Environmental enrichment in the form of a synthetic analogue of the bovine appeasing pheromone to increase the overall welfare of female dairy calves from birth through to weaning

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DECLARATION

I declare that the work contained in this thesis is entirely my original work, and has not been previously accepted for a degree application. I have acknowledged every source of information and assistance that have been utilized in the preparation of this dissertation.

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ABSTRACT

This thesis investigated the effects of a synthetic analogue of the bovine appeasing pheromone (BAP) on the welfare of female dairy calves, assessing its impact on growth performance, physiological stress, and behaviour from birth through weaning, during disease episodes, disbudding procedures, and cognitive testing.

Key findings indicated that BAP treatment contributed to improved growth rates and better stress coping mechanisms during weaning, as evidenced by enhanced average daily gain (ADG) and heart rate variability (HRV) parameters. BAP-treated calves demonstrated reduced salivary cortisol levels and more consistent resting patterns, suggesting diminished stress levels compared to the placebo group. While the overall disease incidence did not differ significantly between groups, BAP-treated calves displayed better autonomic nervous system regulation during and after disease episodes, contributing to faster post-illness recovery and resilience.

During disbudding, despite sedation and analgesia being administered to all calves, BAP-treated calves exhibited HRV parameters indicative of better autonomic balance and lower stress indices, suggesting potentially superior pain management and quicker recovery. Behavioural metrics showed reduced restlessness and more balanced activity levels post-procedure in BAP-treated calves.

The study also explored cognitive performance and emotional states through cognitive bias tests. While no significant improvements in learning rate were observed, calves receiving BAP displayed more nuanced physiological responses and a potential increase in emotional resilience, reflected in altered latency responses to ambiguous cues.

These results support BAP's role in enhancing calf welfare by modulating stress responses during critical management procedures and environmental challenges. Future research should focus on larger, diverse samples, include male calves, and investigate long-term effects to validate findings and explore BAP’s cost-effectiveness in commercial dairy operations.

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CHAPTER 1. LITERATURE REVIEW

1. The history of dairy

1.1. Cattle domestication and natural behaviour

To understand the behaviour and needs of modern cattle it is important to understand the concept of natural behaviour. This term refers to the behaviour that has evolved with a species to adapt and maintain an adequate state of fitness. Therefore, the behaviour of wild ancestors, feral animals of the same species, or the behaviour of domestic species when put in comparable environmental conditions as those faced by their wild ancestors are all sources of knowledge on domesticated animals' natural behaviour (Keeling and Jensen, 2017).

For about 200 000 years, modern humans (*Homo sapiens*) were hunter-gatherers, and only recently, 10 000 years ago, they made the transition to live in sedentary communities, and start producing food from agricultural, horticultural, and pastoral practices through domestication of plants and animals (Higman, 2012). Domestication is the process through which humankind begins to maintain animals in captivity while keeping complete control over their food supply, breeding, and territory. It demands genetic isolation of a few animals from their wild relatives and their gradual adaptation to human presence. This in turn requires behavioural adjustments from the animal's side for them to adapt to their new environment and social relationships (Clutton-Brock, 2012; Hall, 2004). The moment these animals start to reproduce in captivity, genetic modifications will occur as a response to the changes in their environment (natural selection) in addition to human intervention for aesthetic, economic or cultural motivations (artificial selection), resulting in a new genetically distinct population (Clutton-Brock, 2012).

The wild ancestor of modern taurine (*Bos taurus*) and Zebu (*Bos indicus*) cattle was the now extinct auroch (*Bos primigenius*). Evidence of the auroch's domestication has been traced through archaeological findings to the fertile crescent (today's Turkey and Syria) between 10300 and 10800 years ago (taurine cattle), and 1500 years later in the Indus Valley (zebu cattle) (Ajmone-Marsan et al., 2010; Felius et al., 2014). However, it was much earlier, around 610 000 and a million years ago that the auroch split into the two distinct species and explains why both species where domesticated separately (Hall, 2004).

As a result of domestication cattle underwent physical modifications and behavioural attenuation. Whereas wild bovines required long horns to protect themselves from predators and competition, evidence of short horned taurine cattle has been found in Mesopotamia as early as 5100 before present (BP) (Felius et al., 2014). Brain size reduction and body configuration including a reduction of size, skeletal changes, muscular hypertrophy, and intramuscular fat deposition also occurred, to facilitate animal handling and survival during harsh conditions (Hall, 2004). Following human needs, artificial selection has resulted in a variety of breeds that differ in coat colour, docility, growth, and horn development, amongst other characteristics (Ajmone-Marsan et al., 2010; Felius et al., 2014)

Aurochs were herbivorous ruminants that grazed on grass. Females lived in herds with calves and young bulls, creating hierarchical ties amongst herd mates and maintaining them through affiliative behaviours (allogroming) and resource sharing (Tucker, 2017). Older males were separated from the herd and roamed the forest, only approaching the herd during breeding season in August and September (Vuure, 2014). Calving took place from May to June, with cows detaching themselves from the herd and heading into the forest, where the cow and her newborn would stay for two to three weeks, with the cow hiding the calf while feeding within its view, and then returning to the herd at pasture at the conclusion of the period (Vuure, 2014). Calves would nurse from the dam during the first few hours after their birth and continue to do so four to ten times each day. When they returned to the herd, the calves stayed in groups while the cows grazed (Tucker, 2017). Aurochs might have lived up to fifteen years before becoming extinct due to human activity around 1627 (hunting and eviction from natural feeding places) (Ajmone-Marsan et al., 2010).

1.2. Cognition and cognitive abilities in cattle

Cognition refers to the mental processes involved in acquiring, processing, and using information, including perception, learning, memory, and decision-making(Shettleworth, 2010). In cattle, cognition plays a crucial role in social interactions, environmental adaptation, and overall welfare. Understanding how cattle perceive and respond to their environment is essential for improving management practices and ensuring high welfare standards in dairy production systems (Nawroth and Rørvang, 2022a).

Cattle have demonstrated a range of cognitive abilities, including social learning, associative learning, and spatial memory. For example, they can recognize individual conspecifics and humans, retain these memories over long periods, and use learned associations to anticipate and avoid negative experiences (Rørvang and Nawroth, 2021). Social learning, where animals acquire behaviours by observing others, is particularly relevant in cattle, as studies have shown that calves can learn from their peers, such as by observing feeding behaviours (Miller-Cushon and Devries, 2015). This ability has implications for welfare, as calves raised in enriched social environments tend to develop stronger cognitive skills and show greater adaptability to novel situations (Zhang et al., 2022).

Memory and problem-solving are also well-documented in cattle. Studies indicate that they possess excellent spatial memory, which allows them to navigate complex environments and recall the locations of resources such as feed and water (Hirata et al., 2016). Lecorps et al., 2022) developed and applied a modified hole-board test to assess cognitive performance in dairy calves by measuring working memory, general working memory, and reference memory. Over an 11-day learning period, calves improved their ability to locate bottles containing milk while avoiding empty ones, indicating learning and memory retention. When the bottle locations were changed, cognitive performance initially declined but improved again over a 7-day re-learning period, demonstrating behavioural flexibility. Furthermore, dairy cows can differentiate between positive and negative human interactions, leading to behavioural changes based on past experiences (Pajor et al., 2000).

1.3. Calf development and natural behaviour

Cows in herds with little human intervention act similarly to their wild ancestors, seeking for remote and isolated areas to calve and keeping a safe distance from the herd for the first several weeks after the calf is born, building the cow-calf relationship by grooming and frequent vocalisation (Cantor et al., 2019). Calves nurse from their mother for an average of 38 minutes per day in ten to twelve feeding bouts, consuming eight to thirteen litres of milk per day (Cantor et al., 2019; Rushen, 2008). Female calves are weaned at the age of seven to nine months, although bulls continue to nurse until they are fourteen months old. The mother-calf relation continues after weaning, with female calves forming lifelong ties with their mother (Rushen, 2008).

Calves spend most of their time lying down while they are young, but this behaviour decreases as they grow older (Tucker, 2017). They sleep at five-minute intervals throughout the course of a twenty-four-hour period, which accounts for 20% of their time budgets (Hänninen and Valros, 2017). When access to grass is available, calves will begin grazing as soon as they are brought to the herd, a behaviour that will become more prominent as the calf ages (Tucker, 2017). After being introduced and assimilated into the herd, social contact with other calves becomes critical for calf development as they begin to distance themselves from their mother and spend more time with other calves, engaging in play behaviour, learning social interactions, stablishing hierarchies, and developing essential skills such as grazing and foraging (Cantor et al., 2019). Grooming behaviour with the dam, as well as pretend fighting and "sexual" engagement with other calves, help to establish secure ties that will last until maturity (Rushen, 2008).

1.3.1. Evolution of the dairy industry and calf rearing systems

The oldest evidence of milk processing can be traced back to the seventh millennium in northwest Anatolia (modern Turkey) from analysing organic residue in shreds of pottery vessels found in the area (Figure 1), in south-eastern Europe, and in the Levant. Proving that milking of ruminants was certainly practice back then (Evershed et al., 2008). Likewise, there is evidence of this same artifacts (Figure 1) in Northern Europe around 5000 BP (Salque et al., 2013).

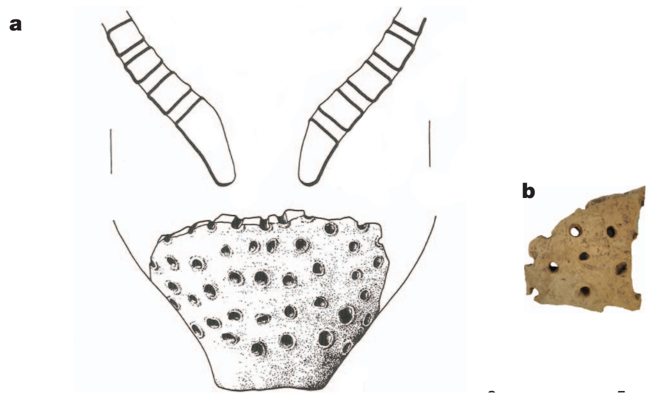

Figure 1. Drawings of pottery reconstructed from fragments found in Poland. (Adapted from: Salque et al., 2013)

However, mammals only consume milk from their own species in infancy and humans are no different, producing the enzyme necessary to digest milk's sugar lactose (lactase) when they are infants and ceasing to produce it after being weaned from nursing. Milk consumption in adulthood is associated with gastrointestinal upset (Wiley, 2015). Therefore, as farming began to replace hunting and gathering in Anatolia and the near east, herders learnt to lower the lactose content in milk by using fermentation to produce cheese, butter, and yogurt (Curry, 2013).

Migration from the near east brought farming and dairying into Europe 9000 years ago (Bramanti et al., 2009). Bone studies from animals from that time suggest that taurine cattle were also carried from the fertile crescent, with little inbreeding occurring with the wild European aurochs (Gerbault et al., 2011a; Leonardi et al., 2012). Around 6000 and 8000 BP a small population of farmers in central and northern Europe were able to consume fresh milk in adulthood. Genetic studies carried out in this area uncovered a mutation of the lactase gene that allowed these humans to continue producing the enzyme lactase throughout their lives. With time this genetic trait underwent positive selection pressure marking the coevolution of dairying and lactase persistence in this geographic location (Gerbault et al., 2011b). Nowadays only 35 percent of human population are able to digest milk in adulthood and they are primarily located in northern Europe and in countries that were colonized by people of European descend (The United States, New Zealand, Australia, and in varying degrees in Latin America) (Wiley, 2015). Other areas of lactase persistence have been discovered in West Africa, the middle East, and South Asia, all of which appear to be connected to different mutations (Curry, 2013).

The gene-culture coevolution of farming and lactase persistence initiated what is now known as the pastoral herding era, where livestock animals were kept in small numbers in pasture and in regular contact with their human caretakers until the beginning of the industrial age (Clutton-Brock, 2012). As demand for milk rose in response to human population growth, dairy facilities were located near metropolitan areas so that milk could be delivered fresh every day to cities and leftover milk could be turned into cheese and butter. However, the requirement for daily market access was reduced with the advent of refrigerated tankers capable of transporting milk long distances to be processed, allowing dairy enterprises to migrate to more remote places with a more favourable climate, more inexpensive land, and abundant water availability. This allowed dairies to expand and thrive without the restraints that come with running an agricultural enterprise near a big city (Douphrate et al., 2013). Following this trend and to maximise efficiency, the dairy industry in developed countries has been shifting into a more intensive production model especially after the Second World War where animals are kept in specialized indoor environments (Clay et al., 2020a; Cronin et al., 2014). This intensification has resulted in a reduction in the number of dairy producers and total number of dairy cows, as well as an increase in herd sizes and milk production per animal (Barkema et al., 2015).

The intensification of livestock production tends to move away from more naturalistic environments, promoting animal management approaches and housing conditions that allow for intensive animal surveillance. This is perceived by farmers as beneficial to detect any approaching health concerns, enhance livestock husbandry, provide enough feed, and more specialised care (Beaver et al., 2019c). As a result, practices that include all year indoor housing, artificial calf rearing and mutilating procedures (e.g., disbudding, tail docking) are more prevalent in today’s industrial dairy farms, potentially harming animal welfare (Nordquist et al., 2017). For instance, all-year indoor housing restricts animals' access to pasture, limiting opportunities to express natural behaviours such as grazing, walking, and social interactions; and can increased lameness, hoof pathologies, and mastitis (Arnott et al., 2017). Additionally, certain routine management procedures, like disbudding and tail docking, are associated with acute and chronic pain if performed without appropriate analgesia and anaesthesia (Stafford and Mellor, 2005). Disbudding, though essential for safety in group housing systems, can lead to tissue damage and prolonged pain responses if pain mitigation is inadequate (Stewart et al., 2009). Tail docking, while traditionally conducted to improve milking hygiene, is now widely debated due to its limited benefits and detrimental effects on pain perception and natural tail use (Eicher et al., 2000; Sutherland and Tucker, 2011).

In 1938 Thomas Olson in his book Elements of Dairying, describes for the first time why the practice to remove the calf from its mother immediately after birth should be employed. His reasons included the fact that it was very challenging to teach a calf how to drink from a container once it had nursed from the dam (Moore et al., 2012). Later, in the early 1950s Moore and Gildow (Moore et al., 2012) argued that allowing calves to nurse in open pens and overfeeding milk raised the incidence of scours, as the excess milk will decomposed in the rumen. In the mid 1950s it was acceptable then to feed calves once a day with an opened pail, as this also reduced labour requirements by as much as 40%. At the same time abrupt weaning of calves at an early age was a common practice, as it stimulated early consumption of dry feed, and it was believed that weaning calves at six or twelve weeks of age had no significant impact on the calves' weight, and good results were obtained when weaning occurred at 21 to 35 days of age (Otterby and Linn, 1981). Up until this time calves and heifers were housed in the same building as the milking herd, however, due to the high incidence of respiratory and enteric diseases with the increase in herd sizes, producers began to use separate facilities to rear calves. Satisfactory criteria for calves' housing facilities included optimal growth, good health, labour efficiency and low costs, and therefore, individual housing was advocated to rear calves during the pre-weaning period (Otterby and Linn, 1981).

Although technological and nutritional advancements have been since the 1950s, the overall concept of artificial rearing of dairy calves has changed very little since then (Kertz et al., 2017). Currently, calves are removed from the dam shortly after birth, housed mostly in individual pens, fed a restricted milk allowance of around 10% of body weight until they are about eight weeks of age when they are weaned from milk and moved to group housing (Cantor et al., 2019).

2. Welfare issues in modern artificial rearing of dairy calves

In the past three decades there have been multiples definitions of animal welfare due to the different interpretations of the term, the understanding of animals' sentiency, the way they perceive their environment, and the impact that internal and external stimuli can have on them (Carenzi and Verga, 2009). High standards of animal wellbeing were initially linked to the animal's health and productivity, as well as its capacity to face physiological challenges like disease (Barnard and Hurst, 1996). Subsequently, the concept of emotional distress and animal suffering became important, as the animal's mental and affective state affects how it copes and adapts to challenges in its environment (Broom, 1991).

More recently, a new dimension of animal welfare was taken into consideration: the "naturalness" of the animal's life or its freedom to express natural and innate behaviours, since the inability to express highly motivated behaviours can negatively impact the animal's physical and emotional wellbeing (Fraser et al., 1997; Musschenga, 2002).

Animal welfare is now widely recognised as a multidimensional subjective condition experienced by animals in response to both internal and external stimuli (Figure 2) (Mellor, 2016). However, dealing with subjective states poses the challenge of determining an animal's welfare, and different methodologies are now in use for this purpose, including health, physiological (activation of the autonomic nervous system and the hypothalamic-pituitary- adrenal axis), and production measures, as well as behavioural observations and tests to determine an animal's emotional state, preferences, motivations, and quality of life, using the three concepts that define animal welfare (Mellor, 2016; Vannier et al., 2014).

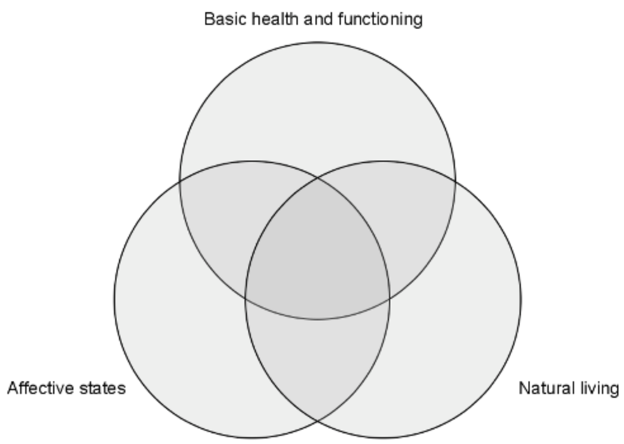

Figure 2 Three-dimensional concept of animal welfare (source: Fraser, 2008)

Both the central nervous system and peripheral tissues are involved in the stress response. Structures in the hypothalamus, the anterior pituitary gland and the adrenal gland all play an essential role and are known as the hypothalamic-pituitary-adrenal axis (HPA) (Figure 3). When exposed to stressful situations, by action of the HPA axis, cortisol is released from the adrenal glands (Smith and Vale, 2006). As there is a clear connection between stress and animal welfare, many efforts have been made to measure adrenocortical hormones and their metabolites. Traditionally adrenocortical output has been measured through blood samples, and it has been considered the gold standard for assessment of the physiological stress response. However, obtaining blood samples is in its own a stressor and can interfere with the assessment of the adrenocortical response (Cook, 2012). To avoid this confounding effect, alternative ways to measure cortisol in mammals have been studied using minimal invasive methods such as urine, faeces, saliva, and hair (Mormède et al., 2007). Blood and saliva concentrations of cortisol give an appraisal of adrenocortical activity at the time the sample was taken, whereas hair, urine and faeces provide an assessment of the adrenocortical response in the past hour (urine) or weeks (hair). Therefore, acute stress is better measured with saliva and urine, whilst chronic stress can be best measured in hair samples. At the same time hair cortisol measurement rounds out the circadian release of cortisol and fluctuations produced by brief episodes of acute stress over a longer period (Cook, 2012).

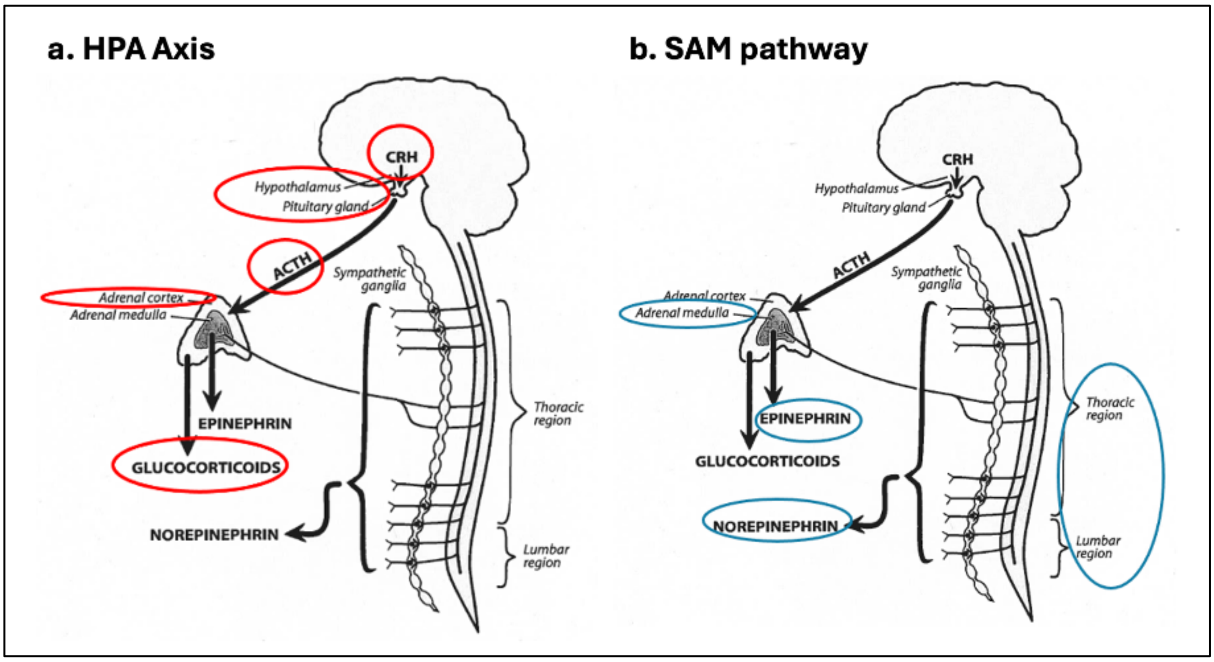

Figure 3 Schematic representation of the physiological stress response, depicting both the a. HPA axis and b. SAM (Sympatho-Adreno-Medullary) pathway. (Source: Adapter from: Fraser, 2008)

When compared to blood, saliva is a less intrusive and frequently low-stress form of sampling. Salivary corticosteroids are a mirror of the physiologically active free hormone in blood, and salivary corticosteroid hormone responses to stress can be significantly higher than total blood concentrations. As a result, salivary cortisol measures have long been thought to be superior to plasma measurements as a marker of adrenocortical activity (Cook, 2012). For the evaluation of long-term stress exposure, hair offers numerous potential benefits over other sample methods. Hair cortisol shows adreno-cortical activity spanning months, but when cut into exact lengths, it represents a shorter time period. Hair can be cut without discomfort, and it can also be collected from the environment for free-ranging animals. The quantity of cortisol detected in the sample is unaffected by the sampling collection procedure, indicating that it is feedback-free (Cook, 2012). Both saliva and hair have been used to measure cortisol levels in cattle with adequate results (Comin et al., 2013; Pagani et al., 2017; Schwinn et al., 2016; Tallo-Parra et al., 2014; Vesel et al., 2020)

To complete the stress response, the sympathetic nervous system and adrenal medulla (SAM pathway, Figure 3) activate noradrenergic neurons in the central nervous system, terminating in the thoracic and lumbar regions, as well as sympathetic neurons ending in the adrenal medulla (Fraser, 2008). Heart rate is regulated by the autonomic nervous system, where basal heart rate reflects parasympathetic activity, and increase activity results from sympathetic regulation. Therefore, heart rate measurement at any given point represents the net interaction between this parasympathetic and sympathetic influences, with increased sympathetic tone reflecting a heightened stress response (Broom and Johnson, 2019). Heart rate variability (HRV) is a non-invasive index that measures the functioning of the autonomic nervous system and is a good indicator of physiological and psychological stress (von Borell et al., 2007). Reduced HRV reflects increased sympathetic tone and has been linked to stress in humans and nonhuman animals (Clapp et al., 2015).

Mohr et al. (2002) and Clapp et al. (2015) demonstrated the usefulness of HRV measurements as a physiological stress indicator in dairy calves. HRV variability can be measured using Holter recorders or fixed systems, and portable heart rate monitors. Most studies conducted in cattle to measure HRV have been done using portable heart rate monitors designed by Polar electronics that detect R-peaks of the electrocardiogram (von Borell et al., 2007). Polar Equine technology portable heart rate monitors are the only commercial heart rate monitors currently validated to measure HRV in cattle (Hopster et al., 1994).

Behavioural observations are very accurate to detect changes in animal behaviour but are labour and time consuming. Another way to measure the behaviour of an animal is using tri axial accelerometers, which automatically obtain information in real time on the movements of the animal wearing the device (J. H. C. Costa et al., 2021). The accelerometer measures changes in velocity over time, this in turn generates a voltage that is used to calculate the movement's speed and direction (Hendriks et al., 2020). Three-axial accelerometers obtain information three dimensionally. When evaluating the welfare of an animal, accelerometers can be useful in detecting specific behavioural patterns associated with the health status of the animal, its wellbeing, and its emotional state (Chapa et al., 2020). Studies have demonstrated the accuracy of accelerometers to measure cattle behaviour. For instance, Robert et al. (2009) observed that classification of lying and standing behaviours by accelerometers fitted to beef calves, had an excellent agreement with video recording data (99.2% and 98% respectively). Nonetheless, the accuracy in classifying walking behaviour was of 67.8%. Likewise, Roland et al. (2018), detected that tri axial accelerometers fitted to pre-weaned dairy cows were successful in detecting lying behaviour, rumination, and feed intake. Tri axial accelerometers have also demonstrated to be useful in detecting early signs of neonatal diarrhoea (Goharshahi et al., 2021) and respiratory infections (Swartz et al., 2017) in dairy calves.

2.1. Early cow-calf separation

In most commercial milk production systems in industrialized nations, it is common practice to separate calves from the dam soon after birth, primarily for economic reasons and with the aim of enhancing calf health (Beaver et al., 2019a). Only 23% of dairy producers in a national study in Canada said they never removed the calf from the dam in the first 30 minutes of life (Winder et al., 2018a). Likewise, 78 % of calves in South Brazil were separated from their mothers within the first twelve hours of life (Hötzel, 2014), 87.2 % did so in the Czech Republic (Staněk et al., 2014), and 60.3 % in Northern Victoria Australia (Phipps et al., 2018). In Austria fewer than 7.8% of farms kept calf and dam together for more than four hours (Klein-Jöbstl et al., 2015).

Those in favour of separating the cow-calf dyad believe that doing so minimises the risk of infectious illnesses spreading to the newborn. In a consensus statement by the American College of Veterinary Internal Medicine (Sweeney et al., 2012), it was recommended that farms infected with *Mycobacterium avium* subsp. *paratuberculosis* (MAP) should remove calves from the dam within the first ten minutes after birth to avoid disease transmission. But then, a systemic review by Beaver et al. (2019a) found no evidence to support the higher risk of calf pneumonia or MAP transmission when keeping cow and calf together; and even more, the prevalence of calf diarrhoea was unchanged or reduced. One of the primary concerns associated with delayed cow-calf separation is the risk of failure of passive transfer of immunity (FPTI). (Beam et al., 2009) found that calves permitted to feed directly from their mother were at a 2.4 times higher risk of FPTI compared to hand-fed calves. Colostrum management plays a crucial role in mitigating this risk, as colostrum is the primary source of immunoglobulins (IgG) that provide passive immunity to calves. Since calves are born agammaglobulinemic, they rely entirely on colostrum intake for early immune protection (Cardoso et al., 2021). The timing, quantity, and quality of colostrum feeding are critical factors in ensuring successful passive transfer. It is recommended that calves receive at least 4 litres (or 10% of their body weight) of high-quality colostrum within the first few hours of life, with a target IgG concentration exceeding 50 g/L (Godden et al., 2019). Delayed or inadequate colostrum intake not only increases susceptibility to diseases like diarrhoea and pneumonia but can also result in poorer growth performance and higher mortality rates during the pre-weaning period (Carter et al., 2021; Crannell and Abuelo, 2023). Colostrum testing and supplementation strategies can help ensure effective passive transfer, even when cow-calf contact is prolonged. Brix refractometers and coloustrometers are practical tools for on-farm colostrum evaluation, with Brix values ≥ 22% indicating good-quality colostrum (Patel and Gibbons, 2014). Furthermore, feeding pasteurised colostrum can reduce pathogen load without compromising immunoglobulin content, thereby mitigating disease transmission risks while supporting calf immunity (Johnson et al., 2011, 2021a).

There is a strong economic incentive to keep separating cow and calves early in the rearing process, as some studies have found that the quantity of saleable milk decreases when calves are allowed to feed from their dam (e.g., de Passillé et al., 2008), the practice can be considered to be more labour intensive (Johnsen et al., 2016) and dry feed consumptions can be lower in calves before weaning (Fröberg et al., 2011).

Yet, early cow calf separation seems to have detrimental impacts on the overall welfare of the calf (Meagher et al., 2019). The study by Stěhulová et al. (2008) investigated how the behavioural and physiological responses of dairy cows and calves to separation were influenced by calf age at separation (1, 4, or 7 days) and the presence or absence of visual and auditory contact post-separation. The results indicated that later separation (e.g., day 4 or 7) led to more intense and prolonged distress behaviours in both cows and calves, with increased standing, vocalisations, and exploratory behaviours such as sniffing and licking walls. Cows allowed visual and auditory contact with their calves after separation exhibited stronger behavioural reactions, including more frequent vocalisations and attempts to interact with their calves. Calves separated later showed prolonged heart rate elevations and more signs of distress immediately after separation but demonstrated improved social competence and quicker habituation to novel social situations at 3 weeks of age (Stěhulová et al., 2008). Furthermore, artificially raised calves put on less weight after weaning and performed more abnormal oral behaviours as opposed to dam-reared calves (Fröberg et al., 2011). Similarly, Roth et al. (2009b) noticed cross suckling to be more common in artificially reared calves. Dam-reared calves were also better prepared to cope with isolation (lower cortisol levels), were more socially competent in new social environments (Wagner et al., 2013) and had lower heart rates (Buchli et al., 2017a).

There is some research looking at the long-term impacts on the practice of early cow-calf separation, and evidence shows that mother-rearing has a favourable influence on calf welfare later in life by promoting better social, behavioural, and physiological development. Calves raised with their dams demonstrate more socially competent behaviours, such as increased affiliative interactions, reduced fear responses, and better adaptability in new social settings. Wagner et al., 2015) found that cows raised by their mother were keener to explore when held in isolation, in a new place, and when exposed to new objects. They also displayed less alterations on the mean heart rate in isolation, and despite their pre-isolation cortisol levels being lower, displayed an adequate acute cortisol increase when exposed to stressful situations. Moreover, these calves are better equipped to establish stable social hierarchies when introduced to unfamiliar peers, and tend to exhibit lower cortisol levels during social isolation tests, indicating improved coping mechanisms and reduced stress sensitivity (Buchli et al., 2017b; Santo et al., 2020).

2.2. Restricted social contact

It is a common practice to house pre-weaned calves individually to decrease the likelihood of disease transmission by limiting contact between animals and by allowing easier health monitoring by farmers (Hulbert and Moisá, 2016). As it is, 86.6 percent of dairy producers in the United States house their pre-weaned calves individually (Urie et al., 2018a), almost 100 percent do so in Belgium and Germany, 63 percent in Canada (Winder et al., 2018a), and 70.2 in South Brazil (Hötzel et al., 2014). Recent data indicates a shift in UK calf housing practices, with the use of individual pens for pre-weaned calves decreasing from 60% in 2010 (Mahendran et al., 2022; Marcé et al., 2010) to 38.4% in a 2022 survey (Mahendran et al., 2022). According to the authors, this decline is attributed to changes in milk buyer policies that encourage pair or group housing to support calf socialisation and welfare, with 87.5% of surveyed farms reporting using some form of group housing pre-weaning. The 2024 update to UK calf management strategies noted increased adoption of automatic milk feeders (AMFs) in group housing setups, which reduce labour demands but require careful weaning management to avoid stress and ensure consistent feed intake (Mahendran, 2024).

Despite the shift, many farmers still favour individual housing due to perceived benefits, such as easier health monitoring and reduced disease transmission.

The most common causes of mortality in dairy calves are infectious diseases, particularly diarrhoea and bovine respiratory disease (BRD) (Johnson et al., 2021). According to Curtis et al. (2016) calves kept in groups had 3.86 times more risk to have diarrhoea and were 5.4 times more likely to develop BRD. Nevertheless, the calves in this study were kept in inadequate living conditions which may have influenced the validity of the results. Svensson et al. (2003) on the other hand, observed that there was no significant difference in the incidence of diarrhoea in large-group, small-group, and individually housed calves, and that group housing was even a protective factor against the development of diarrhoea. In this study, small groups of calves had the same risk of developing BRD as individually housed calves (OR 0.93 and 1.0 respectively), whereas calves housed in larger groups had an increased risk of contracting the disease (OR 2.2). Jensen and Larsen (2014) found no changes in the health status of pair housed and individually housed calves, confirming prior results.

Because cattle are sociable beings, social isolation may be seen as unpleasant by dairy calves, resulting in a detrimental effect on their growth and development. Early social isolation has a negative impact on mammalian development, as shown by studies in other species such as rats and primates, which have shown to affect the animal's social perception, a rise in abnormal behaviours and aggressiveness (reviewed by Cantor et al., 2019). Individually housed calves exhibited more non-nutritive suckling and self-grooming behaviours than paired housed calves and the latter tended to gain more weight throughout the weaning phase (Pempek et al., 2016). Similarly, individually housed veal calves showed greater oral behaviours and self-grooming than group housed calves, according to Bokkers and Koene (2001). Using an Animal Need Index the researchers were able to determine that the overall wellbeing of individually housed calves was lower than in group housed calves. de Paula Vieira et al. (2010, 2012) and Jensen and Larsen (2014) found that individually housed calves showed more reactivity in new environments and during social contact with unknown calves and vocalised more during and after weaning than paired housed calves. In addition, individually housed calves showed a stronger cortisol response in an Adrenocorticotropic hormone (ACTH) test when exposed to new situations (Raussi et al., 2003).

2.3. Restricted diet and weaning distress

Calves ingest between eight and thirteen litres of milk per day in numerous bouts under semi-natural raising circumstances, corresponding to 20 percent of their body weight gain per day, and weaning usually occurs after they are seventh month of age (Khan et al., 2016). To decrease rearing expenses and reduce weaning age, artificially raised calves often have a limited diet (10 percent body weight increase per day), both in terms of the amount of milk/milk replacer they receive and number of feeds they are offered per day, which promotes early solid feed consumption (Costa et al., 2019).

In a survey conducted in Canada, farmers responded that the largest amount of milk supplied to calves in a 24-hour period was 8.2 litres, and 33% of participants said they fed the calves less than six litres per day (Winder et al., 2018a). Feeding twice per day was the most common practice, and abrupt weaning was the norm in 16.5 percent of the farms, with age being the key criterion for weaning (Vasseur et al., 2010). Similarly, in the United States the estimated feeding volume per day was 5.6 litres, provided twice a day in 87.6 percent of farms. Based solely on age, calves were weaned at an average of 65.7 days in 98.1% of cases (Urie et al., 2018a).

In the UK, similar trends in milk feeding and weaning practices have been observed, though with notable differences. The recent survey by (Mahendran et al., 2022) found that restricted milk feeding remains highly prevalent, with 45.4% of surveyed farms providing only 4–6 litres per day, despite evidence supporting higher milk allowances for improved growth and welfare (Knauer et al., 2018; Rosenberger et al., 2017). Most UK farms (87.5%) still follow a twice-daily milk feeding schedule, consistent with findings in North America. However, feeding volumes remain lower than in Canada, where some farms provide up to 8.2 litres daily (Winder et al., 2018b). Weaning in the UK is primarily based on age, with the most common weaning age being 8 weeks (32.9% of farms), though weaning ranges from 6 to 12 weeks depending on management practices. Only a small percentage of UK farms use ad libitum milk feeding, despite research showing that calves voluntarily consume an average of 9.6 litres per day under such systems (Mahendran, 2024). Additionally, abrupt weaning remains common, although there is increasing interest in gradual weaning strategies to minimise stress and maintain growth rates.

Calves on limited diets (milk provided at 10% of body weight per day) have been reported to have greater signs of hunger, less weight gain before and after weaning, and reduced milk outputs later in life (Cantor et al., 2019). For instance, (Korst et al., 2017) found that calves fed a limited diet of 6.78 kg/day milk replacer visited the feeding location more frequently than ad libitum fed calves, with 70% of the visits going unrewarded (no milk was dispensed). In terms of growth, calves fed ad libitum had significantly higher average daily gains (ADG) during the first 4 weeks **(**ADG was 450 g/day in restricted calves and 648 g/day in ad lib calves. When all calves were switched to a restricted diet from day 28 to 69, previous ad libitum-fed calves experienced a temporary dip in growth, likely reflecting a post-restriction growth setback. In the study by de Paula Vieira et al. (2008) feed restricted calves (4.2–4.8 milk replacer L/day) had poorer weight gains (ad libitum-fed calves gaining 0.53 kg/day, whereas restricted-fed calves gained only 0.11 kg/day), spent longer periods standing and exhibited higher suckling times in rewarded visits even when no extra milk was administered

Cross suckling was observed more commonly in calves with a higher energy deficit, and it diminished as the energy balance was corrected, implying that the behaviour was probably due to hunger (Roth et al., 2009b). Additionally, Thomas et al. (2001) discovered that calves being feed-restricted vocalised more and stood for longer periods of time compared to non-restricted calves. All these findings suggest that feed restriction causes hunger and distress, negatively impacting dairy calves' overall welfare.

2.4. Disease challenges

The early life stage of dairy calves, from birth through weaning, is a critical period that significantly influences their future health, productivity, and overall welfare (Bach, 2011; A. Costa et al., 2021). During this phase, young calves are particularly vulnerable to a range of infectious diseases that can lead to substantial morbidity and mortality due to the immaturity of their immune systems and the physiologic stress of transitioning from intrauterine to extrauterine life (Hulbert and Moisá, 2016). The welfare of these animals is closely tied to their ability to withstand and recover from these disease challenges (Doeschl-Wilson et al., 2021).

Acute neonatal diarrhea and pneumonia are the most common health challenges faced by preweaned dairy calves (McGuirk, 2008; Urie et al., 2018b). Enteritis, often caused by pathogens such as Escherichia coli, rotavirus, and Cryptosporidium, is the leading cause of mortality in calves less than 30 days old (Lorenz et al., 2011b; Yimer et al., 2015). Pneumonia, on the other hand, tends to emerge as a predominant issue in calves older than one month (Cummings et al., 2022).

Apart from immediate mortality, calves that survive severe disease episodes often experience impaired growth, delayed weaning, and reduced future milk production, which can have lasting economic consequences for dairy operations (A. Costa et al., 2021; Stanton et al., 2012). The cost of managing disease in young female calves includes not only the direct expenses of veterinary care and medications but also the long-term costs associated with reduced productivity and the need for additional replacements (Buczinski et al., 2021; Overton, 2020). Estimates suggest that the economic burden of disease during the preweaning period can range from USD $0.50–687.80 per animal, with overall costs to the operation potentially reaching thousands of dollars annually (Richter et al., 2017).

The negative impacts of disease in preweaned dairy calves extend beyond individual welfare and economic concerns; they also pose significant challenges to the sustainability of dairy production systems. Disease-related growth setbacks and reduced performance compromise the long-term productivity of affected animals, leading to higher replacement rates and increased resource use per unit of milk produced (Buczinski et al., 2021; Stanton et al., 2012). Calves that experience early-life illness, particularly neonatal diarrhoea and pneumonia, often exhibit lower average daily gains (ADG), delayed first calving, and reduced milk yield in first and subsequent lactations (Dunn et al., 2018; Virtala et al., 1996). These inefficiencies increase the environmental footprint of dairy production, as diseased calves require more feed, veterinary interventions, and management resources to reach maturity, ultimately reducing the efficiency of nutrient conversion and increasing greenhouse gas emissions per unit of milk output (Džermeikaitė et al., 2024; Overton, 2020). Furthermore, the higher mortality and culling rates associated with disease lead to increased replacement heifer rearing, which is resource-intensive and costly (Overton and Dhuyvetter, 2020). Reducing disease incidence and improving resilience in young dairy calves is, therefore, not only critical for enhancing animal welfare and farm profitability but also for improving the overall sustainability of the dairy industry (Buczinski et al., 2021).

Preweaning management is critical for illness prevention (Lorenz et al., 2011c). Passive immunity, the main defence against early-life illnesses, requires proper colostrum management (Godden et al., 2019). Disease prevention also depends on housing conditions including bedding, ventilation, and space allocation (Gorden and Plummer, 2010; Lorenz et al., 2011a). Successful illness management requires timely vaccines, consistent monitoring, and early intervention (Lorenz et al., 2011c; Maier et al., 2022). However, despite the well-documented risks and management strategies, many dairy operations continue to struggle with high morbidity and mortality rates in their preweaned dairy calves (Su et al., 2023). This persistence of disease emphasizes the need for a more comprehensive understanding of the factors that contribute to disease susceptibility and the effectiveness of various intervention and mitigation strategies (Arlington Headley et al., 2024; Ollivett, 2020; Robi et al., 2024). It also underscores the importance of adopting a welfare-centric approach to dairy calf management, where the health and well-being of the animal are prioritized.

2.5. Painful procedures

Dehorning or disbudding is a routine management practice in the cattle industry performed on young calves, aimed at preventing the growth of horns, with up to 81% of dairy farms in the European Union having dehorned/disbudded cattle (Cozzi et al., 2015), 83% in the United Kingdom and around 52% in the United States (Urie et al., 2018a). It is believed that horns in cattle pose risks of injury to other animals and handlers, can increase the incidence of aggressive behaviour, and complicate management in confined settings such as feedlots and milking parlors (Kling-Eveillard et al., 2015; Knierim et al., 2015). While dehorning/disbudding offers management and safety benefits, it is increasingly recognized as one of the most controversial animal husbandry practices from a welfare standpoint (Canozzi et al., 2019; Costa et al., 2019; Stafford and Mellor, 2005). Disbudding refers specifically to the destruction of the horn-producing cells in young calves, typically performed before the calves are two months old, while dehorning involves the removal of fully developed horns in older cattle (American Veterinary Medical Association (AVMA), 2014).

The methods employed for these procedures include surgical amputation, hot iron cautery and chemical caustic paste. Surgical amputation is typically reserved for older animals where the horns are more developed. Hot iron cautery refers to the procedure where a heated iron is applied to the horn buds to prevent their growth; it is widely use in Europe and to a less extend in North America (Cozzi et al., 2015; Gottardo et al., 2011). On the other hand, chemical caustic paste involves the application of a chemical that burns and destroys the horn buds, a technique mostly used in North America compared to Europe (Saraceni et al., 2021). Regardless of the age at which they are performed, all these techniques are associated with significant pain and stress in calves, raising critical concerns about their impact on animal welfare (Kupczyński et al., 2014; Marquette et al., 2023).

Pain is a complex phenomenon that can be categorized into three primary types: (1) acute nociceptive pain resulting from initial tissue injury; (2) inflammatory pain that may remain for days or weeks until the tissue damage is healed; and (3) neuropathic pain arising from damage to the somatosensory nervous system, which can persist indefinitely (Adcock and Tucker, 2017).

The pain associated with disbudding is a significant concern, as it can lead to both immediate and long-term welfare issues for the calves. Research indicates that calves experience acute nociceptive pain and inflammatory pain during and after the procedure, which can manifest in behavioural changes such as increased vocalization and altered feeding patterns (Stafford and Mellor, 2011; Stock et al., 2013). Immediate analgesic and anaesthetic interventions, such as local anaesthesia and non-steroidal anti-inflammatory drugs (NSAID), are effective at reducing acute pain indicators, including elevated cortisol levels and pain-related behaviours such as head rubbing, ear flicking, and restlessness during and immediately after disbudding and dehorning procedures (Stafford and Mellor, 2005; Stock et al., 2021). However, these interventions have limitations in their duration of action and do not consistently manage the prolonged pain that can persist for weeks following the procedure (Adcock and Tucker, 2018; Drwencke et al., 2023; Jimenez et al., 2019a, 2019b). Long-term pain, which may not be as apparent immediately post-procedure, can manifest in subtle behavioural changes such as reduced play behaviour, increased time spent lying, altered weight distribution (favouring the head), and reduced social interactions. (Stafford and Mellor, 2011, 2005; Tschoner, 2021). These prolonged pain responses are likely due to the deep tissue damage and inflammation associated with horn tissue removal, which is not fully alleviated by standard pain management protocols (Tschoner, 2021).

Furthermore, a study by Vidondo et al. (2019) evaluated potential histopathological effects of cautery disbudding on the cornual nerve in calves, particularly in relation to chronic pain. The study included 21 Holstein bull calves were included, with some undergoing disbudding at 7 days and others at 28 days, while a control group received sham procedures. Chronic pain was assessed through subjective evaluations and neurophysiological tests, alongside morphological analysis of nerve samples collected post-slaughter. Results indicated that while four disbudded calves showed signs of chronic pain, there were no significant morphological differences in nerve structure or cellular markers between disbudded calves with and without chronic pain. This suggests insufficient evidence to support neuropathic changes resulting from the disbudding procedure, which could be a consequence of the small sample size.

The duration, intensity, and quality of pain are influenced by numerous factors beyond the magnitude and type of tissue injury. Including an individual's prior and simultaneous encounters with pain and stress; cognitive, social, and emotional factors; the quality and duration of analgesics administered before, during, and after the procedure; and the presence or absence of complementary nonpharmacological interventions (Adcock and Tucker, 2017). Cattle attentiveness and arousal levels can affect pain perception. Distractions and competing motivations like food can lessen procedure pain. Contextual factors like threats and conspecifics also alter pain responses. Addressing these cognitive and emotional modulators is crucial to creating effective pain management solutions and improving the welfare and pain experiences of calves (Adcock and Tucker, 2017).

3. Environmental Enrichment

Environmental enrichment refers to changes in an animal's surroundings or management practices that improve its physical and affective state, encourages the performance of species-specific behaviours, enhance the ability to cope with stressors, beyond the minimum management standards (Mandel et al., 2016; Wells, 2009). There are different categories of environmental enrichment including social (direct or indirect contact with conspecific or humans), occupational (e.g., interactive toys), nutritional (offering a variety of foods and using different methods for food delivery), sensory and physical (e.g., exercise) (Mandel et al., 2016). Some enrichment practices combine different categories (Bolt and George, 2019).

Research has shown the positive effect of environmental enrichment in improving captive animals' wellbeing, as is the case in laboratory animals (Baumans and van Loo, 2013), zoo animals (Mason and Latham, 2004) and farm animals (Bolt and George, 2019), including laying hens (Campbell et al., 2019), broiler breeders (Leone and Estévez, 2008; Riber et al., 2018) and pigs (Beattie et al., 2000; Crone et al., 2021; de Weerd and Ison, 2019). In beef cattle there is evidence indicating environmental enrichment improves productivity and has positive physiological effects (Ishiwata et al., 2006; Park et al., 2020). Moreover, it has also been observed to reduce aggression and agonistic behaviours (Matković et al., 2020; Park et al., 2020).

In dairy cattle, social enrichment in the form of pair housing has been shown to improve the integration process of heifers into a new herd. Heifers introduced in pairs exhibited longer lying times and increased time spent in the feeding area compared to those introduced individually, suggesting that pair housing aids in the habituation process (Gygax et al., 2009). Occupational enrichment in the form of exercise in an open paddock increased activity levels and exploring behaviour in dairy cows, with decrease agonistic interactions and a positive impact in claw conformation (Loberg et al., 2004).

3.1. Environmental enrichment for dairy calves

3.2. Social enrichment

As social animals, cattle should be provided contact with conspecifics. It has been demonstrated that social contact has a positive impact on cognition, fitness, and affective state in dairy calves (Costa et al., 2016). Moreover, calves choose to have full social contact with a peer as opposed to just head contact through metal bars, when given the option (Holm et al., 2002).

It has been observed that the presence of a partner reduces the level of stress in calves exposed to challenging circumstances (social buffering) and increases the likelihood to initiate a particular behaviour when observing others engaged in that activity (social facilitation) (Costa et al., 2016). For instance, Overvest et al. (2018) observed that paired housed calves had significantly higher feed intake than individually housed calves during the weaning process. Similarly, Costa et al. (2015) observed not only increased feed intake but higher weight gain in pair housed calves during the same period. These findings suggest that the presence of a companion eases the transition from milk replacer to solid feed intake. In addition, researchers also found that calves housed with other calves were more likely to try new foods compared to individually housed calves (Costa et al., 2014).

When considering the effect of social enrichment on the affective state of calves, Bučková et al. (2019) found that calves housed in pairs exhibited a more optimistic response in a judgement bias test compared to individually housed calves. Similarly, Duve et al. (2012) observed that pair-housed calves engaged in more play behaviour than individually housed calves, suggesting a more positive affective state. Social enrichment also has a positive effect on calves' cognition. Gaillard et al. (2014) found that pair housed calves had better performance in a reversal learning test. These findings suggest that isolation has a negative impact in certain types of learning leading to a lack of behavioural flexibility, essential when coping with new environments and challenges (Langenhof and Komdeur, 2018).

3.3. Nutritional enrichment

Abnormal oral behaviours such as cross-suckling and fixture suckling has been observed in calves reared artificially due to their high motivation to suckle and due to a restricted diet (De Passille, 2001; Roth et al., 2009). Lidfors et al. (2010) observed that calves who are fed *ad libitum* by the dam do not exhibit oral abnormal behaviour. Equally, Margerison et al. (2003) noticed that cross-suckling was less common when calves were permitted to suckle for fifteen minutes a day from their dam or foster cow, and when allowed higher planes of nutrition.

Artificial teat feeding, as compared to bucket feeding, has shown positive results in reducing abnormal oral behaviours in artificially reared calves, as observed by Horvath and Miller-Cushon (2017). They found that teat fed calves displayed less non-nutritive suckling directed at the pen than bucket fed calves. Ad libitum hay was also found to minimise aberrant oral behaviours in this trial. This reduction is likely because the teat provided an outlet for calves' innate sucking motivation, which is often left unsatisfied when milk is fed from a bucket. Dry teats have also been recommended to decrease cross-suckling in dairy calves kept in groups. For instance, when group housed calves fed by an automated teat feeding system were given access to dried teats after each meal, there was a substantial reduction in cross suckling behaviour compared to calves that did not have access to a dry teat. The opportunity to suckle on dry teats after milk meals satisfied calves' innate sucking motivation, thus reducing the likelihood of cross-sucking. The presence of a stray net also provided additional oral enrichment, though it was less popular than the dummy teats, suggesting that sucking is a primary behavioural need in calves, particularly after milk meals (Ude et al., 2011).

3.4. Occupational enrichment

Some interactive objects can serve as both occupational and sensory enrichment. Brushes are by far the most common type of occupational enrichment researched in dairy calves. Pempek et al. (2017) added stationary brushes and other objects to some individually housed calves, and observed that compared to calves without enrichment objects, animals in the intervention group displayed more locomotor play after feeding. Nonetheless, no significant variations were observed in growth rates or when the calves were exposed to a novelty test. Zobel et al. (2017) also found that pair housed calves that had access to a rope and a brush tended to display more play behaviour.

Horvath and Miller-Cushon (2017) noticed that the presence of an automated brush increased self-grooming behaviours in grouped housed dairy calves. Velasquez-Munoz et al. (2019) used a mechanical brush in calves during the weaning period and observed a tendency to illness in calves with no access to the brush and increase in activity levels and feeding in calves in the intervention group. Self grooming, increased activity levels and feeding behaviour were interpreted in these studies as indicators of better welfare status.

3.5. Sensory enrichment

Sensory enrichment is defined as any stimulus that can trigger one or more of an animal's senses (Wells, 2009). Although olfaction is the least understood of the senses in cattle, it is known that as microsmatic animals (animals with a highly develop sense of smell) they have a very sensitive sense of smell compared to humans (Adamczyk et al., 2015). Semiochemicals are chemical substances produced by animals and released into the environment as volatile molecules or deposited in inanimate objects through the skin, urine, saliva, etc, allowing other individuals from the same or different species to gain chemosensory information through the olfactory system (Wyatt, 2012). Pheromones are a type of semiochemicals that allow chemiosensory communication between individuals of the same species, giving key information on the reproductive state of a conspecific or its levels of stress (Adamczyk et al., 2015).

Pheromones are detected by the receiving individual by two chemiosensory systems located in the nasal cavity of all mammals, the main olfactory system and the vomeronasal system (Stowers and Kuo, 2015). These systems contain peripheral primary sensory neurons that express different types of receptors able to bind pheromones, and each system connects with second order neurons in the main olfactory bulb (main olfactory system) and accessory olfactory bulb (vomeronasal system) Figure 4 (Tirindelli et al., 2009). As observed in Figure 4, the second-order neurons project to the pririform cortex and cortical amygdala (main olfactory bulb), as well as to the medial amygdala and posteromedial area of the cortical amygdala (Accessory olfactory bulb), eventually reaching the hypothalamus (Tirindelli et al., 2009).

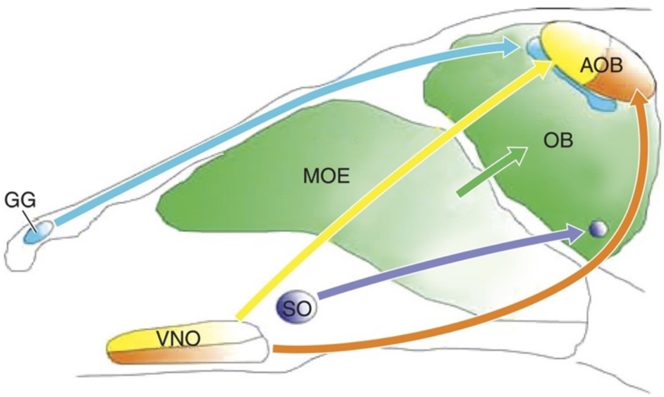

Figure 4 Primary sensory neurons projections to the olfactory bulbs (MOE = Main olfactory system, VNO = Vomeronasal organ, OB = Main olfactory bulb, AOB = Accessory olfactory bulb (From Tirindelli et al., 2009).

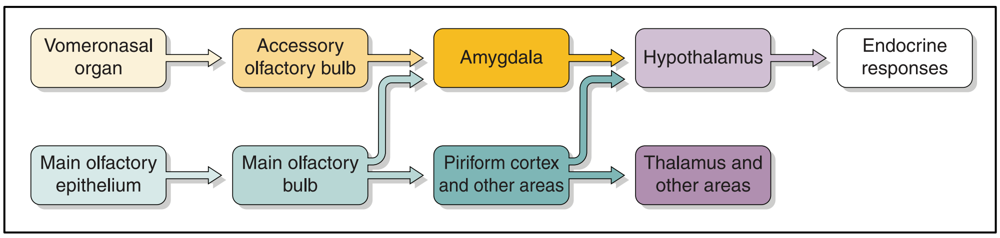

Figure 5 Anatomical pathways of the rodent vomeronasal and main olfactory systems *(From Tirindelli et al., 2009)*

When a pheromone binds to its receptor either in the vomeronasal organ or main olfactory epithelium, it initiates a cascade of electrical and molecular events that ultimately modulate various aspects of the target individual's social behaviour (Francia et al., 2014). There are two types of pheromones according to their function, releaser pheromones evoke short latency behavioural responses, and are associated to recognition of conspecifics (e.g., mating, territory marking). Primer pheromones generate delayed responses through activation of the neuroendocrine system (Tirindelli et al., 2009).

Most studies related to pheromone activity in mammals are centred in reproductive behaviours, and this is no different in cattle (Archunan et al., 2014). However, there are other types of pheromones associated with different aspects of mammals' social interactions. For instance, there has always been interest in understanding olfactory interaction between mammalian mothers and their offspring. Pageat (Pageat - US Patent 6 and 2000, 2000) isolated a pheromone from the mammary gland of a sow and observed that it had calming and appeasing effects on the piglets. The same kind of pheromone was then observed in other female mammals including dogs, horses, cats, sheep, cattle, and rabbits, with different concentrations of oleic acid, palmitic acid and linoleic acid and other components depending on the species (Pageat and Gaultier, 2003). These appeasing pheromones are produced around the skin of the mammary glands a few days after a female mammal gives birth, and since these substances are not too volatile, they require 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 (Pageat and Gaultier, 2003).

Synthetic analogues of these appeasing pheromones have been used in several domestic animals exposed to aversive or uncertain environments. Although their exact mechanism of action remains unclear, they are believed to induce an optimistic cognitive bias by influencing the emotional processing of the target individual. A cognitive bias is when individuals consistently make decisions that aren't based on logic or facts. They do this by viewing information through their own subjective lens, which is usually shaped by past experiences, feelings, or mental shortcuts. These biases can influence perceptions, decisions, and behaviours without conscious awareness, often as a way for the brain to simplify complex information and respond more efficiently (Mendl et al., 2009). Cognitive biases can manifest in various ways, such as confirmation bias, where individuals seek information that supports their pre-existing beliefs while ignoring contradictory evidence, or negativity bias, where negative experiences are given more weight than positive ones (Baciadonna and McElligott, 2015). In animals, cognitive biases have been studied as indicators of emotional states, with pessimistic biases often linked to negative affective states and optimistic biases to positive welfare conditions (Mendl et al., 2009). A positive cognitive bias makes animals feel less threatened by their surroundings and reduces stress levels, rather than directly triggering a behavioural change (Dube et al., 2012).

Most of the research in appeasing pheromones has been carried out in companion animals, mostly dogs and cats, for the treatment of behavioural disorders. Frank et al. (2010)

conducted a systematic review of the use of pheromones (including the cat facial pheromone and the dog appeasing pheromone) for the treatment of undesirable behaviours in both species. The authors included 14 studies of which seven were related to the canine appeasing pheromone. Most of the studies had methodological weaknesses, including randomization issues, and only one yielded sufficient evidence of the effect of the pheromone in reducing fear-anxiety in dogs during training. However, there are a few studies that were not included in this systematic review that have promising results. For instance, Gaultier et al. (2009) conducted a triple blinded, randomised placebo-control trial evaluating the effect of the appeasing pheromone in newly adopted puppies. They observed that 15 days after treatment significantly fewer puppies receiving the treatment showed signs of fear when facing unfamiliar people compared with puppies in the control group.

Research in farm animals often allows for greater control over confounding variables compared to studies on companion animals, as farm animals are typically managed under standardized, controlled environments with consistent feeding, housing, and husbandry practices. In contrast, companion animals often live in diverse, less-controlled settings with varying diets, activity levels, and social interactions, making it more challenging to isolate specific factors influencing the outcomes of behavioural or physiological studies. There are a few numbers of studies testing the effect of an appeasing pheromone in pigs. Mcglone and Anderson (2002) observed that the application of a synthetic analogue of the porcine appeasing pheromone at weaning, reduced fighting and other agonistic behaviour in pigs treated with the substance compared to placebo (144 replicates). Increased exploring behaviour and play behaviour and had a positive impact on body weight gains and food efficiency ratios. Similarly, in another study, although the sample size was small (12 replicates), food neophobia and aggressive interactions were reduced after weaning, in pigs receiving the pheromone (Temple et al., 2016).

Mixed effects have been observed whilst testing the porcine appeasing pheromone during simulated transport of pigs. On one hand, Driessen et al., (2008) observed a positive effect of the pheromone on heart rate parameters in pigs exposed to transport vibration. Specifically, pigs treated with the pheromone exhibited lower minimum, mean, and peak heart rate values compared to control pigs, although only the reduction in minimum heart tare reach statistical significance. Additionally, the number of ventricular ectopic beats (VEB) was higher in pheromone treated pigs during vibration, suggesting increased cardiac excitability. However, no differences were found in the amount of time pigs spent lying down, indicating that the pheromone did not significantly alter resting behaviour. On the other hand, Lewis et al. (2010) found that although the application of a maternal pheromone reduced the rate of fatigued pigs during transport by approximately 39%, this effect was not statistically significant. Furthermore, the pheromone treated pigs displayed increased handling difficulty, requiring more prodding and vocalizing more during unloading; suggesting that whilst the pheromone may have had some stress-mitigating effects, it did not consistently improve ease of handling or transport outcomes.

In horses, Alves de Paula et al. (2019) tested the effects of the equine maternal pheromone on the behaviour and physiologic response of hoof trimming in a small sample of 20 foals, but no statistically significant changes were observed. In contrast, Falewee et al. (2006), in a double blinded, placebo-controlled study, evaluated the effects of a synthetic analogue of the equine appeasing pheromone. Saddled horses walked through a fringed curtain, that were treated with the pheromone, displayed less stress related behaviour and a reduced negative impact on their heart rate response compared to the placebo group when exposed to the task.

So far studies in cattle have focused on evaluating the effect of the bovine appeasing pheromone (BAP) on milk production and weaning (separation from the dam and diet change, with both occurring simultaneously when the calves are around six months of age) of beef calves. For instance, 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 when the synthetic analogue of the pheromone compared to those treated with placebo. Colombo et al. (2020), Cooke et al. (2020) and Schubach et al. (2020) demonstrated that the administration of the synthetic analogue of the bovine appeasing pheromone during weaning and transport of beef calves reduced stress levels whilst the substance was active. This was evidenced by lower levels of cortisol found in hair and blood samples. Furthermore, lower blood haptoglobin levels compared to the control calves, improved feed efficiency and growth rates of the treated calves.

Only one study has tested the effect of the synthetic analogue of the bovine appeasing pheromone on the welfare of dairy calves. Angeli et al. (2020) evaluated the effects of this pheromone on performance, disease incidence and pharmacological costs in Dairy Fir x Holstein calves prior to weaning from a milk diet. Results showed that BAP-treated calves had significantly greater body weight (BW) at weaning (BAP 94.6 kg compared to 90.8 kg in the placebo group). Calves in the BAP group also tended to have higher BW at day. Average daily gain (ADG) was higher in BAP calves from days 42 to 56 and tended to be greater from days 56 to weaning. Although the overall incidence of disease (diarrhoea, pneumonia) was not significantly different between the two groups ($p = 0.92$), BAP-treated calves diagnosed with disease had higher ADG than sick controlled calves. Additionally, pharmacological costs per head were significantly lower in BAP-treated calves, particularly for diarrhoea treatments, where costs were reduced by 42% ($1.91 in control vs. $1.11 in BAP), and for calves diagnosed with both diarrhoea and pneumonia, where costs were reduced by 31% ($6.20 in control vs. $4.28 in BAP). These findings suggest that while BAS administration did not reduce disease incidence, it may have helped mitigate the negative impact of illness on growth and reduced the financial burden of medical interventions. The study highlights the potential of BAP as a cost-effective strategy to improve performance and resilience in pre-weaning dairy calves, although further research is needed to understand its physiological and immunological mechanisms.

To the researcher's knowledge no studies have been carried out to evaluate the effects of BAP on dairy calves' behaviour and physiological measures of stress from the moment they are born, through separation from the dam, weaning from milk and all the welfare challenges imposed by artificial rearing methods, such as weaning, disease and disbudding. At the same time, no studies have been carried out to observe the long-term effects on production, physiological stress and behaviour of dairy calves treated with the bovine appeasing pheromone.

4. Thesis Objectives

To evaluate the effects of a synthetic analogue of BAP on weight gain, as well as physiological and behavioural indicators of stress, in dairy calves from birth through milk weaning in a commercial setting.

To provide an in-depth analysis of the pheromone’s effects on the welfare of diseased dairy calves.

To evaluate the effects of a synthetic analogue of BAP on production, neuroendocrine response and behaviour when applied to dairy calves during disbudding in combination with adequate anaesthesia and analgesia.

To investigate the effects of a synthetic analogue of the bovine appeasing pheromone on dairy calves on their cognitive abilities and their emotional states.

CHAPTER 2. MATERIALS AND METHODS

1. Calves, Experimental design, and treatments

The experiments described in this thesis were done using Holstein Friesian dairy calves born between December 2021 and October 2023 at the Harper Adams University dairy farm. Calves were randomly assigned to either treatment A or B 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 analogue of BAP (SecureCattle® SIGNS Labs, France) or a placebo (2-[2-ethoxyethoxy] ethanol), the same vehicle used in SecureCattle® without the active compound), and 5 ml of either BAP or the placebo (measured using a syringe) were 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 by the same researcher every two weeks depending on each calf's date of birth, with no consideration to a specific time in the day, as recommended by the manufacturer. No restraining of the animals was needed for the application of treatments (P Pageat - US Patent 6 and 2000, 2000). Researchers were blind to treatments, as treatment bottles used during the study were labelled as "A" or "B" and unblinding occurred only after statistical analysis of the data in each experiment was carried out.

After birth, calves were separated from the dam and received a minimum of 4 L of defrosted high-quality colostrum (spectrometry 28-30%) in their first 12 hours 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 6).

Calves were fed milk replacer (Milkivit, Galloway & MacLeod, UK) using teat bottles twice a day, and milk weaning adhered to the farm guidelines (Figure 7): from birth to six weeks of age 3.6L twice daily; between six to seven weeks 2.6L twice daily; and between seven weeks and weaning at approximately eight 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. It was 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 three times a week.

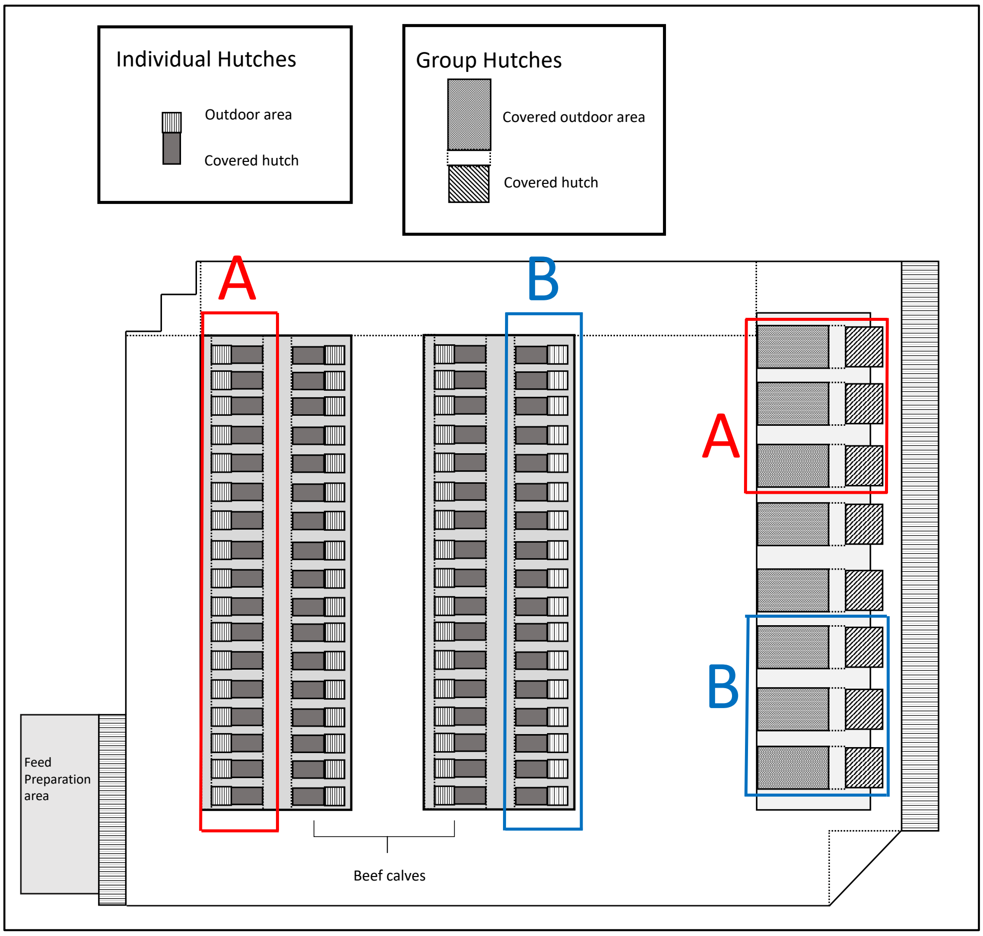

Figure 6 Calf unit layout and treatment allocation in individually and group housed calves.

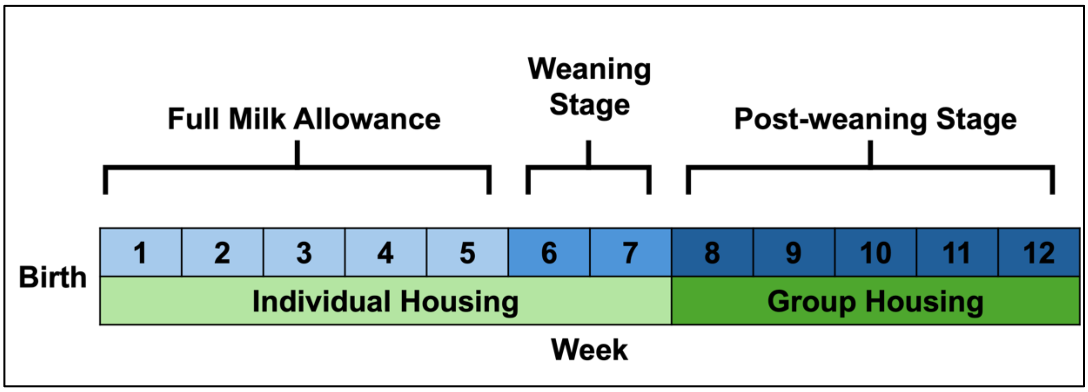

Figure 7 Calf milk weaning protocol

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 6 and Figure 7), with up to five calves per hutch. Clean water and concentrate were offered *ad libitum*, and clean bedding (straw) was provided three times per week. Calves stayed in this setting for around four weeks until they were moved to join the youngstock herd.

All calves were vaccinated for calf pneumonia at two weeks of age with intranasal Bovalto® (Boehring Ingerlheim Animal Health UK Ltd, Bracknell, UK) and hot-iron disbudded at four 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.

2. Productivity Measurements

Calves were weighed at birth and every week afterwards, using a calibrated walk-on scale that was brought to each calf; and average daily weight gain was determined by calculating the difference between two consecutive weights and dividing it by the number of days between the measurements.

3. Neuroendocrine activation variables

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

3.1. Saliva and hair sample collection, sample processing and analysis.

Saliva was collected from each calf by inducing the calf to suck on a stick sponge for three minutes. Time of sample collection depended on the researcher availability; however, the aim was to collect samples only in the morning. 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 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 two-week interval. This approach, 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 three minutes. The supernatant was separated by decantation and the process was repeated once. The hair samples were then left to dry completely for 48 hours 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 minutes. 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 minutes and incubated on a shaker for 18 hours, 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 hours. Samples were reconstituted with 100 μL of phosphate-buffered saline (PBS) and shaken for 30 seconds before quantification of cortisol with an enzyme immunoassay kit (Salimetrics®); cortisol concentration was again obtained using a spectrometer plate reader.

3.2. HRV a 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., 2019b; Kovács et al., 2014). Polar Equine technology portable heart rate monitors (HRM) were used to collect HRV measurements from all calves used in the experiments, as these have been validated and used to measure HRV in cattle (Hopster et al., 1994). The device (H10 Polar HRM) was tightly fitted around each calf thorax using a Polar equine belt and HRV was measured for different periods (from 10 minutes to 24 hours) according to the experiment being conducted. 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 two consecutive R waves in the electrocardiogram) and analysed 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).
HRV parameters, assessed through frequency domain analysis, measure how much heart rate varies over time and how this variability is distributed across different frequency bands: The high-frequency (HF) band, typically ranging from 0.15 to 0.4 Hz, reflects parasympathetic (vagal) activity, while the low-frequency (LF) band, ranging from 0.04 to 0.15 Hz, is influenced by both sympathetic and parasympathetic inputs. The very low-frequency (VLF) band, spanning 0.0033 to 0.04 Hz, is associated with thermoregulation and other physiological processes (von Borell et al., 2007). (Mohr et al., 2002) found that these HRV parameters decreased during stress when calves experience stress, such as during handling or isolation, the autonomic nervous system prioritizes sympathetic activation to manage the perceived threat, indicating a reduction in overall heart rate variability. This reduction is primarily attributed to decreased parasympathetic activity, which is responsible for rest-and-digest functions, alongside increased sympathetic dominance, associated with the fight-or-flight response. In terms of frequency domain metrics, this shift may present as reduced HF power, reflecting diminished parasympathetic modulation, and an increased LF/HF ratio, indicating heightened sympathetic activity.

4. Behavioral measures and data processing

Calves were fitted with triaxial accelerometers at birth (IDS i-QUBE, Peacock Technology Limited) on one of the hind legs, by the researcher, and it was removed when the calves were moved to the young herd. Raw data was uploaded automatically from the accelerometers into the CowAlert 2.7.1 Software (Peacock Technology Limited) where it was analysed; obtaining daily data on lying time, lying bouts, step counts and Motion index (a measure of how active the animal is calculated by the software).

Triaxial accelerometers are devices that measure acceleration along three perpendicular axes (X, Y, and Z) enabling detailed monitoring of movement and orientation. These sensors often utilize piezoresistive technology, where acceleration causes a change in electrical resistance within the sensor's material. This change is then processed to quantify the acceleration experienced by the device (Bouten et al., 1997).

In the of farm animals, the use of triaxial accelerometers has been validated for monitoring behaviours such as lying and standing (Chapa et al., 2020). Studies in dairy calves evaluating triaxial accelerometers attached to the calves reported that the sensor accurately detected lying and standing behaviours, demonstrating its potential for reliable monitoring in bovine calves with high accuracy (>99%) (Finney et al., 2018).

CHAPTER 3. EFFECTS OF A SYNTHETIC ANALOGUE OF THE BOVINE APPEASING PHEROMONE ON THE OVERALL WELFARE OF DAIRY CALVES FROM BIRTH THROUGH WEANING

1. 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., 2020b; 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., 2019b). 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., 2017b). Although social housing and teat feeding fulfil essential needs for calves as discussed in chapter 1, 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 modifications that enhance welfare and promote natural behaviours 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 analogues 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). A more detailed explanation on the mechanism of action of appeasing pheromones can be found in Chapter 1, section 3.1.4.

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 six months 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 analogue of the pheromone compared to 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 analogue of BAP during weaning and transport of beef calves reduced distress indicators whilst the substance was active. This was evidenced by lower levels of cortisol found in hair and blood samples, lower blood haptoglobin levels compared to 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 analogue 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 prior to weaning from a milk diet. They observed an improvement in body weight gain in BAP-treated calves compared to 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 the author's 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 was to evaluate the effects of a synthetic analogue of BAP on weight gain, as well as physiological and behavioural indicators of stress, in dairy calves from birth through milk weaning in a commercial setting. It was hypothesized that calves receiving the pheromone would have greater weight gain, less activation of the neuroendocrine system evidenced by lower levels of hair and saliva cortisol, and higher heart rate variability; and increased resting time compared to calves receiving a placebo.

2. 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).

2.1. 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' management and treatments were described in Chapter 2 section 1. The calves were moved to the experimental setup immediately after birth, and treatments were applied at this point and reapplied every two weeks until the calves were moved to the young herd, approximately four weeks after weaning. Additionally, following thorough cleaning of the individual hutches, treatment sites were changed midway through the experiment to eliminate location as a confounding factor.

2.2. Productivity measurements

Calves were weighed at birth and every week until weaning using a walk-on scale as described in Chapter 2. They were then weighed before being put in the group hutches and again before being moved to the youngstock herd.

2.3. Neuroendocrine activation variables

Physiological stress was measured by observing the activation of the neuroendocrine system through cortisol analysis, 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).

2.4. Saliva and hair sample collection, sample processing and analysis.

Saliva was collected from each calf at birth and every other week depending on each calf's birth date through to weaning weaning, by inducing the calf to suck on a stick sponge for three minutes. The researcher's aim was to only collect saliva samples in the morning, however, due to logistical constraints, some samples had to be taken in the afternoon, which was then considered in the statistical analysis and interpretation of the results. 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. Description of the method use to process and analyse hair and saliva samples can be find in Chapter 2 section 3.1.

2.5. HRV data collection and processing

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 in their individual pen using a Polar equine belt for 24 hours starting on the calves' second week of life and every week afterwards until the end of the experiment. Data processing can be found in Chapter 2 section 3.2.

2.6. Behavioural 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 using a Velcro strap designed and validated to be used in calves by the accelerometer's manufacturer. Each device remained attached to each calf until the end of the experiment. 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.

2.7. 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). Each calf was used as the experimental unit and data analysis was divided in three different parts: (1) assessing the effect of treatment according to the weaning stage, (2) assessing the effect of treatment according to housing condition (i.e., individual or group housing), and (3) assessing the effect of treatment by age of the animal.

This division was necessary for statistical reasons to ensure model stability, improve convergence, and avoid overparameterization, which could otherwise lead to unreliable estimates. A single, overly complex model incorporating all these factors simultaneously would have increased the risk of collinearity issues, model convergence failure, and inflated variance in parameter estimates, making it difficult to draw robust conclusions. The aim of separating the analyses was to enhance the reliability of statistical estimates and detect treatment effects more accurately within each context.

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

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, Figure 6)	ADG Salivary cortisol Hair cortisol SDNN RMSSD Minimum HR Mean HR Maximum HR SI	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

	Lying Time Lying bouts Motion Index		
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

General Linear Mixed Models (GLMM) were produced as a first intention for all outcome variables using the Ime4 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 behavioural 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 three variables, so negative binomial models were ultimately selected.

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.

3. Results

A summary of treatment effects on all the outcome variables depending on the weaning stage and social housing can be found in tables 2 and 3.

Table 2 Summary of the results of the Interaction effects of BAP vs. Placebo and weaning stage for 72 dairy calves. Descriptive statistics are expressed in mean and standard deviation. P stands for probability of the interaction effect.

Variables	Not weaned		Partially weaned		Weaned		Model	Probability
	BAP	Placebo	BAP	Placebo	BAP	Placebo		
Hair cortisol (µg/dL)	0.56 (0.24)	0.54 (0.21)	0.54 (0.33)	0.52 (0.30)	0.34 (0.29)	0.27 (0.23)	GLMM (log-transformed)	P=0.3751
Salivary cortisol (µg/dL)	0.09 (0.07)	0.10 (0.07)	0.10 (0.05)	0.09 (0.07)	0.11 (0.10)	0.12 (0.10)	GLMM (log-transformed)	P=0.0805t
Weight (kg)	52.37 (7.85)	52.89 (8.86)	69.77 (7.33)	69.94 (7.03)	96.67 (13.42)	96.25 (13.52)	GLMM (Box-Cox transformed)	P=0.9868
ADWG (kg)	0.62 (0.31)	0.66 (0.27)	0.73 (0.28)	0.68 (0.28)	0.90 (0.34)	0.76 (0.22)	GLMM	P=0.0295*
SDNN (ms)	20.80 (8.36)	22.52 (19.38)	24.77 (9.20)	22.82 (8.41)	16.82 (8.05)	15.00 (5.40)	GLMM (log-transformed)	P=0.1100
RMSSD (ms)	7.23 (2.84)	7.14 (2.79)	8.91 (3.06)	8.20 (2.81)	6.06 (2.74)	5.70 (1.82)	GLMM (log-transformed)	P=0.2717
Mean HR (bpm)	112.25 (21.29)	116.97 (19.16)	95.68 (17.55)	96.35 (15.69)	97.73 (18.43)	94.33 (19.6)	GLMM	P=0.0676t
Max HR (bpm)	149.35 (31.06)	154.98 (32.77)	135.19 (44.39)	127.98 (27.34)	126.90 (33.46)	116.32 (27.92)	GLMM (log-transformed)	P=0.0063**
Min HR (bpm)	92.75 (20.86)	96.10 (21.05)	79.73 (15.73)	81.24 (14.94)	85.37 (18.21)	83.83 (17.79)	GLMM	P=0.5279
SI	23.37 (7.36)	23.88 (7.37)	19.30 (6.53)	20.37 (6.17)	25.54 (8.42)	26.37 (8.04)	GLMM (log-transformed)	P=0.6874
LF/HF	9.29 (4.43)	9.57 (6.74)	11.61 (6.19)	6.27 (10.35)	12.37 (8.37)	12.34 (7.57)	GLMM (log-transformed)	P=0.8691
Lying (S)	1077.97 (98.13)	1083.02 (92.47)	1009.44 (70.72)	1031.45 (77.94)	965.77 (56.60)	947.02 (75.46)	Negative binomial	P=0.0068**
Standing (S)	362.29 (98.04)	356.96 (92.46)	430.56 (70.72)	408.55 (77.94)	474.23 (56.60)	492.98 (75.46)	Negative binomial	P=0.0405*
Steps	816.95 (406.82)	850.95 (396.42)	877.54 (330.84)	877.45 (319.31)	705.18 (294.81)	773.98 (324.97)	Negative binomial	P=0.1933
Motion index	4454.55 (2242.30)	4483.51 (1948.91)	5286.41 (1998.03)	5320.62 (1876.48)	4605.13 (1970.52)	5054.76 (2143.16)	GLMM	P=0.076t
Lying bouts	17.22 (4.80)	17.69 (4.59)	21.07 (4.92)	22.36 (4.69)	20.87 (5.39)	22.15 (5.58)	GLMM	P=0.3853

Probability symbols: * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001, t p >0.5-0.1

Table 3 Summary of results of the interaction effects of BAP vs. Placebo and social housing for 72 dairy calves. Descriptive statistics are expressed in mean and standard deviation.

Variable	Housed individually		Housed grouped		Model used	Probability
	BAP	Placebo	BAP	Placebo		
Hair cortisol (µg/dL)	0.56 (0.29)	0.53 (0.26)	0.36 (0.29)	0.30 (0.25)	GLMM (log-transformed)	P=0.2136
Salivary cortisol (µg/dL)	0.09 (0.07)	0.10 (0.07)	0.11 (0.09)	0.11 (0.09)	GLMM (log-transformed)	P=0.4833
Weight (kg)	57.39 (11.05)	57.88 (11.40)	97.28 (13.15)	97.93 (12.10)	GLMM (Box-Cox transformed)	P=0.8256
ADWG (kg)	0.66 (0.30)	0.66 (0.27)	0.90 (0.35)	0.76 (0.22)	GLMM	P=0.0528t
SDNN (ms)	22.19 (9.11)	23.10 (17.05)	18.11 (8.16)	15.97 (6.57)	GLMM (log-transformed)	P=0.0399*
RMSSD (ms)	7.81 (3.15)	7.55 (2.79)	6.51 (2.71)	6.06 (2.26)	GLMM (log-transformed)	P=0.4017
Mean HR (bpm)	107.54 (21.77)	112.12 (20.16)	97.64 (18.17)	93.78 (18.33)	GLMM	P=0.0064**
Max HR (bpm)	146.02 (37.30)	149.54 (32.66)	127.85 (33.08)	116.23 (26.83)	GLMM (log-transformed)	P=0.0003***
Min HR (bpm)	88.89 (20.68)	92.35 (20.72)	84.64 (17.46)	82.93 (16.95)	GLMM	P=0.1614
SI	22.21 (7.62)	22.61 (7.15)	24.21 (8.03)	25.54 (8.01)	GLMM (log-transformed)	P=0.3071
LF/HF	10.13 (5.68)	10.10 (6.71)	11.85 (7.31)	12.18 (7.24)	GLMM (log-transformed)	P=0.4654
Lying Time (seconds)	1060.93 (92.96)	1074.26 (87.48)	966.07 (54.23)	955.53 (74.98)	Negative binomial	P=0.0498*
Standing Time (seconds)	379.24 (92.88)	365.73 (87.47)	473.93 (54.23)	484.47 (74.98)	Negative binomial	P=0.1409
Step counts	745.85 (300.35)	787.40 (302.72)	857.38 (408.50)	889.43 (405.71)	Negative binomial	P=0.4654
Motion index	4776.77 (1956.01)	5069.15 (1992.16)	4727.51 (2289.73)	4807.16 (2113.65)	GLMM	P=0.1759
Lying bouts	21.30 (5.25)	22.35 (5.29)	17.55 (4.62)	18.60 (4.91)	GLMM	P=0.8757

Probability symbols: * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001, t p >0.5-0.1

3.1. Productivity Measures

3.2. 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, no significant treatment effect on ADG between calves receiving BAP (0.68 ± 0.32 kg) or placebo (0.67 ± 0.69 kg) was detected. 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 to weaned calves treated with placebo (p=0.04) as observed in Figure 8a. 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 to when housed individually (p=0.07) (Figure 8b). Furthermore, calves treated with BAP had higher ADG during the group housing phase compared to calves treated with the placebo (p=0.05) (Figure 8b).

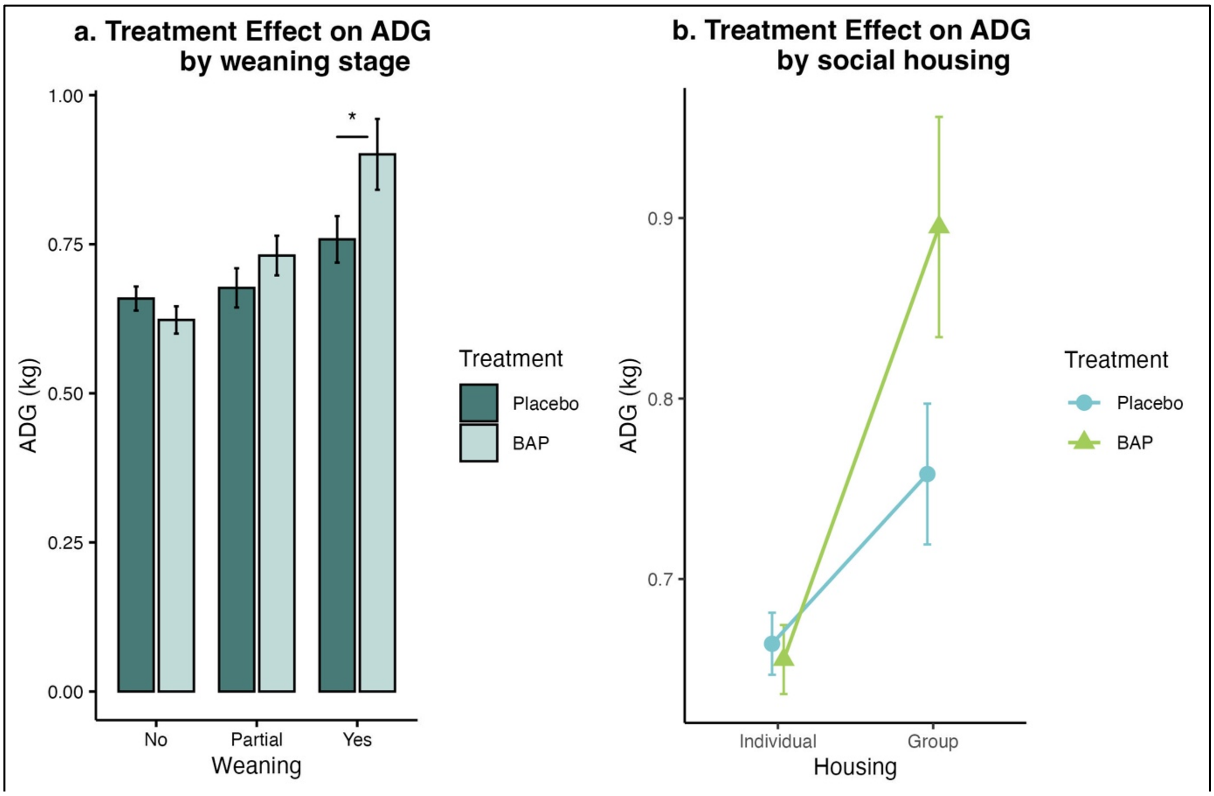

Figure 8 Average daily weight gain (ADG) by housing and weaning stage of 72 calves receiving the bovine appeasing pheromone (BAP) or placebo. Probability symbols: * p ≤ 0.05. Error bars represent ± SEM.

3.3. Physiological Measures

3.4. 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 to during weaning (p=0.03), whereas in the BAP group this difference was not significant (Figure 9). 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 to calves treated with BAP.

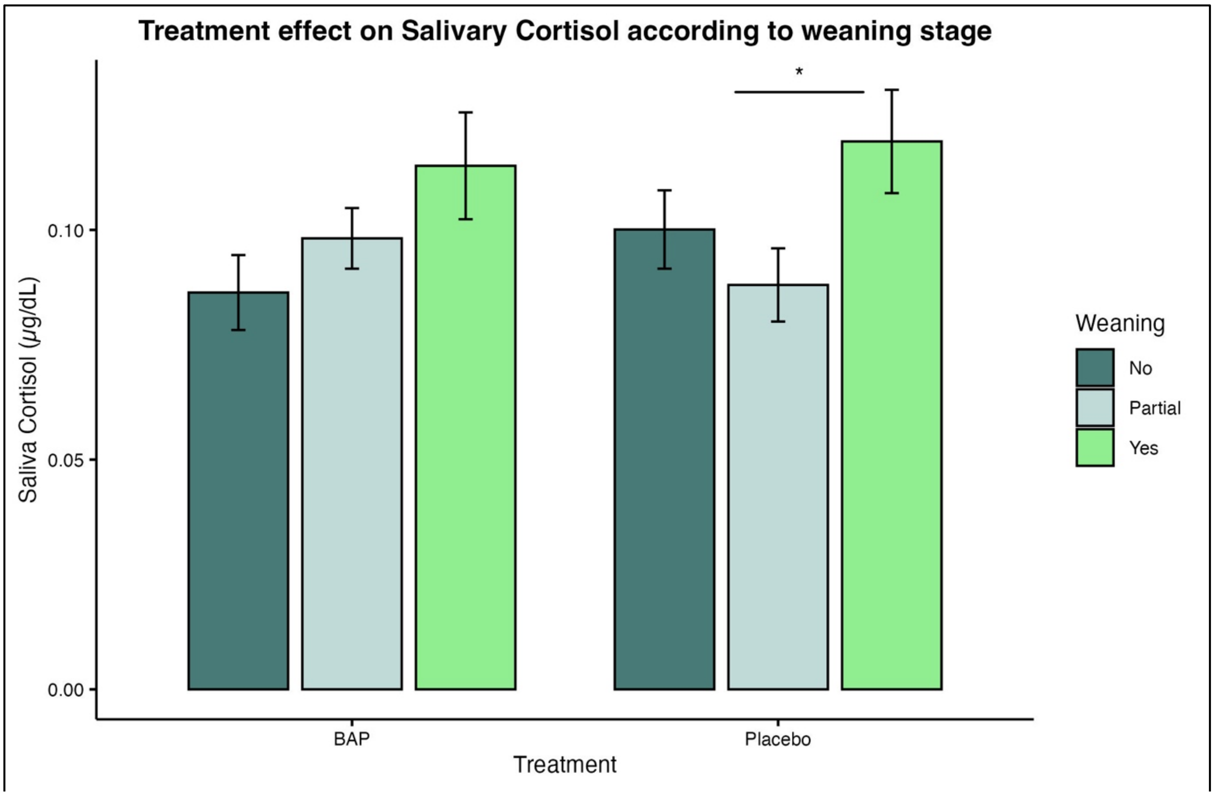

Figure 9 Saliva Cortisol of 72 calves receiving bovine appeasing pheromone (BAP) or placebo depending on Weaning stage and Age. Probability symbols: * p ≤ 0.05. Error bars represent ± SEM.

3.5. Hair Cortisol

No statistically significant differences in hair cortisol levels between treatment groups were observed, 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.

3.6. Standard deviation of beat to beat of normal sinus beats (SDNN)

No treatment effect on SDNN was seen between the treatments (BAP 20.64 ± 8.97 milliseconds (ms), placebo 20.22 ± 14.23 ms). When the treatment effect was analysed 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 to the group housing (p <0.01). This difference was not significant in calves receiving BAP (Figure 10). 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 seven (p=0.05), eight (p=0.02) and nine (p <0.01) weeks of age, compared to calves receiving placebo.

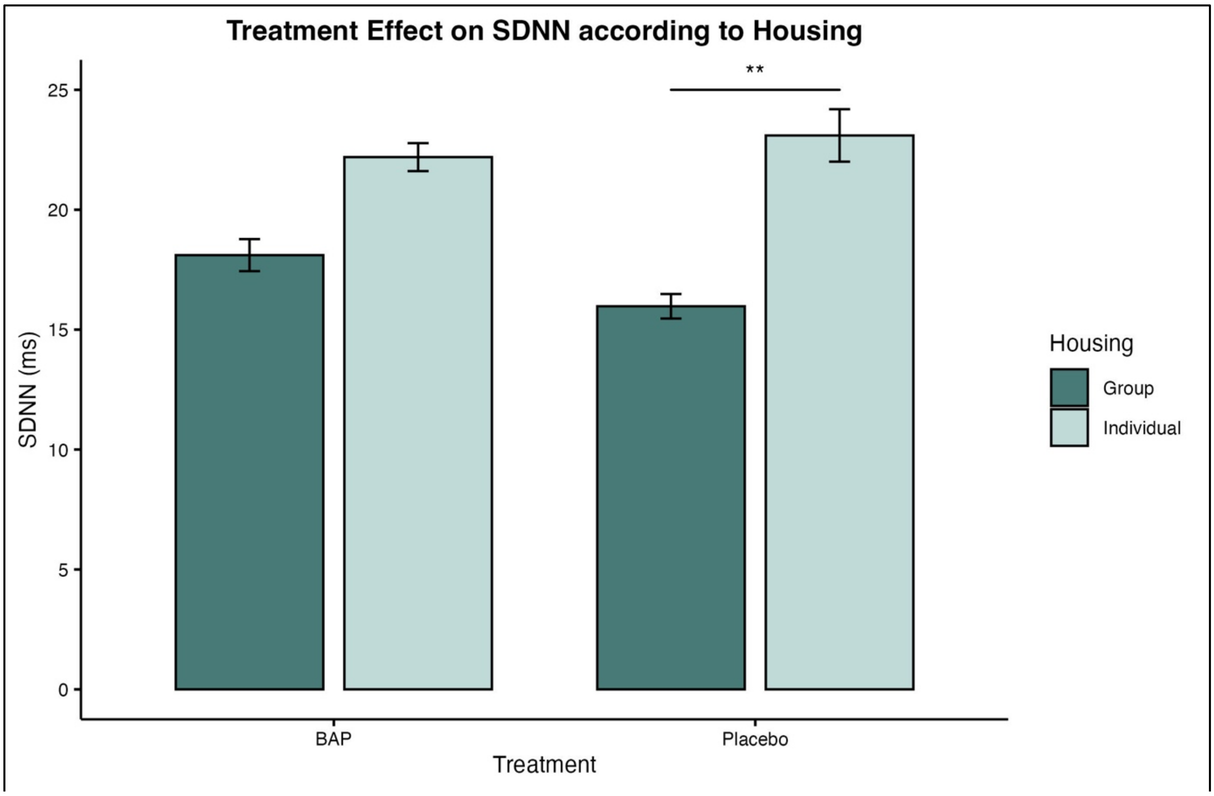

Figure 10 Treatment effect on the standard deviation of beat to beat of normal sinus beats (SDNN) of 72 heifers receiving bovine appeasing pheromone (BAP) or placebo, depending on social hopusing. Probability symbols: ** p ≤ 0.01. Error bars represent ± SEM.

3.7. RMSSD

No overall effect on RMSSD between calves treated with BAP (7.32 ± 3.05 ms) or placebo (6.95 ± 2.69 ms) was observed. Nevertheless, when the treatment effect on RMSSD was analysed 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 seven (p=0.05), eight (p=0.07) and nine (p=0.09) weeks of age (Figure 11).

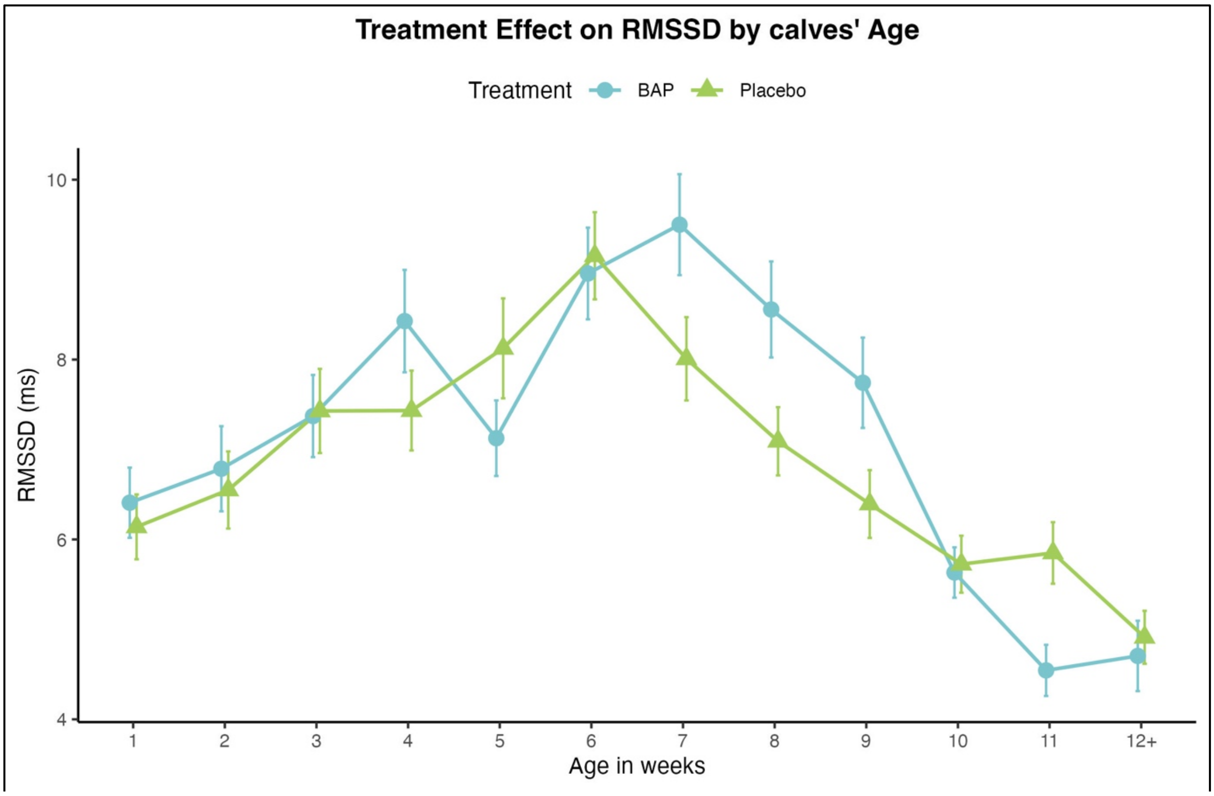

Figure 11 Treatment Effect of the bovine appeasing pheromone (BAP) and placebo on the root mean squares of successive differences (RMSSD) according to Age. Error bars represent ± SEM.

3.8. Mean Heart Rate

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

3.9. Maximum Heart Rate

A significant treatment x weaning interaction for maximum HR was detected (X^2=10.13, df=2, p <0.01). Calves administered with the placebo had a 21% higher maximum HR before weaning started compared to 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 12a). 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 analysed 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 to those receiving a placebo (p <0.01), this difference, however, was not seen when calves were housed in individual hutches (Figure 12b).

It was 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 nine (p <0.001) and ten (p=0.07) weeks of age compared to heifers in the placebo group.

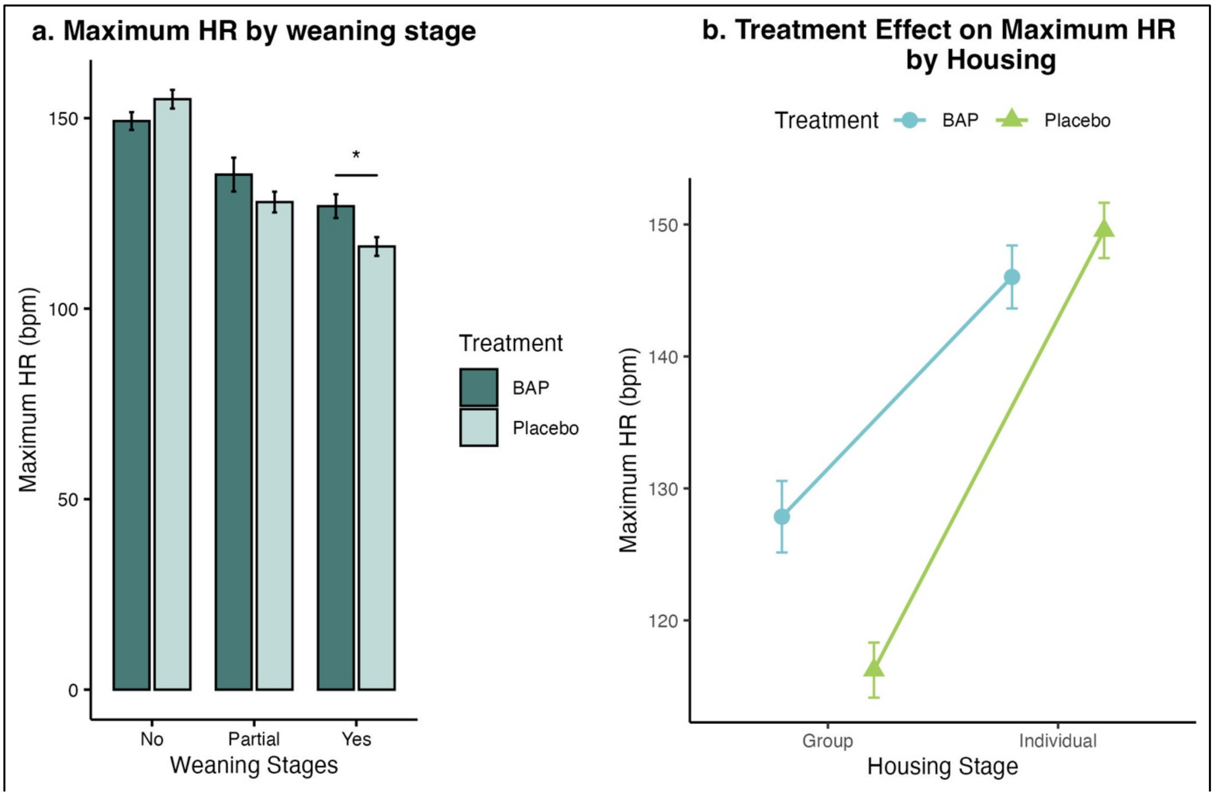

Figure 12 Treatment Effect on Maximum heart rate in 72 calves receiving bovine appeasing pheromone (BAP) or placebo according to Weaning Stage and Social Housing. Probability symbols: * p ≤ 0.05. Error bars represent ± SE

3.10. Stress Index

The Stress Index calculation has been described in Chapter 2, section 3.2. 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 to calves receiving BAP.

3.11. Behavioural Measures

3.12. 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 to 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 13a). When lying time was analysed 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 13b).

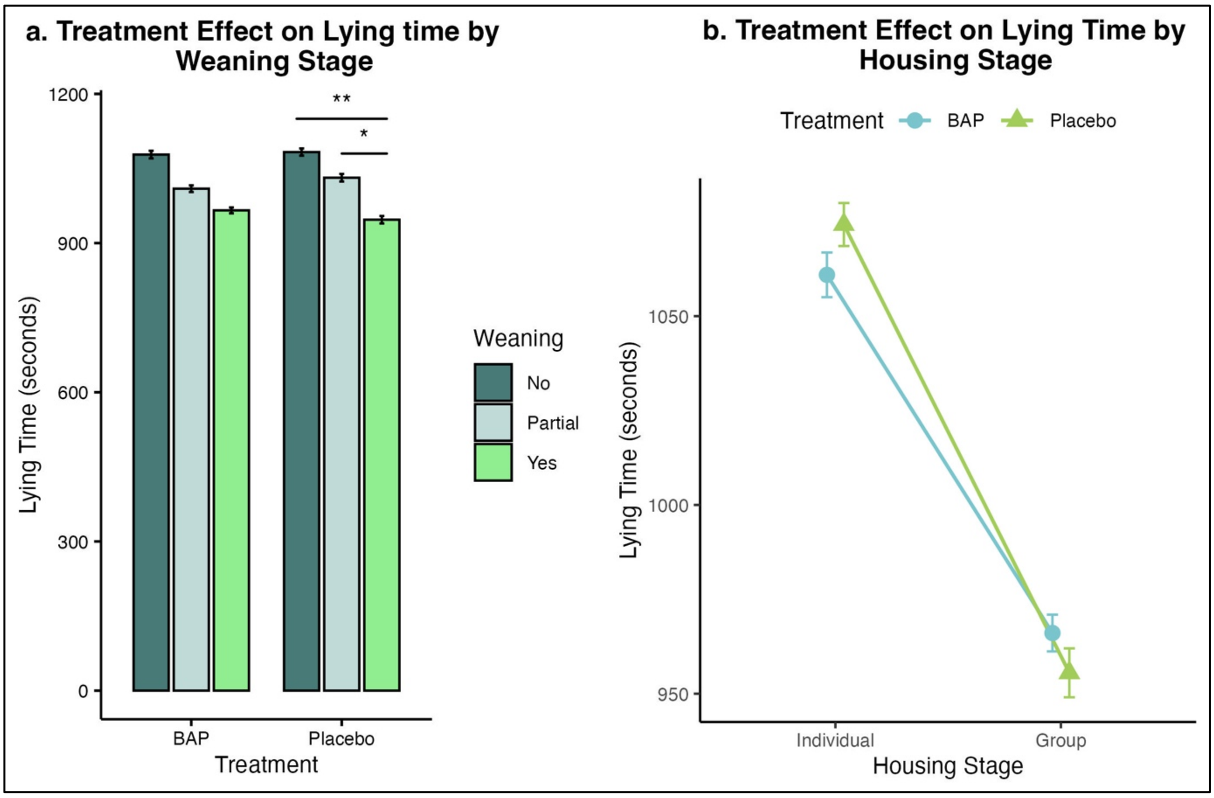

Figure 13 Lying Time of 72 dairy heifer receiving BAP or placebo by Weaning and Housing Stages. Probability symbols: * p ≤ 0.05, ** p ≤ 0.01. Error bars represent ± SE

4. 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, it is known that weaning has a detrimental effect on weight gain (De Passillé et al., 2011; Eckert et al., 2015). In this study, it was observed that completely weaned calves treated with BAP displayed a 0.15 kg higher ADG compared to 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 two months of life or during the milk stage (Kertz et al., 1998). In this study a tendency for BAP to boost ADG in group-housed calves (over eight weeks of age) was observed, 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 to 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 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 to 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. In the current study, HRV parameters (SDNN and RMSSD) remained higher in BAP-treated calves during the weaning and post-weaning stages, suggesting a reduced stress response. This contrasts with findings from Scoley et al. (2019b), who reported a decline in RMSSD post-weaning, even in group-housed calves, indicating increased physiological stress despite gradual weaning. This suggests the pheromone may help buffer calves against isolation stress and weaning stress.

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 to 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.

Behavioural 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-hour 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 observed for lying time suggests that resting time was significantly higher in placebo treated calves before and during weaning, compared to their post weaning values. This phenomenon however was not seen in calves treated with the appeasing pheromone. This finding may imply that weaning stress was higher in placebo calves compared to 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 to 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 behaviours are indicative of a positive coping response and suggest that BAP may mitigate stress during the weaning process, thereby promoting more adaptive behaviour in calves. This aligns with our findings, where we observed similar trends in stress reduction and improved performance in calves treated with BAP. These behavioural 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 behavioural 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 signalling 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 production performance in dairy calves.

5. Strengths, weaknesses, and future recommendations

The sample size was relatively robust as a formal sample size calculation was performed before data collection to ensure there would be sufficient power to find a treatment effect for variables related to performance, physiology, and behaviour. However, 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). Adjustment for potential confounding factors such as weather conditions and location was carried out in the analysis; 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.

Individual milk replacer and solid feed intake were not specifically measured during the individual housing period. Whilst 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, it was 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 two 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 behavioural indicators such as play, which has been used in previous studies as a behavioural 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.

6. 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 to 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, behavioural 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.

CHAPTER 4. EFFECTS OF A SYNTHETIC ANALOGUE OF THE BOVINE APPEASING PHEROMONE ON DAIRY CALVES DURING EPISODES OF DISEASE: DOES IT IMPROVE RESILIENCE?

1. Introduction

The early life stage of dairy calves, from birth through weaning, is a critical period that significantly influences their future health, productivity, and overall welfare (Bach, 2011; A. Costa et al., 2021). During this phase, young calves are particularly vulnerable to a range of infectious diseases that can lead to substantial morbidity and mortality due to the immaturity of their immune systems and the physiologic stress of transitioning from intrauterine to extrauterine life (Hulbert and Moisá, 2016). The welfare of these animals is closely tied to their ability to withstand and recover from these disease challenges (Doeschl-Wilson et al., 2021).

Acute neonatal diarrhoea and pneumonia are the most common health challenges faced by preweaned dairy calves (McGuirk, 2008; Urie et al., 2018b). In the UK, a cohort study by (Johnson et al., 2021c), diarrhoea was diagnosed in 48.2% of calves during their first nine weeks of life, with the highest incidence observed in the second week, with 28.2% calves affected. The overall incidence rate was 7.8 cases per 100 calf-weeks at risk. Bovine respiratory disease (BRD) was observed in 45.9% of calves within the same nine-week period with an overall incidence rate of 10.1 cases per 100 calf-weeks at risk. Enteritis, often caused by pathogens such as Escherichia coli, rotavirus, and Cryptosporidium, is the leading cause of mortality in calves less than 30 days old (Lorenz et al., 2011b; Yimer et al., 2015). Pneumonia, on the other hand, tends to emerge as a predominant issue in calves older than one month (Cummings et al., 2022).

Acute neonatal diarrhoea in calves presents with a range of clinical signs that can vary in severity depending on the underlying cause, duration, and calf's immune status. One of the most noticeable signs is loose or watery faeces, with colour and consistency changes depending on the causative agent. Dehydration is another common sign, evident through sunken eyes, dry gums, and reduced skin elasticity. As dehydration worsens, calves may become lethargic and exhibit cold extremities due to impaired circulation. Electrolyte imbalances are also frequently observed, particularly involving sodium, potassium, and chloride loss. This imbalance can lead to metabolic acidosis, with affected calves showing signs such as muscle weakness, staggering, or even recumbency in more severe cases (Meganck et al., 2014; Todd et al., 2010). A reduced appetite is also a consistent sign, with calves frequently displaying a weakened or absent suckling reflex. Weight loss and poor growth are commonly seen in calves suffering from prolonged or severe diarrhoea due to nutrient malabsorption and increased fluid loss. Alongside these symptoms, calves may show an increased heart rate and respiratory rate, abdominal discomfort may also be present, with calves showing behaviours like kicking at their abdomen, bloating, or general restlessness (Todd et al., 2010).

Clinical signs of calf pneumonia often include increased respiratory rates, elevated rectal temperatures, nasal and ocular discharge, coughing, and signs of depression or lethargy. Early detection is crucial, as initial symptoms may be subtle, such as a slight reduction in feed intake or general dullness. As the disease progresses, more pronounced signs like laboured breathing and purulent nasal discharge may become evident. Implementing regular health monitoring and employing standardized respiratory scoring systems can aid in the timely identification and management of BRD in calves (McGuirk and Peek, 2014).

Apart from immediate mortality, calves that survive severe disease episodes often experience impaired growth, delayed weaning, and reduced future milk production, which can have lasting economic consequences for dairy operations (Costa et al., 2021; Stanton et al., 2012). The cost of managing disease in young female calves includes not only the direct expenses of veterinary care and medications but also the long-term costs associated with reduced productivity and the need for additional replacements (Buczinski et al., 2021; Overton, 2020). Estimates suggest that the economic burden of disease during the preweaning period can range from USD $0.50–687.80 per animal, with overall costs to the operation potentially reaching thousands of dollars annually (Richter et al., 2017). Whilst specific economic data from the UK are limited, studies from other European countries provide insight into the potential financial impact. For instance, a study involving 57 dairy farms across France, Belgium, and the Netherlands reported an average annual loss of €3,000 per farm due to calf diarrhoea, equating to approximately €94 per affected calf. The primary cost components included additional labour (42%), health-related expenses (35.6%), and losses from mortality (22.4%) (Roblin et al., 2023).

The negative impacts of disease in preweaned dairy calves extend beyond individual welfare and economic concerns; they also pose significant challenges to the sustainability of dairy production systems. Disease-related growth setbacks and reduced performance compromise the long-term productivity of affected animals, leading to higher replacement rates and increased resource use per unit of milk produced (Buczinski et al., 2021; Stanton et al., 2012). Calves that experience early-life illness, particularly neonatal diarrhoea and pneumonia, often exhibit lower average daily gains (ADG), delayed first calving, and reduced milk yield in first and subsequent lactations (Dunn et al., 2018; Virtala et al., 1996). These inefficiencies increase the environmental footprint of dairy production, as diseased calves require more feed, veterinary interventions, and management resources to reach maturity, ultimately reducing the efficiency of nutrient conversion and increasing greenhouse gas emissions per unit of milk output (Džermeikaitė et al., 2024; Overton, 2020). Furthermore, the higher mortality and culling rates associated with disease lead to increased replacement heifer rearing, which is resource-intensive and costly (Overton and Dhuyvetter, 2020). Reducing disease incidence and improving resilience in young dairy calves is, therefore, not only critical for enhancing animal welfare and farm profitability but also for improving the overall sustainability of the dairy industry (Buczinski et al., 2021).

Preweaning management is critical for illness prevention (Lorenz et al., 2011c). Passive immunity, the main defence against early-life illnesses, requires proper colostrum management (Godden et al., 2019). Disease prevention also depends on housing conditions including bedding, ventilation, and space allocation (Gorden and Plummer, 2010; Lorenz et al., 2011a). Successful illness management requires timely vaccines, consistent monitoring, and early intervention (Lorenz et al., 2011c; Maier et al., 2022). However, despite the well-documented risks and management strategies, many dairy operations continue to struggle with high morbidity and mortality rates in their preweaned dairy calves (Johnson et al., 2021c; Su et al., 2023). This persistence of disease emphasizes the need for a more comprehensive understanding of the factors that contribute to disease susceptibility and the effectiveness of various intervention and mitigation strategies (Arlington Headley et al., 2024; Ollivett, 2020; Robi et al., 2024). It also underscores the importance of adopting a welfare-centric approach to dairy calf management, where the health and well-being of the animal are prioritized. This approach may involve practices such as providing sufficient high-quality colostrum shortly after birth to support immune function, ensuring clean and comfortable bedding, minimizing handling stress through gentle and consistent interactions, and promoting social contact by housing calves in pairs or small groups where appropriate. Additionally, regular health monitoring, prompt treatment of illnesses, and environmental enrichment can further support calf welfare and long-term productivity (Godden et al., 2019; McFarland et al., 2024).

Environmental enrichment practices have been shown to positively impact the welfare of dairy calves. Research indicates that enrichment can enhance overall health and potentially aid in recovery of disease (Colditz et al., 2024; Veissier et al., 2024; Wells, 2009). For instance, a study by (Occhiuto et al., 2025) demonstrated that providing stationary brushes to dairy calves increased their activity levels and play behaviour, suggesting improved welfare. Calves with access to brushes exhibited more frequent grooming and interactive behaviours, which are indicative of positive health outcomes. These behaviours can be particularly beneficial during recovery from illness, as increased activity and engagement may support immune function and overall vitality. Additionally, a review on environmental enrichment for indoor-housed dairy cows and calves highlighted those enrichments, including social and physical stimuli, can improve biological functioning and help animals cope with environmental stressors. Implementing such enrichments may reduce frustration and promote positive affective states, which are crucial for the recovery and overall well-being of diseased calves (Mandel et al., 2016).

The effect of environmental enrichment in the form of BAP on the welfare of diseased calves has not been deeply studied. Hervet et al. (2021) investigated the effects of bovine appeasing pheromones on stress, respiratory problems, and immunological transcript expression in young bulls during fattening have also been studied. This investigation showed that pheromone therapy increased respiratory clinical symptoms on Day 8 but decreased them by Day 30 in bovines. On Day 8, the pheromone-treated group had increased interleukin 8 transcripts, indicating an improved immunological response. In Vieira et al's. (2023) experiment, BAP therapy dramatically boosted calves' immunological responses to parainfluenza-3 and BVDV-1 vaccinations. BAP improved humoral immunity against these viruses but not infectious bovine rhinotracheitis or BVDV-2. The study by Angeli et al. (2020) mentioned in previous chapters, examined how the pheromone affected performance, illness, and pharmaceutical costs in Dairy Gir x Holstein female calves before weaning from milk. The treatment reduced pharmaceutical expenses but did not affect illness incidence. The ADG for ill BAP-treated animals was identical to that of their healthy counterparts, unlike the control group.

As current knowledge on the effects of BAP on disease incidence and its welfare impact shows potential positive outcomes, the research on the topic is still limited. Therefore, the aim of this study, is to provide an in-depth analysis of the pheromone's effects on the welfare of diseased dairy calves, and the potential benefits of the use of appeasing pheromones on overall welfare, production, neuroendocrine response and behaviour of diseased calves when applied in a commercial setting. It was hypothesized that calves receiving the pheromone would display lower distress levels during bouts of disease demonstrated by greater weight gain, less activation of the neuroendocrine system (higher heart rate variability), and reduced impact on activity levels, compared to calves receiving a placebo.

2. Materials and Methods

The experiment procedure for this randomized controlled trial has been described in Chapter 2. The present study has focused specifically on bouts of disease among the study calves. The experimental 33 female dairy calves were housed 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).

2.1. Calves, experimental design, and treatments

Calves' management and treatments in this study have been described in Chapter 2 section 2 and Chapter 3 section 2.1.

2.2. Disease definition, monitoring and episode recording

The Calf Health Scoring Scale from the University of Wisconsin-Madison was utilized to assess clinical signs of disease (Figure 14). Disease episodes were defined using a threshold score of ≥1 on the scale, or if the calf was given any medication by the farm technicians to treat clinical symptoms not listed on the scale (e.g., lameness). If a calf exhibited more than one episode of disease with at least a week between positive scores, each instance was treated as a separate event. On the other hand, if there was less than a week time between scores, the events were considered as belonging to the same disease episode.

Blood samples were taken by veterinary surgeons from random calves, 48 hours after birth to measure total serum protein (TSP) values as a metric of passive immune transfer from the colostrum feeding, following farm established protocols and not for the sole purpose of this study.

Calf Health Scoring Criteria			
0	1	2	3
Rectal temperature			
100-100.9	101-101.9	102-102.9	≥103
Cough			
None	Induce single cough	Induced repeated coughs or occasional spontaneous cough	Repeated spontaneous coughs
Nasal discharge			
Normal serous discharge	Small amount of unilateral cloudy discharge	Bilateral, cloudy or excessive mucus discharge	Copious bilateral mucopurulent discharge
Eye scores			
Normal	Small amount of ocular discharge	Moderate amount of bilateral discharge	Heavy ocular discharge
Ear scores			
Normal	Ear flick or head shake	Slight unilateral droop	Head tilt or bilateral droop
Fecal scores			
Normal	Semi-formed, pasty	Loose, but stays on top of bedding	Watery, sifts through bedding

Figure 14 The Calf Health Scoring Scale from the University of Wisconsin-Madison. Source: https://fyi.extension.wisc.edu/heifermgmt/files/2015/02/calf_health_scoring_chart.pdf. Accessed: December 2021.

2.3. Data collection and welfare measurements

In this study, data collection focused on heart rate variability (HRV), ADG, and activity levels, with measurements taken weekly from birth through weaning. To comprehensively assess the impact of disease episodes on calf welfare, data were systematically collected at multiple critical time points: two weeks and one week prior to the onset of clinical signs, on the day of the event (specifically for activity levels), and one week and two weeks post-event. These specific intervals were deliberately chosen to capture a clear trajectory of the calves' baseline physiological and behavioural state, their immediate response to the disease, and their subsequent recovery phase. This methodological approach is designed to provide a detailed temporal profile of how disease episodes influence physiological parameters, behaviour, and overall productivity, enabling an in-depth analysis of both the immediate and lingering effects of health challenges on calf welfare (Bowen et al., 2021; Duthie et al., 2021).

2.4. Measures of productivity

Calves were weighed at birth and weekly until weaning using a walk-on scale, once before being moved to group housing, with a final weight recorded at the conclusion of the experiment prior to their transfer to the young herd stock. ADG was determined by calculating the difference between two consecutive weights and dividing it by the number of days between the measurements.

2.5. Collection and processing of HRV

Physiological stress was assessed by examining the activation of the neuroendocrine system through heart rate variability (HRV) analyses conducted before and after each clinical disease episode (von Borell et al., 2007). Reduced HRV indicates heightened sympathetic tone and has been associated with stress in both human and nonhuman animals (Clapp et al., 2015; Kovács et al., 2014), including dairy calves (Jimenez et al., 2019a; Kovács et al., 2014). Portable heart rate monitors (HRM) from Polar were utilized to gather HRV measurements and secured around each calf's thorax with a Polar equine belt for a duration of 24 hours, commencing in the second week of life and continuing weekly until the conclusion of the experiment. Data processing has been described in Chapter 2 section 3.2.

2.6. Behavioural metrics and data analysis

Triaxial accelerometers (IDS i-QUBE, Peacock Technology) were affixed to one hind leg of the calves immediately post-birth. The accelerometers automatically uploaded raw data into the CowAlert 2.7.1 Software (Ice Robotics Ltd) for analysis. Daily data on average lying time, lying bouts, step counts, and Motion index (a measure of animal activity calculated by the software) were collected for each calf until the conclusion of the experiment.

2.7. Statistical Analysis

Prior to the enrollment of calves, a sample size was calculated using G*Power Software based upon effect size estimates on ADG and cortisol levels from previous studies (Angeli et al., 2020; Schubach et al., 2020). All subsequent statistical analyses were performed using R software (2024.04.2+764 by Posit Software, PBC).

To make sure any differences between groups were not due to variation in passive immune transfer form colostrum feeding, TSP values were analysed for both treatments using a linear regression model.

Three primary measures of disease occurrence were used to assess the health outcomes in calves: Cumulative Disease Incidence (CDI), Disease Incidence Rate (DIR), and Mortality Rate (MR) (Alemu et al., 2022; Donlon et al., 2023). CDI was calculated to estimate the proportion of calves that experience at least one episode of disease during the study period. This measure provided insight into the overall disease burden within the population over time. CDI was calculated using the following formula:

$$\frac{Number\ of\ calves\ that\ experienced\ at\ least\ one\ episode\ of\ disease\ during\ the\ study\ period}{Total\ number\ of\ calves\ at\ the\ start\ of\ the\ study\ period}$$

In the above calculation, each calf was counted only once, regardless of whether it experienced multiple episodes of the disease.

The DIR was calculated to measure the frequency of new disease episodes in the population over time, accounting for the total number of episodes and the time each calf was at risk. DIR was calculated using the following formula:

$$\frac{Total\ number\ of\ new\ disease\ episodes\ during\ the\ study\ period}{Total\ time\ at\ risk\ (animal\ months)}$$

Each calf was followed for a period of approximately of 3 months, and the total time at risk was calculated as the number of calves (n = 72) multiplied by the follow-up time (3 months), yielding 216 calf months.

The MR was calculated to assess the rate of calf deaths during the study period. This measure reflected the number of deaths per unit of time and provided insight into calf survival.

MR was calculated using the following formula:

$$\frac{Number\ of\ calf\ deaths\ during\ the\ study\ period}{Total\ time\ at\ risk\ (calf\ months)}$$

The number of deaths was recorded for each calf, with the total observed period amounting 216 calf-months, based on a 3-month follow-up for each of the 72 calves. The mortality rate was expressed as the number of deaths per calf month.

By reporting both CDI and DIR, this study aimed to capture both the overall proportion of calves affected and the rate at which disease occurs, including recurrent episodes. CDI provides a snapshot of disease burden, while DIR offers a dynamic view of disease frequency over time, accounting for repeated illnesses in the same individuals.

To determine whether there were significant differences in CDI and DIR between treatment groups a chi-squared test was used to compare the CDI, and a Poisson regression model was used to compare the DIR between treatment groups, adjusting for the total time at risk for each calf.

For the primary outcome measures HRV (RMSSD, SDNN and SI), ADW and for activity levels, total lying time in second, motion index, step counts and the number of lying bouts, the primary experimental unit was the individual calf episode, with repeated measures taken from the same calf over different episodes and time points. This structure accounted for within-subject correlation and allowed for the assessment of individual and collective responses to disease episodes over time. Descriptive statistics were calculated for each outcome variable using the 'psych' package in R.

Data analysis was performed fitting linear mixed effects models to handle the possible interactions and repeated measures of the data, using the 'lme4' package. Initial models for each outcome included treatment as a fixed effect and calf, season, location, calf's age, sample periods, social housing (individual vs. group) and weaning stateg as random effects to control for potential confounding variables. In a second step, the period of measurement related to the disease event and season effects were considered as fixed effects to analyse any interaction effects.

Model diagnostics included residual plots, Q-Q plots for normality assessment, and tests for homoscedasticity (Breusch-Pagan test 'performance' package). The Shapiro-Wilk and Kolmogorov-Smirnov tests were used to assess the normality of residuals, and in cases where model assumptions were violated, data transformations or alternative distributions (e.g., Poisson, negative binomial for count data) were considered. For the main effects of treatment, ANOVA Type III Wald chi-square tests were performed. Post hoc analyses using least squares means (LSMeans) with multiple testing adjustments were conducted to explore specific group differences when significant main effects or interactions were detected. For some outcome variables such as MI and step counts, we did not find a global treatment effect or any significant interactions; however, given the complexity of linear mixed-effects models and their tendency to be conservative when detecting fixed effects in the presence of small or non-significant random effect variances, we further conducted multiple comparisons using the Genz and Bretz method (Bretz et al., 2001). This post-hoc analysis allowed for a more focused investigation of specific time-period contrasts and seasons.

This approach is consistent with the findings by (Chen et al., 2017; Matuschek et al., 2017a), which suggest that overly complex maximal models, while effective at controlling Type I error, may reduce statistical power and obscure true effects when random effects are small. To address this, a post-hoc analyses was conducted as a complementary approach to the linear mixed model. This method allowed for greater sensitivity in detecting treatment effects over time while maintaining appropriate control for Type I error through multiple comparison adjustments.

Results are reported as significant at $p < 0.05$, and tendencies when P values were between ≥ 0.05 and ≤ 0.10.

3. Results

3.1. Disease Incidence

Mean TSP values were not significantly different between the two treatments (BAP 5.84 ± 0.49 gr/dL, placebo 5.97 ± 0.72 gr/dL, p = 0.61).

Table 4 summarizes the CDI and DIR by disease, age at symptom onset and treatment.

There were a total of 44 disease episodes observed across 33 animals, with an overall CDI of 45.83% and a DIR of 0.68%. CDI was not significantly different in calves receiving BAP compared to calves receiving placebo (BAP 25.00% vs Placebo 20.80%, X^2=0.27, df=1, p=0.60). Similarly, the were no significant differences in the DIR of both treatments (BAP 0.37% vs Placebo 0.31%, p=1.00). The overall mortality rate was 0.93%, with one fatality per group. Acute neonatal diarrhoea was the most common cause of disease in this study affecting calves at an average of 11.90 ± 6.57 days old. Pneumonia was the second main cause affecting calves at 40.40 ± 18.10 days old.

Table 4 Summary of the main causes of disease in dairy calves during the first months of life with their specific cumulative disease incidence, treatment and age at onset of symptoms. Descriptive statistics are expressed in mean and standard deviation.

Symptom/Disease	CDI	DIR	Age	BAP		Placebo	
				Cases	Age	Cases	Age
Navel Infection	2.78%	0.031%	27.00 (7.07)	1	22.00	1	32.00
Gastrointestinal infections	26.40%	0.29%	11.95 (6.57)	11	11.64 (5.30)	8	12.38 (8.40)
Others (Fever, lameness, anorexia)	19.40%	0.22%	21.07 (17.50)	7	14.14 (6.47)	7	28.00 (22.50)
Respiratory infections	20.5%	0.14%	40.44 (18.10)	5	42.60 (18.60)	4	37.80 (19.90)

Tables 5 and 6 summarize the effects of both treatments on the different indicators of welfare metrics.

3.2. Productivity Measures

Overall, no significant treatment effect on ADG between diseased calves receiving BAP (0.52 ± 0.36 kg) or placebo (0.63 ± 0.67 kg) was observed. Nor did any significant interactions between treatment and other confounding factors such as season or location were detected.

3.3. Physiological Measures

3.4. SDNN

A treatment effect on SDNN was seen (X^2=6.34, df=1, p=0.01), with ill female calves receiving BAP having higher SDNN values (18.74 ± 8 ms) than placebo-treated ones (16.11 ± 5.91 ms) (Figure 15a). We observed a significant interaction between treatment and the period of measurement (X2=7.94, df=3, p<0.05). Specifically, calves treated with BAP exhibited higher SDNN values two weeks before the onset of clinical symptoms compared to those treated with a placebo (p=0.01). Furthermore, two weeks after the initial symptoms of disease appeared, BAP-treated also calves displayed higher SDNN values than their placebo counterparts (p<0.01), as shown in Figure 15c.

Calves treated with the placebo had significantly lower SDNN levels during the winter months compared to the BAP treated calves (p=0.0.1) (Figure 15b). Furthermore, within the placebo treatment group, we observed that SDNN values tended to be higher in winter compared to spring (p=0.08) and autumn (p=0.06), but significantly lower compared to the summer months (p=0.04). On the other hand, the SDNN levels on the BAP group remained constant throughout all seasons.

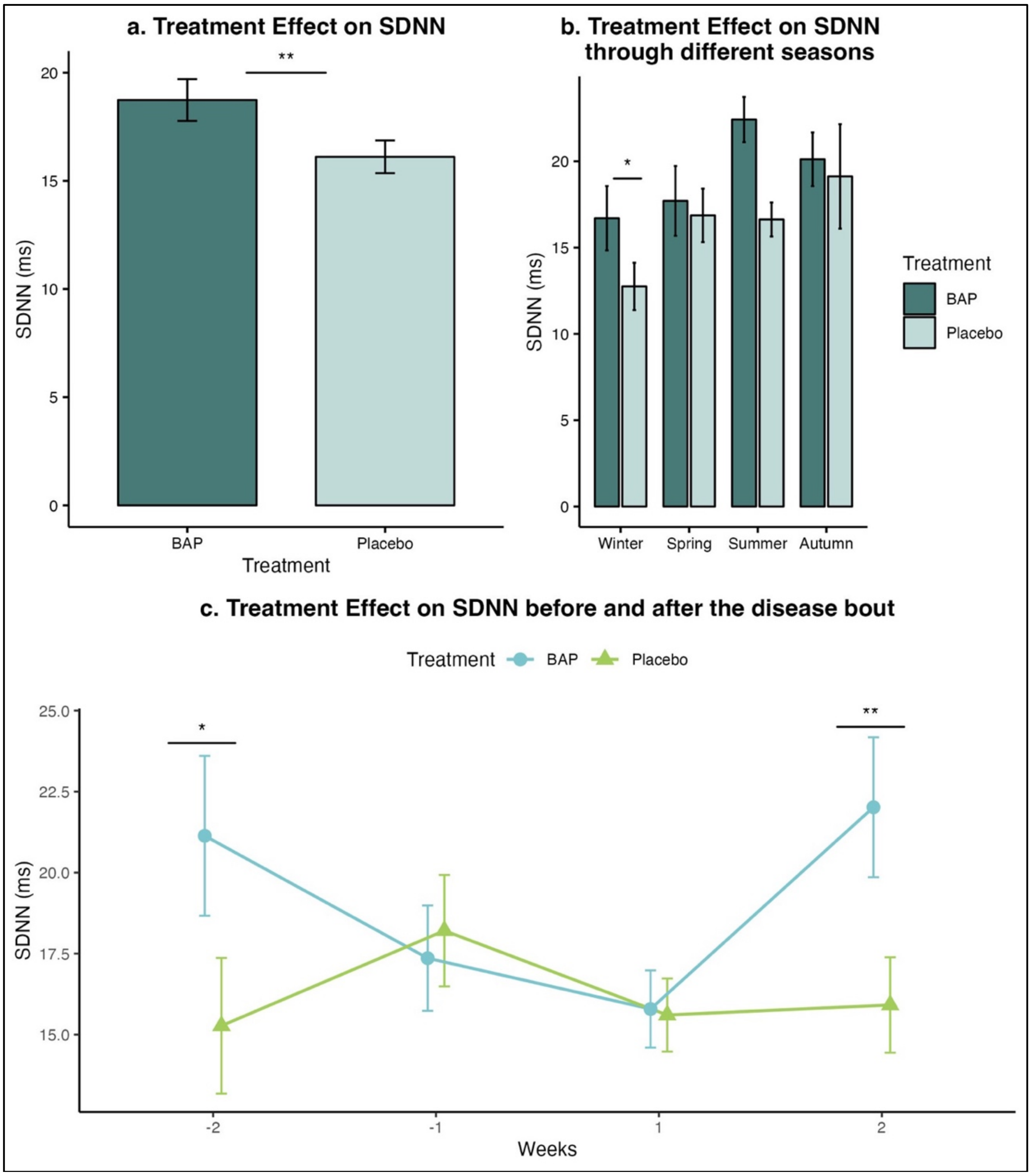

Figure 15 Treatment effect on the standard deviation of beat to beat of normal sinus beats (SDNN) of dairy calves during disease bouts, receiving bovine appeasing pheromone (BAP) or placebo. Probability symbols: * p<0.05, ** p ≤ 0.01. Error bars represent ± SEM.

RMSSD

An overall treatment effect on RMSSD (X^2=4.50, df=1, p=0.04) was detected, with diseased calves treated with BAP having higher RMSSD (6.63 ± 2.42 ms) compared to those treated with placebo (5.81 ± 1.93 ms) (Figure 16a). This difference was more noticeable two weeks before (p=0.02) and two weeks after (p=0.09) the onset of clinical symptoms (Figure 16c). A treatment x season interaction was also observed for RMSSD (X^2=7.92, df=3, p=0.05); calves treated with the pheromone had higher RMSSD compared to those receiving a placebo during the winter (p=0.02) (Figure 16b) and tended to have higher values in the spring (p=0.10). We also observed a variation of RMSSD in the placebo group with levels being lower during the winter compared to autumn (p=0.04), which was not observed in the calves receiving BAP.

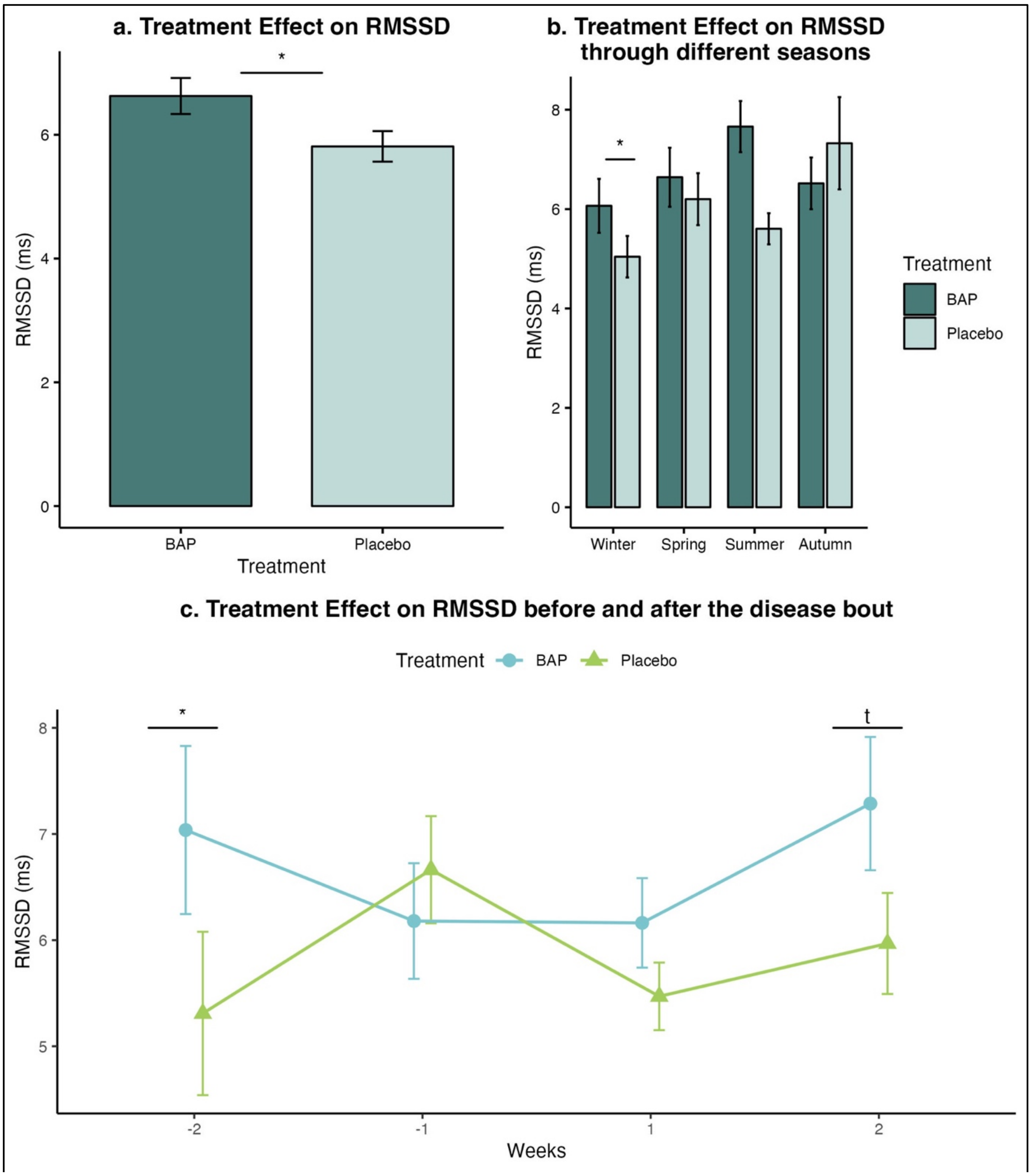

Figure 16 Treatment effect on the root mean squares of successive differences (RMSSD) of dairy calves during disease bouts, receiving bovine appeasing pheromone (BAP) or placebo. Probability symbols: * p<0.05, t p>0.05-0.10. Error bars represent ± SEM.

3.5. Mean, Maximum and Minimum heart rate

A tendency was observed in mean HR for the overall treatment effect (X^2=3.17, df=1, p=0.08). Calves in the placebo group tended to have higher mean HR (115.57 ± 23.79 bpm) than calves in the BAP group (109.68 ± 26.56) as observed in Figure 17a. This difference between treatments tended to be more noticeable a week after the onset of disease symptoms (BAP 107.27 ± 17.18, Placebo; 117.95 ± 24.76, p=0.08) (Figure 17b). No treatment effect on minimum and maximum HR, nor any interactions were observed.

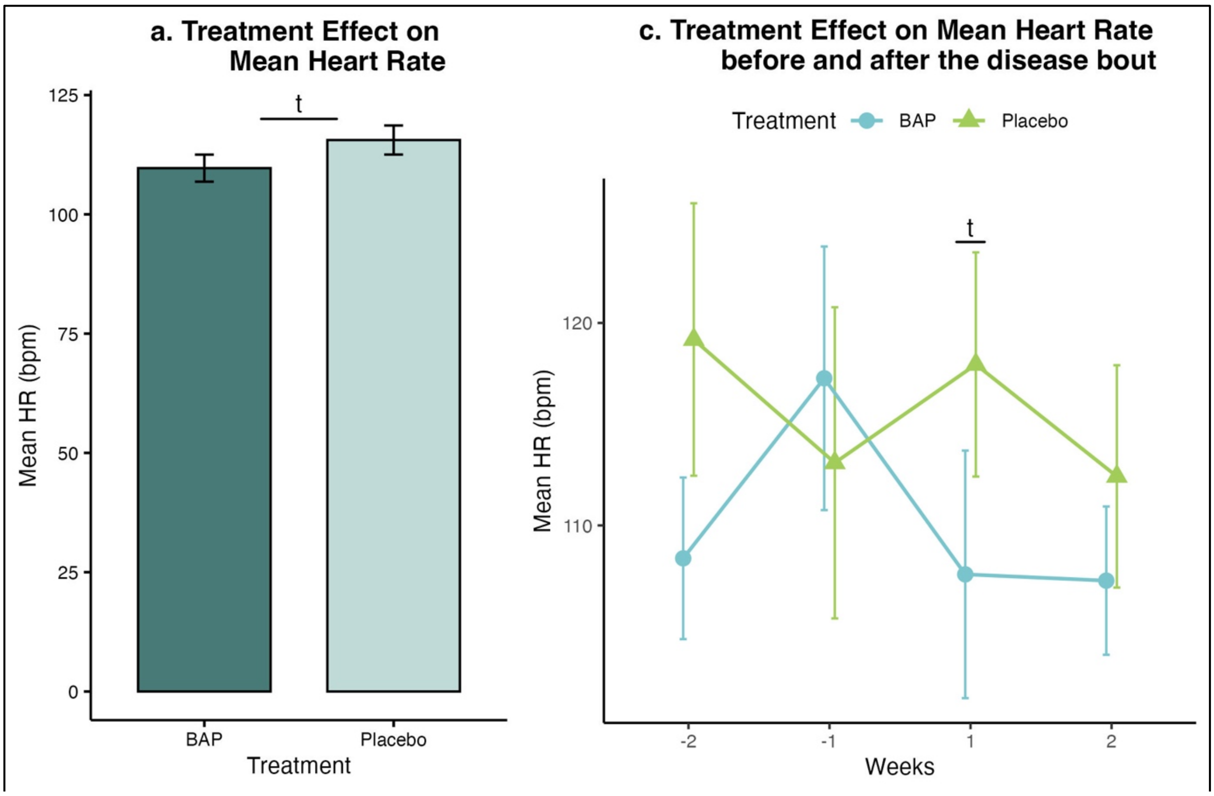

Figure 17 Treatment effect on the mean heart rate (HR) of dairy calves during disease bouts, receiving bovine appeasing pheromone (BAP) or placebo. Probability symbols: t p>0.05-0.10. Error bars represent ± SEM.

3.6. Stress Index

The global treatment effect was significant for SI (X^2=6.03, df=1, p=0.01), with diseased calves treated with the placebo displaying a higher value of SI (27.49 ± 7.18) compared to heifers receiving BAP (24.73 ± 7.68) (Figure 18a). The impact of the BAP treatment on SI was especially pronounced two weeks before the onset of clinical symptoms (p=0.02) and two weeks after (p=0.03) (Figure 18c). There was also a tendency observed in the BAP group with SI being higher in the week after disease symptoms appeared compared to two weeks after (p=0.08); this tendency was not seen in the placebo group where SI remained constant through time (Figure 18c).

A treatment x season interaction was also observed for SI (X^2=13.02, df=3, p=<0.01). During the winter months the SI was significantly higher in calves treated with a placebo compared to those treated with BAP (p=<0.01) (Figure 18b). Similarly, within the placebo treatment, SI tended to be higher during the winter months compared to other seasons (summer p=0.08, spring p=0.07, autumn p=0.02); this SI variation was not observed in the calves treated with BAP.

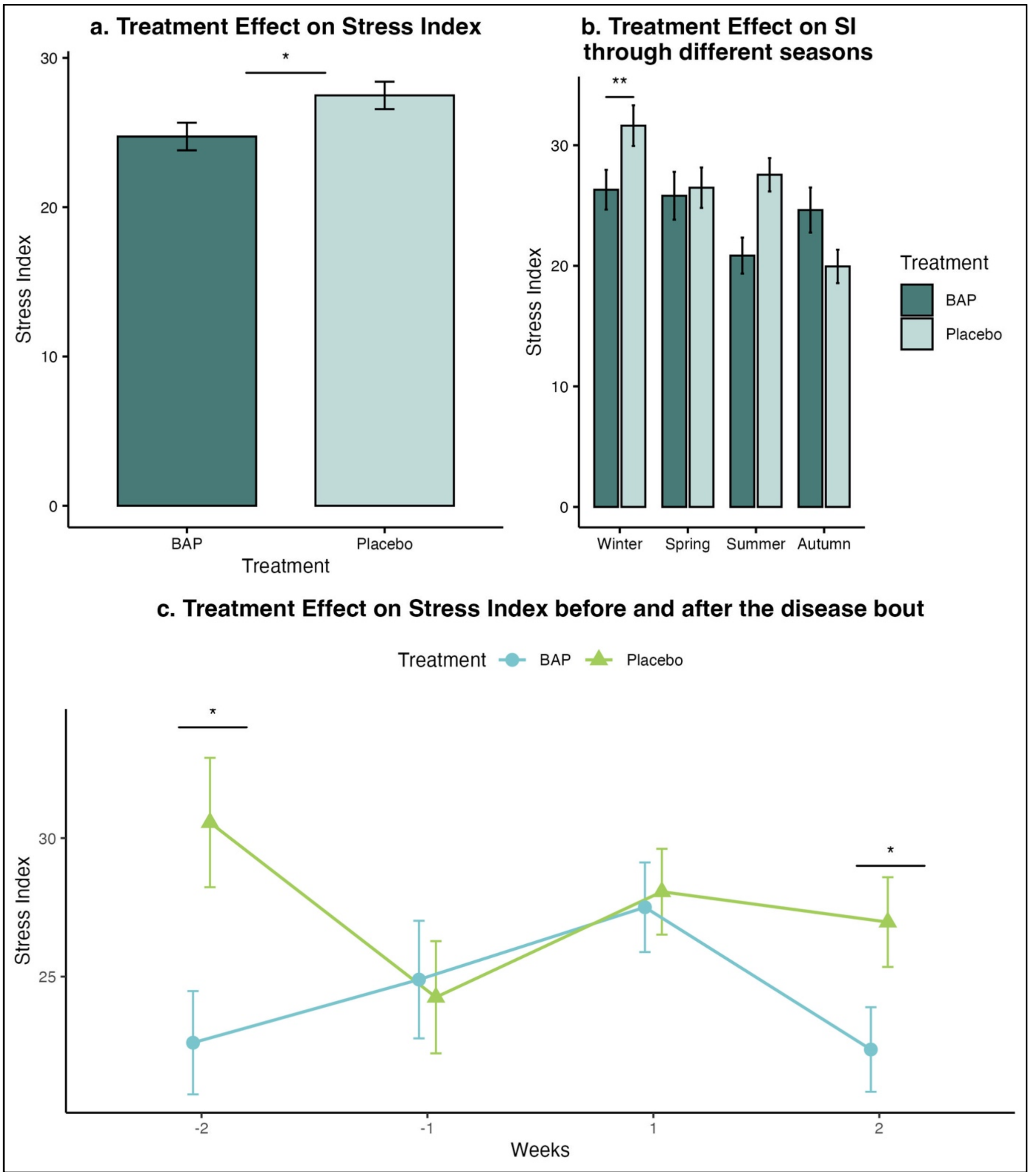

Figure 18 Treatment effect on the on the Baevsky Stress Index (SI) of dairy calves during disease bouts, receiving bovine appeasing pheromone (BAP) or placebo. Probability symbols: * p<0.05, **p<0.01. Error bars represent ± SEM.

3.7. Behavioural Measures

3.8. Motion Index

No treatment effect was observed for MI overall (X^2=0.89, df=1, p=0.35), nor was there a significant treatment x week interaction (X^2=3.21, df=4, p=0.52); however, Tukey-adjusted multiple comparisons revealed that, in within-calves treated with BAP the MI was significantly higher two weeks (p=0.03) and one week (p<0.01) before the calves started to show clinical signs of disease, a phenomenon that was not seen in the within analysis of the placebo treated calves (Figure 19a). At the same time, it was observed that the MI recovered in the first (p<0.001) and second (p< 0.0001) weeks after the onset of the symptoms in the BAP-treated calves (Figure 19b); whereas a similar recovery on MI was only observed in the second week in the calves receiving the placebo (p=0.01). In addition, BAP calves tended to show a lower MI value than their placebo counterparts on the day clinical symptoms appeared (p=0.09).

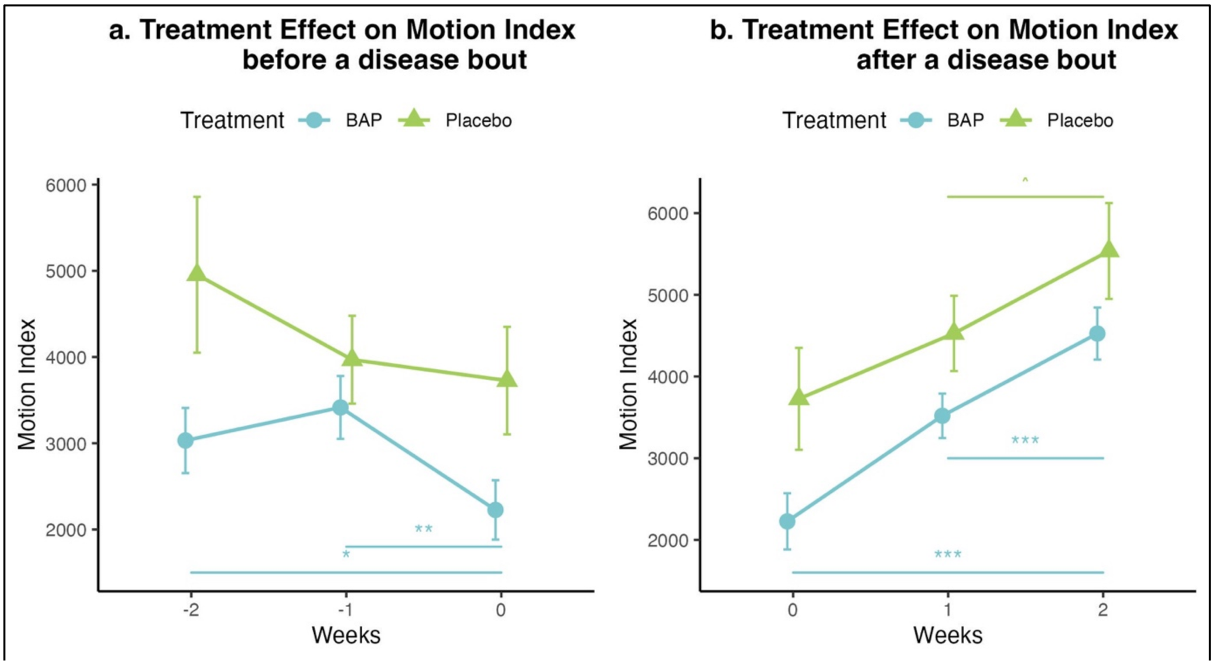

Figure 19 Treatment effect on the Motion Index of dairy calves before and after disease bouts, receiving bovine appeasing pheromone (BAP) or placebo. Probability symbols: * p<0.05, **p<0.01. Error bars represent ± SEM.

A significant treatment x season interaction was observed for MI (X^2=15.18, df= 3, p<0.01). Calves in the placebo group had higher levels of MI during the summer season compared to the spring (p<0.001), autumn (p=0.06) and winter (p<0.001); whereas BAP treated calves only displayed a tendency to have higher MI during the summer compared to winter (p=0.06), but no other differences were observed (Figure 20). Similarly, placebo-treated calves had higher MI values during the summer months compared to the BAP calves in this same period (p=0.01) (Figure 20).

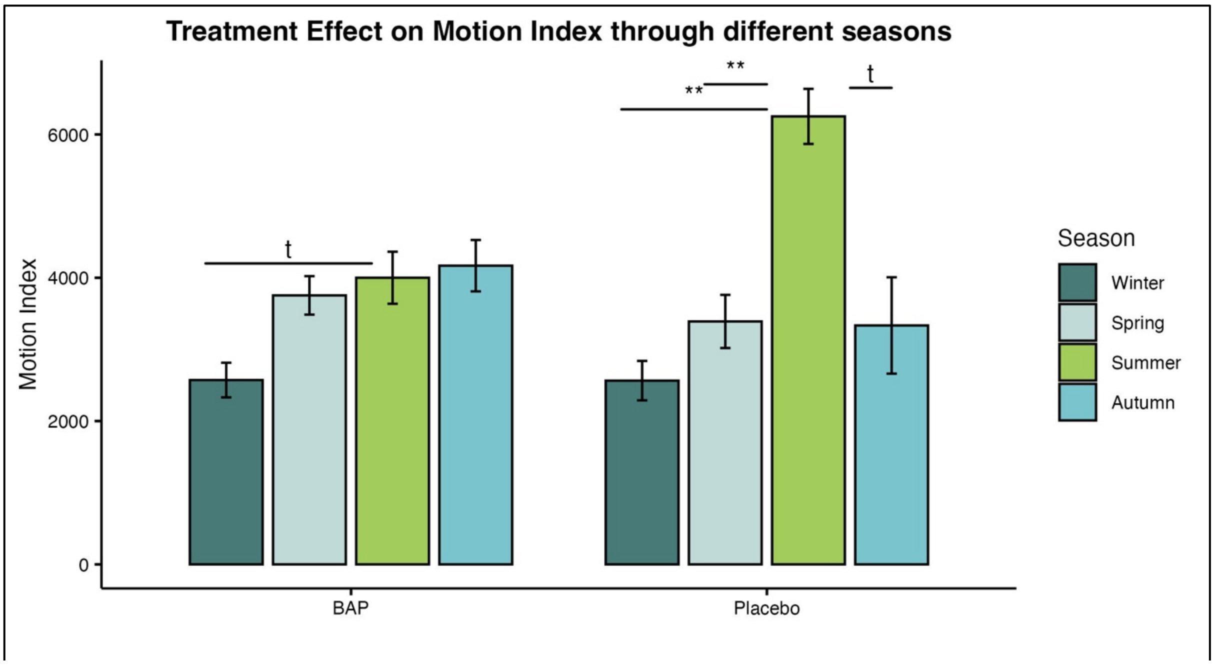

Figure 20 Treatment effect on the Motion Index of dairy calves during different seasons, receiving bovine appeasing pheromone (BAP) or placebo. Probability symbols: t p>0.05-0.10, **p<0.01. Error bars represent ± SEM.

3.9. Step Count

There was no overall treatment effect on the step count (X^2=1.08, df=1, p=0.30) nor was there a significant treatment x week interaction (X^2=3.28, df=4, p=0.51); however, the within-treatment multiple comparison analysis approach revealed that the step count of calves receiving BAP was higher a week before the onset of symptoms (p=0.03) but not in calves receiving the placebo. The step count after the disease bout was significantly higher the first (p<0.01) and second (p<0.001) week after symptoms began in the BAP treated calves; however, this was only evident after the second week in calves receiving the placebo (p=0.05). It was also observed that the step count tended to be higher in the placebo group when the disease was detected compared with their BAP counterparts (p=0.10).

A treatment x season interaction for step count (X^2=14.02, df=3, p<0.01) was observed. Calves in the placebo group had a higher step count during the summer months compared to the calves receiving BAP (p=0.02). Calves treated with the placebo also displayed a higher step count during the summer compared to the spring (p<0.01) and winter months (p<0.01), whereas calves receiving BAP display tended to have higher step count in the winter months compared to the spring season (p=0.07).

3.10. Lying time and bouts

There were no treatment differences or interactions for lying time or lying bouts.

*Table 5 Summary of results of the interaction effects of BAP vs. Placebo with period of measurement for diseased dairy calves. Descriptive statistics are expressed in mean and standard deviation. * p ≤ 0.05*

Variable	Timepoint	Treatment		Model	X^2	df	P values
		BAP	Placebo				
Lying Time (s)		65990.89 (6427.7)	64339.98 (8115.9)		2.90^{-1}	1	0.59
	Week -2	71081.84 (6670.5)	64761.69 (8463.6)				
	Week -1	67598.48 (5858.7)	67675.65 (6594.0)				
	Event	66961.71 (6145.5)	64618.8 (12590.7)	GzLMM NB	3.71	4	0.45
	Week 1	63088.54 (4917.4)	63826.65 (4952.7)				
	Week 2	62351.46 (5077.15)	60787.05 (4463.61)				
Step count		523.37 (271.78)	696.32 (399.45)		1.08	1	0.30
	Week -2	466.32 (251.15)	756.14 (551.95)				
	Week -1	515.87 (277.74)	613.46 (345.26)				
	Event	360.21 (276.23)	572.5 (408.00)	GzLMM NB	3.28	4	0.51
	Week 1	558.54 (195.98)	707.42 (308.66)				
	Week 2	703.71 (243.47)	861.27 (381.26)				
Motion Index		3356.83 (1746.56)	4500.86 (2595.80)		0.89	1	0.35
	Week -2	3031.81 (1648.81)	4954.73 (3259.89)				
	Week -1	3415.43 (1747.54)	3969.31 (2277.69)				
	Event	2226.75 (1684.46)	3726.95 (2790.24)	GLMM Log transformed	3.21	4	0.52
	Week 1	3519.08 (1332.21)	4527.59 (2062.34)				
	Week 2	4525.82 (1561.84)	5536.37 (2556.59)				
Lying Bouts		18.03 (5.40)	19.53 (6.38)		0.063	1	0.80
	Week -2	17.01 (5.88)	19.96 (6.57)				
	Week -1	18.28 (5.77)	20.02 (5.98)				
	Event	17.38 (5.85)	16.85 (5.81)	GzLMM NB	4.63	4	0.33
	Week 1	17.92 (4.87)	20.66 (5.76)				
	Week 2	19.36 (4.79)	20.37 (7.6)				
SDNN (ms)		18.74 (8.00)	16.11 (5.91)		6.34	1	0.01*
	Week -2	21.14 (6.98)	15.27 (6.95)				
	Week -1	17.36 (6.30)	18.21 (5.71)	GLMM Log transformed	7.94	3	<0.05*
	Week 1	15.79 (5.93)	15.61 (5.04)				

	Week 2	22.02 (10.13)	15.92 (6.42)				
RMSSD (ms)		6.63 (2.42)	5.81 (1.93)	GLMM Log transformed	4.50	1	0.03*
	Week -2	7.04 (2.24)	5.31 (2.55)		5.75	3	0.13
	Week -1	16.18 (2.11)	6.66 (1.67)				
	Week 1	6.16 (2.07)	5.47 (1.42)				
	Week 2	7.29 (2.95)	5.97 (2.08)				
Mean HR (bpm)		109.68 (23.56)	115.57 (23.79)	GLMM Log transformed	3.17	1	0.08 t
	Week -2	108.38 (11.29)	119.18 (22.32)		0.84	3	0.84
	Week -1	117.27 (25.21)	113.09 (25.49)				
	Week 1	107.58 (29.95)	117.95 (24.76)				
	Week 2	107.27 (17.18)	112.42 (23.93)				
Stress Index		24.73 (7.68)	27.49 (7.18)	GLMM Log transformed	6.03	1	0.01*
	Week -2	22.61 (5.27)	30.56 (7.75)		5.13	3	0.16
	Week -1	24.89 (8.22)	24.26 (6.72)				
	Week 1	27.5 (7.93)	28.07 (6.93)				
	Week 2	22.37 (7.16)	26.97 (7.06)				
ADG (kg)		0.515 (0.36)	0.631 (0.67)	LMM	2.53	1	0.11
	Week -2	0.52 (0.25)	0.61 (0.32)		3.58	3	0.31
	Week -1	0.37 (0.40)	0.69 (0.31)				
	Week 1	0.47 (0.43)	0.45 (0.23)				
	Week 2	0.70 (0.22)	0.78 (0.37)				

*Table 6 Table 4 Summary of results of the effects of BAP vs. Placebo on different measured variables for diseased dairy calves. Descriptive statistics are expressed in mean and standard deviation. * p ≤ 0.05*

Variable	Treatment		Model	Chi Squared	df	p-value
	BAP	Placebo				
Lying Time (s)	65990.89 (6427.65)	64339.98 (8115.87)	Negative binomial	2.8960^{-1}	1	0.5904
Steps	523.37 (271.78)	696.32 (399.45)	Negative binomial	1.0838	1	0.2978
Motion Index	3356.83 (1746.56)	4500.86 (2595.80)	GLMM (log-transformed)	0.8915	1	0.3451
Lying Bouts	18.03 (5.40)	19.53 (6.38)	Negative binomial	0.063	1	0.8018
SDNN (ms)	18.74 (8.00)	16.11 (5.91)	GLMM (Log-transformed)	6.339	1	0.0118*
RMSSD (ms)	6.626 (2.42)	5.811 (1.93)	GLMM (log-transformed)	4.4971	1	0.0340*
Mean HR (bpm)	109.68 (23.56)	115.57 (23.79)	GLMM (log-transformed)	3.1744	1	0.0748t
Maximum HR (bpm)	147.75 (36.87)	148.74 (37.91)	GLMM	0.0094	1	0.9229
Minimum HR (bpm)	89.52 (22.68)	97.02 (24.42)	GLMM	2.4053	1	0.1209

Stress Index	24.73 (7.68)	27.49 (7.18)	GLMM (Log-Transformed)	6.0339	1	0.0140*
ADG (Kg)	0.515 (0.36)	0.631 (0.67)	GLMM	2.5277	1	0.1119

4. Discussion

The passive immune transfer values in this study were adequate, with both treatment groups having TSP values greater than 5.5 gr/dL (Fischer et al., 2019), and were not significantly different between treatments. Therefore, the variations observed in other metrics measured in this study between treatment groups were not due to failure in passive immune transfer.

The cumulative disease incidence (CDI) in our study was 45.83%, which falls within the range reported globally (Johnson et al., 2011, 2017a), but specific patterns vary by region. In the United Kingdom, a comprehensive analysis of cattle birth and death records from 2011 to 2018 revealed an on-farm mortality rate of 3.87% for calves up to three months of age, with male calves experiencing higher mortality (4.32%) compared to female calves (3.45%) (Hyde et al., 2020). Notably, dairy calves exhibited a higher mortality rate of 6% within the first three months, whereas non-dairy (beef) calves had a lower rate of 2.86% (Hyde et al., 2020). These figures indicate that while the cumulative disease incidence (CDI) in our study was 45.83%, aligning with global reports (Johnson et al., 2011, 2021a), regional variations are evident. For instance, in the United States, the National Animal Health Monitoring System reported a morbidity rate of 33.9% in pre-weaned heifers, which is somewhat lower than our CDI, but a higher mortality rate of 5.0% compared to our 0.93% (Urie et al., 2018c). Similarly, Australian studies reported calf morbidity and mortality rates of 23.8% and 5.6%, respectively, lower than our CDI but closer to U.S. mortality rates (Abuelo et al., 2019). German and Canadian studies have also reported mortality rates ranging from 2.6% to 7.8% and 6.8%, respectively, influenced by production systems and management practices (Falkenberg et al., 2022; Winder et al., 2018b). This suggests that while morbidity in this study is slightly higher compared with global reports, this mortality rate remains much lower than those reported in the literature. The intensive surveillance techniques in this study, which included recognizing subtle symptoms like milk refusal as disease markers, may have increased disease detection. This heightened vigilance may allow the researcher to uncover milder cases of sickness that may not be documented in studies using less intense monitoring procedures or narrower disease definitions. Studies have shown that the implementation of sensor technologies enables the early detection of subtle physiological and behavioural changes, potentially indicating the onset of disease before observable clinical symptoms appear in dairy calves (Gardaloud et al., 2022; Sun et al., 2021).

Acute neonatal diarrhoea was the most common illness observed in this study, particularly affecting calves around 11.90 ± 6.57 days of age, consistent with previous research identifying gastrointestinal infections as a leading cause of morbidity in young calves (Al Mawly et al., 2015; Johnson et al., 2021d; Reiten et al., 2018; Urie et al., 2018d; Windeyer et al., 2014). Respiratory disease, notably pneumonia, was observed predominantly in older calves (40.40 ± 18.10 days old), a trend supported by findings from other studies, which indicate a shift from gastrointestinal to respiratory diseases as calves age (Barry et al., 2020; Dubrovsky et al., 2019; Johnson et al., 2017b).

The lack of significant differences in both CDI and DIR between treatment groups suggests that while BAP may have benefits, these did not translate into significant clinical improvements in the current setting, a finding that was also observed by (Angeli et al., 2020). However, Hervet et al. (2021) observed, in their study on bovine appeasing pheromones (BAP) in young bulls, that BAP treatment increased respiratory clinical signs on Day 8, indicating an initial increase in respiratory sensitivity, but by Day 30, the BAP-treated group had significantly fewer clinical signs of bovine respiratory disease (BRD) than the control group, suggesting a delayed beneficial effect.

Interestingly, while BAP administration did not significantly alter overall disease incidence or mortality, it was observed its potential role regulating stress responses during disease episodes. From previous studies we know that HRV is affect by disease states (Aoki et al., 2020). In our study, differences in physiological stress indicators such as HRV and SI were observed, highlighting the role of BAP in modulating the autonomic nervous system's response during episodes of illness. Specifically, calves treated with BAP displayed higher values of SDNN and RMSSD, both of which are commonly used HRV parameters that reflect greater parasympathetic activity and lower overall stress (Kovács et al., 2014; Linstädt et al., 2024; von Borell et al., 2007). These findings align with previous research demonstrating the appeasing effects of synthetic pheromones in calves during other stressful situations like weaning and transportation (Colombo et al., 2020; Schubach et al., 2020).

Furthermore, the treatment effect on HRV parameters two weeks before and two weeks after the onset of disease symptoms suggests that BAP-treated calves may experience a more stable and adaptive stress response to disease episodes. This temporal pattern in HRV has important implications, as previous studies have shown that the recovery period following stress exposure is critical for overall animal welfare (Arndt et al., 2022; Colditz and Hine, 2016; Salak-Johnson and McGlone, 2007). The improved parasympathetic response in BAP-treated calves, evidenced by higher SDNN and RMSSD, and lower SI values, may contribute to a quicker recovery from disease-related stress. This could indicate that BAP enhances the calves' ability to cope with stressors during illness, possibly by reducing the impact of sympathetic activation typically associated with chronic conditions.

A significant treatment x season interaction for SDNN, RMSSD, and SI was also observed, with BAP-treated calves displaying more consistent HRV values across different seasons compared to placebo-treated calves, and a significant difference between treatments, particularly during the winter months. The higher SI and lower HRV values observed in placebo-treated calves during the winter months are likely reflective of increased thermoregulatory demands (Silva and Bittar, 2019). This suggests that BAP may help buffer the physiological stress effects caused by environmental factors such as seasonal temperature changes, which are known to impact calf health and welfare (Roland et al., 2016; Tripon et al., 2014; Wang et al., 2020). The lack of significant seasonal variation in HRV measures among BAP-treated calves may further support the idea that BAP can provide a stabilizing effect on autonomic nervous system function, irrespective of external and internal environmental stressors. The findings on HRV are also supported by observations on the mean HR in this study, which tended to be higher in the placebo group, especially a week after the onset of symptoms. Similar HR elevations have been reported in cattle exposed to transportation (Gebresenbet et al., 2012), heat stress (Idris et al., 2021), and other environmental stressors(Kovács et al., 2018), suggesting that disease episodes may induce physiological responses comparable to those triggered by external stressors.

Beyond the observed seasonal variation, the differences in SDNN between BAP- and placebo-treated calves may also reflect differences in their autonomic nervous system responses to disease episodes. SDNN, as a measure of overall HRV influenced by both sympathetic and parasympathetic activity, was consistently higher in BAP-treated calves following disease onset compared to placebo calves. This suggests that BAP may have supported a more adaptive autonomic response to illness by buffering the sympathetic activation typically associated with disease-related stress. Higher SDNN values are commonly associated with greater physiological resilience and stress recovery capacity, as reported in previous studies examining stress responses in calves (Kovács et al., 2018; von Borell et al., 2007). Therefore, the consistent HRV values across both seasonal and disease-related challenges support the hypothesis that BAP treatment can modulate stress responses and improve autonomic stability.

While BAP showed potential in modulating stress responses, no significant effects on growth performance were observed, consistent with Angeli et al. (2020); however, in their study, ADG in the placebo group was significantly affected during the disease episodes compared to calves receiving BAP. In this study, the lack of impact of ADG may be attributable to the smaller sample size in our study, limiting the power to detect significant differences in growth metrics.

Illness typically reduces the level of activity in cattle (Eckelkamp, 2019; Puig et al., 2022; Stangaferro et al., 2016). In this study, although we did not observe any overall treatment effects on behavioral measures, a tendency was observed for the MI x season interaction and a significant interaction was also observed between step count and season, with the calves treated with the placebo displaying higher seasonal variations on their activity levels compared to the calves receiving BAP. This suggests that BAP may help mitigate the impact of environmental stressors on calf activity. However, the finding that placebo-treated calves had a significantly higher MI and step count during the summer months compared to their BAP-treated counterparts seems contrary to expectation, given that heat stress is known to reduce activity in calves (Wang et al., 2020). One possible explanation for the observed differences could be that while BAP helps maintain stable activity levels under warmer temperatures, placebo-treated calves might exhibit sporadic bursts of activity, perhaps as a behavioural response to discomfort or compensatory efforts to cool down. As observed by Dado-Senn et al. (2022), where heat-stressed dairy calves engage in modified thermoregulatory behaviours, such as altered standing and lying times, to cope with heat stress. Furthermore, the review by (Laporta, 2021) discusses how early-life exposure to hyperthermia impacts physiological and productive outcomes in young dairy cattle, indicating that without BAP's mitigating effects, calves might experience greater fluctuations in response to environmental stressors like temperatures up to 35.7°C seen in Shropshire in the summer of 2022. Additionally, the study by (Dado-Senn et al., 2024) provides evidence that actively ventilating calf hutches can lead to improved microclimate conditions and more controlled behavioural responses, suggesting that environmental management through active ventilation plays a significant role in moderating the physiological impacts of heat stress, thus explaining the higher variation in activity among the placebo group.

Through a multiple comparison analysis, it was also observed that both MI and step count recovered faster in the BAP treated calves compared to those treated with the placebo. This rapid return to normal activity levels may reflect improved resilience to cope with physiological stressors such as disease processes. As demonstrated in a study by Cantor et al. (2022), dairy calves showing recovery from Bovine Respiratory Disease exhibited significant improvements in feeding behaviour and activity levels, which are associated with successful recovery and overall resilience against the disease.

The results of this study showed that calves treated with BAP exhibited a faster recovery in activity measures, such as motion index and step count, following disease episodes. This pattern corresponds with observations by Angeli et al. (2020), who reported improved recovery in calves treated with bovine appeasing pheromones. Whist this study did not specifically measure veterinary interventions or immune responses, the observed differences in activity patterns observed in this study also align with findings from Hervet et al. (2021), who reported that appeasing pheromones modulated immune responses and reduced clinical signs of bovine respiratory disease complex. Similarly, (Vieira et al., 2023) demonstrated that the application of appeasing substances improved behaviour and immune function in Bos indicus calves during weaning. The increased activity levels and more rapid return to baseline behaviour observed in the BAP-treated calves in the present study may reflect similar stress-mitigation effects, suggesting that these pheromones could influence calves' physiological response to illness. However, further research is needed to directly assess immune function and veterinary intervention requirements.

4.1. Limitations and future directions

Some limitations should be considered when interpreting the results of this study. The relatively small sample size may have restricted our ability to detect more nuanced differences in disease outcomes and growth performance. Additionally, the study exclusively focused on female dairy calves, leaving the question open as to whether BAP's effects would be consistent in male calves or other cattle breeds. Future research should aim to address these gaps by exploring the effects of BAP in larger, more diverse cattle populations to better capture potential other benefits of the pheromone treatment.

Despite these limitations, the findings of this study contribute to the growing body of evidence supporting the use of synthetic pheromones to improve stress management in livestock. The observed stabilization of autonomic nervous system responses and activity patterns suggests that BAP has the potential to enhance calf resilience and welfare during periods of environmental and disease-related stress, warranting further investigation into its broader applications in cattle health management. We also suggest a cost-effective analysis of BAP in a commercial setting.

5. Conclusions

This research offers important perspectives into the role of bovine BAP in modulating stress responses during disease episodes in dairy calves. While BAP did not significantly reduce disease incidence or mortality, it demonstrated clear benefits in regulating physiological stress, as evidenced by more stable HRV parameters and quicker recovery of both parasympathetic and activity levels post-illness. These findings suggest that BAP may help calves better cope with environmental and physiological stressors, enhancing their welfare during periods of illness.

CHAPTER 5. EFFECTS OF A SYNTHETIC ANALOGUE OF THE BOVINE APPEASING PHEROMONE ON THE WELFARE OF DAIRY CALVES AFTER DISBUDDING

1. Introduction

Dehorning or disbudding is a routine management practice in the cattle industry performed on young calves aimed at preventing the growth of horns, with up to 81% of dairy farms in the European Union having dehorned/disbudded cattle (Cozzi et al., 2015), 83% in the United Kingdom and around 52% in the United States (Urie et al., 2018a). It is believed that horns in cattle pose risks of injury to other animals and handlers, can increase the incidence of aggressive behaviour, and complicate management in confined settings such as feedlots and milking parlors (Kling-Eveillard et al., 2015; Knierim et al., 2015). While dehorning/disbudding offers management and safety benefits, it is increasingly recognized as one of the most controversial animal husbandry practices from a welfare standpoint (Canozzi et al., 2019; Costa et al., 2019; Stafford and Mellor, 2005). Disbudding refers specifically to the destruction of the horn-producing cells in young calves, typically performed before the calves are two months old, while dehorning involves the removal of fully developed horns in older cattle (American Veterinary Medical Association (AVMA), 2014).

The methods employed for these procedures include surgical amputation, hot iron cautery and chemical caustic paste. Surgical amputation is typically reserved for older animals where the horns are more developed. Hot iron cautery refers to the procedure where a heated iron is applied to the horn buds to prevent their growth; it is widely use in Europe and to a less extend in North America (Cozzi et al., 2015; Gottardo et al., 2011). On the other hand, chemical caustic paste involves the application of a chemical that burns and destroys the horn buds, a technique mostly used in North America compared to Europe (Saraceni et al., 2021). Regardless of the age at which they are performed, all these techniques are associated with significant pain and stress in calves, raising critical concerns about their impact on animal welfare (Kupczyński et al., 2014; Marquette et al., 2023).

Pain is a complex phenomenon that can be categorized into three primary types: (1) acute nociceptive pain resulting from initial tissue injury; (2) inflammatory pain that may remain for days or weeks until the tissue damage is healed; and (3) neuropathic pain arising from damage to the somatosensory nervous system, which can persist indefinitely (Adcock and Tucker, 2017).

The pain associated with disbudding is a significant concern, as it can lead to both immediate and long-term welfare issues for the calves. Research indicates that calves experience acute nociceptive pain and inflammatory pain during and after the procedure, which can manifest in behavioural changes such as increased vocalization and altered feeding patterns (Stafford and Mellor, 2011; Stock et al., 2013). Long-term welfare concerns may include chronic pain, altered pain sensitivity, and persistent stress-related behaviours, which are discussed in more detail later in this chapter (Adcock and Tucker, 2017; Tschoner, 2021; Vidondo et al., 2019). Immediate analgesic and anaesthetic interventions, such as local anaesthesia and non-steroidal anti-inflammatory drugs (NSAID), are effective at reducing acute pain indicators, including elevated cortisol levels and pain-related behaviours such as head rubbing, ear flicking, and restlessness during and immediately after disbudding and dehorning procedures (Stafford and Mellor, 2005; Stock et al., 2021). Sedation with agents such as xylazine can reduce both the behavioural and physiological stress responses associated with the procedure by inducing a state of calm and muscle relaxation (Faulkner and Weary, 2000; Grondahl-Nielsen and al, 1999). Whilst sedation does not provide analgesia on its own, it can enhance the efficacy of analgesic interventions by reducing the animal's stress response and facilitating safer, more effective application of local anaesthetics (Stock et al., 2013). This combination approach has been found to improve calf welfare during painful procedures like disbudding, particularly when used alongside NSAIDs to address inflammatory pain (Bates et al., 2016, 2015). However, these interventions have limitations in their duration of action and do not consistently manage the prolonged pain that can persist for weeks following the procedure (Adcock and Tucker, 2018; Drwencke et al., 2023; Jimenez et al., 2019a, 2019b). Long-term pain, which may not be as apparent immediately post-procedure, can manifest in subtle behavioural changes such as reduced play behaviour, increased time spent lying, altered weight distribution (favouring the head), and reduced social interactions. (Stafford and Mellor, 2011, 2005; Tschoner, 2021). Research indicates that these pain-related changes can persist for several weeks to months following disbudding, depending on the effectiveness of pain management interventions (Stafford and Mellor, 2011, 2005; Stock et al., 2021; Tschoner, 2021). These prolonged pain responses are likely due to the deep tissue damage and inflammation associated with horn tissue removal, which is not fully alleviated by standard pain management protocols (Tschoner, 2021). Furthermore, a study by Vidondo et al. (2019) evaluated potential histopathological effects of cautery disbudding on the cornual nerve in calves, particularly in relation to chronic pain. The study included 21 Holstein bull calves were included, with some undergoing disbudding at 7 days and others at 28 days, while a control group received sham procedures. Chronic pain was assessed through subjective evaluations and neurophysiological tests, alongside morphological analysis of nerve samples collected post-slaughter. Results indicated that while four disbudded calves showed signs of chronic pain, there were no significant morphological differences in nerve structure or cellular markers between disbudded calves with and without chronic pain. This suggests insufficient evidence to support neuropathic changes resulting from the disbudding procedure, which could be a consequence of the small sample size.

The duration, intensity, and quality of pain are influenced by numerous factors beyond the magnitude and type of tissue injury. Including an individual's prior and simultaneous encounters with pain and stress; cognitive, social, and emotional factors; the quality and duration of analgesics administered before, during, and after the procedure; and the presence or absence of complementary nonpharmacological interventions (Adcock and Tucker, 2017). Cattle attentiveness and arousal levels can affect pain perception. Distractions and competing motivations, such as the provision of food, can reduce the perception of pain during procedures by diverting the animal's attention from the aversive stimulus, a strategy commonly recommended in veterinary settings to alleviate distress (Lomb et al., 2021; Navratilova and Porreca, 2014). Additionally, contextual factors, such as the presence of conspecifics, can alter pain responses through social buffering, as observed in sheep where familiar individuals modulate behavioural pain expression (Guesgen et al., 2014). Similarly, social buffering has also been seen to alter pain responses in disbudded calves, where pair-housed individuals exhibited increased feeding post-procedure, suggesting improved coping ability due to the calming presence of a social conspecific (Bučková et al., 2022). Addressing these cognitive and emotional modulators is crucial to creating effective pain management solutions and improving the welfare and pain experiences of calves (Adcock and Tucker, 2017).

Environmental enrichment in the form of appeasing pheromones have been used in perioperative setting. Siracusa et al. (2010) evaluated the effects of synthetic dog-appeasing pheromone on the behavioural, neuroendocrine, immune, and acute-phase stress responses in dogs undergoing elective surgeries, specifically orchiectomy and ovariohysterectomy. Behavioural observations were recorded, and blood and saliva samples were collected to assess various stress-related parameters, including serum prolactin, cortisol, and white blood cell counts. The results indicated that dogs exposed to the appeasing pheromone showed increased postoperative alertness and visual exploration behaviours, along with a smaller decrease in serum prolactin levels, suggesting a positive effect on the lactotropic axis. However, it did not significantly influence the hypothalamic-pituitary-adrenal axis, immune response, or acute-phase response. These findings suggest that the appeasing pheromone may enhance the recovery and welfare of dogs during perioperative settings by modulating specific behavioural and neuroendocrine stress responses. Similar benefits have been observed in rabbits exposed to a rabbit appeasing pheromone (RAP), which reduced signs of agitation and increased exploratory behaviour during routine clinical procedures and vaccinations, suggesting improved stress adaptation in a veterinary setting (Asproni et al., 2024). Furthermore, Pennington et al. (2023) demonstrated that dogs recovering from spinal surgery in an enriched environment with dog-appeasing pheromones exhibited earlier food intake, fewer doses of rescue pain medication, and reduced anxiety compared to those in a standard environment, highlighting the potential of pheromone-based enrichment in perioperative care.

To the author's knowledge there are no studies on BAP's impact on dairy calves' during painful procedures. Therefore, the aim of this study is to evaluate the effects of a synthetic analogue of BAP on production, neuroendocrine response and behaviour when applied to dairy calves during disbudding in combination with adequate anaesthesia and analgesia. It is hypothesized that calves receiving the pheromone would have faster recovery in the activation of the neuroendocrine system evidenced by higher heart rate variability (HRV) after disbudding and less restlessness, with no negative impact on ADG compared to calves receiving a placebo.

2. Materials and Methods

The experimental procedure for this randomized controlled trial has been reported elsewhere in Chapter 2. The present study has focus specifically on disbudding among the study calves. Calves in this study were housed 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).

2.1. Calves, experimental design, and treatments

This research examined seventy-two female Holstein Friesian dairy calves born from December 2021 to October 2022 at the Harper Adams University dairy farm. Since 2 calves died before being disbudded (from factors unrelated to treatment) and 13 were born polled., disbudding data were collected for the remaining 57 calves (31 in the BAP group and 26 in the placebo group). Calves' management and treatments are described in Chapter 2 section 1.

Calves were disbudded at approximately 4 weeks of age as a current standard practice on the university farm (i.e. not for the purpose of this study), using a hot iron cautery. The procedure was done by a veterinary surgeon using sedation with and intramuscular injection of xylazine 0.05-0.3 mg/kg bodyweight (Chanazine 2% injection ®, Chanelle Pharma), local anaesthesia through a cornual nerve block with Procaine, subcutaneous analgesia with Meloxicam 0.5 mg/kg bodyweight single dose, and antimicrobial protection using topical Terramycin spray, following farm protocols.

2.2. Data Collection and preparation, and welfare measurements

Measurements of HRV, ADG, and activity levels were collected weekly from birth through weaning. Data points for these variables were chosen according to the timing of disbudding to capture baseline, immediate, and recovery responses in physiological, behavioural, and productivity metrics. A baseline for activity levels was established by averaging the measures from the 10 days preceding the procedure, together with data from the day of the procedure and daily assessments up to 10 days post-disbudding. The baseline for ADG and HRV was established using the average of the two measures prior to disbudding, with subsequent data points comprising the following two measurements post-procedure.

2.3. Productivity measures

Calves were weighed at birth and weekly until weaning using a walk-on scale, and subsequently weighed again at the conclusion of the experiment prior to their transfer to the young herd stock. ADG was determined by calculating the difference between two successive weights and dividing it by the interval in days between the measurements.

2.4. HRV data collection and processing

Physiological stress was measured by observing the activation of the neuroendocrine system through analyses of the HRV before and after disbudding (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).

HRV measurement has been recognized as a valuable tool in assessing the autonomic nervous system response during painful procedures in dairy calves (Bergamasco et al., 2021; Stafford and Mellor, 2011; Tschoner, 2021). Polar Equine technology portable heart rate monitors (HRM) were employed to gather HRV readings. The H10 Polar HRM device was strapped to each calf's thorax with a Polar equine belt for a duration of 24 hours, commencing in the second week of life and continuing weekly until the conclusion of the trial. Raw data processing has been described in Chapter 2 section 3.2.

2.5. Behavioural measures and data processing

Calves were equipped with triaxial accelerometers (IDS i-QUBE, Peacock Technology) on one hind leg immediately after birth. Raw data was automatically uploaded from the accelerometers into the CowAlert 2.7.1 Software (Ice Robotics Ltd) for analysis; daily and weekly data on average lying time, lying bouts, step counts, and Motion Index (a metric of the animal's activity calculated by the software) were collected for each calf until the experiment's conclusion. Research indicates that triaxial accelerometers exhibit a high accuracy rate above 99% in identifying movement and resting behaviour (Chapa et al., 2020); furthermore, accelerometers are an appropriate instrument for evaluating pain in calves by quantifying their activity and its variations, and they are reported to be more sensitive than video analysis or direct behavioural observations of calves (Sutherland et al., 2018; Tschoner, 2021).

2.6. Statistical Analysis

Before calf enrolment, a sample size was determined using G*Power Software based on effect size estimates from previous research (Angeli et al., 2020; Schubach et al., 2020). All subsequent statistical analyses were conducted utilizing R Software (2024.04.2+764 by Posit Software, PBC). The complete R code and associated data can be obtained upon request.

For the primary outcome measures HRV (RMSSD, SDNN, SI, low frequency, high frequency and low frequency – high frequency ratio (LF/HF ratio)), ADG and for activity levels, total lying time in second, motion index, step counts and the number of lying bouts, the primary experimental unit was the individual calf, with repeated measures taken from the same calf over different time points. This structure accounted for within-subject correlation and allowed for the assessment of individual and collective responses to disbudding over time.

Descriptive statistics were computed for each outcome variable utilizing the 'psych' package in R. Data analysis was conducted by applying linear mixed effects models to accommodate the data format, with the 'lme4' package. Each outcome incorporated treatment as a fixed effect, whereas calf, season, location, calf's age, weaning, housing, and sample intervals were treated as random factors. In the subsequent stage, the dataset points of measurement related to the disbudding procedure was fitted as a fixed factor to analyze potential interaction effects.

Model diagnostics included residual plots, Q-Q plots for normality assessment, and tests for homoscedasticity (Breusch-Pagan test ‘performance’ package). The Shapiro-Wilk and Kolmogorov-Smirnov tests were used to assess the normality of residuals, and in cases where model assumptions were violated, data transformations or alternative distributions (e.g., Poisson, negative binomial for count data) were considered. For the main effects of treatment, ANOVA Type III Wald chi-square tests were performed. Post hoc analyses using least squares means (LSMeans) with multiple testing adjustments were conducted to explore specific group differences when significant main effects or interactions were detected. We further conducted multiple comparisons using the Gentz and Bretz method to analyze differences between the baseline measurements, with metrics obtained the day of the procedure and post disbudding parameters (Bretz et al., 2001). Matuschek et al.'s (2017) research supports the use of planned comparisons in a focused investigation of treatment effects. By conducting post-hoc analyses of planned comparisons, specifically between the different timepoints in relation to the disbudding event, the researcher aimed to improve sensitivity and identify potentially obscured treatment effects in the broader mixed-effects model, while maintaining appropriate control for Type I error through adjustment for multiple comparisons.

Results are reported as significant at $p < 0.05$, and tendencies when P values were ≤ 0.05 and ≤ 0.10.

3. Results

Calves in this study were disbudded at an age of 36.68 ± 14.18 days. There was no significant difference between treatments for disbudding age (X^2=1.80, df=1, p=0.18, BAP 39.19 ± 13.24 d, Placebo 33.69 ± 14.93 d).

3.1. Productivity Measures - ADG

No Baseline differences were observed between the two treatment groups (BAP 0.61 ± 0.15 kg, Placebo 0.71 ± 0.75, p = 0.15). We did not observe any significant treatment effect on ADG between calves receiving BAP (0.70 ± 0.25 kg) or placebo (0.67 ± 0.22 kg) during the disbudding period. However, when analysing the treatment effect according to the different measurement points, an interaction tendency was observed (X^2=5.62, df=2, p=0.06) as seen in figure 21. Calves receiving the pheromone had a less negative impact on ADG after disbudding compared to those treated with the placebo.

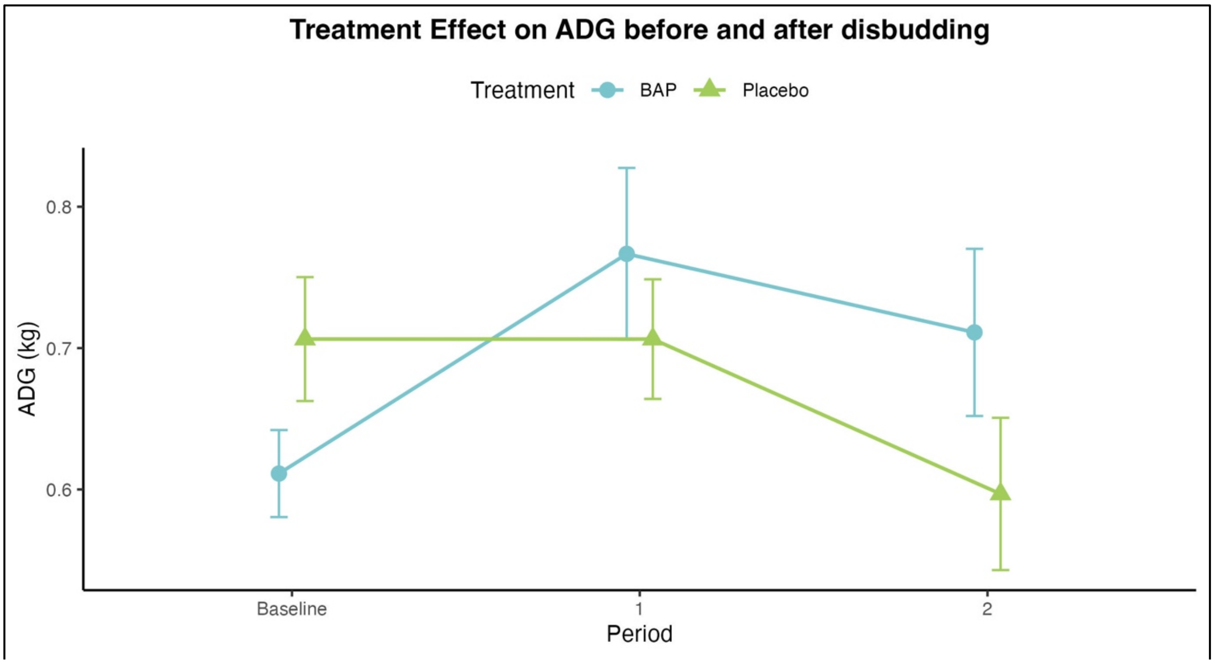

Figure 21 Average daily weight gain (ADG) of calves receiving the bovine appeasing pheromone (BAP) or placebo before and after disbudding. Error bars represent ± SEM.

3.2. Physiological Measure – HRV

3.3. Time domain results

3.4. SDNN

Calves in the pheromone group tended to have higher values of baseline SDNN compared to the ones in the placebo group (BAP 24.58 ± 8.49, Placebo 20.55 ± 8.49, p = 0.08). Calves treated with the pheromone also tended to have higher values of SDNN around disbudding compared to their placebo counterparts (BAP 22.16 ± 8.08 ms, placebo 20.88 ± 8.34 ms, X^2=3.68, df=1, p=0.06) as observed in figure 22. No interactions between treatment and other confounding variables were detected.

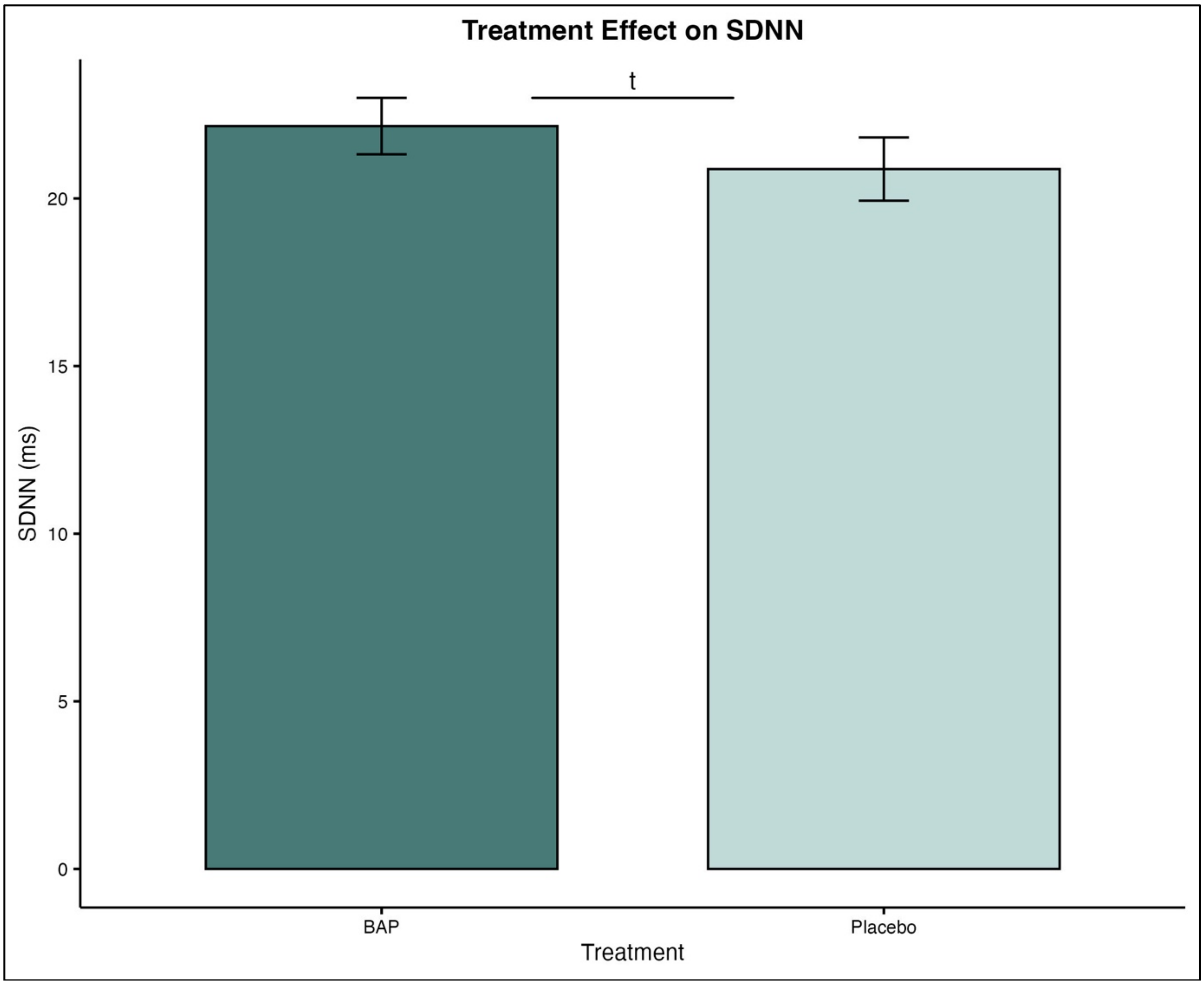

Figure 22 Treatment effect on the standard deviation of beat to beat of normal sinus beats (SDNN) of calves receiving bovine appeasing pheromone (BAP) or placebo during disbudding. Probability symbols: t p >0.05 to 1.10. Error bars represent ± SEM.

3.5. RMSSD

Baseline RMSSD values were not significantly different between both treatment groups (BAP 8.47 ± 3.03, Placebo 7.50 ± 2.30, p = 0.26). No treatment effect on RMSSD was detected between calves treated with BAP (7.79 ± 2.86 ms) or placebo (7.44 ± 2.65 ms) around disbudding, nor did any treatment interactions were observed.

3.6. Stress Index

Baseline values for SI tended to be higher in the placebo group (BAP 21.00 ± 6.08, Placebo 24.32 ± 8.00, p<0.10). A treatment effect on SI was observed with calves treated with BAP (22.3 ± 7.45) tending to have lower SI values compared to those treated with a placebo (23.10 ± 7.08) (X^2=3.39, df=1, p=0.07) during disbudding (figure 23). No treatment interaction effects were observed.

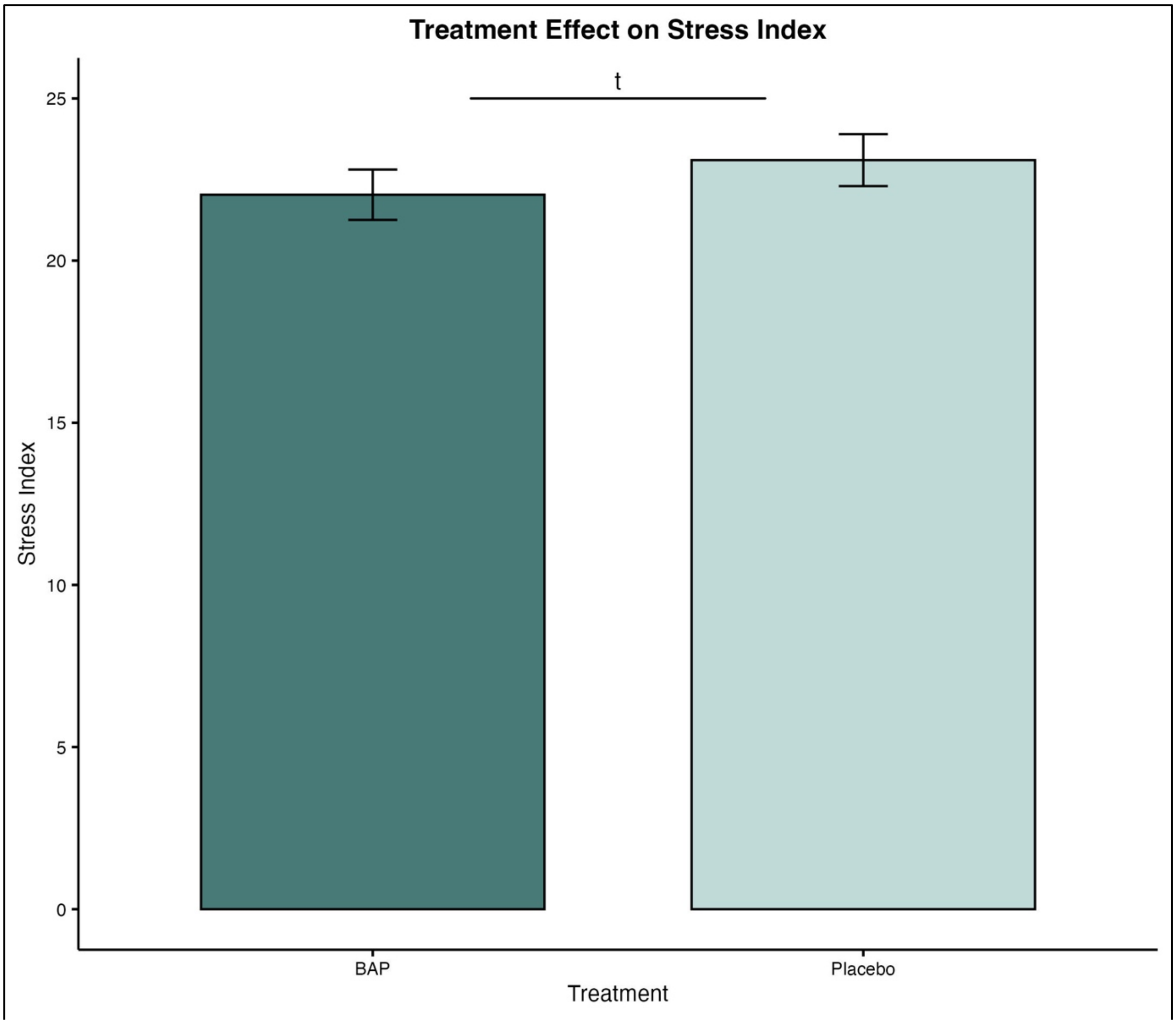

Figure 23 Treatment effect on the Stress Index of female calves receiving bovine appeasing pheromone (BAP) or placebo during disbudding. Probability symbols: t p >0.05 to 1.10. Error bars represent ± SEM.

3.7. Frequency domain metrics

3.8. Low frequency

Baseline values for this variable tended to be higher in BAP treated calves (BAP 449.79 ± 272.87, Placebo 358.56 ± 387.73, p = 0.09). A treatment tendency was detected for the Low Frequency measurement (Figure 24). Calves on the pheromone group had higher levels of Low frequency values compared to those in the placebo group (BAP 374.54 ± 270.68, Placebo 340.01 ± 350.72, X^2=3.40, df=1, p=0.07). No significant interactions were observed.

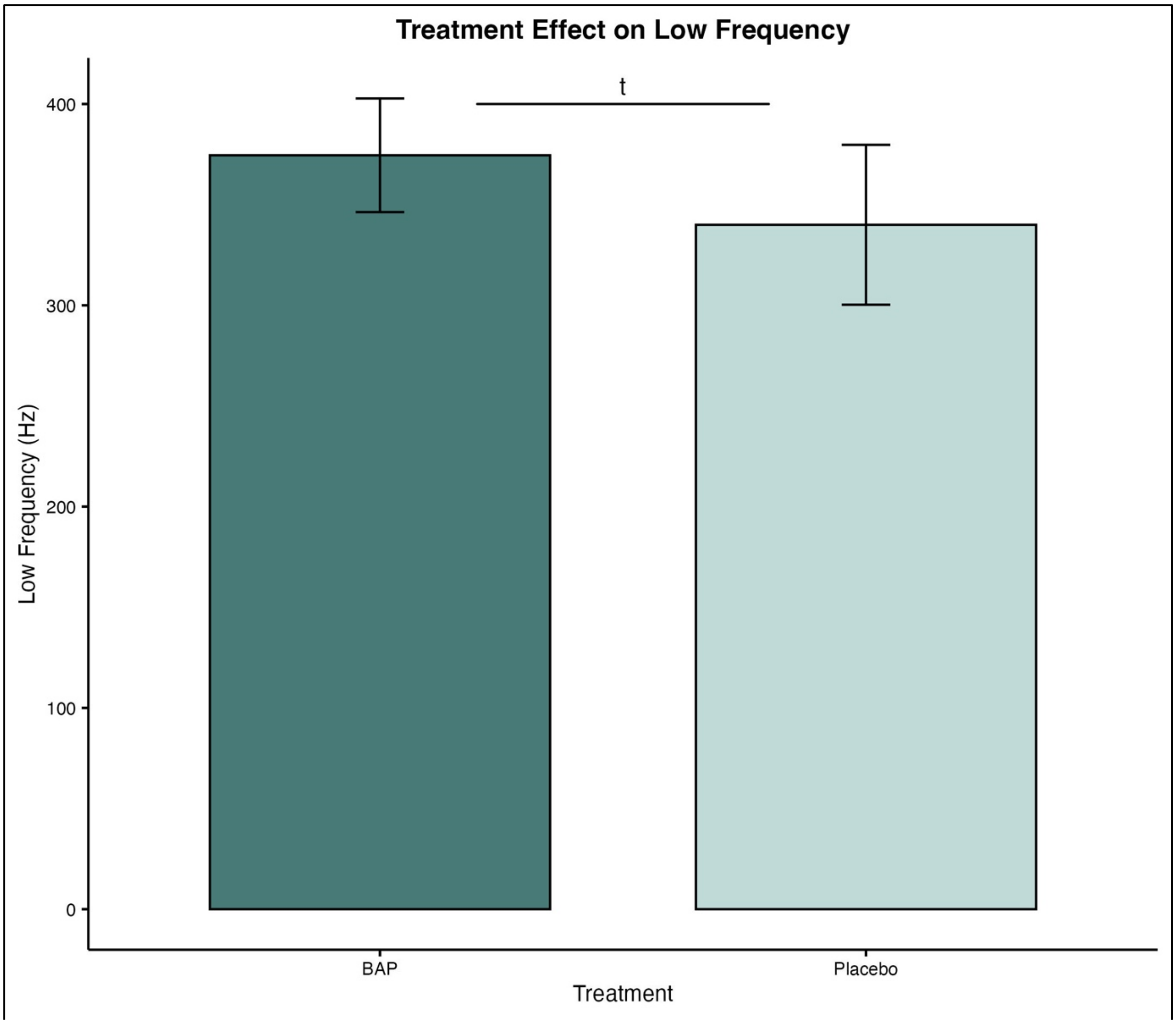

Figure 24. Treatment effect on the Low Frequency domain of female calves receiving bovine appeasing pheromone (BAP) or placebo during disbudding. Probability symbols: t p >0.05 to 1.10. Error bars represent ± SEM.

3.9. High frequency

No baseline differences were observed for this parameter (BAP 44.44 ± 30.56, Placebo 40.21 ± 35.90, p = 0.52). No treatment effect was observed for this parameter around disbudding, and no significant interactions were detected.

3.10. LF/HF Ratio

Baseline values tended to be higher in the pheromone group compared to their placebo counterpart (BAP 10.68 ± 3.76, Placebo 8.77 ± 4.80, p = 0.07). A significant treatment effect was detected for this frequency domain ratio, with calves in the pheromone group having higher ratios compared to those on the placebo group (BAP 10.57 ± 5.19, Placebo 9.31 ± 5.79, X^2=4.07, df=1, p=0.04) (Figure 25). No significant interactions were observed.

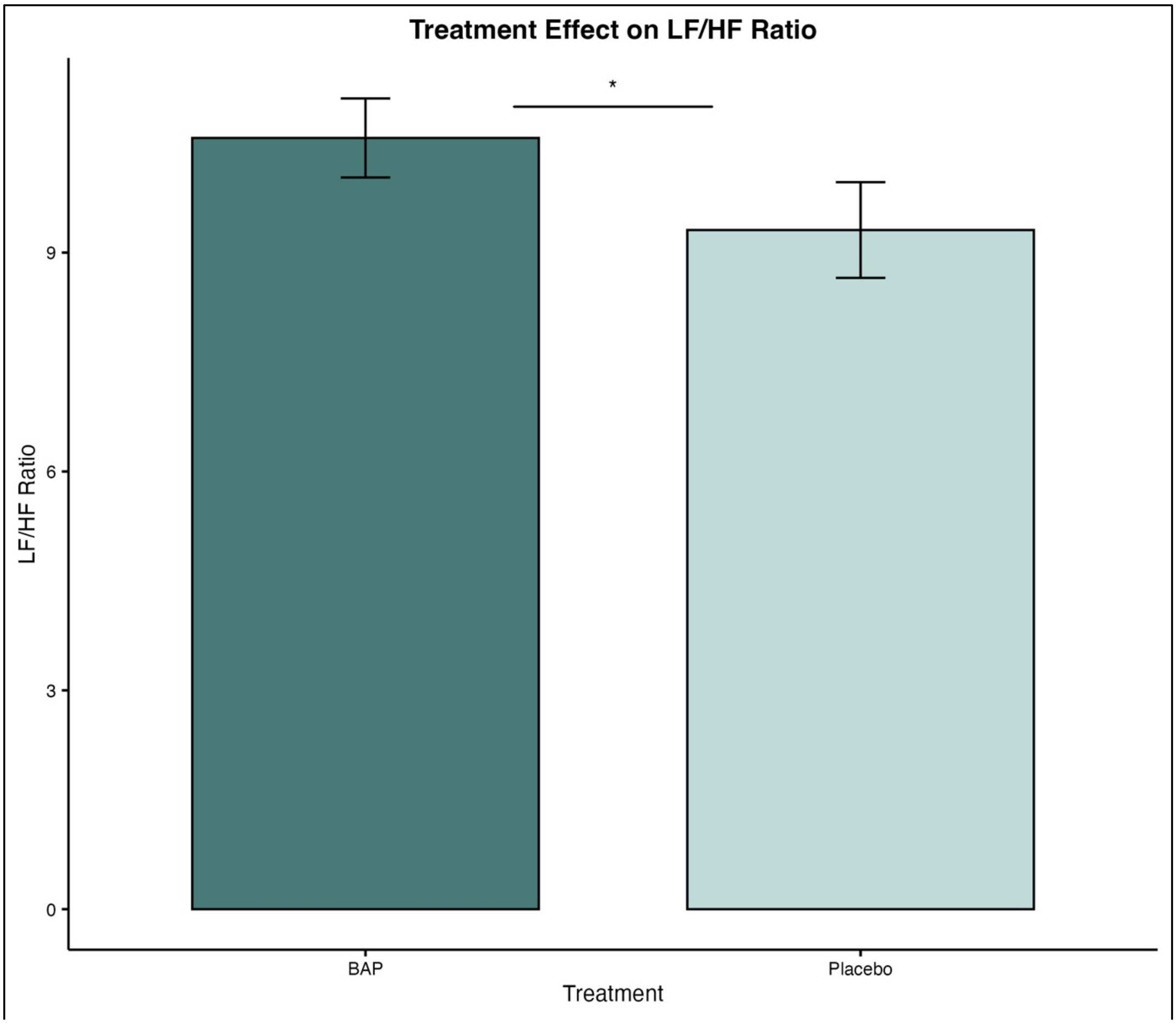

Figure 25. Low Frequency – High Frequency Ratio (LF/HF ratio) of calves receiving bovine appeasing pheromone **(BAP)** or placebo during disbudding. Probability symbols: * p ≤ 0.05. Error bars represent ± SEM.

3.11. Behavioural Measures

3.12. Motion Index

The baseline measurements for MI were not significantly different between treatments (BAP 5451.60 ± 1322.57, Placebo 5523.79 ± 1858.63, p = 0.89). No treatment effect was observed between calves treated with the pheromone compared to those receiving the placebo (BAP 5274.15 ± 2442.77, Placebo 5436.13 ± 2630.79, X^2=0.02, df=1, p=0.89). When analysing post disbudding values with the baseline, we observed that in both treatments the baseline was significantly higher compared to the day of disbudding (p <0.0001), however, on day one post disbudding, MI values were significantly higher on the placebo group (p=0.04) but not on the calves receiving BAP (p=0.50). Furthermore, MI values on day 1 post disbudding were significantly higher in the placebo group compared to those in days 2 to 10 after the procedure. Nevertheless, in the pheromone group, this trend was only observed for days 3 to 8 after disbudding (Figure 26).

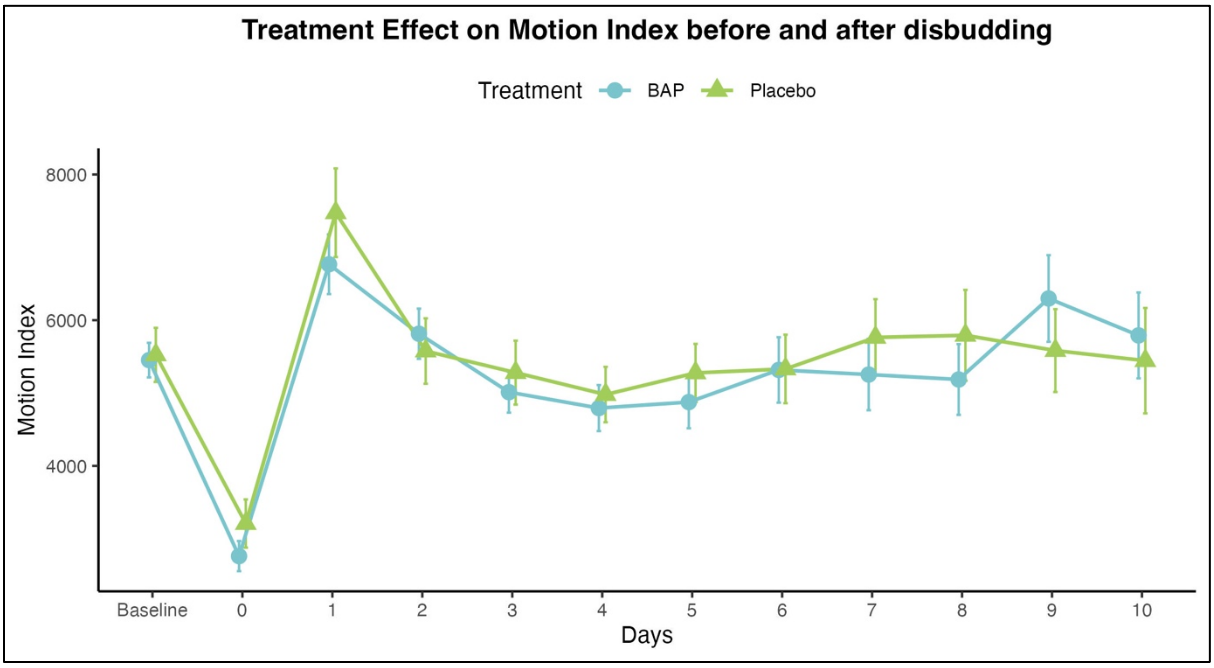

Figure 26 Motion Index of dairy calves receiving bovine appeasing pheromone (BAP) or placebo during and after disbudding. Error bars represent ± SE.

3.13. Step count

Baseline step count was not significantly different between treatments (BAP 876.27 ± 231.34, Placebo 874.69 ± 258.78, p=0.81). Similarly, no significant treatment effect was detected between calves administered the pheromone and those given the placebo (BAP 867.99 ± 425.32, Placebo 876.80 ± 403.73, X^2=0.06, df=1, p=0.81). Upon evaluating post-disbudding values against the baseline, it was noted that in both treatments, the baseline was markedly elevated compared to the disbudding day ($p < 0.0001$). However, on the first day post-disbudding, step count values were significantly greater in the placebo group compared to the baseline ($p < 0.01$), whereas no significant difference was observed in calves administered BAP ($p = 0.48$). Moreover, step count values on the first day post-disbudding were substantially elevated in the placebo group compared to those observed from the second to the tenth day following the treatment. However, in the pheromone group, this was observed solely from days three to eight post-disbudding (Figure 27)

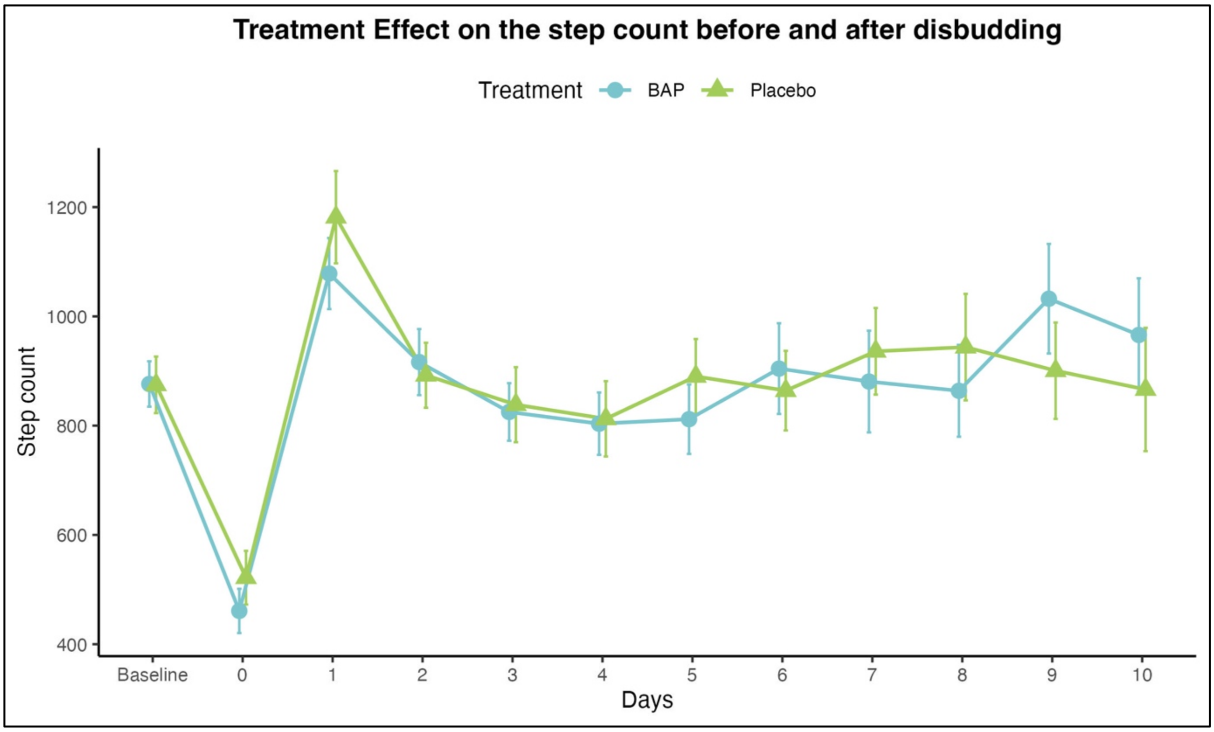

Figure 27 Step count of dairy calves receiving bovine appeasing pheromone (BAP) or placebo during and after disbudding. Error bars represent ± SE.

3.14. Lying Time

The lying time baseline for both treatments was not significantly different (BAP 61848.47 ± 3173.84, Placebo 62032.80 ± 3488.34, p = 0.70), and no overall treatment effect was observed for this parameter during disbudding (BAP 60667.24 ± 6852.28, Placebo 61263.01 ± 8695.67, p =0.70). A treatment x day interaction was observed (X^2=22.05, df=11, p=0.02). Calves in the pheromone group did not exhibit any significant differences on lying time between baseline with disbudding day or any other day after the procedure. In this same group, lying time was significantly higher the day of disbudding compared to the 1st day after the procedure (p<0.001). On the other hand, lying times in the placebo group were significantly higher the day of the event compared to the baseline values (P<0.01), and significantly higher during the day of the procedure compared to the 10 days post disbudding, and did not normalize until the tenth day post procedure. When comparing both treatments, lying times tended to be higher in the BAP treated calves compared to those receiving the placebo on days 5 (p=0.07), 7 (p=0.07) and 8 (p=0.06) post procedure (Figure 28).

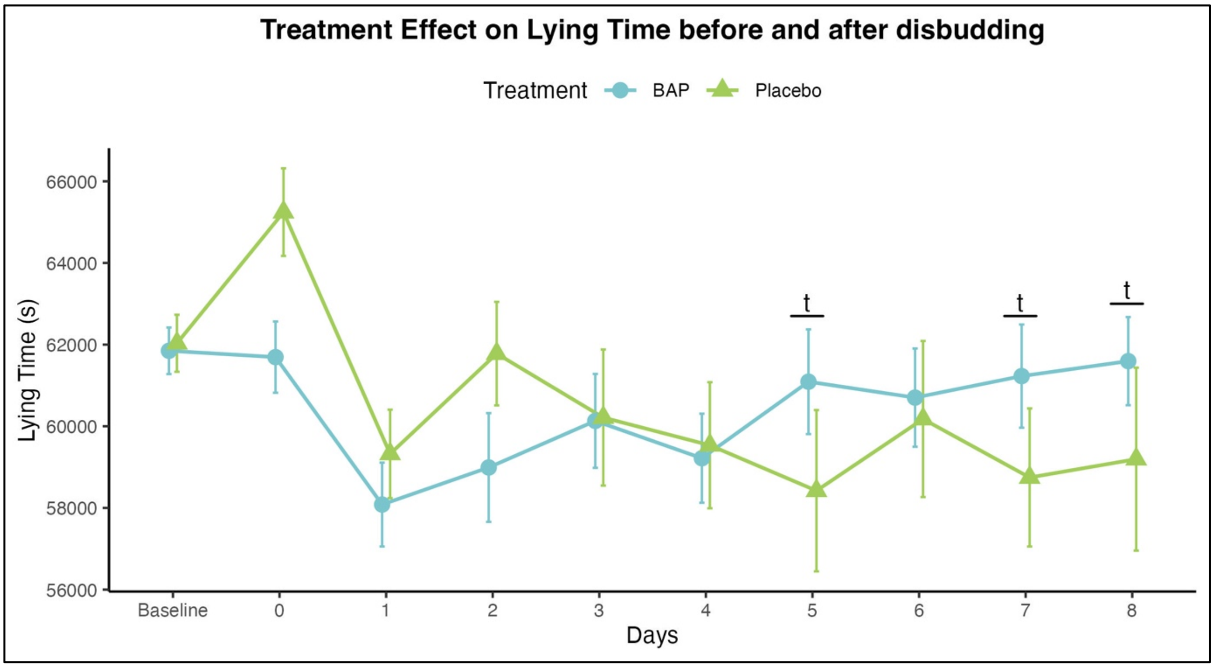

Figure 28 Lying time of calves receiving bovine appeasing pheromone (BAP) or placebo during disbudding. Probability symbols: t p > 0.05 - ≤ 0.10. Error bars represent ± SEM.

3.15. Lying bouts

The baseline for lying bouts for treatments BAP and placebo was not significantly different (BAP 21.51 ± 4367, Placebo 21.47 ± 5.34, p = 0.68). At the same time, no treatment effect on lying bouts (BAP 21.09 ± 5.96, Placebo 21.58 ± 7.26, X^2=0.17, df=1, p=0.68) or any significant interactions were observed.

4. Discussion

In the current study the effects of the synthetic analogue of BAP versus a placebo were investigated, used in addition to adequate sedation, anaesthesia and analgesia during the disbudding of female dairy calves.

The similarity in disbudding ages between the two groups ensured that any observed differences in outcomes were confidently attributed to the treatments rather than age discrepancies, since research has demonstrated that disbudding can either be more painful in younger calves, or has no effect on the level of pain experienced by the animals before 8 weeks of age (Adcock and Tucker, 2018; Alessandro et al., 2018; Caray et al., 2015).

There were no overall differences in ADG, a critical productivity measure, between the treatment and control groups. However, there was a tendency for interaction effects over time, suggesting that calves receiving BAP might have experienced less impact on growth post-disbudding. From previous research we know that using sedation, local anaesthesia and analgesia after disbudding has a positive effect on ADG in the first two weeks after disbudding in dairy calves, compared to not using any pain relief (Bates et al., 2016, 2015; Cuttance et al., 2019). The fact that both control and treatment calves in this study had the same disbudding protocol using anaesthesia, analgesia and sedation, suggest the effect on ADG observed could be attributed to a stress-mitigating effect of BAP, which merits further investigation.

From results observed in the experiment described in Chapter 3 we know that BAP has a positive effect on HRV of dairy calves during the weaning period; therefore, the tendency for better baseline HRV parameters in the BAP group is to be expected, as calves received the pheromone every 2 weeks from birth. However, this trend was still present for the BAP group when the effect of treatment was analysed overall the disbudding procedure. Disbudding pain has a negative effect on HRV (Stock et al., 2013), The improved HRV parameters observed in the BAP group after disbudding, including higher SDNN, lower Stress Index (SI), and increased low-frequency power, suggest a beneficial role of the pheromone in modulating autonomic nervous system activity. Specifically, higher SDNN indicates greater overall heart rate variability, which is associated with enhanced autonomic balance and resilience to stress (Kovács et al., 2014; von Borell et al., 2007). Increased SDNN reflects a more adaptive autonomic response, as it signifies a well-regulated interplay between sympathetic and parasympathetic inputs, helping animals recover more efficiently from acute stressors (Mohr et al., 2002)). The lower SI values further support this interpretation, suggesting that BAP-treated calves maintained better autonomic stability compared to placebo-treated calves. These findings align with research showing that increased HRV, particularly higher SDNN, is associated with improved stress coping ability and overall physiological well-being in cattle (Tschoner, 2021).

In previous research (Byrd et al., 2019; Stewart et al., 2009, 2008) it has been observed that the use of a combination of local anaesthetics and NSAID during disbudding influences heart rate variability (HRV) parameters compared to the use of local anaesthesia alone. For instance, Stewart et al. (2009) observed that calves receiving both a local anaesthetic and an NSAID exhibited increased low-frequency (LF) power and higher LF/HF ratios compared to those given local anaesthesia alone. These changes suggest a more balanced autonomic nervous system response, potentially indicating improved pain management or reduced pain perception. In the current study, pheromone-treated calves demonstrated significantly higher LF/HF ratios, lower Stress Index (SI), increased LF power, and elevated SDNN measurements compared to placebo-treated calves, despite all subjects receiving combined local anaesthesia, NSAID, and a sedative agent. These findings imply that the administration of bovine appeasing pheromone (BAP) may further modulate autonomic responses, potentially enhancing pain mitigation beyond the effects of standard analgesic protocols (Pennington et al., 2023; Siracusa et al., 2010). The observed increase in LF/HF ratios and LF power in BAP-treated calves suggests a shift towards sympathetic dominance or a more adaptable autonomic response, which could be associated with better coping mechanisms during stressful procedures like disbudding. Additionally, higher SDNN values reflect greater overall heart rate variability, indicative of enhanced autonomic flexibility and resilience to stressors.

Research has shown that calves become restless after disbudding, displaying pain-related behaviours such as ear flicking, head shaking and head scratching; and although pain relief, local anaesthesia and sedation has shown to reduce these behaviours they are still observed after the effect of these medications wane (Alessandro et al., 2018; Cuttance et al., 2019; Heinrich et al., 2010; Stewart et al., 2009). Increase in MI and step count indicates restlessness (Krieger et al., 2019), and in the current study, although there were no direct measurements of disbudding-specific behaviours, several patterns regarding these parameters and the effects of treatments (BAP and placebo) on calves post-disbudding were observed. Initially, there was no significant difference in baseline MI or step count values between treatments, indicating that both groups started from a similar level of activity prior to treatment. However, recovery trends in MI and step count differed between treatments; both metrics were significantly higher on day 1 post-disbudding in the placebo group compared to baseline, suggesting a quicker rebound in activity which may indicate more discomfort or reduced capacity to cope with pain (Stafford and Mellor, 2005). This increase was not observed in the BAP group, where the recovery in MI and step count was less pronounced. Furthermore, the placebo group's MI and step count was significantly higher on day 1 compared to days 2 to 10 post-disbudding, suggesting an initial surge in activity followed by a normalization (Stafford and Mellor, 2005). In contrast, the BAP-treated group displayed elevated MI and step count from days 3 to 8 post-disbudding, suggesting a different recovery pattern potentially influenced by better management of post-procedural discomfort or stress. These differences imply that BAP treatment may alter the typical post-disbudding activity pattern, potentially by mitigating stress and discomfort, leading to a more gradual increase in activity as calves recover. This contrasts with the placebo group, where activity levels spiked immediately, possibly due to acute discomfort, before normalizing.

It is recognized that there is a notable reduction in lying time observed immediately after disbudding compared to the pre disbudding period, even when pain relief is used (Cui et al., 2024; Sutherland et al., 2018). Calves in the BAP group showed no significant differences in lying times from baseline through the days following disbudding. However, lying times were notably higher on the day of disbudding compared to the first four days post-procedure. This suggests that while the initial reaction to disbudding under BAP was to rest more, this effect was short-lived, with a return to normal resting levels relatively quickly. For the placebo group, lying times were higher on the day of the procedure compared to the baseline. Following this initial observation, calves in the placebo group had lying times significantly higher the day of the procedure compared to the days post-disbudding and took nearly ten days to return to normal levels which is consistent with the findings by Cui et al. (2024). This pattern may indicate a prolonged recovery or adjustment period needed without the aid of BAP. Furthermore, trends toward higher lying times in BAP-treated calves on days 5, 7, and 8 post-procedure compared to the placebo group, support a faster recovery time compared to the placebo treated calves.

These results suggest that while both groups were managed effectively during the disbudding procedure, the variability in recovery patterns and the subtle differences observed on specific days post-disbudding suggest that BAP may offer benefits in terms of enhancing comfort or reducing recovery times. The discussion around marginal p-values and near-significant trends noted in this study raises crucial considerations about the biological relevance of these findings, beyond their mere statistical representations. This results, whilst interesting from a research perspective, show very slight numerical differences between the treatment and placebo groups across the measured parameters. These differences are statistically detectable but may not be biologically meaningful in terms of impacting the welfare or management practices significantly, and therefore, further evidence of larger-scale benefits is needed.

4.1. Strengths, weaknesses, and future recommendations

For this study a proper sample size calculation prior to data collection was conducted to guarantee adequate power for identifying treatment effects concerning performance, physiology, and behaviour; however, it must be acknowledged that this sample size, although adequate for initial explorations of treatment effects, may be insufficient for detecting smaller, yet clinically significant, variations during the disbudding period. This limitation raises concerns about Type II errors, where true effects of BAP treatment might not be detected. Moreover, the homogeneity in treatment groups, while reducing confounding variables, might not accurately reflect the broader population of dairy calves, potentially limiting the generalizability of the findings. Future studies could address these issues by including a larger and more diverse sample of calves, which would enhance the statistical power and help validate whether the marginal trends observed here hold true in varied farm conditions and across different calf populations.

Owing to sample size constraints, the chemical components of the placebo (2-(2-[ethoxyethoxy] ethanol) served as the baseline, avoiding the inclusion of an extra entirely untreated control group. We accounted for possible confounding variables in our analysis, including weather conditions, weaning stage, and location; nevertheless, due to the study being conducted on a commercial farm, it was not feasible to guarantee that all animals were disbudded at precisely the same age. Even so, it was ensured that these differences were not statistically significant between treatment groups, and age at disbudding was also controlled for the analysis.

The amounts of the pheromone in the air post-application could not be measured, thereby preventing the quantification of any cross-contamination effects. To counteract these potential impacts, we ensured that several rows of calves not involved in the trial were put between the treatment groups.

Finally, although our study concentrated on female dairy calves for future research objectives, it would be worthwhile to examine potential BAP-mediated responses of artificially reared male calves to painful procedures to the treatments. Future research may investigate this element to further the understanding of BAP's impacts across various sexes.

5. Conclusions And Applications

This study evaluated the effects of a synthetic analogue of BAP compared to a placebo, alongside typical pain management measures (sedation, anaesthesia, and analgesia), during the disbudding of female dairy calves. The findings indicate that although immediate post-procedural pain and discomfort were adequately addressed for all calves, BAP may provide supplementary advantages. These advantages include improving comfort or decreasing recovery durations, particularly noticeable in varying recovery patterns and varied differences observed on specific days following disbudding, implying superior management of post-procedural discomfort or stress.

The study highlights potential benefits of BAP in mitigating stress-related impacts on physiological and behavioural parameters post-disbudding, which could have implications for improving animal welfare in dairy practices. Future research should explore larger sample sizes to confirm these findings and extend investigations to different livestock populations, including male calves; and the cost-effective benefits of using the pheromone in commercial setting. Additionally, further studies might examine the impacts of a unique dose of the pheromone before the disbudding procedure.

CHAPTER 6. CAN AN ANALOGUE OF THE BOVINE APPEASING PHEROMONE AFFECT DAIRY CALVES' LEARNING AND DOES IT MAKE CALVES MORE OPTIMISTIC?

1. Introduction

Recent decades have seen an increased focus on cognition in farm animals, particularly dairy calves, due to its relevance for animal welfare and management practices (Nawroth et al., 2019). Cognitive abilities involve the processes associated with learning, memory, decision-making, and problem-solving, which affect the adaptability of animals to their environment (Rørvang and Nawroth, 2021). Traditionally assessed in terms of productivity, understanding the cognitive capacities of dairy calves provides insights into their emotional and mental states, which is increasingly acknowledged as essential for enhancing animal welfare (Gaillard et al., 2014b; Nawroth and Rørvang, 2022b).

Dairy calves encounter multiple management practices that can influence their cognitive development, including early weaning or restricted diets (Lecorps et al., 2023), isolation (Gaillard et al., 2014b), and handling (Schütz et al., 2012). Cognitive tests enable researchers to evaluate the influence of these factors on the calf's learning, memory, and adaptability (Nawroth et al., 2019). Calves raised in enriched environments (those that provide stimulation and opportunities for exploration) tend to exhibit better cognitive performance, suggesting that environmental complexity enhances learning and memory capabilities (Lecorps et al., 2022).

For instance, discrimination learning tasks involve training animals to differentiate between stimuli, such as colours or shapes, associated with rewards or punishments. Reversal learning assesses cognitive flexibility by reversing these associations after initial learning. Gaillard et al. (2014c) conducted a study to determine the effects of individual versus social housing on cognitive performance in dairy calves. Holstein calves were either housed individually in standard pens or kept in pairs using double pens. The calves were tested in a Y-maze, where they were trained to discriminate between two colours (black and white) associated with milk rewards. After reaching a learning criteria, the associations were reversed. The study found that pair-housed calves outperformed individually housed ones in reversal learning tasks, indicating that social housing enhances cognitive flexibility.

Similarly, providing hay as environmental enrichment, can influence cognitive development in dairy calves. Horvath and Miller-Cushon, (2020) explored how hay provision and presentation affect cognitive performance. Calves were assigned to different treatments: no hay, hay provided in a rack, or hay spread on the floor. The cognitive ability of the calves was assessed using a learning task conducted in a T-maze. The experiment involved two main stages, an initial learning stage and a reversal learning stage. Although there was no effect of dietary treatment on performance during the initial learning stage, during the reversal learning stage calves provided with hay (either mixed with starter or offered separately) performed better in terms of cognitive flexibility compared to those that received only a pelleted starter diet. The ability to relearn the task during the reversal learning stage indicates that dietary factors can play a crucial role in cognitive development in young calves.

Furthermore, cognitive test can be considered as a form of environmental enrichment (Clark, 2017). In a study by Meagher et al. (2020), aimed to assess the motivation of Holstein heifers to engage in learning tasks, evaluated the impact of cognitive challenges on the calves' welfare. Using a yoked design, thirty heifers were divided into Learning and Control groups, with the former trained to perform operant responses for rewards. Results showed that learning heifers approached tasks faster and performed more responses, indicating higher motivation; and displayed more play behaviour. These findings suggest that cognitive tests serve as effective environmental enrichment, enhancing animal welfare by providing opportunities for learning and control, ultimately promoting positive behaviours such as play and engagement in training activities. The study by Heinsius et al. (2024) corroborates such results. The main objective of the study was to test the effect of positive reinforcement training (PRT) on anticipatory and play behaviours in dairy heifers. The researchers aimed to determine whether heifers trained with this technique would show more anticipatory and play behaviours compared to control heifers during the period before gaining access to a chute. PRT heifers exhibited more anticipatory behaviours and engaged in more locomotory play. Specifically, PRT heifers performed more behavioural transitions, jumped more frequently and spent more time running in the start box than control heifers. These results indicate that PRT training was associated with a more positive emotional state in the heifers during the anticipation of handling.

Therefore, implementing cognitive enrichment strategies can enhance positive emotional experiences in farm animals. These experiences may include providing cognitive challenges that promote positive anticipation and reward control, or problem solving opportunities, which can lead to improved resilience and empowerment in animals (Boissy et al., 2007; Clark, 2017). Furthermore, cognitive tests are a suitable way of assessing the long-term affective states of farm animals (Kremer et al., 2022). Cognitive bias tests help assess animal wellbeing by describing how emotions affect cognition. By using judgment prejudice, it is possible to determine the emotional state. Animals that are in a positive emotional state are more likely to interpret ambiguous stimuli positively, whilst those in a negative state may interpret the same stimuli negatively. This relationship allows researchers to infer the emotional state of the animal based to its responses to specific cues (Mendl et al., 2009).

Cognitive bias tests typically involve distinct phases. The first phase is *training*, where animals are conditioned to associate specific cues or locations with positive (reward) and negative (non-reward or mild punishment) outcomes. This training ensures that the animals learn to reliably respond with a "go" (approach) response for positive cues and a "no-go" (avoid) response for negative ones (Mendl et al., 2009). Following successful training, the *testing phase* begins, where animals are presented with ambiguous cues that fall between the known positive and negative stimuli. The aim is to observe whether the animals' behaviour indicates optimism or pessimism based on their willingness to approach or avoid these intermediate cues(Kremer et al., 2022).

Most research done using cognitive bias tests in dairy calves has focused on detecting negative emotional states. For instance, the study by Neave et al. (2013) reveals that after undergoing hot-iron disbudding, calves exhibit a 'pessimistic' bias, indicating that their pain affects how they perceive ambiguous situations. Similarly, Lecorps et al. (2020) found that female Holstein calves exhibit a significant decline in sweet solution consumption following hot-iron disbudding, with an average reduction of 48.4%. Calves identified as more pessimistic showed a greater decrease in intake, indicating that their negative outlook exacerbates the effects of pain and leads to a more pronounced state of anhedonia. The study by Lecorps et al. (2019) investigated the effects of pain on mood-based decision-making in calves using a novel probability-based judgment bias test. The findings revealed that calves exhibited increased latencies to approach locations associated with higher probabilities of reward shortly after undergoing painful procedures, specifically hot-iron disbudding. This response indicated a shift towards pessimism and suggested the presence of anhedonia, as the calves showed reduced motivation to seek out rewards during the period of acute pain. The results imply that calves may experience depression-like symptoms following painful experiences. Daros et al. (2014) also found that separation from the dam significantly affected the judgment bias of dairy calves, leading to a decline in their "GO" responses to ambiguous stimuli after separation. Specifically, calves exhibited a negative judgment bias, similar to that observed in animals experiencing pain, indicating a low mood following maternal separation.

As described in previous chapters appeasing pheromones have been used as sensory enrichment to improve the welfare of many domestic species. However, to the author’s knowledge only one study has used a cognitive bias test to evaluate the emotional state and cognition effects of appeasing pheromones in animals. The study by Mengoli et al. (2014) aimed to explore the cognitive-emotional interactions in horses during learning, recall, and reversal tests, with a focus on the effect of synthetic equine appeasing pheromone (EAP) on these processes. A total of 34 horses were divided into two groups, with one group receiving EAP and the other a placebo. The methodology included a series of cognitive tasks where horses were trained to choose between two geometric figures to test learning and recall, followed by a reversal session requiring them to adapt to a change in the correct choice. Parameters assessed included performance (correct/incorrect choices), physiological measures (HRV), and behavioural responses (e.g., attention and stress indicators). Results indicated that the EAP group showed improved performance, making more correct choices with fewer errors, particularly during the recall and reversal sessions. Physiologically, both groups demonstrated significant increases in heart rate during the tasks, reflecting cognitive effort, but the EAP horses exhibited higher SDRR (Standard Deviation of RR Intervals) compared to the placebo group. Higher SDRR is an indicator of greater variability HRV, suggesting a calmer state. The study concluded that EAP administration modulated emotional responses, enhancing attention and cognitive performance in horses during mental tasks without significant physical effort.

This study aims to comprehensively investigate the effects of a synthetic analogue of the bovine appeasing pheromone on dairy heifers to determine its influence on their perception of the environment and overall well-being, particularly in mitigating stress associated with artificial rearing. Specifically, it will assess cognitive bias by comparing heifers treated with the pheromone to those receiving a placebo, with the objective of discerning whether the pheromone alters their interpretation of ambiguous stimuli and thus affects their emotional state. It is hypothesized that heifers treated with the synthetic pheromone will demonstrate a more optimistic cognitive bias compared to the placebo group, indicated by shorter latencies to approach ambiguous stimuli. Additionally, the study will evaluate learning performance, with the hypothesis that pheromone-treated heifers will exhibit enhanced learning ability, as measured by faster task acquisition and fewer errors than those in the placebo group. Physiological stress responses will also be monitored to test the hypothesis that heifers exposed to the pheromone will show reduced physiological stress during training, evidenced by increased heart rate variability and lower salivary cortisol concentrations before and after training sessions compared to placebo-treated heifers. Heart rate variability will be measured before, during, and after training sessions to evaluate differences in autonomic nervous system activity, while salivary cortisol will be collected pre- and post-training to assess stress response. Through these measures, the study aims to provide insights into the potential stress-mitigating and welfare-enhancing effects of the synthetic bovine appeasing pheromone.

2. 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 (0426-202306-PGMPHD-CO2) and in collaboration with the Research Institute for Semiochemistry and Applied Ethology (IRSEA) (Quartier Salignan, France).

2.1. Calves, experimental design, and treatments

Twenty-four dairy female calves born between July 2023 and October 2023 at the Harper Adams University dairy farm were included in the study. Calves were randomly assigned to either treatment A (12) or B (12) using Microsoft Excel (Randbetween function) at the time of birth. The treatments represented a synthetic analogue 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 labelled as "A" or "B" and unblinding occurred only after statistical analysis of the data was carried out.

Calf management and treatments application has been described in Chapter 2.

2.2. Calf training

Training started when the calves were approximately 14 days old, 24 hours after applying the pheromone, and a modified training session used by Daros et al. (2014) and Whistance et al. (2009) was employed. The calves in this study were trained using a secondary reinforcer in the form of a clicker, which sounded immediately after the required event and the reward was a 2L electrolyte solution Life-aid xtra ® (Norbrook Laboratories Limited, Corby, UK), (the researcher previously observed that the calves were highly motivated to consume the electrolyte solution), offered to the calves right after the auditory cue of the clicker.

The training was conducted in four phases.

Phase 1

Calves were conditioned in their individual pen to correlate the sound of the clicker with the introduction of the electrolyte bottle for consumption. This was done for 15 minutes a day or until the animals drank the entire electrolyte bottle. The process was repeated in multiple session until the calves responded by looking for the electrolyte bottle every time they heard the clicker.

Phase 2

The calves were trained in their own pen to respond to the clicker sound by touching a target paddle (with multiple colouring) 20 cm away from the calf's head with the trainer's left hand. The calves were then presented with the paddle until they performed the expected response. The behaviour regarded as a correct response was the calf touching the paddle with the nose and, after hearing the sound of the clicker, moving its head confidently away from the paddle and looking expectantly in the direction of the reward. Once the calf's response was consistently correct, the paddle was then presented at different positions, up, down, left, and right, in a random sequence to prevented them from predicting the position in which the paddle would next appear. The criteria for success was the calf touching the paddle every time it was presented with it.

Phase 3

Calves were assigned randomly a positive colour, red or white. The calves were then trained in their individual hutch using a go/no-go task to discriminate between both colours, displayed on training paddles. The Go response entailed approaching their positive colour on the paddle, and the animal was rewarded with the electrolyte solution after the sound of the clicker. On the other hand, when the animals approached the negative colour on the paddle, no reward was given (no-go response). Each training session lasted until the animal consumed the entire bottle of electrolytes. The calves were considered fully trained when making no mistakes in an entire session.

Phase 4

The calves were taken to a training hutch (roofed, 4m wide x 4m length x 2.4m high pen, figure 29) where the training in phase three was repeated but the colours were displayed attached to the back wall of the training pen. The calves remained next to the trainer on the front of the hutch until the colour cue was displayed on the back wall of the pen. Calves were expected to approach and touch with their nose the colour that represented their positive cue, then after the sound of the clicker, return to the trainer’s side to receive the reward. If calves approached and touched the negative cue colour, no reward was given. The training lasted until the calves chose the correct colour for one entire session.

2.3. Cognitive bias test

Only healthy calves were part of the cognitive bias test, where cues with the positive, negative, and ambiguous probes were presented to the calves in one testing session after the training phases had been completed; to determine the response rate to the ambiguous colours as go or no-go. The ambiguous probes were designed by adjusting the saturation level of red (25%, 50% and 75%). All the colours were added in a sequence randomly assigned using R, red and white cues were displayed six times each, and every ambiguous colour twice. Latency was assessed for each colour, whether the calves decided to touch or not touch the displayed colour; and it was measured from the moment the cue was presented to the moment the calves returned to the trainer's side after looking at the cue and touching it or not. Only the response to touch the positive cue was rewarded.

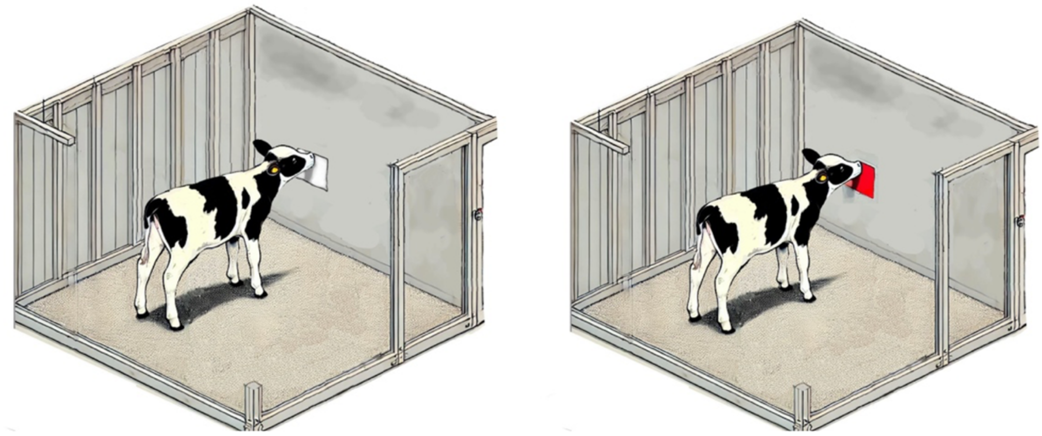

Figure 29 Training hutch setup for dairy calves. The image depicts a training hutch measuring 4m x 4m x 2.4m with colour cues on the back wall.

2.4. Neuroendocrine activation variables

Physiological stress was measured by observing the activation of the neuroendocrine system through cortisol analysis. Saliva cortisol as a measures of acute stress was obtained before every training session and cognitive bias test to use a baseline measurement, and after every training session and test to analyze the physiological stress response generated by the training/test (Pagani et al., 2017; Schwinn et al., 2016). HRV was used as a metric of autonomic activation (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).

2.5. Saliva sample collection, processing and analysis

Saliva was collected from each calf before and after every training session, and before and after the cognitive bias test, by inducing the calf to suck on a stick sponge for three minutes. Samples were then frozen at -20°C until sample processing was carried out. Sample processing has been described in Chapter 2.

2.6. HRV data collection and processing

Polar Equine technology portable heart rate monitors were used to collect HRV measurements. The device (H10 Polar HRM) was fitted around each calf thorax using a Polar equine belt before, during and after every training session, and before, during and after the cognitive bias test to analyze the stress response elucidated by each session/test, by comparing it to a basal measurement; and to evaluate the recovery of the stress response afterwards. HRV data processing has been described in Chapter 2.

2.7. Statistical analysis

The sample size was chosen following the usual number of animals recruited in other cognitive bias test-based studies and based on practical considerations related to the experimental design and the need to ensure that the calves could be adequately trained, monitored and tested (Daros et al., 2014a; Lecorps et al., 2019; Neave et al., 2013b).

2.7.1. Training phase

Data analysis was performed using R software, focusing on evaluating the impact of treatment (BAP or placebo) and training phase on calves' learning performance, stress indicators, and age during training.

To analyse the number of sessions required for calves in each treatment and phase to reach the expected behaviour, a LMM was used to assess the main effects of treatment, with number of sessions as the response variable, treatment and phase of training as fixed effects, and positive colour and age as random effects.

For the statistical analysis of SC and HRV. LMM for each phase of training were initially created, with salivary cortisol, SDNN, RMSSD, Mean HR, SI, LF and LF/HF ratio as the response variables, treatment and time point measurement (Before, during and after) as fixed effects, and cow, age and positive colour as random effects. Additionally, a LMM was created to analyse all the training phases together.

Residuals from the models were evaluated for normality using histograms, Q-Q plots, the Shapiro-Wilk test and the Kolmogorov-Smirnov test. Heteroscedasticity was checked through graphical analysis and statistical tests. When normality and homoscedasticity assumptions were not met, a log transformation was fitted and if this model didn't comply with normality and homoscedasticity either, a negative binomial model was tested to handle overdispersion. ANOVA Type III was conducted on the final model to evaluate the significance of treatment and phase effects, followed by post-hoc pairwise comparisons using least-squares means (lsmeans) to explore specific differences between training phases and time points of measurements (Bretz et al., 2001).

2.7.2. Cognitive bias test

The statistical analysis carried out for the cognitive bias test followed the recommendations highlighted by Gygax (2014), on cognitive judgment bias testing in animals. This framework emphasizes a structured approach to modelling data from cognitive bias experiments, considering the repeated measurements, potential dependencies within the data, and appropriate handling of various response variables such as go/no-go reactions and latency.

Calves' choice to touch or not the different cue colours displayed during the test, using a go/no go approach, and the time it took the animals to make this decision (latency) were the primary outcome variables measured. To carry this analysis, the data was separated and analysed according to the response to each colour cue (red, white, ambiguous 25%, 50% and 75%). Since each calf was presented with each cue multiple times, a LMM was fitted using choice/latency as the response variable, with treatment and positive colour as fixed effects (plus choice when the latency was analysed), and age, cow and the order in which the cue was presented as random effects.

For the salivary cortisol and HRV metrics, LMM were also created, with salivary cortisol, SDNN, RMSSD, Mean HR, SI, LF and LF/HF ratio as the response variables, treatment and time point measurement (Before, during and after) as fixed effects, and cow, age and positive colour as random effects.

As with the training phase, residuals from the models were evaluated for normality using histograms, Q-Q plots, the Shapiro-Wilk test and the Kolmogorov-Smirnov test. Heteroscedasticity was checked through graphical analysis and statistical tests. When normality and homoscedasticity assumptions were not met, a log transformation was fitted and if this model didn't comply with normality and homoscedasticity either, a negative binomial model was tested to handle overdispersion. ANOVA Type III was conducted on the final model to evaluate the significance of treatment, choice, timepoints and positive colour, followed by post-hoc pairwise comparisons using least-squares means (lsmeans) to explore specific differences between the different choices (Bretz et al., 2001). Results were considered significant with P values <0.05 and tendencies when P values were between 0.05 and 0.10 inclusive.

3. Results

3.1. Training stage

Table 7 summarises the results for all the outcome variables in the training stage. Calves required on average 16.67±2.95 training sessions to achieve the desired response, of always choosing to touch the positive colour they were trained to respond too, and never to touch the negative colour. The number of training sessions was not significantly different between calves treated with a placebo (16.76±2.85) or BAP (16.58±1.00) (X^2=0.10, df=3, p=0.99).

Table 7 Summary of the results for the training stage. Descriptive statistics are expressed in mean and standard deviation

Variable	Phase	Treatment BAP	Treatment Placebo	Model	X^2	df	P value
Age (days)	1	13.58 (1.24)	13.92 (1.24)	LMM Log transformed	1.40	3	0.71
	2	21.75 (2.53)	21 (3.13)				
	3	28.67 (2.27)	27.50 (3.71)				
	4	40.83 (4.67)	40.67 (4.54)				
Training sessions	1	3.33 (0.65)	3.42 (0.90)	Negative Binomial	0.10	3	0.99
	2	2.58 (1.00)	2.50 (7.98)				
	3	5.17 (0.84)	5.42 (0.67)				
	4	5.50 (1.51)	5.42 (1.83)				
SDNN (ms)	1	21.02 (5.90)	18.91 (5.68)	LMM Log transformed	2.17	1	0.14
	Before	21.50 (5.11)	18.88 (5.37)				
	During	20.96 (6.92)	20.19 (5.28)		4.65	2	<0.10 t
	After	20.60 (5.63)	17.67 (6.19)				
	2	24.39 (14.17)	22.10 (5.41)	LMM Log transformed	1.14	1	0.71
	Before	22.43 (5.32)	22.22 (5.43)				
	During	30.05 (22.68)	23.55 (5.79)		2.92	2	0.23
	After	20.68 (4.63)	20.54 (4.70)				
	3	27.48 (31.92)	25.42 (7.10)	Negative Binomial	0.17	1	0.68
	Before	23.31 (7.09)	24.20 (5.95)				
	During	35.65 (53.25)	27.92 (7.17)		8.54	2	0.01*
	After	23.35 (8.48)	24.23 (7.53)				
	4	23.06 (7.83)	23.89 (20.75)	LMM Log transformed	0.15	1	0.70
	Before	23.90 (7.30)	22.71 (7.65)				
	During	23.90 (9.58)	23.33 (8.91)		1.77	2	0.41
	After	21.33 (6.00)	25.62 (33.85)				
RMSSD (ms)	1	7.06 (2.02)	6.51 (1.78)	LMM	1.66	1	0.20
	Before	7.17 (1.18)	6.29 (1.51)				
	During	6.98 (2.38)	7.13 (1.95)		6.14	2	<0.05 *
	After	7.02 (1.92)	6.11 (1.71)				
	2	7.59 (1.88)	7.20 (1.77)	LMM	0.69	1	0.41
	Before	7.77 (1.59)	7.32 (1.89)				
	During	7.93 (2.23)	7.52 (1.85)		0.08	2	0.96
	After	7.08 (1.72)	6.77 (1.45)				
	3	8.56 (2.82)	8.85 (5.98)	Negative Binomial	1.44	1	0.23
	Before	8.18 (2.68)	9.47 (9.75)				
	During	9.53 (9.10)	8.67 (2.62)		4.89	2	0.09 t
	After	7.96 (2.63)	8.23 (2.48)				
	4	8.12 (2.58)	7.50 (2.62)	LMM	0.54	1	0.46

	Before	8.96 (1.93)	8.45 (2.26)				
	During	7.47 (3.31)	7.47 (3.28)		1.25	2	0.54
	After	7.92 (2.09)	7.92 (2.14)				
Mean HR (bpm)	**1**	130.41 (21.56)	128.02 (20.76)		0.06	1	0.81
	Before	120.40 (13.93)	120.05 (14.82)	LMM Log			
	During	147.03 (20.98)	141.85 (24.55)	transformed	1.16	2	0.56
	After	123.80 (18.81)	122.17 (14.01)				
	2	128.81 (24.35)	133.97 (25.46)		1.05	1	0.31
	Before	114.94 (16.75)	119.73 (18.52)	LMM			
	During	154.20 (17.07)	157.47 (21.58)		0.69	2	0.71
	After	117.29 (15.65)	124.70 (17.62)				
	3	121.42 (31.08)	127.50 (30.51)		1.09	1	0.30
	Before	102.15 (18.38)	109.29 (16.60)	Negative			
	During	154.53 (18.62)	160.57 (21.83)	Binomial	0.75	2	0.69
	After	107.12 (23.19)	113.62 (21.12)				
	4	117.26 (42.56)	117.51 (40.37)		1.04	1	0.31
	Before	85.75 (18.31)	89.29 (19.49)	LMM Log			
	During	170.58 (18.33)	166.61 (25.30)	transformed	5.58	2	0.06 t
	After	95.60 (21.01)	97.72 (17.38)				
Stress Index	**1**	24.78 (9.52)	25.22 (5.80)		1.06	1	0.30
	Before	22.06 (4.13)	23.06(4.76)	Negative			
	During	29.10 (14.22)	27.22 (6.43)	Binomial	4.55	2	0.10 t
	After	23.19 (5.31)	25.38 (5.44)				
	2	25.84 (23.51)	28.20 (33.87)		3.22	1	0.07
	Before	20.89 (7.49)	28.62 (44.65)	Negative			
	During	30.23 (31.58)	26.83 (5.01)	Binomial	3.38	2	0.19
	After	26.40 (24.41)	29.17 (38.71)				
	3	20.75 (8.33)	22.68 (19.96)		<0.01	1	0.99
	Before	18.88 (8.26)	18.81 (4.04)	Negative			
	During	23.30 (7.92)	23.78 (24.25)	Binomial	5.58	2	0.06 t
	After	20.04 (8.30)	25.39 (33.28)				
	4	21.75 (9.24)	21.90 (8.59)		1.70	1	0.19
	Before	16.16 (3.73)	17.22 (3.92)	LMM Log			
	During	30.02 (10.57)	28.82 (10.65)	transformed	4.95	2	0.08 t
	After	19.12 (4.98)	19.81 (4.67)				
LF (Hz)	**1**	309.07 (224.7)	243.53 (180.8)		2.96	1	0.09t
	Before	331.15 (172.0)	244.68 (132.5)	LMM Log			
	During	279.83 (289.9)	244.68 (202.9)	transformed	5.92	2	0.05
	After	316.23 (197.6)	241.51 (202.5)				
	2	362.15 (296.9)	340.69 (221.5)		0.25	1	0.61
	Before	315.71 (138.2)	356.90 (199.6)	LMM Log			
	During	482.10 (402.6)	377.50 (282.4)	transformed			
	After	288.65 (139.7)	287.67 (162.5)		1.41	2	0.49
	3	438.51 (394.2)	451.40 (358.5)		0.09	1	0.77
	Before	361.34 (223.2)	376.56 (184.4)	Negative			
	During	572.31 (545.0)	546.95 (481.5)	Binomial	0.74	2	0..69
	After	379.98 (307.9)	432.80 (333.1)				
	4	371.27 (398.7)	363.52 (289.7)		0.01	1	0.92
	Before	335.59 (213.9)	349.97 (264.7)	LMM Log			
	During	471.50 (615.6)	411.65 (381.8)	transformed	0.99	2	0.61
	After	306.56 (203.4)	330.23 (191.6)				
LF/HF ratio	**1**	6.22 (4.01)	6.36 (4.37)		0.26	1	0.61
	Before	7.39 (3.43)	8.01 (6.69)	LMM Log			
	During	4.18 (3.43)	4.46 (4.02)	transformed	0.21	2	0.90
	After	7.10 (4.39)	6.62 (3.69)				
	2	6.79 (4.04)	6.80 (4.48)		0.24	1	0.63
	Before	7.68 (3.61)	8.50 (3.63)	LMM Log			
	During	5.43 (5.10)	5.08 (5.53)	transformed	2.33	2	0.31
	After	7.24 (2.84)	6.81 (3.45)				
	3	7.78 (4.75)	7.82 (5.11)		0.02	1	0.90
	Before	10.13 (3.48)	10.00 (4.27)	Negative			
	During	4.10 (4.80)	4.38 (1.76)	Binomial	0.48	2	0.79
	After	9.15 (3.43)	8.98 (4.15)				

	4	16.07 (111.31)	9.06 (5.72)	Negative Binomial	2.51	1	0.11
	Before	10.55 (3.81)	11.71 (5.62)				
	During	27.50 (193.22)	4.31 (4.25)		2.37	2	0.31
	After	10.16 (4.10)	11.04 (3.99)				
SC (µg/dL)	**1**	0.16 (0.19)	0.17 (0.19)	LMM Log transformed	1.98	1	0.16
	Before	0.07 (0.05)	0.07 (0.04)				
	After	0.25 (0.24)	0.28 (0.21)		1.51	1	0.22
	2	0.18 (0.19)	0.19 (0.21)	LMM Log transformed	0.21	1	0.65
	Before	0.07 (0.05)	0.08 (0.08)				
	After	0.29 (0.21)	0.29 (0.24)		0.62	1	0.43
	3	0.21 (0.21)	0.27 (0.28)	LMM Log transformed	0.91	1	0.34
	Before	0.11 (0.1)	0.16 (0.09)				
	After	0.31 (0.24)	0.38 (0.34)		0.22	1	0.64
	4	0.27 (0.34)	0.28 (0.36)	LMM Log transformed	0.40	1	0.53
	Before	0.11 (0.37)	0.11 (0.17)				
	After	0.43 (0.23)	0.46 (0.42)		1.49	1	0.22

3.1.1. Phase 1

The mean age of the calves at the beginning of training phase 1 was 13.75±1.24 days, and there were no significant differences between treatments (BAP 21.75±2.5 days, Placebo 21±3.13 days, p=0.60). The number of sessions required for calves in both treatments to achieve the expected response was not significantly different either (BAP 3.33±0.6, Placebo 3.42±0.90, p=0.91).

3.1.1.1. Salivary Cortisol

Salivary cortisol concentrations were not significantly different between the two treatment groups (BAP 0.16±0.19 µg/dL, Placebo 0.17±0.19 µg/dL, P=0.16). The salivary cortisol baseline for both treatment groups was not significantly different (p=0.87). Although, both treatment groups had significantly higher levels of cortisol after the training sessions compared to the baseline (p<0.0001), the difference between treatments was not significantly different (p=0.19).

3.1.1.2. SDNN

Overall SDNN values were not significantly different between treatments (BAP 21.02±5.90 ms, Placebo 18.91±5.68 ms, X^2=2.17, df=1, p=0.14). When analysing the interaction between treatment and measuring point, a tendency was observed (X^2=4.65, df=2, p<0.10). Calves in the placebo group tended to have lower levels of SDNN after the training session compared to the levels measured duning the training session. This difference was not observed in the BAP group.

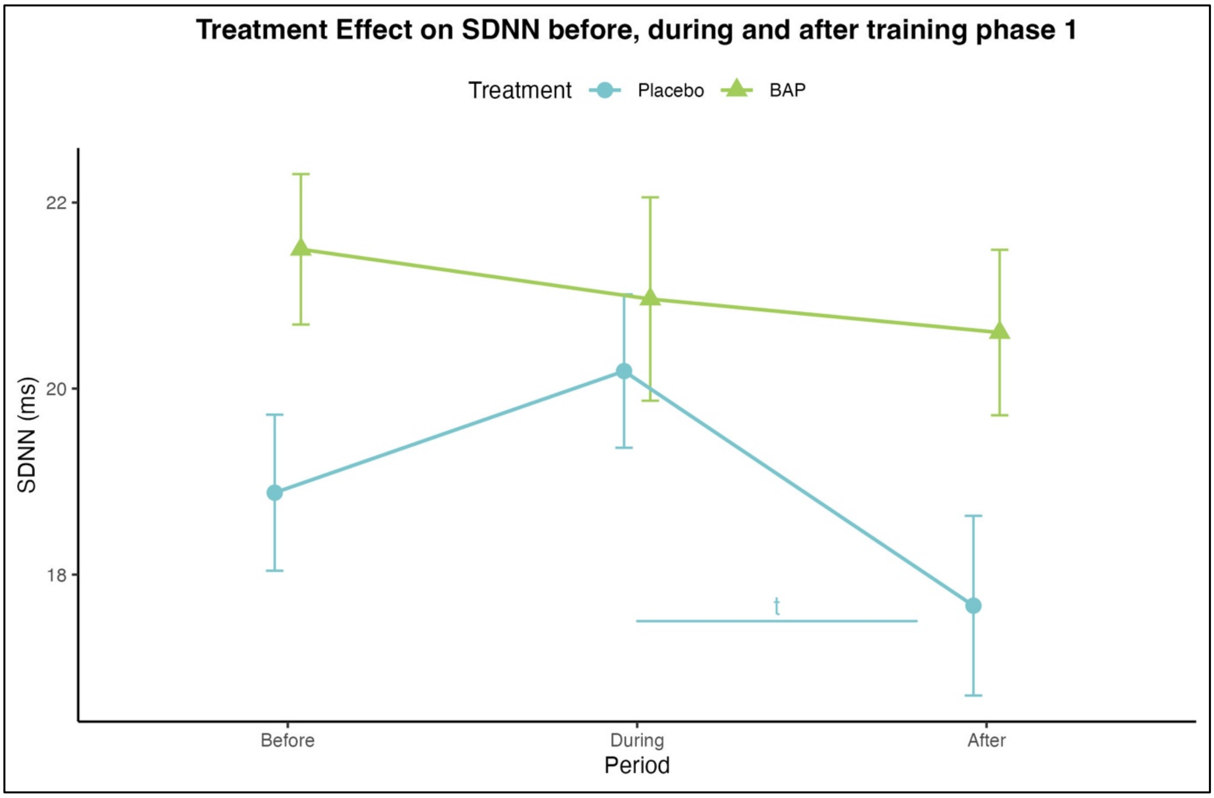

Figure 30 Treatment effect on the standard deviation of beat to beat of normal sinus beats (SDNN) of calves receiving bovine appeasing pheromone (BAP) or placebo before, during and after training phase 1. Probability symbols: t p >0.05 to 1.10. Error bars represent ± SEM.

3.1.1.3. RMSSD

The overall treatment effect on RMSSD was not significantly different between the BAP (7.06±2.02 ms) and Placebo (6.51±1.78 ms) groups (X^2=1.66, df=1, p=0.20). However, an interaction was observed for the treatment x point of measurement interaction (X^2=6.14, df=2, p<0.05). Calves in the placebo group had lower RMSSD values before training compared to the values during training (p=0.04), and the after levels were also lower than the levels obtained during the training session (p <0.01). These findings were not observed in the BAP groups (figure 31).

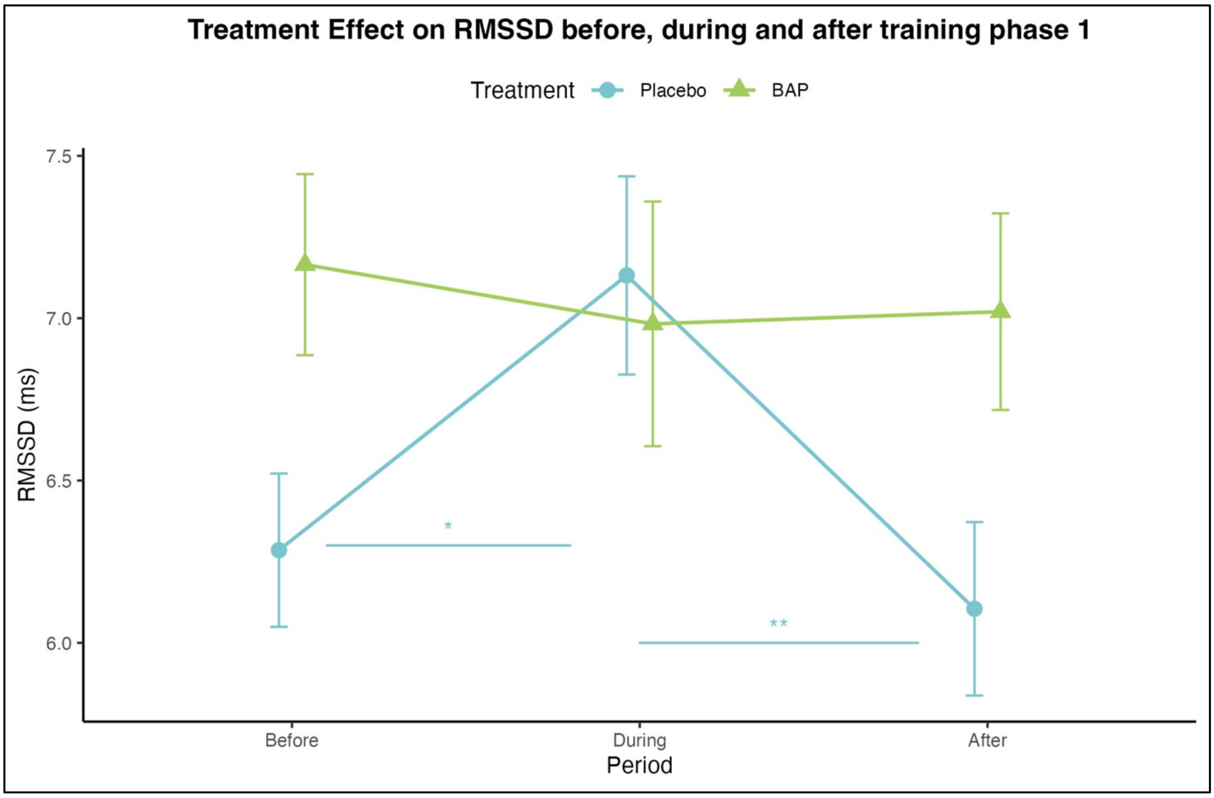

*Figure 31 Treatment effect on RMSSD values of calves receiving bovine appeasing pheromone (BAP) or placebo before, during and after training phase 1. Probability symbols: * p<0.05, ** p<0.01. Error bars represent the SEM)*

3.1.1.4. Mean Heart Rate

No significant effects or interactions were observed for mean heart rate during training phase 1.

3.1.1.5. Stress Index

In general, there was no significant treatment effect on Stress Index metrics between both treatment groups (BAP 24.78±9.52, Placebo 25.22±5.80, X^2=1.06, df=1, p=0.30). None the less, a treatment x period of measurement interaction was observed (X^2=4.55, df=2, p=0.10). Calves receiving the pheromone had significantly lower levels of SI Before training sessions compared to during the training (p<0.0001). Similarly, the levels measured after training sessions was also lower compared to those obtained during training (p<0.001). In the placebo group this difference was only observed between the levels measured before and after the training sessions (p<0.01) as observed in figure 32.

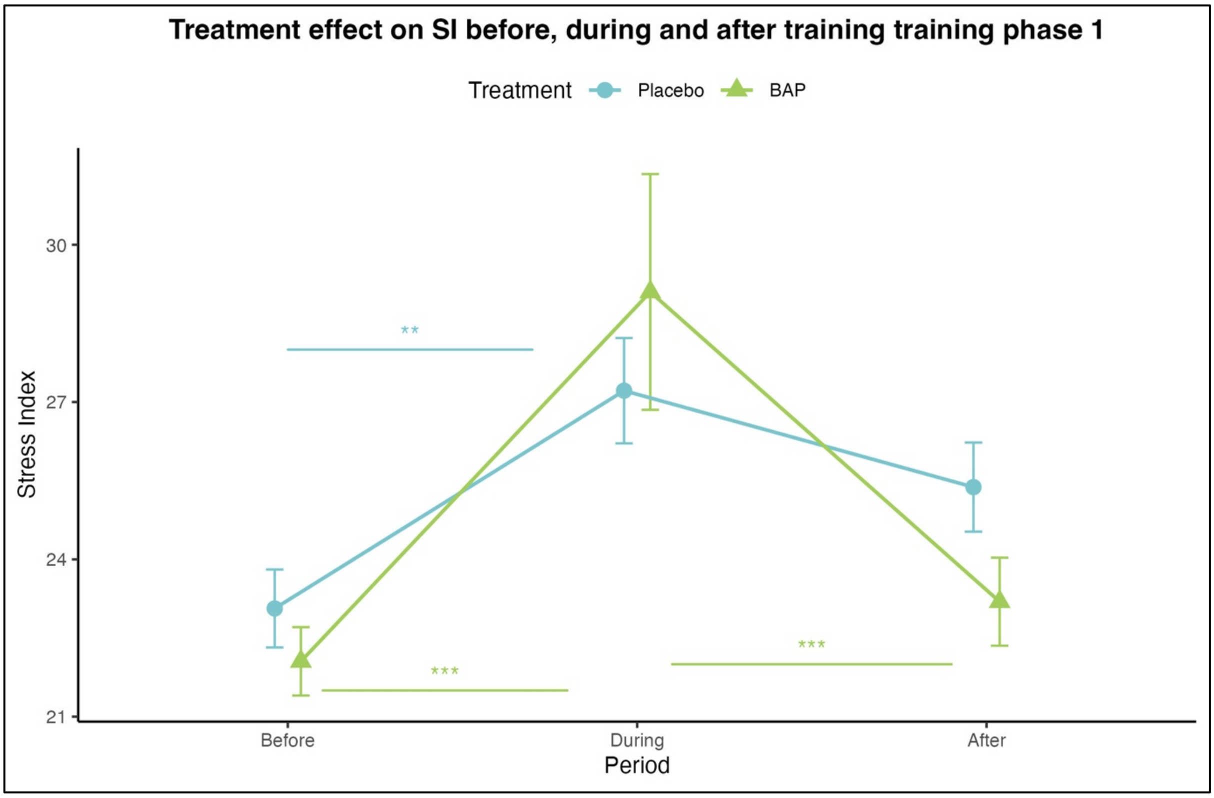

*Figure 32 Treatment effect on the Stress Index of calves receiving bovine appeasing pheromone (BAP) or placebo before, during and after training phase 1. Probability symbols: * p<0.05, ** p<0.01. Error bars represent the SEM).*

3.1.1.6. Low Frequency

A treatment effect tendency was observed for low frequency metrics. Calves in the BAP group (309.07±224.7 Hz) had higher lower frequencies than calves receiving the placebo (243.53±180.8) (X^2=2.96, df=1, p=0.9, figure 33a). Similarly, a treatment x period of measurement interaction was also observed (X^2=5.92, df=2, p=0.05), with calves administered the pheromone having higher values of low frequency both before (p<0.01) and after (p=0.02), compared to values measured during the training sessions (figure 33b).

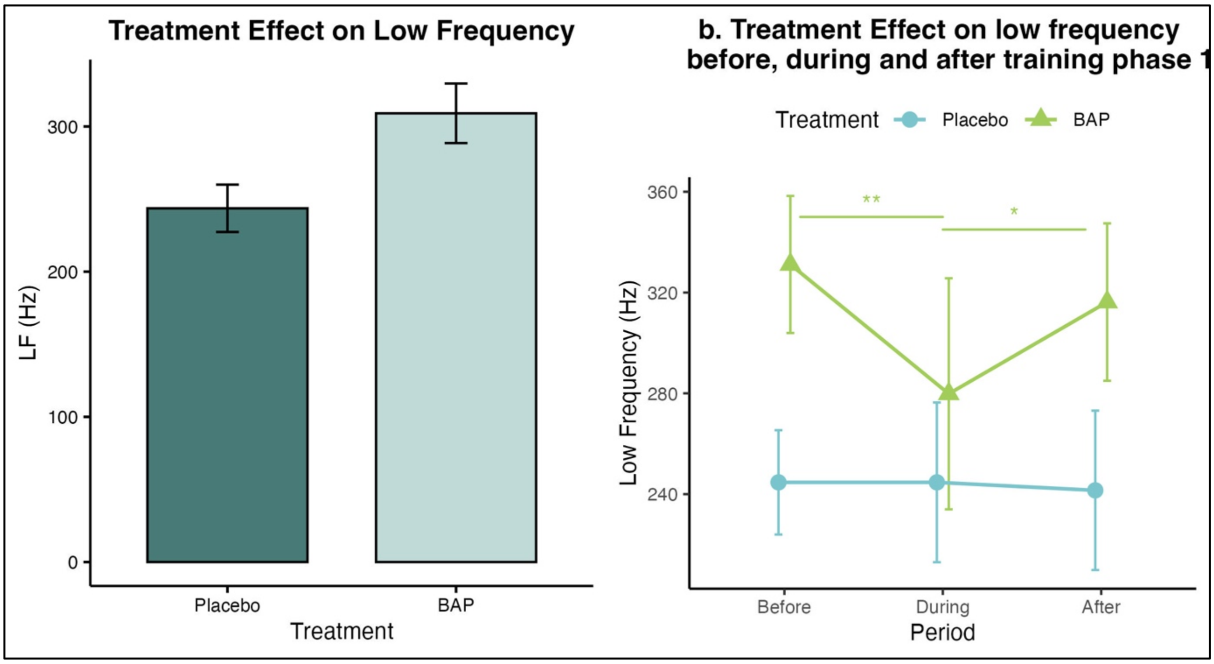

*Figure 33 Treatment effect on Low frequency values of calves receiving bovine appeasing pheromone (BAP) in training phase 1. Probability symbols: t p>0.05, ≤0.10, * p<0.05, ** p<0.01. Error bars represent the SEM)*

3.1.1.7. LF/HF Ration

Overall, no treatment effects or interactions were observed for LF/HF ratio during training phase 1.

3.1.2. Phase 2

The average age of the calves at the beginning of training phase 2 was 13.21.38±2.83 days, and no significant differences were observed between treatments (BAP 21.75±2.53 days, Placebo 21±3.13, p=0.43). Calves in both treatments required on average 2.84±4.49 sessions to achieve the expected response, value that was not significantly different between treatments (BAP 2.58±1.00, Placebo 2.50±7.98, X2=0.10, df=3, p=0.90).

3.1.2.1. Salivary Cortisol

Salivary cortisol concentrations were not significantly different between the two treatment groups (BAP 0.18±0.19 µg/dL, Placebo 0.19±0.21 µg/dL, X^2=0.21, df=1, p=0.65). Both groups had significantly higher levels of cortisol after the training sessions compared to the baseline (p<0.0001) but the difference between treatments was not significantly different (p=0.43).

3.1.2.2. SDNN

SDNN values were not significantly different between treatments overall (BAP 24.39±14.17 ms, Placebo 22.10±5.41 ms, X^2=1.14, df=1, p=0.71). Although no interaction between treatment and measuring point was observed (X^2=2.92, df=2, p=0.23). when doing comparisons within treatments for the different measuring periods, it was observed that calves receiving BAP had lower levels of SDNN before (p<0.001) and after (p<0.001) compared to the levels measured during the training sessions. This difference was not observed in the placebo group.

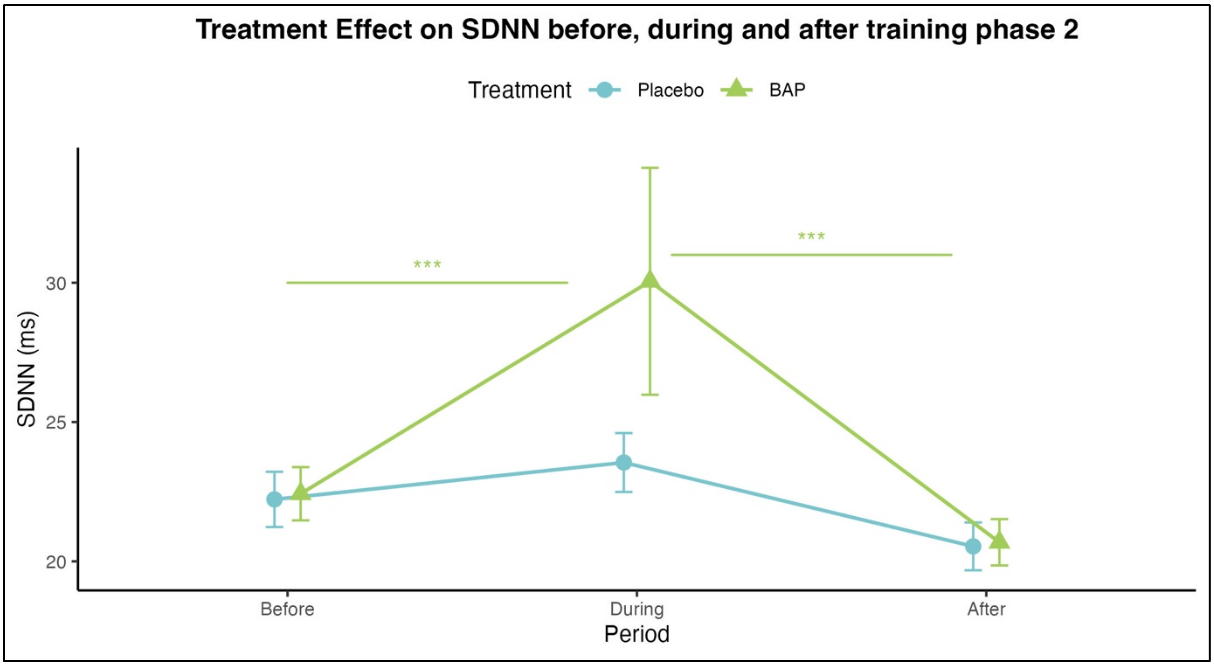

*Figure 34 Treatment effect on the standard deviation of beat to beat of normal sinus beats (SDNN) of calves receiving bovine appeasing pheromone (BAP) or placebo before, during and after training phase 2. Probability symbols: *** p >0.001. Error bars represent the SEM.*

When SDNN was analysed in all training phases during the training sessions, calves in the placebo groups had significantly lower levels of SDNN compared to calves receiving the pheromone, p=0.02 (Figure 35).

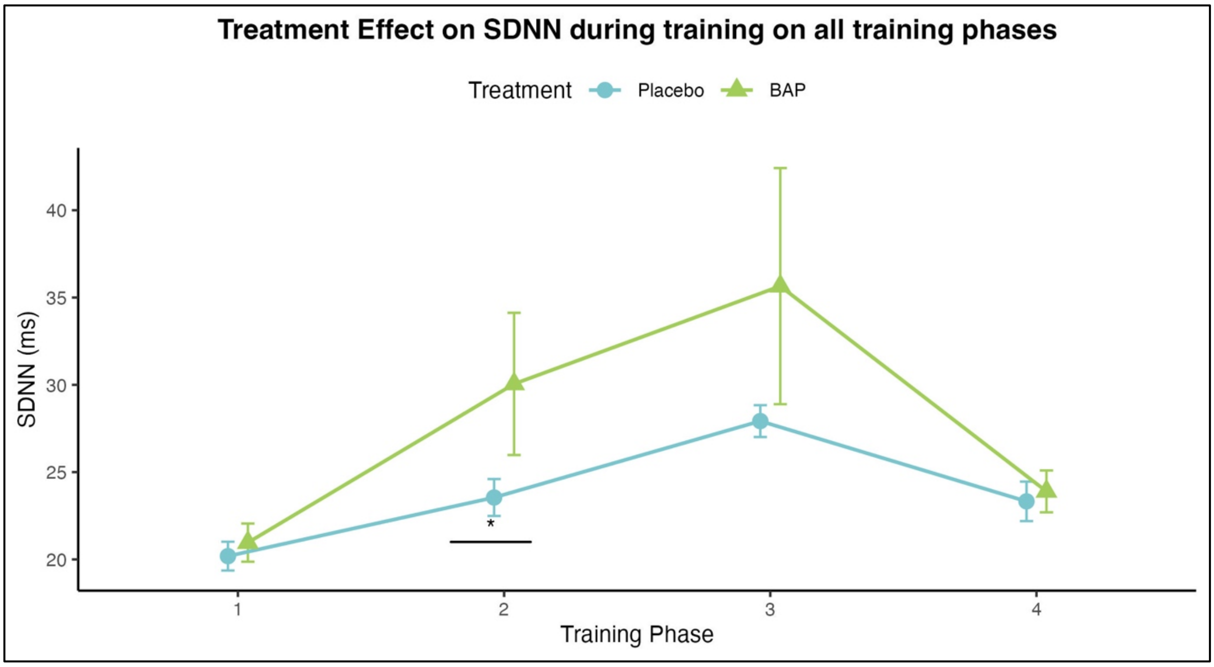

*Figure 35 Treatment effect on the standard deviation of beat to beat of normal sinus beats (SDNN) of calves receiving bovine appeasing pheromone (BAP) or placebo before, during training on all training phases. Probability symbols: t p>0.05 - ≤0.10, * p >0.05. Error bars represent the SEM*

3.1.2.3. RMSSD

No treatment effect on RMSSD was observed between the BAP (7.79±1.88 ms) and Placebo (7.20±1.77 ms) groups (X^2=0.69, df=1, p=0.41), and no interaction was observed for the treatment x point of measurement interaction either (X^2=0.08, df=2, p=0.96). However, when analysing the within treatment comparisons between the different periods of measurement, a tendency was observed for the calves receiving the pheromone. RMSSD values tended to be higher during than after the training sessions, as observed in figure 36 (p=0.06).

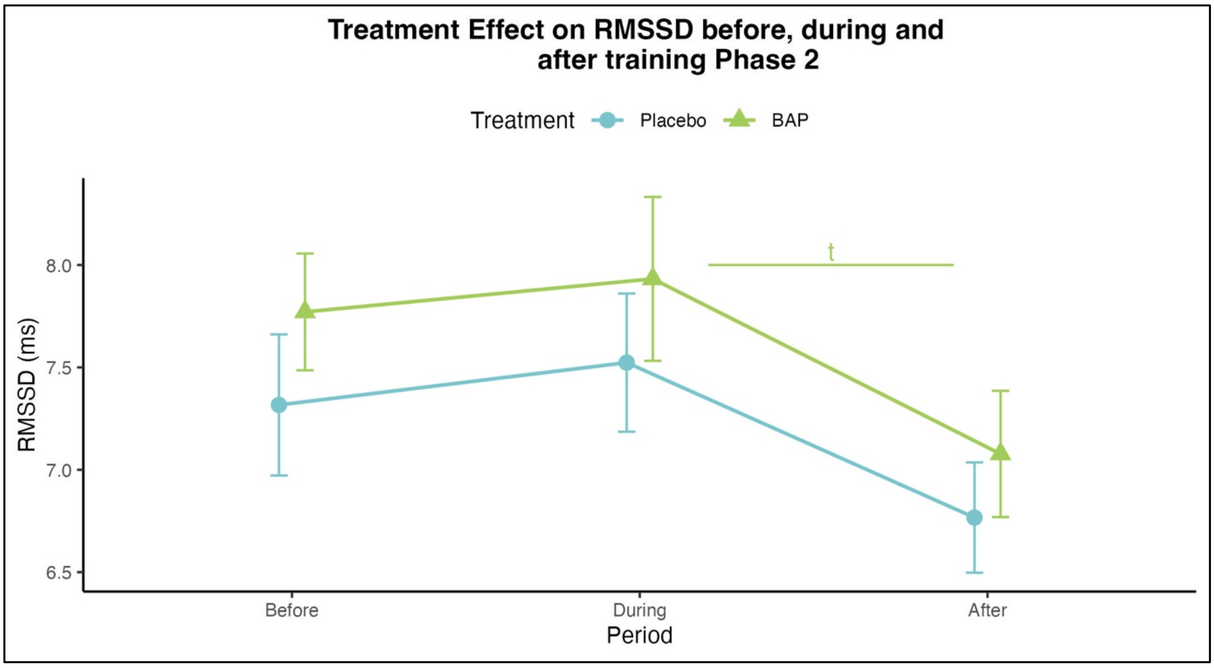

Figure 36 Treatment effect on RMSSD values of calves receiving bovine appeasing pheromone (BAP) or placebo before, during and after training phase 2. Probability symbols: t >0.05 - ≤ 0.10. Error bars represent the SEM

3.1.2.4. Mean Heart Rate

No significant effects or interactions were detected for mean heart rate during training phase 2.

3.1.2.5. Stress Index

A treatment effect tendency was observed for SI between the two treatment groups (BAP 25.84±23.51, Placebo 28.20±33.87, X^2=3.22, df=1, p=0.07). Calves receiving the placebo tended to have higher SI values than calves treated with BAP (Figure 37a). Although, no treatment x period of measurement interaction was observed (X^2=3.82, df=2, p=0.19). when analysing the levels of SI between the different treatment periods, it was observed that calves treated with the placebo tended to have baseline SI values higher than calves receiving the pheromone (p=0.07, figure 37b). Similarly, baseline levels in the placebo group were significantly lower to those observed during the training session (p=0.03, figure 37b).

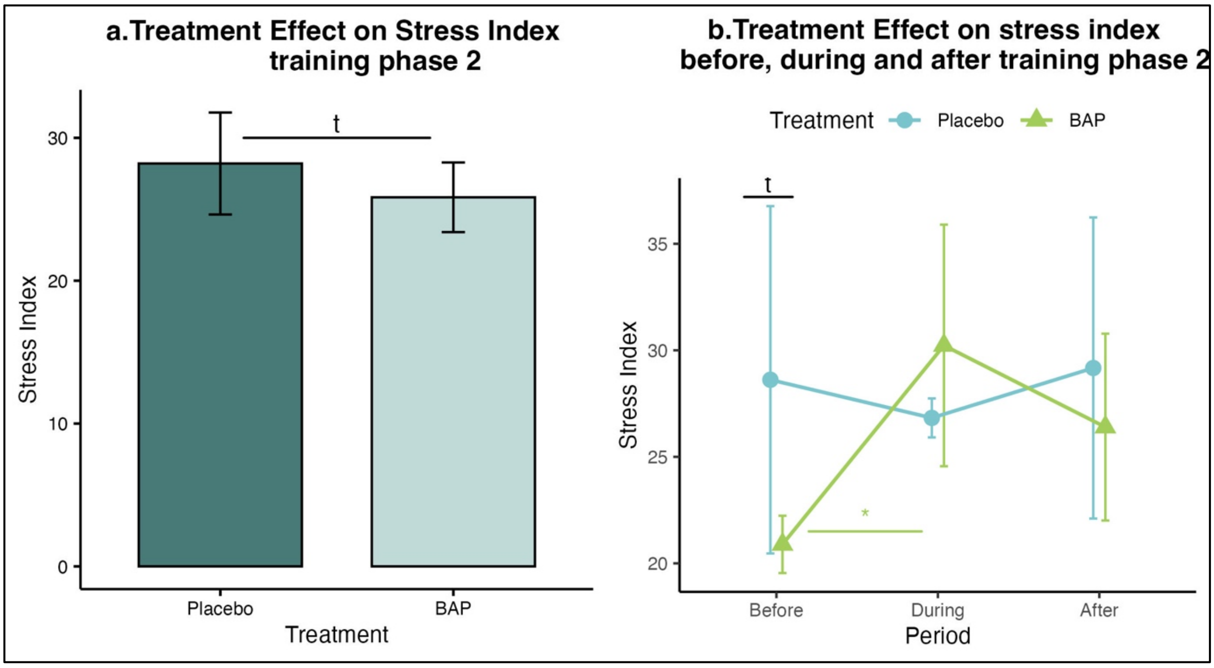

*Figure 37 Treatment effect on the Stress Index of calves receiving bovine appeasing pheromone (BAP) or placebo in training phase 2. Probability symbols: t p >0.05 - ≥0.10, * p<0.05. Error bars represent the SEM).*

3.1.2.6. Low Frequency and LF/HF ratio

No treatment effect or interactions were observed for LF and LF/HF ration during the second phase of training.

3.1.3. Phase 3

Calves' mean age at the beginning of training phase 3 was 28.09±2.99 days, and no significant differences were observed between treatments (BAP 28.67±2.27 days, Placebo 27.50±3.71, p=0.32). On average, calves required 5.30±0.76 sessions to achieve the expected response, and no treatment differences were observed between calves treated with BAP (5.7±0.87) or the placebo (5.42±0.67) (p=0.79).

3.1.3.1. Salivary Cortisol

In general, no treatments effect differences were observed between calves on the BAP (0.21±0.21 µg/dL) and placebo groups (Placebo 0.27±0.28 µg/dL), X^2=0.91, df=1, p=0.34). Both groups had significantly higher levels of cortisol after the training sessions compared to the baseline (p<0.0001)

3.1.3.2. SDNN

No overall treatment effect was detected for SDNN values (BAP 27.48±31.92 ms, Placebo 25.42±7.10 ms, X^2=0.17, df=1, p=0.68). An interaction between treatment and measuring point was observed (X^2=8.54, df=2, p=0.01). During the training session, calves receiving

BAP had significantly higher levels of SDNN compared to calves treated with the placebo (p=0.03) (Figure 38). At the same time calves in the pheromone had significantly lower SDNN metrics before (p<0.0001) and after (p<0.0001), compared to those observed during the training sessions (Figure 38). On the other hand, calves in the placebo group only tended to have SDNN levels lower before training (p=0.08) and after (p=0.08) compared to the values obtained during training (Figure 38).

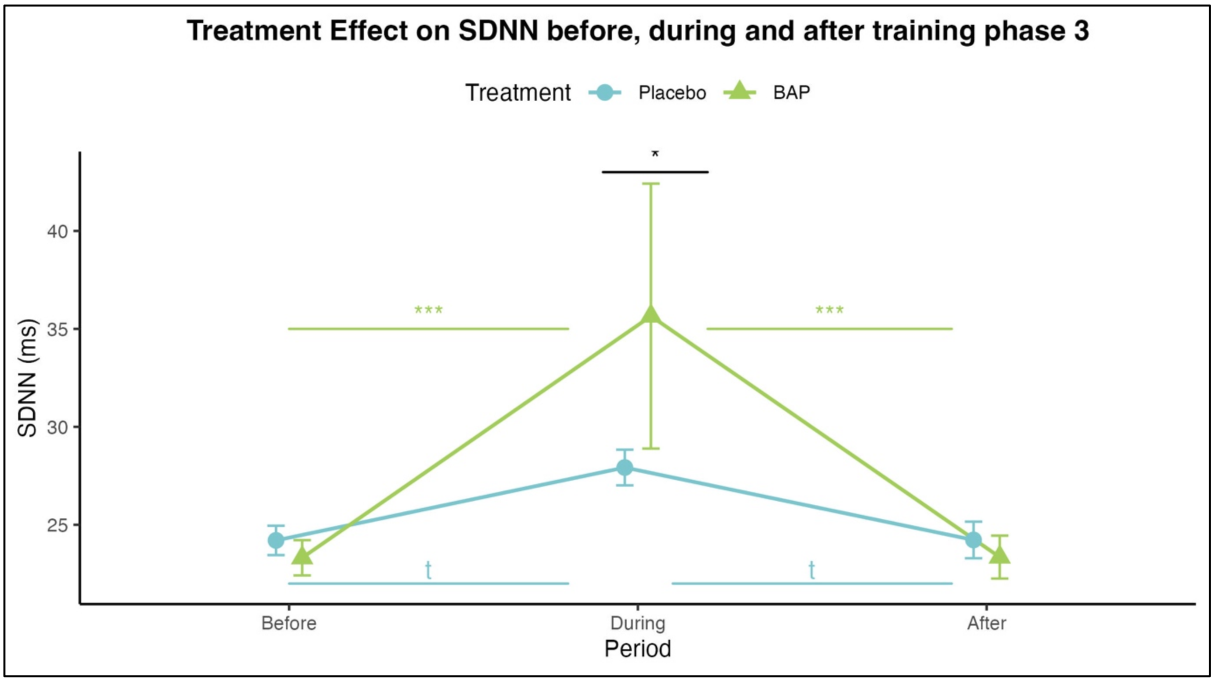

*Figure 38 Treatment effect on the standard deviation of beat to beat of normal sinus beats (SDNN) of calves receiving bovine appeasing pheromone (BAP) or placebo before, during and after training phase 3. Probability symbols: t p>0.05 - ≤0.10, * p<0.05, *** p<0.001.*

3.1.3.3. RMSSD

Overall, no treatment effect on RMSSD was observed between the BAP (8.56±2.82 ms) and Placebo (8.85±5.98 ms) groups (X^2=1.44, df=1, p=0.23). A tendency was observed for the treatment x point of measurement interaction (X^2=4.89, df=2, p=0.09). Calves treated with BAP tended to have lower RMSSD values before training compared to the values observed during training (p=0.07); and the levels of RMSSD were significantly higher during the training sessions compared to the values detected after (p=0.03) (Figure 39).

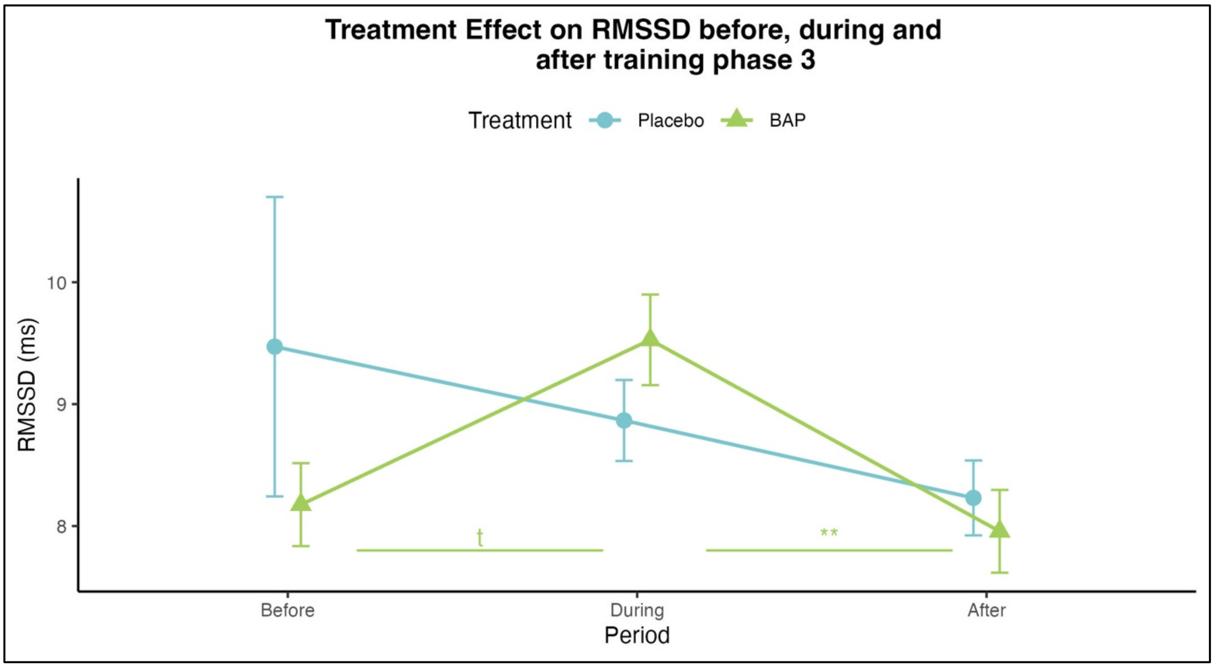

*Figure 39 Treatment effect on RMSSD values of calves receiving bovine appeasing pheromone (BAP) or placebo before, during and after training phase 3. Probability symbols: t >0.05 - ≤ 0.10, ** p<0.01). Error bars represent the SEM*

3.1.3.4. Mean Heart Rate

No significant effects or interactions were detected for mean heart rate during training phase 3.

3.1.3.5. Stress Index

No overall treatment effect was observed for SI between the two treatment groups (BAP 20.75±8.33, Placebo 22.68±19.96, X^2=<0.01, df=1, p=0.99). A treatment x period of measurement interaction tendency was observed (X^2=5.58, df=2, p=0.06). Calves in the pheromone group had significantly lower levels of SI after the training sessions compared to their placebo counterparts (p=0.04) (Figure 40). Calves in this same group had lower SI baseline values compared to those during the training sessions (p<0.01), and levels after tended to be lower compared to values during training (p=0.06) as observed in figure 39. On the other hand, calves in the placebo group had SI levels significantly higher after both baseline (p<0.01) and during training sessions (p<0.001) (Figure 40)

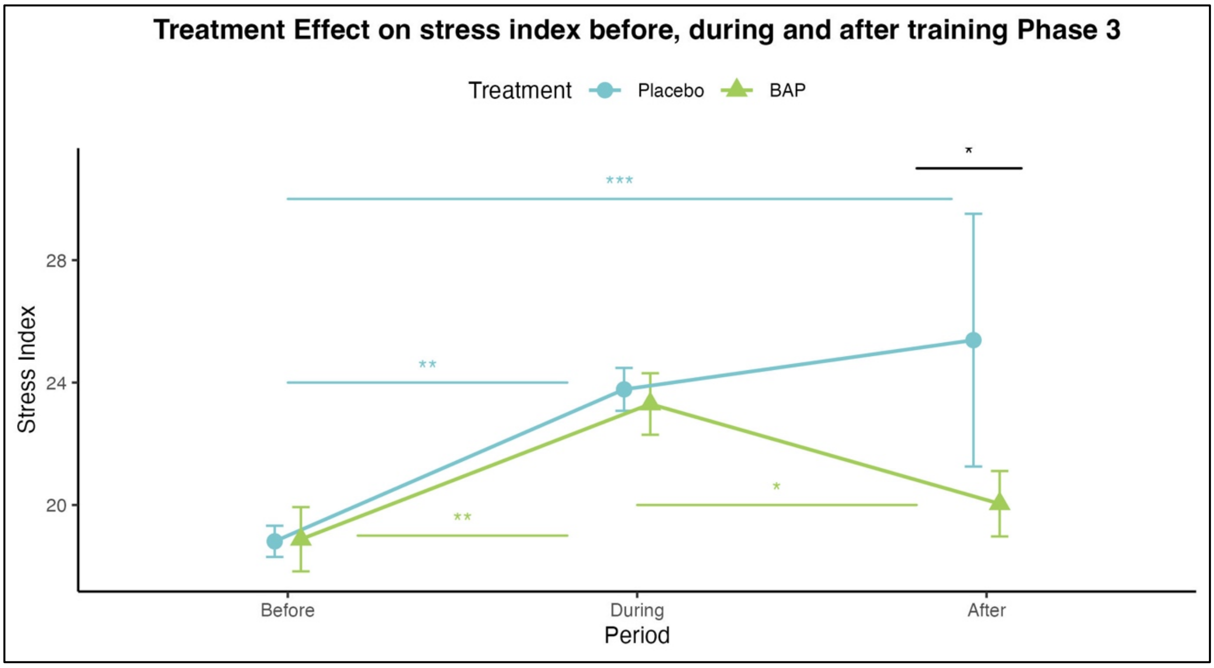

*Figure 40 Treatment effect on the Stress Index of calves receiving bovine appeasing pheromone (BAP) or placebo before, during and after in training phase 3. Probability symbols * p<0.05, ** p<0.01, *** p<0.001. Error bars represent the SEM.*

3.1.3.6. Low Frequency and LF/HF ratio

No treatment effect or interactions were observed for LF and LF/HF ration during training phase 3.

3.1.4. Phase 4

Calves were around 40.75±4.61) days old at the beginning of training phase 4., and no significant differences were observed between treatments (BAP 40.83±4.67 days, Placebo 40.67±4.54, p=0.79). Calves required on average 5.46±1.67 sessions to achieve the expected response, and no treatment differences were observed between calves treated with BAP (5.50±01.51) or the placebo (5.42±1.83) (p=0.93).

3.1.4.1. Salivary Cortisol

No treatment effect were observed between calves on the BAP (0.27±0.34 µg/dL) and placebo groups (Placebo 0.28±0.36 µg/dL), X^2=0.40, df=1, p=0.53). Both groups had significantly higher levels of cortisol after the training sessions compared to the baseline (p<0.0001).

3.1.4.2. SDNN

No overall treatment effect was detected for SDNN values (BAP 23.06±7.83 ms, Placebo 23.89±20.75 ms, X^2=0.15, df=1, p=0.0.71), And no interaction between treatment and measuring point was observed (X^2=1.77, df=2, p=0.41).

3.1.4.3. RMSSD

No treatment effect on RMSSD was observed between the BAP (8.12±2.58 ms) and Placebo (7.50±2.62 ms) groups (X^2=0.54, df=1, p=0.46) overall. Although no treatment x period of measurement interaction was detected (X^2=1.25, df=2, p=0.54), it was observed in the within treatment comparison that calves treated with BAP had lower RMSSD values during training sessions compared to the values observed after training (p=0.0.03). RMSSD values were also lower after training compared to the baseline in the pheromone group (p<0.001). In the placebo group RMSSD values tended to be lower during the training sessions compared to the baseline (p=0.06) (Figure 41).

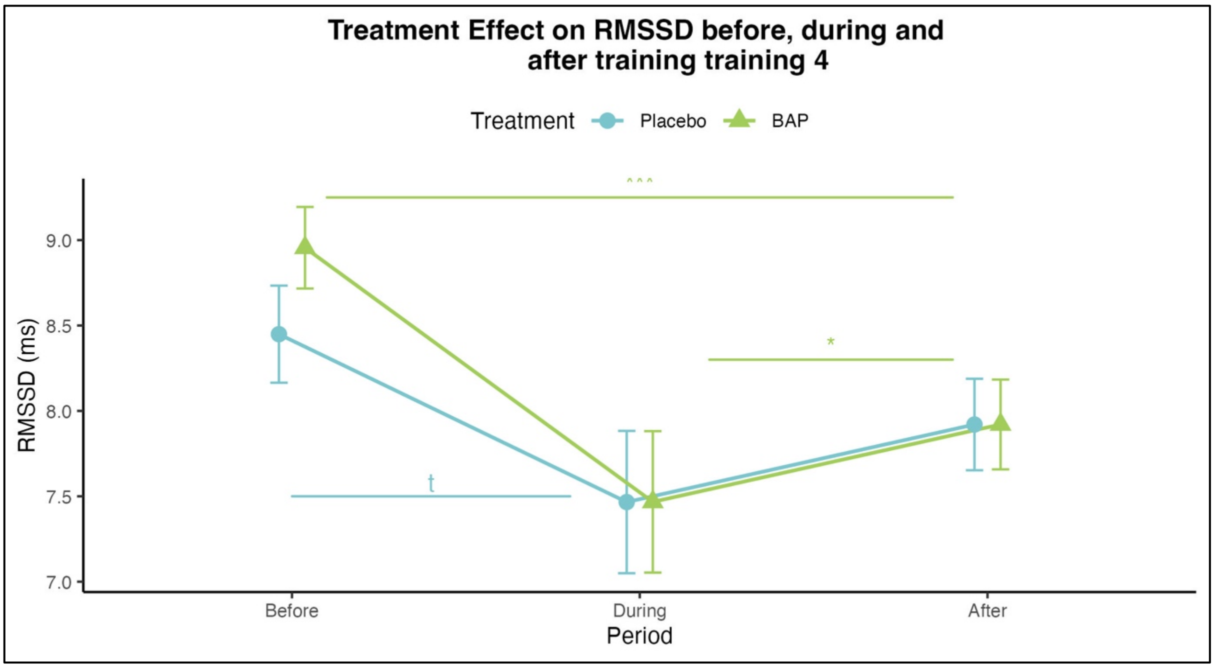

*Figure 41 Treatment effect on RMSSD values of calves receiving bovine appeasing pheromone (BAP) or placebo before, during and after training phase 4. Probability symbols: t >0.05 - ≤ 0.10, * p<0.05, *** p<0.001. Error bars represent the SEM*

3.1.4.4. Mean Heart Rate

No significant effects or interactions were detected for mean heart rate during training phase

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3.1.4.5. Stress Index

No treatment effect or interactions were observed for SI duing training phase 4.

3.1.4.6. Low Frequency and LF/HF ratio

No treatment effect or interactions were observed for LF and LF/HF ration during training phase 3.

3.2. Cognitive bias test

Table 8 summarises the results for the Go /No go responses to the different colour cues.

Table 8 Summary of Go / No go responses of 24 calves during a cognitive bias test. Descriptive statistics are expressed in mean and standard deviation

Cue colour	Outcome Variable	Positive colour	Treatment				Model	X^2	P value
			BAP		Placebo				
			Go	No Go	Go	No Go			
Red	Response	Red	36 (50%)	0	36 (50%)	0	GLMM NB	0	1.00
		White	10 (13.89%)	26 (36.11%)	8 (11.11%)	28 (38.89%)			
	Latency	Red	4.85 (2.55)	NA	4.71 (4.45)	NA	LMM LT	0.90	0.34
		White	3.31 (1.23)	4.68 (2.44)	5.33 (4.98)	5.01 (3.26)		0.17	0.68
White	Response	Red	4 (5.56%)	32 (44.44%)	2 (2.78%)	34 (47.22%)	GLMM NB	0	1.00
		White	36 (50%)	0	36 (50%)	0			
	Latency	Red	4.68 (2.62)	5.36 (2.88)	9.17 (5.91)	5.02 (3.41)	LMM LT	0.33	0.57
		White	3.75 (3.18)	NA	3.99 (1.98)	NA		0.99	0.32
Ambiguous 75%	Response	Red	10 (41.67%)	2 (8.33%)	10 (41.67%)	2 (8.33%)	GLMM NB	0.12	0.73
		White	2 (8.33%)	10 (41.67%)	3 (12.5%)	9 (37.5%)			
	Latency	Red	4.02 (2.07)	11.30 (1.52)	3.25 (1.41)	4.00 (0.21)	LMM	6.51	0.01*
		White	3.34 (0.52)	4.15 (2.02)	3.23 (0.10)	4.37 (1.86)			
Ambiguous 50%	Response	Red	9 (37.5%)	3 (12.5%)	6 (25%)	6 (25%)	GLMM NB	0.07	0.79
		White	5 (20.83%)	7 (29.17%)	3 (12.5%)	9 (37.5%)			
	Latency	Red	4.28 (1.77)	5.92 (2.56)	3.86 (1.97)	3.83 (1.62)	LMM	0.18	0.68
		White	3.49 (0.58)	4.25 (0.97)	3.65 (0.67)	4.18 (1.86)			
Ambiguous 25%	Response	Red	4 (33.33%)	8 (16.67%)	4 (33.33%)	8 (16.67%)	GLMM NB	0.07	0.79
		White	8 (33.33%)	4 (16.67%)	9 (37.5%)	3 (12.5%)			
	Latency	Red	8.04 (5.97)	5.99 (2.82)	6.76 (3.15)	5.35 (3.73)	LMM LT	0.29	0.59
		White	2.6 (1.04)	3.82 (1.83)	5.66 (3.57)	5.43 (2.51)			

3.2.1. Ambiguous 75% cue

As observed in table 8 calves with positive colour red chose the Go response for the ambiguous 75% cue significantly more than the No Go response, and this choice was seen equally in both treatment groups (BAP and Placebo). However, the latency for the No Go response was significantly lower for calves in the placebo group compared to those receiving BAP ($p<0.01$).

For calves trained to respond positively to the colour white, those on the pheromone group tended to choose the No Go response more, but this observation was not statistically significant. Latency times were not significantly different between treatments either.

3.2.2. Ambiguous 50% cue

No treatment effect was observed between the BAP and placebo groups for the Go/No Go response to ambiguous 50% cue, and the same can be same for latency times.

3.2.3. Ambiguous 25% cue

No differences were observed on the Go / No Go response for calves in both treatment groups. However, when analysing the latency times, for calves who were trained to respond positively to the colour white, those on the placebo group took longer to choose the Go response compared to the calves treated with the pheromone ($p=0.03$).

3.2.4. Red cue

Calves who were trained with red as the positive colour always displayed a Go response to the red cue, and this was the same for both calves treated with the placebo and the pheromone. For those calves trained to respond positively to the colour white, 13.89 % of calves in the pheromone group and 11.11% in the placebo group chose the Go response, even though they were taught that touching this colour would get them no reward. However, they were no significant differences between treatments for both Go / No Go responses or latency times.

3.2.5. White cue

Calves trained to respond with a Go response to the white colour, always chose the Go response during the cognitive bias test and latency times were not significantly different between treatment. Calves whose positive colour was red, chose mostly the No Go response, but some mistakes were made. Go / No go responses and latency times were not significantly different between treatments.

3.2.6. Neuroendocrine activation variables

Table 9 summarises the results for outcome variables depending on the period or timepoints of measurement and treatment.

Table 9 Neuroendocrine outcome variables obtained from 24 dairy calves in a cognitive bias test. Descriptive statistics represent the mean and standard deviation.

Variable	Timepoint	Treatment		Model	X^2	df	P value
		BAP	Placebo				
SDNN (ms)		22.87 (7.54)	23.50 (8.92)	LMM	0.51	1	0.48
	Before	20.79 (6.21)	23.04 (9.53)				
	During	28.19 (7.79)	26.73 (10.90)		0.74	2	0.69
	After	19.63 (5.92)	20.74 (4.87)				
RMSSD (ms)		8.21 (2.07)	8.48 (2.58)	LMM Log transformed	0.24	1	0.63
	Before	8.70 (1.77)	9.27 (2.36)				
	During	8.10 (2.73)	8.37 (3.54)		0.18	2	0.92
	After	7.84 (1.63)	7.80 (1.39)				
Mean HR (bpm)		103.03 (38.18)	107.81 (38.55)	LMM Log transformed	1.77	1	0.18
	Before	73.42 (14.52)	83.83 (24.33)				
	During	151.17 (11.72)	142.58 (31.15)		6.78	2	0.03*
	After	84.50 (20.77)	97.00 (33.07)				
Stress Index		19.18 (5.89)	25.93 (34.71)	GLMM NB	3.81	1	0.05t
	Before	15.41 (3.42)	32.63 (60.33)				
	During	23.49 (6.13)	24.62 (9.00)		2.73	2	0.26
	After	18.63 (4.98)	20.61 (5.54)				
LF (Hz)		385.64 (327.6)	371.25 (279.7)	LMM Log transformed	1.81	1	0.18
	Before	253.42 (177.0)	398.92 (340.7)				
	During	687.50 (386.3)	475.42 (299.6)		5.11	2	0.08t
	After	216.00 (104.9)	239.42 (107.0)				
LF/HF ratio		9.37 (5.11)	10.10 (5.19)	LMM Log transformed	0.01	1	0.91
	Before	11.13 (5.43)	10.60 (2.91)				
	During	7.46 (6.10)	7.42 (5.60)		0.33	2	0.85
	After	9.51 (3.02)	12.27 (5.72)				
SC (µg/dL)		0.18 (0.15)	0.24 (0.10)	LMM Log transformed	0.41	1	0.52
	Before	0.10 (0.13)	0.07 (0.05)				
	After	0.25 (0.15)	0.37 (0.26)		0.68	1	0.41

3.2.6.1. SDNN

No overall treatment effect was observed for SDNN values during the cognitive bias test. When within treatments analysis were conducted, it was observed that calves receiving the pheromone tended to have lower baseline SDNN values compared to those observed during the test (p=0.06), and the levels after the test were significantly lower compared to those obtained during the test (p=0.02) as observed in Figure 42. This phenomenon was not observed in the calves treated with the placebo.

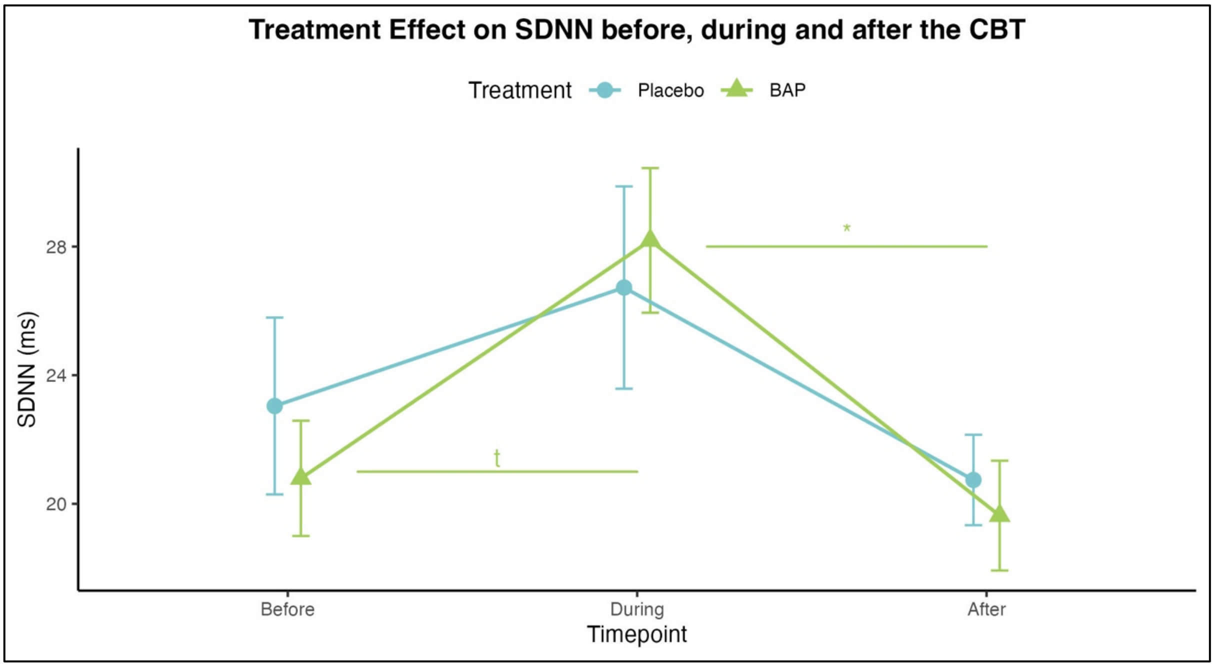

*Figure 42 SDNN values for 24 calves, before, during and after a CBT. Probability symbols: t p>0.05 - ≤0.10, * p<0.05.*

3.2.6.1. RMSSD

No treatment effect or interactions were detected for RMSSD values.

3.2.6.2. Mean heart rate

No overall treatment effect was observed for mean heart rate metrics. However, a treatment x period of measurement interaction was detected (X2=6.78, df=2, p=0.03). Both, calves receiving placebo and BAP had significantly higher mean HR during the cognitive bias test. A tendency was observed to in the placebo group (p=0.10), where the final mean HR was higher than the baseline values as observed in Figure 43.

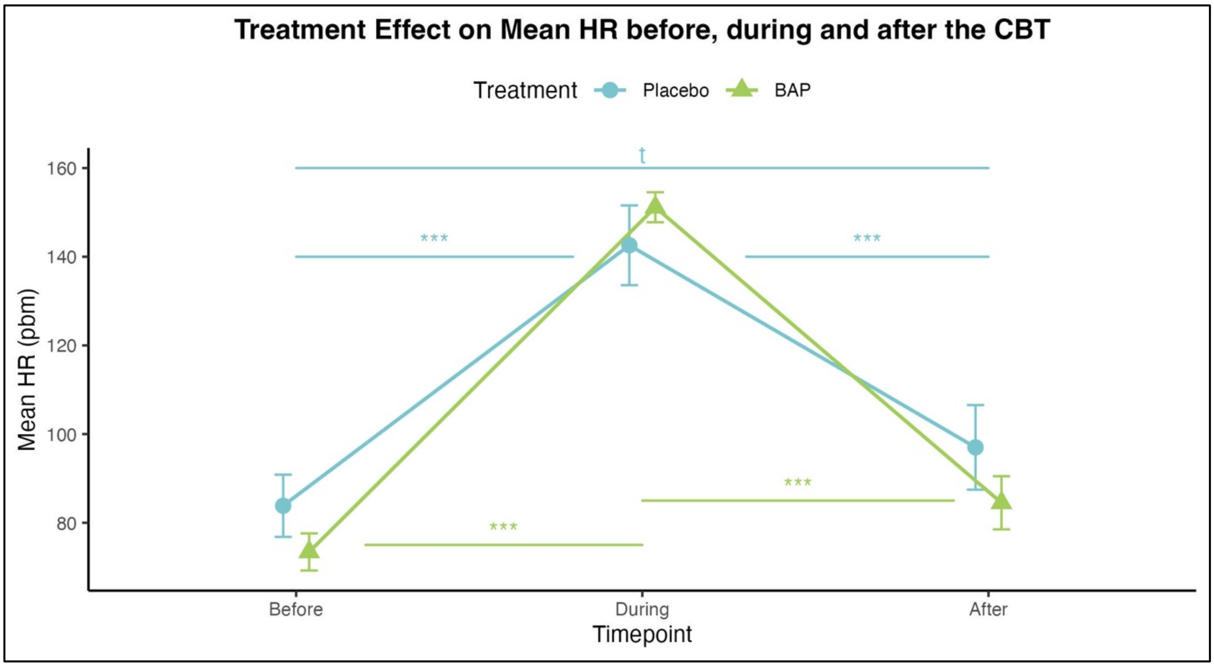

*Figure 43 Mean heart rate of 24 calves, before, during and after a CBT. Probability symbols: t p>0.05 - ≤0.10, *** p<0.001.*

3.2.6.3. Stress index

A tendency for treatment effect was observed for the SI (X^2=3.81, df=1, p=0.05). As observed in Figure 44a, calves treated with the placebo tended to have higher SI levels than those treated with the pheromone. This tendency was due to a tendency in baseline levels, where calves on the placebo group having higher levels of SI compared to their BAP counterpart (p=0.05) (Figure 44b).

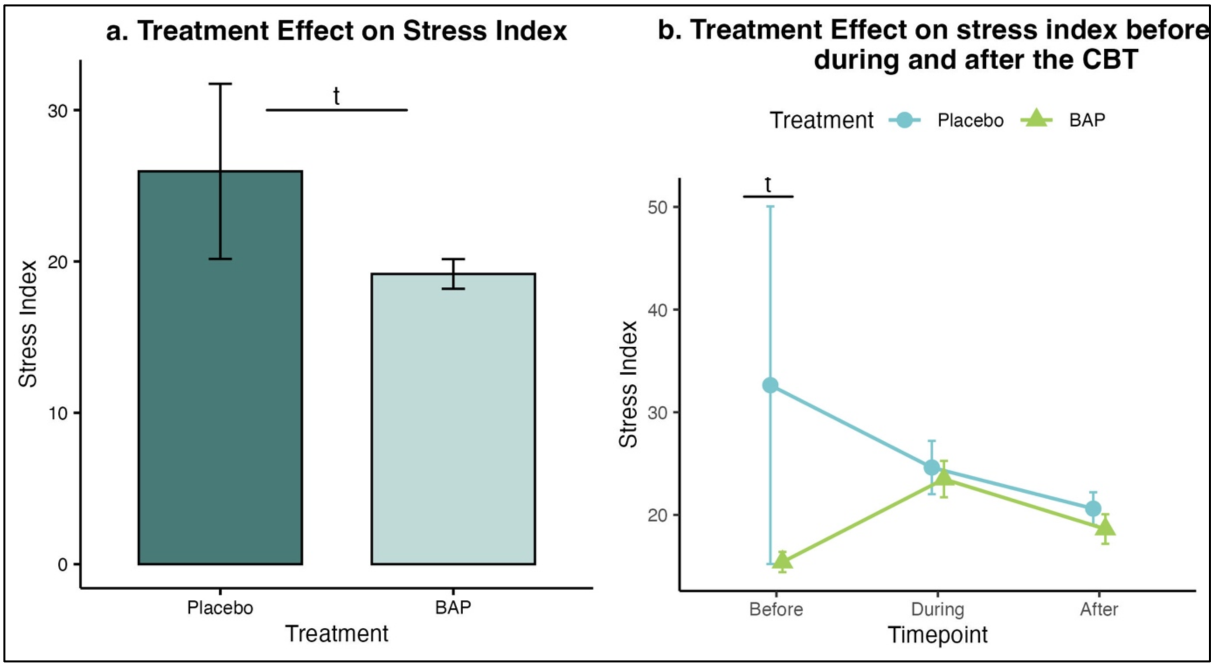

Figure 44 Stress Index of 24 calves, before, during and after a CBT. Probability symbols: t p>0.05 - ≤0.10.

3.2.6.4. Low frequency

In general, no treatment effect was observed for Low frequency values. When a within treatment analysis was conducted, it was observed that calves receiving BAP had higher low frequency levels during the CBT compared to the baseline ($p<0.001$) and the values obtained after the test ($p<0.001$). In the placebo group on the other hand, only a tendency was observed with values after the test being higher to those obtained during the CBT ($p=0.05$), as observed in Figure 45.

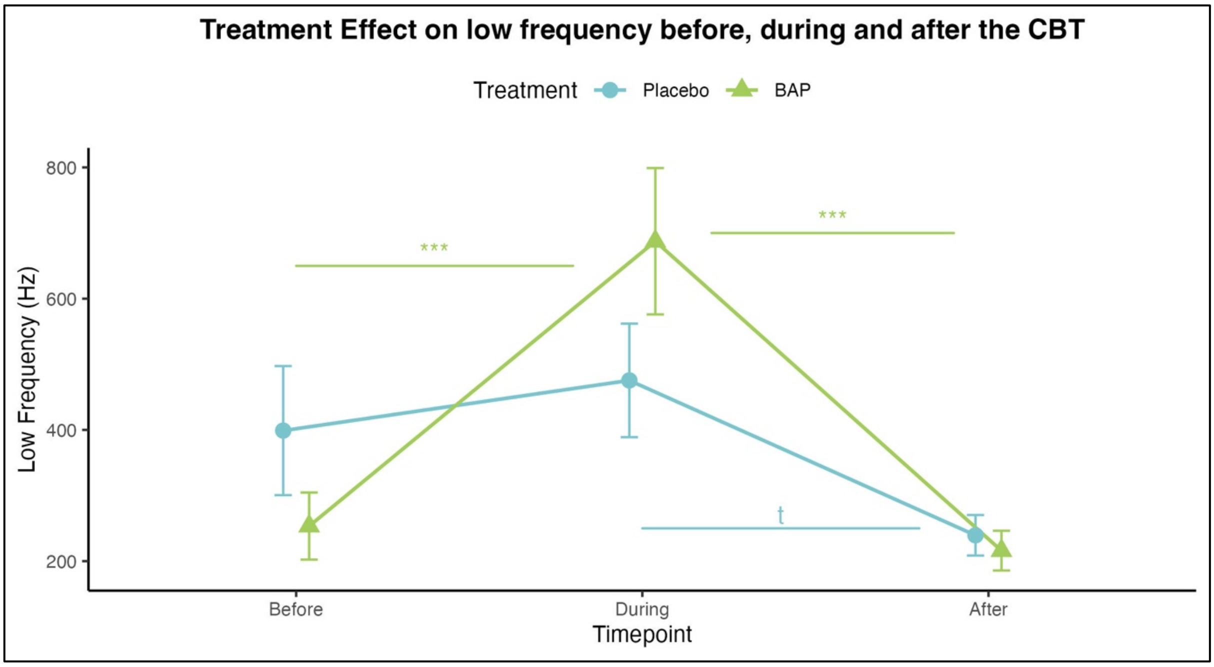

*Figure 45 Low frequency values of 24 calves, before, during and after a CBT. Probability symbols: t p>0.05 - ≤0.10, *** p<0.001.*

3.2.6.5. LF/HF ratio

No treatment effect was detected for LF/HF ratio.

3.2.6.6. Salivary cortisol

Although no overall treatment effect was detected for salivary cortisol, during the within treatments analysis it was observed that even though both the BAP ($p<0.01$) and placebo ($p<0.001$) groups had higher levels of salivary cortisol after the CBT, the level of significance was higher for the calves receiving the placebo (Figure 46).

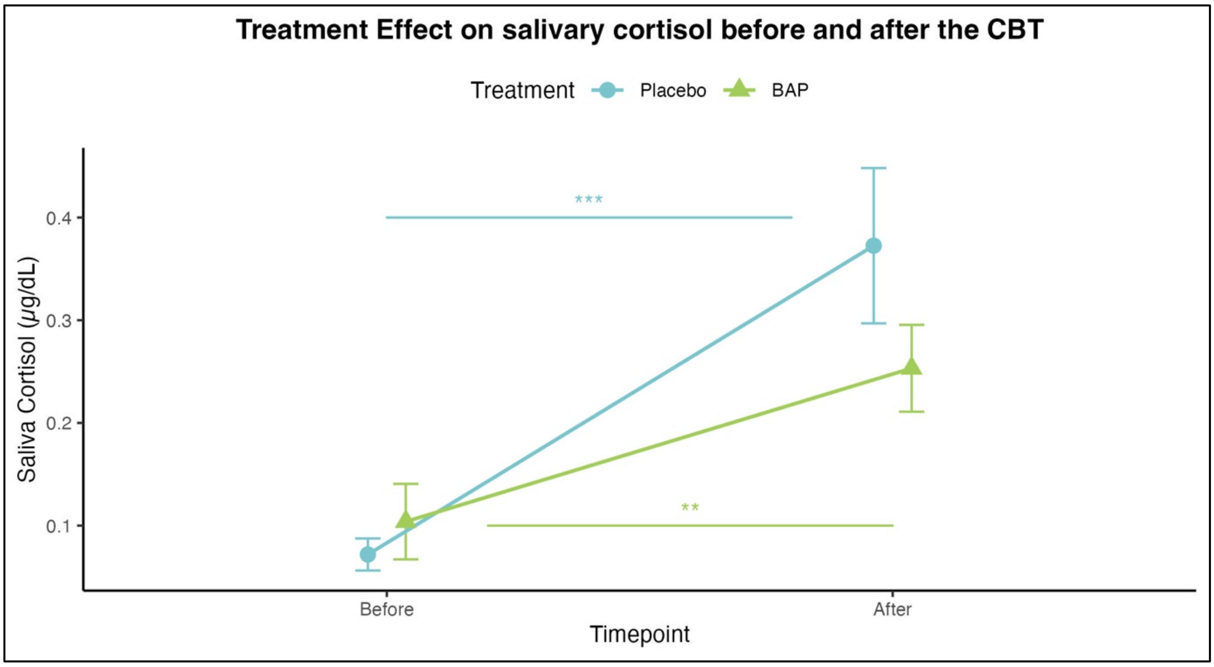

*Figure 46 Salivary cortisol concentrations of 24 calves, before, during and after a CBT. Probability symbols: *p<0.01, *** p<0.001.*

4. Discussion

An important consideration in the interpretation of the findings in this study, is the potential influence of calf age on learning and stress responses (Nawroth and Rørvang, 2022c; Rørvang and Nawroth, 2021). In this study, age was assessed as a potential confounding factor during all training phases, and no significant differences in the age of calves between the BAP and placebo treatment groups were found. This consistency indicates that age did not interfere with the outcomes related to training performance, cognitive bias responses, or physiological stress indicators. The similarity in age distribution across treatment groups strengthens the internal validity of the results by confirming that observed differences in learning, emotional states, or stress responses were not due to age-related variability. Therefore, the effects noted in the study can be more confidently attributed to the treatment itself rather than differences in developmental stages among the calves. This finding aligns with previous research that highlights the importance of controlling for age in studies involving young animals, as age can influence cognitive capabilities and stress resilience (Gaillard et al., 2014b; Lecorps et al., 2020). By ensuring that age did not vary significantly between groups, this study provides a clearer interpretation of the specific impact of BAP on dairy calves' learning and emotional state.

4.1. Training stage

The training phase of this study aimed to evaluate the impact of a synthetic analogue of BAP on calves' learning progression, as measured by the number of training sessions needed to reach the learning criteria. Calves in this study required around 17 sessions to reach the expected response, which is similar to finding in other studies were calves had to learn to differentiate between two different cues (Daros et al., 2014a; Lecorps et al., 2020). Overall, there were no significant differences between BAP and placebo groups in the number of sessions required to achieve the expected responses across all training phases. This finding is consistent with prior studies that have observed limited or no direct effects of appeasing pheromones on the rate of cognitive performance (Mengoli et al., 2014). However, Gaillard et al. (2014) observed, that whilst both individually housed and pair-housed calves were able to learn the initial colour discrimination task at similar rates, pair-housed calves demonstrated better performance in the reversal task, adapting more easily when the training stimuli were reversed. This is an interesting finding that could be further investigated with BAP.

Key physiological stress indicators such as salivary cortisol levels and HRV metrics (SDNN, RMSSD, mean heart rate, frequency domains and stress index) provided information into the calves' stress responses throughout the training. While no overall significant treatment effect was found for salivary cortisol levels, it was evident that both groups experienced increased cortisol after training, aligning with findings from other species, where training, even if using only positive reinforcement techniques, (Hambrecht et al., 2021) induces acute stress responses. However, it is important to consider the limitations of interpreting elevated cortisol solely as a negative indicator of stress. While cortisol is commonly associated with physiological stress responses, its increase during training may not necessarily reflect distress. Instead, it could represent a state of heightened arousal, engagement, or learning, as cortisol can also be released in response to cognitively challenging or stimulating experiences (Otovic and Hutchinson, 2015). For instance, elevated cortisol levels have been observed in animals during positive, rewarding activities that require attention and effort, suggesting that increases in cortisol might reflect an adaptive response to training demands rather than negative stress (Novak et al., 2013). Consequently, while salivary cortisol remains a valuable tool for assessing physiological responses, it should be interpreted alongside behavioural observations and other physiological indicators to distinguish between negative stress and positive engagement in learning contexts.

Both SDNN and RMSSD values showed interesting results during the different training phases, calves in the BAP group demonstrated higher SDNN and RMSSD values during some training phases compared to baseline and post-training periods. For instance, calves receiving a placebo tended to have lower SDNN levels after training during phase one compared to values observed during training, phenomenon that was not observed in calves treated with BAP. On the other hand, calves in the BAP group had significantly higher SDNN levels during training compared to baseline and post-training levels in training phases 2 and 3, compared to calves receiving a placebo. In regards to RMSSD levels, calves treated with a placebo had significantly higher RMSSD levels during training in training phase one, whereas, BAP treated calves had similar RMSSD levels before, during and after the training session. In training phase 2, calves receiving BAP tended to have higher RMSSD values during training compared to levels measured afterwards. Similar results were observed in training phase 3, with BAP treated calves having significantly higher RMSSD levels during training. These observations however, were not seen in calves treated with the placebo. In training phase 4, calves in the BAP group had higher RMSSD values both before and after the training session, whereas the calves treated with a placebo only displayed a higher tendency on RMSSD levels before the training session. Overall, calves in the BAP group showing higher SDNN and RMSSD during training suggest enhanced parasympathetic regulation and possibly better stress resilience compared to the placebo group (von Borell et al., 2007). Based on these observations, tt seems that BAP may enhanced the parasympathetic activity more effectively during the active phases of training, which could be indicative of better adaptation to physical stress or a more robust stress response system in these calves. These findings align with the results obtained by Mengoli et al. (2014), where the fouls treated with EAP showed higher standard deviation of RR intervals (SDRR), which is a measure of HRV, compared to the placebo group. This suggests that the EAP treatment may have contributed to a more balanced autonomic nervous system response during the cognitive tasks, potentially indicating a more relaxed state despite the cognitive demands. In contrast, the placebo group exhibited lower HRV, which could be associated with increased sympathetic nervous system activity and stress during the cognitive tasks. Additionally, in the current study, whilst no overall treatment effect was detected for stress index parameters, there was a notable interaction observed in Phase 3, where calves receiving BAP exhibited lower SI values after training compared to the placebo group. This points to the potential of BAP to aid in post-training recovery, thereby modulating stress responses more effectively. Furthermore, baseline SI levels in the placebo group were significantly higher than during training, indicating that calves not exposed to BAP might have experienced greater pre-training stress. This also supports the potential effect of the pheromone on mitigating the activation of the SAM response pathway during challenging situations (Schubach et al., 2020).

Mean heart rate did not show significant treatment effects across training phases, which may imply that while BAP influenced variability in HRV metrics, it did not substantially impact the overall heart rate. This could suggest that BAP's effects are more nuanced, enhancing autonomic regulation rather than altering heart rate directly.

The low-frequency component of HRV reflects both sympathetic and parasympathetic activity (Mohr et al., 2002; von Borell et al., 2007). A significant treatment effect was observed for LF in some phases, suggesting that calves treated with BAP had higher LF values before and after training compared to during training. This could indicate that BAP-treated calves maintained a better balance between sympathetic and parasympathetic nervous activity, potentially contributing to their resilience during training sessions.

4.2. Cognitive Bias Test

The cognitive bias test aimed to determine the influence of BAP on calves' emotional states by assessing their responses to ambiguous stimuli. Overall, calves demonstrated consistent "Go" responses to their trained positive colours, and no significant treatment effects were found in the Go/No-Go responses for most cues. However, the responses to ambiguous cues revealed subtle differences that merit discussion.

Calves treated with BAP and assigned red as the positive colour exhibited a higher latency compared to calves in the placebo group, when choosing a No Go response to the 75% ambiguous cue. This might suggest that calves receiving the pheromone took more time to decide not to touch the 75% ambiguous cue as they were in a more positive emotional estate than the placebo calves, evidenced by Mengoli et al. (2014) who reports in their study using the equine appeasing pheromone, the researchers interpreted the longer latency of choice observed in the EAP group during incorrect choices as indicative of a more deliberate decision-making process, suggesting that horses in the EAP group may have taken more time to evaluate the ambiguous cues before making a choice, displaying higher emotional regulation that allowed the horses to better assess their options before acting. On the other hand, for the 25% ambiguous cue, calves trained to respond positively to white in the placebo group exhibited longer latencies for the Go response compared to the BAP group. This could suggest that BAP may reduce hesitation or indecision when faced with choices more similar to the colour they were trained to positively respond to, potentially indicating a less negative emotional state during ambiguous situations. This faster response to correct cue was also observed in the study by (Kremer et al., 2022; Mengoli et al., 2014) This observation is relevant as latency times can reflect the level of cognitive and emotional conflict an animal experiences when faced with uncertainty (Kremer et al., 2022). However, in Lecorps et al.’s, (2020) study, calves that were more pessimistic displayed greater overall latencies to touch the ambiguous locations, which they interpreted as an indication of a more negative emotional state. The study quantified this response by calculating a pessimism score based on the latencies recorded during the tests. None the less, calves in this study were tested after they were disbudded with a hot iron and, therefore, were experiencing pain; whereas the calves in both the current study and Mengoli et al.’s, (2014) research where not exposed to negative situations.

Salivary cortisol levels showed an increase in both groups post-CBT, but the elevation was more pronounced in the placebo group, indicating a higher arousal levels. This supports the notion that BAP may modulate stress responses during cognitively demanding tasks, contributing to reduced physiological arousal. The HRV data further reinforced this, with significant differences in mean heart rate and SI, particularly in baseline measurements. BAP-treated calves maintained more consistent mean heart rates compared to placebo-treated calves, which displayed higher variability and stress levels. SDNN values during CBT for BAP-treated calves were significantly higher during the test compared to baseline and post-test periods, suggesting that BAP might enhance autonomic stability during challenging situations. This was not observed in the placebo group. Although the overall treatment effects on the LF/HF ratio were not significant, within-group analyses revealed that BAP-treated calves had higher low-frequency HRV during the CBT which may suggest enhanced sympathetic and parasympathetic activity regulation during cognitive challenges. The stress index showed a tendency for higher baseline values in placebo-treated calves, reinforcing the hypothesis that BAP supports emotional resilience. This pattern on physiological stress responses has also been observed in the studies mentioned in previous chapters.

5. Conclusion, implications and Future Directions

This study's findings emphasise the complex nature of BAP in regulating stress and emotional conditions during training and cognitive assessments. Although direct improvements in learning performance were not apparent, BAP seemed to affect physiological stress indicators and responses in uncertain circumstances, which could promote emotional resilience and optimism. Further research should examine the long-term effects of BAP and assess how these findings might be incorporated into animal welfare policies, especially in different environmental and social contexts. Increasing the sample size and doing multi-phase cognitive assessments may provide more conclusive understanding into the subtle impacts of appeasing pheromones on animal cognition and emotional well-being.

CHAPTER 7. GENERAL DISCUSSION

6. Introduction

The modern practice of dairy calf rearing is heavily influenced by intensive farming practices aimed at optimizing productivity, often involving artificial rearing systems where calves are separated from their dams shortly after birth. While this approach facilitates disease control and allows for individualized management (Medeiros et al., 2022), it introduces stressors such as early separation, social isolation, restricted diets, and exposure to routine painful procedures like disbudding (Barkema et al., 2015; Costa et al., 2019). These conditions are known to adversely impact the well-being of calves, posing significant challenges for animal welfare (Cantor et al., 2019; Moore et al., 2012).

One innovative approach to mitigating stress in livestock is the use of appeasing pheromones, which have shown promise in various domestic species (Mengoli et al., 2014). However, research on the effects of bovine appeasing pheromone (BAP) in dairy calves remains sparse. This thesis aimed to fill this gap by investigating whether BAP can modulate stress responses, promote emotional stability, and potentially improve learning capabilities in dairy calves subjected to standard artificial rearing practices.

To assess the welfare of the animals, different stress indicators were used, such as performance, both on growth rate and response to disease. The physiological stress response in calves is primarily measured through biomarkers such as salivary cortisol and heart rate variability (HRV), which reflect activation of the hypothalamic-pituitary-adrenal (HPA) axis and the autonomic nervous system (von Borell et al., 2007), and behavioral indicators in the form of activity levels. These indicators provide comprehensive insight into how stress impacts health outcomes. Furthermore, behaviour-based assessments, including cognitive bias tests, enable evaluation of emotional states and decision-making processes, contributing to an understanding of the animal's overall welfare (Lecorps et al., 2020; Nawroth et al., 2019).

7. Original contribution to knowledge

This thesis makes several notable contributions to the current understanding of calf welfare and the role of BAP in managing stress and improving behavioural outcomes:

BAP has been shown to modulate stress in livestock, particularly during stressful management practices such as weaning and transport. For instance, research has demonstrated that BAP administration can lead to reduced cortisol levels and improved stress markers in beef calves during weaning and transport periods (Colombo et al., 2020; Cooke et al., 2020b; Schubach et al., 2020). In dairy calves, Angeli et al. 2020) observed that BAP-treated calves exhibited better weight gain prior to weaning compared to those receiving a placebo. Additionally, while disease incidence did not change, pharmacological costs were reduced, and ill BAP-treated calves maintained growth performance similar to healthy counterparts, suggesting improved recovery and resilience.

In chapter 3 we investigated the effects of BAP on growth rates, physiological stress indicators, and behavioural responses in dairy calves from birth through weaning. Previous studies had shown that weaning is a critical stress point for calves, often resulting in weight loss or slowed growth (De Passillé et al., 2011; Eckert et al., 2015). In this study, BAP-treated calves displayed a higher ADG post-weaning, with a 0.15 kg increase in ADG compared to the placebo group. This finding aligns with previous research demonstrating that maternal pheromones can mitigate weaning stress and enhance growth rates in both dairy and beef calves (Angeli et al., 2020; Colombo et al., 2020; Schubach et al., 2020). The study also highlighted the physiological effects of BAP on stress modulation, particularly through cortisol and HRV metrics. While no overall differences were observed in salivary cortisol levels, BAP-treated calves exhibited lower cortisol levels post-weaning than placebo-treated calves, suggesting a calming effect of the pheromone during stressful transitions. This aligns with studies in which BAP reduced cortisol responses in beef calves during weaning (Colombo et al., 2020). Additionally, the HRV metrics, specifically, the higher SDNN and RMSSD values in BAP-treated calves; further supported BAP's stress-reducing effects. These measures, positively associated with parasympathetic activity (Mohr et al., 2002; von Borell et al., 2007), indicated a decrease in sympathetic arousal among BAP-treated calves.

Behavioural observations also provided insights into BAP's effects on calf welfare during weaning. Previous research has shown that weaning reduces resting time (Budzynska and Weary, 2007; Eckert et al., 2015; Jasper et al., 2008). In this study, placebo-treated calves exhibited a marked reduction in lying time during and after weaning, a trend not observed in BAP-treated calves. This suggests that BAP may mitigate the disruptive effects of weaning on resting behaviours, potentially indicating lower stress levels. These findings align with previous studies where BAP facilitated stress coping behaviours in beef calves (Schubach et al., 2020). Collectively, these findings suggest that BAP can modulate stress responses, improve growth performance, and positively influence behaviour, enhancing welfare during weaning.

In chapter 4 we evaluated the effects of BAP on the welfare of calves during disease episodes. The passive immune transfer values observed in this study were adequate for both treatment groups, with total serum protein (TSP) levels exceeding 5.5 g/dL (Fischer et al., 2019). This indicates that any differences found between the BAP and placebo groups in other measured outcomes were not influenced by passive immune transfer deficiencies. The study found no significant differences in CDI or disease incidence rates (DIR) between treatment groups, mirroring findings by Angeli et al. (2020). However, there is evidence from Hervet et al. (2021) that BAP can reduce respiratory signs over time, suggesting potential delayed benefits of BAP. The observed lack of immediate clinical impact contrasts with the physiological findings that showed BAP-treated calves had more stable HRV parameters, indicating a stress-buffering effect. HRV metrics like SDNN and RMSSD, known for their associations with lower stress and higher parasympathetic activity (Kovács et al., 2014; von Borell et al., 2007), were consistently higher in BAP-treated calves, supporting previous research on BAP's calming effects (Schubach et al., 2020). Notably, HRV parameters showed that BAP-treated calves maintained more adaptive stress responses during illness, a critical welfare aspect given the importance of recovery from stress (Colditz and Hine, 2016; Salak-Johnson and McGlone, 2007). Seasonal interactions also revealed that BAP had stabilizing effects on HRV during winter, reducing the stress impacts related to thermoregulation (Roland et al., 2016; Silva and Bittar, 2019). Behavioural data indicated that whilst no overall treatment effects were found on activity levels, there were significant seasonal variations in activity measures. BAP-treated calves displayed less variability in activity, suggesting better stress management under environmental challenges. The placebo group exhibited higher motion index and step counts during summer, possibly linked to discomfort or behavioural responses to heat stress, aligning with studies on thermoregulatory behaviour in calves (Dado-Senn et al., 2022). In summary, while BAP did not significantly alter disease incidence or growth rates, it demonstrated benefits in stress regulation and recovery, as evidenced by stable HRV and activity patterns. These findings indicate that BAP can support better stress management in calves, particularly under challenging conditions, enhancing welfare and resilience.

In chapter 5 we investigated the impact of BAP compared to a placebo on the recovery and welfare of female dairy calves during disbudding, in addition to standard sedation, anaesthesia, and analgesia. The similarity in age at disbudding between the treatment groups ensured that any observed differences could be attributed to the treatment effects rather than age discrepancies, aligning with research that highlights age-related variations in pain perception during disbudding (Adcock and Tucker, 2018; Alessandro et al., 2018). No significant differences ADG were observed between treatment and control groups. However, a tendency for interaction effects over time suggested that BAP-treated calves may have experienced less growth disruption post-disbudding. Previous studies have shown that adequate pain management during disbudding positively influences ADG in dairy calves (Bates et al., 2015; Cuttance et al., 2019). The consistency in ADG across treatment groups, both subjected to the same anaesthesia, analgesia, and sedation protocol, suggests that any slight differences may be related to the stress-mitigating effects of BAP. Physiological stress markers, particularly HRV parameters, indicated potential benefits of BAP treatment. Consistent with earlier findings in Chapter 3, where BAP improved HRV during weaning, BAP-treated calves in this study exhibited better HRV parameters throughout the disbudding process. Improved HRV, including higher SDNN and RMSSD values, reflects enhanced parasympathetic activity and reduced stress (Stock et al., 2013; von Borell et al., 2007). The presence of higher low-frequency (LF) power and LF/HF ratios in BAP-treated calves implies a more balanced autonomic response, suggesting reduced pain perception and stress (Stewart et al., 2009). Behavioural indicators of pain and discomfort, such as increased motion index and step count, were analysed to infer restlessness post-disbudding. While no significant differences were observed in baseline MI or step counts between groups, the placebo group displayed significantly higher MI and step counts on day 1 post-disbudding, indicating heightened discomfort or reduced pain tolerance (Stafford and Mellor, 2005). Conversely, BAP-treated calves showed more gradual recovery patterns in MI and step count, potentially suggesting better post-procedural stress management. Lying time is another critical behaviour affected by (Sutherland et al., 2018). The BAP group maintained consistent lying times post-disbudding, unlike the placebo group, which exhibited increased lying times on the procedure day followed by a prolonged recovery period of up to 10 days. This finding suggests that BAP may support quicker recovery and enhanced comfort after painful procedures. Overall, while both treatment groups received effective pain management, the subtle trends in recovery patterns and physiological data indicate that BAP may offer additional stress-buffering benefits. Although the numerical differences observed were slight, they highlight BAP's potential role in managing post-procedural discomfort.

Finally, in Chapter 6 we studied the effects of BAP on the learning capabilities of dairy calves and its effect on their emotional state. No significant differences in age were observed between the BAP and placebo treatment groups, ensuring that any differences in training performance, cognitive bias responses, or stress indicators could be confidently attributed to the treatment itself rather than age variability (Lecorps et al., 2020; Nawroth and Rørvang, 2022c). This age consistency bolstered the internal validity of the study, confirming that developmental stage did not confound the findings. During the training phase, the study evaluated whether BAP influenced learning progression, measured by the number of sessions required to reach learning criteria. No significant treatment effect was noted between the BAP and placebo groups in session count, consistent with research showing limited direct impacts of appeasing pheromones on cognitive performance (Mengoli et al., 2014; von Borell et al., 2007). However, BAP-treated calves exhibited improved HRV measures, such as higher SDNN and RMSSD during training phases, suggesting better autonomic stability and stress regulation (von Borell et al., 2007). In contrast, the placebo group displayed more fluctuating HRV patterns, indicating increased stress (Mohr et al., 2002). The cognitive bias tests revealed nuanced differences in emotional responses. Calves treated with BAP took longer to respond to a 75% ambiguous cue compared to the placebo group, when trained to respond to red as the positive colour, potentially reflecting a more deliberate, positive emotional state (Mengoli et al., 2014). This observation aligns with findings that longer response times may indicate more thoughtful decision-making and higher emotional regulation (Kremer et al., 2022). Conversely, calves trained to associate white as the positive cue in the placebo group exhibited longer latencies for Go responses to the ambiguous 25% cue, suggesting a more negative emotional state during ambiguous situations (Lecorps et al., 2020). Physiological stress indicators such as salivary cortisol and HRV further supported BAP's impact. Although both groups experienced elevated cortisol post-CBT, the placebo group had higher increases, implying a greater stress response. This reinforces the idea that BAP could reduce physiological arousal during cognitive challenges. Mean heart rate analyses showed that BAP-treated calves maintained more consistent rates compared to their placebo counterparts, indicating better autonomic regulation. While no significant treatment effects were noted for the LF/HF ratio, within-group analyses highlighted higher low-frequency HRV during CBT in BAP-treated calves, suggesting balanced sympathetic and parasympathetic activity.

Overall, the findings suggest that whilst BAP did not markedly change cognitive learning rates, it did appear to enhance stress resilience and emotional regulation during challenging cognitive tasks.

8. Strengths, Weaknesses, and Future Recommendations

The studies conducted provided a robust foundation for understanding the effects of bovine appeasing pheromone (BAP) on dairy calf welfare. However, certain limitations warrant attention. Although the sample size was sufficient for initial treatment evaluations, it may not have been large enough to capture smaller, yet clinically significant, differences, particularly during procedures like disbudding. This raises concerns about potential Type II errors and underscores the need for larger sample sizes in future studies to detect more subtle variations.

The homogeneity within the treatment groups minimized confounding variables but may limit the broader applicability of findings. Future research should include more diverse and larger samples to confirm trends observed and ensure generalizability across varied farming conditions and different breeds, including male calves. Additionally, although efforts were made to control for potential confounders such as weather, age, and location, conducting the study on a commercial farm presented inherent challenges. Age standardization among calves could not be perfectly controlled, and the concentrations of pheromone post-application were not measured.

The inability to consistently sample hair from a single body site may have introduced variability in cortisol measurements, affecting interpretations of chronic stress. This issue should be addressed by refining sampling techniques to improve the accuracy of hormonal analyses.

Despite these limitations, the findings support the potential role of BAP in modulating physiological stress and promoting recovery during weaning and post-procedural stress. Future studies should consider the inclusion of untreated control groups and measurements of pheromone concentration in the environment to assess treatment exposure fully. Moreover, it is essential to examine the impacts of BAP on various physiological and behavioural parameters in different rearing conditions and across male calves to explore potential sex differences in response to treatments.

9. Conclusions and applications

The research presented in this thesis highlights the potential of BAP in managing stress and promoting welfare in dairy calves. Key findings include improved autonomic stability and better behavioural responses in BAP-treated calves, particularly during stressful periods such as weaning and post-disbudding. Although BAP did not consistently improve cognitive learning performance, its influence on stress regulation was evident.

These results suggest that BAP may support the emotional resilience of calves, promoting a more adaptive response to challenging situations. Whist the study did not show significant differences in disease incidence or overall growth rates, the positive trends observed in recovery metrics and stress-related indicators suggest that BAP may enhance the coping mechanisms of calves, potentially leading to better welfare and productivity outcomes.

The application of BAP in commercial settings could be valuable, especially when combined with existing welfare practices such as appropriate sedation, analgesia, and housing conditions. Future research should explore long-term effects on growth and productivity, age at first calving, and milk yield, as well as cost-benefit analyses to determine the economic feasibility of BAP use. Investigating the cumulative impact of early-life BAP application and its potential to enhance resilience against diseases and painful procedures remains a promising avenue for further study.

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