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Effects of a synthetic analogue of the bovine appeasing pheromone on the overall welfare of dairy calves from birth through weaning

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ABSTRACT

Environmental enrichment in the form of synthetic analogs of appeasing pheromones have shown promising results in improving the welfare of domestic animals including dogs, pigs, horses, and cattle. The main objective of this study was to determine if the use of the bovine appeasing pheromone **(BAP)** would improve the welfare of dairy calves; therefore, in this randomized controlled trial, 72 Holstein Friesian dairy calves were housed in individual hutches after birth and were randomly allocated to receive BAP or a placebo once every 2 weeks from birth through weaning. After weaning, calves were moved to group hutches according to treatment for 4 additional weeks. It was hypothesized that dairy calves treated with BAP would display fewer signs of stress compared with calves receiving the placebo during the weaning process. To operationalize stress, calves were fitted with triaxial accelerometers on the hind leg after birth, and activity levels were monitored throughout the experiment. Data on live weight gain **(ADG)** and cortisol levels in saliva and hair were also obtained. Calves were fitted with heart rate monitors every week for at least 24 h to assess heart rate variability **(HRV)**. The use of BAP had a positive effect on ADG after weaning and during group housing and resulted in increased resting time after weaning. Moreover, BAP, was associated with a reduction in the activation of the neuroendocrine system evidenced by higher HRV parameters after weaning, including increased standard deviation of beat to beat of normal sinus beats **(SDNN)** and root mean squares of successive differences **(RMSSD)**. These results suggest a potential welfare benefit of the use of BAP during the artificial rearing of dairy calves.
Keywords. Bovine Appeasing pheromone, dairy cattle, calf raising, welfare, animal wellbeing

INTRODUCTION

Artificial calf rearing systems (i.e., where the calf is removed from the dam and fed milk by hand or automatic feeder) have gained popularity in the dairy sector of industrialized countries in the past century (Medeiros et al., 2022). This is due to the increased intensity of food production systems, which aim to meet the rising global demand for dairy products while maintaining a sustainable business model (Clay et al., 2020; Cronin et al., 2014). Artificial rearing of dairy calves allows for intensive animal surveillance with the aim of limiting transmission of infectious disease and improving performance (Beaver et al., 2019). However, it is often associated with practices such as separating cow and calf at an early stage, social isolation, restricted planes of nutrition, accelerated milk weaning and the introduction of painful procedures (e.g., disbudding) (Moore et al., 2012). These practices have been shown to cause stress in the calves and have negative effects on their overall well-being (Barkema et al., 2015; Cantor et al., 2019; Costa et al., 2019).

Environmental enrichment has been defined as changes beyond the minimum standards in the animal's environment, or management practices that have a positive effect on physical and affective states (Newberry, 1995; Wells, 2009), and has been proven to mitigate some of the negative welfare effects of artificial rearing of calves (Mandel et al., 2016). Examples include social enrichment by housing calves with conspecifics (Costa et al., 2016; Overvest et al., 2018), nutritional enrichment including artificial teat feeding methods (Horvath and Miller-Cushon, 2017) or allowing the calf to suckle from the dam or a foster cow (Lidfors et al., 2010; Margerison et al., 2003) and occupational enrichment with the use of ropes and balls (Zobel et al., 2017). Although social housing and teat feeding fulfil essential needs for calves, individual housing and bucket feeding remain common practices in many farming systems. Thus, some would argue that social housing and teat feeding align with the definition of environmental enrichment, as they serve as

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

modifications that enhance welfare and promote natural behaviors beyond baseline requirements.

Sensory enrichment refers to any stimuli that can trigger one or more of an animal's senses and includes the use of music and brushes (Bolt and George, 2019). This category includes pheromones, which are semiochemicals (substances that carry a chemical message among animals, enabling the detection and discrimination of various molecules with different structures; Tirindelli et al., 2009)). These pheromones bind to a receptor in the vomeronasal organ or main olfactory epithelium of a target individual of the same species, generating a cascade of both electrical and molecular reactions in the thalamus, amygdala, and hypothalamus, producing a behavioral change by the activation of the neuroendocrine system (Francia et al., 2014; Tirindelli et al., 2009).

Appeasing pheromones were initially isolated from the mammary gland of lactating sows and were observed to produce a calming effect on the piglets (P Pageat - US Patent 6 and 2000, 2000). Produced by sebaceous glands around the skin of the mammary glands a few days after a female mammal gives birth, the substance requires a rise in the skin temperature by increasing blood circulation to this area, and the action of local bacteria to allow the substance to evaporate and reach the olfactory epithelium of newborn mammals (Pageat and Gaultier, 2003). This same substance was also seen to be produced by other mammal species with different concentrations of oleic, palmitic, and linoleic acids (Pageat and Gaultier, 2003). Since then, synthetic analogs of these appeasing pheromones have been used in several domestic species to improve their welfare; it is believed that appeasing pheromones reduce stress by generating an optimistic cognitive bias on the target individual through an intrinsic effect on its emotional processing, making the animal feel less threatened by its surroundings (Dube et al., 2012).

Most research carried out on the effects of appeasing pheromones has been conducted in companion animals for the treatment of behavioral disorders (Frank et al., 2010) and as an adaptation aid in stressful situations (Gaultier et al., 2009), with promising results. In farm animals, appeasing pheromones have been used to improve the welfare of pigs (McGlone and Anderson, 2002; Temple et al., 2016) and horses (Alves de Paula et al., 2019; Falewee et al., 2006) with mixed results.

So far studies in cattle have focused on evaluating the effect of the bovine appeasing pheromone **(BAP)** on milk production in dairy cows and weaning of beef calves (separation from the dam and diet change, with both occurring simultaneously when the calves are around 6 mo of age). Osella et al. (2018) observed a significant increase in milk yield during the environmental transition from indoor to outdoor housing of Valdostana dairy cows treated with the synthetic analog of the pheromone compared with those treated with a placebo. Other authors (Colombo et al., 2020; Cooke et al., 2020; Schubach et al., 2020) demonstrated that the administration of the synthetic analog of BAP during weaning and transport of beef calves reduced distress indicators while the substance was active. This was evidenced by lower levels of cortisol found in hair and blood samples, lower blood haptoglobin levels compared with the control calves, and improved feed efficiency and growth rates of the treated calves.

Only one study to date has tested the effect of the synthetic analog of BAP on the health status and growth of dairy calves, both key components of the biological dimension of animal welfare. Angeli et al. (2020) evaluated the effects of this pheromone on performance, disease incidence, and pharmacological costs in Dairy Gir x Holstein female calves before weaning from a milk diet. They observed an improvement in body weight gain in BAP-treated calves compared with placebo-treated calves. Although disease incidence was not affected by the treatment, pharmacological costs were reduced. Additionally, performance measures were not significantly impacted, with the average daily gain (ADG) for diseased BAP-treated animals comparable to the ADG of their healthy counterparts, a pattern not observed in the control group. To our knowledge, no research has been carried out to study the effect of BAP on the overall welfare of dairy calves from birth through weaning. Therefore, the aim of this study is to evaluate the effects of a synthetic analog of BAP on weight gain, as well as physiological and behavioral indicators of stress, in dairy calves from birth through milk weaning in a commercial setting. We hypothesized that calves receiving the pheromone would have greater weight gain and less activation of the neuroendocrine system evidenced by lower levels of hair and saliva cortisol, and higher heart rate variability, compared with calves receiving a placebo.

MATERIALS AND METHODS

The study was carried out at the calf unit at Harper Adams University's dairy farm (Shropshire, UK), with previous ethical approval from the University's ethics committee (0235–202103-PGMPHD) and in collaboration with the Research Institute for Semiochemistry and Applied Ethology **(IRSEA)** (Quartier Salignan, France).

Calves, experimental design, and treatments

Seventy-two Holstein Friesian dairy calves born between December 2021 and October 2022 at the Harper

Adams University dairy farm were included in the study. Calves were randomly assigned to either treatment A (36) or B (36) using Microsoft Excel (Randbetween function) at the time of birth. Random assignment of female calves to treatments did not consider factors such as birth weight or parity of the dam. Confounding factors such as season, location and time of sample collection were included as covariates in the statistical models used for analysis, ensuring that their influence was appropriately controlled for in the results. The treatments represented a synthetic analog of BAP (SecureCattle® SIGNS Labs, France) or a placebo (2-[2-ethoxyethoxy] ethanol), the same vehicle used in SecureCattle® without the active compound). Researchers were blind to treatments, as treatment bottles used during the study were labeled as "A" or "B" and unblinding occurred only after statistical analysis of the data was carried out.

After enrollment, calves received a minimum of 4 L of high-quality colostrum (spectrometry 28–30%) in their first 12 h of life. Calves were then ear-tagged and moved to clean, individual, outdoor hutches (1.87m long, 1.18m wide and 1.38m high: outdoor space 1.35m long and 1.25m wide) with straw bedding, as per the University farm protocol. The calf and hutch allocation within sites A and B started from bottom to top, with beef calves (bred in the same unit but not included in the study) allocated in the inner rows to avoid any potential cross-contamination between the pheromone and placebo groups (Figure 1). Following thorough cleaning, treatment sites were changed midway through the experiment to eliminate location as a confounding factor. When calves were moved to the experimental setup, treatment A or B was applied to the nuchal skin area of each calf based on their assigned treatment group (Angeli et al., 2020; Colombo et al., 2020). The treatment was reapplied every 2 weeks,

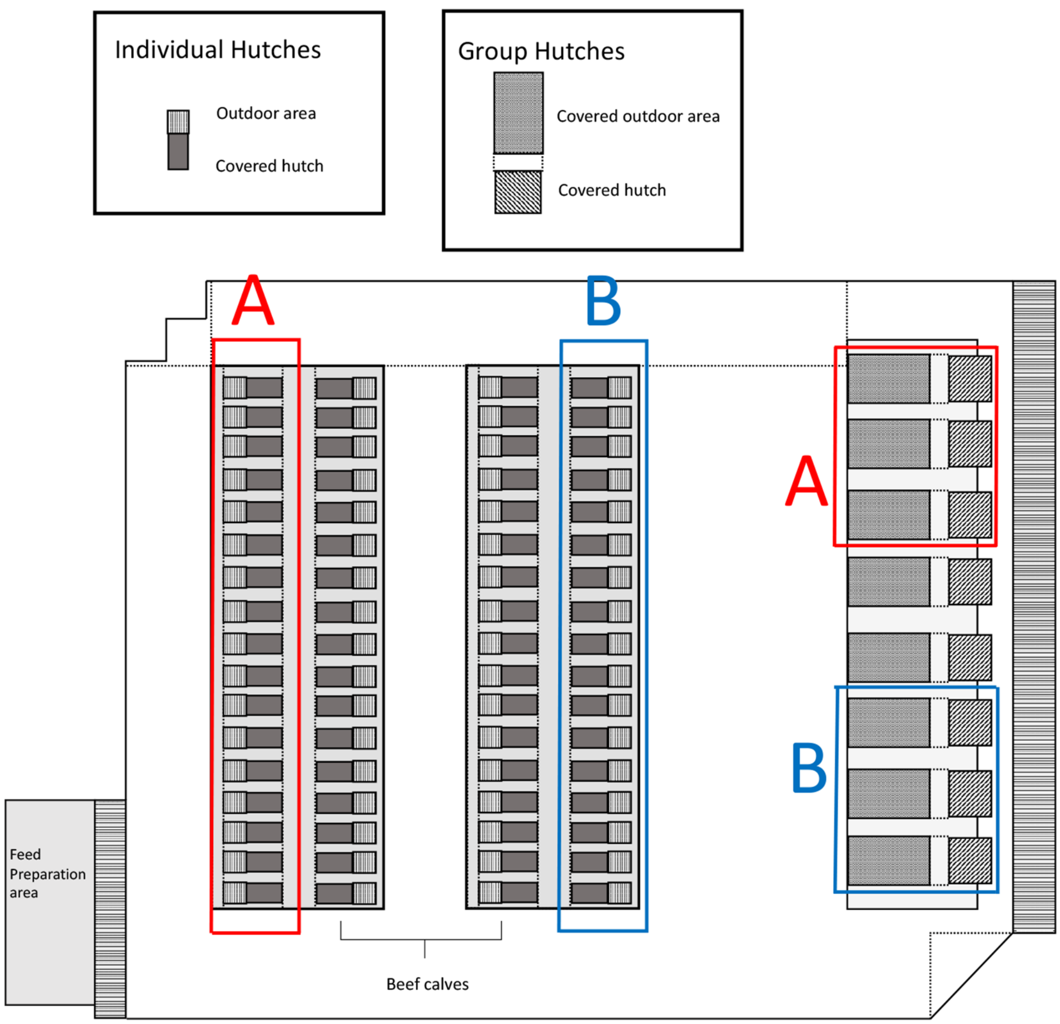

Figure 1. Calf unit layout and treatment allocation in individually and group housed calves.

as recommended by the manufacturer (P Pageat - US Patent 6 and 2000, 2000), and continued until the calves were moved to the young herd, approximately 4 weeks after weaning.

Calves on both treatment groups were fed milk replacer (Milkivit, Galloway & MacLeod, UK) using teat bottles twice a day, and milk weaning adhered to the farm guidelines (Figure 2): from birth to 6 weeks of age 3.6L twice daily; between 6 to 7 wk 2.6L twice daily; and between 7 weeks and weaning at approximately 8 weeks of age 2.6L only in the morning. Readiness to wean was determined by concentrate intake (at least one kg per day). Concentrate (Wynnstay Rearer 18, UK) was offered *ad libitum* in addition to clean and fresh water throughout the study. While individual milk and solid feed intake were not specifically measured, farm technicians recorded any milk refusals. We ensured that calves consumed at least one kilogram of concentrate at the time of weaning to meet the readiness criteria. This approach allowed for uniform management across all study groups, ensuring that all calves received adequate nutrition. The bedding on each individual hutch was topped up 3 times a week.

After weaning, calves were moved to group hutches (2.08m long, 2.59m wide and 1.80m high, with an outdoor space of 2.8m wide and 4.6m long) according to treatment (Figure 1 and Figure 2), with up to 5 calves per hutch. Clean water and concentrate were offered *ad libitum*, and clean bedding (straw) was provided 3 times per week. Calves stayed in this setting for around 4 weeks until they were moved to join the youngstock herd.

All calves were vaccinated for calf pneumonia at 2 weeks of age with intranasal Bovalto® (Boehring Ingerlheim Animal Health UK Ltd., Bracknell, UK) and hot-iron disbudded at 4 weeks of age by a veterinary surgeon using sedation and local anesthesia, followed by a dose of an anti-inflammatory medication, according to the farm protocol.

Productivity Measurements

Calves were weighed at birth and every week until weaning using a walk-on scale. They were then weighed before being put in the group hutches and again before being moved to the youngstock herd.

Neuroendocrine activation variables

Physiological stress was measured by observing the activation of the neuroendocrine system through cortisol analysis using methods that have been validated for cattle samples, ensuring the accuracy and reliability of the results; saliva cortisol as a measure of acute stress (Pagani et al., 2017; Schwinn et al., 2016), hair cortisol as a measure of chronic stress (Comin et al., 2013; Cook, 2012), and heart rate variability **(HRV)** (von Borell et al., 2007), as reduced HRV reflects increased sympathetic tone and has been linked to stress in humans and nonhuman animals (Clapp et al., 2015; Kovács et al., 2014).

Saliva and hair sample collection, sample processing and analysis. Saliva was collected from each calf at birth and every other week afterward until weaning, by inducing the calf to suck on a stick sponge for 3 min. Samples were then frozen at −20°C until sample processing was carried out. For sample processing, the sponges were thawed to room temperature and processed using a bovine cortisol ELISA kit (Salimetrics®) (validated for use in cattle by Gholib et al., 2020; Moya et al., 2013) following the manufactures' instructions, and saliva cortisol concentrations were calculated using a spectrometer reader.

Hair samples were collected at birth and then every other week until the end of the experiment using scissors as close as possible to the skin from different areas of the animal's back end. Due to the slow rate of hair regrowth in calves, it was necessary to collect new hair from different areas each time, as regrowth hair was not sufficient within the 2-week interval. This approach,

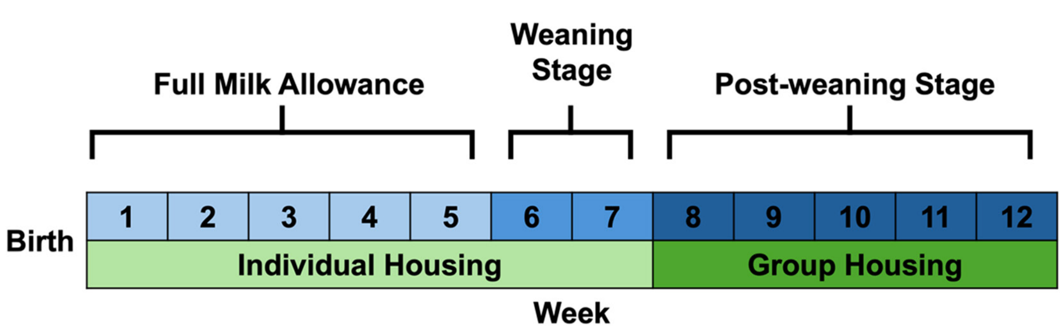

Figure 2. Graphic Representation of Housing conditions and Weaning Stages Arranged by Calves' Age

while necessary, could introduce some variability in cortisol measurements as different areas were sampled over time (Heimbürge et al., 2020). As hair color has shown to impact cortisol concentrations (Vesel et al., 2020), where possible, a sample of white hair was collected. If white hair was not available, a sample of black or mixed hair was collected instead. The hair color of the sample was recorded and included in the analysis. The hair was processed using a modified protocol following Moya et al. (2013) and Tallo-Parra et al. (2014). Each hair sample was washed by adding 5mL of Isopropanol and vortexed for 3 min. The supernatant was separated by decantation and the process was repeated once. The hair samples were then left to dry completely for 48 h at room temperature and under a fume hood. Samples were put in 25 mL metallic cylinders with a 12mm mill ball, and ground with a mixer mill (TissueLyser II) at 22 Hz for 5 min. After this was completed, 20 mg of the ground hair was placed in a 2mL Eppendorf tube, and 1mL of Methanol was added. The samples were sonicated for 30 min and incubated on a shaker for 18 h, at 50°C and 100 rpm. A total of 0.8 mL of the supernatant was pipetted off and evaporated in a block heater at 40°C under a fume hood for 24 h. Samples were reconstituted with 100 µL of phosphate-buffered saline (PBS) and shaken for 30 s before quantification of cortisol with an enzyme immunoassay kit (Salimetrics®); cortisol concentration was again obtained using a spectrometer plate reader.

HRV data collection and processing. HRV measurement has been recognized as a valuable tool in assessing the autonomic nervous system response during stressful conditions in dairy calves (Jimenez et al., 2019; Kovács et al., 2014). Polar Equine technology portable heart rate monitors **(HRM)** were used to collect HRV measurements, as these have been validated and used to measure HRV in cattle (Hopster et al., 1994). The device (H10 Polar HRM) was fitted around each calf thorax using a Polar equine belt for 24 h starting on the calves' second week of life and every week afterward until the end of the experiment. Raw data was extracted using a Bluetooth device and the Polar Flow Software, and imported to Excel where the heart rate per second was converted to an RR interval (Distance between 2 consecutive R waves in the electrocardiogram) and analyzed and corrected using the Kubios HRV Premium software to obtain the root mean squares of successive differences **(RMSSD)**, the standard deviation of beat to beat of normal sinus beats **(SDNN)** and the Baevsky Stress index **(SI)** (Scoley et al., 2019a; Shaffer and Ginsberg, 2017; von Borell et al., 2007).The SI derived from HRV analysis utilizing the mode amplitude, mode RR interval, and the standard deviation of the RR intervals, provides an objective assessment of stress levels by offering insights into the autonomic nervous system activity (Sahoo et al., 2019; Ugarte et al., 2019).

Behavioral measures and data processing

Calves were fitted with triaxial accelerometers (IDS i-QUBE, Peacock Technology Limited) on one of the hind legs right after birth. Raw data was uploaded automatically from the accelerometers into the CowAlert 2.7.1 Software (Peacock Technology Limited) where it was analyzed; and weekly data on average lying time, lying bouts, step counts and Motion index (a measure of how active the animal is calculated by the software) were obtained for each calf until the end of the experiment. Studies have shown high accuracy (>99%) of triaxial accelerometers in detecting movement and resting behavior (Chapa et al., 2020).

Statistical Analysis

The sample size was calculated using effect size estimates from previous studies on ADG and cortisol level (Schubach et al., 2020) to determine the minimum number of calves needed to obtain significant results (P values <0.5) with a power of at least 80% (provided a difference truly exists) using G*Power Software (Mayr et al., 2007; Nakagawa and Cuthill, 2007; Wilson Vanvoorhis and Morgan, 2007).

Data analysis was conducted using R (Version 2023.12.1+402). The full R code and accompanying data are available upon request. Each calf was used as the experimental unit and data analysis was divided in 3 different parts: assessing the effect of treatment according to the weaning stage; assessing the effect of treatment according to housing condition (i.e., individual or group stage) and assessing effect of treatment by age of the animal. These 3 parts were analyzed separately to improve model convergence and increase statistical power.

Data analysis was performed using mixed models to take into account the longitudinal nature of the data and several other random effects (Table 1).

General Linear Mixed Models (GLMM) were produced as a first intention for all outcome variables using the lme4 package in R. Normality and homoscedasticity of model residues were then assessed using graphical representation and normality tests. When these assumptions were violated, a transformation of the data was applied. For behavioral data such as lying time/bouts, standing time/bouts and step count, it was not possible to meet the assumptions of GLMM even after transformation. For this reason, Generalized Linear Mixed Models (GzLMM) for counting data were used. The Poisson model presented overdispersion for the 3 variables, so negative binomial models were ultimately selected.

Table 1. Summary of data analysis models, outcome variables, fixed, and random effects

Model	Outcome Variables	Random Effects	Fixed Effects
Effect of treatment by weaning stage (not weaned, partially weaned, and weaned)	ADG Salivary cortisol Hair cortisol SDNN RMSSD Minimum HR Mean HR Maximum HR SI Lying Time Lying bouts Motion Index	Animal Season Location Social housing Age in weeks Hair color for hair cortisol Time of sample collection for saliva cortisol	Treatment Weaning stage Weaning stage -Treatment interaction
Effect of treatment by social housing (Individual or group housing)	ADG Salivary cortisol Hair cortisol SDNN RMSSD Minimum HR Mean HR Maximum HR SI Lying Time Lying bouts Motion Index	Animal Season Location Weaning Stage Age in weeks Hair color for hair cortisol Time of sample collection for saliva cortisol	Treatment Social housing Social housing - Treatment interaction
Effect of treatment by age of the animal in weeks (zero to twelve)	ADG Salivary cortisol Hair cortisol SDNN RMSSD Minimum HR Mean HR Maximum HR SI Lying Time Lying bouts Motion Index	Animal Season Location Weaning Stage Social housing Hair color for hair cortisol Time of sample collection for saliva cortisol	Treatment Age in weeks Age in weeks – Treatment interaction

In all cases, when multiple comparisons were necessary, the p-values were adjusted using the Genz and Bretz algorithm for multivariate normal probabilities as there were convergence issues with other methods of multiple comparisons (Bretz et al., 2001). For hair cortisol, salivary cortisol and weight, relevant baseline variables (such as hair color, time of day, and birthweight, respectively) were included in the models as random effects.

Results were considered significant with P values < 0.05 and tendencies when P values were between 0.05 and 0.10 inclusive.

RESULTS

A summary of treatment effects on all the outcome variables depending on weaning stage and housing can be accessed at https://data.mendeley.com/datasets/r82dsnnprt/2

Productivity Measures.

ADG The mean body weight at birth of all calves enrolled in the study was 39.96 ± 4.66 kg, and no significant differences were observed between treatments (BAP 40.36 ± 4.12 kg, Placebo 39.55 ± 5.13, X^2 = 0.54, df = 1, P = 0.46). Overall, we did not observe any significant treatment effect on ADG between calves receiving BAP (0.68 ± 0.32 kg) or placebo (0.67 ± 0.69 kg). However, when analyzing the treatment effect according to the weaning stage, a significant interaction was observed (X^2 = 7.04, df = 2, P = 0.03). Treatment effect did not differ in pre-weaned or partially weaned calves, yet weaned calves receiving BAP had an ADG 0.15 kg higher compared with weaned calves treated with placebo (P = 0.04) as observed in Figure 3a. A treatment × housing interaction tended to be observed for ADG (X^2 = 3.75, df = 1, P = 0.05). Calves treated with BAP tended to have higher ADG when housed in groups compared with when housed individually (P = 0.07) (Figure 3b). Furthermore, calves treated with BAP had higher ADG during the group housing phase compared with calves treated with the placebo (P = 0.05) (Figure 3b).

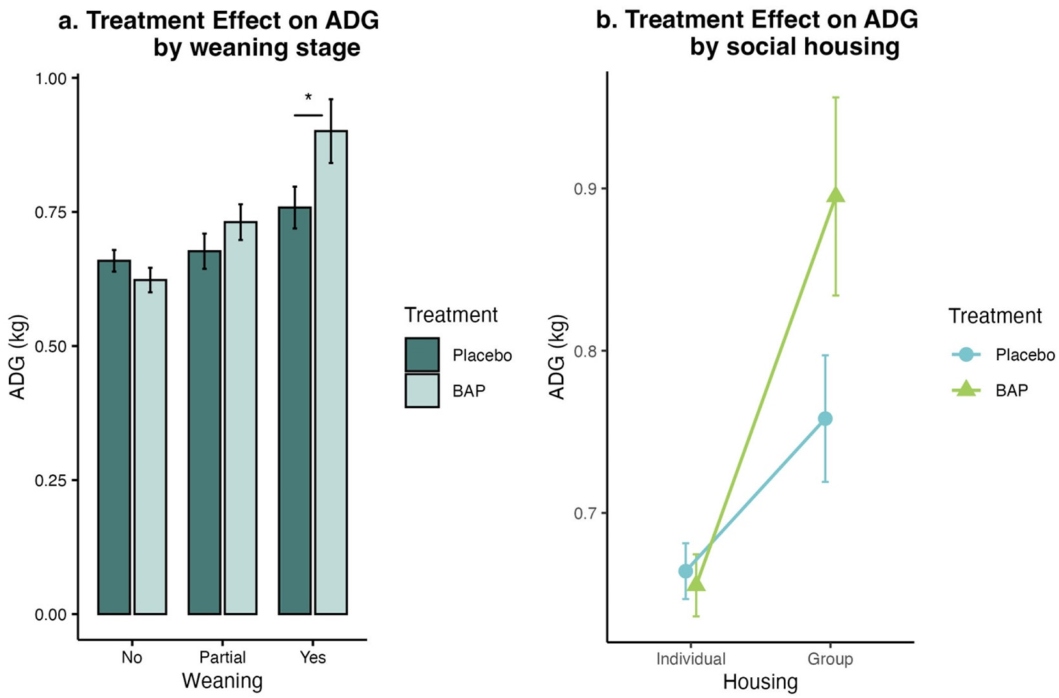

Figure 3. Average daily weight gain **(ADG)** by housing and weaning stage of 72 calves receiving the bovine appeasing pheromone **(BAP)** or placebo. Probability symbols: * $P \leq 0.05$. Error bars represent ± SEM.

Physiological Measures.

Saliva Cortisol There were no significant differences in salivary cortisol concentrations in calves given the placebo (0.10 ± 0.82 µg/dL) or BAP (0.10 ± 0.08 µg/dL). Nonetheless, when treatment effect was analyzed according to the weaning stage, a tendency was observed (X^2 = 5.04, df = 2, P = 0.08). Calves in the placebo group had higher saliva cortisol concentrations after being weaned compared with during weaning (P = 0.03), whereas in the BAP group this difference was not significant (Figure 4). Another tendency was observed for the treatment x age interaction (X^2 = 11.05, df = 6, P = 0.09), where at 10 weeks of age calves treated with placebo had higher levels of salivary cortisol compared with calves treated with BAP.

Hair Cortisol We did not observe any statistically significant differences in hair cortisol levels between treatment groups, and as shown on Tables 2 and 3, nor were there any significant interactions between treatments and other variables such as weaning stage or social housing.

SDNN No treatment effect on SDNN was seen between the treatments (BAP 20.64 ± 8.97 ms **(ms)**, placebo 20.22 ± 14.23 ms). When the treatment effect was analyzed according to the housing conditions, a statistically significant interaction was observed (X^2 = 6.78, df = 1, P = 0.04). In calves treated with placebo, SDNN was 26.7% higher during the individual compared with the group housing (P < 0.01). This difference was not significant in calves receiving BAP (Figure 5). A significant treatment x age interaction was also observed (X^2 = 27.21, df = 11, P < 0.01). Calves receiving BAP had higher SDNN at 7 (P = 0.05), 8 (P = 0.02) and 9 (P < 0.01) weeks of age, compared with calves receiving placebo.

RMSSD We did not find any overall effect on RMSSD between calves treated with BAP (7.32 ± 3.05 ms) or placebo (6.95 ± 2.69 ms). Nevertheless, when the treatment effect on RMSSD was analyzed per animal's age, we observed a significant treatment x age interaction (X^2 = 25.41, df = 11, P < 0.01), where calves receiving BAP had higher RMSSD than calves receiving placebo at 7 (P = 0.05), 8 (P = 0.07) and 9 (P = 0.09) weeks of age (Figure 6).

Mean Heart Rate **(HR)** A tendency was observed in mean HR for the treatment x weaning stage interaction (X^2 = 5.39, df = 2, P = 0.07). Calves in both treatment groups had significantly higher mean HR before weaning started compared with partially weaned (BAP P = 0.03, placebo P < 0.01) and completely weaned (BAP P = 0.01, placebo < 0.001). Even though mean HR decreased significantly for both treatment groups between pre-weaned and fully weaned stages, this decrease tended to be greater in the placebo group. A significant treatment x housing interaction effect was also observed for mean HR (X^2 = 7.43, df = 1, P < 0.01). Calves that received BAP showed a considerably lower average heart rate

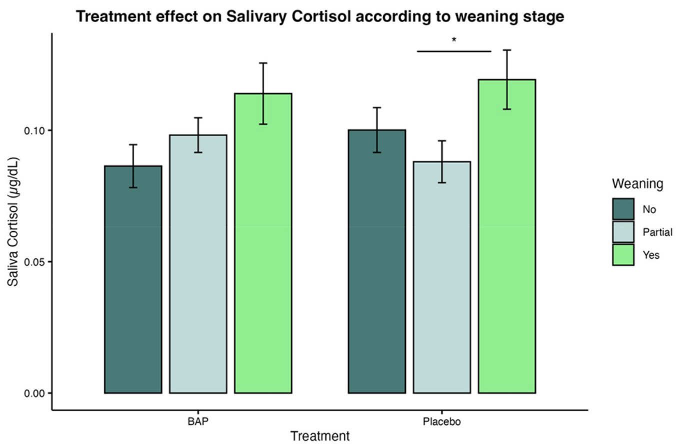

Figure 4. Saliva Cortisol of 72 calves receiving bovine appeasing pheromone **(BAP)** or placebo depending on Weaning stage and Age. Probability symbols: * $P \leq 0.05$. Error bars represent ± SEM.

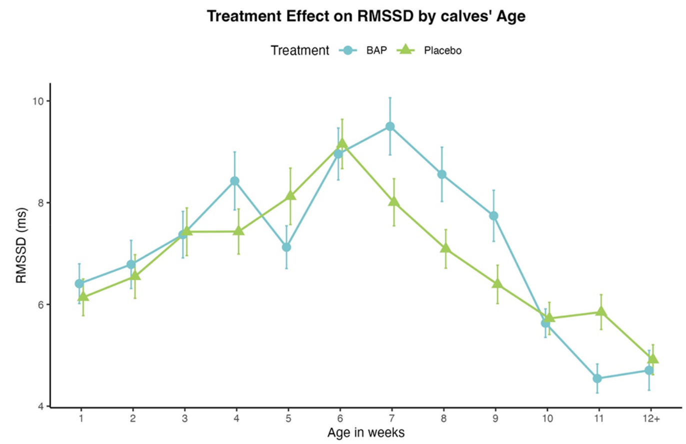

Figure 6. Treatment Effect of the bovine appeasing pheromone **(BAP)** and placebo on the root mean squares of successive differences **(RMSSD)** according to Age. Error bars represent ± SEM.

when housed in groups compared with when they had been housed individually ($P = 0.02$), whereas this difference was not significant in calves receiving the placebo.

Maximum HR We detected a significant treatment x weaning interaction for maximum HR ($X^2 = 10.13$, df = 2, $P < 0.01$). Calves administered with the placebo had a 21% higher maximum HR before weaning started compared with partially weaned ($P = 0.03$), and 30% higher between partially weaned and fully weaned animals ($P = 0.05$). This difference was not significant in calves receiving BAP (Figure 7a). In weaned calves, those receiving BAP had 8.75% higher maximum HR than calves treated with placebo ($P = 0.01$).

When the treatment effect was analyzed according to housing conditions, a significant interaction was observed ($X^2 = 13.09$, df = 1, $P < 0.001$). When calves were in group housing, those treated with BAP had a 9.9% higher maximum HR compared with those receiving a placebo ($P < 0.01$), this difference, however, was not seen when calves were housed in individual hutches (Figure 7b).

We also observed that treatment effect had a significant interaction with age ($X^2 = 27.97$, df = 11, $P < 0.01$). Calves in the BAP group had a higher maximum HR, after weaning at 9 ($P < 0.001$) and 10 ($P = 0.07$) weeks of age compared with heifers in the placebo group.

Stress Index Treatment x age interaction was significant for SI ($X^2 = 27.65$, DF = 11, $P < 0.01$). Calves treated with the placebo had significantly higher SI at age 7 ($P = 0.01$), 8 ($P = 0.09$) and 9 ($P < 0.01$) weeks old compared with calves receiving BAP.

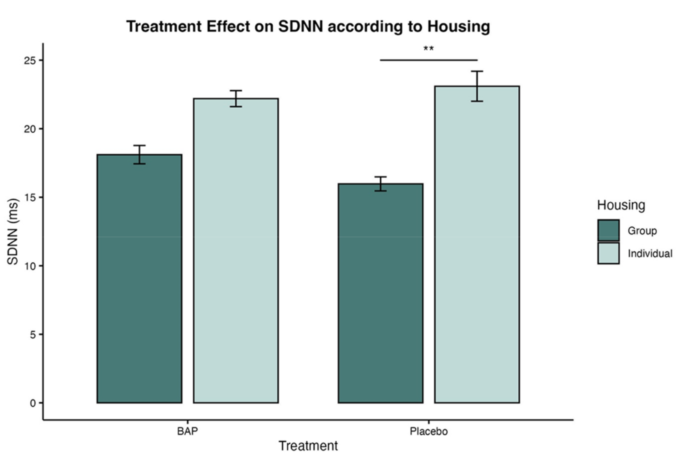

Figure 5. Treatment effect on the standard deviation of beat to beat of normal sinus beats **(SDNN)** of 72 heifers receiving bovine appeasing pheromone **(BAP)** or placebo, depending on social hopusing. Probability symbols: ** $P \leq 0.01$. Error bars represent ± SEM.

Behavioral Measures.

Lying Time A significant treatment x weaning interaction for lying time was observed ($X^2 = 9.98$, df2, $P < 0.01$). Before weaning calves in the placebo treatment spent 6.3% more time lying down compared with weaned calves ($P < 0.01$), and 4.45% more time than partially weaned calves ($P = 0.04$); these differences were not significant in the BAP group (Figure 8a). When lying time was analyzed based on treatment and housing conditions, a significant treatment x housing interaction was also observed ($X^2 = 3.85$, df = 1, $P = 0.05$). Calves receiving both BAP and the placebo had higher lying times housed individually than when housed in social groups (BAP $P < 0.001$, placebo $P < 0.0001$), however this difference was more pronounced in the placebo group (Figure 8b).

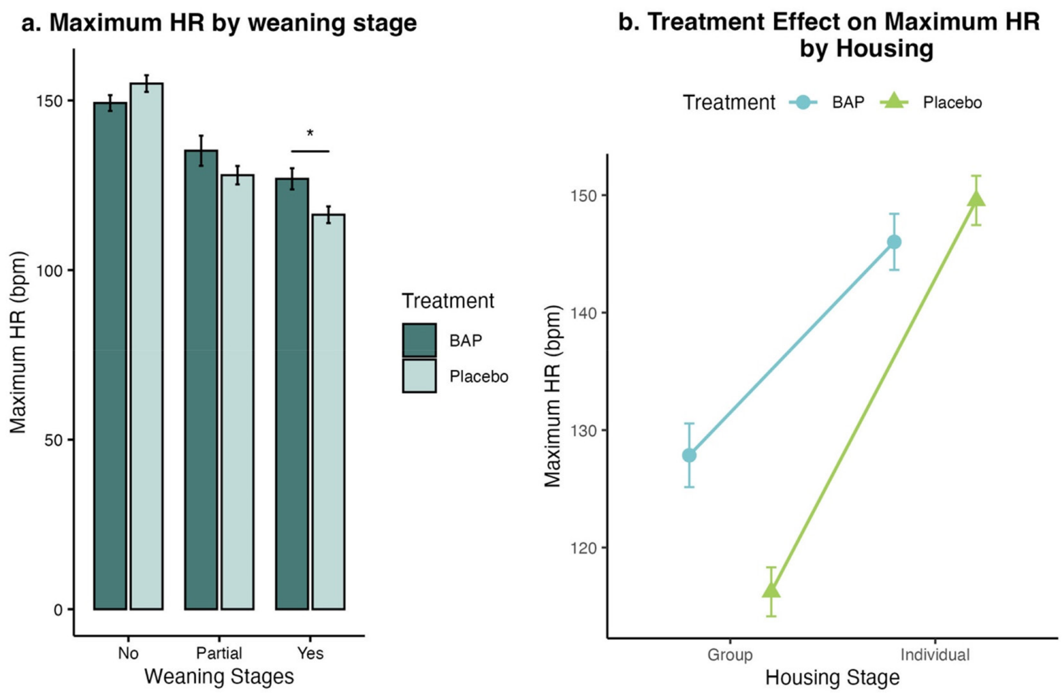

Figure 7. Treatment Effect on Maximum heart rate in 72 calves receiving bovine appeasing pheromone **(BAP)** or placebo according to Weaning Stage and Social Housing. Probability symbols: * $P \leq 0.05$. Error bars represent ± SE

DISCUSSION

The present study investigated the effects of administering bovine appeasing pheromone (BAP) on growth rates, physiological stress indicators, and behavioral responses in calves from birth through weaning. Several notable findings emerged regarding BAP's impacts on mitigating weaning stress and social restriction.

In terms of growth performance, according to the scientific literature, the ideal daily weight gain of Holstein dairy heifers in the first months of life is 0.8 kg (Zanton and Heinrichs, 2005). The ADG in this study was 0.68 ± 0.29 Kg, which is slightly lower than what Hyde et al. (2021) observed in their study including 30 commercial dairy farms in the UK, but notably higher than the 0.12 kg/d obtained by Bazeley et al. (2015). From previous studies, we know that weaning has a detrimental effect on weight gain (De Passillé et al., 2011; Eckert et al., 2015). In this study, we observed that completely weaned calves treated with BAP displayed a 0.15 kg higher ADG compared with placebo-treated cohorts. This aligns with prior work showing maternal pheromone exposure can help minimize the negative effects of stress on growth during the weaning period both in dairy and beef calves (Angeli et al., 2020; Colombo et al., 2020; Schubach et al., 2020).

Dairy calves have their fastest growth stage in their first 2 mo of life or during the milk stage (Kertz et al., 1998). In this study we observed a tendency for BAP to boost ADG in group-housed calves (over 8 weeks of age), even though previous studies suggest that weight gain is also affected in group housed calves (Scoley et al., 2019b). This finding suggests that BAP has the potential to promote weight gain after weaning, which in this case could be partially attributed to social facilitation, as observed by Costa et al. (2016) and Knauer et al. (2021). Further research is needed to disentangle these effects and to explore whether targeted BAP administration at weaning could provide comparable benefits to every other week administration.

Physiological measures provided insights into how BAP may modulate the calves' stress responses to weaning. Previous research has shown that weaning increases cortisol levels in calves (Black et al., 2017). While no overall differences in salivary cortisol were detected in our study, placebo-treated calves tended to exhibit higher cortisol levels after complete weaning compared with partially weaned calves, a finding that was not observed in BAP-treated calves; this difference likely reflects some effect of the pheromone on stress modulation during the weaning process. At the same time, salivary cortisol tended to peak, especially after weaning in calves receiving the placebo, reinforcing the hypothesis that BAP may indeed decrease the endocrine stress response to weaning. Similar findings were observed by other beef-weaning trials where plasma cortisol was higher in

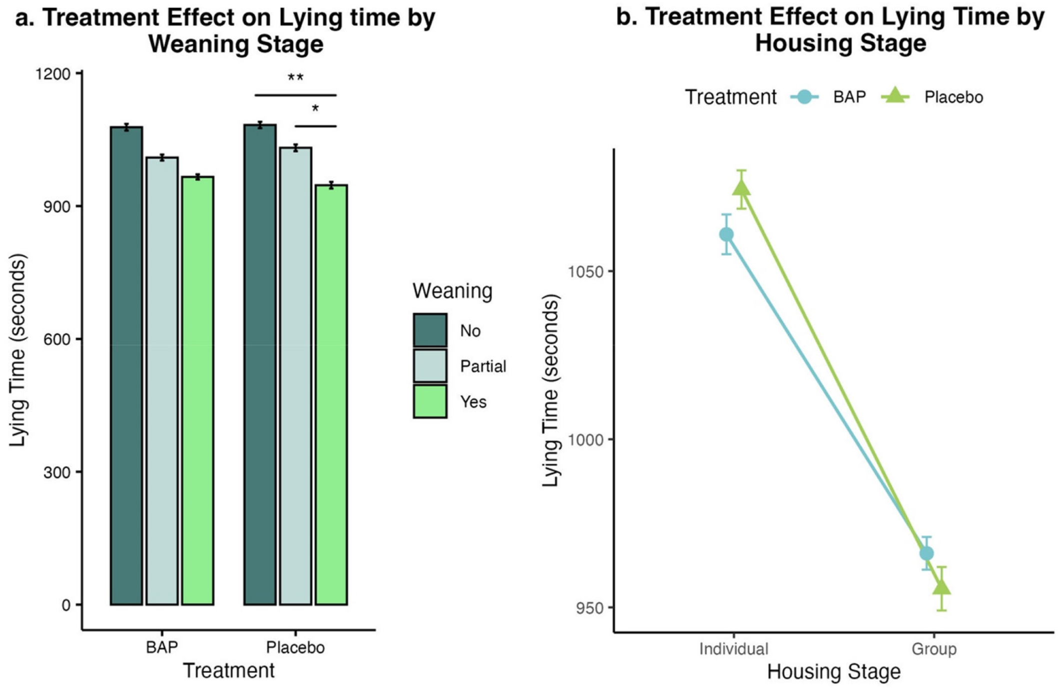

Figure 8. Lying Time of 72 dairy heifer receiving BAP or placebo by Weaning and Housing Stages. Probability symbols: * $P \leq 0.05$, ** $P \leq 0.01$. Error bars represent ± SE

calves treated with a placebo in the initial stages of the weaning process (Colombo et al., 2020).

Differences in the HRV parameters SDNN and RMSSD, which are inversely associated with sympathetic tone and positively correlated with parasympathetic activity (Mohr et al., 2002; von Borell et al., 2007), further support BAP's stress-reducing effects. BAP-treated calves showed higher SDNN and RMSSD values at multiple sampling points compared with those receiving the placebo, especially when the weaning process was occurring, suggesting lowered sympathetic arousal. These findings are also reinforced with higher levels of SI during the weaning and postweaning period. Moreover, individually housed placebo calves displayed markedly elevated SDNN compared with their group-housed counterparts, while housing condition had less influence on SDNN in the BAP group. This suggests the pheromone may help buffer calves against isolation stress. These findings are supported by Scoley et al. (2019b) who reported RMSSD was reduced in the postweaning stage in grouped housed calves that underwent gradual weaning.

The reduced mean heart rate observed in group-housed, BAP-treated calves relative to their individually housed counterparts provides additional evidence that BAP can potentiate the effects of social buffering during stressful situations (Bolt et al., 2017; Bolt and George, 2019). At the same time, mean heart rate was considerably higher in calves before weaning started in both groups compared with partially weaned and fully weaned calves, and this is probably explained by the normal immaturity of the autonomic regulation of the mammalians' heart after birth (Quevedo et al., 2019; Silva et al., 2016). However, differences in mean HR seem to be stronger in placebo-treated calves, which could suggest a stress buffering effect of the pheromone during this weaning stage in calves treated with BAP.

Maximum heart rate revealed divergent results, with BAP-treated weaned calves displaying higher peak heart rates than placebo controls. While seemingly counterintuitive, this could reflect a greater metabolic demand to support the improved growth rates seen with BAP treatment after weaning. Overall, it seems that maximum heart rate was less stable in calves receiving placebo during the weaning stages which could support the hypothesis that BAP has modulated the stress response in BAP treated animals.

Behavioral analyses shed further light on BAP's influence on the weaning experience in calves. Previous studies have demonstrated that lying time is crucial for dairy heifers as they are highly motivated to lie for extended periods of time during a 24-h cycle (Jensen et al., 2005); and that weaning reduces resting time in dairy calves (Budzynska and Weary, 2007; Eckert et al., 2015; Jasper et al., 2008). In the present study, the treatment interaction we observed for lying time suggests that resting time was significantly reduced in placebo treated calves during and after weaning, whereas this phenomenon was not seen in calves treated with the appeasing pheromone. This finding may imply that weaning stress was higher in placebo calves compared with BAP treated heifers. Calves in both groups seemed to be more active when housed in groups, perhaps reflective of their age, space allocation and/or opportunity for social interaction. Nonetheless, considering that the weaning process was finalized when calves were moved to group housing, the housing change is likely to reflect a differential stress response between the treatment groups to some degree. This finding is supported by previous studies where group-housed calves were more active after abrupt weaning versus progressive weaning (Scoley et al., 2019b). In light of the observed treatment interaction, where resting time was significantly reduced in placebo-treated calves during and after weaning but not in calves treated with the appeasing pheromone, it is plausible to infer that the placebo group experienced higher weaning stress compared with the BAP-treated heifers. This hypothesis is supported by previous research by Schubach et al. (2020) where beef calves treated with BAP displayed active engagement in feeding and social interactions and were keen to escape and explore their new environment after weaning. These behaviors are indicative of a positive coping response and suggest that BAP may mitigate stress during the weaning process, thereby promoting more adaptive behavior in calves. This aligns with our findings, where we observed similar trends in stress reduction and improved performance in calves treated with BAP. These behavioral changes likely reflect the broader effects of BAP on reducing weaning-related stress, which in turn may enhance the ability to interact with the environment and conspecifics more positively.

Collectively, the performance, physiological and behavioral data indicate that administration of the bovine appeasing pheromone modulates stress coping mechanisms in ways that could enhance calf welfare and productivity around weaning.

These findings build upon previous research validating the positive impacts of maternal pheromone signaling especially when used with other best management practices such as social housing (Angeli et al., 2020; Colombo et al., 2020; Schubach et al., 2020). In summary, supplementation with bovine appeasing pheromone appears to mitigate stress and facilitate healthy production in dairy calves.

Strengths, weaknesses, and future recommendations

Our sample size was relatively robust as we performed a formal sample size calculation before data collection to ensure we had a sufficient power to find a treatment effect for variables related to performance, physiology, and behavior; however, we acknowledge that this sample size may not have been sufficient for detecting potential smaller differences in disease incidence. Due to sample size considerations, the components of the placebo (2-(2-[ethoxyethoxy] ethanol) represented the baseline (i.e., we could not include an additional completely untreated control group). We adjusted for potential confounding factors in our analysis such as weather conditions and location; however, since the study was conducted on a commercial farm, it was not possible to ensure that all animals were weaned, disbudded, and moved to group housing exactly at the same age.

We acknowledge that individual milk replacer and solid feed intake were not specifically measured during the individual housing period. While this may limit our ability to assess detailed feed intake patterns, the use of a consistent feeding protocol across all study groups mitigated potential variations in nutrition. Future studies could benefit from including precise intake measurements to provide further insights into the effects of the treatments on calf growth and development.

It was also not possible to measure the concentrations of the pheromone in the air after application, and therefore any effect of cross contamination could not be quantified. To mitigate these potential effects, we ensured that several rows of calves not included in the study were placed between the treatment groups.

Due to the slow rate of hair regrowth in calves, it was necessary to collect hair from different areas of the back end of each calf every 2 weeks, rather than using regrowth hair from the same spot. As (Heimbürge et al., 2020) have indicated, this approach can introduce variability in cortisol concentrations, as different body areas may exhibit different cortisol levels. Consequently, the lack of consistent sampling from a single site could have affected the accuracy and reliability of our cortisol measurements, potentially influencing the overall interpretation of chronic stress levels in the calves.

Additionally, while our study focused on female dairy calves for practical reasons, it would be interesting to investigate how male calves reared artificially would respond to the treatments. Future studies could explore this aspect to provide a more comprehensive understanding of BAP's effects across different sexes.

Finally, due to the reduced space of the individual hutches we could not effectively assess certain behavioral indicators such as play, which has been used in previous studies as a behavioral indicator of welfare (e.g., (Krachun et al., 2010; Mintline et al., 2013; Papageorgiou and Simitzis, 2022). Most of the treatment differences we observed were when the animals were partially or completely weaned, but it is currently unknown whether application of the pheromone in early life has a cumulative effect. This research question, as well as possible long-term effects of the pheromone later in life, particularly on age at first service and milk yield, warrants future investigation. A financial cost-benefit analysis of the commercial used of BAP is also needed, in addition to an exploration of potential beneficial effects on animal's resilience after disease and painful procedures.

CONCLUSIONS AND APPLICATIONS

The present study demonstrated that administering bovine appeasing pheromone (BAP) can mitigate the negative impacts of weaning stress on dairy calves. Key findings include improved growth performance, as BAP-treated calves exhibited higher ADG compared with placebo-treated calves when group-housed following weaning. Additionally, BAP administration was associated with lower salivary cortisol levels, higher heart rate variability (SDNN and RMSSD), and lower stress index scores, indicating a reduction in stress and sympathetic arousal during the weaning process. The study also suggests that BAP may enhance social buffering effects, as BAP-treated calves benefited more from social housing conditions. Furthermore, behavioral observations revealed that placebo-treated calves showed increased restlessness, evidenced by significant reductions in lying time during and after weaning, while BAP-treated calves maintained more consistent resting patterns.

These findings suggest that BAP administration can support stress coping mechanisms in calves during weaning, potentially enhancing their welfare and productivity. Future research should explore the long-term effects of BAP on post-weaning growth rates, puberty attainment, milk yield in the first lactation, and overall herd stability, as early-life interventions are known to influence the long-term performance of dairy cows.

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Abbreviations: BAP = Bovine appeasing Pheromone, BPM = Beats per minute, HR = Heart rate, HRM = Heart rate monitor, HRV = Heart rate variability, IRSEA = Research Institute for Semiochemistry and Applied Ethology, ms = Milliseconds, RMSSD = Root mean squares of successive differences, SDNN = Standard deviation of beat to beat of normal sinus beats, SI = Baevsky Stress index

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