction of dairy cows with a milk yield of over 20 kg/day at dry off, revealed that the cows reacted with an increase in FGCM concentrations of 129% relative to the individual baseline at the second day after the sudden dry-off (Bertulat et al., 2013). Similarly, dairy cows which were exposed to heat stress ($\text{THI} \geq 72$) on a single day reacted to this stressor with an increase of 155% in FGCM concentrations relative to individual baseline (Rees et al., 2016). Thus, the FGCM_{rel} concentrations in our study were lower in the post-weaning period than during the pre-weaning period, but post-weaning levels were similar to those found in stressed adult cows. There are several possible factors that may have contributed to this observed higher increase in FGCM_{rel} levels when the calves were on the 'milk' diet compared to the 'solids' diet in our study, which will be discussed in the following sections.

4.1 Validity of results

A major limitation with this study is the small sample size of only five animals. This included all calves from the barn that were born close enough to each other to allow for weaning at about the same age. Whilst it is true that statistical analyses with a small sample have a reduced power,

the advantage of this design, however, was that all animals were the same age and were raised with the exact same husbandry and management conditions right from birth, as they lived not only in the same barn but in the same pen. Thus, for the experiment they had all exactly the same hay quality, TMR and milk composition, group size and unavoidable stressors due to farm routines. Additionally, this study aimed mainly to assess whether a standardized stressor would be reflected at all by FGCMs in pre-weaned dairy calves and results showed that the peak FGCM increase of $233.2 \pm 25.3\%$ at 9-12 hours after transportation on the pre-weaning diet was significantly different from all other intervals but the adjacent ones (Fig. 2). This statistical significant difference of the peak reveals that the sample size of our study was sufficient for answering our main question. Also, the design that each animal served as its own control partly counteracts the limitations of a low number of individuals.

Regarding the influence of a circadian rhythm, controversy exists whether there is also a diurnal rhythm of plasma cortisol in cattle and other ruminants like sheep and deer (see e.g. Alila-Johansson et al., 2003 for a discussion on this). For pre-weaned calves there is, to our knowledge, only one study available, which found that diurnal changes in plasma cortisol were characterized by sharp postprandial decreases, which was followed by a regular increase of cortisol concentrations (Gardy-Godillot et al., 1989). Thus, it seems likely that in the pre-ruminant calf the cortisol secretion is rather dependent on food intake (and peaks before meals), than on a diurnal rhythm. Thus, the peaks after transport can unlikely be explained by a circadian rhythm, but additional sampling for 24 h without transport would have strengthened our study.

Additionally, it has to be taken into account that there were great individual differences among the studied calves as a considerable nuisance factor. Results of the individual progression over time per calf show that calf 4 had generally higher FGCM concentrations than the rest of the studied calves, whereas calf 2 showed overall the lowest FGCMs (Fig. 1). Additionally, the FGCM values of some individuals returned to baseline levels before the end of our sampling period, but for other calves, these were still elevated even 24 hours after the stressor during both study periods (see Fig. 1). Also, in three calves the peak FGCM concentrations occurred about 1.5 hours earlier and in one calf about 5.5 hours earlier in the post-weaning phase compared to the pre-weaning phase. In calf 2, this was even reversed and the peak occurred about 2.5 hours later in the post-weaning phase (see also Fig. 1). However, for the latter this was probably mostly a result of the missing clear peak during the pre-weaning phase.

The found individual variability is in line with results from the literature, as for example Palme et al. (1999) also reported a great inter-animal variation in the concentration of FGCMs in baseline values (range: 10 to 135 ng/g feces) and in peak values (range: 227 to 594 ng/g feces, original data converted to the units used here) in cattle after an ACTH challenge. Equally, cows that were subjected to a transport stressor showed pronounced differences in the baseline (range: 16 to 86 ng/g feces) and peak FGCM concentrations (range: 105 to 700 ng/g feces) between individuals (Palme et al., 2000).

Regarding the time lag until peak concentrations can be measured, there is also high individual variation in adult cattle, as for example, peak FGCM concentrations occurred about 10 hours (range of 6-18.7 hours) after respective plasma cortisol peaks following ACTH injection (Palme et al., 1999). Equally, FGCM peaks were reported between 8-9.5 hours in lactating dairy cows

and 14-18 hours in non-lactating dairy cows after an ACTH injection (Morrow et al., 2002), about 9 hours after the fixation for claw trimming (Pesenhofer et al., 2006), as well as after 12 hours after a transport stressor in lactating dairy cows (Palme et al., 2000).

In our study, the presented individual differences in the stress response could potentially be related to the personality and temperament of the calves, as calf 4, which showed higher FGCM values than the rest of the calves, was generally described by the barn staff as a sensitive and easily excitable animal that occasionally showed tongue rolling. This theory is supported by a study on Brahman cows, which revealed that temperamental cows had higher blood serum cortisol concentrations before a transport event than cows that were classified as calm or of intermediate temperament (Price et al., 2015). Alike, Lecorps et al. (2018) demonstrated that dairy calves which were classified as more fearful in standardized tests (open field, novel object, human reactivity and social motivation test) show higher increases in maximum eye temperature in response to transportation. Collectively, the amplitude of the stress response is not only related to the intensity of the stressor per se, but also to the animal's personality (Finkemeier et al., 2018; Lecorps et al., 2018) and perception of the stressor (Henry, 1992; Veissier and Boissy, 2007). Thus, it is crucial to consider individual differences when evaluating challenging situations, as animals can vary considerably in their reaction to a stressor. Therefore, Palme et al. (1999) already recommended to either include a large sample of animals in the study or to use each animal as its own control, as done in our study by calculating the percentage increase relative to the individual baseline and by subjecting all calves to both treatments.

Generally, this study aimed mainly to assess whether a standardized stressor would be reflected at all by FGCMs in young dairy calves pre-weaning, as this method for stress assessment was never validated and applied in pre-weaned calves before. Our study found a significant peak increase in FGCMs, which was evident in all five animals at around the same time span despite the sex differences of the calves, showing the validity of FGCMs to reflect a stressor also in calves on a predominantly milk diet. Thus, despite the small sample size, the study can provide helpful information for future studies that want to utilize FGCMs in pre-weaned calves.

4.2 Habituation to transport

One additional confounding factor to consider in our study is the level of familiarity with transportation during the second journey. At the first transport during the pre-weaning phase all calves were transport-naïve, whereas for the post-weaning transport, they had already experienced this stress once before, which might have led to a reduced stress response already in blood cortisol levels and consequently also to reduced $FGCM_{rel}$ concentrations during the post-weaning sample collection.

It is widely known that animals can habituate to the transport procedure with repeated transportation (e.g. Roussel et al., 2006; Schmidt et al., 2010; Wickham et al., 2012). However, the literature rather varies with regard to the extent of habituation after only one single transport. In a study by Fell and Shutt (1986) on dairy calves of 0.5 to two months of age, which were repeatedly transported, no significant differences were found in salivary cortisol levels between the first and second trip. On the other hand, repeated exposure of female Holstein calves to

three transport simulations caused a progressively less marked increase in the blood cortisol response of the calves, but peak values during the second transport were still about two thirds of the level measured for the first transport (Locatelli et al., 1989). This degree of reduction of about one third between first and second transport falls roughly into the same range as values for gestating Brahman cows with intermediate or excitable temperament reported by Price et al. (2015). In contrast to the aforementioned, a study on four month old Holstein calves that were repeatedly transported, blood plasma cortisol levels after four hours of the journey were actually higher during the second transport than during the first transport (Adams, 2012), which rather contradicts a habituation effect after a single transport.

Taken together, the above studies imply that transport habituation underlies a high individual, and quite likely also situational, variation, but can already occur in part after one single transport. However, if habituation from first to second transport was present, the measured reduction in cortisol levels was at maximum around one third of the values of the first transportation. During our study, the mean FGCM peak value of the first transport was 618 ± 121 ng/g, whereas the peak in the second transportation was only 233 ± 21 ng/g, which represents thus a considerably stronger reduction in comparison to the dimensions reported in the literature after a single transport.

Additionally, a clear tendency of lower post-weaning values was already present in the baseline values before transportation had started and thus habituation would have affected the results. This shows that in addition to the habituation, other factors must have contributed to the reduced FGCM_{rel} concentrations in our study during the post-weaning period.

4.3 Dilution of FCM concentrations by fecal mass & changes in bile flow rates

One possible explanation for the lower FGCM concentrations during the post-weaning phase in our study might be related to the observation that all studied calves defecated more often when they were on the solids diet with a high amount of roughage compared to the pre-weaning phase with a high milk proportion (table 1). This is in line with results from Vaughan et al. (2014), which also reported significantly more defecation events after weaning for female Holstein calves. The higher frequency of defecation might be related to an increased feed intake, higher amount of dietary fiber and higher fecal mass, as for example a study by Hirata et al. (2001) on heifers found a positive correlation of the defecation frequency with dry matter intake and fecal dry matter output.

In former studies on other species, it has been shown that increased dietary fiber can lead to a reduction of excreted progesterone (Wasser et al., 1993) as well as corticosterone and testosterone metabolite concentrations (Goymann, 2005). This has led to the hypothesis that a higher fecal mass can lead to a dilution of FGCMs, since when the same amount of hormone metabolites per time unit is placed into a larger amount of feces per time unit, this will result in a lower concentration of hormone metabolites in the fecal sample (as the concentration is typically expressed with reference to fecal mass; Goymann, 2012). This theory of a dilution of FGCM concentrations in consequence of an increased fecal mass would also make sense for our post-weaning period, as the calves were consuming more fiber, which results in increased fecal volume and could thus have led to a lower FGCM concentration per unit of fecal sample.

On the other hand, a study by de Souza et al. (2022) in brown brocket deer found that a high dietary fiber content led to a higher quantity of fecal dry matter as well as higher mean fecal androgen metabolite levels compared to a diet with a low fiber content, which rather contradicts the dilution theory, at least for these ruminant mammals.

The crucial point in this regard seems to be that a dilution of FGCM concentrations in consequence of higher fecal volume, necessitates a constant bile flow into the duodenum per time unit as a prerequisite, as FGCMs are excreted via the bile. However, even if ruminants seem to have a comparably more constant bile secretion (Symonds et al., 1982; Gooneratne et al., 2013) than monogastrics, it is questionable whether the bile flow rates of our tested calves during the two study periods were comparable as they underwent a severe dietary change. To our knowledge, there are no studies which have directly compared bile flow rates in dairy calves pre- and post-weaning. However, it has been reported that mean bile flow rates of pre-ruminant calves on a whole milk diet are considerably greater than those of calves on a skim milk diet (Davis et al., 1985), adult steers (Symonds and Mallinson, 1982) or rates known for other domestic animals such as pigs or sheep (Debarre et al., 1979). It has been discussed that these differences in bile flow rates might be caused by a higher fat intake of calves fed whole milk compared to other domestic animals (Debarre et al., 1979) as well as the fact that a calf fed whole milk (~4% fat) would consume twice as much fat as calves on a skim milk diet (~2% fat) and 8-10 times as much fat as adult steers (Davis et al., 1985). It seems likely that the bile flow rates of the calves in our study were equally influenced by the pre- and post-weaning diet, which was identical, except that during the pre-weaning phase the calves had an additional milk allowance. Thus, the bile flow rates were potentially greater in our calves on the pre-weaning diet with a high milk content and thus higher fat content. As the FGCMs are excreted with the bile, a higher bile flow rate during the pre-weaning period could partly explain the increased metabolite concentrations found in the feces of our calves in the pre-weaning period compared to the post-weaning period.

4.4 Changes in the gut microbiome with the diet

An additional factor could be that the change from a primarily milk-based diet (high fat, low fiber) pre-weaning to a diet mainly based on roughage with additional concentrate (low fat, high fiber) post-weaning has led to major changes of the gastrointestinal tract and in particular of the microbiome in the gut of our studied calves. Given that bacteria in the gut can influence and alter the metabolism and hormone secretion of the host (Lee and Hase, 2014) and that gut microbiota possess enzymes for metabolism of steroids (Kunc et al., 2016), a change in the gut microbiome will greatly impact on the measured FGCM concentrations.

It is generally known, that the gut microbiome is not stable but changes depending on temperature and available substrates in the digestive tract (Hussain et al., 2021). For dairy calves during the weaning process it has been described that the altered carbohydrate composition of the diet shifts the dominant phyla in the rumen from *Bacteroidetes* in pre-weaned calves, to *Firmicutes* in post-weaned calves, regardless of their weaning age (Li et al., 2012; Rey et al., 2014; Meale et al., 2016, 2017). Thus, the change in diet from mainly milk to roughage has very likely led to a substantial change in the type and abundance of bacteria in the gut microbiome of our calves between the pre- and post-weaning data collection. This will

have affected the measured FGCM concentrations, as metabolites of steroid hormones are products of extensive metabolism by the liver and further modification by enzymes of bacteria in the gut (Brownie, 1992; Palme et al., 2005). In Meishan piglets for example, it was shown that weaning caused an alteration in the colonic microbiome and that the colonic metabolites differed between suckling and weaned piglets, with some metabolites being negatively and others positively related to the abundance of specific genera of bacteria (Jiang et al., 2020). Consequently, the type and amount of formed cortisol metabolites will differ between animals, as well as within the same individual, depending on the diet and the associated gut microbiome composition.

In addition to that, it has to be taken into consideration that the gut passage time of the food pulp will vary with the type of diet, which may influence the exposure time of cortisol metabolites to bacterial processing. As discussed in Van Gastelen et al. (2021) the gut passage time depends on many factors, such as for example particle size of the solid feed (Poppi et al., 1980) or feed intake level of the animal relative to body weight and size (Colucci et al., 1990). In Holstein veal calves it has been found that the passage time of digesta through the total digestive tract averaged 12.4 hours for milk replacer in contrast to 21.4 hours for concentrates, 36.8 hours for long hay and 59.1 hours for chopped straw (Van Gastelen et al., 2021). This is in line with results from other studies reporting a total tract retention time of 12.2 hours for milk replacer in veal calves (Gilbert et al., 2017) or 24.7 hours for rapeseed meal concentrate in dairy cows (Ahvenjärvi et al., 2010). Thus, if the gut passage time of milk and milk replacer is shorter than that of roughage and concentrate, it seems likely that also the food pulp in our study at the post-weaning stage may have taken longer to pass through the digestive tract than during the pre-weaning phase. Consequently, there will have been a higher chance of further bacterial processing of FGCMs during the post-weaning phase in contrast to the pre-weaning stage, where bacteria had less time for metabolism. This is further supported by a study of Lexen et al. (2008) in which sheep were infused with ^{14}C cortisol and the polarity of the radioactive FGCMs increased with longer retention time inside the animal.

Overall, it seems likely that the dietary change from milk to mainly roughage of the calves in our study led to a change in the gut microbiome, especially the ratio of *Bacteroidetes* to *Firmicutes*, as well as gut passage time and thus exposure time of FGCMs to bacterial transformations. Consequently, with the change in gut microbiome, the kind of formed cortisol metabolites will differ as well as the measured FGCM concentrations, as the employed EIAs for laboratory analysis of FGCMs are not specific, but always measure groups of metabolites (Möstl et al., 2002). The EIA employed in our study only picks up cortisol metabolites with a $5\beta\text{-}3\alpha\text{-ol-}11\text{-one}$ structure, and thus, depending on the structure of the produced FGCM metabolites during the pre- and post-weaning stage, these will have been reflected to different extents in results of the laboratory analysis. This might partly explain the measured differences in our FGCM concentrations between the pre- and post-weaning diet as well as the presented divergence between the baseline values of the two stages in our study, even if the latter only tended to differ.

5. Conclusion

The results of our study showed a significant increase with a clear peak in FGCM concentrations in the pre-weaning phase, which demonstrates that FGCMs can be used to evaluate the stress response of young calves that are still on a milk diet, the same way they can be used for older, weaned cattle.

The type of diet seems to have an influence on the measured time lag of FGCM excretion after a stressor, with an earlier peak occurring on the solid-based diet and a later one on the primarily milk-based diet. This was not statistically significant, but should nevertheless be considered in the design of future studies.

Also, peak FGCM_{rel} concentrations tended to be higher in unweaned calves on a primarily milk-based diet in comparison to weaned calves, likely due to a combination of effects of habituation to transport and dietary effects on bile flow rates and the composition of gut bacteria. This combination of factors does not allow to estimate the exact degree of difference in FGCM_{rel} concentrations between a primarily milk- and a solid-based diet, but it highlights that FGCM_{rel} concentrations of weaned calves tend to be lower than those of unweaned calves, and are thus not directly comparable.

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Conflict of interest

The authors declare to have no conflicts of interest that are relevant to the content of this article.

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Study II

Can a gradual weaning and separation process reduce weaning distress in dam-reared dairy calves? A comparison with the two-step method

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Can a gradual weaning and separation process reduce weaning distress in dam-reared dairy calves? A comparison with the two-step method.

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Abstract

The weaning and separation phase remains one of the biggest challenges for cow-calf-contact systems, but a gradual process that better mimics the naturally occurring reduction in milk intake has not yet been scientifically investigated. Therefore, the aim of our study was to compare behavioral and physiological indicators of distress in 3-months-old dam-reared dairy calves (with previous full-time cow-calf contact) weaned and separated either via gradual reduction of contact time with the dam (GR, 1 week half day contact, 1 week morning contact, 1 week fence-line contact before complete separation, n=18) or via two-step weaning using a nose flap (NF, 2 weeks access to dam with a nose flap, 1 week fence-line contact before complete separation, n=18). Behavior was recorded 1 week before (or for lying 3 weeks before) weaning start and during the 3 week weaning and separation period with direct observations on 4 days per week or via accelerometers (locomotor play, lying behavior). Blood and fecal samples were taken twice per week from weaning start until 3 weeks after weaning start. Calves were weighed weekly. Statistical analysis was conducted using (generalized) linear mixed models. Over the whole weaning and separation phase, NF calves showed a stronger decrease in the number of lying bouts, amount of locomotor play and average daily weight gains, as well as a higher increase in total mixed ration feeding time compared to GR calves, whereas GR calves vocalized more often and showed more searching behavior than NF calves. Also, the neutrophil:lymphocyte ratio of NF calves was elevated on day 3 after insertion of the nose flaps compared to baseline, but showed no change for GR calves on any sampling day. Overall, results point towards a favorable effect of a gradual weaning strategy on reduction of weaning and separation distress in dam-reared dairy calves, but the method requires further improvement from the protocol used in our study.

Keywords: cow-calf-contact systems, weaning, separation, stress

1. Introduction

Weaning and separation distress is one of the biggest challenges in cow-calf contact systems as reported by 87% of farmers practicing cow-calf-contact across Europe (Eriksson et al., 2022). For the calf, the weaning and separation process is not only associated with loss of milk as main nutritional source and loss of contact to the dam, but often also with the loss of familiar peers, mixing with new conspecifics and changes in physical environment (Weary et al., 2008; Lynch et al., 2019). These multifactorial stressors can result, among others, in reduced weight gains (Haley et al., 2005; Sweeney et al., 2010), a strong increase in vocalizations (Haley et al., 2005; Loberg et al., 2008), increased pacing and seeking behavior (Enriquez et al., 2010), reduced play behavior (Enriquez et al., 2010) and reduced lying times (Haley et al., 2005; Budzynska and Weary, 2008), as well as neutrophilia (O'Loughlin et al., 2011, 2014) and an increase in cortisol levels (Loberg et al., 2008; O'Loughlin et al., 2014) in abruptly weaned calves. As separation from the dam further induces a pessimistic judgement bias in calves (Daros et al., 2014), indicative of a negative affective state, the aforementioned behavioral and physiological responses to weaning thus reflect that calves experience distress, in the sense of stress that adversely affects an animal's welfare (Moberg, 2000), during the process.

In order to reduce the weaning distress, different methods have been investigated in the past that separate the loss of the milk from the loss of social contact to the dam. These include for example weaning via two-step separation, which means that suckling is prevented with so-called 'nose flaps' prior to permanent total separation (Sirovnik et al., 2020), as well as fence-line weaning, which allows partial physical contact to the dam without suckling (Sirovnik et al., 2020). Beef and dairy calves that were weaned with nose flaps showed reduced behavioral reactions (Haley et al., 2005; Loberg et al., 2008; Enriquez et al., 2010) as well as reduced physiological reactions at the time of separation (Loberg et al., 2008) compared to abrupt weaning. Thus, nose flaps have been proposed as a low stress weaning method for beef calves (Haley et al., 2005) and are also considered as an alternative for dairy cow-calf-contact systems (Sirovnik et al., 2020; Schneider and Ivemeyer, 2021; Barth et al., 2022). The major advantage of two-step weaning with nose flaps is the high practicability for farms, as in contrast to other weaning and separation methods it requires no modifications of the barn (Barth et al., 2022) or adjustments of on-farm routines for animals or personnel. Additionally, farmers value the lower number of vocalizations by cows and calves during weaning with nose flaps not only as a sign of reduced distress, but also because frequent vocalizations during weaning are emotionally challenging for them and lead to additional worries that others may mistake these as an indication for mistreatment of the animals (Waiblinger and Hebesberger, 2023; personal communication S. Waiblinger, University of Veterinary Medicine, Vienna, Austria). On the other hand, the use of nose flaps results in diminished weight gains compared to abrupt or fence-line weaning (Boland et al., 2008; Enriquez et al., 2010; Wenker et al., 2022) and can lead to severe nasal abrasions and open wounds with or without bleeding and secretion (Lambertz et al., 2015; Valente et al., 2022), which potentially even initiate pituitary abscesses (Fernandes et al., 2000), and leave nose flaps questionable with regard to animal welfare. Thus, there is a need for further improvement or implementation of new weaning strategies.

One possible approach is a gradual weaning and separation method, in which the cow-calf contact time prior to permanent separation is gradually reduced. To date, this weaning method has not yet been scientifically investigated, however, a gradual reduction of contact time could also be a promising weaning and separation method for cow-calf contact systems for several reasons. Firstly, it resembles more the naturally occurring reduction of milk intake during natural weaning, which is typically a gradual process in which the number of suckling bouts declines significantly with age of the calf over the course of several months (Reinhardt and Reinhardt, 1981). Secondly, Weary et al. (2008) suggested that during the increased times which cow and calf spend apart before weaning, the calves are potentially able to habituate to the periods of separation and increase their intake of solid feed, which eases the transition at weaning. Thirdly, gradual weaning is commonly used as a standard procedure for artificially reared dairy calves, either by diluting the milk/milk replacer with water or by feeding the calves less often or with smaller amounts of milk during the end of the milk feeding period. These practices showed that gradual weaning leads to reduced vocalizations (Jasper et al., 2008; Bittar et al., 2020), increased lying times (Scoley et al., 2019), as well as increased starter intake pre-weaning, and can alleviate part of the compromised post-weaning weight gains compared to abrupt weaning (Khan et al., 2007; Sweeney et al., 2010; Omidi-Mirzaei et al., 2015). Therefore, a gradual reduction of the daily cow-calf-contact time might cause less distress than weaning with a nose flap.

Accordingly, the aim of our study was to compare the behavioral and physiological distress responses of 3-months-old dam-reared dairy calves during weaning and separation either via gradual reduction of contact time between dam and calf or via two-step weaning using a nose flap. We hypothesized that a gradual process would ease the psychological and physiological adaption of calves to being separated from the mother and thus predicted a lower weight loss and less distress related behaviors, that is less vocalizations and searching behavior, but higher lying times, reduced number of lying bouts and more play behavior in gradually weaned calves compared to using a nose flap. Also, we expected that gradually weaned calves will react with less pronounced changes in hematological parameters and lower cortisol levels to the weaning and separation process compared to calves weaned with a nose flap.

2. Materials and methods

2.1 Animals, housing and feeding

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). The study was conducted from November 2019 till March 2020 with 36 dam-reared calves at the Thünen Institute of Organic Farming in Germany. All calves were of the German Holstein breed, but belonged to two different herds (19 horned: 8 female/11 male calves, 17 genetically polled: 8 female/9 male calves), that were kept in identically mirrored parts of an open-sided free stall barn (see Wagner et al., 2012 for a scheme of the barn). Calves stayed about 5 days with their mothers in an individual calving pen and were afterwards kept in a calf section with full-time access to the cow herd, including their mothers, until the weaning and separation process started. For calves which were born during the grazing period (up from

August until mid November 2019, applies for all but 2 calves) this included also pasture access with the cows during the pre-weaning phase. After the grazing period ended, the calves were kept completely indoors in a separate calf section of 96.5m², which was directly connected to the cow section via an automatic transponder-controlled selection gate. Calves were free to enter the cow section and suckle their mothers the whole day except for the milking hours (05:15-08:30am and 3:30-6:45pm) and during times when cows were feeding, as calves had no access to the milking parlor and the cows' feeding area. Consequently, the cow-calf contact was always initiated by the calf.

In the calf section the calves had ad libitum access to water, hay and a total mixed ration (TMR, composed of about 63.6% grass silage, 30.0% corn silage, 6.2% concentrate feed in the form of coarse grain and 0.2% mineral feed), which was provided freshly once a day in the afternoon. Additionally, calves had access to a concentrate feeder (Förster Technik GmbH, Germany) with an allowance of 1.5 kg concentrate per animal and day distributed in portions of 50 g. No additional milk was fed from buckets or an automatic feeder to the calves beside the milk that could be suckled from the mothers or other cows. The calf section was equipped with an automatic calf brush (Schurr Gerätebau GmbH, Germany) in the walking area (rubber-coated and concrete floor, 71.9m²) as well as two straw bedded lying areas (12.6m² and 12.0m²). In addition, the walking alleys and cubicles of the cows (490.0m²) could be entered by the calves as well. Group size of the calves was dynamic and varied from 15-23 calves (horned herd) and 14-20 calves (polled herd), due to new calvings or regrouping of fully weaned calves into the youngstock barn. In calf sections of both herds, the number of study animals that were simultaneously at any stage of the weaning and separation process ranged from 2 to 7 calves. Thus, in addition to the study animals there were always other younger calves present. Group size of the cow herd varied from 45 to 49 cows (horned herd) and 43 to 48 cows (polled herd) throughout the experiment.

2.2 Experimental design and treatments

All calves were about 3 months old when the weaning and separation process started. The calves were randomly allocated, balanced for sex (male/female) and herd affiliation (horned/polled), to one of the following two treatments: The nose flap calves (NF, n=18 calves, 8 female, 10 male) were weaned and separated with a two-step method using a nose flap (Quiet wean, JDA Livestock Innovations, Canada). Calves were equipped with the nose flap for 2 weeks, allowing them whole day contact to their dam without suckling. After these 2 weeks, the nose flap was removed, and the calves were kept in the calf section for one more week, i.e. they were not allowed to access the cow barn but had 'fence-line' contact to their mother via the selection gate and the pen boundaries. This still allowed visual, auditory, olfactory, and limited tactile contact to their mothers without suckling. After these 3 weeks the weaning and separation process was completed by moving the calves to the youngstock herd in a different barn (table 1). In the gradual reduction treatment (GR, n=18 calves, 8 female, 10 male) the weaning and separation took place by gradually reducing the daily cow-calf contact time. For this purpose, access to the cow section was controlled automatically via transponders on the calves' collars. When the weaning and separation process started, the cow-calf contact time was reduced from full-time contact pre-weaning to half day contact between milkings during the day (~7 hours,

from about 8:30 a.m. - 3:30 p.m). During the evening and night the calves had no access to the cow herd. After one week of half day contact, the cow-calf contact time was further reduced to ~3.5 hours from the end of morning milking till noon. Following this week of morning contact, the GR calves had one more week of fence-line contact to their dam like the NF calves, before they were moved to the youngstock barn (table 1).

The weaning and separation process was always initiated on the same day of the week (Tuesday) and started for half of the calves in both treatments with 12 weeks of age (exact age of calves: NF_{12w}: range, mean \pm S.D.; 83-89, 87.8 \pm 2.0 days, GR_{12w}: 83-89, 85.0 \pm 2.2 days at weaning start) and for the other half of the calves with 14 weeks of age (NF_{14w}: 98-102, 100.0 \pm 1.3 days, GR_{14w}: 97-103, 99.2 \pm 1.9 days old at weaning start, n=9 each). This was done to counterbalance the fact that, due to the nature of the treatments, the complete milk loss happened at treatment day 0 for calves in the NF treatment, but not until introduction of fence-line separation from the dam on day 14 in the GR treatment, thus with a 2-week delay (table 1). By staggering the starting age by two weeks in each treatment, calves with similar age at weaning start, at milk loss (NF_{14w}: 98-102, 100.0 \pm 1.3 days old at milk loss; GR_{12w}: 97-103, 99.0 \pm 2.2 days old at milk loss) and at introduction of fence-line separation from the dam were included in both treatments (see supplementary table S1 for details, available at <https://data.mendeley.com/datasets/zfxty2pxd8/1>). Consequently, age of all calves in the NF treatment ranged from 83-102 days (mean \pm S.D. 93.9 \pm 6.5 days) and age of all GR calves ranged from 83-103 days (92.1 \pm 7.6 days at weaning start) and this age was later included in the statistical analysis to best reflect the existing variation.

2.3 Data collection

For assessment of the calves' distress responses to the weaning and separation process a combination of physiological and behavioral indicators was used. All sampling types and time points are given in table 1.

2.3.1 Physiological assessment

2.3.1.1 Average daily weight gains (ADG)

Calves were weighted once per week between days -21 till +21 relative to weaning start with a commercial cattle weighing scale (Patura Wiegekäfig, Wiegeset S1, Accuracy \pm 1%). All calves were weighed routinely every week starting from birth and were thus accustomed to being moved onto the weighing scale.

2.3.1.2 Fecal cortisol metabolites (FGCM)

Between days 0 till +21 relative to weaning start, fecal samples were collected twice per week in the morning at about 07:30 a.m. (\pm 60 min). A minimum of 5 g of feces was collected by rectal stimulation, homogenized, and transferred into a commercial fecal sampling tube. Sampling tubes were immediately transferred into a cooling box and placed within 30 min into a freezer at -20°C , where they were kept until further analysis. Analysis of fecal samples in the laboratory followed the established protocol (Palme et al., 2013) with an 11-oxoetiocholanolone enzyme immunoassay (EIA), as this EIA was shown to be superior for FGCM analysis in calves (Vogt et al., 2023).

2.3.1.3 Hematological variables

As O'Loughlin et al. (2014) emphasized neutrophil number as a robust biomarker of weaning distress in beef calves, blood samples of calves were taken twice per week between days 0 till 21 relative to weaning start. 2 ml of blood was collected via jugular venipuncture always by the same qualified experimenter into ethylenediaminetetraacetic acid (K EDTA) coated sampling tubes (S-Monovette by Sarstedt) and kept refrigerated at 8°C until daily collection by a courier. The hemogram was done at the same day by an external medical diagnostics provider with laboratory services for veterinary medicine (SYNLAB.vet GmbH, Hamburg).

2.3.2 Behavioral assessment

2.3.2.1 Direct observation of behavior

Direct observation of calves' behavior was done always by the same observer on 4 consecutive days per week between days -7 till +21 relative to weaning start. All areas of the cow and calf section were visible to the observer at all times, except for the calves' lying and feeding area, which were observed via video recordings. Recorded behaviors are described in table 2, though locomotor, social and object play behavior were combined into 'total play behavior' afterwards. Observation times included: (a) 2 hours following morning milking, (b) 1 hour before the start of evening milking and (c) 2 hours following evening milking. These times were chosen, as cows and calves were accustomed to being separated and reunited around milking and showed thus the most activity during these periods. Observations after the end of milkings (a and c) started in the exact moment the cows and calves were allowed to reunite. As there were 2 herds for observation, the observer switched between herds every 30 min, thus totaling 2.5 hours daily observation time per calf. Observation start was alternated between the horned herd in even - and the polled herd in odd calendar weeks and was also swapped each day within a week.

2.3.2.2 Video analysis of behavior

Analysis of TMR feeding time and brush use (table 2) was done via video observation on the same 4 consecutive days when direct observation took place, using Jovision Infrared Network Cameras with Jovision NVR System Software (Version 2.0.1.49). Cameras automatically switched to infrared mode when light conditions were not sufficient for color mode anymore. For better identification of calves during the infrared mode, photos of the calves' individual

coat patterns were used. Analysis was conducted by 4 different observers for TMR feeding time (Cohen's kappa $\kappa_{\text{TMR}} \geq 0.91$) and 3 different observers for brush use ($\kappa_{\text{Brush use}} \geq 0.88$; one observer (with part of data for 4 and all data for 3 calves) had to be excluded due to too low inter-observer reliability for brush use) with the software BORIS (Friard and Gamba, 2016, Version 7.9.22). All behaviors were recorded continuously in intervals of 6 hours (night: 00:00-06:00h, morning: 06:00-12:00h, afternoon: 12:00-18:00h, evening: 18:00-00:00h) and specific intervals on days of treatment change were subsequently deleted from the data set (i.e. the night interval of day 0 for NF calves as the nose flap was not inserted until the morning).

Table 2) Ethogram of behaviors accessed by direct or video observation.

Behavior	Definition	Recording rule	Mode
Vocalization	Calf produces a clearly audible sound through the mouth (Loberg et al., 2008)	Continuous (freq)	Direct
Searching behavior	Calf is moving parallel to, within 1m of, the pen partition up and down, or standing with its head through the selection gate or standing beside the pen partition (radius 2.5m) with head elevated with eyes and ears focused in the direction of the cow's section and scanning (adapted from Loberg et al., 2008; Enriquez et al., 2010)	Interval	Direct
Suckling attempt	Calves attempt to nuzzle the udder but did not obtain milk (because of nose-flap or cow rejection) (Enriquez et al., 2010)	Interval	Direct
Locomotor play	Calf is galloping, jumping, leaping, bucking or buck-kicking (after Jensen et al., 1998)	Continuous (dur)	Direct
Social play	Two calves are standing front to front, pushing, rubbing or butting head against head/neck without force, often including rotating head movements (adapted from Jensen et al., 1998)	Continuous (dur)	Direct
Object play	Calf is butting water bowl, hayrack or bars in the pen, standing up. Or calf is butting straw or rubbing head, throat or neck in straw, kneeling down on the two forelegs (Jensen et al., 1998)	Continuous (dur)	Direct
Socio-positive behavior	Calf is sniffing, licking or rubbing against another calf (Loberg et al., 2008)	One-zero	Direct
Rumination	Chewing after regurgitating boluses of feed (Enriquez et al., 2010)	Interval	Direct
Mounting	Calf lifts its front legs from the ground, supporting itself on another calf. The act of an animal raising the anterior part of its body generally onto the posterior part of another animal (Solano et al., 2005)	Continuous (freq)	Direct
Cross-sucking	Calf sucking any part of another calf (Hepola et al., 2006)	One-zero	Direct
TMR feeding time	Calves' head is placed through the feeding rack (marker: ears in front of the rack)	Continuous	Video
Brush use	Calf having physical contact with the brush with any body part while being (automatically) brushed or scratching itself against the brush (adapted from Horvath and Miller-Cushon, 2019)	Continuous	Video

Continuous=continuous behavior sampling, Interval=scan sampling every 3 minutes, One-zero=One-zero sampling every 3 minutes, with 1 denoting the presence of a behavior in that period and 0 denoting the absence of the behavior in that period; Freq= frequency, Dur= duration.

2.3.2.3 Automatic assessment of behavior

2.3.2.3.1 Concentrate feed intake

Output from the concentrate feeder (Förster Technik GmbH) was automatically recorded with Förster CalfCloud Software (Förster Technik GmbH). Daily allowance per calf was 1.5 kg concentrate, however, in the course of the experiment it was frequently observed that calves other than the intended recipient ate up (parts of) the portion. Therefore, a precise allocation of the concentrate intake per calf was not possible and concentrate intake was not further considered for analysis.

2.3.2.3.2 Lying behavior

For assessment of lying times and number of lying bouts 28 of the 36 calves (14 per treatment) were fitted with an accelerometer (Hobo Pendant G data logger, Onset Computer Corporation). The 3-axis accelerometers were placed directly above the metacarpal joint on the medial side of the left hind leg and tilted by 90° to allow for a better fit to the calves' legs, such that the y-axis was parallel to the ground and the x-axis was perpendicular to the ground pointing downward. Data loggers recorded g-force and degree of tilt of the x-axis at 60s intervals from day -28 till +21 relative to weaning start, with days -28 till -22 used as habituation period. The accelerometers were replaced every 14 days due to limited storage capacities. Data was downloaded using HOBOWare software (Onset Computer Corporation, Version 3.7.17). Recordings at the time of fitting or replacement of loggers, i.e. between 07:00 - 10:00 a.m. on respective days, were omitted due to their potential nuisance effect on the data. The degree of vertical tilt (x-axis) was used to determine the lying position of the calf, such that readings $\leq 120^\circ$ indicated the calf lying down, whereas readings $> 120^\circ$ indicated the calf standing up (adjusted from Ito et al., 2009 for tilted logger position). Afterwards recordings were edited with an event filter, which converted single outlier readings (e.g. a single lying event that was preceded and followed by a standing event, or vice versa) to the behavior that preceded it, as these were potentially erroneous readings of lying or standing events (Ledgerwood et al., 2010). From these data, total lying time in percent per day and the number of lying bouts per day was calculated.

2.3.2.3.3 Locomotor play

For automatic assessment of locomotor play, 28 of the 36 calves (all but 4 identical to those calves used for assessment of lying behavior, NF= 13/GR= 15 calves) were fitted with a second accelerometer for 9 hours (08:00 a.m. - 05:00 p.m.) on the medial side of the right hind leg on days -4, +3, +10, +17 relative to weaning start. Loggers were set to measure g-force of the vertical axis (x-axis, as loggers were tilted by 90° again) at a rate of 1 Hz as previously validated by Luu et al. (2013). Readings were exported with HOBOWare and analyzed using the peak acceleration method (Größbacher et al., 2020), i.e. counts of acceleration peaks from the data logger determine the counts of play events of the calf. For validation, 30 min video sections (from continuous video recordings in the calf section) were selected in which the calves showed locomotor play (running, bucking or single kick) during the times the 'play accelerometer' was worn (available for 16 of the 28 calves). Then a Pearson correlation was calculated for different

upper and lower threshold values of peak acceleration between counts of observed play events from video and counts of peak acceleration measurements from the data loggers. Our data showed the best results for peak threshold values of $\geq +3.0g$ and $\leq -2.0g$ ($r=0.92$), which was used for further analysis. Thus, our data are reported in counts of peak acceleration measurements (CPA) from HOB0-loggers per 9 hour sampling period for peak threshold values of $\geq +3.0g$ and $\leq -2.0g$.

2.4 Statistical analysis

2.4.1 Exclusion criteria

Data points from collection days at which calves showed clinical signs of ill health or gained access to the cow area during periods in which they were supposed to be separated from their dam (named thereafter 'illegitimate access'), e.g. GR calves during the night in the half day phase, were excluded from analysis as described in the following paragraph.

2.4.1.1 Illegitimate cow-calf contact

Video-monitoring of the selection gate where calves entered the cow section was used to examine if any weaner calves obtained illegitimate access to their dams, e.g. by forcing themselves through the gate along with younger calves. Percentage of time a calf entered the cow section from the total time it was not permitted in the cow section anymore was calculated per 6 hour interval (morning, afternoon, evening, night) to account for the different permission times in the course of the GR treatment. Error score 0 was given when a calf had 0-2% of time illegitimate access to its dam, error score 1 was given when a calf had >2% illegitimate access to its dam per interval. If error score 1 was given for an interval, the calf automatically obtained this score for the subsequent interval as well, as we expect calves that had just suckled their dams to have a lower motivation (or vice versa less distress) to enter the cow section and were thus not representative for weaning distress examination during that period. For physiological indicators (except ADG) and automatically measured behaviors, the highest error score of the 4 intervals from that day was used for correction of data. For weight gains which were measured only once per week, the 2% threshold was used as well, i.e. calves which entered the cow section for >2% of time illegitimately within a week were not included in ADG analysis for that week. Only observations with error score 0 were used in all statistical analyses.

21 of the 36 study calves entered the cow section illegitimately (6 NF/ 15 GR calves). On average (\pm S.D.) these 21 calves entered the cow section for $18.66\pm 18.12\%$ (Median: 13.5%, range 0.28 – 86.4 %) of time per 6 hour observation illegitimately, however, this was mainly due to 5 prominent calves (all of the GR treatment). Within the GR treatment the majority of observations with illegitimate access was recorded in the third week (55.61% of occurrences, 13 calves), followed by the first week (23.98%, 13 calves, 11 calves identical to week 3) of the weaning and separation process. NF calves could only enter the cow section illegitimately during the third week (100% of occurrences of illegitimate access, 6 calves).

2.4.1.2 Health monitoring

Health status of the calves was monitored in detail twice per week before collection of blood samples by the experimenter. Assessment included general condition, nasal and ocular discharge, coughing, ear position, navel health, lesions, cleanliness of hindquarters, fecal consistency and measurement of rectal temperature (adapted from Roth et al., 2009). A calf was considered to have fever, if rectal temperature was above 39.1°C. Health problems were scored with a 0-1-2 system, with 0 equaling no health problems and a 2 equaling a severe symptom (see Roth et al., 2009), e.g. fever, liquid feces or all conditions which made medications and/or veterinary care necessary. A 1 equaled a minor symptom, e.g. sporadic coughing or superficial lesions. Total health score for the day equaled the highest score the calf got on one of the single parameters on the specific day. If an animal was scored a 2 in its total health score (i.e. it scored a 2 in any of the health measurements) on a specific day, it was completely excluded from analysis for this day as well as the preceding and subsequent day. Only data for weight gain were based on a data set which included calves with scours (fecal consistency scored 2) on single days. This was the case, since the weight measurements were taken only once every week and single days with scours in between will be masked by the other days. Furthermore, deleting calves with nutritional scours due to high milk consumption could have led to an underestimation of the ADG.

For FGCM analysis the health and error score data from the day before data collection was used, as in calves FGCMs peak about 8 - 10 hours after the occurrence of a stressor (Vogt et al., 2023), and thus our samples from 07:30 a.m. reflect the calves' FGCM concentrations from the previous evening (about 9 p.m. till midnight).

Remaining data points after correction for health problems and illegitimate cow-calf-contact are shown in supplementary figures S1 and S2.

2.4.2 Data analysis

Statistical analysis was performed with SAS Version 9.4. An a priori reduction of explanatory variables was done by checking for correlations between variables. For all behavioral parameters observed at several intervals per day (TMR feeding times, rumination, vocalizations, searching behavior, total play behavior and brush use) the means per week for each individual calf were calculated before analysis. Data were analyzed according to their respective distribution (Gaussian: ADG (kg/d), rumination (% of scans), FGCM (ng/g), neutrophils (% of total leukocytes), lymphocytes (% of total leukocytes), vocalizations (frequency per 30min), searching behavior (% of scans), total play behavior (s per 30min), brush use (min per 6h), lying times (% of 24h) and suckling attempts (% of scans); beta distribution (logit-link): N:L ratio; Poisson distribution (log-link): locomotor play (CPA per 9h) and lying bouts (number per 24h); geometric distribution (log-link): TMR feeding time (min per 6h) with a linear or generalized linear mixed effects model with repeated measures. FGCM data was log-transformed before analysis and back-transformed using the omega method. Vocalizations, searching behavior, total play behavior and suckling attempts were square root-transformed before analysis and back-transformed using the delta method. The (generalized)

linear mixed effects model included treatment (GR/NF), phase (baseline phase/weaning and separation phase), the interaction between treatment and phase, as well as sex of the calf (male/female) as fixed effects. Age of the calf at start of the weaning procedure (in days) was included as continuous variable. The calf ID nested within herd (horned/polled) was included as random effect. Additionally, the calendar week merged with herd was included as cross-classified random effect to account for the weekly changing group composition in the calf section of each herd throughout the study (Cafri et al., 2015). Correction for multiple testing of several parameters (in total 14) was calculated according to the Benjamini-Hochberg false discovery rate (FDR) correction (Glickman et al., 2014, see supplementary table S2).

In case that the treatment x phase interaction showed significance ($p \leq 0.05$) or a tendency ($p \leq 0.1$) after correction for multiple testing, an extended model was run which included the exact week (Baseline, Week 1, Week 2, Week 3 of the weaning and separation phase) instead of the whole weaning and separation phase as the sampling time point. For all models, model requirements (normal distribution and homoscedasticity of residuals) were checked graphically. Pairwise differences of different treatment x phase interactions (or for extended analysis treatment x week interactions) were calculated using a Tukey-Kramer post-hoc test. All results are presented as (back-transformed) least square means \pm S.E. Confidence intervals for the estimates are presented in supplementary tables S4 and S5.

Exceptions to this procedure were FGCM concentrations, hematological variables and suckling attempts. For FGCM data only the extended model with the specific weeks was run, since a comparison of the whole weaning and separation phase is not valid due to the different nutritional basis in the course of the treatments, which was shown to heavily influence FGCM levels (Vogt et al., 2023). For hematological variables the exact day of treatment was used as the sampling time point instead of the week in the extended analysis, since literature showed that changes in hematological parameters are transient and can return to baseline levels within just 3 days after an abrupt weaning stressor (O'Loughlin et al., 2012, 2014). For suckling attempts of the NF calves we used the same mixed model as above, except that it contained only treatment day, age at weaning start and sex of calf as fixed factors, as all calves were of the same treatment.

Analysis of socio-positive behavior, mounting behavior and cross-sucking was not possible due to a too low occurrence of these variables during our selected observation times. Cross-sucking was observed once in 4 calves (2 NF, 2 GR) during the whole behavioral observation.

3. Results

3.1 Average daily weight gains (ADGs)

Weight gain (kg/d) differed between treatments depending on the phase (interaction treatment x phase, table 3). GR calves gained more weight than NF calves during the three-week weaning and separation phase (pairwise post-hoc test: $t_{77} = 3.6$, $p = 0.004$, table 3). However, both treatments showed lower ADGs during the weaning and separation phase compared to their individual baseline before start of the treatment (GR: $t_{81} = 7.8$, $p < 0.001$; NF: $t_{60} = 12.9$, $p < 0.001$, table 3).

The extended model revealed the lowest gains for NF calves compared to baseline in the first week immediately after insertion of the nose flap ($t_{137}=-9.3$, $p<0.001$), but ADGs remained at a relatively low level for the second and third week as well (table 4). In contrast, GR calves showed increasing weight gains over the weaning and separation period with highest ADGs in the third week, which also did not differ from the GR baseline anymore ($t_{164}=-2.4$, $p=0.28$, table 4, fig. S1A).

3.2 Total mixed ration (TMR) feeding

Duration of feeding on TMR differed between treatments depending on phase (interaction treatment x phase, table 3). Throughout the weaning and separation phase, NF calves spent more time feeding on TMR than GR calves ($t_{31}=-6.7$, $p<0.001$). Both treatments showed an increase in TMR feeding duration during the weaning and separation phase compared to their individual baseline before the weaning procedure started (GR: $t_{81}=-6.5$, $p<0.001$; NF: $t_{74}=-13.5$, $p<0.001$), but this increase was stronger for NF calves (table 3).

The extended model revealed that NF and GR calves each showed a significant increase in the duration of TMR feeding with each week of the weaning and separation procedure (table 4), except for NF calves from week 2 to week 3 ($t_{63}=-2.3$, $p=0.33$). In general, NF calves showed a noticeably stronger increase in TMR feeding duration compared to GR calves, as they fed significantly longer from the TMR in each weekly comparison (table 4, fig. S1B).

3.3 Rumination behavior

There was no treatment x phase interaction for the percentage of scans in which calves showed rumination behavior, but a main effect of the treatment phase (table 3). Rumination behavior increased in all calves during the weaning and separation phase as compared to the baseline phase (table 3).

3.4 Vocalizations

The frequency of vocalizations differed between treatments depending on phase (interaction treatment x phase, table 3). GR calves showed an increase in frequency of vocalizations from baseline to the weaning and separation phase ($t_{128}=-4.1$, $p=0.001$), which was in contrast not the case for the NF calves ($t_{128}=-0.2$, $p=0.99$, table 3). Consequently, GR calves showed more vocalizations during the weaning and separation phase compared to NF calves ($t_{48}=4.7$, $p<0.001$, table 3).

Additionally, the extended model revealed a difference in temporal distribution of vocalizations, as GR calves vocalized most frequently during the third week of the treatment with fence-line contact and NF calves most frequently during the first week, which differed significantly from another ($t_{122}=5.9$, $p<0.001$, table 4, fig. S2A). These both represented more vocalizations compared to the respective baseline value for both of the treatments (GR: $t_{95}=11.4$, $p<0.001$; NF: $t_{68}=4.6$, $p<0.001$, table 4) and was for both treatments the time point when milk loss happened. Introduction of half day (week 1) and morning contact (week 2) did not significantly increase vocalizations compared to baseline in GR calves (table 4, fig. S2A).

Table 3) Model output of the main model for the effect of weaning and separation of calves with either two-step weaning using a nose flap (NF) or gradual reduction of contact time to the dam (GR) on behavioral and physiological indicators of distress. Values are expressed as least squares means (LSM) \pm standard error (S.E.). A Benjamini-Hochberg false discovery rate calculation for the TxP interaction confirmed the p-values in bold for rejection of the null-hypothesis with an $\alpha \leq 0.05$, while p-values in bold and italics correspond to an $\alpha \leq 0.1$.

Variable			Phase		p-values (F-value _{Num DF, Den DF})		
	T	N	Baseline	Treatment	T	P	T x P
ADG (kg/d)	NF	18	1.50 \pm 0.07 ^a	0.26 \pm 0.07 ^{bx}	0.003	<0.001	0.028
	GR	18	1.58 \pm 0.07 ^a	0.68 \pm 0.09 ^{by}	(F _{1,28} =10.6)	(F _{1,72} =198.7)	(F _{1,70} =5.1)
TMR feeding time (min per 6h)	NF	18	6.81 \pm 0.74 ^a	29.25 \pm 2.56 ^{bx}	<0.001	<0.001	<0.001
	GR	18	6.51 \pm 0.71 ^a	13.04 \pm 1.15 ^{by}	(F _{1,31} =14.1)	(F _{1,93} =186.6)	(F _{1,58} =26.9)
Rumination (% of scans)	NF	18	9.95 \pm 2.25	16.31 \pm 1.76	0.74	0.001	0.50
	GR	18	11.72 \pm 2.27	16.05 \pm 1.73	(F _{1,29} =0.1)	(F _{1,89} =10.9)	(F _{1,72} =0.5)
Vocalizations ¹ (frequency per 30min)	NF	18	0.50 \pm 0.15	0.61 \pm 0.10 ^x	0.12	0.003	0.007
	GR	18	0.16 \pm 0.05 ^a	3.21 \pm 0.47 ^{by}	(F _{1,87} =2.5)	(F _{1,128} =9.2)	(F _{1,128} =7.4)
Searching behavior ¹ (% of scans)	NF	18	0.32 \pm 0.09	0.50 \pm 0.08 ^x	0.09	<0.001	<0.001
	GR	18	0.01 \pm 0.00 ^a	3.62 \pm 0.53 ^{by}	(F _{1,90} =3.0)	(F _{1,127} =15.7)	(F _{1,114} =12.2)
Total play behavior ¹ (s per 30min)	NF	18	1.54 \pm 0.26	0.19 \pm 0.02	0.01	<0.001	0.71
	GR	18	2.59 \pm 0.43	0.80 \pm 0.09	(F _{1,44} =7.7)	(F _{1,90} =36.5)	(F _{1,67} =0.14)
Locomotor play (CPA per 9h)	NF	13	34.77 \pm 7.40 ^a	12.17 \pm 2.14 ^{bx}	0.11	<0.001	0.007
	GR	15	31.38 \pm 6.10	26.10 \pm 4.45 ^y	(F _{1,19} =2.9)	(F _{1,43} =14.0)	(F _{1,38} =8.1)
Brush use (min per 6h)	NF	16	3.57 \pm 0.68	3.77 \pm 0.54	0.73	0.67	0.25
	GR	17	4.18 \pm 0.61	3.62 \pm 0.49	(F _{1,11} =0.13)	(F _{1,51} =0.2)	(F _{1,38} =1.4)
Lying times (% of 24h)	NF	15	66.69 \pm 0.71	62.11 \pm 0.73	0.47	<0.001	0.047
	GR	14	66.64 \pm 0.75	63.53 \pm 0.83	(F _{1,25} =0.6)	(F _{1,248} =86.9)	(F _{1,279} =4.0)
No. of lying bouts (per 24h)	NF	15	18.52 \pm 0.75 ^a	11.50 \pm 0.50 ^{bx}	0.31	<0.001	<0.001
	GR	14	17.28 \pm 0.74 ^a	13.89 \pm 0.66 ^{by}	(F _{1,25} =1.1)	(F _{1,201} =255.3)	(F _{1,211} =37.4)
Neutrophils (%)	NF	18	19.84 \pm 2.43	25.05 \pm 1.53	0.12	0.46	0.016[#]
	GR	17	27.49 \pm 2.60	24.74 \pm 1.75	(F _{1,39} =2.6)	(F _{1,97} =0.6)	(F _{1,89} =5.9)
Lymphocytes (%)	NF	18	71.85 \pm 2.57 ^a	65.76 \pm 1.60 ^b	0.12	0.32	0.012
	GR	17	63.64 \pm 2.74	66.25 \pm 1.83	(F _{1,41} =2.5)	(F _{1,95} =1.0)	(F _{1,85} =6.5)
N:L ratio	NF	18	0.30 \pm 0.06	0.41 \pm 0.04	0.21	0.62	0.045[#]
	GR	17	0.46 \pm 0.07	0.40 \pm 0.04	(F _{1,43} =1.6)	(F _{1,102} =0.2)	(F _{1,87} =4.2)

Superscript letters a,b indicate a difference between baseline and the weaning/separation phase within a treatment, superscript letters x, y indicate a difference between the NF and GR treatment within a phase with $p \leq 0.05$. #individual pairwise comparisons were no longer significant or only tended to be significant after Tukey-Kramer adjustment. T= treatment, P= phase, T x P = interaction of treatment x phase. DF= degrees of freedom. Baseline= last 3 weeks before weaning start for ADG, lying times and lying bouts, 1 week before weaning start for hematological responses and all other behaviors, Treatment phase= the 3-week weaning and separation phase. ADG= average daily gains, TMR= total mixed ration, CPA= counts of peak acceleration measurements, N:L ratio= neutrophil: lymphocyte ratio. ¹LSM and S.E. back-transformed from square-root-transformation.

3.5 Searching behavior

The percentage of scans in which calves showed searching behavior differed between treatments depending on phase (interaction treatment x phase, table 3). During the three-week weaning and separation phase, GR calves showed more searching behavior than NF calves ($t_{52}=6.2$, $p<0.001$, table 3). The highest increase in scans with searching behavior compared to baseline was seen in GR calves during the first and especially the third week of the weaning and separation process, whereas NF calves showed no significant increase in searching behavior compared to their baseline in any treatment week (table 4, fig. S2B). Generally, GR calves showed significantly more searching behavior during the third week with fence-line contact than during any other week of the weaning and separation process (table 4). Equally, the amount of searching behavior of GR calves in the third week was also higher than that of NF calves in any week of the weaning and separation period, including the first week in which the milk loss happened for NF calves (table 4, fig. S2B).

3.6 Total play behavior (direct observation)

There was no interaction of treatment x phase for the duration of total play behavior of the calves, i.e. locomotor, social and object play behavior combined, but a main effect of phase (table 3). During the weaning and separation phase all calves played less in comparison to the baseline phase (table 3).

3.7 Locomotor play (automatic assessment)

Counts of peak acceleration (CPA per 9h), indicative of locomotor play, differed between treatments depending on the phase (interaction treatment x phase, table 3). During the three-week weaning and separation phase, calves weaned with a nose flap showed a decrease in locomotor play compared to their baseline phase ($t_{37}=4.5$, $p<0.001$), which was however not the case for GR calves ($t_{47}=0.9$, $p=0.82$, table 3). Consequently, GR calves showed more locomotor play during the weaning and separation phase than NF calves ($t_{19}=3.5$, $p=0.007$, table 3).

The extended model revealed that compared to baseline, locomotor play behavior of NF calves was mainly reduced in the first and second week while wearing the nose flap (week 1: $t_{33}=-3.5$, $p=0.03$; week 2: $t_{35}=-4.1$, $p=0.007$), but not during the third week with fence-line contact anymore ($t_{39}=-2.8$, $p=0.13$, table 4, fig. S2C). Contrarily, no significant decline in locomotor play was observed for GR calves from baseline to any treatment week (table 4, fig. S2C). Nonetheless, GR calves showed a numerical reduction in play behavior compared to baseline after introduction of fence-line contact to the dam in the third week (table 4; note however that this was based on data of only 3 calves, denoted by the diamonds in Supplemental Fig. S2C).

3.8 Brush use

There was no interaction of treatment x phase for the duration of brush use of the calves and no main effect either (table 3).

Table 4) Model output of the extended model for the effect of weaning and separation of calves over three weeks with either two-step weaning using a nose flap (NF) or gradual reduction of contact time to the dam (GR) on behavioral and physiological indicators of distress. Values are expressed as least squares means (LSM) \pm standard error (S.E.).

Variable			Week				p-values (F-value _{Num DF, Den DF})		
	T	N	Baseline	Week 1	Week 2	Week 3	T	W	T x W
ADG (kg/d)	NF	18	1.51 \pm 0.07 ^a	0.07 \pm 0.13 ^c	0.41 \pm 0.13 ^{bc}	0.27 \pm 0.14 ^c	<0.001	<0.001	0.03
	GR	18	1.58 \pm 0.07 ^a	0.64 \pm 0.18 ^{bc}	0.51 \pm 0.16 ^{bc}	1.07 \pm 0.21 ^{ab}	(F _{1,52} =17.3)	(F _{3,112} =67.2)	(F _{3,113} =3.2)
TMR feeding time (min per 6h)	NF	18	6.05 \pm 0.63 ^{ab}	21.52 \pm 2.30 ^d	30.56 \pm 3.08 ^c	37.61 \pm 3.84 ^c	<0.001	<0.001	<0.001
	GR	18	5.64 \pm 0.58 ^a	9.09 \pm 0.95 ^b	13.21 \pm 1.39 ^c	18.06 \pm 1.89 ^d	(F _{1,29} =32.0)	(F _{3,66} =116.3)	(F _{3,49} =13.8)
Vocalizations ¹ (frequency per 30min)	NF	18	0.25 \pm 0.05 ^a	3.68 \pm 0.80 ^b	0.12 \pm 0.24 ^a	0.04 \pm 0.01 ^a	<0.001	<0.001	<0.001
	GR	18	0.30 \pm 0.06 ^a	1.20 \pm 0.24 ^{ab}	0.85 \pm 0.17 ^a	13.78 \pm 2.91 ^c	(F _{1,30} =31.9)	(F _{3,71} =19.8)	(F _{3,68} =45.6)
Searching behavior ¹ (% of scans)	NF	18	0.14 \pm 0.03 ^{ab}	1.61 \pm 0.40 ^{bc}	0.02 \pm 0.01 ^a	0.90 \pm 0.22 ^{ac}	<0.001	<0.001	<0.001
	GR	18	0.05 \pm 0.01 ^a	2.30 \pm 0.53 ^c	0.70 \pm 0.16 ^{ac}	15.40 \pm 3.73 ^d	(F _{1,26} =38.7)	(F _{3,71} =31.2)	(F _{3,65} =21.5)
Locomotor play (CPA per 9h)	NF	13	35.66 \pm 7.25 ^a	11.71 \pm 3.49 ^b	11.12 \pm 2.78 ^b	15.68 \pm 4.08 ^{ab}	0.36	0.001	0.004
	GR	15	29.25 \pm 5.51 ^{ab}	25.45 \pm 6.17 ^{ab}	31.52 \pm 6.34 ^a	7.42 \pm 3.90 ^{ab}	(F _{1,23} =0.9)	(F _{3,34} =6.7)	(F _{3,31} =5.4)
Lying times (% of 24h)	NF	15	66.69 \pm 0.71	62.68 \pm 0.86	62.89 \pm 0.85	60.90 \pm 0.86	0.49	<0.001	0.19
	GR	14	66.66 \pm 0.74	64.19 \pm 0.97	64.48 \pm 0.99	60.49 \pm 1.20	(F _{1,30} =0.5)	(F _{3,346} =35.5)	(F _{3,328} =1.6)
No. of lying bouts (per 24h)	NF	15	18.54 \pm 0.73 ^a	12.02 \pm 0.62 ^{bc}	11.26 \pm 0.57 ^c	11.25 \pm 0.58 ^c	0.11	<0.001	<0.001
	GR	14	17.35 \pm 0.72 ^a	14.69 \pm 0.78 ^b	14.07 \pm 0.78 ^{bc}	11.57 \pm 0.86 ^c	(F _{1,31} =2.7)	(F _{3,285} =91.9)	(F _{3,276} =12.6)
FGCM ^{2,3} (ng/g)	NF	18	234.76 \pm 38.72 ^{ac}	255.02 \pm 28.07 ^a	146.50 \pm 17.05 ^c	151.06 \pm 17.29 ^{bc}	0.11	0.006	0.045
	GR	17	276.62 \pm 48.22 ^{ab}	221.57 \pm 27.03 ^{ac}	214.47 \pm 28.69 ^{ac}	211.47 \pm 33.99 ^{ac}	(F _{1,25} =2.7)	(F _{3,80} =4.4)	(F _{3,72} =2.8)

Superscript letters (a-e) indicate statistically significant differences at $p \leq 0.05$ (all post-hoc pairwise comparisons between treatment x week combinations for a variable considered to allow also for individual relevant comparisons like e.g. the week of milk loss in both treatments). T= treatment, W= sampling time point (week), T x W = interaction of treatment x week. DF= degrees of freedom. Baseline= comprised for ADG and lying times the last 3 weeks before weaning started, for FGCM and all other behaviors only 1 week before the weaning start. ADG= average daily gains, TMR= total mixed ration, CPA= counts of peak acceleration measurements, FGCM= fecal cortisol metabolites. ¹LSM and S.E. back-transformed from square-root-transformation, ²LSM and S.E. back-transformed from log-transformation. ³Note that for FGCM levels only weeks with identical feeding base are reasonably comparable between treatments and within a treatment.

3.9 Lying times and number of lying bouts per day

Daily lying times of calves tended to differ after correction for multiple testing between treatments depending on the phase (interaction treatment x phase, table 3). Numerically though, there was no difference in the percentage of total lying time per day between treatments over the whole three-week weaning and separation phase (table 3) and the extended model revealed no difference between treatments in any weekly comparison either (table 4). However, there was a main effect of the treatment phase on lying times (table 3), since lying times of both treatments decreased during the three-week weaning and separation phase in comparison to the individual three-week baseline before treatment start (table 3).

The number of lying bouts per day differed between treatments depending on phase (interaction treatment x phase, table 3). Compared to baseline values, the number of lying bouts declined in both treatments, but stronger in NF calves ($\approx 40\%$ fewer bouts) than in GR calves ($\approx 20\%$ fewer bouts, table 3). Thus, GR calves showed more lying bouts than NF calves over the whole three-week weaning and separation phase ($t_{38}=3.0$, $p=0.02$, table 3). The extended model revealed no difference in the number of lying bouts between treatments in any weekly comparison (table 4).

3.10 Suckling attempts and nasal injuries of nose flap calves

It is noteworthy reporting that calves in the NF treatment which wore the nose flap over a duration of 2 weeks all showed some tissue alterations caused by the nose flaps. Out of 18 calves in the NF treatment, 12 calves had pressure marks at the nasal septum from the nose flap, the other 6 had injuries: 2 calves showed tissue bleeding, 3 calves had a purulent inflammation and in one calf the nose flap caused bleeding and a purulent inflammation at the tissue of the nasal septum at the contact site of the nose flap.

Suckling attempts of NF calves decreased considerably over the two-week period while wearing a nose flap (table 5). The percentage of scans in which suckling attempts were observed showed no significant difference from zero suckling attempts anymore at the second treatment day and all days of the second treatment week (table 5).

Table 5) Number of unsuccessful suckling attempts with the nose flap of calves weaned via two-step weaning.¹

Variable	Treatment day								p-value (F-value _{Num} DF,Den DF)
	0	1	2	3	7	8	9	10	D
Suckling attempts (% of scans) ²	9.31 $\pm 3.34^a$	6.73 $\pm 2.42^a$	0.77 ± 0.24	2.84 $\pm 0.89^a$	1.39 ± 0.48	0.12 ± 0.04	0.34 ± 0.09	0.16 ± 0.44	<0.001 (F _{8,81} =9.2)

¹Data are presented as percentage of scans (LSM \pm S.E.) in which suckling attempts occurred during direct observation with scan sampling every 3 minutes. Treatment day 0 represents the day of insertion of the nose flap.

²LSM and S.E. back-transformed from square-root-transformation.

^aIndicates a statistically significant difference from zero behavioral observations with suckling attempts at $p \leq 0.05$. D= sampling time point (Treatment day). DF= degrees of freedom.

3.11 Hematological responses

Neutrophil and lymphocyte percentage differed between treatments depending on phase (interaction treatment x phase) and tended to do so after correction for multiple testing for the N:L ratio (table 3). NF calves showed a tendency for an increased neutrophil percentage ($t_{87}=-2.4$, $p=0.09$) and an accompanying decreased lymphocyte percentage in blood ($t_{84}=2.6$, $p=0.048$) during the weaning and separation phase compared to the NF baseline phase (table 3). This was also reflected in a numerically increased N:L ratio of NF calves ($t_{88}=-1.9$, $p=0.25$, table 3). In contrast, no significant changes in neutrophil percentage, lymphocyte percentage or N:L ratio from baseline to the weaning and separation phase were observed for GR calves (table 3).

Extended models with individual treatment days showed a numerical increase in neutrophil percentage and a numerical decrease in lymphocyte percentage in NF calves from day 0 to day 3, which was the first sampling point after insertion of the nose flap. This resulted in an elevated N:L ratio of NF calves on day 3 compared to the NF baseline on day 0 ($t_{64}=-3.9$, $p=0.02$, table 6). For none of the other treatment days significant changes compared to baseline became evident in any of the two treatments (table 6).

Table 6) Effect of the weaning and separation procedure on total neutrophil and lymphocyte percentage and the neutrophil: lymphocyte (N:L) ratio of calves weaned with two-step weaning using a nose flap (NF) or gradual reduction of contact time to the dam (GR). Values are expressed as least squares means \pm standard error.

Variable			Treatment day							p-values (F-value _{Num DF, Den DF})		
	T	N	0	3	7	10	14	17	21	T	D	T x D
N (%)	NF	18	21.37 ± 2.48	28.54 ± 2.22	23.36 ± 2.26	24.57 ± 2.06	24.62 ± 2.71	25.11 ± 2.17	21.81 ± 2.54	0.58 (F _{1,26} =0.3)	0.49 (F _{6,76} =0.9)	0.17 (F _{6,69} =1.6)
	GR	17	27.52 ± 2.66	24.75 ± 2.45	25.67 ± 2.56	24.81 ± 2.42	28.52 ± 3.36	24.31 ± 3.48	21.94 ± 3.21			
L (%)	NF	18	70.52 ± 2.57	62.15 ± 2.30	69.58 ± 2.35	66.90 ± 2.13	65.93 ± 2.86	65.47 ± 2.24	66.55 ± 2.62	0.67 (F _{1,26} =0.2)	0.36 (F _{6,72} =1.1)	0.10 (F _{6,65} =1.9)
	GR	17	63.34 ± 2.75	65.31 ± 2.53	65.37 ± 2.65	66.06 ± 2.51	63.66 ± 3.49	66.59 ± 3.61	70.28 ± 3.34			
N:L ratio	NF	18	0.30 ^a ± 0.05	0.48 ^b ± 0.05	0.34 ± 0.04	0.37 ± 0.04	0.36 ± 0.05	0.36 ± 0.04	0.34 ± 0.05	0.56 (F _{1,14} =0.4)	0.19 (F _{6,59} =1.5)	0.05 (F _{6,63} =2.2)
	GR	17	0.46 ± 0.06	0.40 ± 0.05	0.36 ± 0.05	0.40 ± 0.05	0.38 ± 0.07	0.40 ± 0.07	0.34 ± 0.07			

Means with different superscript letters (a-b) differ between sampling days within a row with $p \leq 0.05$. No further comparisons were significant. T= treatment, D= sampling time point (Treatment day), T x D = interaction of treatment x treatment day. DF= Degrees of freedom.

3.12 Fecal cortisol metabolites (FGCMs)

There were no significant differences in FGCM concentrations (ng/g) between the two treatments during the baseline phase or the third week with fence-line contact (table 4). Within treatments, NF calves showed the highest increase in FGCM concentrations in reaction to insertion of the nose flap in week 1 compared to the other two treatment weeks (week 2: $t_{83}=4.3$, $p=0.001$, week 3: $t_{39}=4.0$, $p=0.004$, table 4). For GR calves, treatment weeks are not reasonably comparable due to a different nutritional basis (Vogt et al., 2023).

4. Discussion

The present study compared weaning and separation of calves through two-step weaning with nose flaps (NF) to a gradual reduction of contact time between cow and calf (GR). Results showed that the weaning and separation procedure induced a considerable distress response in calves regardless of the weaning method, but revealed differences between the two methods in behavioral as well as physiological indicators (table 3). Considering the whole three-week weaning and separation phase, NF calves showed a stronger decrease in the number of lying bouts, locomotor play and ADG, as well as a higher increase in TMR feeding time compared to baseline than GR calves, whereas GR calves vocalized more often and showed more searching behavior than NF calves. Also, a tendency for an increased neutrophil percentage and a significantly decreased lymphocyte percentage compared to baseline could be found for NF, but not for GR calves during the weaning and separation phase. Overall, in line with our hypotheses, results point towards a favorable effect of the GR method on reduction of weaning and separation distress in calves, which will be discussed in the following sections.

4.1 Physiological adaption to the new diet

Calves of both treatments showed only a modest roughage intake during the baseline phase, when milk was still available ad libitum, and hence reacted with a decrease in ADG once the weaning process started. This is in line with results of beef suckler calves weaned with nose flaps (Haley et al., 2005; Boland et al., 2008; Enriquez et al., 2010) as well as of artificially reared dairy calves in which the increased starter intake could not compensate for the reduced milk intake during gradual weaning (Sweeney et al., 2010). The lower ADG of the NF calves compared to our GR treatment over the whole weaning and separation phase was expected, since GR calves were still able to suckle milk from their dams for 2 weeks of the weaning phase, whereas the NF calves abruptly lost the milk and had to meet their nutritional requirements solely from solid feed. Accordingly, the TMR feeding time of NF calves over the whole weaning and separation phase was about twice as high as for GR calves. Additionally, these high TMR feeding times could result from an altered, potentially more cautious, feeding behavior of calves with the nose flap, leading to a lowered feeding rate at the feeding rack. Thus, even if the feeding duration differed between our treatments, the actual feed intake might have been the same, which is also supported by the similar rumination times of calves in both treatments.

Importantly however, NF calves lacked an increase in ADGs during the third week with fence-line contact, despite the highest TMR feeding times at that point and without impairment through the nose flap. On the contrary, GR calves increased their TMR feeding and weight gains during the weaning and separation process and showed the highest ADGs in the third week, which did not differ from their baseline anymore. This might be explained by an improved adaption of the gastrointestinal tract (GIT) to the changed diet in the two treatments, including a potentially smoother transition of the gut microbiome from pre- to post-weaning state (Li et al., 2012; Rey et al., 2014; Meale et al., 2016, 2017) with the gradual weaning method, as compared to the abrupt change in NF calves. In this regard Steele et al. (2017) discussed that increased starter intake in gradually weaned calves can potentially accumulate in the rumen as substrate for microbial fermentation, creating a greater nutrient pool to support growth. Accordingly, studies in artificially fed dairy calves showed that post-weaning weight

gains of gradually weaned calves are usually higher than those of abruptly weaned calves and prevent the typical depression in post-weaning weight gains (e.g. Khan et al., 2007; Sweeney et al., 2010; Omid-Mirzaei et al., 2015). In contrast to this, the abrupt loss of milk through the nose flap has likely not allowed enough time for a sufficient transition of the gut microbiome for an effective maintenance of body weight from solid feed after abrupt cessation of milk feeding. This is in line with results by Niekerk et al. (2021) who reported a delay in rumen structural development, inadequate short chain fatty acid absorption and reduced ruminal pH (acidosis) for up to 2 weeks post-weaning in calves that were weaned in one step from a high milk replacer diet. In addition, it has been shown that hay consumption from hay racks is restricted by the nose flap (Barth et al., 2015) which might have additionally reduced ruminal pH and delayed adaption of the GIT in our NF calves, since hay intake during weaning stimulates rumen development with a positive effect on ruminal pH (Khan et al., 2011; Castells et al., 2013; Pazoki et al., 2017), ruminal bacterial diversity and abundance (Kim et al., 2016) and thus affects the establishment of its fermentative functions. An impeded adaption of the GIT in our NF calves is further supported by results of our hematological variables. There was a tendency for an increased neutrophil percentage and a significantly decreased lymphocyte percentage during the whole weaning and separation phase in NF calves, whereas we found no significant change in relation to baseline values in GR calves at any sampling day. Such an increased neutrophil percentage and decreased lymphocyte percentage, resulting in an increased N:L ratio, was equally reported for beef calves in response to abrupt weaning (e.g. Hickey et al., 2003; de Souza Teixeira et al., 2021), while no neutrophilia and lymphopenia was found in gradually weaned dairy calves fed at an automatic milk feeder (AMF, Johnston et al., 2016), which is in line with our findings. This increase in inflammatory blood markers can result from different weaning stressors leading to elevated glucocorticoid levels in the bloodstream (discussed in Burton et al., 2005; de Souza Teixeira et al., 2021), but might in this regard be especially likely to reflect a temporary hindgut acidosis of the calves during the weaning process as hypothesized by Steele et al. (2016). Thus, our hematological markers may mainly reflect an inflammation of the GIT in consequence of the abrupt change in diet in NF calves, or vice versa the better nutritional adaption through the GR treatment. This is further supported by the reduced lying bout frequency and play behavior of NF calves compared to our GR treatment, which might also be related to the different energy levels of the calves in consequence of a different speed of adaption of the GIT. Generally, calves of both treatments showed reduced lying times compared to baseline values during the weaning and separation process, which is in line with literature, as increased activity levels and reduced lying times have been frequently reported for beef (Budzynska and Weary, 2008; Hötzel et al., 2010) and dairy calves (Jasper et al., 2008; Eckert et al., 2015) in response to weaning. In our study, this was likely caused by conversely increased standing times at the feeding rack, as well as increased time spent with searching behavior in the GR treatment. The reduced number of lying bouts in NF calves compared to our GR treatment was, however, contrary to expectations as we predicted more but shorter lying bouts in hungrier calves due to enhanced restlessness. Nonetheless, our results are in line with a study by Black et al. (2017), that reported a reduced lying bout frequency in dairy calves following abrupt weaning. Potentially, this could be a consequence of the energy deficit of calves during weaning, as increased bout duration reduces the number of necessary raisings and conserves energy. This is partly supported by the fact that dairy cows show less but longer lying bouts during cold conditions compared to hot or

thermoneutral climate (Lovarelli et al., 2020). Also, the reduced amount of locomotor play of NF calves compared to GR calves during the weaning and separation phase, supports this assumption as past research has repeatedly shown that the amount of locomotor play in calves decreases with a low energy intake (Krachun et al., 2010; Duve et al., 2012; Rushen et al., 2016). The high levels of locomotor play of GR calves in the first two weeks of the weaning process while still being able to suckle milk are in line with this.

Lastly, we found the highest increase in FGCM concentrations within the NF treatment in the first week compared to the other weeks of the weaning and separation process, which is in line with hematological responses and equally points towards a heightened distress response in NF calves directly after insertion of the nose flap. However, it has to be kept in mind here that the composition of the gut microbiome was probably profoundly different in the first week of wearing the nose flap compared to afterwards, due to the progressive transition towards a solid diet with time. As we have shown in a previous study that FGCM concentrations in the same calves will be higher when the calves are fed a primarily milk-based diet in comparison to a diet based on solids (Vogt et al., 2023), the high FGCM concentrations in NF calves in the first week without milk are especially noteworthy. Nevertheless, the change in diet brings nuisance to the FGCM results and complicates comparability not only between weaning methods but also between weaning stages. Therefore, it is concluded that FGCM concentrations are of limited use as a marker for weaning distress, which should be considered in the design of future studies.

4.2 Lack of vocalizations seems not equivalent to absence of distress

Contrary to our hypothesis and also contrary to the aforementioned results, we found significantly more vocalizations and searching behavior in GR calves compared to the NF treatment over the whole weaning and separation period. For the NF calves this is in line with literature as a low frequency of vocalizations has been repeatedly reported in studies on beef (Haley et al., 2005; Enriquez et al., 2010) and dairy calves (Loberg et al., 2008) weaned with a nose flap. However, in contrast to the generally accepted view, we argue that this low frequency of vocalizations does not indicate that the NF calves experienced less distress during the weaning and separation procedure, as our remaining indicators do not support this assumption, but rather reflects the different situation regarding the necessity of vocal communication between cow and calf. In general, contact calls of cow-calf pairs seem to be linked to the desire to reunite as well as the need to obtain milk or have milk removed from the udder, as calls are often followed by reunion and nursing events (de la Torre et al., 2015) and increasing time spans between nursing events increase the probability that one or both members of a cow-calf pair vocalizes (Watts, 2001). Also, calf vocalizations are likely to be linked to hunger, as bottle fed calves call less frequently and at a lower fundamental frequency when fed higher amounts of milk (Thomas et al., 2001). This makes sense in the view of the honest signaling theory, as the energy cost and risk of being caught by predators during vocalizing is high, and thus the frequency of calling must be balanced against the urgency of obtaining resources from the dam (discussed in Weary and Fraser, 1995; Enriquez et al., 2011). Interestingly however, De Paula Vieira et al. (2008) reported a lack of vocalizations in dairy calves fed at an AMF whose nutritional needs were verifiably not met. It might therefore be reasonable to assume that calves' vocalizations are not simply an expression of hunger, but more an attempt to regain contact to

the dam or communicating with a caretaker that has been previously associated with milk provision (De Paula Vieira et al., 2008). Accordingly, there is less need for vocalizations by the calf when direct contact to the cow is already re-established, which was the case for our NF treatment. Similarly, searching behavior in this study covered only standing next to or walking up and down the pen barrier or standing with the head through the selection gate, for which NF calves had a reduced necessity due to access to the cow herd for most part of the treatment. In line with this, beef calves weaned with a nose flap showed less fence-line pacing compared to abruptly or fence-line weaned calves (Enriquez et al., 2010), which might equally be explained by their possibility of direct contact to the dam. Altogether it might therefore be stated, that calves which show searching behavior with or without vocalizations are often likely to be hungry and/or wish to reunite with the dam for other reasons, but that vice versa it seems not reasonable to assume that calves which are not showing these behaviors are not stressed and that their nutritional needs are satisfied.

4.3 Frustration of behavioral needs during weaning

Although the higher frequency of vocalizations and proportion of searching behavior in GR calves compared to NF calves over the whole weaning and separation period seem to reflect mainly the differing necessity for vocal communication with the dam over a distance, it is noteworthy that NF calves showed almost no searching behavior and vocalizations in response to fence-line separation from the dam in the third week, whereas GR calves reacted to this step with the highest increase in these behavioral responses, despite the presumably better nutritional adaption. This signals a high motivation of GR calves to reunite with the dam, which is supported by the comparatively high proportion of GR calves that gained illegitimate access to the cow area over the whole weaning period, but especially during the third week with fence-line contact. The main explanation here might be that suckling of the dam was still possible for GR calves until the fence-line separation commenced, which has been shown to lead to oxytocin release in calves (e.g. Lupoli et al., 2001; Uvnäs-Moberg et al., 2001) and does reinforce the strength of the cow-calf bond. Particularly, dairy calves which had only partial contact to their dam, without suckling, up from birth showed little distress in response to debonding (Wenker et al., 2022), and also cows showed a greater motivation for calf contact in the days following parturition when suckled compared to non-suckled controls (Wenker et al., 2020). Thus, the prevention from suckling through the nose flap has probably loosened the cow-calf bond in the NF treatment, whereas the continued possibility of GR calves to suckle has presumably maintained the bond to the dam and consequently led to frustration when access to their dam was denied in the third week. This is in line with results in beef calves, in which fence-line separation has equally been shown to cause increased frustration levels (Enriquez et al., 2010). Given the comparably high weight gains of GR calves in the third week, the high increase in vocalizations and searching behavior is more likely a reaction to the loss of access to the dam per se, than to the loss of milk, which is supported by the fact that abrupt maternal separation leads to a pessimistic judgement bias in dairy calves, even when the calves are nutritionally independent (Daros et al., 2014). With regard to the NF calves, it can be assumed that the inability to obtain milk from the dam during the weeks with the nose flap has equally caused a state of frustration in the NF calves, due to the fact that they were not able to suckle milk from the dam despite the perceived potential for access to the udder (Enriquez et al., 2010, 2011). In

fact, exposure of fasted animals to situations in which they previously learned to expect food, but could unexpectedly not obtain it, has been used as an experimental technique to create a state of frustration in study animals in the past (Lewis, 1999; Mason and Burn, 2011). This assumption is supported by the observation that the NF calves in our study as well as beef calves weaned with a nose flap (Enriquez et al., 2010) show a high number of unsuccessful suckling attempts for 2-4 days after the nose flap is inserted. Such repeated attempts to consume an inaccessible food have been described as a typical behavioral reaction in situations where strong motivations are frustrated (Mason and Burn, 2011). Furthermore, the severe drop in locomotor play behavior in the two weeks while wearing the nose flap, which has been found in our study and also in beef calves (Enriquez et al., 2010), equally points towards a possible negative affective state in these calves, but is likely confounded with reduced energy intake as discussed above. Generally, play behavior is discussed as an indicator of positive welfare in animals (Boissy et al., 2007; Held and Spinka, 2011) and a reduction of play behavior in calves indicates i.a. impaired welfare such as pain (Mintline et al., 2013) or social deprivation (Jensen et al., 2015). As the nose flap likely caused discomfort or potentially even pain in calves and has potentially impeded several behaviors such as self- or allo-grooming as well as hay intake and milk consumption, it is probable that wearing the nose flap created a state of frustration in NF calves, which was reflected in the reduced play levels. Vice versa, removal of the nose flap would have caused an improved affective state due to a relief-like affect, which could partly explain the enhanced play levels of NF calves in the third week despite the presumably low energy level as reflected in ADG. In line with this, studies on other ruminants showed that the release from different stressors (restraint, isolation, shearing) creates a positive judgement bias in animals (Doyle et al., 2010; Sanger et al., 2011). Differences in space allowance also strongly impact play levels in calves (Waiblinger et al., 2020), but provide no explanation for the changes in locomotor play within the NF treatment or the differences to the GR calves, since calves with the longest access to the additional space in the cow section (NF calves in week 1 and 2) played the least. Lastly, the reduced lying bout frequency of NF calves compared to their baseline might additionally hint towards a negative affective state in NF calves, as reduced activity levels have been discussed to be related to a depression-like state in non-human animals as well (Fureix and Meagher, 2015). Hence, it seems possible that the low levels of vocalizations and searching behavior of NF calves in response to fence-line separation from the dam could not only point towards a weakened cow-calf bond, but might additionally stem from the negative affect of frustration which further persisted in the third week and decreased their motivation to enter the cow section. Importantly however, NF calves showed no reduction in brush use, which has been reported in dairy cows in reaction to stressors like acute metritis, heat stress, regrouping or cow and calf separation (Mandel et al., 2013, 2017; Lecorps et al., 2020, 2021) and is discussed to indicate an anhedonia-like affective state in cows (Lecorps et al., 2020, 2021). The lack of reduction in brush use in response to weaning with the nose flap in our study is contrary to our other indicators and suggests that brush use might not be a similarly reliable indicator for stress in calves like it is for older cattle, at least not for weaning distress. One possible alternative could be that brush use during weaning in dam-reared calves could act as a partial substitution for the loss of maternal grooming like suggested for artificially reared calves by Zobel et al. (2017). Future studies are encouraged here to enhance our understanding of brush use in dairy calves during stressful events.

4.4 General evaluation of the two weaning methods

On the whole, results of our study suggest that both types of weaning and separation methods caused a distress response in the calves. However, the abrupt change in diet during two-step weaning with the nose flap apparently compromised adaptation of the GIT and led to a greater energy deficit in NF calves compared to the gradual weaning method. The fact that the high TMR feeding times of NF calves were not reflected in respective high weight gains, gives reason for concern as it indicates major impairment of feed intake by the nose flap. Next to the energy deficit, the severe reduction in lying bouts and locomotor play behavior in addition to the high number of suckling attempts with the nose flap, also hints towards a negative affective state in these calves. As the nose flap also caused pressure marks and partly even injuries in the nasal septum of calves, two-step weaning with nose flaps cannot be classified as a high-welfare weaning method. This is in line with recent results from Wenker et al. (2022) who compared different separation methods for cow-calf contact systems with varying types of contact and concluded that for dairy calves with full contact to the dam, the nose flap was the less effective method to reduce weaning distress compared with fence-line weaning. That said, it should be considered that classical fence-line weaning also leads to an abrupt termination of milk feeding and probably causes similar problems within the GIT, which merits further research.

Notwithstanding, our study demonstrated that the gradual weaning and separation method still provoked distress and frustration in the GR calves, especially in the third week with fence-line separation from the cow. Thus, even if the GR treatment seemed favorable compared to two-step weaning with a nose-flap, it requires further refinement from the protocol used in our study. In this regard, future studies should investigate the effect of enabling a closer contact between cow and calf during the last step of the gradual weaning and separation procedure, since separation by the selection gate used for the fence-line phase in our study provided the pairs with only very limited visual and tactile contact. Furthermore, a gradual reduction treatment with smaller steps will potentially be more successful, as suggested by a study in lambs that showed little stress response during definite weaning after a gradual increase in separation time from the dam from 2-23 hours per day over a period of 2 months (Orgeur et al., 1998). Nevertheless, many small reductions in cow-calf contact time might be problematic with regard to on-farm practicability as this may be associated with a considerable workload as long as there are no technical solutions such as automatic selection gates that are both affordable for farmers and reliably avoid unauthorized access of calves.

An additional factor to consider with the gradual weaning and separation method is that the results of the milk yields of the dams during evening milking (Barth et al., 2021) suggest that the calves in our GR treatment drank comparable amounts of milk during the day-time in the morning contact phase (week 2) as during the day-time of the half day contact (week 1) and baseline phase. This implies that the calves partly compensated for the reduced contact time by drinking higher amounts of milk during the shortened access times during the day, but not enough to compensate the lost suckling time during night. Consequently, the reduction in milk consumption of the GR calves only truly happened by prevention of suckling during the night and was therefore not as gradual as planned, but rather stepwise from baseline to week 1. This is in line with results from Roadknight et al. (2022) who compared a full-time to a half day

cow-calf contact system and reported that half day contact calves suckled twice as long around milking as calves with full-time access to their dams and that weight gains were similar between treatments. In general, it was a prominent result of our study that the highest changes in behavioral and physiological indicators of weaning distress of the calves occurred constantly at the time point of cessation of suckling and accompanying milk loss in both treatments (NF week 1 and GR week 3, table 4). This was less clear for the GR calves as the separation from the dam happened at the same week and thus effects of milk loss and loss of the dam cannot be examined separately for this treatment. However, for the NF calves our indicators point towards a higher distress response after the removal of milk compared to separation from the dam, and indicate that the former needs to be alleviated in order to achieve a comparably mild weaning and separation procedure. This is supported by studies on dam-reared dairy calves provided with additional access to an AMF, which were shown to spend more time playing (Rushen et al., 2016; Johnsen et al., 2018), but less time close to the separation barrier and to produce fewer vocalizations during or after separation from the dam than calves with no additional access to an AMF (Johnsen et al., 2018). Equally, Wenker et al. (2022) found that dairy calves which had partial contact to their dams without suckling, but were bucket fed, showed minimal signs of distress during the debonding process from the dam compared to calves with full cow-calf contact. Altogether, these results from the literature in conjunction with results from our NF calves underline that calves' weaning and separation distress is largely affected by the nutritional dependency from the dam. Therefore, a gradual reduction of contact time as weaning and separation method will probably be more successful in alleviation of weaning and separation distress, if it can be ensured that calves really reduce suckling times and do not compensate for the reduced contact time by spending more time suckling and drinking larger amounts of milk in the remaining time.

4.5 Limitations

A limitation of our study was that no data collection took place after actual permanent total separation from the cow when calves were moved to a different barn, which is a main stressor during the weaning and separation process. However, data collection after movement to the youngstock barn would have been heavily influenced by introduction into a new herd, loss of known peers, new surroundings with different germs and different feed. These confounders would have left results not clearly attributable to the particular treatment and therefore, we limited our data collection to direct comparison of calves' distress responses in the third week in which cows and calves were kept separated in adjacent pens with 'fence-line' contact through pen boundaries. This way, external factors were kept stable and we could ensure that calves' behavioral and physiological distress responses were caused by loss of direct contact to the dam, rather than e.g. an unfamiliar, dominant conspecific in the youngstock herd. Also, this way all direct behavioral observations were conducted by exactly the same observer. Future studies are encouraged, which are able to control such external stressors after permanent total separation from the dam.

5. Conclusion

Results of our study showed that both weaning and separation methods led to behavioral and physiological distress responses and likely caused frustration in calves. However, a gradual reduction of contact time to the dam was favorable compared to weaning with a nose-flap in most indicators, foremost allowing a better adaptation to the dietary change. This included higher weight gains in gradually weaned calves, even in the week after complete milk loss, and a lower decrease in lying bouts and locomotor play levels compared to baseline in gradually weaned calves, indicative of higher energy levels and a potentially less compromised affective state. Moreover, an increase in inflammatory blood markers was present in reaction to nose-flap weaning but not to gradual weaning. Therefore, it is concluded that a gradual weaning strategy is a promising weaning and separation method for dam-reared dairy calves, but requires further improvement from the protocol used in our study. It is important to note that this recommendation is based on effects on calves and needs to be confirmed regarding effects on the dams, as a weaning and separation method must always consider both partners of a cow-calf pair.

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Conflict of interest

The authors declare to have no conflicts of interest that are relevant to the content of this article.

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Supplementary Material

Table S1) Scheme on the events during the weaning and separation process in the two treatments according to the age of calves at start of the weaning and separation process. Exact age of calves is given in mean \pm S.D. and as minimum to maximum age at weaning start.

Calf age (Week)	Nose flap treatment (NF)		Gradual reduction of contact time treatment (GR)	
	Start at 12 weeks 87.8 \pm 2.0, 83-89 days (n=9)	Start at 14 weeks 100.0 \pm 1.3, 98-102 days (n=9)	Start at 12 weeks 85.0 \pm 2.2, 83-89 days (n=9)	Start at 14 weeks 99.2 \pm 1.9, 97-103 days (n=9)
1 – 11	Full time contact	Full time contact	Full time contact	Full time contact
12	 Full time contact with nose flap	Full time contact	Half day contact (till 3:30pm)	Full time contact
13	Full time contact with nose flap	Full time contact	Morning contact (till 12pm)	Full time contact
14	Fence-line contact	 Full time contact with nose flap	 Fence-line contact	Half day contact (till 3:30pm)
15	Move to youngstock barn	Full time contact with nose flap	Move to youngstock barn	Morning contact (till 12pm)
16		Fence-line contact		 Fence-line contact
17		Move to youngstock barn		Move to youngstock barn


 label = displays the time point of complete milk loss for the calves

Table S2) Correction for multiple testing of the 14 analyzed parameters according to the Benjamini-Hochberg false discovery rate correction (Glickman et al., 2014). Bold printed p-values remained significant for an $\alpha \leq 5\%$ after correction for multiple testing, bold and italics p-values remained significant at $\alpha \leq 10\%$ after correction for multiple testing. TxP = treatment x phase interaction of the main model.

No. Tests	Variable	P-value of the TxP Interaction	Significance level cutoff for $\alpha \leq 5\%$	Significance level cutoff for $\alpha \leq 10\%$
1	TMR feeding time (min per 6h)	<0.001	0.004	0.007
2	No. of lying bouts (per 24h)	<0.001	0.007	0.014
3	Searching behavior (% of scans)	<0.001	0.011	0.021
4	Locomotor play (CPA/ 9h)	0.007	0.014	0.029
5	Vocalizations (frequency per 30min)	0.007	0.018	0.036
6	Lymphocytes (%)	0.012	0.021	0.043
7	Neutrophils (%)	0.016	0.025	0.050
8	ADG (kg/d)	0.028	0.029	0.057
9	N:L ratio	0.045	0.032	0.064
10	FGCM (ng/g)	0.045*	0.036	0.071
11	Lying times (% of 24h)	0.047	0.039	0.079
12	Brush use (min per 6h)	0.25	0.043	0.086
13	Rumination (% of scans)	0.50	0.046	0.093
14	Total play behavior (s per 30min)	0.71	0.050	0.1

TMR= total mixed ration, N:L ratio = neutrophil: lymphocyte ratio, CPA= counts of peak acceleration measurements from the accelerometer, ADG= average daily weight gain

FGCM= fecal (glucocorticoid) cortisol metabolites

* p-value from the extended model, as the main statistical model was not valid for FGCM data.

Table S3) Extended model results for variables not reported in the main paper due to missing significance of the treatment x phase interaction in the main model. Displayed is the effect of weaning and separation of calves over three weeks with either two-step weaning using a nose flap (NF) or gradual reduction of contact time to the dam (GR) on behavioral stress indicators. Values are expressed as least squares means (LSM) \pm standard error (S.E.).

Variable			Week				p-values (F-value _{Num DF, Den DF})		
	T	N	Baseline	Week 1	Week 2	Week 3	T	W	T x W
Rumination (% of scans)	NF	18	9.57 \pm 2.23	14.43 \pm 2.42	14.90 \pm 2.29	19.42 \pm 2.34	0.87	0.005	0.41
	GR	18	11.42 \pm 2.25	14.65 \pm 2.26	17.43 \pm 2.31	16.25 \pm 2.38	(F _{1,26} =0.0)	(F _{3,75} =4.6)	(F _{3,63} =0.97)
Total play behavior (s per 30min)	NF	18	1.57 \pm 0.25 ^{ab}	0.10 \pm 0.02 ^c	0.34 \pm 0.06 ^c	0.11 \pm 0.02 ^c	0.005	<0.001	0.006
	GR	18	2.39 \pm 0.38 ^a	1.84 \pm 0.29 ^{ab}	0.61 \pm 0.10 ^{bc}	0.24 \pm 0.04 ^c	(F _{1,31} =9.3)	(F _{3,73} =19.9)	(F _{3,58} =4.7)
Brush use (min per 6h)	NF	16	3.67 \pm 0.70	3.36 \pm 0.77	3.78 \pm 0.69	4.07 \pm 0.72	0.99	0.96	0.34
	GR	17	4.13 \pm 0.63	3.85 \pm 0.65	3.70 \pm 0.65	3.23 \pm 0.70	(F _{1,9} =0.0)	(F _{3,41} =0.1)	(F _{3,36} =1.2)

Superscript letters (a-c) indicate statistically significant differences at $p < 0.05$ (all pairwise comparisons of treatment x week combinations for a variable considered). T= treatment, W= sampling time point (week), T x W = interaction of treatment x week. DF= degrees of freedom. Baseline= comprised 1 week before the weaning start. % of scans= percent of scans the behavior was recorded during direct observation with scan sampling every 3 minutes.

Table S4) Confidence intervals for the results of the main model for the effect of weaning and separation of calves with either two-step weaning using a nose flap (NF) or gradual reduction of contact time to the dam (GR) on behavioral and physiological stress indicators.

Variable	Phase					p-value (F-value _{Num} DF,Den DF)
	Baseline		Treatment		T x P	
	T	Lower 95% CI	Upper 95% CI	Lower 95% CI		Upper 95% CI
ADG (kg/d)	NF	1.36	1.64	0.12	0.40	0.028 (F _{1,70} =5.1)
	GR	1.45	1.72	0.50	0.87	
TMR feeding time (min per 6h)	NF	5.49	8.44	24.53	34.88	<0.001 (F _{1,58} =26.9)
	GR	5.25	8.07	10.93	15.55	
Rumination (% of scans)	NF	5.48	14.42	12.79	19.84	0.50 (F _{1,72} =0.5)
	GR	7.22	16.22	12.57	19.53	
Vocalizations ¹ (frequency per 30min)	NF	0.02	1.65	0.22	1.20	0.007 (F _{1,128} =7.4)
	GR	0.03	0.94	2.23	4.36	
Searching behavior ¹ (% of scans)	NF	0.00	1.32	0.16	1.01	<0.001 (F _{1,114} =12.2)
	GR	0.25	0.46	2.59	4.82	
Total play behavior ¹ (s per 30min)	NF	0.83	2.46	0.04	0.45	0.71 (F _{1,67} =0.14)
	GR	1.63	3.76	0.45	1.26	
Locomotor play (CPA per 9h)	NF	22.68	53.30	8.49	17.46	0.007 (F _{1,38} =8.1)
	GR	21.25	46.32	18.41	36.99	
Brush use (min per 6h)	NF	2.21	4.93	2.67	4.86	0.25 (F _{1,38} =1.4)
	GR	2.96	5.41	2.61	4.62	
Lying times (% of 24h)	NF	65.25	68.13	60.64	63.58	0.047 (F _{1,279} =4.0)
	GR	65.13	68.15	61.87	65.19	
No. of lying bouts (per 24h)	NF	17.04	20.12	10.53	12.55	<0.001 (F _{1,211} =37.4)
	GR	15.82	18.86	12.61	15.29	
Neutrophils (%)	NF	15.02	24.66	21.94	28.17	0.016 (F _{1,89} =5.9)
	GR	22.33	32.66	21.20	28.29	
Lymphocytes (%)	NF	66.76	76.93	62.48	69.04	0.012 (F _{1,85} =6.5)
	GR	58.20	69.08	62.54	69.97	
N:L ratio	NF	0.18	0.42	0.33	0.48	0.045 (F _{1,87} =4.2)
	GR	0.33	0.59	0.31	0.48	

T= treatment, T x P = interaction of treatment x phase, DF= degrees of freedom, CI= confidence interval.

Baseline= last 3 weeks before weaning start for ADG, lying times and lying bouts, 1 week before weaning start for hematological responses and all other behaviors, Treatment phase= the 3-week weaning and separation phase. ADG= average daily gains, TMR= total mixed ration, CPA= counts of peak acceleration measurements, N:L ratio= neutrophil: lymphocyte ratio. ¹CI back-transformed from square-root-transformation.

Table S5) Confidence intervals for the results of the extended model for the effect of weaning and separation of calves over three weeks with either two-step weaning using a nose flap (NF) or gradual reduction of contact time to the dam (GR) on behavioral and physiological stress indicators.

Variable	Week									p-value (F-value _{Num DF, Den DF})
	T	Baseline		Week 1		Week 2		Week 3		
		95% LC	95% UC	95% LC	95% UC	95% LC	95% UC	95% LC	95% UC	
ADG (kg/d)	NF	1.37	1.64	-0.19	0.34	0.15	0.67	-0.01	0.54	0.03
	GR	1.45	1.72	0.30	0.99	0.19	0.83	0.65	1.48	(F _{3,113} =3.2)
TMR feeding time (min per 6h)	NF	4.92	7.45	17.39	26.63	24.98	37.38	30.67	46.13	<0.001
	GR	4.60	6.94	7.38	11.19	10.71	16.28	14.66	22.26	(F _{3,49} =13.8)
Vocalizations ¹ (frequency per 30min)	NF	0.01	0.80	2.20	5.54	0.00	0.56	0.05	0.40	<0.001
	GR	0.02	0.89	0.49	2.23	0.27	1.77	10.84	17.07	(F _{3,68} =45.6)
Searching behavior ¹ (% of scans)	NF	0.01	0.68	0.60	3.11	0.11	0.36	0.22	2.02	<0.001
	GR	0.05	0.50	1.14	3.88	0.14	1.68	11.86	19.40	(F _{3,65} =21.5)
Locomotor play (CPA per 9h)	NF	23.68	53.68	6.43	21.32	6.72	18.38	9.31	26.42	0.004
	GR	20.03	42.70	15.65	41.39	21.00	47.29	2.54	21.66	(F _{3,31} =5.4)
Lying times (% of 24h)	NF	65.26	68.12	60.95	64.41	61.20	64.59	59.19	62.62	0.19
	GR	65.16	68.16	62.26	66.12	62.52	66.45	58.11	62.86	(F _{3,328} =1.6)
No. of lying bouts (per 24h)	NF	17.12	20.10	10.85	13.32	10.17	12.46	10.15	12.47	<0.001
	GR	15.93	18.88	13.21	16.33	12.59	15.72	9.98	13.40	(F _{3,276} =12.6)
FGCM ^{2,3} (ng/g)	NF	169.75	324.67	204.90	317.39	116.34	184.48	120.33	189.66	0.045
	GR	196.38	389.62	174.00	282.13	164.61	279.44	153.92	290.51	(F _{3,72} =2.8)

T= treatment, T x W = interaction of treatment x sampling time point (week). LC= lower confidence interval, UC= upper confidence interval, DF= degrees of freedom. Baseline= comprised for ADG, lying times and lying bouts the last 3 weeks before weaning started, for FGCM and all other behaviors only 1 week before the weaning start. ADG= average daily gains, TMR= total mixed ration, CPA= counts of peak acceleration measurements, FGCM= fecal (glucocorticoid) cortisol metabolites. ¹CI back-transformed from square-root-transformation, ²CI back-transformed from log-transformation. ³Note that for FGCM levels only weeks with identical feeding base are reasonably comparable between treatments and within a treatment.

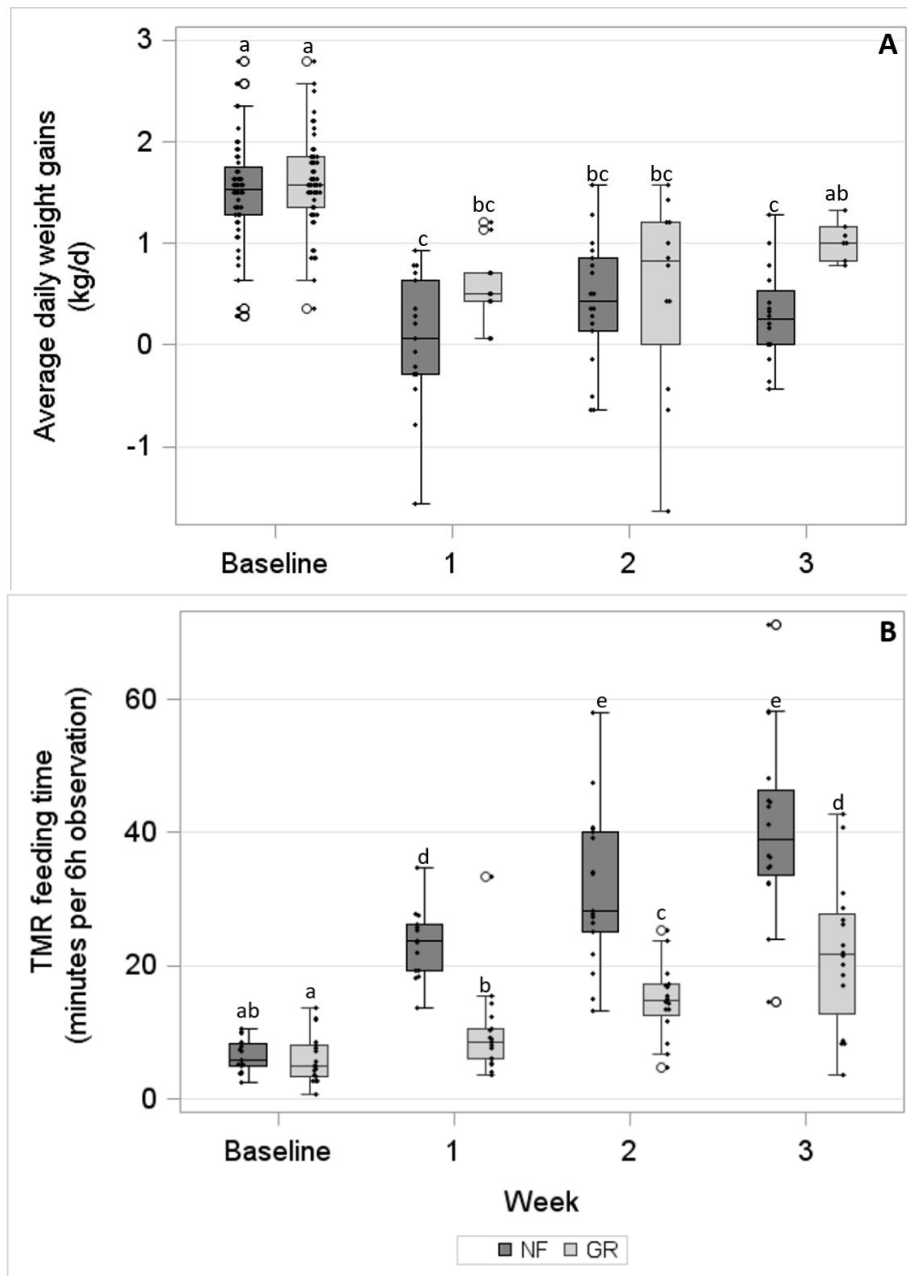


Figure S1) Boxplot graphs of (A) average daily weight gains in kg/d and (B) TMR feeding time in minutes per 6h video observation (previously calculated as means per week) for calves weaned and separated via two-step weaning using a nose flap (NF) or gradual reduction of contact time between cow and calf (GR). 'Baseline' comprised for (A) the last three weeks before weaning started and for (B) observations from one week before start of the weaning and separation procedure. Week 1-3 display the three week weaning and separation phase. Lowercase letters (a-e) indicate statistically significant differences at $p \leq 0.05$. Diamonds display the values of individual calves in each week. Graphs are based on original data and display the number of remaining data points after correction for health problems and illegitimate contact to the dam.

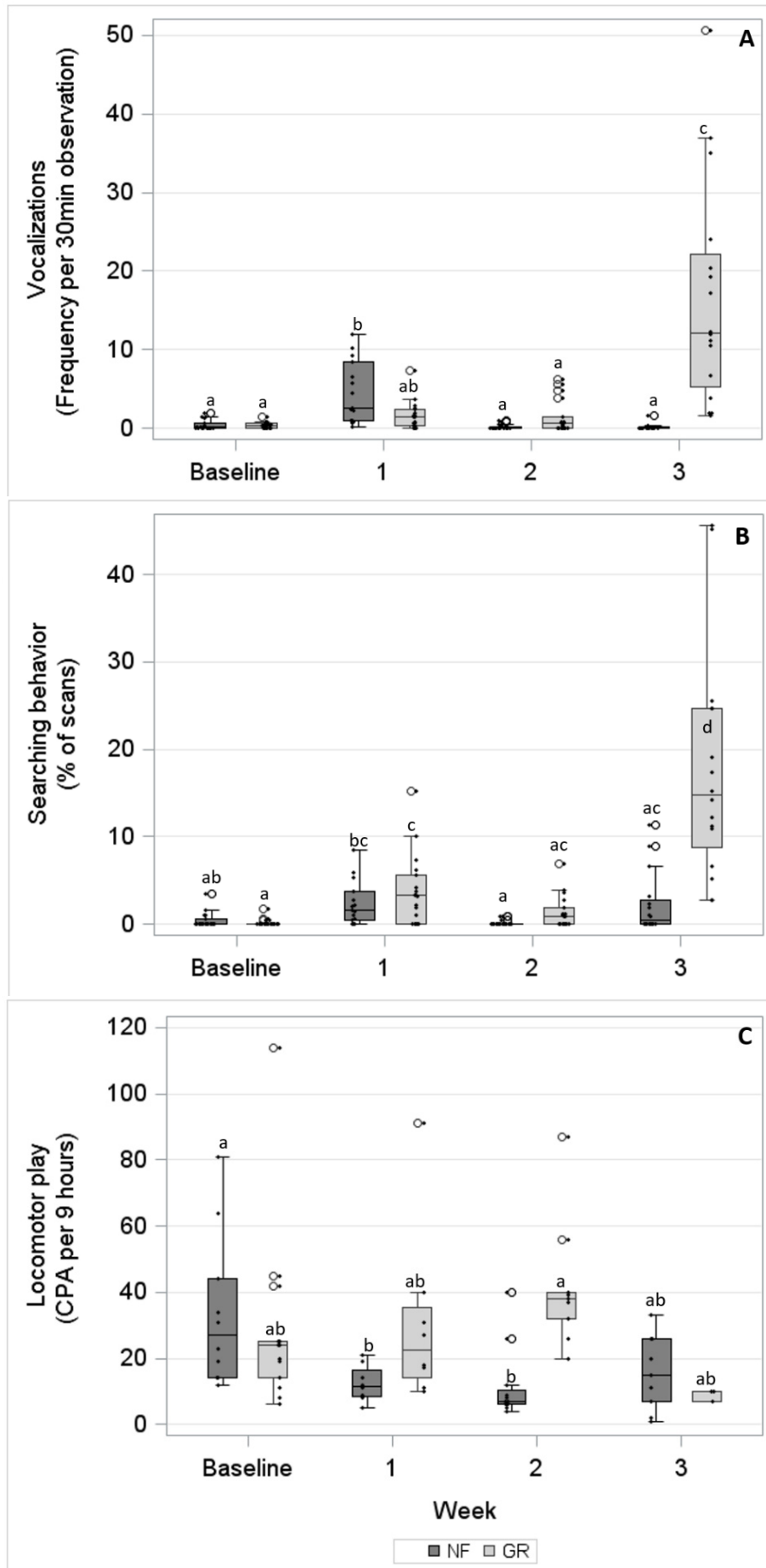


Figure S2) Boxplot graph of (A) frequency of vocalizations per 30min direct observation, (B) searching behavior of the calf (% of scans, observed per 30min direct observation with scan sampling in 3 minute intervals) and (C) locomotor play (counts of peak acceleration measurements (CPA) per 9 hour sampling period from accelerometers) for calves weaned and separated via two-step weaning using a nose flap (NF) or gradual reduction of contact time between cow and calf (GR). For (A) and (B) means per week for each individual calf were calculated before analysis. 'Baseline' comprises the week before the weaning and separation process started, but note that for (C) each sampling week contained only one sampling day each (assessment on day -4, 3, 10 and 17). Lowercase letters (a-d) indicate statistically significant differences at $p \leq 0.05$. Diamonds display the values of individual calves in each week. Graphs are based on original data and display the number of remaining data points after correction for health problems and illegitimate contact to the dam.

Study III

Dairy calves' time spent in the cow herd in a calf-driven cow-calf contact system during two-step separation with a nose flap

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Dairy calves' time spent in the cow herd in a calf-driven cow-calf contact system during two-step separation with a nose flap

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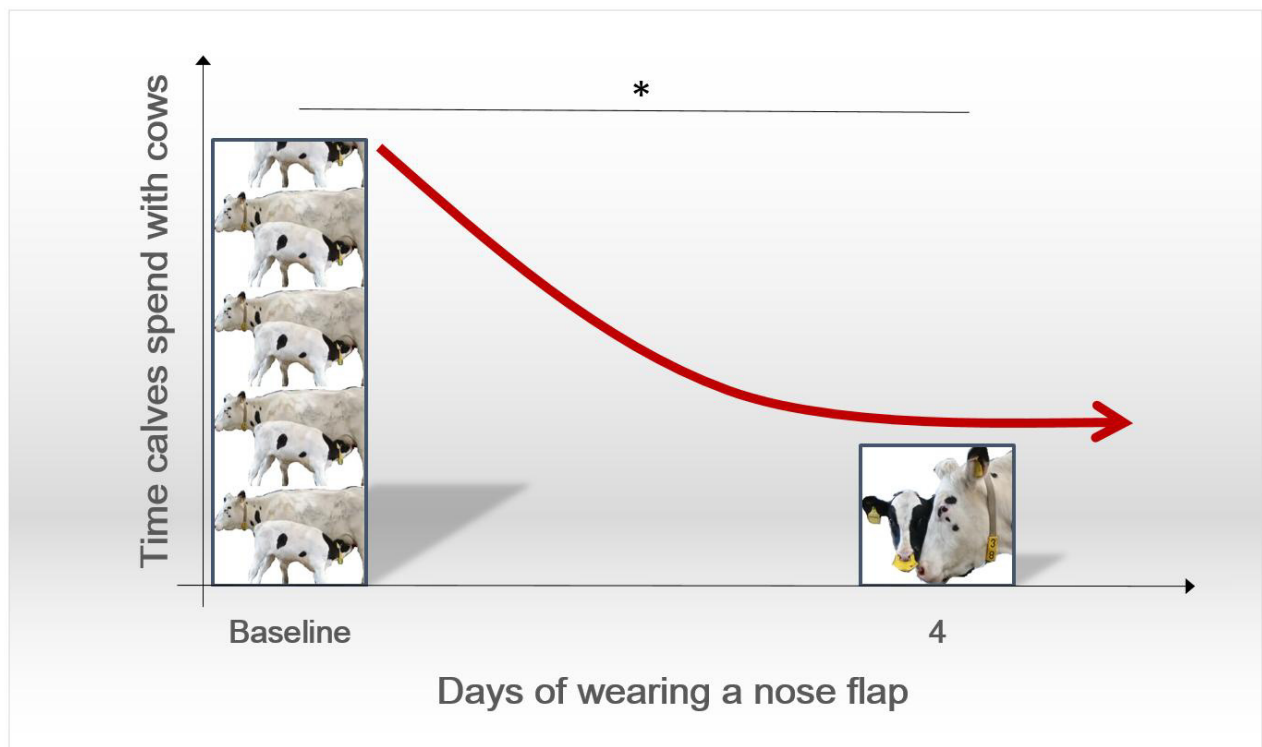
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Graphical Abstract



Abstract

Although originally developed for weaning of beef calves, nose flaps are also used on dairy farms nowadays to wean calves which are reared with contact to their dam or a foster cow. These calves are often weaned younger than beef calves and the time they wear nose flaps is highly variable, since it is unknown for how long younger calves need to wear the nose flap in order to induce an effective weaning. The aim of our study was therefore to track changes in dam-contact initiated by dairy calves after insertion of a nose flap in order to determine the minimum duration a nose flap needs to be worn to effectively reduce suckling motivation of calves. The study was conducted in two sequential experiments using 3-month-old dairy calves that were reared with full-time dam contact. Calves were weaned with a nose flap over either 14 (experiment I, 9 calves in herd A and 9 calves in herd B) or 7 days (experiment II, 11 calves in herd A and 12 calves in herd B) before they were fence-line separated from their dams. The total time a calf spent within the cow herd (TIC) per day was determined from continuous video recordings during 7 days before nose flap insertion (baseline), as well as during the 7 or 14 days in which calves had free access to the cow area while wearing the nose flap. Statistical analysis was conducted using linear mixed effects models. Results from both experiments showed that calves reduced their TIC at the fourth day of wearing the nose flap compared to their individual baseline ($p=0.03$ and $p=0.001$ respectively). In experiment I, calves showed a further numerical reduction in TIC from day 4 to day 5, while in experiment II, calves showed no further reduction in TIC after the 4 days of wearing the nose flap. After these 4 or 5 days respectively, TIC of calves in both experiments stayed at a constant low level of several hours per day. Taken together, these results indicate that in 3-month-old dairy calves it needs at least 4 days until the motivation to spend time with the cow decreases after insertion of a nose flap, while there seems to be no further decrease in motivation when using a nose flap longer than this time. However, results were subject to considerable inter-individual variability already during the baseline week and need replication in a pasture-based setting with less space-restrictions.

Highlights

- Nose flaps are increasingly used for weaning of dam-reared dairy calves
- Times calves entered the cow area (TIC) with a nose flap were monitored
- TICs were reduced after 4 days of nose flap usage compared to baseline
- There was no further reduction in TIC when using the nose flap longer than 4-5 days
- Baseline TIC of calves showed a considerable inter-individual variability

Keywords: nose flap, two-step separation, weaning, dairy calves, cow-calf contact

Introduction

Two-step weaning and separation of cow and calf entails that prior to permanent total separation of the pair, suckling by the calf is prevented with a so-called nose flap (defined by Sirovnik et al., 2020). This way the calves' stress of losing milk as their main nutritional source and losing contact to the dam is dispersed over two time points, which is why this method is regarded as an advantageous alternative to abrupt weaning and separation in beef calves (Haley et al., 2005). With the emergence of dairy cow-calf contact (CCC) systems, two-step weaning with nose flaps is also discussed as an option for dairy calves which were reared with contact to their dam or a foster cow (Barth et al., 2022; Schneider and Ivemeyer, 2021; Sirovnik et al., 2020) and could be especially valuable for weaning of such calves in pasture-based environments where options for alternative weaning strategies are more limited. For example, supplementary milk feeding with an automatic milk feeder during the hours of separation from the dam in combination with fence-line (Johnsen et al., 2018) and gradual (Johnsen et al., 2024) separation methods successfully reduced weaning distress in the past and will be more challenging in a pasture-based system, which equally applies to separation of pairs via a gradual reduction of cow-calf contact time with the assistance of automatic selection gates (Vogt et al., 2024). However, in the dairy CCC systems that practice two-step weaning with nose flaps so far, the calves are typically weaned at a much younger age than beef suckler calves for which the nose flaps were originally developed, and the time span calves wear the nose flaps is highly variable, ranging from a few days up to a month (Barth et al., 2022). In this context it is important to consider that nose flaps have been shown to cause reduced weight gains in beef calves (Boland et al., 2008; Enriquez et al., 2010; Valente et al., 2022), and for parts of the animals, lesions, bleeding and/or inflammation at the calves' nasal septum was evident after usage for 5-7 days (Lambertz et al., 2015; Taylor et al., 2020; Valente et al., 2022). Additionally, the calves' welfare might already be reduced even before lesion development, e.g. through discomfort or pain as well as interference with self-grooming or exploratory behavior, which has to our knowledge not been systematically measured in the aforementioned studies or elsewhere yet.

Regarding the duration of nose flap usage, Alvez et al. (2016) reported no difference in weight gains or behavioral indicators of distress in beef calves that wore nose flaps for 7 or 21 days prior to permanent separation from the dam. Equally, no differences in vocalizations after total separation from the dam were found in beef calves wearing the nose flap for 3 or 14 days beforehand (Haley et al., 2005). Given these results, nose flap usage for short time periods appears favorable and might potentially also be an option to prevent lesion development in the calves, but up until today it is unknown how long it actually takes for the suckling motivation to cease after insertion of a nose flap. Therefore, the aim of our study was to track changes in dam-contact initiated by the calves after insertion of a nose flap in order to determine the minimum time period the nose flap needs to be worn until the calves show reduced motivation to spend time within the cow herd as a proxy for suckling motivation. We hypothesized that calves would reduce their time spent with the cows around 5 days following insertion of the nose flap, given that Price et al. (2003) reported that fence contact to the dam declined significantly over 5 days following fence-line weaning in 7 month old beef calves.

Material and Methods

The study consisted of two sequential experiments, which were both carried out in the experimental barn of the Thünen Institute of Organic Farming in Germany. There, cows and calves are kept in two separate herds (A and B) in identically mirrored parts of the same barn (see Wagner et al., 2012 for a scheme of the barn). Experiment I was conducted from 11/2019 - 03/2020 as part of a larger study by Vogt et al. (2024), which compared calves' stress responses during different weaning and separation methods and a detailed description of the methods can be found there. Experiment II was conducted over the following two years (08/2020 – 04/2021 and 08/2021 – 06/2022) as part of the study by Schneider et al. (2024), which compared the effects of whole-day contact to half-day contact between cow-calf pairs. During the first year of experiment II, pairs from herd A had whole-day contact and pairs from herd B had half-day contact, which was reversed in the second year, so that pairs from herd B had whole-day contact and pairs from herd A had half-day contact. For the present study, we only included pairs with whole-day contact at the weaning stage from the study by Schneider et al. (2024).

The required sample size for the study of Vogt et al. (2024) 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%. For the study by Schneider et al. (2024), a post-hoc power analysis was conducted using R (Version 4.3.1) and setting $\alpha = 5\%$, which returned a high power (≥ 0.98).

Animals and housing

There were 18 calves included in experiment I (9 calves in herd A and 9 calves in herd B) and 23 calves in experiment II (11 calves in herd A and 12 calves in herd B, see table 1). All calves and their dams were of the German Holstein breed. The animals were kept in two separate herds (A and B) in an open sided free-stall barn. Each part of the barn is comprised of a lying area separated into 51 straw-bedded cubicles (128.0 m² plus additional 64.0m² headspace), a feeding area (237.5m²) and a rubber-coated walking area (298.0 m²) for the cows.

Calves were housed in their own separate calf area, which was directly connected to the cow area of the respective herd via automatic selection gates. In the calf area, calves were provided with water, hay, a total mixed ration, concentrate feed, a calf brush (Schurr Gerätebau GmbH, Germany), a walking area (71.9m²) and a straw bedded lying area (12.6m²; and an additional 12.0m² during the whole study period of experiment I and for part of the study period of experiment II in both herds whenever this was required due to a too high number of calves). All calves were reared with full-time contact to their dam up from birth, i.e. calves were free to enter the cow area through the selection gate during the whole day, except for the two daily milking times (starting at about 5 a.m. and 4 p.m.). In the cow area, calves had access to the lying and walking space of the cows, but could not enter the cows' feeding area. Group size of the cow herd in experiment I varied from 43 to 48 cows (herd A) and 45 to 49 cows (herd B) throughout the experiment. In experiment II, group size of the cow herd ranged from 32 to 47 cows (herd A) and 28 to 46 cows (herd B). This was due to inclusion of primiparous cows into the herd and/or selling and culling of older cows.

Experimental treatment

All calves were equipped with a nose flap (Quiet Wean, JDA Livestock Innovations, Canada) for initiation of the weaning process when they were about 3 months old (details see table 1). During experiment I the nose flap remained in the nostrils for 14 days, while in experiment II, the calves were equipped with the nose flap only for 7 days. During the days when the calves wore the nose flap, they continued to have full-time access to the cow area including their mothers via the selection gate. Afterwards, the nose flap was removed and calves had one more week of fence-line contact to their dams through the pen boundaries between the cow and the calf area, before they were fully separated by moving the calves to the Youngstock barn. In both experiments the nose flap was inserted at around 7:30 a.m.

Table 1) Overview of attributes of calves included in the study

	Experiment I Herd A	Experiment I Herd B	Experiment II Herd A	Experiment II Herd B
Term of experiment	11/2019 - 03/2020	11/2019 - 03/2020	08/2020 - 04/2021 [#]	08/2021 - 06/2022 [#]
No. of calves	9 calves	9 calves	11 calves	12 calves
Sex of calves	4 female/5 male	4 female/5 male	4 female/7 male	7 female/5 male
Horn status	all polled	all horned	all polled	all horned
Age of calves at day of nose flap insertion (range, mean \pm S.D.)	83-102 days (92.7 \pm 7.2 days)	89-101 days (95.1 \pm 5.9 days)	90-96 days (93.5 \pm 2.3 days)	91-96 days (93.0 \pm 2.0 days)
Number of days wearing the nose flap	14 days	14 days	7 days	7 days

[#] weaning of the calves happened in the period between 12/2020-03/2021 (herd A) and 11/2021-05/2022 (herd B)

Data collection and analysis

As described above, calves were free to enter the cow area voluntarily during the pre-weaning phase as well as when wearing the nose flap. The selection gate which allowed calves to change sides from the calf area into the cow area was video monitored for 24 hours per day. From these videos, the total duration a calf spent within the cow herd (time in cow herd, TIC) was determined per day using BORIS software (Friard and Gamba, 2016, Version 7.9.22) during one week before the nose flap was inserted (baseline), as well as the one (experiment II) or two weeks (experiment I) while the calves wore the nose flap. A calf was considered to have entered or exited the cow area, if the calf's rear end, including the tail, was behind the top bar (videos were recorded from above) of the selection gate. Video analysis in experiment I was done by two different observers (Cohen's kappa κ =0.99) and in experiment II by six different observers (Fleiss' kappa κ =0.92).

For further analysis the mean TIC of a calf during the baseline week was calculated to define the individual baseline value per calf. This baseline value was then subtracted from the TIC of a given day of wearing the nose flap (hereafter called treatment day) to calculate the difference to baseline for each treatment day. Statistical analysis was done separately for both experiments using linear mixed effects models with repeated measures (Littell et al., 1998) in SAS Version 9.4, with differences to baseline as the dependent variable. As fixed effects, the models included treatment day (days 1-7 or days 1-14 for experiment II or I respectively), sex of the calf (male/female) and parity group of the cow (1st lactation/2nd-3rd lactation/4-7th lactation), as well as the continuous variables age when the nose flap was inserted (in days) and size of the cow herd (heads of animals in the herd at the observation day). The calf ID nested within herd was included as random effect and additionally, the calendar week of the year merged with herd was included as a cross-classified random effect to account for the dynamic group composition in the calf and cow area of each herd throughout the studies (Cafri et al., 2015). The covariance structure between repeated measures was set to autoregressive. In order to test for significant changes in TIC during the treatment days compared to baseline, it was tested whether the differences to baseline differed from zero. In order to test for differences between treatment days, all possible post-hoc pairwise comparisons between treatment days were calculated using a Tukey-Kramer post-hoc test (Rafter et al., 2002). Model requirements (normal distribution and homoscedasticity of residuals) were checked graphically. Confidence intervals for the estimates of TIC for each treatment day are presented in supplementary tables S1 and S2. During the studies, calves' health status was routinely checked daily by the trained barn personnel of the research station according to a specific checklist. Calves which were scored as severely sick, i.e. had fever or needed medical and/or veterinary treatment, were excluded for the respective day as well as one day before and afterwards each, before statistical analysis. Additionally, days with technical failures of the videos were excluded, e.g. when some hours of a day were missing on videos. In total 35 out of 378 data points were excluded in experiment I, and 26 out of 322 data points were excluded in experiment II.

Results

Experiment I

The time that calves spent in the cow area during the baseline week ranged from 2.6 to 16.6 hrs/day (mean±S.D.: 5.4 ±3.9 hrs/day) in herd A and from 0.9 to 8.3 hrs/day (mean±S.D.: 1.9 ±1.8 hrs/day) in herd B (see Fig. 1). Within a calf, differences between the shortest and longest TIC per day during the baseline week ranged from 2.2 to 12.1 hrs/day (5.9±3.1 hrs/day) in herd A and from 1.2 to 6.1 hrs/day (2.4±1.5 hrs/day) in herd B, but exceeded ≥ 6 hours only for 5 of the 18 calves (4 calves from herd A and 1 calf from herd B, see Fig. 1).

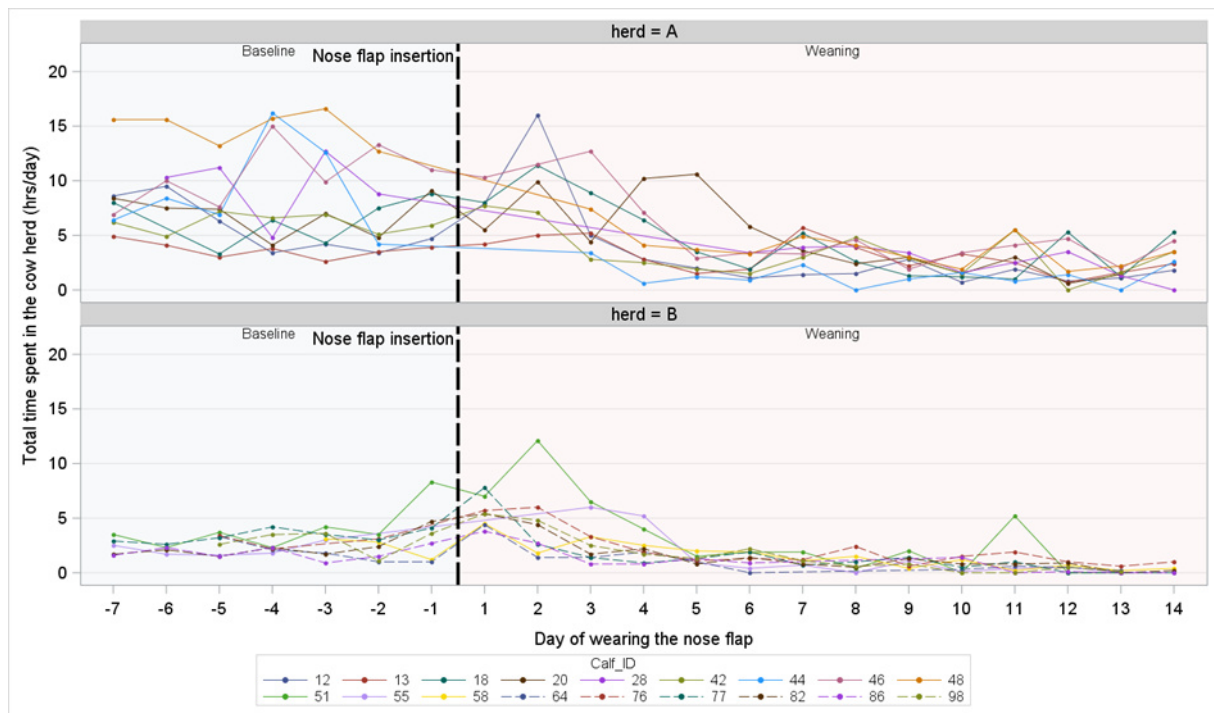


Fig 1) Individual daily time spent within the cow herd (TIC) including the calves' mothers in hours per day of dam-reared dairy calves during one week before and two weeks while wearing a nose flap (experiment I). Data are displayed separately for the two herds in the barn ($n=9$ calves per herd). The nose flap was inserted at day 1 in the morning (dashed lined was offset to leave data points on day 1 visible). Missing data points for single days refer to days at which calves were classified as sick or contact times could not be fully retraced due to technical failure ($n=35$).

The number of days the nose flap was worn influenced the time the calves spent in the cow herd ($F_{13,163}=16.8$, $p<0.0001$). During the first (least square means (LSM) \pm S.E.: $+0.98\pm 0.8$ hrs/day, $t_{24}=1.2$, $p=0.24$) and second day ($+1.71\pm 0.8$ hrs/day compared to the individual baseline, $t_{24}=2.1$, $p=0.04$) of wearing the nose flap, the calves increased their TIC compared to baseline, however, this increase was only significant at the second day (Fig. 2). Up from day 3, TIC of calves decreased and the calves spent significantly less time within the cow herd compared to their individual baseline for the first time at the fourth day of wearing the nose flap (-1.9 ± 0.8 hrs/day, $t_{22}=-2.4$, $p=0.03$, Fig. 2). There was a further reduction in calves' TIC on the fifth day of wearing the nose flap (day 5: -3.0 ± 0.8 hrs/day compared to individual baseline), that was significantly lower than on day 3 ($t_{176}=4.2$, $p=0.004$), but only numerically lower compared to day 4 ($t_{150}=2.4$, $p=0.49$, Fig. 2). Up from the 5th day of wearing the nose flap there was no further reduction in TIC until the end of the observation period (pairwise comparisons of days 5-14 all $p > 0.05$, Fig. 2). The mean time (mean \pm S.D.) that calves still spent in the cow area at day 4 was 3.4 ± 2.6 hrs/day and at day 5 was 2.3 ± 2.3 hrs/day.

Sex and weaning age of the calf, as well as parity of the cow or group size of the cow herd did not significantly influence TIC (all $p>0.1$). On the other hand, the random effect of calf ID nested in herd significantly influenced the measured time that calves spent in the cow herd ($Z=2.3$, $p=0.01$, see also Fig. 1).

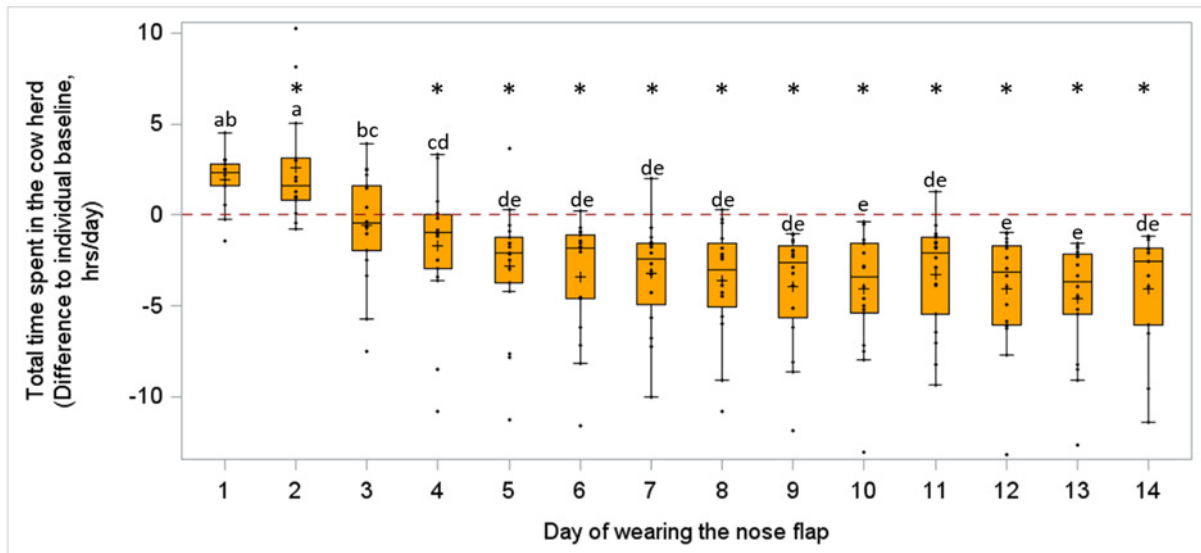


Fig. 2) Time spent within the cow herd (TIC) including the calves' mothers expressed as difference to the individual mean baseline in hours per day of dam-reared dairy calves (n=18) after insertion of a nose flap in the morning of day 1 (experiment I). Asterisks indicate differences of days of wearing a nose flap from baseline at $p < 0.05$. Lowercase letters (a-e) indicate differences between days of wearing a nose flap at $p < 0.05$. The plus symbol indicates the mean. Diamonds display the values of the individual calves at each day.

Experiment II

Baseline TIC between calves ranged from 1.7 to 19.1 hrs/day (mean \pm S.D.: 11.0 \pm 4.4 hrs/day) in herd A and from 0.8 to 16.9 hrs/day (mean \pm S.D.: 4.5 \pm 3.7 hrs/day) in herd B (see Fig. 3). Within calves, differences between the shortest and longest TIC per day during the baseline week ranged from 2.6 to 12.3 hrs/day (6.1 \pm 3.0 hrs/day) in herd A and from 1.2 to 14.7 hrs/day (5.5 \pm 3.4 hrs/day) in herd B, but exceeded ≥ 6 hours only for 8 of the 23 calves (4 calves from herd A and 4 calves from herd B, see Fig. 3).

The number of days that calves wore the nose flap influenced the time the calves spent in the cow herd ($F_{6,80}=10.5$, $p < 0.0001$). Compared to baseline, the calves numerically increased their TIC in the first two days after insertion of the nose flap (day 1: +0.23 \pm 0.8 hrs/day, $t_{44}=0.3$, $p=0.76$, day 2: +0.26 \pm 0.8 hrs/day compared to individual baseline, $t_{49}=0.3$, $p=0.74$, Fig. 4). Afterwards TIC of calves decreased and was significantly reduced compared to baseline up from the fourth day of wearing the nose flap (-2.78 \pm 0.74 hrs/day, $t_{40}=-3.8$, $p=0.001$, Fig. 4). Calves showed no further reduction in TIC after the 4th day of wearing the nose flap (pairwise comparisons of days 4-7 all $p > 0.05$, Fig. 4). The mean time (mean \pm S.D.) that calves still spent in the cow area at day 4 was 5.4 \pm 5.8 hrs/day.

Group size of the cow herd significantly influenced TIC of calves ($F_{1,21}=5.8$, $p=0.03$), since the time that calves spent in the cow area decreased (-0.41 \pm 0.17 hrs/day) per head of animals that the cow herd increased. Also, the measured TICs were significantly influenced by the random effect of calf ID nested in herd ($Z=2.0$, $p=0.02$, see also Fig. 2).

Sex and weaning age of the calf, as well as parity of the cow did not significantly influence TIC (all $p > 0.1$).

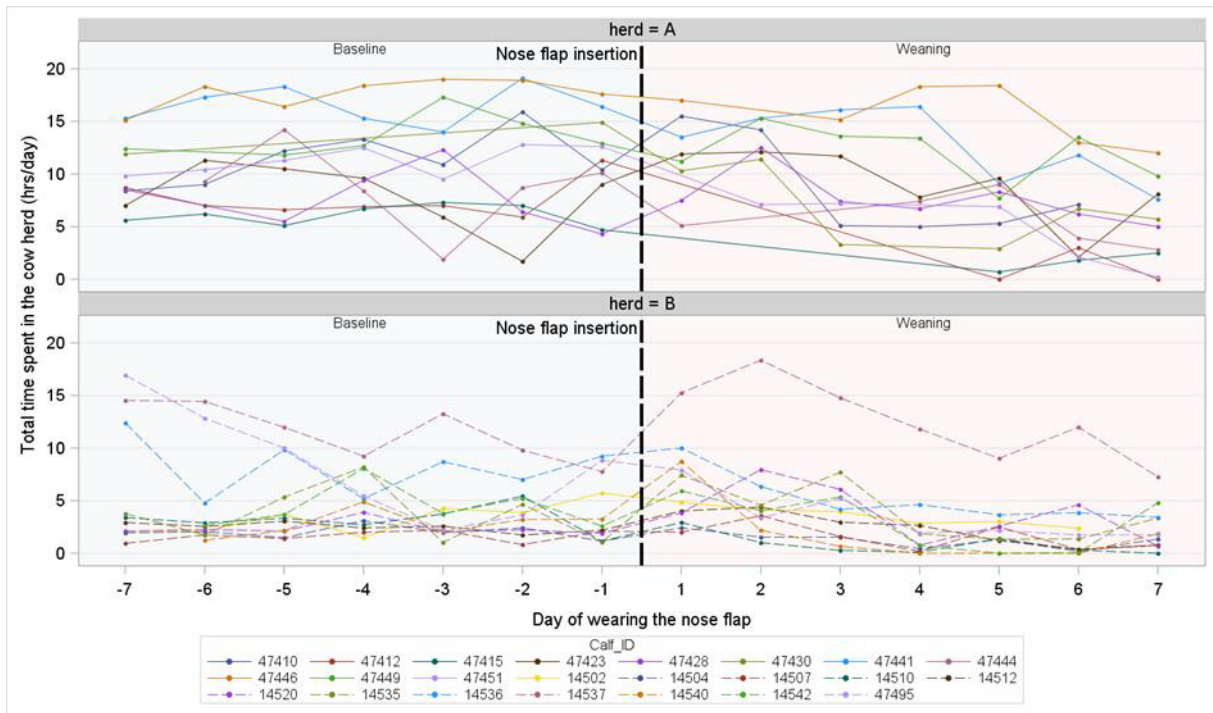


Fig. 3) Individual daily time spent within the cow herd (TIC) including the calves' mothers in hours per day of dam-reared dairy calves during one week before and one week after insertion of a nose flap (experiment II). Data are displayed separately for the two herds in the barn (herd A: n= 11 calves, herd B: n = 12 calves). The nose flap was inserted at day 1 in the morning (dashed lined was offset to leave data points on day 1 visible). Missing data points for single days refer to days at which calves were classified as sick or contact times could not be fully retraced due to technical failure (n=26).

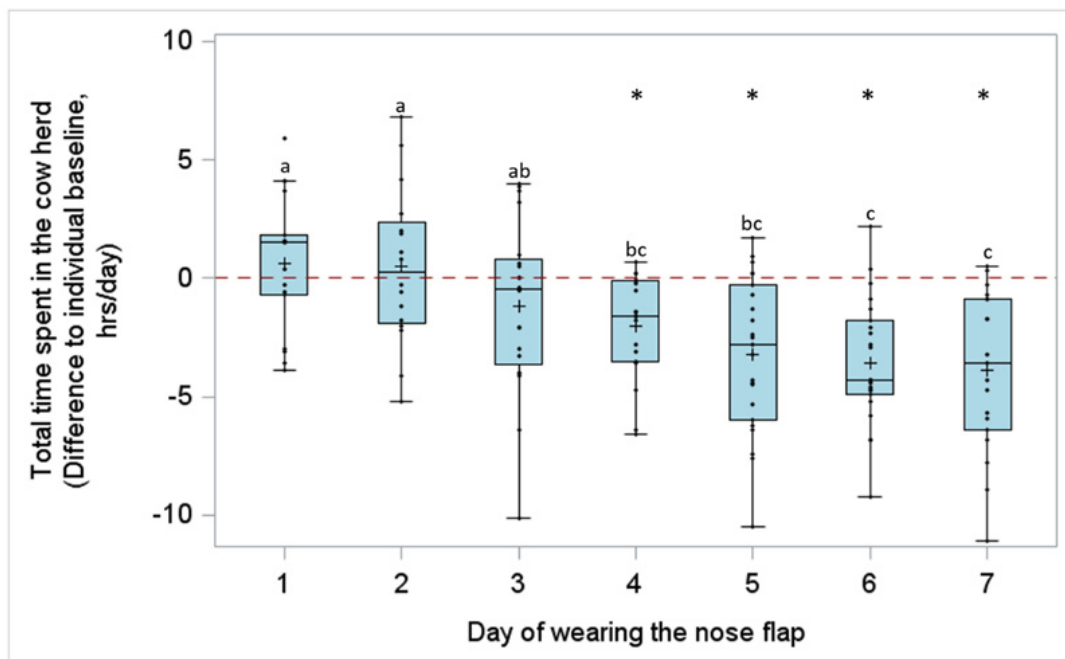


Fig. 4) Time calves spent within the cow herd (TIC) including the calves' mothers expressed as difference to the individual mean baseline in hours per day of dam-reared dairy calves (n=23) after insertion of a nose flap in the morning of day 1 (experiment II). Asterisks indicate differences of days of wearing a nose flap from baseline at p < 0.05. Lowercase letters (a-c) indicate differences between days of wearing a nose flap at p < 0.05. The plus symbol indicates the mean. Diamonds display the values of the individual calves at each day.

Discussion

Results of this explorative study showed that insertion of a nose flap significantly altered the time that calves spent within the cow herd, which was measured as a proxy for suckling motivation. Whilst there was a considerable individual variability in TIC already during the baseline period between calves, we still found a general pattern in both experiments that calves increased their TIC, at least numerically, during the first two days after insertion of a nose flap, followed by a reduction in TIC that remained at a constant, reduced level starting from day 4-5 after insertion of the nose flap with no further decrease afterwards. In both experiments, calves spent significantly less time within the cow area after 4 days of wearing a nose flap compared to their individual baseline, which implies that the motivation for dam contact of these calves was reduced after this time without milk provision. Largely in line with this, fence-line separated beef calves show a significant decline in time spent within 3m of the fence over 5 days following introduction of the fence-line (Price et al., 2003). Similarly, Enriquez et al. (2010) found a significant reduction in percentage of beef calves observed within 5m of the fence over the first 3 days after start of fence-line weaning, with no further reduction at days 4 and 5. In the same study, beef calves that were not weaned via fence-line separation but with nose flaps, showed a slight increase in vocalizations after fitting of the nose flaps, but this vocal response had returned to zero at the 5th day after nose flap insertion as well (Enriquez et al., 2010). Furthermore, it is interesting to note that calves in experiment I showed a significant increase in TIC at the second day of wearing the nose flap, which is in agreement with results of fence-line separated beef calves which also showed the highest behavioral reaction 48 hours after separation through the fence (Pérez-Torres et al., 2016; Solano et al., 2007). In general, increased walking activity in calves for the first 1-2 days after fitting of the nose flap, which quickly decreases afterwards, was reported in several studies with dairy and beef calves (de la Cruz-Cruz et al., 2021; Hötzel et al., 2012; Lambertz et al., 2015; Loberg et al., 2008). Additionally, the same calves as used in experiment I (Vogt et al., 2024) as well as beef calves weaned with a nose flap (Enriquez et al., 2010) showed a high number of unsuccessful suckling attempts for 2-4 days after the nose flap was inserted. This indicates a persistently high suckling motivation of calves for the first days after nose flap insertion, which is likely caused by hunger due to insufficient nutrient supply from solid feed at this time point. Taken together, our results in conjunction with literature indicate that calves' behavioural responses to two-step separation with a nose flap are predominantly present for about 3-5 days after fitting of the nose flap, after which the animals seem to adjust somewhat to the new situation.

Reasons for the reduction in TIC are likely multifactorial

The reduced need and/or motivation for contact to the dam after insertion of the nose flap is probably caused by an interplay of several factors. First, suckling of the dam's udder leads to the release of oxytocin in calves (e.g. Lupoli et al., 2001; Uvnäs-Moberg et al., 2001), which is known to be involved in reinforcement of the mother-young social attachment. Also, the receipt of milk has been reported to lead to the release of endogenous opioids in young animals of several species (reviewed in Nelson and Panksepp, 1998; Newberry and Swanson, 2008). In addition, Weary et al (2008) already discussed that milk could potentially also act as a source of exogenous opioid to the calf, as specific tetra peptides in the cow's milk can also bind to opioid receptors (Yoshikawa et al., 1986). Thus, milk ingestion and the act of suckling itself

both strengthen attachment to the dam, while prevention of this will lead to a diminished motivation for social contact to the dam, as part of the rewarding hormonal effect is missing. At the same time as the attachment becomes weaker, calves will increase their nutritional independence from the dam by progressive adaptation of the gastrointestinal-tract (GIT) to a diet based on solids. This is supported by observations of significantly increased eating and rumination times by the fourth day, compared to first day, after fitting of nose flaps in 5-week-old dairy calves (Haley, 2006). Likewise, the calves from experiment I showed a significant increase in the duration of TMR feeding time compared to their baseline in both weeks while wearing the nose flap (Vogt et al., 2024). However, in the latter study, it remained uncertain, if this was due to an actual increase in roughage intake or simply due to an altered, slower, feeding behavior with the nose flap since the actual feed intake of calves was not measured and weight gains of calves remained low (Vogt et al., 2024).

Furthermore, not only the physical dependence, but also the calves' general motivation for dam contact might have become weaker after cessation of milk supply in our study. Generally, the number of suckling bouts per day (Reinhardt and Reinhardt, 1981a), as well as the time cow and calf spend within close distance from another (Lidfors and Jensen, 1988) decreases with age of the calf under extensive conditions. Up from the age of about 3 weeks, calves also form groups with other calves, so called crèches, which are accompanied by only 1-2 guardian cows while the mothers are away to graze (Reinhardt and Reinhardt, 1981b). Thus, calves become socially more independent of the mother with increasing age. Since the design of both experiments lacked a control group in which calves were dam-reared and not separated from their mothers, it is possible that the reduced TIC of calves might also have been partly a result of the fact that calves simply got older with each day of wearing the nose flap. However, the age range of almost 3 weeks (83-102 days of age at weaning start) of calves during experiment I showed no significant effect on TIC of calves. Thus, we infer that during the one or two week period that the nose flap was worn in the two experiments of this study, the age effect is not a dominant factor and that consequently, the individual baseline times from the week directly before nose flap insertion give a reasonable enough indication of TICs that could have been expected for the respective one or two following weeks in case calves would have remained without a nose flap. This assumption is further supported by the fact that the decline in suckling bouts naturally takes place over several months instead of days and that calves under semi-natural conditions are not weaned until an average age of 9-11 months (Reinhardt and Reinhardt, 1981a).

More likely than the age effect is that the outer conditions in the barn reduced the motivation of calves to enter the cow area and thus further enhanced the observed reduction in calves' TICs after insertion of the nose flaps. Since observations of TIC during both experiments took place in an artificial barn setup during the winter months, i.e. without pasture access, calves were limited to spend time with their dams in the cow area of the barn. Consequently, they had to share the restricted walking area and cubicles with the cow herd and might have been repeatedly displaced by cows other than their dam (Waiblinger et al., 2020), while on the other hand they had all necessary resources available in the calf area. This is especially likely for calves of cows with lower social rank in the herd and for the time periods with a high group size of the cow herd of 48 or 49 heads for the 51 available cubicles. This is supported by the result that during

experiment II, the TIC of calves was reduced by about 25 min/day per increase in herd size by one cow. As a result, motivation of calves to continue to reunite for other purposes than suckling after nose flap insertion might not have been as strong in our studied calves as it would have been under natural conditions. In any case however, TIC never decreased to zero in our study, but the calves still visited the cows after nose flap insertion for a decreased but consistent level of about 2.3 to 5.4 hours/day (experiment I and II respectively). This implies that our calves were nonetheless still motivated to reunite with their dams for purposes other than suckling, just in decreased amount. This is in line with observations of cattle under semi-natural conditions where weaning is never accompanied by complete separation, but dams form grazing and licking associations with their (eldest) offspring that remain constant for several years after weaning (Reinhardt and Reinhardt, 1981b).

In this context it might also be worth noting that the dams of the calves used in experiment I, showed no obvious decline in interest in their calves over the first four days after calves got the nose flap inserted, as the amount of strong vocal responses by cows recorded during direct observations was highest on days 3 and 4 after nose flap insertion compared to the first two days (see supplementary material of Vogt et al., under review). This points towards the fact, that the observed reduction in calves' TIC within the 4 days is probably not a counter-reaction to a reduced interest from the dams after their calves discontinued nursing, but seems more so to be due to a reduced motivation for dam contact by the calves themselves. However, this theory requires verification in future studies.

Individual variation

There was a considerable inter-animal variation in baseline TICs of the calves during both experiments, which likely contributed to the difference of about 2.3 vs. 5.4 hrs/day that calves continued to spent time in the cow area at the 4th or 5th day after cessation of the suckling opportunity in the two experiments. In this regard, descriptive analysis of TIC in both experiments showed that baseline TICs of calves from herd A were in general considerably higher than from herd B, while calves from herd B showed often quite constant, low TICs already during the baseline week of the study and thus no considerable further decrease in TIC after insertion of the nose flap (see Fig. 1 and 3). The most obvious difference between the herds was the horn status, as herd A included predominantly polled cows, whereas all cows in herd B were horned. As reviewed by Knierim et al. (2015), there is only limited knowledge on social behavior in horned compared to polled cattle herds, but it seems evident that horned cattle maintain greater inter-individual distances and show less frequent agonistic physical interactions compared to polled cattle, leading to generally more stable herds of horned cattle. It is thus possible, that due to the greater inter-individual distances kept in horned cattle, the calves from herd B were less likely to use the lying spaces of the cows if another cow was already lying close by and rather limited the time they spent in the cow herd to shorter activities like suckling. This needs however to be confirmed in future studies specifically monitoring cubicle use in both herds. Next to the horn status, there are several further factors which may have influence the individual (baseline) TIC of calves which we did not control for in our study, including for example personality of the calf (Finkemeier et al., 2018; Veissier and Boissy, 2007), milk yield and milk composition of the cow (Ungerfeld et al., 2009), the resulting

weaning weight of the calf (Lidfors and Jensen, 1988) and in general the calves' establishment on solid feed and thus nutritional independence from the dam at the point of weaning start (Johnsen et al., 2018). Regarding the latter it has, for example, been reported that dairy calves which had access to a supplementary milk feeder and were thus nutritionally independent from their dams, spent less time close to the separation barrier which separated their pen from the dams' pen during the day within the nursing phase (calves had night-time access to their dams) and also vocalized less during the separation phase compared to calves that were nursed by their dams (Johnsen et al., 2018). Hence, it seems likely that calves with a higher level of nutritional independence in our study might have also been the ones with lower TICs during the baseline week. With regard to personality of the calves, it can be suggested that individuals which are very sociable (a description of the sociability trait can be found in Lecorps et al., 2018b) are potentially the ones which expressed higher TICs during the baseline in our study, since more sociable calves are generally more motivated to remain in close proximity to conspecifics (Lecorps et al., 2018a). Also, there seems to be a link between personality of the calf and the level of nutritional independence at weaning, since a recent study reported that calves which are scored more 'explorative' and 'active' show higher willingness to try new feed and have higher solid feed intakes pre-weaning compared to less exploratory calves (Neave et al., 2018), which again might have influenced how motivated calves were for cow contact during the baseline. It is additionally possible that the yield of the dams also influenced baseline variability in TIC of calves in a way that cow-calf pairs with high yielding dams had more contact to each other. At least in beef calves it has been reported that individuals which are heavier at weaning showed more vocalizations during the weaning process than lighter calves, which could be related to the fact that they lost access to higher yielding dams and thus a higher milk supply (Stěhulová et al., 2017). However, a possible influence of the milk yield of dams on motivation of calves for dam contact during the baseline was not verified during our study as parity of the cow had no significant influence on the measured TICs. Lastly, maternal care of the dam towards the calf will affect the strength of the cow-calf bond and has been reported to be influenced by several factors such as personality and body condition of the dam (Stěhulová et al., 2013), age of the dam (Green, 1993; Stěhulová et al., 2017) as well as sex and weight of the calf (Stěhulová et al., 2013). Hence, all of these factors could have played an additional role in the high variability in baseline TICs found between calves in our study.

But not only the inter-animal variation was considerable, also intra-animal variation between days within the baseline period was also quite large for single individuals. This might be explained by further factors not accessed in our study, such as e.g. high restlessness in the cow herd at single days due to cows in oestrus. For calves with a generally high intra-animal variation already during the baseline, it is a limitation of our study that we compared the TIC on single days during the weaning period to the average of the baseline week, as this average might not be a good representation of the actual time these calves would still have spent within the cow herd, if no nose flap had been worn. Nonetheless, we found quite consistent results in the general pattern of the calves' response to nose flap insertion over the two experiments included for this study, despite the comparably low sample size and considerable inter- and intra-animal variation. On the whole, the 4 days seem to give a reasonably good indication for the time point when calves' motivation to spend time within the cow herd, and thus likely also their suckling motivation, is decreased after insertion of a nose flap in an artificial barn setting

after calves were kept with 3 months of full-time contact to their dams, but of course this can never apply to all individuals. More research is needed here to determine which factors mainly contribute to the found considerable differences between and within individuals as well as between herds in the present study.

Limitations and future directions

To the best of our knowledge this is the first study that attempted to track changes in dam-contact initiated by dairy calves after insertion of a nose flap so far. Hence the result from this explorative study in 3-month-old Holstein calves in a barn with a separate calf area needs verification in different husbandry systems as well as with calves of different weaning ages, different levels of nutritional dependence and also from dual-purpose breeds. Like discussed above, space-availability seemed to be a relevant confounder of TIC and thus results might differ in open pasture settings which have typically less space-restrictions, as well as in more spacious barn conditions. Also, the separate calf area, where calves had access to all essential resources but were undisturbed from the cows, likely influenced TIC in our study. Therefore, future studies that track cow-calf interactions initiated by the calf over the days after nose flap insertion in joint deep-bedded loose housing systems, as well as in pasture-based rearing systems with and without a calf creep area would help our understanding of this confounding factor.

Next to the low generalizability of our results, it is also a limitation that it was not possible to systematically score the integrity of the calves' nasal septum over the course of our two experiments. Since tissue alterations or even lesions occur frequently at the contact site where the nose flap rubs against the skin of the nasal septum, it would be essential to assess whether lesions in the calves' nostrils are also present after the suggested 4-5 days of nose flap usage. In this context, Valente et al. (2022) reported open wounds at the nasal septum of 8-month-old beef calves after 5 days of usage in all tested animals (n=41). Compared to that, we found partly only pressure marks (n=12) and partly lesions with bleeding and secretion (n=6) in the 3-month-old calves used in experiment I when the nose flap was removed after the longer period of 14 days (results presented in Vogt et al., 2024; in experiment II lesion scoring was unfortunately not possible). A daily scoring would have been desirable, but since pressure marks and lesions are covered up by the nose flap as long as it sits inside the nose and a frequent removal and re-insertion of the nose flap is highly stressful to the animals and might also cause lesions, an inspection of the nasal septum was only possible after the final removal of the flap after 14 days. Thus, it is unfortunately unknown, if the lesions found in the 6 calves from experiment I would already have been present after 4 days of nose flap usage as well.

The current literature suggests in this regard however, that lesions occur consistently in dairy and beef calves regardless of age or duration of nose flap usage (Lambertz et al., 2015 (7.5 mo old calves, 7 days of usage); Freeman et al., 2021 (8 mo, 7 days usage); Valente et al., 2022 (8 mo, 5 days usage); Wenker et al., 2022 (2 mo, 14 days usage); Kirk and Tucker, 2023 (7 mo and different weights, 7 days usage); Vogt et al., 2024 (3 mo, 14 days usage)). Therefore, the result of our study that calves need to wear the nose flap for a minimum duration of 4 days in order to effectively reduce suckling motivation in conjunction with results by Valente et al. (2022) imply that a functional weaning of calves with nose flaps might adversely affect the

physical integrity of some calves. However, systematic studies that specifically investigate nasal septum injuries through the nose flap with standardized scoring systems (e.g. as recently developed by Kirk and Tucker, 2023) in younger calves after a short-period of 4 days are still lacking and are encouraged here.

Lastly, other negative welfare consequences of 4-5 days of nose flap usage need to be examined as well. In this context, the two recent comprehensive studies (Vogt et al., 2024; Wenker et al., 2022) which assessed different types of alternative weaning methods for 2 to 3-month old dairy calves in full-time cow-calf contact systems with regard to the calves' stress responses and performance are rather discouraging for the nose flaps. Compared to fence-line separation, two-step separation with nose flaps over 14 days led to lower weight gains during the weaning period and also to higher activity levels in the post-weaning period, so that the authors concluded that fence-line separation is the more effective method to reduce weaning and separation stress for dam-reared dairy calves (Wenker et al., 2022). Also, the results of the larger study where calves of experiment I were part of, suggested that the two-step separated calves had problems with adaptation of the GIT, resulting in greater energy deficits, compared to gradually weaned calves and also showed indicators of a more compromised affective state than gradually weaned calves (Vogt et al., 2024). Next to this and like already mentioned in the introduction, no comprehensive studies have to our knowledge assessed the potential discomfort and pain that could be caused by the nose flaps, as well as the impact of nose flaps on self-grooming or exploratory behavior so far. Anecdotal results of two 10-week-old calves that were weaned with a nose flap over 14 days, reported increased self-grooming frequency of the calves in the days after total separation from the cow (Verwer and Kok, 2012). The authors discussed that this might be an attempt to compensate for the loss of grooming through the mother after separation (Verwer and Kok, 2012), but likewise this could also be a re-bounce behavior after the preceding prevention of self-grooming by the nose flap, since this behavior is also important for ensuring hygienic body maintenance (Kohari et al., 2009). Importantly, all of the aforementioned was assessed in calves weaned with nose flaps over 14 days, but at least part of these effects are likely also present in the first 4-5 days of nose flap usage, so that a thorough consideration of these negative welfare consequences is necessary before employing the nose flap method, even for shorter time periods. Nonetheless, results of our study suggest that there is little benefit of using nose flaps for longer periods than 4-5 days, which might give a valuable indication for those farms which currently still use the nose flap due to its high practicability and local restrictions for implementation of other weaning methods. Furthermore, a time period of 4-5 days per step could also be useful for the design of future alternative stepwise weaning protocols.

Conclusion

Results of our study indicate that in 3-month-old dairy calves that were raised with full-time contact to the dam, it needs at least 4 days until the motivation to spend time with the cow decreases after insertion of a nose flap. When using the nose flap for longer than 4-5 days there was no further reduction in contact times, and contact times remained at a relatively constant level of several hours per day despite the loss of the suckling opportunity. However, there was a considerable variability between individuals already during the baseline and thus, the result

of this explorative study can only give an indication and needs replication, foremost in a pasture-based environment with less space-restrictions.

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Conflict of interest

The authors declare to have no conflicts of interest that are relevant to the content of this article.

Notes

This study received no external funding.

All experiments were performed in accordance with the German Animal Welfare Act (Federal Republic of Germany, 2020). Animal experiment number for experiment I: V244-51520/2019, institution of approval: MELUND Schleswig-Holstein.

For experiment II, the local Animal Welfare Committee decided that no ethical approval was required as calves were only monitored using commercially available sensors and video recordings while kept with their standard husbandry and living conditions in the barn. For weaning, nose flaps were used by default in the barn.

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Supplementary Material

Table S1) Confidence intervals (CI) for the results of the linear mixed model analysis of the individual daily time that calves spent within the cow herd (as differences to individual baseline, in hours per day) during the two weeks after insertion of a nose flap (**experiment I**, n= 18 calves).

Day after insertion of the nose flap	Estimate	Standard error	Lower 95% CI	Upper 95% CI
1	0.98	0.81	0.70	2.65
2	1.71	0.81	0.05	3.38
3	-0.76	0.79	-2.40	0.87
4	-1.87	0.79	-3.51	-0.24
5	-3.01	0.79	-4.64	-1.38
6	-3.38	0.78	-5.01	-1.76
7	-3.01	0.79	-4.67	-1.37
8	-3.44	0.80	-5.09	-1.79
9	-3.76	0.80	-5.40	-2.11
10	-4.11	0.78	-5.74	-2.49
11	-3.32	0.78	-4.95	-1.70
12	-4.10	0.78	-5.73	-2.48
13	-4.62	0.78	-6.25	-2.99
14	-3.56	0.82	-5.24	-1.87

Table S2) Confidence intervals (CI) for the results of the linear mixed model analysis of the individual daily time that calves spent within the cow herd (as differences to individual baseline, in hours per day) during one weeks after insertion of a nose flap (**experiment II**, n= 23 calves).

Day after insertion of the nose flap	Estimate	Standard error	Lower 95% CI	Upper 95% CI
1	0.23	0.76	-1.30	1.75
2	0.26	0.78	-1.30	1.82
3	-1.47	0.74	-2.97	0.03
4	-2.78	0.74	-4.28	-1.28
5	-3.38	0.71	-4.82	-1.93
6	-3.72	0.71	-5.17	-2.27
7	-4.22	0.74	-5.71	-2.72

Study IV

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

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Dairy cows' responses to two 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 months of full-time contact with either the two-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 days/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/wk from day -1 till +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 two separation methods in any of the examined behavioral and physiological response variables. However, a significant increase in vocalizations and searching behavior compared to baseline was present with both methods. Further, 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 inter-individual 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 wks while calves wore the nose flap. Milk yield was higher in NF than GR cows during the 2 wks while GR calves had time-restricted access to their dams and NF calves were prevented from suckling, but showed no difference afterwards anymore. 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 wks in 3 steps, as well as with the NF method, but differences between individuals were considerable.

Keywords: cow-calf contact systems, gradual separation, stress, cows

1. Introduction

Dairy cow-calf contact (CCC) systems, that allow contact between calves and their dam or a foster cow for an extended time period (Sirovnik et al., 2020), receive increasing interest and demand from society (Placzek et al., 2021; Sirovica et al., 2022), scientists (EFSA AHAW Panel, 2023; Cook and von Keyserlingk, 2024) and farmers (Bertelsen and Vaarst, 2023; Hansen et al., 2023). Dairy cows are highly motivated for contact to their calf (Wenker et al., 2020) and form strong maternal bonds irrespective if 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, weaning and separation is typically done earlier, on average when calves are 17 weeks old (for Europe, Eriksson et al., 2022), than under natural conditions when the termination of suckling takes place at about 8-12 months (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, in order to ensure high levels of welfare of 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-months-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 two-step separation method with nose flaps which 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 to the two-step separation method with a nose flap, foremost allowing a more gentle physiological adaption to the dietary change (Vogt et al., 2024b). However, it is unknown, if a gradual method is equally favorable for the dams, when compared to two-step separation. While 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 one month 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 to cows that had full-time contact to their calves (Neave et al., 2024a).

On the contrary, a two-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 two-step separation with nose flaps leads to decreased vocalizations, pacing and walking behavior, but to increased grazing duration, after complete separation from calves compared to abrupt separation (Haley, 2006a; Ungerfeld et al., 2015), even when the beef suckler calves were weaned as early as 60 days of age (Ungerfeld et al., 2016). Similarly, dairy cows separated from their 5-week-old calves (Haley, 2006b) or 12-week-old foster calves (Loberg et al., 2007) show reduced behavioral responses to complete separation from their calves with two-step weaning compared to 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). In addition to the aforementioned, there are reports that non-suckled dairy cows which had only partial contact, that means restricted contact as affiliative interactions and/or nursing is 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 to 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 two-step separation with nose flaps on weight gains (Boland et al., 2008; Enriquez et al., 2010; Wenker et al., 2022), nasal tissue integrity (Valente et al., 2022 (8 mo old calves); Wenker et al., 2022 (2 mo); Kirk and Tucker, 2023 (7 mo and different weights); Vogt et al., 2024b (3 mo)), 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 two-step separation with nose flaps especially for pasture-based systems, we generally do not encourage this method as a welfare friendly alternative for weaning and separation in dairy CCC systems with regard to calf welfare by now (Vogt et al., 2024a,b). Nonetheless, a detailed comparison of dairy cows' reactions to gradual separation from their calf to two-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, in order to avoid these.

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 two-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 two-step method compared to a gradual reduction in contact time to the calf.

2. 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).

2.1 Animals, housing and feeding

36 dairy cows (mean age \pm S.D.: 4.5 ± 2.0 years, range 2.2-9.4 years at start of the separation process; see supplementary table S1 for more details) 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 two different herds according to their horn status: 19 cows were from the horned herd and 17 cows belonged to the genetically hornless (polled) herd. From the latter, only 12 cows were truly hornless, since the polled herd was in a transition phase from formerly horned into a completely polled herd and the 5 animals which had horns were remaining older cows. 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 two herds were housed in identically mirrored parts of the same open-sided free stall 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 two 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 total mixed ration (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 05:15-08:30am and 3:30-6:45pm. Water was provided ad libitum.

Cows calved in individual calving pens, where cow and calf stayed together for approximately 5 days to allow the cow-calf bond to establish. Afterwards, 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. Since cows were integrated into the herd about 5 days after calving, all experimental cows had been part of their herd for about 3 months when the separation treatments began.

2.2 Treatment groups

The separation process started when the related calf of a cow reached the age of 3 months (mean \pm S.D. 93 ± 6.9 days at treatment start). Half of the cow-calf pairs was assigned to the two-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 about 30m away.

In the **NF treatment**, calves were equipped with the nose flap (Quiet wean, JDA Livestock Innovations, Canada) for 2 weeks, 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 weeks, the nose flap was removed and calves remained in the calf section for one more week, i.e. 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 three weeks 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 **GR treatment**, the CCC time was reduced from full-time contact pre-weaning to half day 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 hours, about 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 hours per day, from the end of morning milking till noon. Following this week of morning contact, cows had one more week of fence-line contact to their calf through the pen boundaries like in the NF treatment. After these three weeks of partial separation, the pair was totally separated by moving the calf to the youngstock barn. Access of calves to the cow section through the selection gate was automatically controlled via transponders in the calves' collars during the whole process.

Allocation of cow-calf pairs to the two 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 two treatments can be found in supplementary table S1. Weaning age of the calves was staggered by two weeks in both treatments to counterbalance the effect of the later milk loss in the GR treatment (milk loss at week 3) compared to the NF treatment (milk loss at week 1, described in detail in Vogt et al., 2024b).

2.3 Data collection

A combination of behavioral and physiological indicators was used to evaluate the distress response of cows to the two 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 two separation methods on changes in milk yield.

	BEGINNING OF PARTIAL SEPARATION													TOTAL SEPARATION																						
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	
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			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	X

Figure 1) Sequence of the separation process within the two separation methods and an overview of sampling time points for all assessed indicators. NF= Cows were separated from their calves via the two-step method using a nose flap for the calves (n=18). GR= Cow-calf pairs were separated by gradual reduction of cow-calf-contact time (n=18). Fence-line contact = limited contact of cow and calf through the 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.

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 weeks. Within this study period, each cow was sampled for a duration of 5 weeks, including one week prior to separation, the 3-week 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.

2.3.1 Direct behavioral observation

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

Since calves reached the required weaning age of 3 months at different weeks within the 18-wk-study period, there was a maximum of 6 cow-calf pairs plus 3 cows whose calves had just been moved to the youngstock barn (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 which were simultaneously at any stage of the separation process within a week and needed thus to be observed simultaneously.

2.3.2 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; 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 till +27 relative to the start of the separation process, but days -14 till -8 were taken as 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 was 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 minutes 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).

Table 1) Ethogram of assessed behaviors

Behavior	Definition	Indicator classification	Mode	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 1m of the pen partition up and down, or is standing at the selection gate or beside the pen partition (radius 2.5m) with head elevated and eyes and ears focused in the direction of the calf's section and scanning (adapted from Enriquez 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 sensors 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 sensors 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 sensors 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/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

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

2.3.3 Ruminantion time

For assessment of the daily ruminantion 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; validation studies and logging rate see table 1) from day -14 till +27 relative to the start of the separation process. Again, days -14 till -8 were taken as habituation period for the cows and not analyzed. Day of removal (day +27) or of necessary replacement of the sensors were also omitted from the data set due to incomplete data for the 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, i.e. pressure changes. The recorded pressure changes were processed with RumiWatch Manager 2 (Version 2.1.0.0.) and then converted into daily ruminantion 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.

2.3.4 Fecal cortisol metabolites (FGCMs)

Fecal samples of the cows were collected twice per week between days -1 till +23 relative to the start of the separation process. Sample collection always took place at about 07:30 a.m. (\pm 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).

2.3.5 Hematological 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 till +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 ethylenediaminetetraacetic acid (K EDTA) coated 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 two cows (NF=1/GR=1).

2.3.6 Milk yield

Cows were milked twice per day (05:15-08:30am and 3:30-6:45pm) in a 2x4 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).

2.4 Data analysis

2.4.1 Exclusion criteria

Data points from sampling days at which cows showed clinical signs of severe sickness, or when cows were observed to be in (pro)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, 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, like for example severe diarrhea, severe lameness, infected lesions. In case that a cow was identified with a severe symptom, i.e. scored a 2 in any of the health indicators on a specific day, and/or was treated with medications by the barn personnel and/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 at which cows were identified to be in proestrus or estrus were deleted from the data as 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 6-17 hours (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, since we expected dams that have just nursed their calves to have a lower motivation to reunite with their calf.

In summary, the final data set contained only data points for observations or collection days at which cows had no severe health problem, were not in (pro)estrous and cows and calves had spent $\leq 2\%$ 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 6-18 hours 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 till midnight of the preceding day. An overview of the remaining data points for all assessed parameter can be found in supplementary table S2.

2.4.2 Statistical analysis

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

2.4.2.1 Indicators of distress: main model

Analyses of cows' distress indicators (vocalizations (frequency/30min), amount of searching behavior (% of scans/30min), lying time (%/24h) and lying bouts (number/24h), rumination time (hrs/24h), FGCM concentration (ng/g), neutrophil number (cells/ μ l), lymphocyte number (cells/ μ l) and N:L 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, as 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/NF), phase (baseline/partial separation/total separation), the interaction between treatment and phase, lactation group of the cow (1st lactation/2nd-3rd lactation/4-7th lactation), sex of the calf (male/female), as well as month of data collection (Nov/Dec/Jan/Feb/Mar). Weaning weight of the calf at the start of weaning (in kg) was included as continuous variable. The cow ID nested within herd (horned/polled) was included as 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, as there was a correlation between weaning weight and weaning age of the calf ($r_p=0.7$, $p<0.001$), 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 towards the smaller values (see supplementary figures S1 and S2) returning funnel shaped residuals. As elimination of zeros (like e.g. implemented by Neave et al., 2024b for the same issue) could not significantly improve model

fit due to a high occurrence of values between 1-10, data were square root-transformed before analysis and afterwards 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 least squares means of the main model underrepresent strong responses (but see section 2.4.2.2). Post-hoc pairwise comparisons were calculated using a Tukey-Kramer test. As 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 (see supplementary tables S3 and S4). For results of the treatment x phase interaction this was not necessary, as there was a lack of significance of the treatment x phase interaction in all indicators. Results are presented as (back-transformed) least squares means (LSM) \pm standard error (S.E.).

2.4.2.2 Indicators of distress: strong behavioral responses

Like described above, 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 least squares means and especially the standard error 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 section 2.4.1, which means that it included the 5 observation time points of 30min 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 30min observation or showed searching behavior in $> 20\%$ of observational scans (during the 30min direct observation with scan sampling every 3 mins, equaling about > 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 three-week 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 section 2.4.2.1, but with a binary distribution (logit-link). Distribution of strong responses in the two treatments over the different weeks of the separation process is given descriptively.

2.4.2.3 Indicators of distress: physiological responses

For the hematological indicators (neutrophil number (cells/ μ l), lymphocyte number (cells/ μ l) and N:L ratio) and FGCM concentrations (ng/g), the main model described in section 2.4.2.1 was additionally run with the exact sampling day instead of only the phase as sampling time point, since previous studies showed that physiological stress responses in cows are transient and can return to pre-weaning values within 48 hours after start of the separation process (Whisnant et al., 1985; Acevedo et al., 2005).

2.4.2.4 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 (% of scans/30min), the amount of cow-calf interactions (% of scans/30min) 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 x 30 min in the 2 hours following morning milking) was included in the data set as this is 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 above (section 2.4.2.1), with the exception that it included the exact week (week 1, week 2, week 3) of the partial separation phase instead of the pooled 3-week partial separation phase as the sampling time point and consequently also the treatment x 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 afterwards.

For analysis of changes in nursing behavior only the GR treatment was included in the analysis, as suckling was not possible anymore after insertion of the nose flap in the NF treatment. Also, the afternoon observation time point (i.e. 1 hour before evening milking) was not considered for analysis, since this time point was not comparable between the week 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 two weeks 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 x week interaction was omitted from the model, as 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.

2.4.2.5 Effect of the separation methods 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 (morning/evening milking; in kg per milking per cow), data was split into sub-sets separated by milking time before running the analysis.

3. Results

3.1 Distress responses of cows to the two separation methods

There was no treatment x 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 two separation methods over the three-week 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), as cows in both treatments vocalized more frequently (post-hoc: t -statistic_{degrees of freedom}: $t_{122} = -3.3$, $p = 0.003$) and showed more searching behavior ($t_{120} = -3.9$, $p = 0.0004$) during the three-week partial separation phase than during the baseline phase (table 2). Once the calves were moved to the youngstock barn, i.e. 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 to baseline anymore (table 2). An overview of results for the remaining main effects included in the models can be found in supplementary table S5.

3.1.1 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 four-week separation phase was considerable, with a large proportion of weak behavioral responses and some very strong responses by cows. There was also a high inter-animal 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 30min 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 30min 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, while 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-week partial separation phase, as well as during the total separation phase was similar in both treatments (treatment x phase interaction: F-test statistic_{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 x 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 week 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 supplementary figures S3 and S4.

Table 2) Model output for behavioral and physiological indicators of distress for cows that were separated from their calves via the two-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)[†].

Variable	Phase					p-values (F-value ^{Num DF, Den DF})		
	T	N	Baseline	Partial separation	Total separation	T	P	T x P
Vocalizations ^{1,2} (frequency per 30min)	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 ^{1,2} (% of scans in 30min)	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 24h)	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 24h)	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 (hrs/24h)	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)
FGCMs (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 (x1000 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 (x1000 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)
N:L 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 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) as there was no significant treatment x phase (TxP) interaction for any of the examined indicators. Baseline phase= the week before weaning start, Partial separation= the 3-week 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, N:L ratio= neutrophil: lymphocyte ratio. Values are presented as (back-transformed) least squares means (LSM) ± standard error (S.E.).

¹Means per week per cow calculated before analysis

²LSM and S.E. back-transformed from square-root-transformation.

^{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).

Table 3) Weekly distribution of strong vocal responses and high amounts of searching behavior by cows in the two 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

Variable	Treatment	Unit		Sampling week											
				Baseline		Week 1		Week 2		Week 3		Total Separation		Sum	
Vocalizations	NF	No. of SR	% of all SR	3	--	37	54.4	17	25.0	9	13.2	5	7.4	68	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 [†]	
	GR	No. of SR	% of all SR	7 [#]	--	21	18.9	8	7.2	54	48.7	28	25.2	111	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 [†]	
Searching behavior	NF	No. of SR	% of all SR	0	--	23	54.8	10	23.8	6	14.3	3	7.1	42	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 [†]	
	GR	No. of SR	% of all SR	3 [#]	--	17	22.0	5	6.5	36	46.8	19	24.7	77	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 [†]	

¹Values 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; **% of all SR**), absolute numbers of strong responses recorded during the morning, afternoon or evening 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 30min 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 cow-calf contact time (n=18). NF= Cows were separated from their calves with the two-step weaning method using a nose flap for the calves (n=18). Observations were conducted on 4 days per week at the following time points: 2x 30min during the 2 h following morning milking (morning), 1x30min during the 1 h before the start of evening milking (afternoon) and 2x30min during the 2 h following evening milking (evening).

[#]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.

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

3.1.2 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 both treatments showed neutrophilia (post-hoc: $t_{103}=3.2$, $p=0.03$) and increased FGCM concentrations at the 2nd day of the separation process ($t_{133}=4.5$, $p=0.0004$) compared to baseline values, but this was only short-lived in both indicators (table 4). FGCM concentrations showed a second peak compared to baseline on day 16 ($t_{156}=-3.3$, $p=0.03$, table 4), i.e. the first sample after introduction of fence-line contact, which was however not evident in neutrophil numbers.

3.2 Changes in social behaviors of cow and calf during the separation process

Changes in nursing behavior was only considered for the GR cows, since NF calves could not suckle anymore 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 to the baseline observations (table 5). However, there was no further increase in the amount of scans with nursing behavior from the first week 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 two separation methods depending on the phase (table 5). However, there was a main effect of treatment, as cow-calf interactions were more frequently observed by GR pairs 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 led to no significant adjustments in the amount of observed cow-calf interactions neither after morning milking compared to 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 two weeks 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 supplementary table S6).

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 30min at which several pairs were observed simultaneously totaling 3518 observations).

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

Variable			Sampling day								p-values (F-value _{Num DF, Den DF})		
	T	N	Baseline	Week 1		Week 2		Week 3		Total Sep	T	D	T x D
			-1	2	6	9	13	16	20	23			
N (x1000 cells/ μ l)	NF	17	2.15 $\pm 0.28^A$	3.12 $\pm 0.28^B$	2.25 $\pm 0.27^{AB}$	2.69 $\pm 0.28^{AB}$	2.47 $\pm 0.29^{AB}$	2.07 $\pm 0.30^{AB}$	2.09 $\pm 0.28^{AB}$		0.13 (F _{1,24} =2.5)	0.03 (F _{6,108} =2.5)	0.96 (F _{6,105} =0.2)
	GR	17	2.61 $\pm 0.30^A$	3.20 $\pm 0.37^B$	2.74 $\pm 0.35^{AB}$	2.92 $\pm 0.34^{AB}$	2.69 $\pm 0.37^{AB}$	2.68 $\pm 0.42^{AB}$	2.48 $\pm 0.45^{AB}$				
L (x1000 cells/ μ l)	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 (F _{1,28} =0.2)	0.13 (F _{6,104} =1.7)	0.23 (F _{6,100} =1.4)
	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				
N:L ratio	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 (F _{1,23} =2.4)	0.07 [#] (F _{6,101} =2.0)	0.87 (F _{6,97} =0.4)
	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				
FGCM (ng/g)	NF	18	20.06 $\pm 2.63^A$	25.97 $\pm 2.67^C$	19.54 $\pm 2.62^A$	18.75 $\pm 2.61^A$	23.83 $\pm 2.61^{AC}$	24.53 $\pm 2.62^{BC}$	18.16 $\pm 2.62^{AB}$	19.21 $\pm 2.61^A$	0.25 (F _{1,30} =1.4)	<0.001 (F _{7,132} =6.0)	0.62 (F _{7,128} =0.8)
	GR	18	22.25 $\pm 2.81^A$	28.80 $\pm 3.15^C$	22.79 $\pm 2.90^A$	23.67 $\pm 2.85^A$	26.62 $\pm 3.07^{AC}$	27.67 $\pm 3.14^{BC}$	25.51 $\pm 3.16^{AB}$	24.20 $\pm 2.71^A$			

Values are expressed as least squares means \pm standard error. T=treatment, D= sampling day, Total Sep= Total separation.

^{A-C}Capital 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).

[#]individual pairwise comparisons were no longer significant after Tukey-Kramer adjustment.

3.3 Changes in milk yields of the cows during the separation process

Daily milk yield (in kg) differed between treatments depending on the week (interaction treatment x week, table 6). 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 anymore 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 to the second week with morning contact ($t_{400}=-6.4$, $p<0.001$). Hence, milk yield of GR cows did not differ anymore from 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 two treatments (NF week 1 vs. GR week 3, $t_{64}=3.0$, $p=0.1$, table 6). Descriptively, milk yield of GR cows was reduced by about 4.3 liters compared to cows separated with the NF method (mean \pm S.D.: GR: 18.49 ± 6.20 ; NF: 22.82 ± 6.38 kg/day) when considered as average over the whole three-week 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 to 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 to the third week with fence-line contact in neither of the two 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 two 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 separation 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 to the week of total separation ($t_{327}=-3.5$, $p=0.02$, table 6). This was not the case for GR cows, since 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 another (GR week 1 vs. week 2: $t_{418}=-1.1$, $p=0.98$). Only when fence-line contact was introduced in the third week, the evening milk yield of GR cows considerably increased compared to baseline ($t_{390}=-11.5$, $p<0.001$) and the other two weeks of the weaning and separation process (week 1: $t_{375}=-13.1$, $p<0.001$, week 2: $t_{409}=-12.5$, $p<0.001$, table 6). Descriptive analysis of the sampling days showed a sharp increase in evening milk yield after introduction of fence-line contact (figure 2), which is contrary to the slow adaptation in morning milk yield described above.

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

Variable	Week					p-values (F-value _{Num DF, Den DF})		
	T	N	Baseline	Week 1	Week 2	T	W	T x 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			

¹Cows were either separated from their calves via two-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 (2x 30min in the 2 hours following morning milking) were included for analysis of both indicators as this is the only observation time point that is comparable between treatments or between weeks within the GR treatment. T= treatment, W= sampling time point (week), T x W = interaction of treatment x week. DF= degrees of freedom. Baseline = the week before start of the separation process. Values are presented as least squares means (LSM) ± standard error (S.E.).

²LSM and S.E. back-transformed from square-root-transformation.

^{A,B}Capital superscript letters indicate significant differences between weeks at $p \leq 0.05$.

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

Variable	Week							p-values (F-value _{Num DF, Den DF})		
	T	N	Baseline	Week 1	Week 2	Week 3	Total Separation	T	W	T x 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 (F _{1,30} =2.3)	<0.001 (F _{4,337} =190.5)	<0.001 (F _{4,314} =25.9)
	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}			
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 (F _{1,27} =1.5)	<0.001 (F _{4,331} =96.1)	0.18 (F _{4,326} =1.6)
	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}			
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 (F _{1,27} =27.4)	<0.001 (F _{4,348} =127.3)	<0.001 (F _{4,344} =76.5)
	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}			

¹ Cows were either separated from their calves via two-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 05:15-08:30am and evening milking from 3:30-6:45pm. T= treatment, W= sampling time point (week), T x W = interaction of treatment x week, DF= 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 least squares means (LSM) ± standard error (S.E.).

^{a-c}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).

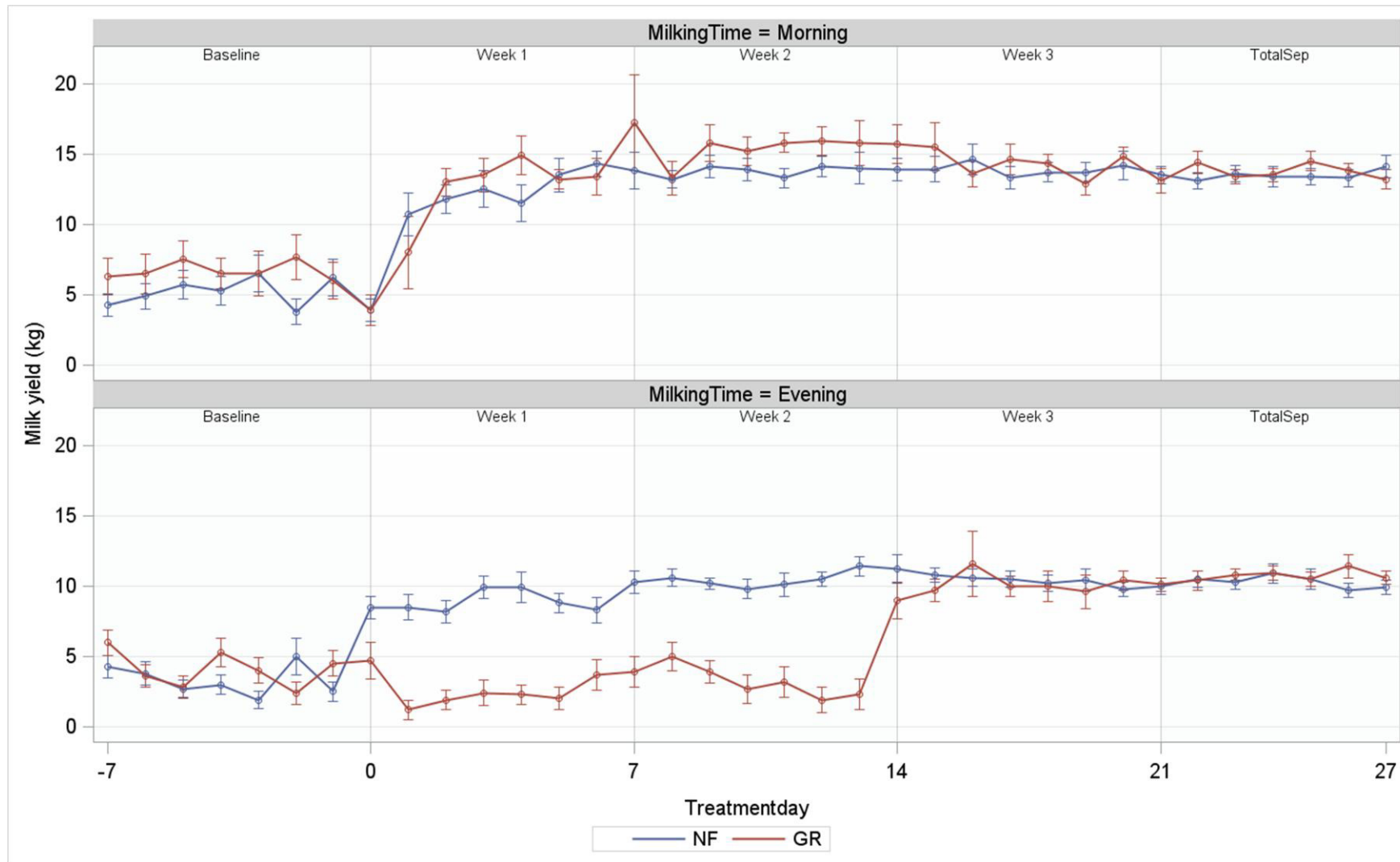


Figure 2) Milk yield of cows (mean \pm S.E., in kg) during morning (approx. 05:15-08:30am) and evening (approx. 3:30-6:45pm) milking in the course of the 3 weeks of partial separation (starting at day 0) and the first week after calves were moved to the youngstock barn (at day 21, total separation (=TotalSep)). Cows were either separated from their calves via two-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).

4. Discussion

With this study, we aimed to compare behavioral and physiological responses of dairy cows to separation from their calf, after 3 months of full-time contact, either via two-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, two-step separation with nose flaps and a gradual reduction of contact time to the calf 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 to baseline, indicating that separating dams from their 3-months-old calves with these methods is still of welfare concern. Descriptive analysis additionally revealed large inter-individual differences between cows as well as a different temporal distribution in occurrence of strong responses in the two treatments. Expectedly, milk yield was higher in NF compared to GR cows during the 2 weeks 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 two 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 contact, indicating that our gradual separation method worked not as gradually as intended.

4.1 Distress responses to the two 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 two-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-month-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 to pre-weaning times, after 4 days of wearing the nose flap to about 3.5 hrs/day (Vogt et al., 2024a). This declined numerically even further to about 2.5 hrs/day 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 hrs/day 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 below.

4.1.1 Expectations matter

Within both separation treatments, part 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 week 1, followed by week 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 are also observed under extensive conditions (Watts, 2001). This is plausible since both partners preferentially respond to calls from their own dam or calf (Marchant-Forde 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 reversed to responses in beef cows, which reacted with more vocalizations and pacing behavior after final separation from their calf compared to prevention of nursing through nose flaps in several studies (Haley, 2006a; Ungerfeld et al., 2015, 2016). This is likely due to different housing conditions, since 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. Since 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 two weeks 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 chose to remain in the calf area.

For the GR treatment, our results are largely in line with findings by Neave et al. (2024b), who used a very similar experimental design to us: Dairy cows in a CCC system were gradually separated from their calves over 2 weeks 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 non-responding animals, but those who vocalized did so when contact time was reduced to 50% and/or when pairs were totally separated (Neave et al., 2024b). 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., 2024b). 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. (2024b). Their findings and the descriptively strong vocal response to fence-line separation of GR cows in our study, both points towards 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 to baseline was expected, since 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 2-3 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 hours of the day (Roadknight et al., 2022). Also, Bertelsen and Jensen (2023) reported that half-day contact calves that were separated from their dam over night, suckled in >90% of cases within 30 min after reunion with the dam, whereas this was only the case in about 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 to 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 two weeks 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 saw during observations before start of evening milking. Hence, the missing 3.5 hours afternoon contact during the second week of the separation process might not have significantly altered nursing times as 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 to the week with half-day contact.

Generally, the two milking times created a routine for the animals in our barn, since 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 week 1, while in week 3 the behavioral reactions were more frequent during the morning and afternoon observations (see table 3 and supplementary figures S3 and S4). 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 days after separation in dairy cows which were used to very limited daily contact to their calf for only 20 min of nursing prior to morning milking, but had likely also formed an expectation to reunite at this time. This points towards 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. (2024b). 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 two 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 to 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. Since 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 response 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. (2024b) for the missing difference to abrupt separation.

4.1.1.1 Towards 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 reduction 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 non-nutritive 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 either be achieved 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 milk suckled 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.

Lastly, 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 days) or long (4 weeks) period, found only slight differences in behavioral responses of the cows between the two 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.

4.1.2. *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 two 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. (2024b) 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 pre-separation 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 inter-individual 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 impact 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.

4.1.2.1 *Towards 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 supplementary table S5), which is in line with results in beef (Stěhulová et al., 2017) and dairy cows (Neave et al., 2024b). However, in other studies multiparous cows reacted with a higher physiological (de Paula et al., 2023, zebu cows) and behavioral (Ungerfeld et al., 2011, beef cows) stress response to abrupt weaning compared to 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 supplementary 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, since 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 which 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, since 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 since 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 pre-weaning 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 impact of the most known personality traits which 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, i.e. 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, since 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 cows, that are likely stronger affected by separation, in the future to support a move towards more individualized separation protocols.

4.1.3 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). Since during the first two weeks 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, the reduced (evening and total) milk yields in the parlor of GR cows compared to NF cows during these weeks are plausible.

Within the GR treatment, cows showed a weekly increase in total milk yield during the 3-week 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 above) 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 about 4 liters in week 1 with half-day contact compared to week 2 with morning contact (table 6) was mainly caused by a lower morning milk yield during the first ~2 days after treatment start within week 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 to the second week. This indicates that cows needed on average about 2 days 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 to the frequent milk removal in the pre-separation period. Whilst 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, like already discussed by others for yields of CCC cows (e.g. Barth, 2020; Churakov et al., 2023). The fact that milk ejection was only transiently impaired for about 2 days 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 two of the seven following milkings. Once the cows in our study habituated to the (half-day) prevention of nursing after about 2 days, 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 days in dairy cows in mid lactation after a change of the milking routine from twice daily to only once-a-day milking, equally pointing towards 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. Since 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 2nd day of the separation process, which was the first sampling time point after treatment start. Whilst 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 i.a. 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 N:L 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. 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. Whilst 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 separation 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, since 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 caused itself 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 towards 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/or 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 to 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.

4.2 Limitations

In order to judge which of our two 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, since previous studies showed reduced lying times in dairy cows within the first 8 – 72 hours after separation from their calves (Loberg et al., 2007; Rhim, 2013; Neave et al., 2024b) as well as reduced lying and rumination behavior in beef cows during the first 72 hours 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 and/or rumination time 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 two-step separation with nose flaps from their calves. These cows were sampled for the first 4 days after initiation of each new step within the separation processes and the results are in accordance with our findings.

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 due to the fact that 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 30min, since there were less distractions and more space on the aisles at this time point. Furthermore, observations of nursing behavior were also not optimal. Whilst we observed nursing frequently, observation was hampered due to the necessity that the observer had to switch between the herds every 30min. In consequence we faced the problem, that cows were often still at the feeding rack in the beginning of the observation (especially those which 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 which happened in parallel at the other herd. Consequently, our data only reflect changes in the 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 inter-individual variability between cows including many observations with zero occurrences of cow-calf interactions. Additionally, our observation mode was not optimal for assessment of all types of cow-calf interactions. While 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-cow/calf contact or 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.

5. Conclusion

Dams reacted with a similar stress response to two-step separation with use of nose flaps for the calves or a gradual reduction of contact time to their calf, potentially since both methods ultimately reduced the daily duration of dam-calf contact and involved a rather abrupt termination of nursing. Within both methods we found large inter-individual differences, but dams in both treatments reacted with a longer-lasting behavioral stress response, as well as with a transitory increase in physiological stress markers and inhibition of milk ejection after initiation of the separation process. Therefore, neither of the two methods proved to be favorable for reduction of separation distress, but since the gradual separation treatment was favorable for calves (Vogt et al., 2024b) this method should be developed further. In this regard, the similar milk yields in the week with half day and morning contact proved, that the weekly reductions in contact time we used for the gradual separation treatment had limited success since they worked in a rather stepwise than gradual manner, which needs future improvement. In addition, descriptive results of our study strongly support preceding demands for development of more individualized separation protocols as well as for consideration of previously formed expectations as important mediators of the distress response during development of future separation protocols.

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Conflict of interest

The authors declare to have no conflicts of interest that are relevant to the content of this article.

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Supplementary material

Table S1) Overview of attributes of cows and their calves in the two separation treatments. Cows were separated from their calves either via two-step separation using a nose flap for the calves (NF) or via gradual reduction of cow-calf contact time (GR).

			NF	GR
No. of cow-calf pairs			18	18
COWS	Herd (No. of experimental cows in herd)	horned	9	10
		polled	9 (3 with horns [†])	8 (2 with horns [†])
	Age (years at treatment start)	range	2.3-9.1	2.2-9.4
		mean± S.D.	4.3±2.0	4.6±2.0
	Lactation number (No. of cows)	1 st	6	4
		2 nd or 3 rd	8	8
4 th – 7 th		4	6	
CALVES	Sex (No. of calves)	males	10	10
		females	8	8
	Age (days of age at treatment start)	range	83-102	83-103
		mean± S.D.	93.9±6.5	92.1±7.6

[†]as the polled herd was in the transition from a formerly horned into a completely polled herd, there were some older, horned cows remaining in the herd.

Table S2) Overview of excluded data points after correction of the dataset for sampling days at which cows showed clinical signs of severe sickness, when cows were observed to be in (pro)estrus or when cows and calves had illegitimate contact for all sampled parameters.

Variable	No. of cows included in the dataset	Number of data points before correction	Number of remaining data points after correction
Directly observed behaviors (i.e. vocalizations, searching behavior, nursing behavior, cow-calf interactions)	36	3600	2730
Lying time and number of lying bouts per day	26	910	689
Rumination time per day	30	1050	775
FGCM concentration	35	280	200
Hematological variables (i.e. Neutrophils, Lymphocytes and N:L ratio)	33	238	168
Milk yield per milking	36	2520	1920

Table S3) Correction for multiple testing of the 9 indicators for separation distress of cows for the main effect of the **treatment** according to the Benjamini-Hochberg false discovery rate correction (Glickman et al., 2014). No p-values remained significant for an $\alpha \leq 5\%$ after correction for multiple testing.

No. Tests	Variable	P-value Treatment	Significance level cutoff for $\alpha \leq 5\%$
1	Searching behavior (% of scans)	0.007	0.006
2	Vocalizations (frequency per 30min)	0.03	0.011
3	Neutrophils (cells/ μ l)	0.14	0.017
4	N:L ratio	0.19	0.022
5	FGCM (ng/g)	0.35	0.028
6	Rumination time (hrs/24h)	0.40	0.033
7	Lying times (% of 24h)	0.49	0.039
8	Lymphocytes (cells/ μ l)	0.77	0.044
9	Lying bouts (number per 24h)	0.94	0.050

FGCM= fecal cortisol metabolites, N:L ratio= neutrophil: lymphocyte ratio.

Table S4) Correction for multiple testing of the 9 indicators for separation distress of cows for the main effect of the **phase** according to the Benjamini-Hochberg false discovery rate correction (Glickman et al., 2014). P-values printed in bold remained significant for an $\alpha \leq 5\%$ after correction for multiple testing.

No. Tests	Variable	P-value Phase	Significance level cutoff for $\alpha \leq 5\%$
1	Searching behavior (% of scans)	0.0002	0.006
2	Vocalizations (frequency per 30min)	0.003	0.011
3	FGCM (ng/g)	0.04	0.017
4	Lying bouts (number per 24h)	0.06	0.022
5	Neutrophils (cells/ μ l)	0.08	0.028
6	N:L ratio	0.11	0.033
7	Rumination time (hrs/24h)	0.38	0.039
8	Lymphocytes (cells/ μ l)	0.61	0.044
9	Lying times (% of 24h)	0.68	0.050

FGCM= fecal cortisol metabolites, N:L ratio= neutrophil: lymphocyte ratio.

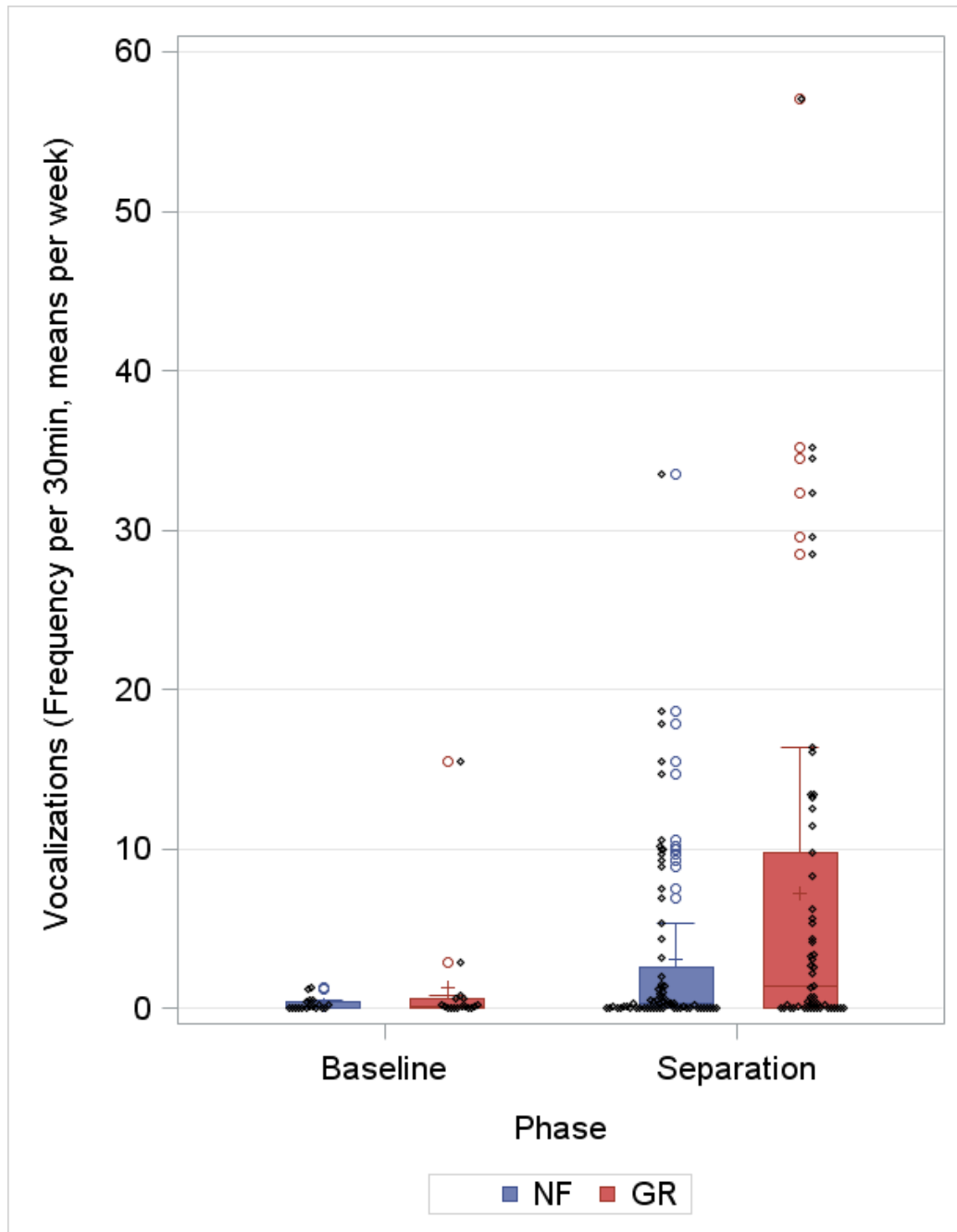


Figure S1) Boxplot graph of frequency of vocalizations by cows per 30min direct observation during the 1 week baseline phase (=baseline) and the 3 weeks of partial separation plus the first week of total separation after calves were moved to the youngstock barn (= separation). Cows were either separated by two-step separation using a nose flap for their calves (NF, n=18) or by gradual reduction of contact time between cow and calf (GR, n=18). The plot is based on the calculated means per week for each cow. Midline = median; plus symbol = mean; edges of boxes = 25th and 75th percentile; whiskers = range of values until minimum and maximum observation that is less than or equal to 1.5 times the interquartile range (close enough not to be considered outliers); circles= outliers; diamonds = the means per week of the individual cows.

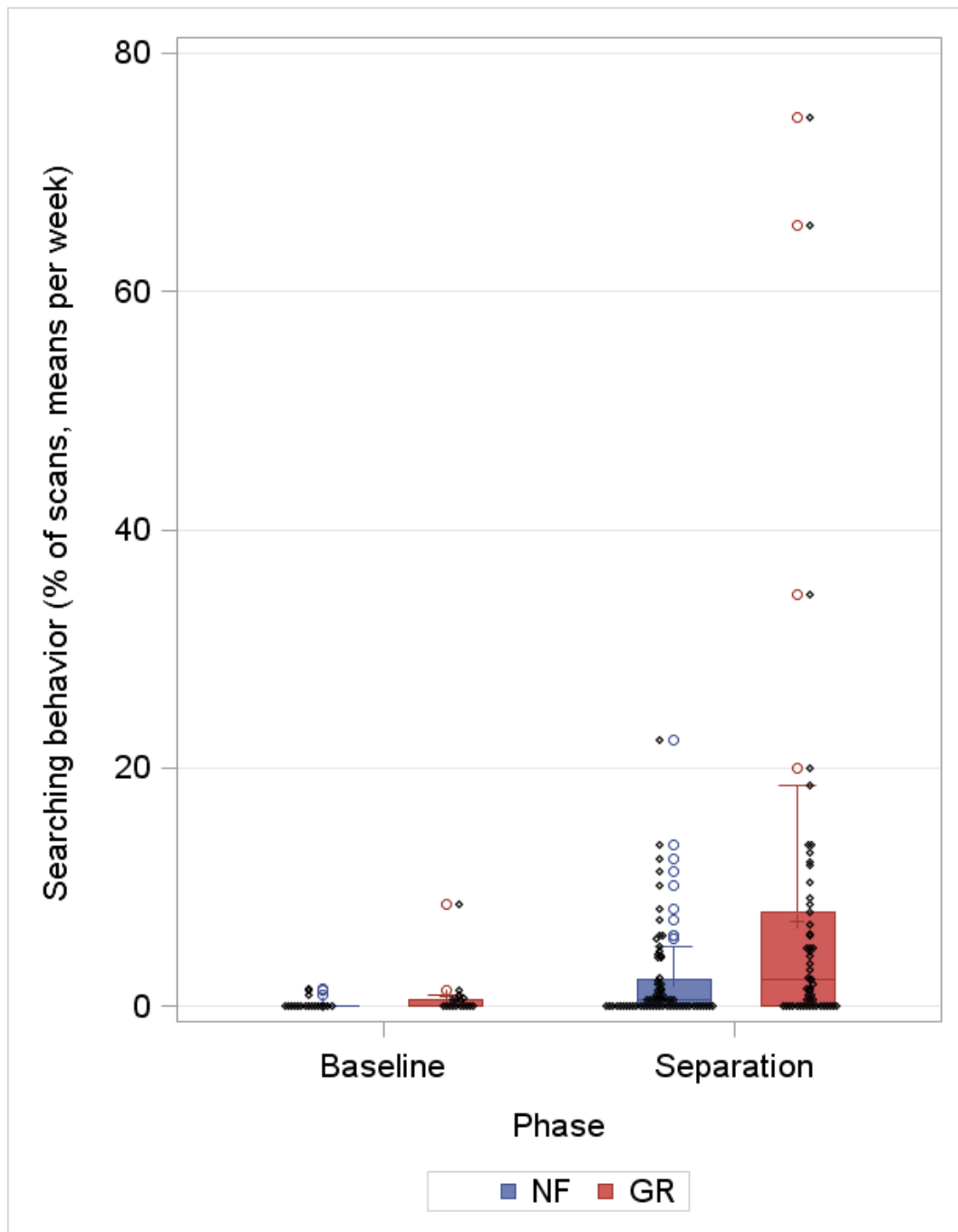


Figure S2) Boxplot graph of the amount of behavioral scans in which cows showed searching behavior (in % of scans, observed during 30 min direct observation with scan sampling in intervals of 3 min) during 1 week baseline (=baseline) and the 3 weeks of partial separation plus the first week of total separation after calves were moved to the youngstock barn (=separation). Cows were either separated by two-step weaning using a nose flap for their calves (NF, n=18) or by gradual reduction of contact time between cow and calf (GR, n=18). The plot is based on the calculated means per week for each cow. Midline = median; plus symbol = mean; edges of boxes = 25th and 75th percentile; whiskers = range of values until minimum and maximum observation that is less than or equal to 1.5 times the interquartile range (close enough not to be considered outliers); circles= outliers; diamonds = the means per week of the individual cows.

Table S5) Overview of results (p-value and F-Values_{Num DF, Den DF}) of the remaining main effects included in the main model for all 9 analyzed indicators of distress (described in section 2.4.2.1).

Variable	Lactation group of the cow	Sex of the calf	Weaning weight of calf at start of weaning	Month of data collection (i.e. season of the year)
Vocalizations (frequency per 30min)	0.25 (F _{2,29} =1.5)	0.87 (F _{1,28} =0.0)	0.03 (F _{1,32} =4.9)	0.02 (F _{3,70} =3.7)
Searching behavior (% of scans)	0.23 (F _{2,27} =1.6)	0.89 (F _{1,27} =0.0)	0.24 (F _{1,31} =1.5)	0.03 (F _{3,71} =3.3)
Lying times (% of 24 hrs)	0.26 (F _{2,20} =1.4)	0.71 (F _{1,19} =0.1)	0.04 (F _{1,20} =4.7)	0.06 (F _{4,122} =2.4)
Lying bouts (number per 24 hrs)	0.80 (F _{2,20} =0.2)	0.99 (F _{1,19} =0.0)	0.71 (F _{1,19} =0.2)	0.23 (F _{4,105} =1.4)
Rumination time (hrs/24h)	0.38 (F _{2,19} =1.0)	0.37 (F _{1,19} =0.9)	0.33 (F _{1,19} =1.0)	0.54 (F _{4,101} =0.8)
FGCM (ng/g)	0.07 (F _{2,30} =2.9)	0.12 (F _{1,30} =2.6)	0.12 (F _{1,31} =2.6)	0.17 (F _{4,55} =1.7)
Neutrophils (cells/ μ l)	0.46 (F _{2,23} =0.8)	0.88 (F _{1,22} =0.0)	0.10 (F _{1,23} =2.9)	0.43 (F _{4,84} =0.9)
Lymphocytes (cells/ μ l)	0.17 (F _{2,27} =1.9)	0.17 (F _{1,27} =2.0)	0.02 (F _{1,28} =6.0)	0.03 (F _{4,87} =2.7)
N:L ratio	0.29 (F _{2,24} =1.3)	0.69 (F _{1,22} =0.2)	0.58 (F _{1,23} =0.3)	0.57 (F _{4,55} =0.7)

- The cows' mean frequency of vocalization was higher, the heavier a calf was at the start of the separation procedure (+0.014 \pm 0.01 vocalizations per 30min per additional kg of calf weight). This was also mirrored in reduced lying times of the cows (by -0.1 \pm 0.05% per day, F_{1,20}=4.7, p=0.04) and reduced lymphocyte number of cows (by -0.02 \pm 0.01 x 1000 cells per μ l, F_{1,28}=6.0, p=0.02) per increase in kg weight of their calf at start of the separation procedure.
- The number of vocalizations was significantly reduced during December (0.43 \pm 0.08) compared to February (2.82 \pm 0.86 vocalizations per 30min, t₅₄=-2.9, p=0.02). This was also reflected in the amount of searching behavior, which was less often observed during December (0.28 \pm 0.06%) than during February (2.30 \pm 0.69% of scans during 30min observation, t₅₅=-2.8, p=0.03). Also, the number of lymphocytes was significantly lower during December (2.86 \pm 0.17) compared to January (3.32 \pm 0.17 x 1000 cells per μ l; t₈₄=-2.8, p=0.04).

Table S6) Overview of the results (p-value and F-Values_{Num DF,Den DF}) for the remaining main effects included in the models for changes in social behavior between cow and calf (described in section 2.4.2.4) and for daily milk yield of the cow (described in section 2.4.2.5).

Variable	Lactation group of the cow	Sex of the calf	Weaning weight of calf at start of weaning	Month of data collection (i.e. season of the year)
Nursing own calf (% of scans per 30 min)	0.23 (F _{2,12} =1.7)	0.48 (F _{1,11} =0.5)	0.48 (F _{1,12} =0.5)	0.25 (F _{3,31} =1.5)
Cow-calf interactions (% of scans per 30 min)	0.87 (F _{2,28} =0.1)	0.01 (F _{1,28} =6.9)	0.15 (F _{1,27} =2.2)	0.08 (F _{3,34} =2.5)
Total milk yield (kg/d)	<0.001 (F _{2,30} =9.8)	0.90 (F _{1,30} =0.0)	0.59 (F _{1,30} =0.3)	<0.001 (F _{4,130} =7.9)

- Cow-calf pairs with female calves showed more cow-calf interactions (3.06 ± 0.05) than pairs with male calves ($1.46 \pm 0.02\%$ of scans per 30min, $t_{28} = -2.6$, $p = 0.01$).
- Milk yield of primiparous cows (lactation group 1) was significantly lower (15.7 ± 1.04 kg/day) than yields of multiparous cows with 2-3 lactations (19.64 ± 0.87 kg/day, $t_{30} = -3.0$, $p = 0.02$) or ≥ 4 lactations (22.08 ± 1.07 kg/day; $t_{30} = -4.4$, $p < 0.001$).
- Milk yields of cows were significantly lower during November (16.75 ± 1.02 kg/day) and December (17.95 ± 0.70 kg/day) than during January (21.02 ± 0.63 kg/day, $t_{134} = 4.2$, $p = 0.0004$ and $t_{106} = -4.95$, $p < 0.001$ respectively) as well as than during February (21.05 ± 0.82 kg/day, $t_{125} = 3.3$, $p = 0.01$ and $t_{117} = -3.3$, $p = 0.01$ respectively for comparison with November and December).

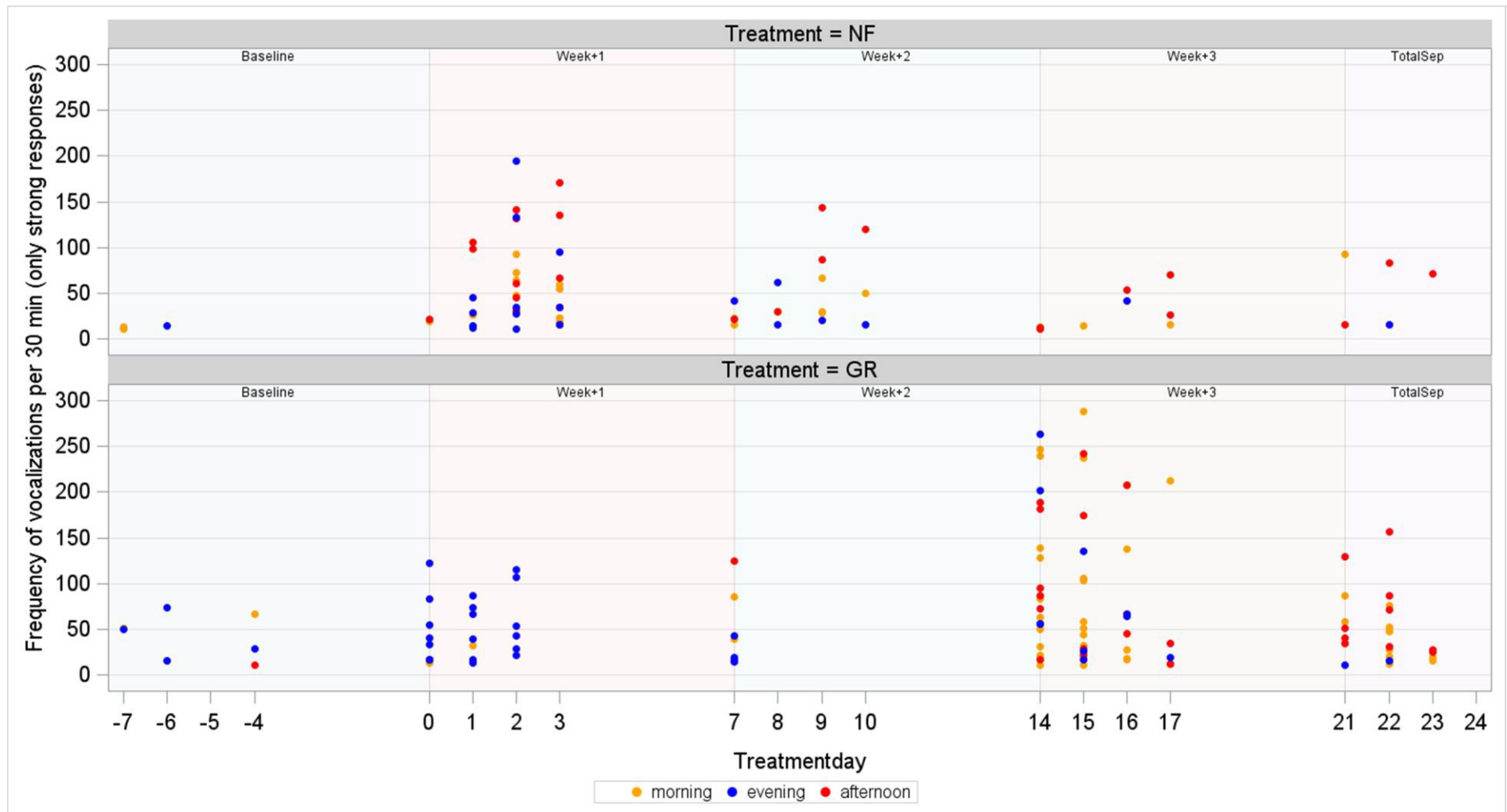


Figure S3) Distribution of strong vocal responses in the two treatments during 1 week baseline and the 3 weeks of partial separation plus the first week after calves were moved to the youngstock barn (=total separation (TotalSep)). Cows were either separated by two-step separation using a nose flap for their calves (NF, n=18) or by gradual reduction of contact time between cow and calf (GR, n=18). A frequency of >10 vocalizations by a cow during 30min of direct observation was classed as a strong response. Observations were conducted on 4 days per week at the following time points: 2x 30min during the 2 h following morning milking (yellow dots), 1x30min during the 1 h before the start of evening milking (red dots) and 2x30min during the 2 h following evening milking (blue dots).

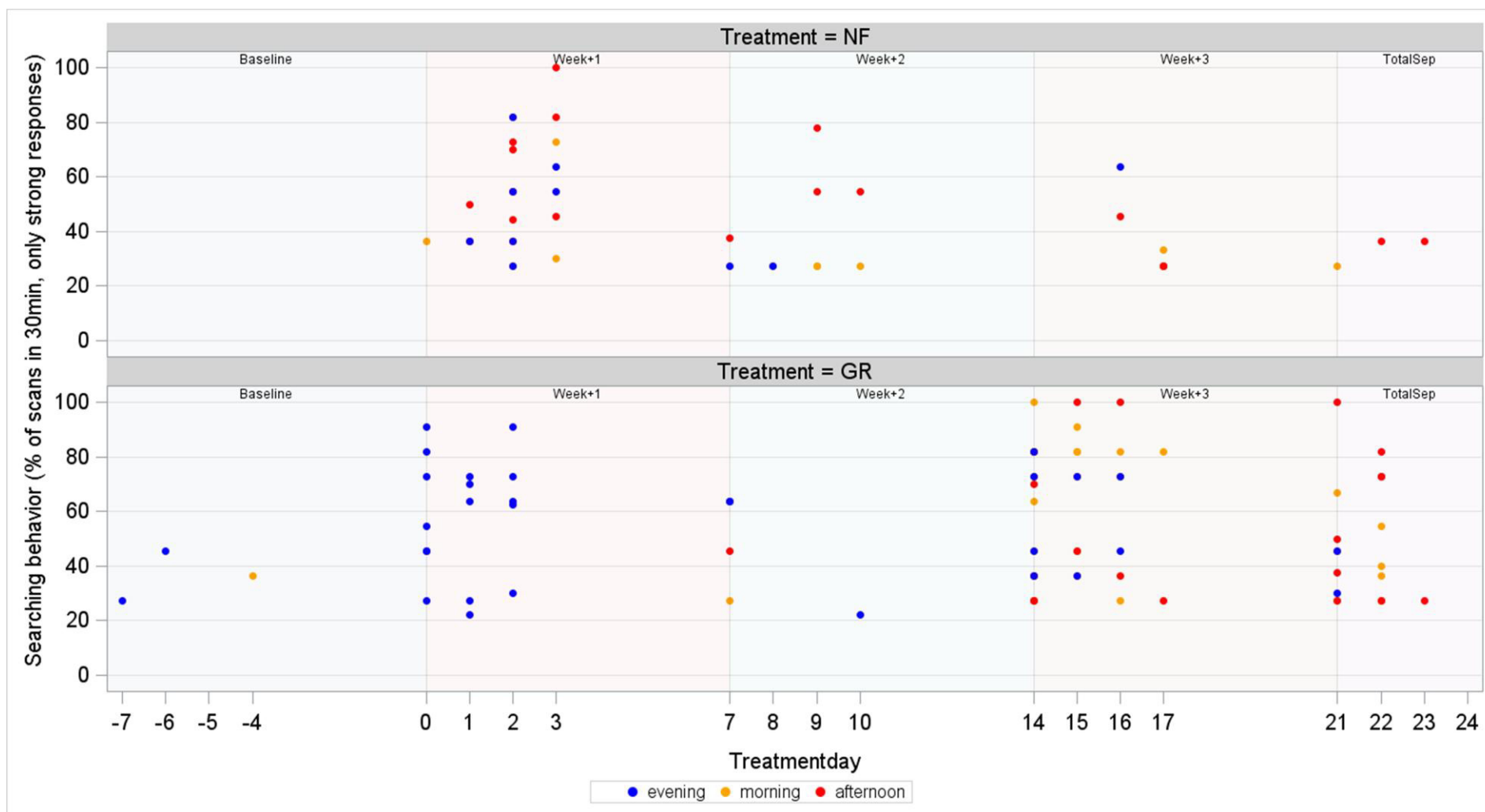


Figure S4) Distribution of high amounts of searching behavior in the two treatments during 1 week baseline and the 3 weeks of partial separation plus the first week after calves were moved to the youngstock barn (=total separation (TotalSep)). Cows were either separated by two-step separation using a nose flap for their calves (NF, n=18) or by gradual reduction of contact time between cow and calf (GR, n=18). A response was considered to be strong, if a cow showed searching behavior in > 20% of observational scans (during the 30min direct observation with scan sampling every 3mins). Observations were conducted on 4 days per week at the following time points: 2x 30min during the 2 h following morning milking (yellow dots), 1x30min during the 1 h before the start of evening milking (red dots) and 2x30min during the 2 h following evening milking (blue dots).

Study V

Relative telomere length in dairy calves and dams undergoing two different methods of weaning and separation

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Relative telomere length in dairy calves and dams undergoing two different methods of weaning and separation after three months of contact

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Abstract

Telomere length (i.e., the length of the repeated sequences of DNA at the end of chromosomes) is a promising indicator of overall stress. Our study aimed to compare the effects of a stress-inducing separation process between dams and their calves, with either a gradual or a nose-flap separation method after a three-months dam-calf contact since calving, on relative telomere length (RTL). Due to their nature, the nose-flap and gradual separation method have different effects on behaviour, stress hormone levels and physical development during and after dam-calf separation, which requires an overall measure of the weaning and separation stress during both procedures. We also investigated correlations between behavioural and other physiological stress indicators on RTL. We found no significant effect of the weaning and separation method on RTL in dairy calves after weaning and separation from their dams, but a tendency for shorter RTL in gradually separated dams compared to nose-flap separated dams. No correlations between behavioural or physiological stress indicators and RTL were found, which may be due to a short interval between the two RTL measurement points. Future studies should aim to analyse the effect of various separation methods over a longer period and preferably include a non-separated group as reference.

Keywords: Telomere length, stress, cumulative welfare, dam-calf separation, nose-flap separation, gradual separation

Highlights

- Telomere length is a promising indicator of overall stress
- We compared gradual and nose-flap separation methods on RTL
- We report no significant effect of separation method on RTL in calves
- A tendency for shorter RTL indicates more overall stress in gradually separated dams
- No correlation between RTL and other stress indicators was found

Introduction

Telomere length is thought to be able to integrate welfare states over time and is thus considered as a cumulative welfare indicator [1]. Telomeres are repetitive sequences of DNA at the ends of chromosomes that protect the cell's genomic stability [2]. While telomere shortening occurs with every cell division [3], evidence from humans indicates that average telomere length declines at an accelerated pace due to negative life experiences in a cumulative and dose-dependent manner [1,4]. Furthermore, stress-related telomere shortening can be mitigated and possibly even reversed by positive lifestyle interventions [5]. Meta-analyses confirm telomere length to be a good indicator of psychological and physiological stress, including social stress, offspring removal and diet, across species [6,7]. In cattle, telomere length is influenced by heat stress [8], but the impact of social aspects including dam-calf separation has not yet been investigated.

Dam-calf separation is a known psychological stressor in cattle. It results in a change of behaviours indicating stress in dams and calves. After separation, effects such as increased locomotion and vocalization, reduced time performing grooming, lying down and feeding can last for days [9,10]. The separation method likely affects welfare of both dams and calves. Dams are more motivated to re-unite with suckling calves than with calves that are prevented from suckling them [11]. Thus, dams may benefit from preventing suckling before permanent physical removal of calves (i.e., permanent total separation) as reported in beef cattle [12,13]. Furthermore, as indicated by behavioural parameters, preventing suckling through either udder-nets [10] or nose-flaps [14,15] reduces post separation stress in dairy and beef calves separated from their dams or foster cows. Thus, fitting calves with nose-flaps before permanent total separation (i.e., nose-flap separation) will likely have a less negative impact on calves undergoing separation, compared to abrupt separation [14,16]. However, it is possible that the nose-flap separation distributes stress over the whole separation period as animals react to each of the two-steps in the separation process [17]. Further, nose-flaps limit physical contact between animals [18], which may lead to frustration. Additionally, just one week of nose-flap use can lead to ulcerative lesions in the nostrils and by means of dissemination of the infection to pituitary abscesses in calves [19]. Thus, even if each step of the nose-flap separation method taken individually seems to induce less stress compared to abrupt weaning and separation, cumulative stress experienced during the transition from full dam-calf contact to total permanent separation needs to be considered.

Another attempt to reduce stress during dam-calf separation is to gradually reduce the amount of the dam-calf contact prior to permanent separation (i.e., gradual separation method). Dams in semi-natural conditions spend less time with their calves with increasing calves' age (compared to soon after birth

[20]). While their dams are away, calves in semi-natural conditions spend time with other calves in creche groups supervised by a familiar cow [20]. This way the calves may already habituate to spending time separated from their dams and get accustomed to solid feed [21]. Thus, gradually reducing the time the calves are able to leave the calf area to have contact with their dams may reduce the separation stress.

Therefore, the aim of the present study was to compare the effects of gradual and nose-flap separation after a three-months period of full-time dam-calf contact, on relative telomere length (RTL) in both calves and dams as an indicator for welfare. This was part of a larger study comparing these two weaning and separation methods with behavioural and physiological indicators [22,23]. We hypothesised lower overall stress and longer RTL in calves separated with gradual separation method as the gradual nature of the separation likely allows for habituation to having less access to the dams, while the nose-flap method may introduce cumulatively more stress due to abrupt prevention of suckling and possible ulcerative injuries. In contrast, dams in the gradual separation method need to adapt to the changes of the time and duration of physical contact with their calves, which is undisturbed with the nose-flap separation method until the permanent separation. Furthermore, preventing suckling may reduce the dams' motivation to re-unite with the calves after the permanent physical separation. Thus, we hypothesised that the nose-flap method would result in less overall stress and longer RTL in dams than the gradual separation method. We further hypothesised that RTL will negatively correlate with elevated levels of behavioural and physiological stress indicators, such as faecal cortisol metabolite levels, neutrophil: lymphocyte (N:L) ratio, vocalisations and searching behaviour. Moreover, telomere length was hypothesised to positively correlate with indicators of reduced stress (e.g., elevated levels of play behaviour in calves and rumination in cows).

Materials and methods

Animals and housing

This study was part of a larger experiment, in which a number of behavioural and physiological measures were taken [22,23]. All experimental procedures were performed in accordance with the German Animal Welfare Act (Federal Republic of Germany, 2020; experiment number V244-51520/2019, MELUND Schleswig-Holstein). The experiment was carried out from November 2019 to March 2020 at the Thünen-Institute of Organic Farming in Trenthorst, Germany. Cows were kept in an open-sided barn with cubicle loose housing. They were fed a total mixed ration, composed of grass silage, corn silage, concentrate feed in the form of coarse grain and mineral feed, which was provided freshly twice a day. Cows used in the experiment were either horned or genetically hornless (polled) Black-and-White German Holstein and housed in two herds in two identical parts of the same barn with 50 cubicles each. In one herd all cows were horned, in the second herd most cows were polled, but some older cows had horns, because the herd was in a transition from formerly all-horned into a completely polled herd (Table 1).

Number of cows per herd varied from 45 to 49 cows in the horned herd and 43 to 48 cows in the predominantly polled herd throughout the experiment. The herd sizes changed, when animals were removed from the herd to be either sold or moved to the calving pens or sick pens, or when primiparous cows were introduced into the herd. Cows were milked twice daily in a tandem parlour.

Table 1. Details on the number of animals sampled. Both separation methods (nose-flap and gradual separation) were additionally both subdivided into early and late separation (with a two-week difference in the separation start). The table shows the number of individuals that were used for the analysis of telomere length.

	<i>Nose-flap</i>		<i>Gradual</i>	
	Early	Late	Early	Late
Calves				
Sex				
Males (all intact)	5	5	5	4
females	4	4	4	4
Dams				
Herd				
horned	4	5	3	5
polled	5 (1 with horns)*	3 (1 with horns)*	4 (0 with horns)*	4 (2 with horns)*
Lactation				
1 st lactation	2	4	2	2
2 nd lactation	3	1	1	1
3 rd lactation	2	2	2	2
4 th lactation	0	0	2	1
5 th lactation	2	0	0	2
6 th lactation	0	0	0	1
7 th lactation	0	1	0	0

* as the polled herd was formerly also horned and at this stage in transition into a completely polled herd, there were some horned individuals remaining in the herd.

For each of the two herds, calves were housed in a calf area from which calves had, depending on the separation method, access to their dams in the adjacent cow barn. Calf access to the cow area was managed automatically via an automatic selection gate connected to a transponder in the neckband of the calves. When calves were restricted to the calf area, they had auditory and limited visual and physical contact with their dams through the bars in the fence separating the cow barn and the calf area (i.e., fence-line contact). The two calf areas were equipped with a resting (deep litter, 24.6 m²) and an activity area (rubber-coated and concrete floor, 71.9 m²). The groups were formed dynamically, i.e. newborn calves were continuously added to the group and older calves were taken out of the group after they had completed the weaning treatment, and group size did not exceed 23 and 20 calves for the horned and polled group, respectively. During the grazing period, calves had access to pasture together with the cow herd. Grazing period lasted until mid-November. Thus, pasture access in calves depended on the date of birth, and all but two calves received some access to pasture.

Each of the two calf areas was equipped with a concentrate feeder (Förster Technik GmbH, Engen, Germany) to which all calves had free access at all times with an allowance of 1.5 kg concentrate per animal and day distributed in portions of 50 g over the day. Individual identification of calves was ensured via a chip in the calves' neckbands. In the calf area the calves had ad libitum access to water and hay. Additionally, a total mixed ration (composition was the same as for cows) was provided freshly once a day in the afternoon. No milk was provided in the calf areas.

Treatments

In total, 36 cows and their calves were included in the larger experiment [22,23]. The 36 cow-calf pairs were evenly distributed amongst two separation methods according to the calves' sex (male/female) and the pair's herd affiliation (horned/predominantly polled). However, blood samples were only collected from $N = 33$ cows and $N = 33$ calves (see Table 1 for details). The age of cows included in the present study ranged from two to nine years, with an average of four years (mean \pm S.D. 4.0 ± 1.98 years) for both herds; 10 animals were primiparous cows. All calves were housed together with their dams in a calving pen for the first five days of life. Afterwards, the dams returned to their respective herd, and the calves were brought to the calf area. In the calf area, they were able to choose to either enter the cow herd and have contact with the dams or to stay in a calf area with their peers (i.e., away from their dams and the cow herd). Thus, they had full time cow-calf contact until the weaning and separation treatments started. Detailed description of all cow-calf related terms can be found in [18].

To separate the calves from their dams we used two methods: (1) **gradual separation** (by stepwise reduction of the time of dam-calf contact) and (2) a **nose-flap separation** method (Table 2). All calves in the nose-flap separation method were fitted with a nose-flap (Quiet wean; JDA Livestock Innovations, Canada) at the beginning of the first week of the three-week separation process. Nose-flap separated calves continued to have whole-day full contact to their dams for two weeks with the nose-flap, after which the nose-flap was removed. Simultaneously with the nose-flap removal, dam-calf pairs in the nose-flap separation only had fence-line contact (i.e., calves could no longer join the cow-herd) in the third week. In the gradual separation method, calves' access to the cow herd was reduced from whole-day contact to half day contact (i.e., 7 hour contact/day between morning and afternoon milking) in the first week and then morning contact (i.e., 3.5 hour contact/day after morning milking to midday) in the second week, and finally in the third week, analogous to the last phase in the nose-flap separation method, fence-line contact only. During access times, calves in the gradual separation method had unrestricted physical contact with their dams.

Dam-calf separation of half of dam-calf pairs in both separation methods began when the calves reached 12 weeks of age (i.e., early separation) and for the other half of the dam-calf pairs separation started at 14 weeks of calves' age (i.e., late separation). These two age groups per separation method were implemented to account for the different ages at full milk loss, which happened in the first week with nose-flap insertion in the nose-flap separation method and at the third week with fence-line contact in the gradual separation method (Table 2). Allocation to a separation time (i.e., early and late separation) was done in a balanced way for the sex of the calves, month of the study and herd of the animals.

Table 2. Timeline of separation method application. Approximately half of the dam-calf pairs (n=16) underwent a gradual separation (by gradually reducing the time of dam-calf contact) method, and the other half of the dam-calf pairs (n=17) a nose-flap separation (with a nose-flap) method. The separation methods were additionally both subdivided into early and late separation (with a two-week difference in the separation start).

<i>Calf's age</i> <i>in weeks</i>	<i>Nose-flap</i>		<i>Gradual</i>	
	Early	Late	Early	Late
≤11	Full time contact	Full time	Full time contact	Full time contact
12	Full time contact* ^{S1} with nose-flap	Full time contact	Half-day contact ^{S1} 8:30AM – 3:30 PM	Full time contact
13	Full time contact with nose-flap	Full time contact	Morning contact 8:30 AM – 12 PM	Full time contact
14	Fence-line contact	Full time contact* ^{S1} with nose-flap	Fence-line contact*	Half-day contact ^{S1} 8:30 AM – 3:30 PM
15	Total separation ^{S2}	Full time contact with nose-flap	Total separation ^{S2}	Morning contact 8:30 AM – 12 PM
16		Fence-line contact		Fence-line contact*
17		Total separation ^{S2}		Total separation ^{S2}

*calf is weaned; ^{S1} and ^{S2} indicate time points of sample collection; ^{S1}:before separation, ^{S2}:after separation.

Data collection

Physiological assessment

Hemogram and telomere analysis. All blood samples were taken by a qualified person and collected from the tail vein (*Vena caudalis mediana*) in cows and jugular vein (*Vena jugularis*) in calves into EDTA-coated sampling tubes. For the **hemogram**, we collected 2ml of blood from cows and calves twice per week over the whole 3-week treatment period (see 22, 23 for details). These samples were stored at 8°C until they were collected daily by a courier to be transported to a service laboratory (SYNLAB.vet GmbH, Hamburg) for haematological analysis on the same day of blood sampling. From the hemogram, the neutrophil-to-lymphocyte (N:L) ratio was calculated by dividing the numbers of neutrophils by the numbers of lymphocytes.

On the day of treatment start (day 0 for calves and day -1 for cows, S1 in Table 2) and at the last day of fence-line contact, i.e. 21 days later (day 21 for calves and day 20 for cows, S2 in Table 2) additional blood was taken for **telomere analysis**. This meant that, telomere samples were collected at 12 and 15 weeks of calves' age in the early separation groups and at 14 and 17 weeks of calves' age in the late separation groups (Table 2). At these two days, we collected the blood into 9 ml EDTA tubes and pipetted 2ml into the smaller EDTA tubes for the hemogram. The remaining EDTA blood was then refrigerated at -20°C within 1 h after collection. Every second week the blood was transferred to a different freezer in which samples were stored at -80 °C until the end of sample collection. Afterwards the samples were shipped to the Institute of Animal Breeding and Genetics at the Justus Liebig University Giessen for DNA extraction and further telomere analyses. Relative telomere length is expressed as the amount of telomeric DNA in relation to the amount of a reference non-variable copy number (non-VCN) gene that is constant in copy number. Relative telomere length of blood samples was estimated using the real time quantitative polymerase chain reaction (qPCR) method first established by Cawthon (2002) [24]. The reference gene *B2M* (*beta-2-microglobulin*), which is known to have a constant copy number [25], was used to quantify baseline DNA levels in samples and thus

allows measurement of the amount of telomeric DNA relative to genomic DNA input (i.e., RTL). For the qPCR two pairs of previously published primers [24,26,27] were used (Table SM1 in supplementary material). Primers were not used in a multiplex approach, but reactions for the reference gene and the amplifications of the telomeres were always run on the same plate for the same individual.

For inter- and intra-run calibration, standards and controls, consisting of a negative control, a calibrator sample and a 1:4 serial dilution of a standard sample, were included in each 96-well qPCR plate. Samples were mixed with primer pairs (*telomere or reference gene B2M*) and the LightCycler 480 SYBR Green I Master mix (Roche Diagnostics, Germany) resulting in a total volume of 20 µl per well. Controls and sample preparations were placed by hand onto the qPCR plate as triplicates, the latter one in a randomized order. However, this was done in a way that a single cow-calf pair from all four separation method and separation time combinations was analysed simultaneously on a given plate, and samples collected before and after exposure to each separation method were randomly assigned to a plate for each individual. This approach avoids potential confounds due to plate effects and run-to-run variation. Sample position remained the same before and after for most samples except for the cases when one sample had to be analysed again due to the failed quality control. This was the case for 6 cow and 8 calf samples. Those samples passed quality control at the second attempt. Run specifications were chosen according to Ilska-Warner et al. [26]. Raw data without baseline correction was extracted from the BioRad software for further analysis with LinRegPCR software [28].

Faecal glucocorticoid (i.e., cortisol) metabolites (FGCM). In both cows and calves, faecal samples (minimum 5g) were collected twice a week at 07:30 a.m. (with a deviation of ±60 minutes) by rectal stimulation (calves) or taken directly from the rectum (cows) from days -1 to 20 and 0 to 21 (cows/calves respectively) relative to the start of the separation methods. These samples were homogenized and transferred into commercial faecal sampling tubes, which were promptly placed into a cooling box and then transferred to a freezer at -20°C within 30 minutes. The samples remained stored at this temperature until further analysis.

The analysis of faecal samples in the laboratory followed a well-established protocol [29] utilizing an 11-oxoetiocholanolone enzyme immunoassay (EIA). This method has been identified as suitable for faecal cortisol metabolite (FGCM) analysis in cows [30] and calves [31].

Average daily gain (ADG, kg/d). Body weight of calves was determined at 7-day intervals between day 0 to 21 relative to the start of the separation methods, using a commercial cattle weighing scale (Patura Wiegekäfig, Wiegeset S1, Accuracy ± 1%). For the analysis, average daily weight gain (kg/d) for every week was calculated by dividing the weekly gain by 7 days.

Daily milk yield. Milk yield of the cows was recorded between days -7 and +27 relative to treatment start twice daily during milking with the Dairy Plan C21 herd management software (GEA Farm Technologies GmbH, Bönen, Germany). Cows were milked from about 05:15-08:30 am in the morning and 3:30-6:45 pm in the evening in a 2×4 tandem parlour (GEA Farm Technologies GmbH, IQ cluster, 38kPa, vibration stimulation, automatic stripping device and cluster removal).

Pathological assessment

Wounds of the nasal septum. As nose-flaps can lead to wounds and infection of the nasal septum [32], calves in the nose-flap separation method were checked for purulent and/or bloody excretion at the nasal septum after the removal of the nose-flaps (i.e., two weeks after the insertion).

Behavioural assessment

Direct observation of calf and cow behaviour was conducted consistently by the same observer on four consecutive days per week between days -7 to $+21$ (calves) and $+27$ (cows) relative to the initiation of the separation methods in all calf and cow areas. However, observations of the calves' lying areas with the calf brush and the calves' feeding areas were conducted via video recordings since these were not simultaneously visible with the rest of the barn during direct observations. The following behaviours were observed through direct observation:

1. **Vocalization:** Frequency was recorded continuously for both calves and cows. Vocalization was defined as clearly audible sounds produced through the mouth of a calf or cow [14].
2. **Searching behaviour:** Occurrence was recorded at intervals of every 3 minutes for both calves and cows. Searching behaviour was defined as movements parallel to, within 1 meter of, the pen partition, standing with its head through the selection gate, or standing beside the pen partition (within a radius of 2.5 meters) with the head elevated and eyes and ears focused in the direction of the calf's or cow's section, including scanning (adapted from [14,17]).
3. **Locomotor play:** Duration was recorded continuously for calves. Locomotor play was defined as galloping, jumping, leaping, bucking, or buck-kicking of a calf (after [33]).
4. **Social play:** Duration was recorded continuously for calves. Social play was defined as two calves standing front to front, pushing, rubbing, or butting head against head/neck without force, often including rotating head movements (adapted from [33]).
5. **Object play:** Duration was recorded continuously for calves. Object play by the calf was defined as butting water bowl, hayrack, or bars in the pen, standing up or butting straw, or rubbing head, throat, or neck in straw, kneeling down on the two forelegs [33].

Locomotor, social, and object play behaviours were combined into '**total play behaviour**' for analysis.

Direct observations occurred 2 hours following morning milking, 1 hour before the start of evening milking, and 2 hours following evening milking. These time points were selected because cows and calves were accustomed to being separated and reunited around milking times, exhibiting the most activity during these periods. Observations following the termination of morning and evening milkings began as soon as the cows and calves were allowed to reunite. The observer alternated between the two herds every 30 minutes, ensuring that each cow-calf pair was observed for a total of 2.5 hours daily. The start of observations alternated between the horned herd and the polled herd, with the schedule alternating between even and odd calendar weeks.

Video analysis of **total mixed ration (TMR) feeding time** and **brush use** in calves was conducted on the same four consecutive days as direct observations. Jovision Infrared Network Cameras with Jovision NVR System Software (Version 2.0.1.49) were utilized for this purpose. Total mixed ration feeding time was defined as the duration during which the calf's head was placed through the feeding rack, with the ears positioned in front of the rack. Brush use was defined as any physical contact with the brush by any body part of the calf while being automatically brushed or while scratching itself against the brush. This definition was adapted from [34]. Continuous observation was employed for both total mixed ration feeding time and brush use analyses. Animal behaviour was continuously recorded in intervals of 6 hours: night (00:00–06:00), morning (06:00–12:00), afternoon (12:00–18:00), and evening (18:00–

00:00). Specific intervals on days of treatment change were subsequently removed from the dataset; for example, the night interval of the day of nose-flap insertion, as the nose-flap was inserted in the morning. Analysis was conducted by four different observers for TMR feeding time (with Cohen's kappa ≥ 0.91) and three different observers for brush use (with Cohen's kappa ≥ 0.88). One observer had to be excluded due to insufficient inter-observer reliability for brush use. The software BORIS ([35], Version 7.9.22) was employed for the analysis of the video recordings. Cameras automatically switched to infrared mode in dark light conditions. For calf identification during the infrared mode, photos of the calves' individual coat patterns were utilized.

Automatic assessment was utilized to analyse the lying behaviour and locomotor play of calves. To assess **lying behaviour**, accelerometers (Hobo Pendant G data loggers, Onset Computer Corporation) were attached to 28 out of the 36 calves (14 per treatment). The 3-axis accelerometers were positioned directly above the metacarpal joint on the medial side of the left hind leg, tilted by 90° to ensure a better fit to the calves' legs. This orientation aligned the y-axis parallel to the ground and the x-axis perpendicular to the ground, pointing downward. Data loggers recorded g-force and the degree of tilt of the x-axis at 60-second intervals from day -28 to $+21$ relative to the start of the separation methods. The period from days -28 to -22 was designated as a habituation period. Due to limited storage capacity, the accelerometers were replaced every 14 days. Data retrieval was performed using HOBOWare software (Onset Computer Corporation, Version 3.7.17). Recordings obtained during the fitting or replacement of loggers, occurring between 07:00 and 10:00 a.m. on respective days, were excluded from analysis. The degree of vertical tilt (x-axis) was then utilized to determine the lying position of the calf. Readings $\leq 120^\circ$ indicated that the calf was lying down, while readings $> 120^\circ$ indicated that the calf was standing up (adapted from [36] for the tilted logger position). Subsequently, the recorded data were refined using an event filter. This filter identified and adjusted single outlier readings. For example, if a single lying event was surrounded by standing events, or vice versa, it was adjusted to reflect the behaviour preceding it. These adjustments were made because such readings were considered potentially erroneous [37]. From this processed data, the total lying time as a percentage per day and the number of lying bouts per day were computed.

For the automatic assessment of **locomotor play**, a subgroup of calves was chosen from the original cohort. Specifically, 28 out of the 36 calves, comprising 13 in the NF treatment and 15 in the GR treatment (all but 4 calves were identical to those used for the assessment of lying behaviour), were equipped with a second accelerometer. This accelerometer was positioned on the medial side of the right hind leg and was worn continuously for 9 hours each day, from 08:00 a.m. to 05:00 p.m., on days -4 , $+3$, $+10$, and $+17$ relative to the start of the separation methods. The accelerometers were tilted by 90° to guarantee more precise measurements. The loggers were programmed to measure the g-force of the vertical axis (x-axis) at a rate of 1 Hz, a methodology validated by Luu et al. (2013) [38]. Subsequently, the recorded data were exported using HOBOWare and analysed utilizing the peak acceleration method, as outlined by Größbacher et al. (2020) [39]. This method entails counting acceleration peaks recorded by the data loggers to identify instances of play events by the calves and thresholds to define peak accelerations were validated via video comparisons [23].

In cows, **lying behaviour** was recorded with Ice Tag leg sensors (Ice Robotics Ltd, Edinburgh, UK), which were attached to the left hind leg of each cow from days -14 to $+27$ relative to treatment start. If a lying bout was <4 minutes, it was deleted from the dataset as erroneous reading. **Rumination time** of cows was measured with RumiWatch noseband pressure sensors (Itin+ Hoch GmbH, Liestal, Switzerland) from day -14 to $+27$ relative to separation start. Days when leg and noseband sensors were fixed or removed (day -14 and $+27$) were omitted from the data since data were only available for part

of these days. Also, days -14 till -8 were omitted from the data set for rumination and lying times as habituation period.

In the final step, all data were corrected for illegitimate contact between cow and calf and ill health of the animals, as well as dams being in oestrus, before conduction of statistical analysis (see 22 and 23 for details).

Statistical analysis

For statistical analysis, we used the statistical software R studio [40] in R statistical program [41]. Sample size was determined a priori using G*Power 3.1, assuming a large effect ($f=0.5$) and accepting an α -error of 5% and a β -error of 10%.

Separation method. We analysed the treatment-related data via ANCOVA, with RTL after separation (final RTL) as the outcome variable, which is a suggested method for analysing RTL data obtained by the qPCR method [42]. Predictor variables included in the global statistical model for calves were separation method (nose-flap or gradual), separation time (early or late) and their interaction, herd (horned or polled), sex, and q-PCR plate number. Predictor variables in the global model for dams were separation method, separation time and their interaction, herd, age, and q-PCR plate number. Parity was not included in the model, because previous research did not show an effect of parity on behavioural indicators in response to separation [43] and due to a correlation between age and parity. There are individual differences in telomere length already at birth and they remain present throughout life. Thus, initial RTL was included as a covariate to correct for the ‘regression to the mean’ [44] in both dams and calves.

We selected best models using AICc and the function dredge (package *MuMIn*), and separation method and initial RTL (i.e., covariate) were fixed terms. The selected models contained separation method, separation time as predictor variables, and initial RTL as covariate. We also checked whether the presence of the purulent and/or bloody nasal septum excretion resulted in shorter RTL. For this we fitted an ANCOVA model for the calves of the nose-flap separation method using status of the nasal septum (binary response: purulent and/or bloody excretion vs. no or transparent excretion) and the covariate initial RTL as predictor variables.

Prior to fitting the models, we inspected the distribution of final RTL, which appeared to be symmetrically distributed. To verify the assumption of the models, residuals were checked for normal distribution using a Shapiro–Wilk test, histogram and qq-plot and for homogeneity of variance using a Levene test and by plotting residuals against fitted values. Also collinearity was not an issue (maximum Variance Inflation Factor: 1.144 [45]). To ensure that initial RTL did not differ between separation methods and separation times [46] we tested for ANCOVA’s assumption of independence of the covariate and fixed factors. This was done with an ANOVA, in which RTL before separation introduction (initial RTL) was treated as independent variable and separation method and separation time as predictor variables. We also visually inspected that there was no correlation between initial RTL and separation method and separation time with boxplots where separation method and separation time were depicted on the x-axis and initial RTL on the y-axis.

To test the significance of the predictors as a whole and avoid ‘cryptic multiple testing’ [47] we compared the fit of the full model with that of the null model comprising only the initial RTL. The estimates of the coefficients were obtained using a ‘summary.lm(model)’ function. Estimated marginal

means (adjusted for initial RTL) and standard errors of final RTL from ANCOVA were calculated using the package *emmeans*.

Behavioural and physiological stress indicators. We separated the data for cows and calves, as well as for each separation method, calculated average values for each measure over a three-week separation period and utilized histograms to inspect the data for normality. Subsequently, we calculated correlations between final telomere length and averages of other physiological and behavioural stress indicators, as well as average daily weight gain and daily milk yield. For this correlation analysis, we used the `pcor.test` function with the Spearman method [48]. Additionally, we included initial telomere length as a covariate to correct for the 'regression to the mean'. This approach helps to account for any baseline differences and strengthens the accuracy of the correlation analysis. Due to multiple comparisons we used `p.adjust` function with a holm method to adjust p-values.

Results

Separation method

The estimated marginal means and standard errors of final RTL (adjusted for initial RTL) of cows and calves in different separation methods and separation times are shown in Table 3 and model outputs are reported in Table SM 2 and 3 in the supplementary material.

In dams, the gradual separation method tended to result in shorter final RTL (1.16 ± 0.08) compared to the nose-flap separation (1.36 ± 0.08 , $T = -1.725$, $P = 0.095$), and separation time did not affect final RTL ($T = 1.536$, $P = 0.135$). In calves, separation method did not affect final RTL (Table 3 and SM 2; $T = -0.310$, $P = 0.759$), but there was a tendency for late separation to result in shorter final RTL (1.03 ± 0.08) than early separation (1.22 ± 0.07 , $T = -1.819$, $P = 0.079$). In calves, the covariate (initial RTL) had a significant effect on final RTL ($T = 2.667$, $P = 0.012$) indicating that the initial RTL had a positive relationship with the final RTL and that it adjusted the model output of the dependent variable (final RTL). There was no effect of initial RTL ($T = 1.071$, $P = 0.293$) on final RTL in dams. Initial RTL was not different for both separation methods and separation times in either dams or calves (Tables SM5 and SM6).

Six out of eighteen calves in the nose-flap separation method developed wounds with purulent and/or bloody excretion of the nasal septum. Numerically, we observed that calves with purulent and/or bloody wounds had shorter final RTL. However, testing for statistical significance revealed no effect of nasal septum wounds on final RTL ($T = -0.850$, $P = 0.409$; Table SM 4 in the supplementary material). Also in this model, the covariate (initial RTL) had a positive relationship with the final RTL ($T = 4.152$, $P = 0.001$).

Table 1. From ANCOVA estimated marginal means (adjusted for initial RTL) and standard errors (SE) of final RTL (TL/non-NVC).

	<i>Cows</i>				<i>Calves</i>			
	<i>n</i>	<i>mean</i>	<i>SE</i>	<i>P</i>	<i>n</i>	<i>mean</i>	<i>SE</i>	<i>P</i>
<i>Separation method</i>				0.095				0.759
<i>Nose-flap separation</i>	17	1.36	0.08		18	1.15	0.07	
<i>Gradual separation</i>	16	1.16	0.08		17	1.11	0.08	
<i>Separation time</i>				0.135				0.079
<i>Early</i>	16	1.17	0.08		18	1.22	0.07	
<i>Late</i>	17	1.35	0.08		17	1.03	0.08	
<i>Nasal septum condition</i>								0.409
<i>Excretion*</i>					5	1.07	0.08	
<i>No excretion</i>					13	1.20	0.13	

*Excretion: bloody or purulent excretion at the nasal septum where the nose-flap had contact.

Behavioural and physiological stress indicators

None of the other physiological or behavioural measures showed statistically significant partial correlations with the final relative telomere length (RTL), as indicated in Table 4. Additionally, daily milk yield in cows (nose-flap: $r_s = -0.016$, $P = 10.953$; gradual: $r_s = 0.341$, $P = 0.213$) and average daily weight gain in calves (nose-flap: $r_s = 0.295$, $P = 0.250$; gradual: $r_s = 0.122$, $P = 0.653$) were also not correlated with the final RTL.

Table 2. Partial correlation estimates (r_s) and P-values between final RTL and the stress measures from the Spearman correlation tests.

<i>Parameter</i>	<i>Cows</i>				<i>Calves</i>			
	<i>Nose-flap</i>		<i>Gradual</i>		<i>Nose-flap</i>		<i>Gradual</i>	
	r_s	P	r_s	P	r_s	P	r_s	P
<i>Physiological stress indicators</i>								
<i>N:L ratio</i>	0.207	0.918	0.207	0.459	-0.133	1	0.228	0.79
<i>FGCM</i> ¹	-0.038	1	-0.039	1	-0.158	1	-0.331	0.422
<i>Behavioural stress indicators</i>								
<i>Vocalisations</i>	-0.187	1	-0.033	1	0.147	1	-0.1	0.714
<i>Searching</i>	-0.172	1	0.011	1	-0.018	1	-0.304	1
<i>Total play</i> ²					-0.424	0.63	0.069	1
<i>Locomotor play</i> ³					0.079	1	-0.243	1
<i>Lying duration</i>	-0.295	1	-0.458	0.915	-0.434	1	0.245	1
<i>Lying bouts</i>	0.255	1	-0.003	1	-0.258	1	0.331	1
<i>Brush use</i>					-0.050	1	-0.157	1
<i>Rumination</i>	-0.554	0.48	-0.315	1				

¹ Faecal glucocorticoid (i.e., cortisol) metabolites

² direct observation

³ automatic assessment

Discussion

This study compared the effects on telomere length of dams and their calves undergoing either a nose-flap or gradual separation method. Although in cattle telomere length has been widely studied mainly in relation to fitness traits associated with survival, longevity, health, and reproductive use [25,26,49], knowledge about the effect of psychological stress on telomere length is lacking in this species. This study is the first step to filling this information gap as dam-calf separation is a known psychological stressor in cattle [9,10]. We hypothesised that compared to the nose-flap separation, gradual separation would result in less overall stress and longer RTL in calves and to be cumulatively more stressful and lead to shorter RTL in dams. Indeed, our study found a tendency for shorter final RTL, indicating higher stress levels, in dams undergoing gradual separation as compared to the nose-flap separation method. However, we identified no significant effect of the separation method on final RTL in calves. We also hypothesised that behavioural and physiological indicators of stress will correlate with telomere length. This hypothesis was not confirmed as none of the measures correlated with the final RTL.

We collected samples for telomere length measurements three weeks apart – immediately before the separation process began and at the end of the fence-line contact period. Studies on other (shorter lived) species showed an effect of stress on telomere length after nine days in young and four weeks in adult animals [1]. Thus, in dams three weeks may have been a too short interval between samplings. Additionally, behaviour analysis of the same animals revealed that differences between separation methods in the number of vocalisations and proportion of searching behaviour in cows and calves were greater during the fence-line contact phase compared to other separation phases (i.e., insertion of nose-flaps and reducing the contact time) [22,23]. Fence-line contact was only introduced one week before the second telomere sample collection. Thus, differences in cumulative stress indicators such as RTL between separation methods as well as correlations between behavioural and physiological stress indicators and RTL would likely be greater at a later sampling time point, i.e., at least nine days and four weeks after introduction of fence-line contact in calves and dams, respectively.

Given that our sampling interval was short, RTL results probably reflect mostly the initial phase of the separation process. The found tendency for shorter final RTL in dams of the gradual separation method compared to nose-flap separated dams, might therefore indicate that the restriction of nursing by the calves was less stressful to the dams than separation from the calf over night and part of the day. This could be explained by the fact that nose-flap calves were still present in the cow area and dams could express other types of affiliative behaviours with their calves. In this regard, it has been previously shown that suckling increases oxytocin levels [50], which is among others involved in the establishment and maintenance of social bonds [51]. Likely related to this, motivation in dams to re-unite with their calves is increased in dams that are suckled by their calves [11]. This could further explain why nose-flap cows which were not suckled but had access to their calves, were less stressed than gradual reduction cows which were still suckled but were temporarily separated from their calves at the measured time point.

Additional to the suckling, various other types of interaction, which could also influence social bonding between dams and their calves, were restricted by the separation methods. For example, play and lying close to the calf were restricted in the gradually separated dam-calf pairs to the time when calves were permitted to visit the dams and nose-flap separation calves were prevented from licking the dams. However, the effects of these types of interactions on separation stress have not been studied so far.

Dams respond to vocalisations of their calves by walking towards the location of vocalisation [52]. Gradually separated calves vocalised more over the whole separation period, but especially during the

last phase of separation (i.e., fence-line contact) [23]. This corresponds with the proportion of searching behaviour, which showed a similar pattern as vocalisations [23]. Thus, it is possible that dams in the gradual separation method experienced stress in response to vocalisations of their calves, especially since they could not reach the calf. The effect was minor, but our results on RTL indicate that preventing calves to lick and suckle the dams in the nose-flap separation method is overall less stressful to the dams than restricting the amount of time they could be in full physical contact with their calves in the gradual separation method.

Milk production is thought to be a physiological stressor [53]. Dams in both separation methods continued to be milked twice a day as before the separation began. Analysis of the milk yield from the dams of the same experiment showed that the nose-flap separation resulted in a higher milk yield during machine milking than gradual separation when considered over the whole 3-week separation period [22]. However, in cow-calf contact systems, milk obtained in the milking parlour is not a good indicator of milk production as it is affected by various factors related to suckling and machine milking prior to weaning, including milk ejection efficiency [54]. Furthermore, milk production has not been shown to affect telomere shortening in cattle [8]. Thus, the difference between the separation methods regarding milk production is an unlikely explanation for this trend in the effect of separation method on RTL.

There was no significant effect of separation method on RTL in calves, which is in accordance with the physiological measures from our animals as reported elsewhere: faecal cortisol metabolite concentrations and differential blood count over the whole separation process did not differ between the separation methods [23]. Behavioural indicators and some physiological measures collected for the same animals showed differing effects of the separation method on animal welfare [23] and do not offer an explanation for our findings. Although both separation methods induced behavioral and physiological distress responses, a gradual reduction of contact time to the dam was favorable compared with weaning with a nose-flap in most indicators [23]. For example, gradually weaned calves showed higher weight gains and a lower decrease in lying bouts and locomotor play levels compared with baseline, indicative of higher energy levels and a potentially less compromised welfare [23]. Moreover, presence of the nasal septum wounds was observed in reaction to nose-flap weaning and not to gradual weaning [23]. As telomere shortening decelerates in response to increased locomotor levels and accelerates in wounded animals [1], it was contra to our expectations that this was not reflected in a shorter RTL in our study. However, the mechanisms of telomere shortening are complex and also may be influenced by other factors such as body weight, as in theory, faster body growth requires more cell divisions and, therefore, bigger sized individuals of the same species have shorter telomeres [55].

The two separation methods likely led to different behavioural needs being unfulfilled. Nose-flaps in the nose-flap separated calves prevented suckling, and possibly restricted the ability to perform social or self-grooming [18], which could negatively affect calves' welfare. Gradual separation limited the time allowed to spend in physical contact with the dams and other cows [18]. With advancing age, calves in semi-natural conditions spend increasingly less time with their dams and more time with other calves [56,57]. However, they stay under supervision of another cow from the herd while the dams are away [56,57]. Being forced to spend time away from any adult cow while not in contact with the dams may thus additionally negatively affect their welfare. Hence, it is likely that calves in both types of separation methods were frustrated due to unsatisfied behavioural needs, which resulted in no significant differences with regard to cumulative stress level (i.e., final RTL). In further studies, it would be interesting to investigate, if allowance of contact to one or more unrelated, adult cows could help to alleviate negative welfare effects of separation from the dam as in horses [58].

We found a tendency for shorter final RTL in calves undergoing late separation compared to early separation, whereas in dams, final RTL was not affected by separation time (early vs. late). Telomeres shorten with each cell division [59] resulting in shorter telomeres in older animals compared to younger individuals [49]. Nevertheless, there was no difference in the initial RTL suggesting that the difference in the final RTL likely originates from the separation stress. This is interesting as other data in the same calves showed that late separated calves likely experienced less stress than early separated calves as seen by a lower decrease in play behaviour compared to before separation [31]. Also non-related studies showed less separation stress in older calves as seen by fewer vocalisations and lower increase in cortisol levels after the separation [60,61]. Thus, the tendency for shorter RTL in calves of the late separation treatment in our study may point at confounding factors related to age such as increasing size and thus space requirements which may be fulfilled decreasingly in the calves' area but are not related to separation and weaning stress itself [62,63]. However, while telomere length generally decreases with age, the rate of telomere shortening can vary [49]. This variation may account for the differences observed between the early and late separation methods.

Diet can affect telomere length in humans [64], but this effect has not been confirmed in adult cattle [8]. No information on calves could be found. Under semi-natural conditions, dams usually nurse their calves for 8–12 months, until shortly before their next calving [65]. Calves in our study were weaned at a much younger age than under semi-natural conditions. Thus, a difference of two weeks as in our study is likely too small to lead to strong differences in calves' welfare due to changes in diet that would be reflected by RTL. Calves were also weaned at an older age compared to most other commercially kept calves, which are usually weaned at 8–12 weeks, but sometimes even as early as 6 weeks of age [66–68]. Future studies should investigate the effects of other weaning ages on RTL.

Dam-calf pairs can be separated after various lengths of contact times and with different methods. We only compared two separation methods. To get more comprehensive information about the effect of a given separation method as well as age at separation on welfare, the separation methods used in this study should be compared to abrupt separation after prolonged dam-calf contact, separation at birth as usually done in conventional dairy farms (negative control) and not separated dam-calf pairs (positive control). Furthermore, additional external factors such as weather conditions and health that were not considered in this study might have confounded the results [1,8]. This study revealed no age or sex effects on telomere length in dams and calves, respectively. Numerically it appears that cows had longer telomeres than calves, however, this was not statistically analysed and due to a high inter-individual variation in telomere length, may not be statistically significant. Telomere shortening slows-down in adult cattle and may be difficult to detect as previous research indicated [49]. Males usually have shorter telomeres compared to females [69], however it seems that the sex effect on telomere length appears later in life [70,71].

Nose-flaps caused wounds with purulent and/or bloody nasal excretion in six calves (23). Inflammations and infections may affect RTL in certain conditions through increased cell division, direct damage to telomeres via oxidative stress, or reduction of telomere repair associated with physical and psychological stress [1]. However, we did not see an effect of nasal septum wounds on final RTL. This may be due to a small number of affected calves. Additionally, it is likely that the stress associated with impaired health was not strong enough to affect RTL within the short period in our study. However, another study showed no effect of udder health and lameness in cows on RTL [8] indicating that RTL may not be a valid indicator of health in cattle.

Quantitative PCR is a standard method to measure telomere length. However, results from southern blotting, the much more laborious gold standard method, have a higher statistical power, e.g., due to lower measurement errors [42]. We cannot exclude that measurement of telomere length by southern blotting and/or in a higher number of animals would have led to higher precision. However, in this study, resources were not available to establish southern blotting. Including a higher number of experimental animals to increase the statistical power was not possible, because this study was part of a larger project on behaviour and physiology, and sample size was planned for these parameters [22,23].

Conclusion

Our study shows no significant effect of weaning and separation method on RTL in dairy calves after weaning and separation from their dams, which is in line with physiological stress indicators in other analyses considered over the whole separation period [23]. However, a tendency for shorter RTL in gradually separated dams compared to nose-flap separated dams could indicate that gradual separation may be overall more stressful for dams. In contrast to previous reports [60,61], our results (i.e., shorter RTL in late separated calves) point to greater overall stress in late separated calves, though the finding was only a tendency and may be confounded with other age-related factors such as calves space requirements [62,63], which should be investigated further. Although telomeres are thought to be a promising indicator of cumulative stress [1], we suggest more precise measurement methods, e.g., southern blotting to be used in future studies on dam-calf separation stress. Further, we suggest that future studies should aim to analyse the effect of various separation methods over a longer period than 21 days, as used in this study, and ideally include a non-separated group as reference.

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Supplementary material

Table SM 1. Primer pairs used to amplify the reference gene and the target (telomere).

Primer name (concentration)	Primer sequence	Reference
Telomer 1b (10 pmol)	5'-CGG TTT GTT TGG GTT TGG GTT TGG GTT TGG GTT TGG GTT-3'	(25,26)
Telomer 2b (10 pmol)	5'-GGC TTG CCT TAC CCT TAC CCT TAC CCT TAC CCT TAC CCT-3'	
B2M 1b (10 pmol)	5'-GCTTCTGACACAACACTGTGTTCACTAGC-3'	(23,25)
B2M 2b (10 pmol)	5'-CACCAACTTCATCCACGTTCCACC-3'	

Table SM 2. Model output for dams.

Response: final RTL	Estimate	SE	T value	P-value
Intercept	0.944	0.296	3.191	0.003
S. method_gradual	-0.199	0.115	-1.725	0.095
S. time_late	0.181	0.118	1.536	0.135
Initial RTL	0.269	0.251	1.071	0.293

Table SM 3. Model output for calves.

Response: final RTL	Estimate	SE	T value	P-value
Intercept	0.879	0.171	5.139	0.000
S. method_gradual	-0.033	0.106	-0.310	0.759
S. time_late	-0.191	0.105	-1.819	0.079
Initial RTL	0.288	0.108	2.667	0.012

Table SM 4. Model output for nose purulent and/or bloody excretion in calves in the nose-flap separation method.

Response: final RTL	Estimate	SE	T value	P-value
Intercept	-0.173	0.337	-0.514	0.615
Excretion_yes	-0.135	0.159	-0.850	0.409
Initial RTL	1.033	0.249	4.152	0.001

Table SM 5. Model output of the independence testing in dams.

Response: initial RTL	Estimate	SE	T value	P-value
Intercept	1.115	0.069	16.234	0.000
S. method_gradual	0.097	0.082	1.183	0.246
S. time_late	0.131	0.082	1.596	0.121

Table SM 6. Model output of the independence testing in calves.

Response: initial RTL	Estimate	SE	T value	P-value
Intercept	1.348	0.147	9.172	0.000
S. method_gradual	-0.158	0.171	-0.924	0.362
S. time_late	-0.029	0.171	-0.172	0.865

General discussion and conclusion

General discussion

1.1 Summary of results

Cow-calf-contact (CCC) systems can contribute to a more sustainable dairy sector that better aligns with public demands (Busch et al., 2017; Sirovica et al., 2022) and improves the animals' overall welfare (Meagher et al., 2019), especially with regard to opportunities to engage in highly motivated behaviors (Wenker et al., 2020, 2021; Johnsen et al., 2021). In order to achieve implementation of CCC systems in practice on a larger scale, there is, however, a high necessity for development of practical and low-stress weaning and separation strategies, since this is one of the major problems reported by farmers already practicing CCC (Eriksson et al., 2022; Hansen et al., 2023).

In order to contribute to the advancement of low stress separation strategies for dairy CCC systems, the present thesis examined two different weaning and separation methods that were previously hypothesized to have considerable potential to reduce stress of cows and calves during the process: two-step weaning and separation with nose flaps and a gradual weaning and separation method. Two-step weaning with nose flaps separates the loss of nursing and complete loss of contact to the cow (or for dams: contact to the calf) in time and is repeatedly mentioned by farmers as a practical approach to reduce separation stress in CCC systems (e.g. "De Öko Melkburen", Germany or "The Ethical Dairy", Scotland). On the other hand, a gradual reduction in cow-calf-contact time partly mimics the naturally occurring reduction of milk intake during natural weaning (Reinhardt and Reinhardt, 1981a) and had not yet been scientifically investigated at the time point of determination of the experimental design for this thesis (in 2019). In order to achieve a comprehensive comparison of these separation methods, this thesis additionally examined the use of fecal cortisol metabolites in calves (**study I**) and relative telomere length in both cows and calves (**study V**) as stress indicators as a secondary aim, since these indicators are hypothesized to allow, to a limited degree, a more cumulative measurement of the stress the animals experience, compared to the commonly used plasma or saliva cortisol measurements (Ashley et al., 2011; Palme, 2012; Bateson, 2015).

Examination of the calves' behavioral and physiological stress responses to the two studied weaning and separation methods revealed that both methods caused stress for the calves, but that overall the GR method was favorable for calves compared to weaning with a nose flap (**study II**). The abrupt change in diet during two-step weaning with the nose flap seemed to compromise adaption of the gastrointestinal tract (GIT), potentially even causing local inflammation, and led to a greater energy deficit in NF calves compared with the gradual weaning method, as indicated by lower weight gains and locomotor play levels as well as an increase in inflammatory blood markers in these calves (**study II**). This parallels reports of a negative effect of two-step weaning with nose flaps on weight gains in 2-month old dam-reared dairy calves (Wenker et al., 2022a) as well as in 6 to 7.5 month old beef calves (Haley et al., 2005; Boland et al., 2008; Enriquez et al., 2010), which underlines that abrupt milk removal is physically stressful regardless of age. Furthermore, a severe reduction in lying bouts and locomotor play behavior during the time when calves' wore the nose flaps along with the high number of unsuccessful suckling attempts in our study, additionally point towards a negative affective state of frustration in these calves. It was of further welfare concern that the nose flap also caused pressure marks and partly even injuries at the nasal septum in the majority of tested

calves when flaps were removed after the 14 day period (**study II**). Since the motivation of our NF calves to spend time with the cows remained at a similar level as during the baseline for 4-5 days after insertion of the nose flap (**study III**), but other studies reported open wounds at the nasal septum already after 5 days of nose flap usage in older beef calves (Valente et al., 2022), it is likely that a functional reduction of the suckling motivation with the two-step method will in some cases simultaneously compromise the physical integrity of calves. This is likely, since results of other studies conducted in the meantime revealed that nasal tissue damage occurs irrespective of calf age, weight or duration of usage (Lambertz et al., 2015 (7.5 mo old calves, 7 days of usage); Freeman et al., 2021 (8 mo, 7 days usage); Valente et al., 2022 (8 mo, 5 days usage); Wenker et al., 2022 (2 mo, 14 days usage); Kirk and Tucker, 2023 (7 mo and different weights, 7 days usage); Vogt et al., 2024 (3 mo, 14 days usage)). Collectively, the results of this thesis suggest thus a compromised physical state for two-step separated calves, due to nasal tissue damage and problems for adaption of the GIT, likely also provoking hunger, as well as a compromised mental state with high levels of frustration, so that a two-step separation with the nose flap cannot be classified as a high-welfare weaning method for calves. In contrast to this, we saw higher weight gains, a lower decrease in lying bouts as well as a lower decrease in locomotor play levels compared to baseline in gradually weaned calves, indicating a better adaption of the GIT to the dietary change as well as a likely less compromised affective state. In line with this, a gradual weaning method (by reduction in milk amounts or dilution of milk with water) is also the generally recommended method compared to abrupt weaning for artificially reared dairy calves, leading i.a. to better weight gains and a better rumen development (Khan et al., 2007a,b; Jasper et al., 2008; Sweeney et al., 2010; Steele et al., 2017). Given that there were no differences in neither the behavioral nor physiological indicators of stress in cows when separated with the two-step or gradual separation method (**study IV**), it can be concluded that a gradual separation is overall favorable for weaning and separation of cow-calf pairs compared to two-step separation with nose flaps.

Nonetheless, there were great individual differences between cows in both treatments and, despite the better physical adaption and higher weight gains of GR calves, a marked behavioral response during the gradual weaning and separation process was evident in the GR pairs. Both cows and calves reacted with a considerable behavioral response in terms of vocalizations and searching behavior to introduction of fence-line contact in the third week of the GR treatment (**study II & IV**) which completely terminated the suckling opportunity and reduced contact to auditory, visual, olfactory and very limited tactile contact through the fence. In calves, we observed an additional, numerical decline in locomotor play levels in this week (**study II**), while dams still showed a behavioral response when calves were moved to the youngstock barn one week later (**study IV**). Additionally, analysis of relative telomere length (RTL) suggested that the gradual weaning and separation method induced more stress in cows compared to the two-step separation (**study V**), but this result was only a very weak tendency and validity of RTL results is limited due to a too short sampling interval (see section 1.3 about indicators for weaning and separation stress). Taken together, this demonstrates that the gradual weaning and separation method still provoked considerable stress and frustration for cows and calves during the process, which is in line with findings by Neave et al. (2023) who used a very similar gradual separation protocol to ours. This clearly calls for future refinement of the GR method from the protocol used in our study and recommendations for this, as highlighted by this thesis and current literature, are given in the section below.

1.2 Possible options for future refinement of the gradual weaning and separation method

1.2.1. Using a higher number of smaller reductions in contact time over a longer period

As already discussed in detail in **study IV**, the high behavioral response of cows and calves to introduction of fence-line contact was probably related to the fact, that our gradual weaning and separation method worked more in a stepwise than gradual manner, since milk yields of cows suggested that calves drank comparable amounts of milk during the week with half-day and morning contact (**study IV**). Thus, prevention of the nursing opportunity in the third week with fence-line contact was likely experienced as very abrupt and stressful since calves still consumed a high amount of about 7-8 liters of milk from the cow at this time point (see milk yields **study IV**). This can partly explain the high number of vocalizations and searching behavior of calves (**study II**) and cows (**study IV**) in the third week. As already elaborated in detail in **study IV**, this might be caused either by compensation of calves, which drank more milk in the remaining hours, or by the fact that the step of reduction of half day- to only morning contact in the GR method did not significantly interfere with established nursing patterns of the pairs, as indicated by the low number of observed nursing bouts during the afternoon observation in the two weeks beforehand. In either case, the reduction of cow-calf contact from half-day to morning contact in our study seemed not to greatly reduce daily nursing time of the pairs. In support of this Hvidtfeldt Jensen (2024a), using the same cow-calf pairs as the study by Neave et al. (2023) that compared abrupt separation to gradually reducing contact time over 2 weeks (50%, then 25% per week of original contact time) before total separation of cow-calf pairs, reported that the reduction in daily contact times did not consistently reduce the measured suckling time in all of their calves. In a companion study, they additionally found that the cows whose contact to the calf was gradually reduced over 2 weeks, pushed higher weights in a 'maximum price paid' test during the restricted suckling period than cows which remained with full-time contact to their calves until abrupt separation (Hvidtfeldt Jensen, 2024b). This indicates that a reduction in daily contact increased the cows' motivation to nurse the calf, rather than decreased it, potentially since it made the access to the calf more valuable to the cows, comparable to other sparse resources in nature (Hvidtfeldt Jensen, 2024b). Together with the partial compensation of suckling time (Hvidtfeldt Jensen, 2024a), this might explain why the employed gradual reduction of contact time in their study was equally not successful in inducing a gentle, gradual weaning and separation of pairs (Hvidtfeldt Jensen, 2024b).

In case that this hypothesis is true and access to the calf gets more valuable with decreasing contact time, as long as pairs have one full nursing bout that maintains the bond, a modification of the gradual weaning and separation method by incorporating a higher number of smaller steps could potentially also not lead to satisfactory results when pairs are completely separated. In a hypothetical scenario in which contact time is reduced by 30min daily over 48 days, it is possible that the majority of contact time reductions will only lead to weak responses from the animals, like seen for our week with morning contact (**study II & IV**), but that the animals will react with a very strong response when the last 30min are taken at which time a full nursing was still possible up to this time point and which was highly valuable to the animals. This is at least what is suggested by the temporal distribution of behavioral responses in the GR treatment of our studied cows and calves (**study II & IV**) and by the previous suggestion that unfulfilled anticipation of reunion events at learned time points plays a major role in mitigation of the stress

response at separation (Neave et al., 2023, discussed in detail in **study IV**). In support of this theory, dairy cows that had only 20 min daily nursing contact to their calf prior to morning milking for 13 weeks, also reacted with a considerable vocal response to separation which remained present for 3 days (Nicolao et al., 2022). In addition, calves can consume more than 10 kg of milk/day when allowed to suckle for short periods of 30mins twice daily (Fröberg et al., 2008), which equally indicates that gradual weaning off milk will likely not take place as long as calves maintain short periods of full-time contact to their dam that allow a full nursing bout. On the other hand, ewes and lambs that were separated by a gradual reduction in contact time over a 12 week time span up from 3.5 weeks of age, showed a considerable vocal response when separated for the first time and when the duration of daily separation was increased to 8 hours, but afterwards there was only a weak vocal response to further reductions in contact time or to complete separation at 15.5 weeks of age (Orgeur et al., 1998). This is partly in line with results in dairy CCC calves gradually separated from the dams over short (10 days) or long durations (28 days), which also showed the strongest vocal response to the first reduction in contact time, after which it declined and remained on a lower level, but still present, in response to subsequent further reductions of contact time (Johnsen et al., 2024). It has to be noted though that these results were influenced by provision of additional milk, up from the beginning of the separation process, to the calves. Nonetheless both studies suggests, that animals can partly habituate to the daily separations and that a subsequent final separation does not lead to an additional strong stress response then. Further research is needed here to determine if there is a necessary minimum time span for such a longer separation process with more frequent steps, which stimulates enough solid feed intake of calves and avoids a compensatory response by the pairs in the remaining short contact times, to successfully induce the weaning and separation process. Only then, the total separation from each other will likely cause comparably lower stress levels in the animals. In order to achieve this, it might especially be worth testing to gradually increase the interval length of separation of the pairs, like done in the study with lambs (Orgeur et al., 1998), since the increasing time without opportunity for suckling will likely better stimulate solid feed intake and better support the transition of the GIT compared to more uniform steps of such a longer contact time reduction. If so, this modification of the gradual weaning and separation process compared to the rather stepwise process employed in this thesis (discussed in **study II & IV**), might be a feasible option for indoor rearing when affordable technical solutions with automatic gates are available to monitor such frequent reductions in contact time and additionally avoid unauthorized access of calves to the cow herd (like described in **study II**).

1.2.2 A fence-line separation process that ensures reduced milk ingestion by calves

A further possible option for refinement of the GR method to achieve a truly gradual reduction in milk intake by calves might be achieved with the help of a fence, where the pairs have an unrestricted nursing opportunity at their usual times through the fence (see fig. 1) as a first step. After this, weaning could be induced by closing the fence off in the middle or driving the cow (or calf) away from the fence, after a designated amount of minutes of nursing, which is gradually decreased. This would prevent a full nursing event and could thus truly reduce milk intake by calves in a gradual manner, which would hopefully stimulate a higher increase of solids. Since cows in the GR treatment showed a high behavioral response when contact to the

calves at the anticipated times was suddenly completely prevented (**study IV**), this gradual reduction in minutes of nursing time at routine time points could potentially also reduce the stress response, as the routines are not completely broken. This assumption needs, however, to be verified in an experimental study.

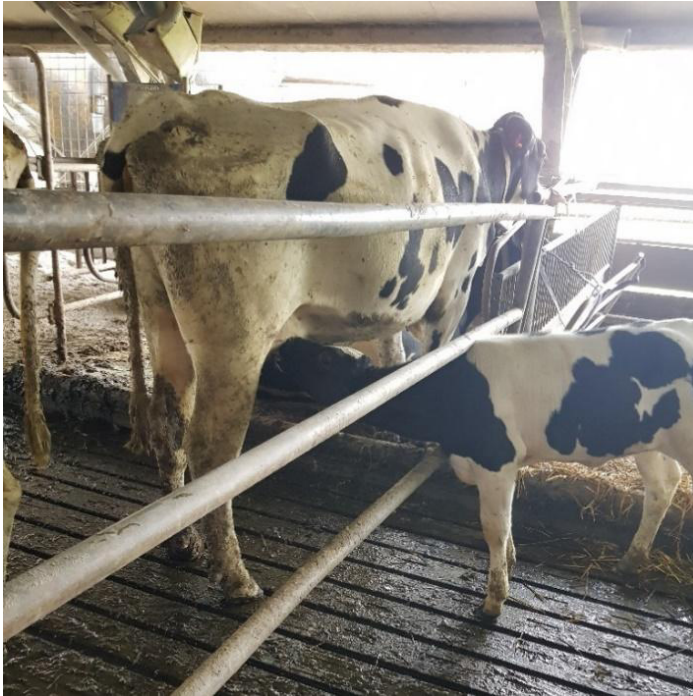


Fig. 1) Fence-line separation with a fence through which calves can suckle

Photo credits: Margret L. Wenker, Source: "Supplementary material S2 – Illustration of debonding strategies" published with the article Wenker et al. (2022) under the terms of the Creative Commons CC-BY 4.0 license (<https://creativecommons.org/licenses/by/4.0/>). No changes were made to the picture.

In a last step, a longer phase of partial contact between cow and calf without the opportunity for suckling should be provided. An abrupt separation at this point is not advisable, since the continued allowance of even the reduced nursing time will likely maintain the bond between cow and calf, as it has been shown that social contact between mother and young, including suckling, leads to oxytocin and opioid release and has a rewarding effect in various species (Nelson and Panksepp, 1998; Lupoli et al., 2001; Uvnäs-Moberg et al., 2001). In cows kept in CCC systems, it has been shown that the nursing opportunity increases the behavioral response to separation after a 6 week nursing phase, compared to not suckled cows (Johnsen et al., 2018) and that cows with partial contact to the calf without a nursing opportunity showed a decreased expression of affiliative behaviors towards the calf compared to dams whose calves could suckle (Wenker et al., 2021). This both underlines that the cow-calf bond is strengthened and maintained through suckling. During the studies conducted for this thesis, prevention of suckling with the nose flaps likewise reduced the behavioral and partly physiological response of cows and calves to fence-line separation in the third week compared to GR pairs which had continued nursing opportunities (**study II & IV**). Thus, even after complete reduction of nursing time, continued partial contact between cow and calf is advisable. This could be achieved by closing the fence off in the middle to prevent any further suckling, but building the fence low enough so that the cow can reach the calf by putting the head above the fence, allowing her to lick and sniff it, similar to a visiting window. With some adjustments this

method could be feasible in a barn setup where the (weaner) calves' pens are somewhere located close to the walkways of cows to and from the milking parlor or with some adjustments to a solid wooden fence, this could potentially even be possible in a pasture-based system.

Future research is necessary here to determine the optimal durations for the specific steps. The first step of termination of the nursing act and thus milk ingestion after a specific amount of minutes by driving the cows away from the fence, could potentially be done in a rather stepwise manner comparable to usual reductions of about 1.5-2L per step during weaning of artificially reared dairy calves. In a recent review on weaning practices for artificially reared dairy calves (Welk et al., 2024), it was pointed out that studies on this issue are scarce, but that the existing evidence suggests that there are no differences in calf performance whether milk reduction is done linearly in many small daily reductions or stepwise in several larger reductions of milk allowance (Welboren et al., 2019; Parsons et al., 2020, 2021). Also, a truly linear reduction will be in the CCC context neither possible and likely also not beneficial, since very low daily milk allowances (e.g. 1L/d) in the final period of linear gradual weaning methods have been suggested to still stimulate suckling motivation and to be potentially responsible for incidents of cross-sucking and a high number of unrewarded visits to the milk feeder, indicative of frustration in artificially reared calves (reviewed in Welk et al., 2024). However an abrupt reduction in milk from about 10L/d to 6L/d negatively affected calf performance and thus sudden, too large reductions should also be avoided (Welboren et al., 2019). In general, there seems to be the conflict that on the one hand, calves show improved starter intake when weaned over longer periods and/or with fewer (but larger) meals (reviewed in Welk et al., 2024 for artificially reared calves), which is beneficial for transition of the GIT, reduces nutritional dependence and thus stress responses of calves when milk supply is completely stopped. On the other hand, calves continue to stay motivated for milk ingestion as long as milk supply continues and will likely experience frustration when milk allowances become very small during a gradual process, which argues against a too linear weaning process. In support of the former suggestion (of improved solids intake), our study also showed that the reduced milk intake by calves of about 4-8 liters (**study IV**) during half-day and morning contact compared to baseline, successfully increased TMR feeding time of the calves (**study II**), which is in line with increased duration of solid feed intake in half-day calves compared to whole-day calves in the study by Bertelsen and Jensen (2023). The latter suggestion (of continued motivation to suckle), was also supported by our study since calves showed a high suckling motivation after prevention of contact during the night (**study IV**), which indicates increased motivation for nursing after longer periods of separation, as long as milk supply is continued. Therefore, identification of optimal time points when calves are ready for reduction or complete cessation of the nursing opportunity is probably complex and would at best be orientated at amounts of individual solid feed intake of calves, so that calves are well established on solid feed when nursing is completely prevented. At least in artificially reared dairy calves, it has been shown that individual solid feed intake of calves helps to identify suitable time points for weaning and can reduce stress responses during and after the process (Roth et al., 2008; de Passillé and Rushen, 2016; Benetton et al., 2019). Automatic monitoring of calves' rumination times, as a proxy for solid feed intake, with ear tags (Borchers et al., 2016) or neck collars (Ambriz-Vilchis et al., 2015) with sensors adapted for calves could in this context be helpful and is more discussed in section 1.2.4., so that future research is encouraged here.

For the last step of complete prevention of nursing, while affiliative behaviors remain possible when the cow reaches over the fence, future research is also needed to determine the necessary minimum duration, but this step should be continued until the pair shows considerably reduced interest in each other, which should then facilitate the total separation. Results presented in **study III** suggest in this context, that calves motivation to spent time with cows was significantly reduced after 5 days of prevention of suckling, but they still continued to enter the cow herd for about 2.5 hours a day afterwards. In beef suckler calves and their dams it was shown that calves showed a preference for their own dam up to day 24 after abrupt weaning, while cows rejected their calves' nursing attempts up from day 20 (when reunited for 2 min at specific days after abrupt weaning, Veissier et al., 1990). Hence, a limited contact period of about 3 weeks might be necessary after termination of suckling until the cows' can easily be separated from their calves. On the other hand, beef cows who stayed in partial contact to their 15 day old calves (restricted from nursing, but allowed other tactile contact between the cow and calf) still displayed a preference for the own calf over an unrelated calf after 4 weeks of this partial separation (Lamb et al., 1997), indicating that the maternal bond was still present. As discussed in detail in **study IV**, it is very likely that the required duration for this last step of the separation will also differ between individual cows and calves depending on their personality and the strength of the cow-calf bond, so that useful indicators for a targeted individualized separation need to be determined. Possible criteria for more individualized separation protocols have already been discussed in detail in **study IV** and warrant further research.

A possible disadvantage of the proposed method is, that calves would need to be weaned in groups, in which all pairs have the same daily allowance of nursing time after which the fence is closed off. This might sometimes not be possible due to management or facility constraints at a farm. Also, a second pen for the calves' in the second step would be necessary to facilitate both stages (open/closed fence stage) for different calves at the same time. An extra pen for pairs with fence-line contact without nursing time would however have the advantage that calves can stay there for the individually necessary time span for the pair before total separation takes place. Furthermore, there could be difficulties with cows that do not stand close enough to the fence to allow calves to suckle. In this case it might be necessary to hold the cow in place for the duration of nursing with an additional gate, which also requires additional work and makes the practice less attractive. Alternatively, if the cow is generally not very interested in her calf, the calf would likely be flexible enough to nurse from a dam of another weaner calf. At least for foster reared dairy calves, it has been reported that calves increase the time suckling from other cows when their designated foster cow is removed and this way maintained an unchanged total suckling duration (Jensen et al., 2023). Also, the required time to stay with the pairs until the nursing event between pairs needs to be terminated by the farmer, might be perceived as causing too much effort. However, since a nursing event usually takes no more than 15min, this time could as well be combined with the routine daily health checks of the animals to make it more time efficient. More importantly than the aforementioned, the proposed method could also bear the risk that calves start non-nutritive sucking or even cross-sucking other calves, when the nursing bout is terminated ahead of normal times. In general, the sucking motivation of calves is elicited by the ingestion of (even small quantities of) milk and persists for about 10 minutes after a meal in artificially reared calves (reviewed by de Passillé and

Rushen, 1997; de Passillé, 2001). Since suckling bouts on the dam last on average also about 7-10 minutes (reviewed in de Passillé, 2001), such an early interruption of nursing could potentially leave calves with continued high suckling motivation for several minutes during which non-nutritive suckling can occur and calves experience high levels of frustration. Furthermore, a disruption of the nursing act could itself potentially induce a negative affective state in calves and especially also the cows, since the social contact between mother and young, including suckling, leads to oxytocin and opioid release and has a rewarding effect in various species (Nelson and Panksepp, 1998; Lupoli et al., 2001; Uvnäs-Moberg et al., 2001), like already described above. Future research is needed here to verify these discussed potential drawbacks and benefits of the proposed fence-line method.

1.2.3 Ensuring reduced milk ingestion of calves through partly pre-emptying of the cows' udders

A further possible weaning and separation strategy to truly reduce the actual milk intake by calves in a gradual manner, could be to partly empty the udder of the cow before pairs can reunite. This could for example be achieved through a short machine milking before reunion in systems where cow-calf contact is normally provided after milking anyway. However, suckled cows frequently show lower oxytocin secretion in response to stimulation by machine milking, leading to an impaired alveolar milk ejection (Lupoli et al., 2001; de Passillé et al., 2008) like discussed in detail in **study IV**. Therefore, a targeted reduction in the remaining milk amounts in the udder through machine milking will likely be difficult. Alternatively, weaner calves could be separated from their own dam via a (closed) fence-line, but continue to have time-delayed access to foster cows, which could for example be the mothers of younger calves of the group. If access of the weaner calves to the foster cows is provided with some time delay, the younger calves will have the opportunity to remove most of the milk from the udder beforehand, so that the available milk supply for weaner calves is reduced compared to the full nursing bout at their own dams pre-weaning. In order to also take sufficient account of the dam, a longer phase of partial contact via a fence-line between the calf and its own dam should be provided simultaneously to the step with calves' access to the foster cows as well as afterwards. This practice has not only the advantage that it would truly reduce the ingested milk amount by the weaner calves, but additionally separates the loss of milk and loss of contact to the own dam in time, which will likely further reduce the calves' stress response. Such a method is already applied by some farmers (e.g. "Hofgut Rengoldshausen", Germany), but a systematic scientific evaluation is lacking so far. Specifically it needs to be determined whether this practice sufficiently reduces the dams' stress response, as it would resemble an abrupt fence-line separation for the dam. Literature on cows' stress responses to abrupt fence-line separation is sparse, but a study in Brahman cows reported a high percentage of cows close to the fence, increased vocalizations and partly increased serum cortisol levels to introduction of fence-line contact, however this stress response was short-lived and decreased 48 hours after initiation of the fence-line separation (Pérez-Torres et al., 2016). In line with this, Johnsen et al. (2015b) reported that the behavioral response of cows was reduced when they were separated with a fence-line compared to a solid wall that allowed only auditory contact, but that a high vocal response was nevertheless evident in the first days following introduction of the fence-line (Johnsen et al., 2015b). Furthermore, it should be tested, whether this method negatively affects

udder health of the foster cows, since in machine milked cows over-milking of an empty udder can cause teat damage and may result in an increased mastitis risk (discussed in Hillerton et al., 2002; Vierbauch et al., 2021). Regarding the calves, it needs to be ensured that their nutritional requirements are still met during the time when access to the foster cows is granted, but calves still consume little solid feed. In an experiment by Nicolao et al. (2022) it was reported that dam-reared dairy calves which were able to suckle their dam for 2.5 hours immediately after morning milking received only little milk amounts of about 3.0kg/day, causing very low weight gains and high morbidity and mortality rates, so that this rearing treatment had to be stopped after 8 weeks. Consequently, determination of the optimal duration of the distinct steps of such a weaning and separation procedure seems crucial and needs to be determined by future research (see section 1.2.2 for some suggestions in this regard).

1.2.4 Inclusion of supplemental milk feeding

As outlined in detail above, a gradual reduction in milk intake is physiologically favorable and likely an essential part of a low stress weaning and separation process. If it is not feasible to gradually wean the calf off milk intake from the udder, there is also the option to transfer the calves first from the cows to an artificial milk feeder (AMF) and later wean them gradually off the milk with the AMF. This also disentangles the stress of separation from the mother from the stress of the shift in diet by separating these two events in time. Results from research by Johnsen et al. show that provision of access to an additional milk source during the separation process, which reduced nutritional dependency of the calf from the dam, also reduced the behavioral response to weaning and separation (Johnsen et al., 2018) and increased calf performance in terms of weight gains after separation (Johnsen et al., 2015a) compared to calves without additional milk intake. These calves were weaned with a short 4 day period of fence-line contact before total separation. Recently it was however also shown that provision of additional milk from an AMF also reduced the behavioral response during a gradual weaning and separation process, especially at the point of complete loss of the nursing opportunity (Johnsen et al., 2024). In this study, contact time between the pairs was gradually reduced (24h, 12 h, 6 h and finally 0 h/day) and calves with lower behavioral responses during separation consumed milk amounts >1.5L per day from the AMF (Johnsen et al., 2024). Milk yields of the cows from this study gradually increased with each step that the contact time was reduced (Sørby et al., 2024a), which suggests that calves which have access to an AMF do not compensate for the reduced contact time by drinking larger amounts of milk from the cow, which is contrary to our results (**study IV**) and likely favorable for mitigating the dam's response to weaning. Indeed, the higher level of nutritional independence, and likely the concurrent reduced number of vocalizations by the less dependent calves, also showed positive effects on the vocal responses by the dams (Johnsen et al., 2018, 2024). Taken together, provision of an additional milk source to calves during and after the separation process from the dam and a subsequent, controlled reduction of milk supply at the AMF seems thus to be a successful option for refinement of the gradual weaning and separation method tested in this thesis, since the higher nutritional independence reduced the behavioral response of cows and calves compared to results found for the gradual weaning and separation protocol used for this thesis (**study II & IV**). Nonetheless, it should be noted that calves in the mentioned study (Johnsen et al., 2024; Sørby et al., 2024b), with gradual reduction of contact time between pairs with support of an

AMF, consumed only very little concentrate until contact to the cow was reduced to 0 hours and weaning off the milk from the milk feeder begun (Sørby et al., 2024b). Also, calves showed low ADGs and partly even a growth check after weaning off milk was completed (Sørby et al., 2024b). In this study, there were some confounding effects of the day of weighing (Sørby et al., 2024b), but in general this indicates that a gradual weaning and separation method with additional milk provision from an AMF is successful in reducing calf hunger and the behavioral stress responses of both cows and calves during separation, but likely leads to a delay in stimulation of solid feed intake of calves compared to a method without the AMF. It would be interesting to explore, if the same reduction in behavioral response could also be achieved by feeding warm water instead of milk to the gradually separated CCC calves. An earlier study by Jasper et al. (2008) reported a significant reduction in the behavioral response to weaning when calves had continued access to the milk feeding system with warm water instead of milk compared to calves that were weaned abruptly from the milk feeder. The authors discussed that the warm water may have provided some satiation by gut fill, but that it might as well be the sucking behavior itself that is rewarding for the calves or an attachment to the milk feeding routine through learned associations between the milk feeding routine and the milk itself (Jasper et al., 2008). Since calves that received the diluted milk consumed about double the amount of solid feed before weaning than calves fed non-diluted milk (Jasper et al., 2008), this could be worth exploring. An additional disadvantage of this method might be that the increased work load by training calves on the AMF, maintenance and cleaning of AMF as well as the additional investment in an automatic feeder could make CCC systems less attractive to farmers who value the reduced work load with calf feeding as a motivator for CCC, as previously discussed by Bertelsen (2023). Lastly, a gradual weaning and separation method with supplemental milk feeding from an AMF is not possible for pasture-based systems, except when calves are kept indoors for the separation phase. Since, pasture-based dairy systems are also under high social demand (Schuppli et al., 2014) and it is likely that numbers of pasture based CCC systems will increase in the future, refinement of gradual weaning strategies that are suitable for pasture-based environments are also necessary and further research on this topic is encouraged.

1.2.5 Better stimulation of solid feed intake by calves

As elucidated in the preceding section, a high nutritional independence from the dam through provision of additional milk reduces the behavioral stress response of cows and calves at separation (Johnsen et al., 2015a, 2018). NF calves of our study reacted with a higher behavioral response to loss of milk than loss of contact to the dam (**study II**) and reduced the times they entered the cow herd within 4 days once suckling was prevented by the nose flaps (**study III**). This suggests that achieving nutritional independence of the calf from the dam is an important mediating factor for the intensity of stress during the separation of pairs, and thus it is crucial to stimulate a high solid feed intake by calves during the weaning and separation process. Like already described in the introduction, a potential better stimulation of solid feed intake was also one of the main arguments to hypothesize a reduced stress response in calves during a gradual weaning and separation process from the dam, since in artificially reared calves, a gradual weaning from milk and feeding restricted milk amounts caused increased solid feed intakes during the pre-weaning period compared to abrupt weaning (Sweeney et al., 2010; Khan et al., 2011; Klopp et al., 2019). Importantly however, this effect was not present when the artificially

reared calves were only about 1 month old when gradual weaning was initiated and calves had already low starter intake at initiation of the gradual weaning process (Bittar et al., 2020). Since artificially reared calves that consume high milk amounts have usually a low starter intake compared to calves on lower milk allowances during the milk feeding period (e.g. Huuskonen and Khalili, 2008; Bach et al., 2013) and dam-reared dairy calves are often able to feed ad libitum from the udder pre-weaning, the initial starter intake in CCC calves might often also be very low, which can delay rumen development (Baldwin et al., 2004). In our study calves of both treatments showed low TMR feeding times during the baseline period, which rapidly started to increase once the milk supply was reduced in GR calves or totally prevented in NF calves (**study II**). Also, in the study of Sørby et al. (2024) who recorded daily weight gains in the same animals used by Johnsen et al. (2024) comparing gradual weaning over a short (10d) or long (28d) duration, the calves showed concentrate intakes close to 0g in the period with full-time access to the dam, that only started to increase once the gradual separation process started (Sørby et al., 2024, supplementary material). Afterwards, calves in both studies showed increased TMR feeding time (**study II**), or intake of concentrate feed respectively (Sørby et al., 2024, supplementary material), with each week of the gradual separation process. Thus, reduction of milk supply seems important to induce higher solid feed consumption also in dam-reared dairy calves, but nonetheless, levels of concentrate intake stayed below 0.2 kg/day until complete denial of access to the cow in the study by Sørby et al. (2024, supplementary material) and also GR calves in our study reached on average not more than 13 min TMR feeding time per 6 hour observation during the period while part-time access to cows was still possible (**study II**). However, increased hay intake will also have played a role, which could unfortunately not be measured in any of the two studies. Interestingly, average daily weight gains of calves weaned and separated with the GR method in our study steadily increased (after an initial drop compared to baseline) which each week that calves' contact time to the cows was reduced (**study II**), but Sørby et al. (2024) found that calves in the long debonding treatment showed a drop in weight gains with each week that contact time to the dam was reduced, which was partly also evident in calves of their short debonding treatment. These decreasing weight gains were evident, despite the fact that the calves had access to an additional milk source. Taken together, this mixed evidence underlines that the gradual weaning and separation method by reduction of contact time stimulates increased solid feed intake by calves, but not necessarily in high amounts and also does not necessarily ensure good weight gains of the calves. Future gradual weaning and separation protocols thus clearly need to be designed with the specific aim to encourage solid feed intake during the separation process and should also add solid feed intake as an outcome measure for success of the protocol, like already proposed by Neave et al. (2023). This could for example be done using automatic rumination activity sensor systems like ear tags (Borchers et al., 2016) or neck collars (Ambriz-Vilchis et al., 2015) with sensors adapted for calves to automatically assess increases in calves' rumination time during the process.

With regard to possible options to increase solid feed intake pre-weaning, recent research has shown that solid feed intake during the pre-weaning period is partly dependent on personality traits of calves, as calves classed as more exploratory have higher concentrate intakes during the pre- and post-weaning period (Neave et al., 2018; Whalin et al., 2022). On the other hand, calves that were more vocal and active during the weaning period, classed as higher in the "sociability" trait, show reduced pre-weaning concentrate intake (Whalin et al., 2022). Thus,

placing solid feed at strategically well-selected locations where calves spend a higher amount of time, could increase ‘accidental’ interaction of less active and exploratory calves with the feed source. At least in artificially reared calves it was reported that placement of solid feed next to the milk feeder increased solid feed intake during the pre-weaning period and led to increased weight gains of the calves (Parsons et al., 2020). Also, in beef calves it was reported that calves which were manually preconditioned to hay for 10 days before weaning, were more often observed eating hay during the first 3 days after weaning compared to calves not preconditioned to hay before (Price et al., 2003), however it was not clearly described how preconditioning in this study was achieved.

Additionally, some farmers practicing CCC report that feeding calves additionally an attractive and enjoyable feed, like for example carrots, during the weaning and separation period leads to distraction and less vocalizations by the animals (personal experience, also reported by 4 famers in Eriksson et al., 2022; and recommended online by MUKA Fachstelle Switzerland, 2024). In this regard, a contributing factor to the found differences in development of ADG in the course of the gradual separation process between our study (**study II**) and the study by Sørby et al. (2024) might simply be that the fed TMR, which had a high silage content, was palatable to the calves and thus better stimulated calves’ solid feed intake. A further and potentially more important difference between our study and that of Sørby et al. (2024) is that all but two of our calves had pasture access together with the cow herd during the pre-weaning period and all calves could also enter the cow herd and had a limited possibility to observe their mothers during feeding in the barn. In the study by Sørby et al. (2024) calves were kept separated from the cow herd and could only come together with their mothers in a designated meeting area. Thus, our calves had more opportunities of social learning of solid feed intake from older herd members in the pre-weaning period, which might have positively affected the roughage intake during the weaning and separation phase. In this context, a study by De Paula Vieira et al. (2012) showed that presence of an older weaned calf stimulates the feeding behavior and weight gains of young calves before and after weaning off milk. Furthermore, provision of an experienced demonstrator conspecific also had a positive effect on the onset of grazing (Costa et al., 2016) and general grazing activity, but not grazing time (Hessle, 2009) in naïve heifers during their first grazing. This is likely even more so the case when dairy calves can learn from the dams on pasture, and thus the magnitude of increase in solid feed intake during the weaning and separation process in dam-reared dairy calves with different contact times to their dam should be examined in future studies.

Lastly, delaying weaning age will also be beneficial for increased pre-weaning solid feed intake of calves. In beef calves (Lambertz et al., 2015b; Pérez-Torres et al., 2016; de Souza Teixeira et al., 2021) and artificially reared dairy calves (Eckert et al., 2015; de Passillé and Rushen, 2016) it has been shown that the stress response is reduced when calves are weaned (and separated for beef calves) at a later age. This is likely linked to a better gastrointestinal and metabolic development of older calves due to higher levels of solid feed intake pre-weaning (Eckert et al., 2015) and thus a better nutritional independence from the dam or milk source. Nonetheless, abrupt weaning and separation still causes a considerable stress response, also physiologically, in beef calves that are weaned at later ages of 7 months (Hickey et al., 2003;

Lynch et al., 2011). This indicates, that a later weaning age is beneficial, but a gradual or stepwise reduction in milk supply and at the same time active stimulation of increased solid feed intake is likely still needed to ensure a low stress weaning process, like already pointed out by Bertelsen (2023). More research on methods how to best stimulate high individual solid feed intake by calves which are simultaneously still feeding on milk is highly encouraged.

In summary, it must be concluded that there is still significantly more research necessary to find successful low stress weaning and separation strategies for cow-calf contact systems. In general it seems mandatory though to efficiently reduce nursing time during gradual weaning and separation procedures in order to achieve a successful reduction in separation stress, since solid feed intake does often only start to substantially increase once milk amounts are reduced. A good establishment on solid feed is necessary to decrease nutritional dependence of the calf from the dam, which has been shown to greatly reduce separation stress in both partners of a cow-calf pair. Additionally, if contact time is reduced but nursing duration stays the same, as seen in this thesis and by Hvidtfeldt Jensen (2024a), the bond of the pair is reinforced and animals will likely react with a high stress response when the nursing opportunity is completely terminated. This can especially be expected at the anticipated time points when pairs normally reunite for nursing, regardless of gradually reduced contact times beforehand. Therefore a true reduction in milk amount, with a simultaneous encouragement of high solid feed intake, is needed in order to initiate the weaning process. Subsequently, it seem reasonable to use calves' individual rates of solid feed intake in order to individually target the optimal time point for complete cessation of the nursing opportunity for a pair. As a last step, a longer period of partial contact of pairs, e.g. through a visiting window or over a fence, should be allowed before total separation of cow and calf, since a strong cow-calf bond is likely maintained as long as even short periods of suckling remain possible.

1.3 Evaluation of indicators for weaning and separation stress

In addition to comparison of the weaning and separation methods, this thesis further highlighted some limitations of the commonly used stress indicators that should be taken into consideration during the design of future studies. Before conducting the study, we hypothesized that compared to plasma or saliva cortisol measures, fecal cortisol metabolites might be a more suitable physiological marker for such gradual weaning protocols with less distinct time points when occurrence of stress can be expected compared to abrupt weaning. This was hypothesized due to the fact that metabolites of the circulating stress hormones are integrated into the feces over a longer time period (discussed in Ashley et al., 2011; Palme, 2012) and allow therefore, to a limited degree, a more long-term measure of stress, that is less dependent on the exact sampling time point compared to plasma or saliva cortisol samples. Results of the validation study for use of fecal cortisol metabolites as a stress indicator in dairy calves proved that FGCM concentrations show a clear peak after a stressor in dairy calves pre- and post-weaning (**study I**), which suggests that FGCM concentrations are a valid marker for stress in younger calves, just as they are for older cattle (e.g. Palme et al., 2000; Pesenhofer et al., 2006; Veissier et al., 2018). However, peak FGCM concentrations tended to be higher in unweaned calves on a primarily milk-based diet in comparison to weaned calves and, furthermore, the time lag of excretion of FGCMs after a stressor was numerically greater while calves were still feeding

primarily from milk compared to the solid-based diet (**study I**). Thus, it became evident that the diet has a strong influence on measured FGCM concentrations, which needs to be considered during the design of future studies and highlights that FGCM concentrations are not a suitable indicator for comparison of pre- and post-weaning states in calves with a change in diet. Future research is needed here to find suitable markers that display the weaning and separation stress of animals over a longer period, especially for gradual weaning and separation protocols during which the stress for the animals might not occur until hours after introduction of a new change in contact time, and thus sampling time points are hard to determine.

As a potential alternative, we examined the use of relative telomere length (RTL) as a stress indicator, since the shortening rate of telomeres has been proposed to integrate positive and negative life experiences by animals over a longer time period and was therefore suggested as such a potential indicator for cumulative welfare (Bateson, 2015). In dairy cows, telomere shortening had so far been shown to reflect heat stress (Seeker et al., 2021), but was never applied in the context of social stressors like cow-calf separation. RTL of animals examined for this thesis showed no difference for calves, despite the decreased weight gains and increased inflammatory markers in NF calves (**study II**), and only a very weak tendency ($p=0.095$) for shorter RTL in cows separated with the gradual weaning and separation method, indicating higher overall stress for GR cows. The latter result is partly in accordance with the numerically higher number of vocalizations and searching behavior of cows in our study (**IV**), however, does not align with the fact that we found no significant differences in neither the behavioral responses nor the other physiological stress indicators measured in the cows over the whole weaning and separation period (**study IV**). In general, it was striking that there was no correlation of RTL with health indicators (visible inflammation of the nasal septum of calves through the nose flap) or any of our other stress indicators (e.g. high FGCM concentrations, high frequency of vocalizations or searching behavior, **study V**). Thus, it does not seem that RTL as sampled in the present thesis is a valid indicator of weaning and separation stress in cattle. Like already discussed in **study V**, this might be either due to the fact that RTL may generally not be a valid indicator for health in cattle, since an effect of udder health and lameness of cows on RTL was also missing in another study (Seeker et al., 2021), which could thus equally apply for separation stress. Alternatively and more likely, our sampling interval of three weeks was likely too short to reliably reflect the experienced stress in RTL, especially since the second RTL sample was only taken a week after introduction of fence-line contact in the treatments (discussed in **study V**). Thus, future research is warranted to examine stress responses using later sampling time points for RTL and/or to assess alternative indicators that are capable to reflect stress that cattle experience over longer time periods.

In any way, this thesis underlined that a combination of behavioral and physiological measures is highly valuable for interpretation of results: During **study II**, the cumulative view of all combined stress markers for NF calves suggested that the reduced number of vocalizations was likely not indicative of reduced stress levels, but that vocal responses of calves during weaning and separation depend on the need for vocal communication and are less likely to occur in situations in which calves already have close contact to their dams. Therefore, a lack of vocalizations during weaning and separation methods which allow close partial contact between pairs should not be taken as evidence of low stress levels in future studies, if not backed by

further indicators. That said, we did not distinguish between high- and low-pitched vocalizations as done by other authors studying weaning and separation stress (Bertelsen and Jensen, 2023; Neave et al., 2023). High-pitched vocalizations are used in cattle to reunite when pairs are separated over greater distances, while low-pitched vocalizations are used to communicate when pairs are in close proximity to each other (Padilla de la Torre et al., 2015). Therefore, the above made suggestion will mostly apply for high-pitched vocalizations.

Lastly, vocal responses and searching behavior of the cows (**study IV**), but also by the calves (**study II**), showed distinct temporal patterns that likely depended on previously established routines and anticipation of reunion (discussed in detail in **study IV**). If behavioral observations would have occurred at time spots were the pairs were not used to reunite, we would have falsely recorded a very low behavioral response to weaning and separation. In line with this Bertelsen and Jensen (2023) reported that calves which were fence-line weaned from their dams vocalized only during morning observations when cows were present in the pen, whereas control calves vocalized mostly during the afternoon when routine milk feeding took place. The authors equally discussed that this might be related to frustrated anticipation of half-day calves to reunite after cows returned from milking or anticipation to be milk-fed at this time of the day by control calves. Also, the authors discussed that they might potentially have overestimated the vocal response of calves with half-day contact to their dam, compared to whole day contact or control calves, due to timing of the behavioral observations (Bertelsen, 2023). Consequently, it is highly recommended for future studies to establish solid knowledge of routines of the animals during baseline observations to find the appropriate time slots for behavioral recordings. Especially during gradual separation protocols, the hour of initiation of (gradually) reduced access time will likely often not match the time point when stress for the animals occurs, as we have seen during the week with morning contact in our study (**study II and IV**), which needs to be considered. Since vocalizations are often used as main indicator for evaluation of separation stress (e.g. Johnsen et al., 2018; Neave et al., 2023) it should be ensured that choosing wrong time points of observation does not lead to a false under- or overestimation of the stress experienced with a tested method.

General conclusion

This thesis aimed to contribute to the advancement of dairy cow-calf contact systems by comparison of two potential low-stress weaning and separation methods for the pairs, and additionally, it aimed to evaluate useful indicators for weaning stress. A gradual reduction of contact time between cow and calf was the more gentle method for physiological adaption of the gastrointestinal tract of the calves compared to the abrupt milk loss during two-step separation with nose flaps. This was reflected in higher weight gains and more locomotor play behavior of gradually separated calves, which might not only be indicative of higher energy levels, but also of a more positive affective state of these calves. On the other hand, two-step separation with nose flaps led to reduced weight gains and locomotor play behavior, as well as a short-lived increase in immune markers and partially also to injuries in the calves' nostrils. Since such injuries were already evident after 5 days of nose flap usage in older beef calves (Valente et al., 2022), but our results suggest that a minimum duration of 4 days is necessary for an effective reduction of the calves' suckling motivation, it remains questionable, if nose flaps can be used without adversely affecting the welfare of calves. Therefore, a gradual method seems collectively favorable for a low stress weaning and separation process of calves and was also not worse for the cows. Nonetheless, cows and calves that were gradually separated with three steps over three weeks reacted with a very high behavioral response to complete cessation of the suckling opportunity. The measured milk yields suggested that the gradual reduction of contact time led to no consistent reduction of ingested milk by the calves, causing a rather abrupt removal of high milk amounts in the week with fence-line separation. Thus, further refinement of the gradual weaning and separation method is strongly encouraged, especially in ways that ensure a truly gradual reduction in milk intake and concurrent increase in solid feed intake of calves to achieve a greater nutritional independence from the dam. Additionally, this thesis found evidence of great inter-individual variation, especially between the dams, so that development of gradual weaning and separation protocols that can be more flexibly adapted to individual cow-calf pairs is encouraged. Also, frustrated anticipation to reunite seemed to substantially influence the stress response in gradually separated pairs, which should be considered during development of future separation protocols. Lastly, FGCM concentrations were found to be no suitable indicator for comparison of calves' pre- and post-weaning stress due to the different diets, and results of relative telomere length were not conclusive due to a likely too short sampling interval. In sum, it is concluded that a gradual weaning and separation method has potential to reduce weaning and separation stress, foremost due to favorable physiological effects, but that refinement of the method used for this thesis is inevitably needed to achieve a higher increase in calves' nutritional independence within the period when pairs have reduced contact time.

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