J. Dairy Sci. 106:2124–2136
https://doi.org/10.3168/jds.2022-22341
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Fecal cortisol metabolites reflect transport stress in 3-month-old dairy calves pre- and postweaning: A pilot study

Anina Vogt,[1]* Uta König von Borstel,[1] Susanne Waiblinger,[2] Rupert Palme,[3]† and Kerstin Barth[4]†
[1]Division of Animal Husbandry, Behaviour and Welfare, Justus Liebig University of Giessen, 35392 Giessen, Germany
[2]Institute of Animal Welfare Science, University of Veterinary Medicine, 1210 Vienna, Austria
[3]Institute of Physiology, Pathophysiology and Experimental Endocrinology, University of Veterinary Medicine, 1210 Vienna, Austria
[4]Institute of Organic Farming, Johann Heinrich von Thünen Institute, Federal Research Institute for Rural Areas, Forestry and Fisheries, 23847 Westerau, Germany

ABSTRACT

Measurement of fecal cortisol metabolites (FGCM) is a well-established, noninvasive method to assess stress in adult dairy cattle. However, this procedure has not yet been validated for unweaned dairy calves, 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 whether 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, 5 German Holstein calves of about 3 mo of age (93 to 102 d preweaning) were exposed to a 45 min transport stressor once before and once after weaning, which was 3 wk apart. All voided fecal samples were collected for 24 h after termination of the transport. Fecal cortisol metabolites were analyzed with an 11-oxoetiocholanolone enzyme immunoassay and changes in FGCM concentrations relative to the individual baseline ($FGCM_{rel}$) were calculated. 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 preweaning diet (at peak: +233 ± 25% increase relative to baseline) in comparison to the postweaning diet (+124 ± 23%). Considering the whole 24 h sampling period, the $FGCM_{rel}$ concentrations for all calves were significantly higher on the preweaning diet than on the postweaning 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 h longer when the calves were on the preweaning diet compared with the postweaning 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.

Key words: dairy calves, glucorticoid metabolites, weaning stress, diet, transport

INTRODUCTION

Measurement of fecal cortisol metabolites (**FGCM**) is a well-established, noninvasive 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, however, it is unknown how the milk diet affects the composition, time lag, and concentration of excreted FGCM. This is of special interest for evaluating weaning distress in young calves, as the diet changes substantially between the pre- and postweaning periods.

In general, there is a considerable variation between species and 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-DOA**) 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

Received May 26, 2022.
Accepted September 29, 2022.
*Corresponding author: anina.vogt@agrar.uni-giessen.de
†These authors contributed equally to this work.

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 h in sheep, 24 h in ponies, and 48 h 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 h (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 FGCM in contrast to weaned calves or adult cattle on a solid diet. In different species, it has been determined that the diet of an animal can have a distinct effect on FGCM concentrations (e.g., Wasser et al., 1993; von der Ohe et al., 2004; Dantzer et al., 2011). In particular, dietary fiber content has been 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). 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, experimental addition of dietary fiber decreased corticosterone metabolite concentrations in droppings of European stonechats (Goymann, 2005) and FGCM 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 DM 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 FGCM 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) preweaning or roughage and concentrate (low fat, high fiber) postweaning.

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.

MATERIALS AND METHODS

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 3 mo old (mean ± SD: 98 ± 3.94 d of age) when the treatment started. These 5 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 68 m^2, of which 12.6 m^2 was a straw-bedded lying area. Next to the 5 study animals, there were 8 other calves present, totaling 13 animals in the calf area. Before the second data collection started, 1 male calf was sold, leaving 12 animals in total in the calf area for the second data collection.

During the preweaning phase, the calves were provided with 12 L of whole milk per day from an automatic milk feeder (Förster Technik) and had additional ad libitum access to hay and 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 per 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 preweaning period. For weaning, milk allowance was reduced continuously over 2 wk by ~0.9 L per day, so that after 2 wk, the calves were weaned off milk completely. Calves were about 3.5 mo (107 to 116 d) old when they were fully weaned off milk. During the postweaning 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.

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) and were transported around the area of the home stable once before weaning and once again after weaning. The transport started at 1000 h at both occasions. The journey lasted 45 min and included short passages of cobblestone (about 1/8 of the total distance) and 1 sudden brake process. The trailer was 2.5 × 6 m in size and no bedding was provided for the 45 min drive, so that calves were transported on the antislip checkered plate of the trailer. Sample collection started immediately after unloading the calves and lasted for 24 h. Transport route and driver were the same for the journey before and after weaning.

Immediately after the first transport and the preweaning sample collection were completed (age of calves: 93 to 102 d), the 2 wk weaning process started. Once they were fully weaned off milk, the calves were given 1 wk to allow for the gastrointestinal tract to adapt to the dietary changes. Then the second transport and sample collection for the postweaning phase took place, which was exactly 3 wk after the first transport and sample collection (age: 114 to 123 d). 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 preweaning phase and about 20°C and a mix of sun and clouds without precipitation for the postweaning transport.

Sample Collection

About 1.5 h 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 weighed and a 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 5 study calves was collected immediately after voidance for a period of 24 h. As soon as 1 of the 5 study calves defecated, about 2 to 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 min and all samples collected within the 30 min period were transferred to a freezer and kept at −20°C until further analysis.

Analysis of Fecal Cortisol Metabolites

Extractions of the samples followed the established protocol (Palme et al., 2013). In brief, defrosted (60°C; 30 min) fecal samples were homogenized, weighed (0.5 g), and extracted with 5 mL of 80% methanol. Fecal cortisol metabolites were analyzed in a portion of the supernatant with an 11-oxoetiocholanolone EIA, previously validated for cattle (Möstl et al., 2002). Intra- and interassay coefficients of variation were below 10% and 15%, respectively. This EIA measures FGCM with a 5β-3α-ol-11-one structure (for details, including cross-reactions of the antibody, see Möstl et al., 2002). With the EIA measuring 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.

Statistical Analysis

Statistical analysis was performed with SAS version 9.4 (SAS Institute Inc.). Baseline concentrations included only pre-transport values, namely the 1 sample taken per animal before a transport started. Differences of the baseline FGCM values between the pre- and postweaning 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 2 diets was analyzed using a Wilcoxon signed-rank test.

For further analysis, following Kleinsasser et al. (2010), the fecal samples collected in the 24 h sampling period after the transport were grouped into 3 h intervals. If more than 1 sample was present in a specific time interval, the mean value for the individual

calf in this interval was calculated. However, not every calf had a sample available in each interval, as defecation was irregular. This way, the mean FGCM value in nanograms per gram wet feces per interval for each calf was obtained. Based on this mean value in nanograms 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 concentrations relative to the individual baseline ($\mathbf{FGCM_{rel}}$) = [(FGCM – baseline value): baseline value] × 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 (preweaning or postweaning), interval, and 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 squares means (LSM) ± standard error (SE).

The differences between the time lags until occurrence of the peak $FGCM_{rel}$ concentrations in feces during the pre- and postweaning 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.

RESULTS

Total Number of Defecations and Individual Progression Over Time Per Calf

The total number of defecations of the calves differed between the 2 types of diet, as all calves tended to defecate less often when they were on the preweaning milk diet (mean ± SE 9.2 ± 0.58 defecations per 24 h collection period) than when they were on the postweaning solid diet [16.4 ± 2.25, signed rank statistic (S) = 7.5, $P = 0.06$, see Table 1].

The FGCM baseline concentrations tended to differ between the 2 diet types (mean ± SE preweaning: 180 ± 28 ng/g; postweaning: 109 ± 10 ng/g, $t_4 = 2.5$, $P = 0.07$). Four calves showed higher baseline concentrations during the preweaning phase than during the postweaning phase. Only 1 individual (calf 2) showed similar baseline concentrations in the 2 phases (see Figure 1).

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

Calf no.	No. of defecations preweaning per 24-h collection	No. of defecations postweaning per 24-h collection
1	7	12
2	10	14
3	10	15
4	10	25
5	9	16

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

Percentage Increase in Fecal Cortisol Metabolite Concentrations Relative to the Individual Baseline

Results showed that the phase had a significant effect on the mean increase in $FGCM_{rel}$, as the mean $FGCM_{rel}$ concentration changes over the whole 24 h sampling period were significantly higher in the preweaning phase (LSM ± SE: +90.6 ± 14.5%) than in the postweaning phase (+46.9 ± 14.2%, $F_{1,47} = 11.6$, $P = 0.001$, Figure 2).

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

The highest increase in $FGCM_{rel}$ concentrations on the postweaning solid feed diet was found at 6 to 9 h 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 h after transportation (all $P > 0.05$, see Figure 2B). In general, the differences between the intervals were less pronounced on the postweaning solid diet than on the preweaning milk diet.

However, although the peak increase was more pronounced and occurred 1 interval later when calves were

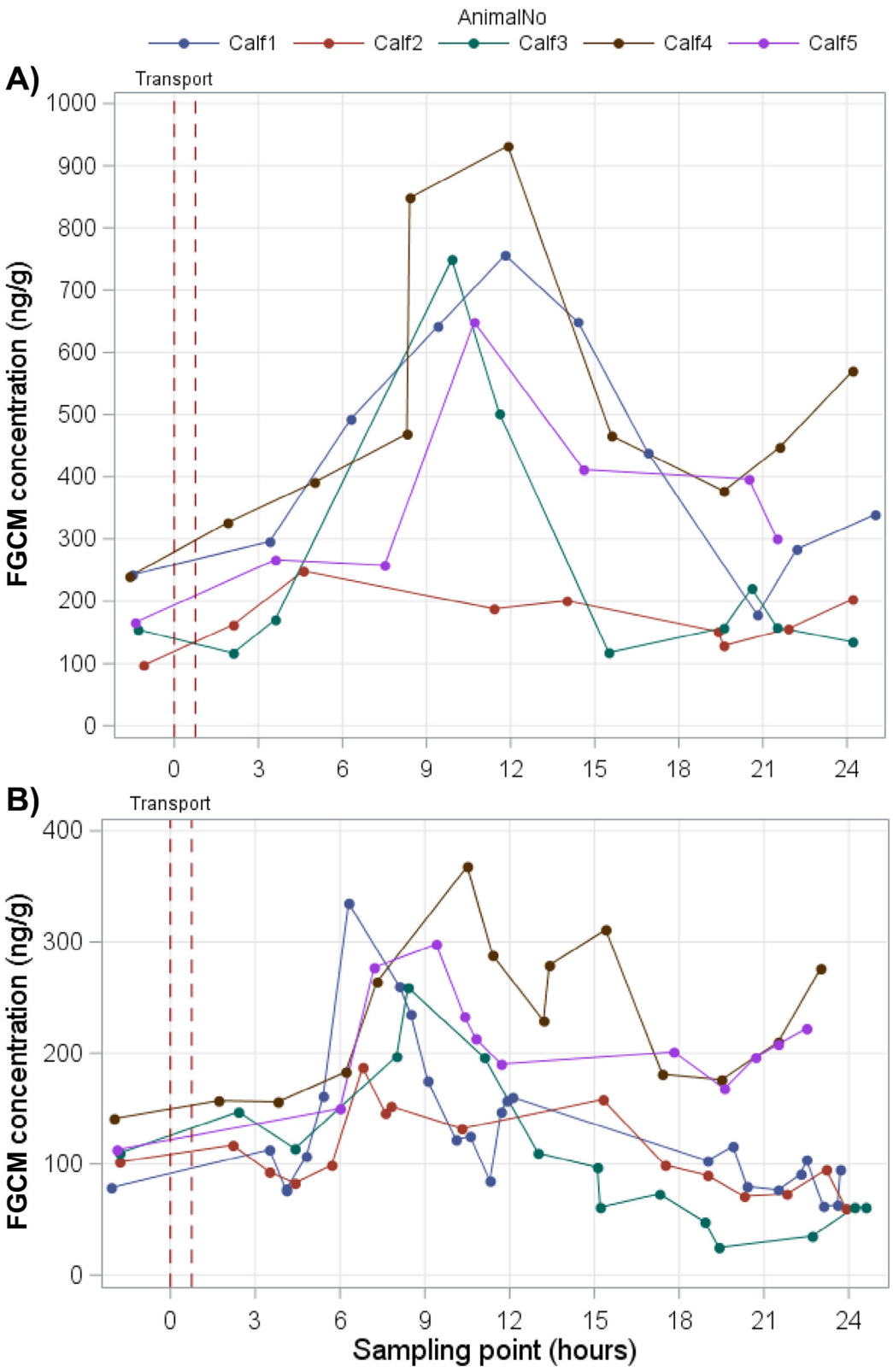

Figure 1. Individual fecal cortisol metabolite (FGCM) concentrations (ng/g wet feces) of 5 dairy calves over a 24-h sampling period following a 45-min transport stressor on (A) the preweaning diet (milk + roughage + concentrate) and (B) the postweaning diet (only roughage + concentrate). Sampling point 0 represents 1000 h 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.

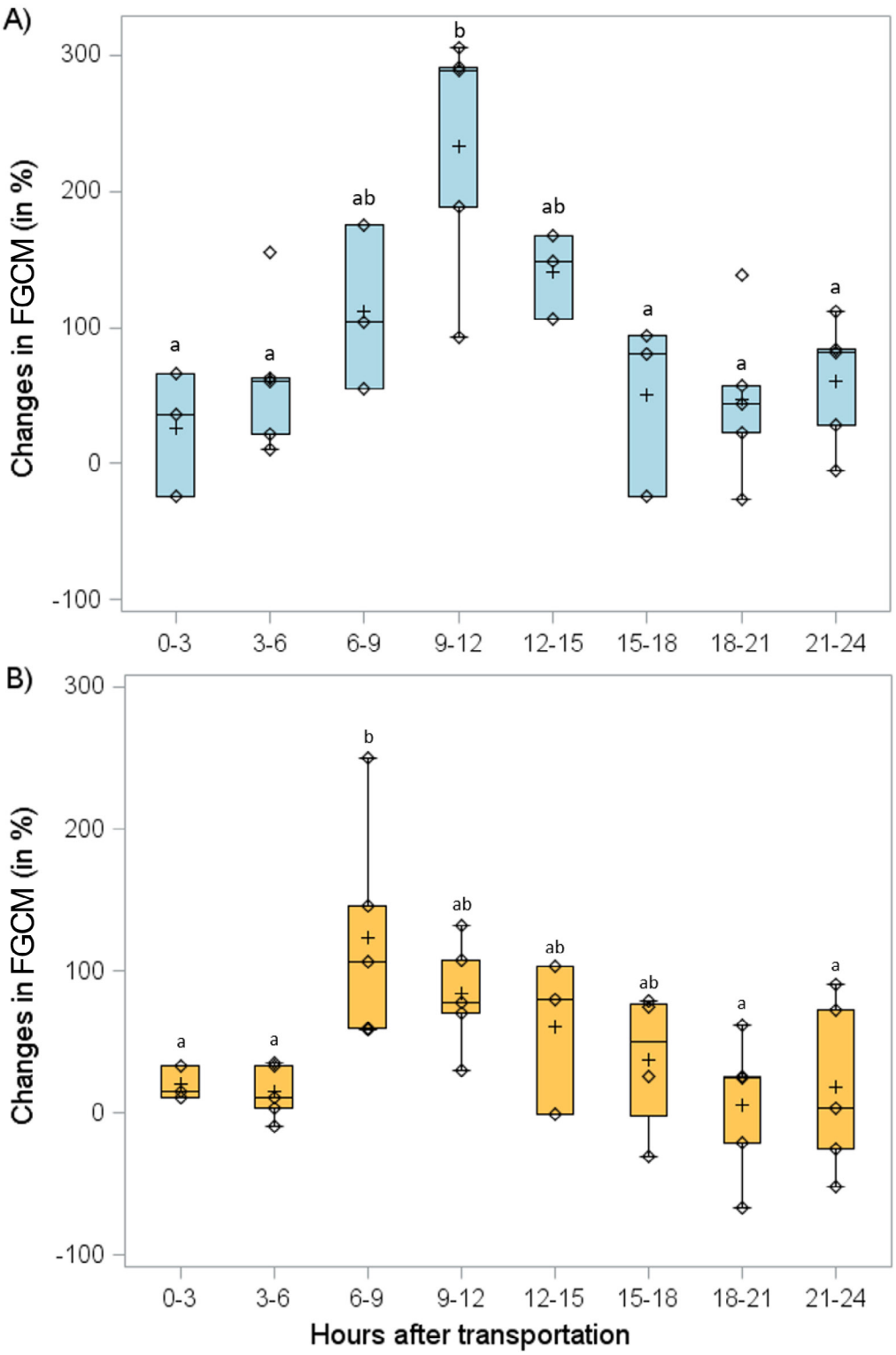

Figure 2. Boxplot graphs of changes (in %) in fecal cortisol metabolite concentrations relative to the individual baseline ($FGCM_{rel}$) per 3-h sampling interval for the 5 dairy calves on (A) the preweaning diet (milk + roughage + concentrate) and (B) the postweaning diet (only roughage + concentrate). Baseline values comprise only 1 sample taken from each calf before the transport stressor started. 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); diamonds = values of the individual calves. Note the small sample size of only 5 calves and that a value for every interval was not present for each calf as defecation was irregular. Different letters (a, b) indicate significant differences at $P < 0.05$.

on the preweaning diet compared with the postweaning diet, the $FGCM_{rel}$ concentrations of the respective peak intervals of the 2 diets (preweaning 9 to 12 h: 233.2 ± 25.3% vs. postweaning 6 to 9 h: 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 h (mean ± SE) longer when the calves were on the preweaning diet (peak after 9.8 ± 1.4 h) than when they were on the postweaning diet (peak after 8.3 ± 0.8 h), but this difference was not significant ($t_4 = 1.2$, $P = 0.29$). In 4 calves, the peak occurred earlier in the postweaning phase than in the preweaning phase. However, in calf 2, where a clear peak was lacking, this was reversed and the peak occurred later in the postweaning phase (see also Figure 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 h (mean ± SE) longer when the calves were on the preweaning diet than when they were on the postweaning 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 (±SE) 618 ± 121 ng/g on the preweaning diet at 9 to 12 h after transport and 233 ± 21 ng/g on the postweaning diet at 6 to 9 h after the transport stressor.

DISCUSSION

In this study, the stress response of young dairy calves to a transport stressor was evaluated before and after weaning by measuring FGCM. Results revealed that FGCM concentrations on the preweaning 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 preweaning milk diet in comparison to the postweaning diet based on solid feed. However, this was only a tendency and not a significant difference between the 2 phases, which was probably caused by the small sample size of only 5 animals and the high individual variability. Considering the whole 24 h sampling period, though, the $FGCM_{rel}$ concentrations were significantly higher for all calves on the preweaning diet than on the postweaning 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 preweaning diet (9.8 ± 1.4 h) than when they were on the postweaning diet (8.3 ± 0.8 h). In a study by Palme et al. (2000), 8 transport-experienced, lactating dairy cows were transported for 2 h on country roads and samples were taken from every spontaneous defecation over a period of 48 h after the transport. Peak concentrations occurred 12 h after the transport and FGCM concentrations were significantly higher from 8 to 16 h after the start of the transport, compared with 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 to 16 h. 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 9 h after the stressor (Pesenhofer et al., 2006).

Although a different EIA was used in our study, the increase in $FGCM_{rel}$ concentrations to 124% relative to individual baseline during the postweaning 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 per d at dry-off revealed that the cows reacted with an increase in $FGCM_{rel}$ concentrations of 129% at the second day after the sudden dry-off (Bertulat et al., 2013). Similarly, dairy cows that were exposed to heat stress (temperature-humidity index ≥72) on a single day reacted to this stressor with an increase of 155% in $FGCM_{rel}$ concentrations (Rees et al., 2016). Thus, the $FGCM_{rel}$ concentrations in our study were lower in the postweaning period than during the preweaning period, but postweaning levels were similar to those found in stressed adult cows. Several possible factors may have contributed to this observed higher increase in $FGCM_{rel}$ levels when the calves were on the milk diet compared with the solid diet in our study, which will be discussed in the following sections.

Validity of Results

A major limitation with this study is the small sample size of only 5 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. While 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 all had exactly the same hay quality, TMR, 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 FGCM in preweaning dairy calves, and results showed that the peak $FGCM_{rel}$ increase of 233.2 ± 25.3% at 9 to 12 h after transportation on the preweaning diet was significantly different from all other intervals but the adjacent ones (Figure 2). This statistically 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 preweaning calves, there is, to our knowledge, only 1 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 depends not on a diurnal rhythm but rather upon food intake and peaks before meals. 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 FGCM (Figure 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 h after the stressor during both study periods (see Figure 1). Also, the peak FGCM concentrations occurred about 1.5 h earlier in 3 calves and about 5.5 h earlier in 1 calf in the postweaning phase compared with the preweaning phase. In calf 2, this was even reversed and the peak occurred about 2.5 h later in the postweaning phase (see also Figure 1). However, for the latter, this was probably mostly a result of the missing clear peak during the preweaning phase.

The found individual variability is in line with results from the literature as, for example, Palme et al. (1999) also reported a great interanimal variation in the concentration of FGCM 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 could be measured, there is also high individual variation in adult cattle as, for example, peak FGCM concentrations occurred about 10 h (range of 6 to 18.7 h) after respective plasma cortisol peaks following ACTH injection (Palme et al., 1999). Equally, FGCM peaks were reported between 8 and 9.5 h in lactating dairy cows and 14 to 18 h in nonlactating dairy cows after an ACTH injection (Morrow et al., 2002), about 9 h after the fixation for claw trimming (Pesenhofer et al., 2006), as well as after 12 h 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. For instance, 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). Similarly, Lecorps et al. (2018) demonstrated that dairy calves that were classified as more fearful in standardized tests (open field, novel object, human reactivity, and social motivation tests) 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 mainly aimed to assess whether a standardized stressor would be reflected at all by FGCM in young dairy calves preweaning, as this method for stress assessment was never validated and applied in preweaning calves before. Our study found a significant peak increase in FGCM, which was evident in all 5 animals at around the same time span despite the sex differences of the calves, showing the validity of FGCM to reflect a stressor 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 FGCM in preweaning calves.

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 preweaning phase all calves were transport-naïve, whereas for the postweaning 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 postweaning 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 1 single transport. In a study by Fell and Shutt (1986) on dairy calves of 0.5 to 2 mo of age that 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 3 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 2/3 of the level measured for the first transport (Locatelli et al., 1989). This degree of reduction of about 1/3 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 4 mo old Holstein calves that were repeatedly transported, blood plasma cortisol levels after 4 h 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 highly individual, and quite likely also situational, variation but can already occur in part after 1 single transport. However, if habituation from first to second transport was present, the measured reduction in cortisol levels was at most around 1/3 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 thus represents a considerably stronger reduction in comparison to the dimensions reported in the literature after a single transport.

Additionally, a clear tendency of lower postweaning 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 postweaning period.

Dilution of Fecal Cortisol Metabolite Concentrations by Fecal Mass and Changes in Bile Flow Rates

One possible explanation for the lower FGCM concentrations during the postweaning phase in our study might be related to the observation that all studied calves defecated more often when they were on the solid diet with a high amount of roughage compared with the preweaning 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. For example, a study by Hirata et al. (2001) on heifers found a positive correlation of the defecation frequency with DMI and fecal DM 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 FGCM, because placing the same amount of hormone metabolites per time unit into a larger amount of feces per time unit 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 postweaning period, as the calves were consuming more fiber, which resulted 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 DM as well as higher mean fecal androgen metabolite levels compared with 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 FGCM 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 2 study periods were comparable as they underwent a severe dietary change. To our knowledge, no studies have directly compared bile flow rates in dairy calves pre- and postweaning. 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 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 with 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 to 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 postweaning diet, which was identical except that the calves had an additional milk allowance during the preweaning phase. Thus, the bile flow rates were potentially greater in our calves on the preweaning diet with a high milk content and thus higher fat content. As the FGCM are excreted with the bile, a higher bile flow rate during the preweaning period could partly explain the increased metabolite concentrations found in the feces of our calves in the preweaning period compared with the postweaning period.

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) preweaning to a diet mainly based on roughage with additional concentrate (low fat, high fiber) postweaning 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 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 preweaned calves to *Firmicutes* in postweaned 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 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 postweaning 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, it must 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 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 h for milk replacer in contrast to 21.4 h for concentrates, 36.8 h for long hay, and 59.1 h 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 h for milk replacer in veal calves (Gilbert et al., 2017) or 24.7 h 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 the food pulp in our study at the postweaning stage may also have taken longer to pass through the digestive tract than during the preweaning phase. Consequently, there will have been a higher chance of further bacterial processing of FGCM during the postweaning phase in contrast to the preweaning 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 FGCM 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 FGCM 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 FGCM 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 5β-3α-ol-11-one structure, and thus, depending on the structure of the produced FGCM metabolites during the pre- and postweaning stages, 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 postweaning diet, as well as the presented divergence between the baseline values of the 2 stages in our study, even if the latter only tended to differ.

CONCLUSIONS

The results of our study showed a significant increase with a clear peak in FGCM concentrations in the preweaning phase, which demonstrates that FGCM can be used to evaluate the stress response of young calves that are still on a milk diet, just as they can 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 us 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.

ACKNOWLEDGMENTS

This study received no external funding. We thank the staff of the research station and the technical coworkers at the Thünen Institute, Westerau, Germany, for their excellent help in sample collection of this study, as well as Edith Klobetz-Rassam from the University of Veterinary Medicine, Vienna, Austria, for fecal cortisol metabolite analysis. The authors have not stated any conflicts of interest.

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ORCIDS

Anina Vogt https://orcid.org/0000-0002-6896-2244
Uta König von Borstel https://orcid.org/0000-0002-7313-1197
Susanne Waiblinger https://orcid.org/0000-0003-3565-4306
Rupert Palme https://orcid.org/0000-0001-9466-3662
Kerstin Barth https://orcid.org/0000-0001-7070-6445