Assessing effects of dietary and milking frequency changes and injection of cabergoline during dry-off on hunger in dairy cows using 2 feed-thwarting tests


ABSTRACT

We investigated the single and combined effects of 2 feeding levels (normal lactation diet vs. energy-reduced diet, both fed for ad libitum intake) and 2 daily milking frequencies (twice vs. once) during 1 wk before the dry-off day (d 0), as well as an intramuscular injection of either a dopamine agonist (cabergoline; Velactis, Ceva Santé Animale; labeled for use only with abrupt dry-off, e.g., no reduction in feeding level or milking frequency before the last milking) or saline after the last milking on d 0 on the feeding motivation of clinically healthy, loose-housed, pregnant, lactating Holstein cows. From d 0, all cows were fed the same dry-cow diet for ad libitum intake. Cows were subjected to 2 feed-thwarting tests, a test in the home pen using their diets (test A: d −6, −1, and 1; during 35 min when the feed bins were filled, but locked) and another test carried out in an adjacent pen in which access to concentrate provided in a familiar plastic box was blocked by a wire-mesh lid (test B: d −5 and 2). In test A, we recorded how often cows attempted to feed per 35 min, whether cows vocalized during the 35-min period, and latency to feed within 300 s after feed bins were unlocked. In test B, we recorded latency to approach either of 2 familiar boxes (the wire-mesh box and an identical open box with a small portion of concentrate) within 600 s and how often cows directed behaviors toward the wire-mesh box (number of occurrences/5 min). On d −6 (test A), no clear differences in feeding motivation among treatments were found. On d −5 and −1, cows previously fed the energy-reduced diet attempted to feed approximately 75% more on d −1 compared with d −6 (test A). On d 2 (test B), cows previously fed the normal diet directed 40% more behavior toward the wire-mesh box than cows previously fed the energy-reduced diet. Reducing feeding level, either before or on the dry-off day, resulted in consistently increased feeding motivation, interpreted as a sign of hunger. No clear effects of change in milking frequency, singly or combined with reduced diet energy density, on feeding motivation were found before d 0. Whereas, on d 2, cows previously milked twice daily were quicker to approach a box than cows previously milked once daily. Cows injected with cabergoline attempted to feed more, but showed lower probability of vocalizing compared with saline-injected cows (d 1; test A), irrespective of treatment before d 0. The effects of cabergoline on feeding motivation are not easily interpreted and warrant further investigation. From a hunger perspective, reducing milking frequency rather than diet energy density seems to be a less negative management to reduce milk production before dry-off.

Key words: dairy cow, dry-off, hunger, welfare, dopamine agonist

INTRODUCTION

The dry period in dairy production refers to the 4-to-8-wk interval between the last day of milking at the end of the lactation period and the onset of the next lactation at calving (Andersen et al., 2005). A dry period aims to safeguard regeneration of udder cells and increase milk production in the subsequent lactation (Capuco et al., 1997). In commercial farms, dry-off is often initiated by abrupt dietary change and milking cessation on the day of last milking (i.e., the dry-off day; Vilar and Rajala-Schultz, 2020). Alternatively, to reduce milk production before the last milking and minimize the potential consequences of accumulating milk in the udder, such as pain induced by udder engorgement and milk leakage (Zobel et al.,...
2015), gradual reduction in feeding level, daily milking frequency, or both may be practiced during days before the dry-off day (Ollier et al., 2014; Rajala-Schultz et al., 2018). Furthermore, cessation of milk production and acceleration of mammary gland involution may be facilitated by use of pharmaceutical dopamine agonists such as cabergoline (e.g., Bach et al., 2015; Boutinaud et al., 2016; Bertulat et al., 2017). None of the available studies examining cabergoline in cows, however, included measures of feeding motivation. The secretion of pituitary prolactin is mostly controlled by the hypothalamus and dopamine neurons, which are responsible for modulation of several physiological and cognitive processes, such as energy balance, feeding motivation, and perception of feed (Berridge, 1996; Freeman et al., 2000; Spruijt et al., 2001; Martins et al., 2017). Changes in feeding motivation are not part of the targeted aim of the pharmaceutical dopamine agonists when used to reduce milk synthesis at dry-off. However, it cannot be ruled out that administration of cabergoline may induce changes in a cow’s feeding motivation, and understanding the consequences of this is important to evaluate the effects of various dry-off management strategies on animal welfare and production.

Either abrupt or gradual, current dry-off management practices challenge the welfare of high-yielding cows (Zobel et al., 2015). For instance, reducing feeding level quantitatively (Tucker et al., 2009) or qualitatively (Odensten et al., 2007; Valizadeh et al., 2008) may result in negative energy balance and a higher rate of vocalizations than in cows not subjected to reduced feeding level. Increased rate of vocalizations might be a way that undernourished cows express their increased feeding motivation (Manteuffel et al., 2004). Given the positive relationship between milk yield and energy demand (Brown et al., 1977), it is possible that a modulation of daily milking frequency, together with dietary changes, may affect feeding motivation of cows. One way to assess the effects of dry-off on feeding motivation of cows can be through feed-thwarting tests. Such test paradigms rely on the recording of the frequency of appetitive feeding behaviors (e.g., search for and attempts to access feed) directed toward an inaccessible feed source during a predetermined period. Earlier studies used similar methodology to assess feed preference and feeding motivation in a range of species, such as rats (Platt and Wike, 1962), dogs (Thompson et al., 2016), and domestic fowl (Duncan and Wood-Gush, 1972).

We investigated the single and interactive effects of dietary and daily milking frequency changes during 1 wk before dry-off, as well as the effect of an injection of cabergoline following the last milking, on the feeding motivation of cows. Before dry-off, we hypothesized that adding straw to a normal lactation diet, thereby reducing its energy content (i.e., qualitative feed restriction), would result in a higher motivation to feed compared with when the cows were fed the normal diet. Additionally, the provision of an energy-reduced diet without simultaneous reduction in daily milking frequency would result in the highest degree of feeding motivation. Conversely, we hypothesized that reducing daily milking frequency from twice to once during 7 d before dry-off would reduce energy demand and, consequently, feeding motivation. After dry-off, cows dried off abruptly (i.e., previously fed a normal lactation diet and milked twice daily) were hypothesized to be more motivated to feed compared with cows on the other treatments, which were dried off gradually, due to a greater negative dietary shift when potentially synthesizing a high amount of milk. Furthermore, we hypothesized that the pharmacological inhibition of prolactin secretion would collaterally reduce feeding motivation in cows.

**MATERIALS AND METHODS**

**Study Population**

The present study was conducted from September 2017 to May 2019 at the Danish Cattle Research Centre, Aarhus University, Denmark, and was part of a large project investigating the effects of dry-off management on performance, metabolism, and welfare of dairy cows, as described in detail by Larsen et al. (2021), which describes the experimental design in detail along with the performance of the population of cows included in the current study. All procedures involving animals were approved by the Danish Animal Experiments Inspectorate in accordance with the Danish Ministry of Environment and Food Act No. 474 (May 15, 2014), approval number 2017–15–0201–01230. The experimental protocol (internal ref. F19–12–1919) was registered in the experimental facility data storage system at Aarhus University. The experimental work was conducted according to Good Clinical Practice Guideline VICH GL19 (VICH, 2001), and the study as well as the unregistered use of cabergoline (Velactis, Ceva Santé Animale) was approved by the Danish Medical Agency (permit no. 2017064040). In countries where Velactis is registered, Velactis is labeled to be used with abrupt dry-off (e.g., no reduction in feeding level or milking frequency before the last milking). Use in other dry-off regimens is off-label.

This study included 119 (72 primiparous and 47 multiparous) loose-housed, lactating, and pregnant
Holstein cows in a randomized block design with repeated measurements. Cows from the resident herd were continuously enrolled into blocks of 8 within parity group, 14 d before the dry-off day. The enrollment occurred biweekly in batches of 1 to 6 cows, depending on available cows, resulting in 36 successive batches [1 nonexperimental companion dry cow was kept with the experimental cow in case of n = 1 (3 batches)]. Thus, 2 to 6 cows were housed in an experimental home pen (Franchi et al., 2021) in the same barn as the resident pens from d −7 to 7 relative to the dry-off day. From d −14 to −8 relative to the dry-off day, cows were fed ad libitum a normal lactation partially mixed ration (PMR; NEL: 6.59 MJ/kg of DM) in computerized feed bins (Insentec B.V.) and were milked at an interval of a minimum of 8 h (7 h for primiparous cows) or 9 kg of milk per milking in an automatic milking system (DeLaval AB). During milking, cows were offered 3 kg/d of a commercial pelleted concentrate (SL395044, DLG; NEL: 6.76 MJ/kg of DM). Within block, cows were randomly allocated to treatment. The PMR and TMR were offered before dry-off and after dry-off, respectively, in computerized feed bins 4 times daily (at approximately 0630, 1030, 1430, and 2000 h). Treatments followed a 2 × 2 factorial arrangement with feeding level [normal lactation diet (NEL: 6.59 MJ/kg of DM) or energy-reduced lactation diet (NEL: 4.96 MJ/kg of DM), both fed for ad libitum intake] and daily milking frequency [twice (0530–0700 h and 1530–1630 h) or once (0530–0700 h)] from d −7 to −1 relative to the dry-off day. The energy-reduced lactation PMR was made by mixing barley straw into a portion of the lactation PMR, targeting an energy density of the dry-cow diet (Larsen et al., 2021). In addition to the experimental PMR, cows were offered either 3 kg/d (normal lactation diet) or 1 kg/d (energy-reduced lactation diet) of concentrate during milking. After the last milking, the study followed a 2 × 2 factorial arrangement with the inclusion of the third factor: a single intramuscular injection of cabergoline (Velactis, Ceva Santé Animale) or saline. In statistical terms, cow was the experimental unit, as treatments were applied at this level. After dry-off, all experimental cows were fed ad libitum a dry-cow diet (NEL: 5.40 MJ/kg of DM) and were not milked. The sample size per treatment was based on the availability of cows in the resident herd and supported by power calculations to detect differences in feeding motivation between treatment groups before dry-off (described in Franchi et al., 2019). Throughout the experimental period, experimenters were blinded to the treatment allocation, daily milking frequency and injection, and partly blinded to the diets because they were visible in the feed bins. A total of 115 cows were used for the testing, as 4 cows were excluded from the study before testing due to lameness (n = 2) or mastitis (n = 2). The 4 excluded cows were evenly distributed among treatments (Franchi et al., 2021). On d −14 relative to the dry-off day, cows weighed (mean ± SD) 778 ± 77 kg, were 225 ± 6 d in pregnancy, and yielded 26 ± 6 kg of milk per day.

Procedures and Testing

Test A: Feed-Thwarting Test in the Home Pen. On d −6, −1, and 1 relative to the dry-off day, test A took place in the experimental home pen following the end of the second daily, fresh feed delivery, lasting (median; range) 36 min; 14 to 76 min. The choice of test days was made to fit the weekly schedule of the large experiment. Allocation of cow to experimental computerized feed bin occurred before moving cows into the pen. Each cow was assigned to only 1 of the 6 feed bins throughout the experimental period, and if there were less than 6 cows in the pen, the unassigned feed bins were empty and locked to all cows. No protocol ensuring that cows learned which bin they were assigned to was adopted before data collection, as all cows had previous experience with the Insentec feed bin system as well as being assigned to another Insentec feed bin. Upon the start of feed delivery, all feed bins in the barn were remotely locked, and remained locked until the completion of feed delivery. Meanwhile, an experimenter switched the experimental feed bins from automatic to manual mode. Hence, when the feed delivery ended, all nonexperimental feed bins elsewhere in the barn were remotely unlocked, and feed was made accessible to the cows, except the experimental feed bins that remained locked for additional 35 min. The 35-min period was chosen as it was the approximate median duration of the second daily feed delivery during pilot testing, and would approximately double the duration of the period without access to feed. Hence, experimental cows had no access to their rations for approximately 71 min per test session (36 min of feed delivery plus 35 min of feed-thwarting test). During this 35-min period, experimental cows could hear nonexperimental feed bins opening and see cows in neighboring pens feed. One neighboring pen was adjacent to the experimental home pen (Franchi et al., 2021), and the second neighboring pen was on the opposite side of the corridor to the automatic milking system. During the 35-min period, 1 familiar experimenter stood silently in front of the feed bins, approximately 4 m away, and recorded attempts to feed and vocalizations of each cow. Attempt to feed was defined as the focal cow putting her head over any locked feed bin containing feed while placing her collar past
the feed bin gate. Vocalization was defined as the focal cow displaying an uninterrupted open-mouth call of at least a 3-s duration while positioned anywhere in the pen. After the 35 min had elapsed, the experimenter unlocked the experimental feed bins and recorded the latency of each experimental cow to open her own feed bin and start eating her respective diet. The time point was recorded to the nearest second with a stopwatch (Select). A maximum latency of 300 s was used, after which latencies were right-censored. This duration was chosen because it was the median time period taken by cows to put their heads over any locked feed bin after the end of the feed delivery during pilot testing. No relationship between the feed delivery duration and frequencies of attempts to feed and vocalizations per 35 min, as well as latency to feed, on each test day were found (Franchi et al., 2021).

**Test B: Feed-Thwarting Test with Concentrate.** Between approximately 0830 and 1100 h on d −5 and 2 relative to the dry-off day, cows were individually subjected to the second feed-thwarting test, which took place in a (length × width) 6.3 × 4 m pen (Franchi et al., 2021) adjacent to the experimental home pen, permitting visual contact to peers. The first test day comprised 2 phases, habituation and testing, and only testing was conducted on the second test day. Initially, each cow was individually moved to the test pen following the ascending order of the identification numbers and offered a small portion of concentrate (150 g; same type as offered during milking) in an open gray plastic box (length × width × height: 36 × 26.5 × 11 cm; Franchi et al., 2021) located 5 m away from the pen entrance. Habituation was defined as the cow sniffing (Table 1), eating the concentrate, or both. No failing criteria was set, as cows were offered unlimited time to respond to the portion of concentrate. All cows fulfilled the habituation criteria within 10 min. Following habituation, the cow was walked back to the home pen to allow for the setting of the testing phase (approximately 2 min). Subsequently, the same cow returned to the test pen where 2 plastic boxes were placed next to each other at the same location as before: the familiar box with a new free 150-g portion of concentrate and another identical box full of concentrate (approximately 5 kg), but covered with a wire-mesh lid with 2.5 × 2.5 cm square gaps (i.e., the concentrate could be seen and smelled, but not reached or eaten; Franchi et al., 2021). After entering the pen (i.e., the whole body was past the pen gate), each cow had 600 s to sniff or make physical contact with either of the 2 boxes. The latency to approach the boxes was recorded to the nearest second with a stopwatch. Cows failing to contact any of the 2 boxes within 600 s were right-censored at 600 s and immediately removed from the test pen. This duration was chosen for consistency with the habituation time period. From the moment of the first response (sniffing, physical contact, or both) directed toward either of the 2 boxes, all behaviors directed toward each of the boxes (Table 1) were recorded during 5 min by an experimenter standing outside the pen, approximately 5 m away. After that, the cow returned to the home pen. On both days, cows were tested in the same ascending order of the identification numbers. After each day, both boxes were emptied and cleaned. The choice of days to conduct test B was made to fit the weekly schedule of the large experiment. A timeline summarizing the testing relative to the dry-off day is illustrated in Figure 1.

**Statistical Analyses**

All variables were analyzed with mixed-effects modeling in R v.3.6.1 (https://www.r-project.org/). P-values

<table>
<thead>
<tr>
<th>Behavior</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biting</td>
<td>Cow encloses her mouthparts around any part of a box.</td>
</tr>
<tr>
<td>Licking</td>
<td>Cow visibly passes the tongue over any part of a box, with physical contact.</td>
</tr>
<tr>
<td>Pushing</td>
<td>Cow moves a box from its original position by pushing with her forehead, nose, or muzzle.</td>
</tr>
<tr>
<td>Sniffing2,3</td>
<td>Cow keeps her nostrils within ~5 cm from a box or touches a box with the nose while the mouth is closed.</td>
</tr>
<tr>
<td>Stepping2,4</td>
<td>Cow raises at least 1 leg, keeping the hoof out of contact with the floor, and sets it down forcefully without any net movement of the body.</td>
</tr>
<tr>
<td>Vocalizing5,6</td>
<td>Open-mouth call directed toward a box lasting at least 3 s.</td>
</tr>
</tbody>
</table>

1Following the first response to either of 2 boxes (an open box containing 150 g of concentrate and another identical box covered with a wire-mesh lid and filled with concentrate) within 600 s from entering the test pen, the behaviors directed toward a box were recorded for 5 min.
2If the interval between bouts of the same interaction was less than 1 s, the interaction was recorded as 1.
3Distance between cow’s nostrils and a box was visually estimated.
4Behaviors were mutually exclusive, except “stepping,” which may occur concurrently with any of the other behaviors.
5If the interval between successive vocalizations was less than 3 s, the vocalizations were recorded as 1.
6Duration of vocalization was measured by approximate counting.
< 0.05 were considered significant, and values of 0.1 > P ≥ 0.05 were considered tendencies. Each variable was analyzed separately for the period before and the period after dry-off. Before dry-off, models of variables in test A included fixed effects of parity (primiparous, multiparous), day relative to dry-off (−6 and −1), feeding level (normal, energy-reduced), daily milking frequency (twice, once), and all possible 2- and 3-way interactions between feeding level, daily milking frequency, and day. Number of cows in the pen (2–6) was included as a covariate. Batch and cow were included as random effects to account for dependency between cows in the same batch and between repeated measures on cows, respectively. Models of variables in test B included fixed effects of parity, feeding level, daily milking frequency, and the 2-way interaction between feeding level and daily milking frequency. Number of cows in the pen was included as a covariate. Batch was included as a random effect. As test B variables were only recorded once before dry-off, the fixed effect of day relative to dry-off and the random effect of cow were not included in the models. After dry-off, all models included the fixed effects parity, feeding level, daily milking frequency, type of injection (cabergoline; saline), and all possible 2- and 3-way interactions between feeding level, daily milking frequency, and type of injection. Injection. Number of cows in the pen was included as a covariate. Batch was included as a random effect. Tukey-adjusted posthoc analyses at a significance level of P < 0.05 were performed for each model on each period using the package emmeans v.1.5.0 (Lenth, 2020).

**Test A Variables.** The first batch containing 5 cows (1 cow fed normal diet, milked twice daily, injected with cabergoline; 2 cows fed normal diet, milked once daily, injected with saline solution; 2 cows fed energy-reduced diet, milked twice daily, injected with cabergoline) was not tested on d 1 due to miscommunication with the barn staff who initiated the feed delivery earlier than planned. Additionally, 1 cow (fed normal diet, milked twice daily, injected with cabergoline and 1 cow fed normal diet, milked twice daily, injected with saline solution) was removed from the analysis due to lameness on d −1, and 2 cows (1 cow fed normal diet, milked once daily, injected with cabergoline and 1 cow fed normal diet, milked twice daily, injected with saline solution) were removed because they had been feed-deprived for 18 h between d −1 and 0 due to feed bin malfunction. Furthermore, a fourth cow (fed normal diet, milked once daily, injected with saline solution) was removed from the study on d 1 due to mastitis. Hence, 115 cows on d −6, 112 cows on d −1, and 106 cows on d 1 were included in the statistical analyses of the test A variables. The frequency of attempts to feed per 35 min was analyzed with a generalized linear mixed-effects model with zero-inflated log-link negative binomial distribution using the package glmmTMB v.1.0.2.1 (Brooks et al., 2017). Due to the high proportion of cows not vocalizing on each test day (Franchi et al., 2021), frequency of vocalizations per 35 min was transformed into a binary variable (yes; no) and analyzed with a mixed-effects logit-link logistic regression using the package glmmTMB v.1.0.2.1. The fit of the models was confirmed by graphical assessment of the residuals using the package DHARMa v.0.3.2.0.

**Figure 1.** Timeline illustrating the days when the feed-thwarting test in the home pen (test A) and the feed-thwarting test with concentrate in an adjacent pen (test B) took place relative to the dry-off day, when cows were milked for the last time and subsequently injected with either cabergoline or saline solution. Before dry-off, diets were either the normal lactation diet or energy-reduced lactation diet (NEₜ = 6.59 or 4.96 MJ/kg of DM, respectively), both for ad libitum intake. Furthermore, cows were milked either twice (0530–0700 h and 1530–1630 h) or once (0530–0700 h) daily. During milking, cows were offered either 3 kg/d (normal lactation diet) or 1 kg/d (energy-reduced lactation diet) of a commercial pelleted concentrate (SL395044, DLG; NEL: 6.76 MJ/kg of DM). After dry-off, cows were fed a dry-cow diet for ad libitum intake (NEₜ = 5.40 MJ/kg of DM) and not milked. Test A occurred on d −6, −1, and 1 relative to the dry-off day, and test B occurred on d −5 and 2 relative to the dry-off day. The choice of days on which to conduct tests A and B was made to fit in the experimental weekly schedule of the large experiment and to give cows the opportunity to behaviorally adapt to their respective experimental treatments.
The latencies to feed within 300 s following end of the 35-min period were analyzed with Cox’s proportional hazards mixed-effects model using the packages \textit{survival} v.3.2–3 (Therneau, 2020a) and \textit{coxme} v.2.2–16 (Therneau, 2020b). Fit of the model was checked by assessing significance of the integrated log-link test. A hazard ratio (HR) $>1$ indicates that the probability of feeding for 1 level of an explanatory categorical variable is higher than for the level with which it is compared. Correspondingly, a HR $<1$ indicates a lower probability of feeding. In test A, cows that were highly motivated to feed were expected to display a high frequency of attempts to feed, an increased probability of vocalizing, and a shortened latency to feed within 300 s.

**Test B Variables.** One cow (fed energy-reduced diet, milked once daily, injected with cabergoline) showed signs of reduced responsiveness before and after testing on d $-5$ and was removed from the analyses on that day. Additionally, the same 4 cows that were removed from test A analyses due to health issues or lack of access to feed were also excluded from the analyses of test B. Hence, 114 cows on d $-5$ and 111 cows on d 2 were included in the statistical analyses of the test B variables. The latencies to approach any of the 2 boxes within 600 s were analyzed with Cox’s proportional hazards mixed-effects model. Fit of the model was checked by assessing significance of the integrated log-link test. As described above, a HR $>1$ indicates higher probability of approaching a box within 5 min, and a HR $<1$ indicates a lower probability of approaching a box within this interval. The breakdown on the numbers of each behavioral element directed toward each box on each test day is reported in Franchi et al. (2021). For the analysis of feeding motivation in test B, only behaviors directed toward the wire-mesh box were considered, as they represented the feed-thwarting situation. The open box containing 150 g of concentrate was made available during testing to provide a free alternative feed source to stimulate feeding motivation. On d $-5$, 87% of all cows fed from the free concentrate. On d 2, 97% of all cows fed from the free concentrate. The frequency of behaviors directed toward the wire-mesh box per 5 min was analyzed with a generalized linear mixed-effects model with zero-inflated log-link negative binomial distribution using the aforementioned R package. Fit of the model was confirmed by graphical assessment of the residuals. In test B, cows highly motivated to feed were expected to display a shortened latency to approach a box within 600 s and direct an increased frequency of behaviors toward the wire-mesh box.

**Interobserver Reliability Assessment.** One main experimenter conducted 80% of test A and 92% of test B. Six different experimenters conducted the remaining test A, and 3 different experimenters conducted the remaining test B. Hence, accounting for this, 1-way random effects and absolute agreement intraclass correlation coefficients (ICC; 1,1) (Koo and Li, 2016) were computed for the frequency of attempts to feed and vocalizations (test A) and the frequency of behaviors directed toward the wire-mesh box (test B). For test A, each of the 6 experimenters performed the test together with the main experimenter 1 to 2 times on different days (21 cows observed in total). Meanwhile, for test B, testing was repeated once for each of the 3 experimenters, together with the main experimenter, on different days (16 cows observed in total). Calculations were performed using the package \textit{irr} v.0.84.1 (Gamer et al., 2019). The ICC for attempts to feed was 0.96 (95% CI: 0.92 < ICC < 0.98), the ICC for vocalizations was 0.96 (95% CI: 0.91 < ICC < 0.98), and the ICC for frequency of behaviors directed toward the wire-mesh box was 0.88 (95% CI: 0.69 < ICC < 0.95). All computed ICC revealed strong agreement (Koo and Li, 2016) among experimenters.

**RESULTS**

The \(P\)-values and test statistics of all factors included in the models are listed in Table 2. For each variable in each period, significant effects or interactions are presented first, and tendencies are presented subsequently.

**Before Dry-Off**

**Frequency of Attempts to Feed Per 35 min (Test A).** An interaction between feeding level and day was found (\(P < 0.01\); Table 2). Cows fed the normal diet did not differ in frequency of attempts to feed between d $-6$ and $-1$ (LSM, 95% CI: 5, 4–7 vs. 5, 4–8, respectively). However, cows fed the energy-reduced diet attempted to feed more often on d $-1$ (7, 6–9) compared with d $-6$ (4, 3–5; \(P < 0.05\)). On each given day, no differences between dietary treatments were found.

**Probability of Vocalizing During the 35-min Feed-Thwarting Period (Test A).** An interaction between feeding level, milking frequency, and day was found (\(P = 0.03\); Table 2). On d $-6$, the descending order of probability of vocalizing tended to be as follows: energy-reduced diet and milked twice daily (probability, 95% CI: 0.8, 0.37–0.95), energy-reduced diet and milked once daily (0.6, 0.23–0.88), normal diet and milked once daily (0.3, 0.07–0.68), and normal diet and milked twice daily (0.2, 0.05–0.61). On d $-1$, cows fed the energy-reduced diet and milked once showed the highest probability of vocalizing (0.8, 0.38–0.95).
These cows differed in probability of vocalizing from cows fed the normal diet and milked twice daily (0.2, 0.04–0.60; \( P = 0.04 \)) and cows fed the normal diet and milked once daily (0.1, 0.01–0.45; \( P < 0.01 \)). However, their probability of vocalizing was not different from cows fed the energy-reduced diet and milked twice daily (0.5, 0.17–0.84).

Latency to Feed Within 300 s (Test A). An interaction between feeding level and day was found (\( P = 0.02 \)), Table 2. On d −6, approximately 33% of the all cows fed within 300 s. Yet, on that day, cows fed the normal diet were quicker to feed (300, 125–300 s; HR = 0.5; 95% CI = 0.31–0.76; \( P < 0.01 \)) compared with cows fed the energy-reduced diet (300, 198–300 s; HR = 0.4; 95% CI = 0.21–0.56; \( P < 0.01 \)). On d −1, approximately 77% of all cows fed within 300 s. On that day, cows fed the energy-reduced diet were quicker to feed (43, 10–135 s; HR = 2.9; 95% CI = 1.87–3.92; \( P < 0.01 \)) compared with cows fed the normal diet (105, 15–300 s; HR = 1.6; 95% CI = 1.00–2.23; \( P < 0.01 \)).

Latency to Approach a Box Within 600 s and Frequency of Behaviors Directed Toward the Wire-Mesh Box Per 5 min (Test B).

Figure 2. Survival plot of the cumulative probability of feeding within 300 s after feed bins were unlocked (test A) of cows fed either the normal lactation diet (n = 54; black lines) or energy-reduced diet (n = 58; blue lines) on d −6 (dotted lines) and −1 (solid lines) relative to dry-off. The y-axis displays the probability of cows to open their particular feed bin and start feeding, and the x-axis displays the time (s) the cows took to feed. The red dashed reference line indicates when 50% of the cows started feeding.

Table 2. Test statistics (\( \chi^2 \) test) and \( P \)-values for frequency of attempts to feed per 35 min, probability of vocalizing during the 35-min period, and latency to feed within 300 s recorded in the feed-thwarting test in the home pen (test A) as well as latency to approach a box within 600 s and frequency of behaviors directed toward the wire-mesh box per 5 min recorded in the feed-thwarting test with concentrate (test B).

<table>
<thead>
<tr>
<th>Test</th>
<th>Variable</th>
<th>Statistic (^2)</th>
<th>F</th>
<th>M</th>
<th>D</th>
<th>F × M</th>
<th>F × D</th>
<th>M × D</th>
<th>F × M × D</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Attempts to feed (no./35 min)</td>
<td>( \chi^2 ) test</td>
<td>0.28</td>
<td>0.51</td>
<td>18.67</td>
<td>0.24</td>
<td>9.89</td>
<td>0.54</td>
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<tr>
<td></td>
<td></td>
<td>( P )-value</td>
<td>0.59</td>
<td>0.47</td>
<td>&lt;0.01</td>
<td>0.62</td>
<td>&lt;0.01</td>
<td>0.46</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>Vocalizations (yes; no)</td>
<td>( \chi^2 ) test</td>
<td>10.47</td>
<td>0.003</td>
<td>0.81</td>
<td>0.04</td>
<td>0.57</td>
<td>0.53</td>
<td>4.51</td>
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<td></td>
<td></td>
<td>( P )-value</td>
<td>&lt;0.01</td>
<td>0.95</td>
<td>0.37</td>
<td>0.83</td>
<td>0.45</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>Latency to feed (s)</td>
<td>( \chi^2 ) test</td>
<td>1.93</td>
<td>0.01</td>
<td>53.55</td>
<td>1.39</td>
<td>5.05</td>
<td>2.14</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td></td>
<td>( P )-value</td>
<td>0.16</td>
<td>0.92</td>
<td>&lt;0.01</td>
<td>0.24</td>
<td>0.02</td>
<td>0.14</td>
<td>0.95</td>
</tr>
<tr>
<td>B</td>
<td>Latency to approach a box (s)</td>
<td>( \chi^2 ) test</td>
<td>21.11</td>
<td>2.53</td>
<td>—</td>
<td>0.15</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td>( P )-value</td>
<td>&lt;0.01</td>
<td>0.11</td>
<td>—</td>
<td>0.09</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>B</td>
<td>Behaviors (no./35 min)</td>
<td>( \chi^2 ) test</td>
<td>16.43</td>
<td>0.12</td>
<td>—</td>
<td>0.04</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td>( P )-value</td>
<td>&lt;0.01</td>
<td>0.72</td>
<td>—</td>
<td>0.83</td>
<td>—</td>
<td>—</td>
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</tr>
</tbody>
</table>

\(^{1}\)Before dry-off, the analyses followed the 2 × 2 factorial arrangement of treatment factors feeding level (F) and daily milking frequency (M) on days (D) −6 and −1 for test A and −5 for test B. After dry-off, the analyses followed the 2 × 2 × 2 factorial arrangement of treatment factors feeding level (F), daily milking frequency (M), and type of injection (I) on d 1 for test A and d 2 for test B. The effects of parity group (primiparous, multiparous) or number of cows in the pen (2 to 6) were not significant (\( P > 0.1 \)) for the presented variables and are therefore not presented in the table.

\(^{2}\)Degrees of freedom = 1; test type = 2.
only 1 cow (fed energy-reduced diet, milked once daily, injected with saline) did not approach a box within 600 s. Cows fed the energy-reduced diet were quicker to approach a box (median, IQR: 13, 5–37 s; HR = 1.7; 95% CI = 1.24–2.13) than cows fed the normal diet (60, 21–124 s; HR = 0.6; 95% CI = 0.42–0.74; P < 0.01; Table 2). A tendency for interaction between feeding level and milking frequency was found (P = 0.09; Table 2). The descending order of probability of vocalizing tended to be: normal diet and milked twice daily (0.7, 0.37–0.87), energy-reduced diet and milked once daily (0.4, 0.18–0.65), energy-reduced diet and milked twice daily (0.4, 0.17–0.62), and normal diet and milked once daily (0.4, 0.15–0.62).

**Probability of Vocalizing During the 35-min Feed-Thwarting Period (Test A).** Cows injected with cabergoline showed a lower probability of vocalizing (probability, 95% CI: 0.3, 0.16–0.45) than saline-injected cows (0.6, 0.44–0.76; P < 0.01; Table 2). A trend for interaction between feeding level and type of injection was found (P = 0.05; Table 2). The descending order of probability of vocalizing tended to be: normal diet and milked twice daily (0.69–2.73), energy-reduced diet and milked once daily (0.44–2.57; P = 0.07), cows fed the normal diet and saline-injected (0.37–0.87), and cows fed the energy-reduced diet and cabergoline-

After Dry-Off

**Frequency of Attempts to Feed Per 35 min (Test A).** Cows injected with cabergoline attempted to feed more (LSM, 95% CI: 8, 6–11) than cows injected with saline (6, 5–8; P < 0.01; Table 2). A tendency for interaction among feeding level, milking frequency, and type of injection was found (P = 0.05; Table 2; Figure 4). Cows injected with cabergoline tended to attempt to feed more than cows injected with saline solution, irrespective of treatment before dry-off, except among cows previously fed the energy-reduced diet and milked twice daily.

![Figure 3](image-url) **Figure 3.** Survival plot of the cumulative probabilities of approaching a box within 600 s (test B) of cows fed the normal lactation diet (black line; n = 56) and cows fed the energy-reduced diet (blue line; n = 58) on d −5 relative to dry-off. The y-axis displays the probability of cows to approach a box, and the x-axis displays the time (s) the cows took to approach and direct the first behavior toward one of the 2 boxes. The red dashed reference line indicates when 50% of the cows approached a box.

![Figure 4](image-url) **Figure 4.** Frequency of attempts to feed during the 35-min period when access to the fresh feed was thwarted (test A) across the 8 treatments (diet energy density × daily milking frequency × type of injection; n = 14 for all treatments) on d 1 after dry-off. The bars represent LSM, and the error bars indicate 95% CI. Red dots illustrate the individual variation in frequency of attempts to feed.
injected (209, 26–300 s; HR = 0.6; 95% CI = 0.22–1.05; P = 0.06).

Latency to Approach a Box Within 600 s and Frequency of Behaviors Directed Toward the Wire-Mesh Box Per 5 min (Test B). On d 2, all cows approached a box within 600 s. Cows previously milked twice daily were quicker to approach a box (median, IQR: 5, 4 to 8 s; HR = 1.3; 95% CI = 1.02–1.64) than cows previously milked once daily (6, 4–16 s; HR = 0.8; 95% CI: 0.59–0.97; P = 0.01; Table 2; Figure 6). Cows previously fed the normal diet directed more behaviors toward the wire-mesh box (LSM, 95% CI: 7, 6–9) than cows previously fed the energy-reduced diet (5, 4–6; P < 0.01; Table 2).

DISCUSSION

This study investigated the effects of dry-off management involving changes in feeding level and daily milking frequency during 1 wk before the dry-off day, as well as administration of cabergoline after the last milking on the dry-off day, on the feeding motivation of cows using 2 different feed-thwarting tests. On d −6 (test A), unclear differences in feeding motivation among treatments were found. On d −5 and −1, cows fed an energy-reduced lactation diet had a higher feeding motivation than cows fed the normal lactation diet as indicated by higher probability of vocalizing and a shorter latency to feed (test A), as well as more behavior directed toward the box and a shorter latency to approach a box. Furthermore, cows fed the energy-reduced diet attempted to feed more on d −1 compared with d −6 (test A). After dry-off, cows previously fed the normal diet directed more behavior toward the wire-mesh box than cows previously fed the energy-reduced diet, and cows previously milked twice daily were quicker to approach a box than cows previously milked once daily (test B, d 2). Additionally, cows injected with cabergoline attempted to feed more, but displayed a lower probability of vocalizing compared with saline-injected cows, regardless of treatment before dry-off (test A, d 1). Results from each period relative to dry-off and overview of tests are discussed below.

Before Dry-Off

On d −6 (test A), the effects of dry-off management on attempts to feed, probability of vocalizing,
and censored latency to feed were unclear and should thus be interpreted cautiously. No differences among treatments were found in the frequency of attempts to feed, which may have been due to a potential lack of habituation with the novel context (e.g., novel pen, group, and management). Cows may have prioritized exploratory behavior over feeding or attempting to feed on d −6 (von Keyserlingk et al., 2008), revealing that this day was rather early to detect treatment effects on this feeding motivation measure. Additionally, among cows milked twice daily, cows fed the energy-reduced diet tended to show a higher probability of vocalizing than cows fed the normal diet. Vocalizations might have been the way cows expressed their refusal to feed from the energy-reduced diet or a response to a thwarted motivation to feed induced by the energy-reduced diet and concomitant high energy demand for milk production, as well as from seeing and hearing nonexperimental cows feeding (Valizaheh et al., 2008; Tucker et al., 2009; Green et al., 2018). Nevertheless, we cannot rule out, across all experimental cows, the possible effects of recent separation from the resident herd and regrouping on this behavior (Fiorevich et al., 2006), which could explain the absence of clear differences in probability of vocalizing among treatments. Cows fed the normal diet were quicker to feed following the end of the feed-thwarting period. During the initial hours after the arrival to the experimental pen and the change from lactation diet to the energy-reduced diet, cows subjected to the qualitative feed restriction might have refused to feed from the lower energy diet and thus may not have been motivated to acquire more of that. Alternatively, these cows might have been hesitant to feed from the energy-reduced diet due to its novelty (Van Os et al., 2017). Irrespectively, this finding has to be interpreted carefully because fewer than 50% of all cows fed within 300 s on that day. Although cows knew how to feed from the Insentec feed bins, most of them might not have learned which experimental feed bin was assigned to them at that point in time. Future studies using this feed-thwarting test paradigm should consider implementing a protocol to ensure that cows are habituated with the novel context before the data collection.

On d −5 and −1, cows fed the energy-reduced diet consistently showed signs of increased feeding motivation as compared with the cows fed the normal diet. Namely, cows fed the energy-reduced diet showed a higher probability of vocalizing (test A), were quicker to feed within 300 s (test A), were approximately 5-fold quicker to approach a box (test B), and directed more behavior toward the wire-mesh box (test B) compared with cows fed the normal diet. During the week before dry-off, cows fed the energy-reduced diet had a lower mean DMI than cows fed the normal diet (12 ± 0.41 vs. 17.6 ± 0.41 kg/d, respectively), and cows fed the energy-reduced diet showed a drop of approximately 50% in energy consumption and consequent negative energy balance (Larsen et al., 2021). Franchi et al. (2019, 2020) tested the feeding motivation of 2 different subgroups of the current study’s population using 2 different test paradigms (operant-based and latency to feed, respectively) conducted outside the home pen and similarly found a consistently increased feeding motivation in cows fed the energy-reduced diet between d −5 and −2 relative to the last milking. Altogether, this evidence suggests that reducing feeding level during a gradual dry-off can increase feeding motivation of cows who may not adapt to this management, despite the reduction in milk yield and, consequently, nutrient demand (i.e., this qualitative restriction of energy supply reduced milk yield by approximately 38% during the week before dry-off; Larsen et al., 2021).

Unexpectedly, reducing the daily milking frequency, singly or concomitantly with dietary changes, had no clear effects on feeding motivation before dry-off. The present findings are similar to what was reported in other parts of the project (Franchi et al., 2019, 2020). Our current tests were conducted approximately 4 h after all cows had been milked, which might have jeopardized the assessment of the effects of milking frequency changes on feeding motivation. Perhaps, had tests A and B been conducted after the second daily milking, cows milked once daily would have had more milk accumulating in the udder, which could have affected their mobility and motivation to attempt to access feed. Otherwise, more than 1 wk of gradual dry-off by reduced milking frequency may be needed to detect effects of daily milking frequency change on feeding motivation. For instance, Rémond et al. (2004) compared the DMI of TMR-fed Holstein cows milked either once or twice daily during the first 14 wk of lactation and reported a substantially lower DMI in cows milked once daily from the seventh experimental week, suggesting a slow translation of decreased milk production into decreased feeding motivation.

**After Dry-Off**

Irrespective of when feeding level reduction occurs, either from 1 wk before the dry-off day or on the dry-off day, this management can increase feeding motivation in cows. In fact, on d 1 (test A), cows previously fed the energy-reduced diet and injected with saline tended to be the quickest to feed within 300 s. We expected that cows fed the energy-reduced diet would only to
some degree show increased feeding motivation due to a minor dietary shift on the dry-off day and a lowered energy demand resultant from a decreased milk production. Knowing that these cows were in negative energy balance (Larsen et al., 2021) due to the energy-reduced diet and continued milking during the week before dry-off, these consistent signs of increased feeding motivation may be interpreted as hunger (i.e., a negative affective state caused by undernourishment and lasting beyond the period between meals or feed deliveries; adapted from D’Eath et al., 2009). Furthermore, cows subjected to the most abrupt feeding level and milking frequency reduction on the dry-off day (i.e., were dried off abruptly) tended to show the highest probability of vocalizing. These cows faced the most dramatic reduction in diet energy density on the dry-off day, which may be related to a thwarted feeding motivation when presented with the dry-cow diet (Valizaheh et al., 2008; Tucker et al., 2009), as well as an engorged udder and increased intramammary pressure (Zobel et al., 2015). Cows dried off abruptly had the highest milk yield during the week before dry-off, and their udders were reportedly the most engorged on d 1 (Larsen et al., 2021). On d 2 (test B), cows previously fed the normal diet directed more behavior toward the wire-mesh box than cows previously fed the energy-reduced diet, which was likely an effect of the negative dietary shift on the dry-off day. Moreover, cows previously milked twice daily showed shorter latency to approach a box in comparison to cows previously milked once daily. Milk synthesis, and consequently energy demand, can be persistently high in the beginning of the dry period (Vilar and Rajala-Schultz, 2020), which may explain this effect of milking frequency on feeding motivation.

As mentioned, the current study is the first to investigate the effects of cabergoline on feeding motivation of dairy cows at dry-off. Few effects of cabergoline administration were found, inconclusively pointing in both directions in terms of feeding motivation. Cows injected with cabergoline attempted to feed more but displayed a lower probability of vocalizing compared with saline-injected cows on d 1 (test A), irrespective of treatment before dry-off. To understand the effects of cabergoline on feeding motivation of cows, further studies are needed that involve variables such as clinical and cognitive measures, automatically monitored feeding behavior data, and preferably data including the first 24 h following injection (Cabergoline’s half-life ranges from 17 and 40 h; Committee for Medicinal Products for Veterinary Use, 2014). Knowing that the DMI of cabergoline-injected cows declined abruptly on the dry-off day (Larsen et al., 2021), had the tests been conducted on that day, the effects of cabergoline on feeding motivation could probably have been interpreted more clearly.

Overview of the Tests

The test paradigms shared strengths and limitations for testing feeding motivation of cows. First, no training of cows was needed, except habituation to the test pen and boxes in test B to avoid fear of situational novelty (Herskin et al., 2004). Therefore, no cows were excluded because of failure to fulfill training criteria. Second, tests were not invasive because they required little or no handling of cows. Third, both tests, but especially the test B, were quick to conduct. Fourth, testing in the home environment or in an adjacent location permitted visual and auditory contact among experimental cows, and thus avoided social isolation, which could have affected cows’ responses during testing (Pedersen et al., 2002). Fifth, both tests stimulated meaningful behavioral responses specifically related to the alleviation of the energy insufficiency in question (Weary et al., 2017). Sixth, tests allowed little or no consumption of feed, and this is specifically critical when using concentrate as a feed reward. Access to large amounts of concentrate would have disrupted the effects of the experimental diets on the cow’s feeding motivation (D’Eath et al., 2009). Thus, we suggest that the feed-thwarting tests are effective ways to assess feeding motivation of cows and to ensure limited feed consumption, limited disruption of the experimental diets, and limited negative consequences to their health. With proper adjustments, the tests can meaningfully assess feeding motivation in other domestic animal species subjected to reduced feeding level, such as gestating sows (e.g., Bench et al., 2013) and broiler breeders (e.g., Arrazola et al., 2020).

In test A, anecdotal observation suggested that some cows displayed high frequencies of attempts to feed and vocalizations for most of the 35-min period, and they appeared to give up trying to open their feed bin toward the end of the testing period and entered the lying area of the pen. Individuals responding in this way would be assigned long latencies to feed even though they had made several attempts to feed during most of the testing period. We can speculate that the intensity of feeding motivation grew during the feed-thwarting period (Van der Harst and Spruijt, 2007) until a point where the efforts to access feed (e.g., attempts to open the bin, vocalizations, walking and standing in the feeding area) might have seemed pointless to some cows, who then prioritized other activities such as lying or drinking. If some cows had ceased to attempt to access feed toward the end of the test, this may have been a potential source
of variation in the results. Hence, the duration of the feed-thwarting period, in addition to the period of feed delivery that varied in duration, might unintentionally have reduced the sensitivity of the behavioral measures. Future studies using this paradigm should determine a cut-off point at which the cows give up getting access to feed to adapt the test duration to below this point to ensure high sensitivity of the measures in terms of feeding motivation. Furthermore, future studies could consider the following points: adding cues signaling to cows the end of the deprivation period and possibility to resume feeding; right-censoring the latency to feed at a later time point (e.g., 600 s, 900 s), permitting a higher likelihood of recording the latency to feed on-site; or simply not right-censoring the latency to feed and have it recorded through footage.

In test B, the use of concentrate, a highly energetic and palatable feed, as feed reward might have further motivated cows to feed. In addition, cows might have associated the presence of boxes with the forthcoming feed reward, which may have stimulated cows and increased their willingness to enter the pen and approach the boxes (Chen et al., 2016; Pajor et al., 2000). Hence, we cannot rule out that feeding motivation of the cows during test B might have been overestimated by the choice of feed reward (D’Eath et al., 2009) and familiarization with the test setting, especially on d 2 when they were exposed to the boxes containing concentrate for the third time. Irrespective, the 2 feed-thwarting tests showed similar results before dry-off and thus provided sensitive measures for assessing the feeding motivation of cows. However, the interpretation of the test outcomes, and especially when repeated testing is used, can be further validated by studies of potential learning as well as effects of testing duration and type of feed reward to increase accuracy and meaningfulness of the feeding motivation measures.

CONCLUSIONS

The results of this study showed that dry-off management affected feeding motivation of high-yielding cows. Specifically, reducing the feeding level, even when the diet was provided for ad libitum intake, increased feeding motivation, herein interpreted as hunger. This was true when feeding level was reduced either during the week before dry-off or on the day of last milking. Clear effect of milking frequency change on feeding motivation was only observed after dry-off, when cows previously milked once daily were less motivated to feed than cows previously milked twice daily. The effects of cabergoline on feeding motivation were present, but unclear, and further research is required to understand the effects of this dopamine agonist on dairy cow feeding motivation. This study adds knowledge about the effects of dry-off on the welfare of dairy cows and suggests that, in terms of feeding motivation, restriction of nutrient supply, either before or on the dry-off day and even when the diet is provided for ad libitum intake, is associated with signs of hunger.

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