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Temperature asymmetries in facial areas as indicators of affective state in dairy calves and horses

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ABSTRACT

The study of emotions in the animal welfare field is essential to increase the understanding of the way animals might perceive and react to different situations and how modifications in the way they are kept may improve their quality of life. In the field of animal studies, several methods looking at physiological, behavioural and cognitive elements of emotion had been developed, but some of them with particular limitations, including the need for laboratory or clinical facilities, their invasivity, subjective interpretation or lack of standardisation for the results. For these reasons, the development of new methods that allow the non-invasive measurement of objective indicators of animal emotion is required. The current dissertation aims to identify the potential of measuring temperature asymmetries as an indicator of emotional valence using infrared thermography as a non-invasive tool. To help achieve this aim, emotions have been categorised in terms of emotional valence (positive or negative) and emotional arousal (high or low). To explain how emotions are processed in the brain a lateralised processing has been hypothesised, i.e. left hemisphere for positive and right hemisphere for negative emotions, and a relationship between the activity of the brain hemispheres and temperature asymmetries in different Regions of Interest (ROIs) has been suggested (difference between temperatures in left and right sides in bilateral areas, like the eyes, nostrils, ears and skin covering the nasal airways). For this purpose, a series of studies in dairy calves and horses looking at situations likely to elicit negative (hot-iron disbudding and separation from the mother in calves) and positive affective responses (rewarding food in horses) were carried out, yielding some interesting results, suggesting a relationship between the direction of the temperature asymmetries in some ROIs and the valence of the emotion elicited in such circumstances, and highlighting the potential of infrared thermography for the study of animal emotions.

Dedication and Acknowledgements

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AUTHOR’S DECLARATION

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's *Regulations and Code of Practice for Research Degree Programmes* and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others is indicated as such. Any views expressed in the dissertation are those of the author.

SIGNED: [redacted] DATE: 04 / 07 / 2021

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List of abbreviations

ROI - Region of Interest

ANS - Autonomous Nervous System

CNS - Central Nervous System

HPA - Hypothalamic-pituitary-adrenal system

HRV - Heart rate variability

LF - Low frequency

HF- High frequency

ECG - Electrocardiogram

SDNN - Standard deviation of normal to normal intervals

RMSSD - Root mean square of successive heartbeat interval differences

EEG - Electroencephalogram

fMRI - Functional magnetic resonance image

CN - Cranial nerve

FPS - Frames per second

ExpObs - Experienced observers

InexObs - Inexperienced observers

THI - Temperature Humidity Index

TMT - Tympanic membrane temperature

NS - Not significant

1. INTRODUCTION

This dissertation aims to study changes in temperature of different Regions of Interest (ROIs) in dairy calves and horses using Infrared thermography, which are likely to reflect changes in the activity of the autonomic nervous system in response to changes in the affective state of the individuals, as well as the suitability of these measurements for the study of emotions in non-experimental settings.

Some of the current measurements of animal emotions involve the use of invasive technology that might disturb the affective state of the animals or require experimental settings that imply several limitations for its use. Other non-invasive methods based on behavioural observation of the animals have the disadvantage of being time-consuming, and there are some downsides regarding the interpretation of the results. For this reason, I looked at the use of infrared thermography with a novel approach (measuring temperature asymmetries) as a non-invasive method to measure temperature data objectively and to identify variation in temperature patterns associated with changes in affective state in real-time.

In Chapter 1, an introduction to the study of animal emotions will be set according to the most recent scientific literature on this area. The topics covered in this introduction include an overview of the definition of emotion and other affective states (Chapter 1.1), the importance of measuring emotions in animals and its relevance to their welfare (Chapter 1.2.), a brief overview of different theories used for the study of emotions (Chapter 1.3), a deeper insight into a componential view of animal emotions (Chapter 1.4.), an overview of the common methods for measuring emotions, the more relevant findings in the species used for this dissertation and the limitations of those methods (Chapter 1.5.); as well as an introduction to the new approach used in this dissertation based on temperature asymmetries as indicators of animal emotions (Chapter 1.6.) and an overview of how the different Chapters of this thesis will address specific research questions (Chapter 1.7.)

In Chapter 2, the general methodology used for the recording of thermal videos (Chapter 2.1.), the selection of the thermograms to be measured (Chapter 2.2.), the measurement of the Regions of Interest (Chapter 2.3.) and the statistical analysis of the data (Chapter 2.4.) will be explained.

Chapters 3 to 6 will be looking at the different experiments carried out for the purpose of this thesis. In those Chapters, the following research questions were addressed: do temperature asymmetries occur in dairy calves (Chapter 3 to 5) and horses? (Chapter 6), in which Regions of Interest can those asymmetries be measured? (Chapter 3 to 6), is there an association between putative negative states and the direction of those asymmetries? (Chapter 3,4 and 6) are there differences in the direction or extent of temperature asymmetries in individuals assumed to be in a positive affective state? (Chapter 3 and 6), is the previous experience of negative situations associated with temperature asymmetries measured whilst observing a conspecific going through a similar situation? (Chapter 3), do environmental and methodological factors (camera position and time within recording) influence the asymmetries measured and if so, are specific ROIs less susceptible to those factors? (Chapter 5), are temperature asymmetries related to the behaviours performed by individuals? (Chapter 3 and 4).

Finally, Chapter 7 will cover a general discussion of the results obtained in the experimental Chapters, addressing how they respond to the research questions stated in the previous paragraph and providing some suggestions of which steps need to be taken for the use and refinement of this method for its further application.

1.1. Emotions and other affective states

The term affective state is generally used to refer to the subjective experience of an emotional state, and it is sometimes used as a synonym for the term emotion. However, the term affective state encompasses both short-living emotional states (“emotions”) and long-term emotional states (“moods”). More broadly, the term affect is considered to involve *"the physiological and behavioural responses that vary both in terms of valence (pleasantness/ unpleasantness) and intensity (arousal) which are the core features of affective and emotional processes"* (Paul et al., 2005).

An emotion is considered as an intense but short-lived response to an event associated with specific body changes, composed of behavioural, autonomic, subjective and cognitive components (Birbaumer and Ohman, 1991; Panksepp, 2004; Scherer, 2005; Mulligan and Scherer, 2012); for an emotion to be elicited it is considered that individuals require to perform intuitive and autonomic evaluations of situations ("appraisals") based on several checks of an event in terms of the previous experience and the innate response of the individual ("instinct"), i.e. relevance and implications of the situations as well as the coping potential of the individuals. The relevance of the situation to elicit a specific emotional response will depend on the levels of familiarity with the situation, the predictability and relevance of the outcomes from the situation, as well as the value of the situation in terms of intrinsic pleasantness. The implications of such situation will be evaluated in terms of the probabilities of different outcomes, the level of discrepancy between the individual's expectations and the actual situation, the feasibility of taking action under the specific situation and the level of urgency to act towards the eliciting stimulus. Finally, the coping potential will depend on the power and control that the individual has over the specific situation (LeDoux, 1995; Désiré et al., 2004; Scherer, 2004).

In contrast to a short-term emotion, a mood is a long-term affective state that does not require a specific stimulus to be elicited. It can be considered that moods result from the repetition of positive or negative emotions, leading to a generalised response to unspecific situations. Two prominent examples of mood are anxiety and depression; the former characterised by a fear response towards future and unknown events and the latter as a negative state associated with low expectations of positive events, which is reflected as an anhedonic response towards typically rewarding events (Frijda, 1986; Willner et al., 1992; Kalueff and Tuohimaa, 2004; Mendl et al., 2010).

For the purposes of this dissertation, I will not be addressing in practice any long-term affective states or “moods”. However, the broader term “affective state” will be used alongside the term “emotion” as I do not aim to address the differences between short- and long-term affective states with the methodology used in my research.

1.2. Importance of studying emotions in animals

To improve the welfare of the animals under human care, it is fundamental to increase our understanding of animal emotions. Aiming to explain how emotions are organised, some contemporary researchers advocate for a componential view, stating that emotions contain physiological, behavioural and cognitive and subjective components (Scherer and Ekman, 1984; Scherer, 2004; Sander and Scherer, 2009). In human psychology, the gold standard for the existence of emotions is considered by some authors to be self-report, as it allows researchers to identify the subjective components of the emotions as experienced by the individuals (LeDoux, 2015; LeDoux and Hofmann, 2018). In contrast, the lack of a common language between humans and animals complicates the attribution and understanding of the subjective component of emotions in animals. However, this componential view identifies common elements between animal and human emotions, including the physiological, behavioural and cognitive components that allow us to infer and interpret animal emotions even with the lack of evidence of a subjective component (discussed in Chapter 1.4.).

From a philosophical perspective, it is not possible to determine at which point in human prehistory or history humans started to attribute the way they felt to other animals. As part of coevolution with other species, it is feasible that the first human ancestors did not identify themselves as different from other species, and therefore self-assignment of superior attributes relative to other animal species occurred much later in history when a line between the cognitive and conscious capabilities of humans and animals was drawn. The oldest cave paintings are from around 40,000 years ago and showing rituals, hunting scenes, and interactions between animals and humans with animal features, evidencing the humans' interest and fascination for the animals surrounding them. The domestication process started around 15,000 years ago with dogs, likely in the form of a commensal relationship between wolves and humans. Later, about 11,000 years ago, with the first human settlements and the development of agriculture, the domestication of animals for farming purposes, like pigs, goats, sheep, and cattle started. With domestication and farming, a stronger bond between humans and animals was formed, and a further understanding of the behaviours and needs of those animals to be kept under human care was developed (Kalof, 2007; Zeder, 2012). In ancient cultures, including Roman, Egyptian and Greek, mythological stories of gods and heroes with animal features, able to suffer, fall in love, or feel anger, provides evidence of the first written zoomorphic/anthropomorphic figures in the human story (Gilhus, 2006; Budge, 2012; Johnston et al., 2016).

From a scientific perspective, Charles Darwin is considered one of the first scientists to write openly about animal feelings in his book “The expression of the emotions in man and animals” in 1872, in which he attributed emotions like happiness, sadness, fear, anger, surprise and disgust to animals, interpreting their facial expressions and behaviours in a similar way to those observed in their human counterparts (Darwin and Prodger, 1998; Ekman, 2009; Hess and Thibault, 2009). However, it was not until 1964 that interest in the animal welfare area increased into broader society with the publication of the Book “Animal Machines” by Ruth Harrison, in which many issues affecting the welfare of farm animals subjected to intensive farming practices were exposed to the public (Harrison, 2013). As a response to the concerns elicited by the publication of the book, the British government appointed a committee chaired by Roger Brambell that produced a document entitled “Report of the technical committee to inquire into the welfare of animals kept under intensive livestock husbandry systems”, reviewing the main concerns relating to the welfare of intensively farmed animals, addressing the suffering and behavioural needs of the animals (Brambell, 1965; Mench, 1998; Rushen, 2008). In 1979 as a continuation to this committee, the UK Farm Animal Welfare Council (FAWC) was created, and the Five Freedoms considered the basis of animal welfare were put forward, i.e., freedom from hunger and thirst, freedom from discomfort, freedom from pain, injury and disease, freedom to express normal behaviour and freedom from fear and distress (Freedoms, 1979). Since then, many animal welfare scientists have worked to increase the knowledge of animal sentience by studying the links between behaviour, welfare and emotions in a broad number of animal species.

In practice, the study of emotions in animals has been mainly focussed on negative affective states identifying the presence of the physiological mechanisms and structures involved in the processing of pain and fear (Gregory, 2004; Sneddon et al., 2014; Sneddon, 2017), the behavioural responses towards negative stimulus (Appleby et al., 2018), the physiological and behavioural responses to pharmacological treatment used for mood disorders like anxiety and depression (Dulawa et al., 2004; Sandem et al., 2006b; Lee et al., 2016; Drozd et al., 2019), and the modification of cognitive responses associated with painful or stressful circumstances (Paul et al., 2005; Burman et al., 2009; Mendl et al., 2009; Neave et al., 2013; Daros et al., 2014; Gaillard et al., 2014; Lee et al., 2016; Lecorps et al., 2019). However, other evidence, like the existence of dopaminergic and opioid systems required to process rewarding stimulus (Schultz, 1998; Glimcher, 2011; Stracke et al., 2017), the preferences or changes of behaviour towards rewarding situations (Schultz, 1998; Hagen and Broom, 2004; Lee et al., 2011), and the benefit of positive events in cognitive responses (Brydges et al., 2011; Douglas et al., 2012; Richter et al., 2012; Keen et al., 2014; Baciadonna et al., 2016; Horvath et al., 2017), highlights the potential of animals to experience positive emotions, which has led to newer approaches to animal welfare in which not only the avoidance of negative affective states is addressed, but also the provision of elements and resources to elicit rewarding and positive experiences.

1.3. Theories of emotions

There are several theories regarding how emotions evolved and how they are organised and processed in the brain; the most basic emotions are thought to be originated from the primitive mechanisms that allowed the animals to seek valuable resources ("rewards") and to avoid stimuli that could represent harm ("punishments") (Panksepp, 1994). The discrete emotions theory suggests several basic emotions that have biological relevance to the survival of different species and are thought to have been selected for through the process of evolution (Ekman, 1992; Ekman and Davidson, 1994). The core affect theory from Russell and Barrett (1999), adapted for the study of emotions in animal models by Panksepp et al. (2002), classifies the different emotions in a bi-dimensional space representing the valence of the emotion ('positive/negative') on the "X" axis, and the arousal levels ('high/low) on the "Y" axis. Allowing the emotions to be allocated within four quadrants ("Q") represents the different arousal and valence combinations. For example, affective states with positive valence and high arousal, like excitement, are allocated in "Q1"; ones with positive valence and low arousal, like calm or relaxed, are allocated in the "Q2"; negative emotions with low arousal, like depression, are in "Q3" and negative emotions with high arousal, like anxiety in "Q4" (Figure 1). The affective state of an animal is dynamic and moves within these axes following the activity of two basic systems: reward acquisition (from Q3 to Q1) and punishment avoidance (from Q2 to Q4) (Mendl et al., 2010).

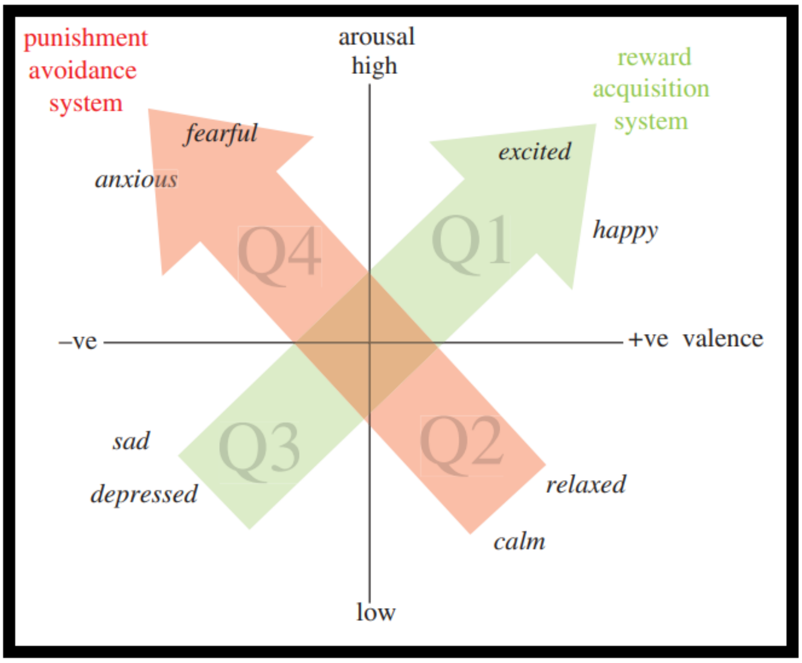


Figure 1. Representation of the two-dimensional space of the Core affect. Q1 and Q2 represent positive affective states with high and low arousal, respectively, and Q3 and Q4 represent negative affective states with high and low arousal. The red arrow represents the punishment avoidance system (from Q2 to Q4), and the green arrow represents the reward acquisition system (from Q3 to Q1). Figure from Mendl et al. (2010).

Other models of emotions like the vector model and the positive activation – negative activation (PANA) model suggest a similar classification of the emotions in terms of valence and arousal, suggesting that low arousal affective states are located more closely to a neutral valence whereas high arousal states are located further away towards the positive and negative valences (Watson and Tellegen, 1985; Bradley et al., 1992). Such models might Imply difficulties for the study of contrasting low arousal emotions like depression and calmness, which are considered by many authors as highly valanced emotions (Fureix et al., 2012; Fureix and Meagher, 2015). Another important model used to classify emotions is the PAD (pleasure-arousal-dominance) model, which adds the level of dominance of the individual over its emotions as a third dimension to classify emotions (Mehrabian and Russell, 1974). It can be considered that the PAD model is the most adequate model to study emotions based on the verbal report of the individuals. But as it is not possible to infer the level of control that animals had over a specific situation it has not been used for animal studies.

For this dissertation's purpose, emotions will be classified in terms of arousal and valence as stated by the core affect theory, alongside a componential view of the emotional structure to label the findings and the methods used for the study of animal emotions, in terms of their physiological, behavioural and cognitive components.

1.4. Components of emotions

As mentioned in chapter 1.2, the componential view will be used to address the structure of the different elements of emotions. In this chapter, physiological, behavioural and cognitive components will be reviewed, along with their relationship with the emotional responses and the limitations of their interpretation.

1.4.1. Physiological components

The physiological responses associated with processing emotions are mainly mediated by the autonomic nervous system (ANS), the central nervous system's (CNS) dopaminergic and opioid systems and the Hypothalamic-pituitary-adrenal system (HPA)(Kreibig, 2010; Wirth, 2011). Traditionally, only the structures from the subcortical areas, like the basal ganglia and the amygdala, were considered to be involved in the processing of emotions (LeDoux, 1993; Gallagher and Chiba, 1996; Bennett and Hacker, 2005). However, more recent research has highlighted the complexity of emotional processing and the involvement of cortical structures, like the temporal cortices and the cingulate cortex, in those processes (Heinzel et al., 2005; Stevens et al., 2011; Mellem et al., 2016).

The sympathetic branch of the ANS is the primary regulator of the “fight and flight” responses of the individuals through the release of epinephrine and norepinephrine, whilst the parasympathetic branch of the ANS is in charge of the “rest and digest” responses mediated mainly by the release of acetylcholine (McCorry, 2007; Gibbons, 2019). Shifts in the balance between those systems generate changes in blood flow and cardiac activity, which can be interpreted in terms of arousal and, to some extent, to emotional valence (Reece et al., 2015). For example, when animals are in a low arousal affective state, a lower heart rate, a dilation of peripheral blood vessels and changes in heart rate variability (HRV) associated with a dominance of the parasympathetic branch of the ANS (discussion of HRV in chapter 1.6.1.) are observed. In contrast, affective states with higher arousal levels will cause immediate vasoconstriction of the peripheral blood vessels, increased cardiac output/heart rate, and patterns of HRV indicative of dominance of the sympathetic branch of the ANS. However, fluctuations in blood flow and cardiac activity are not exclusively controlled by the autonomic nervous system. The adrenal glands release catecholamines (epinephrine and norepinephrine), whilst the adrenal medulla increases aldosterone levels through the renin-angiotensin-aldosterone system (Freel and Connell, 2004), the adrenal cortex releases cortisol through the HPA axis (Kelly et al., 1998). All of which can raise central blood flow either by peripheral vasoconstriction of the blood vessels or an increased cardiac output (Goldstein, 2005; Ayada et al., 2015), which might cause an increase of deep body temperature, reducing peripheral temperature in associated to high levels of stress rather than an infectious fever, which is known as emotional or psychogenic fever (Vinkers et al., 2013; Oka, 2015; Olivier, 2015).

As mentioned previously, the way in which emotions are processed in the brain is very complex and several structures are involved at different levels of the processing. However, as this work does not aim to investigate the processing of emotions at a structural level, it is possible to simplify the brain's reward acquisition and punishment avoidance systems. The former mainly overseen by the mesolimbic dopaminergic (in control of the anticipatory behaviours) and opioid systems (in control of the consummatory behaviours), as well as by the medial orbitofrontal cortex, the nucleus accumbens and the ventral pallidum; and the latter considered to be regulated by the serotonergic circuits, the amygdala, the lateral habenula, the anterior insula and the lateral orbitofrontal cortex (Ressler, 2004; Boissy et al., 2007; Mendl and Paul, 2020; Gueguen et al., 2021).

Physiological changes associated with the activity of the ANS and HPA system in terms of emotional valence are not always clear or straightforward and must be done cautiously. Even though changes in those systems had been generally considered indicators of a classic stress response, stimulus triggering emotions from an opposite valence are likely to elicit similar physiological responses; for example, an increase in the heart rate of an individual can be a response to a threatening stimulus in the same way that can be associated with the expectation of a reward, or as a product of increased physical activity (Paul et al., 2005; Kreibig, 2010). For this reason, it is essential to consider other factors such as the behaviour of the individuals and the context of the situation to provide a better interpretation of those physiological components (Mauss and Robinson, 2009).

1.4.2. Behavioural components

In terms of emotions, it can be considered that behaviour performed by individuals is usually driven by motivational systems driving reward-acquisition and punishment-avoidance and based on positive or negative appraisals of the stimuli eliciting an emotion (read chapter 1.3.). This may then allow interpretation of some behaviours in terms of emotional valence (positive or negative) (Gray, 1970; Davidson et al., 1990). Similarly, certain behaviours might be interpreted in terms of arousal in an emotional context; for example, low-energy behaviours like lying down or staying still can generally be considered indicators of a low arousal emotional state; whilst more active behaviours like running or jumping can be regarded as indicators of higher arousal. The interpretation of these behaviours can give a general picture of the affective state in relationship to core affect theory (arousal and valence). However, under several circumstances, interpretation of behaviours is not always straightforward; for example, individuals showing certain levels of inactivity in response to a specific stimulus can be regarded as an indicator of calmness or relaxation (low arousal/positive emotion) (Lehmann, 1979). Still, they could also be interpreted as an indicator of sadness or boredom (low arousal/negative emotion), making the interpretation of the behaviour unreliable if the context of the situation and other components of the emotion are not taken into account (Fureix and Meagher, 2015).

To aid interpretation of behavioural components in relation to affective states, it is not only important to understand the behaviours that occur as an immediate response to the presence of the stimuli eliciting the emotional response; the “anticipatory” and “consummatory” behaviours observed before and after the initial response towards the stimulus might also help us to make an adequate interpretation of the emotion elicited (Spruijt et al., 2001; Mellor, 2015). Anticipatory behaviours are the ones that occur during the expectation of the occurrence of an event. They are generally characterised by the active seeking or avoidance of a specific stimulus, whilst consummatory behaviours occur directly towards the eliciting stimuli with the aim to fulfil the need that triggered the response (Spruijt et al., 2001). Anticipatory behaviours are common in individuals expecting to be fed or to receive a reward; for example, rats trained through Pavlovian conditioning to access rewarding situations (sexual partner, play area or a sucrose reward) had an increase in general activity and exploratory behaviours in the period prior to getting access to the rewarding stimuli (Spruijt et al., 2001; van der Harst et al., 2003), which could be interpreted as an indicator of a high arousal emotion (Špinka, 2012). In a similar context, an example of consummatory behaviour will be a reduction in locomotion and general activity during the ingestion of the rewarding food (Galarce et al., 2007), which can be interpreted as an indicator of an individual under an emotion of lower arousal levels.

There are few examples of behaviours that can be unambiguously related to particular affective states. Such behaviours often occur only in specific situations and therefore may be reliable indicators of the affective states likely to occur in those situations. For example, play behaviour seems to be a self-rewarding behaviour that occurs mainly when an animal has fulfilled their basic needs, and the situation or environment allows the animals to perform it; for these reasons, it is generally considered an indicator of positive emotional states (Lawrence, 1987; Fraser and Duncan, 1998; Spinka et al., 2001). However, even this behaviour might lead to difficulties in interpretation; in most species it is an age specific behaviour found in young animals which generally offer less value as indicator of positive state as the individuals grow. Furthermore, there are some situations in which play behaviour is performed at higher levels under unfavourable circumstances (Mintline et al., 2012b). For example, animals that have been kept in barren or restricted environments show higher levels of play behaviour when the opportunity to perform them increases in a novel situation ("rebound effect") (Liu et al., 2020). This response could be misinterpreted if the general context of the situation is not taken into account that could lead to the wrong interpretation that animals from barren environments are in a more positive emotional state than those kept in enriched ones.

1.4.3. Cognitive components

The term cognition can be broadly defined as the process through which information is acquired, stored and manipulated (Shettleworth, 2010). Studies in humans and animals suggest that the relationship between cognition and emotion is a bidirectional process, in which long-term affective states (moods) influence the way that cognitive processes occur and vice-versa. For example, affective states being influenced by cognitive functions involved in attention and decision-making mechanisms (appraisals) (Paul et al., 2005).

As mentioned previously, the lack of self-report available when studying emotions in animals prevents us from understanding how they experience emotions subjectively. However, it is possible to indirectly infer affective states through the measurement of cognitive processes such as whether animals pay more attention to positive or negative elements in a specific situation ("attention bias"), whether positive or negative memories are recalled in certain circumstances ("memory bias") and how animals respond to neutral/ambiguous situations ("judgement bias"). These processes may indicate to us the underlying affective states of an individual, as it is expected that animals with an underlying negative affective state will be more pessimistic in their expectations towards ambiguous or uncertain cues and be more attentive to potential threatening stimuli compared to individuals with a more positive underlying affective state. Those differences in information processing can be assessed using cognitive bias tests or paradigms (Mendl et al., 2009). Those paradigms have been used to assess different affective states in the context of the effects of environmental enrichment (Brydges et al., 2011; Douglas et al., 2012; Richter et al., 2012; Destrez et al., 2014; Horvath et al., 2017), husbandry (Neave et al., 2013; Daros et al., 2014; Lecorps et al., 2019; Lecorps et al., 2020a), drug manipulations (Kis et al., 2015; Stracke et al., 2017; Drozd et al., 2019), animal-human interactions (Baciadonna et al., 2016), among others.

The term brain lateralisation refers to the physiological process of having bilateral structures in the brain that process information from different sources. Brain lateralisation is thought to have arisen during evolution to avoid the duplicate processing of information and generate a faster response of individuals to their surroundings (Levy, 1977; Rogers, 2000). In animals and humans, several processes have been attributed to a specific brain hemisphere; for example, the left hemisphere is predominant in the processing of familiarity, language, and motor skills, whereas the right hemisphere predominantly processes novelty, conspecific faces and temporospatial cues (Rogers, 2000; Daselaar et al., 2006; Hervé et al., 2013). There are several hypotheses about whether and how emotions are processed bilaterally in the brain; the right hemisphere hypothesis states that all emotions are processed in the right hemisphere of the brain (Gainotti, 1972). The approach/withdrawal hypothesis states that emotions that are characterised by an active approach of the individual to the stimulus are processed mainly by the left hemisphere; whilst emotions resulting in avoidance of the stimulus are processed with more intensity on the right hemisphere (Davidson et al., 1990; Sobotka et al., 1992a). Similarly, the valence hypothesis states that all positive emotions are processed mainly in the left hemisphere and negative ones on the right (Silberman and Weingartner, 1986; Cameron and Rogers, 1999; Lee et al., 2004; Leliveld et al., 2013).

For this dissertation's purpose, I will discuss the processing of emotions from the perspective of both the valence and approach withdrawal hypothesis, as it can be difficult to separate these two hypotheses. In general, it is expected that a stimulus that generates a positive emotion will provoke a behavioural approach, whilst a stimulus likely to cause a negative emotion will generate an aversive or withdrawal response; However, under certain circumstances, an active approach towards a negative stimulus can also be observed, for example, when an active fight response occurs towards a predator in response to a threatening situation.

In conclusion, the interpretation of an emotion based solely on the physiological, behavioural or cognitive components might lead to confusion or a general misinterpretation of the animal's affective state. For this reason, it is essential to use an integrative approach, looking at the different components of the affective response and the general context of the situation eliciting the response.

1.5. Evidence of emotions in animals

In this chapter, some of the evidence for the presence of affective states in the species studied in this dissertation will be discussed from the componential view of emotions.

It is important to highlight that it is possible to address the study of affective states in animals through two different perspectives; (i) by measuring changes in potential indicators of affective states during situations that are assumed to elicit specific affective states, for example, measuring behaviours in a situation assumed to be negative and suggesting that significant changes in behaviour observed during that situation might be good indicators of that affective state; or (ii) suggesting that there are strong and reliable indicators of an affective state that can be used as a 'ground truth' to infer the affective state of an individual, for example, that a behaviour 'x' only occurs when an animal is in a relaxed state, and therefore it is possible to assume that an animal performing behaviour 'x' is relaxed. As I consider that most of the physiological, behavioural and cognitive indicators used for the study of emotions are not highly specific, I will address the scientific evidence and my research by the first approach (i).

1.5.1. Physiological evidence

Physiological responses linked to negative affective states have been found in dairy cows and calves; for example, when separation between the calf and the mother occurs, an increase in heart rate, cortisol and noradrenaline concentrations has been found in both of them (Hickey et al., 2003; Stěhulová et al., 2008a; Stilwell et al., 2012; Hernandez et al., 2014; Appleby et al., 2018). Cows with different temperaments showed diverse patterns on hearth rate variability (HRV), indicating that more reactive/impulsive animals had stronger sympathetic responses towards unfamiliar persons at milking, suggesting that those individuals might perceive unfamiliar stimulus in a more negative way (Kovács et al., 2015). The balance between sympathetic and parasympathetic activity has been measured using HRV to assess pain and stress levels in horses, finding that horses undergoing higher levels of exercise and untreated pain showed increases in the low frequency power (LF) power and the low frequency / high frequency ratios (LF/HF ratio), which are hypothesised to indicate increased activity of the sympathetic branch of the ANS (Rietmann et al., 2004a; Rietmann et al., 2004b). Similarly, when calves are subjected to painful procedures like hot-iron disbudding or castration, physiological parameters indicative of pain or distress, like a drop of eye temperature (assessed with Infrared thermography), increased cortisol levels, and changes in HRV (increase in LF/HF ratio) can be found (Stewart et al., 2008b; Stewart et al., 2010a; Stilwell et al., 2010; Stilwell et al., 2012).

Less work has been done in positive affective states in those species. Still, evidence of brain lateralisation through ECG measurements has shown that horses had higher activation of the left hemisphere when listening to voices of humans that had been associated with positive experiences, whilst activation of the opposite hemisphere occurs when listening to voices of humans associated with negative experiences (d'Ingeo et al., 2019). Similarly changes in HRV associated with higher parasympathetic/vagal dominance(decreased LH/HF ratio) occurred when horses were groomed by a familiar human in contrast to being groomed by an unfamiliar handler, suggesting differences in positive affective states dependent on the familiarity of the situation.

1.5.2. Behavioural evidence

Research in human-animal interaction has suggested that cattle and horses are able to identify different humans and conspecifics, and they differ in their behaviours towards them according to their previous positive or negative experiences with them (Munksgaard et al., 2001; d'Ingeo et al., 2019; Trösch et al., 2020). For example, cows from the study of Munksgaard et al. (2003) and horses from Trösch et al. (2020) observed an aversive and a gentle human handling a conspecific, after which they behave differently towards them, spending significantly longer close to the gentle handler. In a similar way in the study of d'Ingeo et al., (2019) mentioned earlier, horses that listened to voices of humans associated with positive experiences turned their head towards the right more often and with their ears towards the front; in contrast when listening to voices associated to negative experiences, in which case no side bias was found, but horses had their ears significantly longer positioned backwards. Similarly, ear position has been identified as a potential indicator of emotions in dairy cattle. Proctor and Carder (2014) found that cows shown a relaxed ear position (ears hanging backwards) while being stroke, which was suggested by the researchers to induce a low arousal positive state. In several studies, personality traits in cattle and horses have been identified and associated to different levels of fear responses through behavioural assessments (reactions to novelty and human approach tests) (MacKay et al., 2014; Kovács et al., 2015) which highlights the individuality in response between conspecifics.

There is some evidence that cattle can recall previous experiences in different places, modifying their behaviour according to whether they experienced positive or negative events in such places. For example, Rushen et al. (1998) found that cows stay significantly longer closer to a human in places where they received a gentle treatment compared to areas they associate with negative experiences, highlighting that animals are capable of making active decisions based on the emotional mechanisms of reward acquisition and punishment avoidance. A preference to gain access or use resources that potentially offer a positive or rewarding experience has been found in cattle and horses (DeVries et al., 2007; Lee et al., 2011; Newby et al., 2013). For example, grooming behaviour increases when an automated brush is provided compared with pens without this resource (DeVries et al., 2007; Newby et al., 2013). Horses have a great need to exercise, but their motivation to access exercise areas depends on the resource available, for example, Lee et al. (2011) found in a small group of horses, that access to an outdoor paddock is preferred against staying in their stalls, particularly if other horses were there. In contrast, a preference to stay in their stall is observed when given a choice to access a treadmill to exercise. Similarly, Lesimple et al. (2020) found a high motivation of horses to exercise freely in paddocks with other conspecifics, which positively affected their stall behaviour, reducing the number of stereotypies observed.

In horses, the study of facial expressions associated with pain has led to the elaboration of a grimace scale and a Facial Action Coding System (EquiFACS) to assess acute pain associated with lameness, dental disorders, and surgical procedures (Dalla Costa et al., 2014; Gleerup et al., 2015b; Wathan et al., 2015; Dalla Costa et al., 2016; Marcantonio Coneglian et al., 2020; Rashid et al., 2020). In cattle, not enough evidence on facial expressions has been found to develop a similar system. However, elevated eyebrows, dilated nostrils and ears in a backward position have been associated with acute pain (Gleerup et al., 2015a; Müller et al., 2019).

1.5.3. Cognitive evidence

In cattle and horses, several studies had evidenced the effect of positive and negative affective states in the cognitive response of those species. Calves that had been separated from their mother or had suffered a painful experience elicited by removing their buds/horns show a more negative response towards ambiguous stimulus than those who have not suffered from such negative experiences. (Neave et al., 2013; Daros et al., 2014; Lecorps et al., 2019; Lecorps et al., 2020a). Calves that are housed in pairs perform better in discrimination tasks and are more optimistic in response to ambiguous cues than single housed calves (Gaillard et al., 2014; Bučková et al., 2019), suggesting that social interactions not only affect their emotional response, but influences directly their learning and cognitive processes. Similarly, housing conditions and social interactions influence the cognitive responses in horses; stabling in social isolation and with limited access to outdoor areas result in horses with a more pessimistic response in cognitive bias tests than horses kept in more naturalistic conditions (living in groups and having access to free-range areas) (Löckener et al., 2016; Henry et al., 2017). As in the case of dairy calves, affective states in horses not only influence the cognitive perception of specific situations, but it affects the performance and flexibility of their cognitive response. For example, horses in a more positive affective state more succesful into achieving an extintion criterion in an instrumental task compared to horses induced into a negative affective state (Fortin et al., 2018).

1.6. Measurements of emotions

In this chapter, the methods used to study the different components of animal emotions are addressed, as well as some of the most relevant results in terms of affective states obtained using those methods.

1.6.1. Physiological components

1.6.1.1. Heart rate variability

An Electrocardiogram (ECG) is a device that records the voltage of the electrical signals of the heart activity using electrodes. Heart rate, interval between heartbeats and the power of the different heart conductivity components (high and low frequencies) can be calculated through heart rate variability measurements. The variation in the power of the high (HF) and low frequencies (LF) has been considered an indicator of parasympathetic and sympathetic activity, respectively. The standard deviation of normal to normal R-R intervals (SDNN) is regarded as an index of general ANS activity, whilst the root mean square of successive heartbeat interval differences (RMSSD) is considered indicative of parasympathetic activity(Mohr et al., 2002; Appelhans and Luecken, 2006; Duarte and Pinto-Gouveia, 2017).

In the context of emotions, HRV has been used to study post-traumatic stress associated with traumatic experiences in early life in rats (Cohen et al., 2007), the effect of different stress loads in cattle (Mohr et al., 2002), the impact of the suddenness and novelty on the presentation of a stimulus on the emotional response in lambs (Désiré et al., 2004); studies where an increase on the LF: HF ratio has been interpreted as an indicator of high arousal emotions (Von Borell et al., 2007).

In dairy calves, A series of studies from the group of Stewart et al. (2010a) analysed the effect of different negative stimulus (castration and disbudding) on sympathetic activity using HRV. They assess the impact of different castration methods, finding that calves castrated without anaesthesia showed an initial response mediated by the sympathetic branch of the ANS, i.e., increased heart rate and elevated catecholamine levels, which was followed by a response from the parasympathetic branch characterised by an increased RMSSD, reduction of the LF: HF ratio and increased eye temperature (Stewart et al., 2010a). Similarly, they found that calves subjected to hot-iron disbudding without local anaesthesia had an initial sympathetic response, i.e., increased heart rate, LF and LF: HF ratio, and reduced HF and eye temperature, followed by an increase in eye temperature that could be associated to a higher parasympathetic response.

Most of the studies using HRV in animals had focussed on situations that putatively elicit a negative emotion, finding variations in the sympathetic and parasympathetic balance that can be interpreted in terms of affective arousal. The lack of studies looking at HRV in positive emotions in animals makes it difficult to know how and which HRV calculations can be used to interpret the valence of animals' emotions. A study of positive affective states showed a decrease in RMSSD in dogs while being petted, suggesting a higher activation of the sympathetic activity, which in association with an increase of active behaviours could be interpreted as a high arousal positive emotion (Kuhne et al., 2014). On the contrary, another study looking at the effect of petting and owner separation in dogs by Katayama et al. (2016) found no difference in RMSDD in the positive situation, but a reduction in the negative situation. However, the behavioural data from this study suggested that the affective states elicited were not balanced in arousal, as much more active behaviours were observed in the dogs separated from their owner (Katayama et al., 2016). Similarly, evidence from human studies suggests that positive emotions generate changes in cardiac activity similar to those found when negative experiences from similar arousal levels are elicited (Shiota et al., 2011; Duarte and Pinto-Gouveia, 2017), suggesting that measurements of cardiac activity might not be valence specific, but rather indicators of emotional arousal.

1.6.1.2. White eye percentage

White eye percentage has been suggested as an indicator of emotion. It reflects the muscular activity in the upper eyelid area, mediated by sympathetic innervation. Therefore, an association between sympathetic activity and increased eye-opening has been found (Sandem et al., 2006b).

In dairy cattle, white eye percentage has been considered a dynamic indicator of frustration and satisfaction by Sandem and Braastad (2005), who studied the effect of calf separation in dairy cows; finding an increase in white eye percentage in cows immediately after the removal of their calf, followed by a reduction in white eye percentage when reunited with their calf. Likewise, this increase in white eye percentage has been associated with food frustration (Sandem et al., 2006a) and in cows responding towards a threatening novel object (umbrella opening) (Sandem et al., 2004). Despite those findings, an increase in white eye percentage has been found as well in cows in a putative high arousal/ positive state, for example, while expecting a rewarding food (Sandem et al., 2006a), which might suggest that white eye-opening is instead an indicator of emotional arousal.

1.6.1.3. Changes in peripheral temperature

Thermal cameras allow us to estimate an object's temperature by measuring the infrared energy emitted by it. Infrared thermography has been used conventionally to measure differences in skin temperature resulting from inflammation mediators, blood perfusion, muscular activity, and heat convection from other organs in humans and animals (Schaefer et al., 2004; Rainwater-Lovett et al., 2009; Stewart et al., 2010a; McCafferty et al., 2011; George et al., 2014; Cook et al., 2015; Digiovani et al., 2016; Sathiyabarathi et al., 2016; Soroko and Howell, 2018).

Changes in skin temperature in specific ROIs have been used as indicators of emotion in animals and humans (Zajonc et al., 1989; Nakayama et al., 2005; Proctor and Carder, 2015). Decreases in nasal temperature have been attributed to negative affective states in Rhesus monkeys while observing videos of facial expressions of conspecifics during agonistic encounters (Nakayama et al., 2005), suggesting a decrease in peripheral temperature associated with peripheral vasoconstriction mediated by the sympathetic branch of the ANS. Similarly, reduction in nasal temperature has been observed in cattle whilst being stroked, a situation that authors considered to elicit a positive emotion in the animals, and was attributed to shifts in emotional valence (Proctor and Carder, 2015). However, as explored in the HRV chapter, those changes associated with the sympathovagal balance might be indicative of emotional arousal.

In the study by Stewart et al. (2010a) mentioned in the HRV chapter, changes in eye temperature were found in calves castrated either with or without local anaesthetics; a drop in eye temperature was observed immediately after castration, which was attributed to peripherical vasoconstriction mediated by the sympathetic branch of the ANS, followed by an increase of temperature above baseline levels, mediated by the parasympathetic ANS. Results corroborated by Stewart et al. (2010b) by measuring the effect of exocrine epinephrine on eye temperature.

In cattle, negative affective states elicited by aversive handling (shouting, startle and hitting) generates a similar response on eye temperature to that observed when calves are disbudded or castrated without anaesthesia (Stewart et al., 2008a), implying that changes in eye temperature involve other affective components not directly associated with pain. However, in other species like sheep and laying hens, a drop in eye temperature has been found in emotions assumed to have positive valence (Moe et al., 2012). Again, eye and nasal temperatures are more likely to be indicative of arousal than of emotional valence.

1.6.1.4. Brain activity

Electroencephalography (EEG) is a technology that allows measure the activity of different areas of the brain, using electrodes to quantify the activity from different electric waves, (i.e. alpha, beta, gamma, delta and theta) that are conducted from the brain to the adjacent structures (skull and skin). This method allows us to measure the activity of those different waves in the brain cortex (frontal, parietal, occipital, temporal lobes and cerebellum). Another technology used to measure brain activity is functional magnetic resonance image (fMRI), which uses a scanner that detects changes in blood flow associated with the different brain structures' activity, giving a three-dimensional image of those areas and their respective level of activity/blood flow. Whilst EEG provides information about the electrical activity on the different cortical regions of the brain, fMRI offers a detailed image of the structures and changes in their blood flow. Using these technologies, it is possible to observe the brain structures activated during different situations, making it possible to identify areas of the brain that are more active during the processing of emotions.

1.6.1.5. Hormones and neurotransmitters

Through histological and biochemical techniques, it is possible to measure the concentration of hormones or neurotransmitters in different tissues and fluids of animals and humans. There are many biomarkers of affective states in animals. Still, epinephrine, norepinephrine, cortisol, dopamine, serotonin, and oxytocin are the most commonly used.

Epinephrine and norepinephrine are neurotransmitters that are transmitted or released during the sympathetic response, causing an increase in heart rate and peripheral vasoconstriction, resulting in a drop of peripheral temperature and increased blood flow towards the hepatic and muscular tissue, allowing the individuals to be ready for an energetic response, reason for which is considered to be an indicator of emotions with high arousal levels, regardless of their valence (Christianson and MjÖRndal, 1985; Toth et al., 2013).

Cortisol is produced in the adrenal glands and is released in association with situations that trigger an adaptative response of the individuals towards challenging or threatening situations (elevated stress levels), whose concentration levels are easily measurable in blood, faeces, saliva or hair; and has been considered one of the main indicators of negative affective states in animals (Het et al., 2012; Burnard et al., 2017).

Dopamine, serotonin and oxytocin are neurotransmitters that are generally associated with the processing of positive emotions: dopamine is directly involved with the processing of pleasure and rewarding systems, serotonin has an inhibitory effect on the brain, stabilizing the mood of the individuals, and oxytocin is associated with the formation of social bonding (Schultz, 1998; Glimcher, 2011; Kis et al., 2015).

1.6.2. Behavioural components

1.6.2.1. Spontaneous behaviour

Through behavioural observation, it is possible to determine the behavioural repertoire of an animal and the time they spend performing different activities. Even though the interpretation of most behaviours does not allows us to make a direct inference of an affective state; the increase or decrease in the duration or frequency of behaviours can be a very useful tool to identify variation in the activity of the animals in response to affective state or health challenges.

Animals that have suffered from a painful procedure or condition usually show some behaviours that can be interpreted as indicators of discomfort, i.e. calves that have been disbudded flick ears and shake their heads more often, and spend more time lying down than control calves that have not been disbudded; also, the frequency of those behaviours was reduced if effective analgesic treatment was provided to the disbudded calves (Graf and Senn, 1999; Faulkner and Weary, 2000; Heinrich et al., 2010b; Stilwell et al., 2010; Coetzee et al., 2012; Stilwell et al., 2012; Theurer et al., 2012). Animals that suffer lameness shift the weight of their body away from the affected limb and show a considerable reduction of their locomotion, alongside with an increase in the time they spent lying down (Galindo and Broom, 2002; Rietmann et al., 2004a; Chapinal et al., 2010; Beer et al., 2016).

Individuals in a positive emotional state tend to have a more varied behavioural repertoire and perform certain behaviours more frequently, like social interactions and play behaviour (Boissy et al., 2007; Duve et al., 2012; Mintline et al., 2012a; Jensen et al., 2015). It is known that long term affective states (“moods”) may influence the behavioural repertoire of the animals. For example, anxious individuals are more responsive to a situation that is not considered to elicit a response from normal individuals, showing an increase of behaviours like restlessness, vigilance, startle response and freezing behaviours, which can be regarded as a negative response to uncertain events (Cohen et al., 2007; Lecorps et al., 2016; Reid et al., 2017). Whereas depressed animals show lower reactivity to situations that are expected to elicit a positive/rewarding response, reducing the capacity of the individuals to process pleasurable experiences (anhedonia), which can be observed as a reduction of active behaviours and an increase of inactivity (Fureix et al., 2012; Fureix et al., 2015; Lecorps et al., 2020b).

Stereotypies or repetitive behaviours are considered indicators of poor welfare and, to some extent, indicative of negative affective states (Broom, 1983). However, some evidence indicates that those animals showing stereotypies might be using those behaviours as coping mechanisms to sub-optimal environments, suggesting that animals not performing those behaviours in similar situations might be in a more negative affective state (Mason and Latham, 2004; Pomerantz et al., 2012; Novak et al., 2016).

1.6.2.2. Behavioural responses to specific situations

Behavioural tests have been used to assess emotions and moods in animals in response to situations that are expected to elicit negative affective states or identify the individuals' response towards novelty, conspecific encounters, or animal-human interactions.

The reaction of an animal towards a novel situation gives an insight into the boldness, fearfulness and other mechanisms that could be linked to long term affective states (Van Reenen et al., 2005; Duclot et al., 2011; MacKay et al., 2014). Many studies have focused on lab animals as models for mood disorders like depression or anxiety (Pollak et al., 2010; Yan et al., 2010; Neumann et al., 2011). However, further interest in behavioural tests in domestic animals has led to the identification of the effect of personality traits, social structure, environmental enrichment and animal husbandry on animal responses towards novel and threatening situations (Munksgaard et al., 2001; Herskin et al., 2004; Miranda-de la Lama et al., 2013; MacKay et al., 2014).

Behavioural tests allow us to identify differences in behaviour in groups of animals subjected to different treatments or husbandry practices towards a specific situation in a more standardised setting. However, the information provided in terms of the affective state of individual subjects is much more limited as each individual's response is associated with its own experiences and individual traits.

1.6.2.3. Behavioural lateralisation

Behaviour lateralisation is the preference or dominance for using a specific appendage or organ to perform a behaviour; it can refer to the tendency to observe or smell a stimulus with a particular eye or nostril, the paw/ hoof preference that an animal uses to perform a behaviour towards a specific stimulus or the side an individual chooses to walk in response to a specific situation (McGreevy and Rogers, 2005a; Siniscalchi et al., 2008; Robins and Phillips, 2010; Siniscalchi et al., 2011; Phillips et al., 2015; Wells et al., 2017).

Behavioural lateralisation is considered an indicator of brain lateralisation. For example, in dairy cattle, submissive cows observed more dominant conspecifics predominantly with their left eye, which is considered to be indicative of higher activation of the right hemisphere. These animals tended to use their left eye predominantly while observing a novel stimulus and behave more restlessly whilst being restrained in a chute (Phillips et al., 2015), suggesting a correlation between eye preference and temperament. As mentioned in the brain laterality chapter, one of the main theories suggests that the right hemisphere has a higher involvement in coordinating the flight and fight response during threatening situations. In cattle, horses and pigs, most of the input that comes from the eye is connected to the contralateral hemisphere of the brain (Herron et al., 1978), suggesting that the left-eye preference observed in the submissive cows from Phillips' study might be associated with higher activation of the right hemisphere, indicating higher levels of neophobia and fear towards conspecifics in those individuals.

It is important to mention that behaviour lateralisation might be influenced by species and individual bias. For example, a right-hand preference at the population level of up to 90% has been reported in humans (McManus, 2002). In contrast, cats and dogs are highly lateralised individually but without a general preference observed at the population level (Ocklenburg et al., 2019). For many other species, including cattle and horses, evidence of some degree of population-level preferences has been found (McGreevy and Rogers, 2005b; Vallortigara, 2006; Austin and Rogers, 2012), but the exact proportion of these processes is still unknown; for this reason, interpretation of behaviour lateralisation must be made cautiously on those species.

1.6.2.4. Facial expressions

The study of facial expressions in animals has generated vital information for the understanding of pain in animals like horses, cats and rats (Sotocina et al., 2011; Leach et al., 2012; Dalla Costa et al., 2014; Gleerup et al., 2015a; Dalla Costa et al., 2016; Evangelista et al., 2019; Müller et al., 2019; Marcantonio Coneglian et al., 2020); that has been used to create pain scales based on pictures, like the grimace scale, which has allowed to score objectively and accurately pain indicators on those species.

Facial expressions had been studied in the context of emotions, showing that animals use facial expressions, body positions, and vocalisations to communicate and identify emotions in their conspecifics (Kemp and Kaplan, 2013; Wathan et al., 2016; Dyson et al., 2017). Moreover, some studies looking at emotional contagion during playful interactions had shown the relevance of facial expressions in animals to communicate emotions between them (Cordoni and Palagi, 2013; Palagi et al., 2019).

1.6.3. Cognitive components

1.6.3.1. Cognitive bias

The change or bias in how the information is processed by an individual associated with mood modifications is known as “cognitive bias”; those biases can be classified as attention, memory, and judgment bias (Paul et al., 2005). The focus that animals have towards different elements of the environment that can be considered threatening or rewarding can be assessed through attention bias tests. For example, animals that are generally in a negative state will be focusing more on the threatening elements of the environment and will modify their behaviour towards the avoidance of such stimulus (Lee et al., 2016; Lee et al., 2018; Monk et al., 2018).

The way individuals experience a particular event will consolidate memories that can be either positive, negative or neutral; those previous experiences can be assessed through memory biases; for example, animals in a positive state will recall more positive memories of a situation than animals with a more negative state (Lensink et al., 2001; Raussi et al., 2003; Burman and Mendl, 2018).

Finally, judgment biases assess the individuals' underlying affective response, looking at their response towards an ambiguous stimulus. For this purpose, animals are trained to learn that some cue is associated with a positive/rewarding event, whereas an opposite cue is associated with a non-rewarding/negative event. After that, the responses of individuals towards intermediate cues are tested, e.g. near positive, ambiguous and near negative cues; in this test, it is expected that animals under negative affective states will respond more ‘pessimistically’ towards ambiguous cues than animals in a more positive affective state (Baciadonna et al., 2016; Henry et al., 2017; Lagisz et al., 2020; Neville et al., 2020).

There is broad research focused on the area of emotions and other affective states in animals. In the next chapter, the main limitations of the current methods for the study of animal emotions will be discussed.

1.7. Limitation of current methods

As explored in the previous chapter, several methods are used to study the different components of the affective states in animals. Physiological and cognitive components are more indicative of the mechanisms underlying the affective response of the individuals whilst behavioural components show us a visible response of the animals towards the stimulus. However, several limitations on the interpretation or applicability of these methods highlight the need for refinement or development of new methods.

The study of behavioural responses in relation to changes in affective states in animals provides valuable data regarding the reaction of the individuals to challenges or situations that are expected to elicit an emotional response in terms of valence and arousal. However, methods that require behavioural observation can be very labour intensive as they may require long periods for behavioural scoring. Furthermore, some issues mentioned earlier (see chapter 1.3.2), like individual coping strategies, the rebound effect of some positive behaviours in novel environments and the lack of specificity of certain behaviours regarding emotional valence (e.g. inactive behaviours), complicate the interpretation of behavioural responses by themselves (Jensen et al., 1998; Mintline et al., 2012b; Fureix and Meagher, 2015).

Cognitive approaches offer insight into how changes in affective states modify the processing of information and may be very helpful in identifying the effect of specific situations on animals, e.g., housing conditions, environmental enrichment, social interaction, negative experiences, drug treatments, etc. However, several limitations complicate its practical application; as mentioned earlier, it can be useful to compare between groups of individuals, but usually, the affective state manipulation has to be attributable to a specific stimuli/situation to make more assertive interpretations. As the methodology of different cognitive tasks lacks standardisation in rewarding schemes, the number of trials or stimulus utilised, and it generally aims to identify the effect of different treatments/manipulations, they do not provide much information at the individual level. Moreover, some cognitive tasks require the animals to be tested individually and in a novel environment, which might cause an affective response by itself. If the cognitive test involves using an operant response, as in the case of cognitive bias paradigms, habituation to the apparatus and a training process is required, which may be time-consuming.

The use of the ECG allows us to measure the different waves of the heart, heart rate frequency and heart rate variability, which gives an insight into the balance between the sympathetic and parasympathetic branches of the ANS, allowing us to make an objective inference of the arousal levels on the animal. However, this method requires the direct attachment of equipment to the animals, which might modify the affective response of the animals due to the handling required for the equipment positioning and/or lack of habituation to wearing the equipment (Varshney, 2020; Herlin et al., 2021); the equipment itself is fragile and sensitive to environmental factors and can be easily destroyed by the animals. Furthermore, ECG data requires trained staff for its interpretation and must be cleaned to remove errors generated during its acquisition. Finally, as mentioned previously, the data obtained from ECG can be useful to assess the arousal levels of an emotion, but it is not completely clear to what extent it provides information on valence.

Methods to assess or visualise brain activity like fMRI allows us to measure directly which areas of the brain are active during specific situations. Therefore they can be considered the most reliable and objective indicator of the central processing of emotions. Through fMRI readings, it is possible not only to identify which structures are involved in emotional processing, but it has the potential to measure the activity of both brain hemispheres and, therefore, identify brain lateralisation processes. The use of fMRI has several limitations; animals need to be trained to stay still during the scanning, the equipment is highly costly and requires clinical facilities to perform brain scans, making this technology very limiting for studying emotions in animals.

To measure hormone concentrations, it is usually necessary to restrain animals to obtain blood or saliva samples, which might cause distress during the process. Experienced staff are required to analyse the samples with access to a laboratory for processing. Furthermore, the synthesis of hormones like cortisol follows a circadian rhythm in some and as it is main role is associated with the metabolism of glucose it is likely to vary in response to exercise, feeding and time of the day. The measurement of neurotransmitters like dopamine and serotonin usually requires more complex techniques and expertise; in animals, analysis of those neurotransmitters has been done mainly using immunohistochemical analysis of brain tissue, meaning that animals must be sacrificed to obtain the samples, implying additional limitations as it is not possible to measure changes over time.

Infrared thermography has been used in humans to identify temperature patterns in ROIs of human faces, associated with specific emotions, like anger, disgust, fear, joy, and sadness (Cruz-Albarran et al., 2017). However, to achieve a high level of accuracy, a calibration system based on the verbal report of emotions is required, suggesting that those patterns are not fixed to all individuals. The main issue to use this approach in animals is the lack of a verbal report, making it more difficult to identify individual differences in response to the emotions elicited. In animals, infrared thermography has been used to identify temperature changes in the nasal and ocular area associated with different emotions. However, there have been disagreements in the interpretation of results, as some researchers have observed increased temperatures in those areas to be a response towards a negative stimulus, whilst others have found a similar reaction towards positive emotions. As mentioned earlier, several studies by the group of Stewart looking at eye temperature reported a decrease in peripheral temperature due to sympathetic dominance of the ANS, followed by an increase in temperature above baseline levels, associated with parasympathetic dominance of the ANS. These studies looked at the effect of aversive handling (Stewart et al., 2008a), hot iron-disbudding (Stewart et al., 2008b), castration (Stewart et al., 2010a) and injection of exogenous adrenaline in cattle (Stewart et al., 2010b); even though all of those studies can be considered to be focused on negative emotions, the association between exogenous catecholamines and decreased eye temperature, suggest that changes in peripheral temperature, might be more an indicator of emotional arousal than of emotional valence.

As has been shown in animal and human studies, infrared thermography has the potential of being a useful tool for the study of emotions. It is a non-invasive method that allows a quantitative measure of temperature dynamics associated with physiological changes in the organism in real time; nevertheless, there are several limitations for its use: to have reliable temperature readings, it is necessary to: avoid direct contact between the sunlight and the surface measured, avoid as much as possible high levels of wind/ventilation, and to measure external parameters, like ambient humidity, ambient temperature and distance between the camera and the object (Church et al., 2014; Fernández-Cuevas et al., 2015; Ijichi et al., 2019). Therefore, it is recommended to use infrared thermography indoors or in conditions that allow the control of those environmental factors. Furthermore, changes in skin temperature are positively correlated with environmental temperature and are likely to reflect changes in blood flow or muscular activity non-related to the processing of affective states.

1.8. A novel approach to the use of thermography for the study of emotions

My PhD aimed to identify if temperature asymmetries in different ROIs in dairy calves and horses could be associated with changes in affective states. As mentioned in the previous chapters, infrared thermography has been a valuable tool to identify changes in temperature associated with the balance between the sympathetic and parasympathetic branch of the ANS, suggesting a relation between peripheral temperature and emotional arousal. However, the interpretation of those results in terms of emotional valence has been contradictory.

The hypothesis to be tested here is whether emotions are processed differently in the brain hemispheres (brain lateralisation process), it is feasible that different areas of the animals' face might show an asymmetric temperature pattern, resulting from local changes in vascular and muscular activity associated with differences in brain activity regulating the autonomic innervation of those areas. Temperature asymmetries refer to the differences between the left and right sides of regions that have bilateral positioning on the animals' heads, like the eyes, nostrils, nasal airways, and ears.

For this purpose, emotions will be categorised as suggested by the core affect theory mentioned in chapter 1.2, considering them to be defined by two major dimensions: valence (positive/negative) and arousal (high/low). As mentioned in chapter 1.4.1.4., there are several hypotheses of how emotions are processed differently in the brain hemispheres. According to these hypotheses, it has been predicted that temperature asymmetries will reflect differences in brain activity that are in line with the emotional valence hypothesis (positive emotions processed mainly in the left-hemisphere and negative emotions primarily processed in the right hemisphere) and/or the approach-withdrawal hypothesis (emotions resulting in behavioural approach are processed in the left hemisphere and the ones resulting in behavioural avoidance occur in the right hemisphere), as both of those predictions are closely related.

There are three main mechanisms by which it can be expected that the activity of the brain hemispheres will influence temperature asymmetries. The first one is that areas that are closely positioned to the brain will increase their temperature due to heat convection from the closest hemisphere, the second mechanism involves an increase of temperature due to increased muscular activity mediated by the brain hemisphere from which the innervation originates, and the last mechanism is due to changes in the blood flow in a specific area mediated by the local activity of the ANS of one of the hemispheres. None of those mechanisms are mutually exclusive, and the predictions for each of the Regions of Interest (ROIs) utilised for this dissertation are presented in the following paragraphs.

For our studies, it was decided to measure temperatures in the ocular and periocular areas, nostrils, ears, and nasal airways/passageways, as those are areas that are placed bilaterally on the animal's head. The maximum temperature of the eye's inner corner was chosen as there is evidence of a high correlation between it and rectal temperature (Johnson et al., 2011; Hoffmann et al., 2013; George et al., 2014). As mentioned in chapter 1.4.1.3, several studies have found differences in the maximum temperature of the inner corner of the eye associated with changes in the sympathovagal balance of the ANS in response to stimulus eliciting high arousal emotions. It was decided to explore differences in the average temperature of the iris, as it is an area easy to identify, allowing us to standardise a region to measure the average temperature of the eye. Considering that temperature asymmetries in those two areas could indicate either the activity of the ipsilateral hemisphere as the eyes are located in close proximity to the brain or the contralateral hemisphere as the optical nerve axons decussate in a percentage between 80 to 90% in horses and cattle, and therefore its movement and irrigation is mainly regulated by the opposite hemisphere (Edmondson, 2008; Schmidt et al., 2019). As it is not completely clear if the asymmetries in the iris and inner corner areas will reflect an ipsilateral or a contralateral relationship with the activity of the brain hemispheres. It was decided to measure two highly vascularised areas surrounding the eye, which are expected to reflect muscular activity and/or blood flow controlled by the contralateral hemisphere. The area denominated “rostral eye surrounding” was allocated in the area of the “angularis oculi vessels”, whereas the “caudal eye surrounding” was allocated in the area of the “inferior and superior lateral palpebral veins” and the “external ophthalmic artery”; the musculature of both areas is innervated by the trochlear, abducens and oculomotor nerves. (Budras and Berg, 2011; Budras et al., 2012).

The nasalis muscles oversee the dilation and contraction of the nostrils and are innervated by the buccal branch of the facial nerve (CN VII). It is considered that the facial nerve controls the muscular activity of the ipsilateral nostril, reason for which asymmetries in nostrils and nasal airway areas are more likely to reflect dominance of the ipsilateral hemisphere (Sasaki et al., 2010; Budras et al., 2012; de Lahunta et al., 2014; Furr and Reed, 2015; Niimi et al., 2020).

The sensory/auditory pathway of the sound from the ears to the brain cortex is considered to be bilateral, albeit significantly more afferent fibres originating from the contralateral hemisphere are found (Firszt et al., 2006; de Lahunta et al., 2014). Similarly, studies looking at direct stimulation of the frontal cortex in macaques and gerbils suggest a contralateral dominance of efferent fibres innervating the ear muscles, which is reflected by the movement of the opposite ear when activating each hemisphere individually (Bon and Lucchetti, 1994; Seto-Ohshima et al., 2001). In humans, asymmetry in tympanic membrane temperatures measured with infrared thermometers had been directly associated with an increase of temperature on the ipsilateral brain hemisphere (Mariak et al., 1994). Similarly, studies looking at affective states in chimpanzees, marmosets, bushbabies and cats suggest an ipsilateral relationship between tympanic membrane temperatures and hemisphere activation (Parr and Hopkins, 2000; Tomaz et al., 2003; Mazzotti and Boere, 2009; Hanbury et al., 2013). Nonetheless, as temperatures were measured on the ear pinnae base, it was suggested that the "base of the ear" temperature would reflect changes in muscle activity. Therefore it was hypothesised that temperature changes in the area might indicate activity from the contralateral hemisphere. Nonetheless, it is possible that heat convection from the activity of the hemisphere located on the same side might influence the temperatures measured in this area.

In Chapter 2, the general methodology will be addressed, followed by the experimental Chapters (Chapter 3 to 6). Chapter 3 addresses the existence of temperature asymmetries in dairy calves subjected to negative emotions elicited by the routinary practice of hot-iron disbudding and the effect of previous negative experience when witnessing this negative situation. Chapter 4 looks at short-term negative emotions elicited in dairy calves separated from the mother between 24 and 48 hours after birth; in this Chapter, the association of some external factors occurring during the separation and temperature asymmetries was addressed. Finally, relationships between the different behaviours performed by the calf during the separation process and the temperature asymmetries observed were addressed.

After corroborating the existence of temperature asymmetries in different regions of interest in dairy calves subjected to negative experiences, Chapter 5 addresses some methodological questions using data from the experiment of Chapter 3, i.e. what is the effect of Ambiental parameters (temperature, humidity and temperature-humidity index), camera position (angle and elevation) and time separation between thermograms. This Chapter aims to identify the suitability of measuring temperature asymmetries within different situations and their possible limitations and determine if some regions of Interest are more suitable than others to be used under those conditions.

In the study looking at negative emotions elicited by hot-iron disbudding, the way calves responded to the researcher / thermal camera yielded interesting results suggesting that the direction of the temperature asymmetries might be influenced by whether the animals actively approach or move away from the researcher. So the idea of using the direction of the asymmetries as an indicator of emotional valence was explored in contrasting situations assumed to induce positive and negative emotions in Chapter 6. Unfortunately, due to COVID-19, our dairy cattle farm was closed to research making it impossible to continue the research with cattle. However, the opportunity to collaborate with a colleague working in horse yards allowed me to perform this study with horses instead of cattle; some challenges and difficulties in interpreting results from different species are discussed in the general discussion chapter of this dissertation (Chapter 7).

2. GENERAL METHODOLOGY

In this chapter, the general methodology used for the recording of thermal videos, selection of thermograms, measurement of the regions of interest and statistical analysis for the different studies included in this dissertation (Chapter 3 to 6) are presented.

2.1. Thermal video recording

All thermal recordings used for this dissertation were recorded using the video function of a FLIR T660^{TM} camera (FLIR Systems, Inc., USA), with a resolution of 640 x 480 pixels, a 30 FPS rate recording, sensitivity of 0.02°C and accuracy of ± 1%. It was decided to use thermal videos instead of shooting still images as it offers the advantage of having several frames (30fps), allowing to select the best images in terms of position, distance and focus (discussed in Chapter 2.2).

In all experiments, an acclimatisation period was taken to allow the animals to get used to the researcher's presence and the thermal camera pointing towards them (more detail in each experimental chapter). The camera was allowed to heat for at least 15 minutes prior to the initial recording. The emissivity of the skin, defined as the ratio of energy radiated by an object or surface to that emitted by a perfect emitter (black body), was set at 0.98 as recommended by other researchers (Montanholi et al., 2009; Hoffmann et al., 2013; Talukder et al., 2014); ambient temperature and humidity were updated every 15 minutes, and the distance between the camera and the subject was set to a distance of two meters.

The researcher recorded the thermal videos at the animals' head level for each recording from a distance between one and two meters. For Studies 1 to 3 videos, thermal data were collected from four different views of the calves' heads (front, back, left and right), whilst for Study 4, only images from the front view of the horses' heads were used.

2.2. Selection of images (thermograms)

Once the videos were recorded, the researcher watched each video and made a pre-selection of three to four images of each view, considered to be exemplars of good quality frames (Experiments 1 and 2). 'Good quality images' for the side views (left and right) were those where calves had their eyes wide open, and the angle between the inner corner of the eye and the camera lens was less than 30° (see Figure 2); for front and back views, they were those of heads in which ears were spread in a symmetrical position, taken at the head level and with an angle deviation of less than 30° from a perpendicular perspective between the head and the camera lens (see Figure 3 and 4). Once images were preselected, the best one for each view in terms of position was selected for analysis.

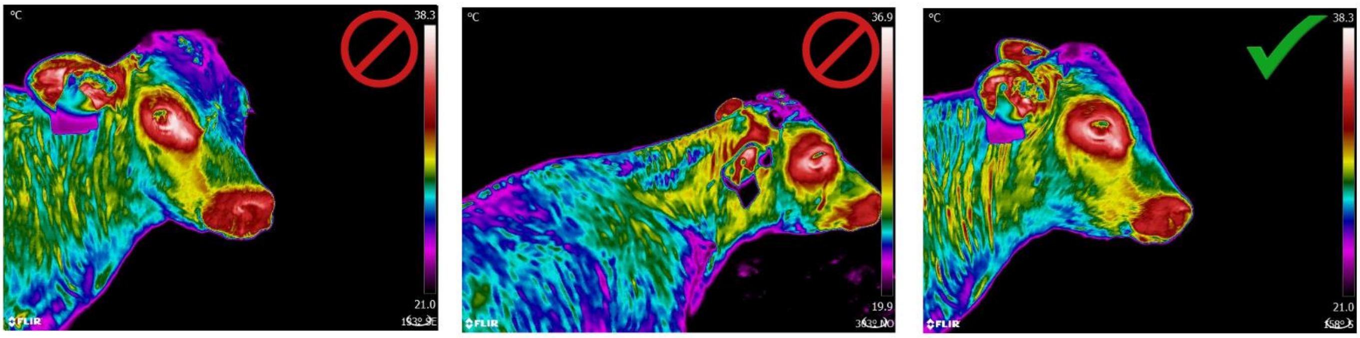


Figure 2. Bad quality and good quality examples of images taken from a side view.

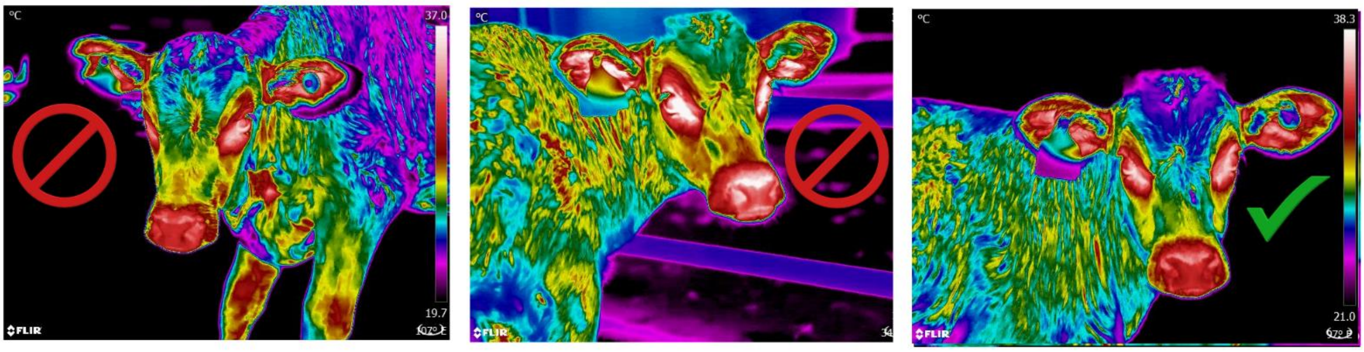


Figure 3. Bad quality and good quality examples of images taken from the front view.

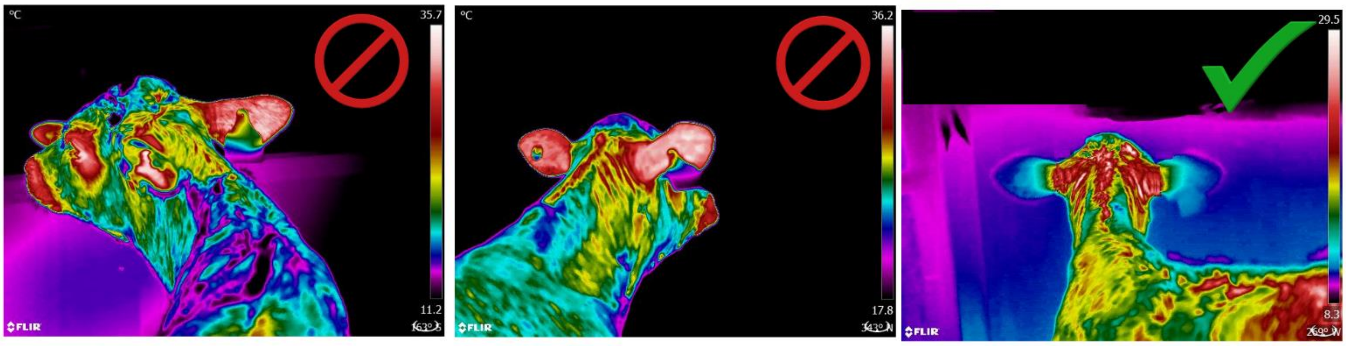


Figure 4. Bad quality and good quality examples of images taken from the back view.

For Experiment 3, images from a different angle deviation (from 0° to 60°) and position were selected to identify the effect of such variations in the thermal readings. For experiment 4, three images from the front view recorded at the head level and a deviation of less than 30° between the nostrils and the camera were selected as described in the previous paragraph.

2.3. Delimitation of the regions of interest on the Thermograms

Delimitation, extraction and calculation of the temperatures of the maximum and average temperatures of the ROIs were conducted using the software from the manufacturer, FLIR tools (6.4.18039.1003). Regions of Interest were those areas of the calves' heads selected to study temperature differences and temperature asymmetries for this dissertation. For ascertaining maximum temperatures, only the warmest pixel in the ROI was used, whereas average temperatures were calculated using all the pixels within the ROI. All the lines used to delimit the ROIs had a width of one pixel.

2.3.1. For calves (chapters 3, 4 and 5)

For studies 3, 4 and 5, images from the four different views were analysed, measuring different ROIs. From the back view, maximum and average temperatures of the base of the ears were measured. From the front view, maximum and average temperatures from the base of the ears were measured, and also from nostrils, nasal airways, muzzle and hair whorl areas. Finally, from the two side views (left and right), maximum temperatures of the inner corner of the eye, rostral eye surrounding, caudal eye surrounding and average temperatures of the eyeball were measured.

2.3.1.1. Back view: Base of the ears

Pixels selected to measure the temperature of the left and right ear bases were those lying within narrow straight lines (Li1, Li2) drawn between the lower point on the superior curve of the ear to the upper point of the inferior curve of the ear, as shown in Figure 5.

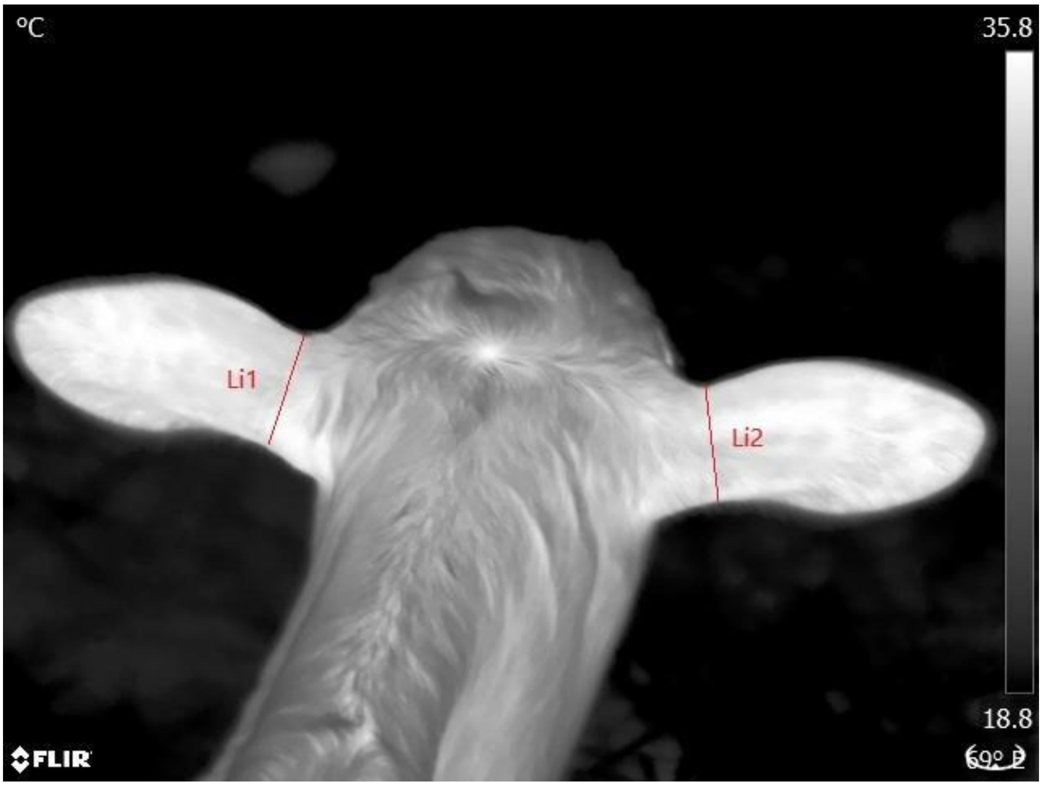


Figure 5. Delimitation of the ear base areas (Li1 and Li2) from an image taken from the back of the calf.

2.3.1.2. Front view: ears, nostrils, nasal airways, muzzle and hair whorl

To delimit the region, and pixels therein, used to calculate the temperatures of the frontal ear base areas, a straight line from the lower part of the upper curve of the ears towards the highest point of the lower curve of the ears was drawn (Li1 and Li2) (see Figure 6).

Nostril areas were delimited by circles with radii extending from the centre of each nostril towards the furthest visible point of the upper rim of the nostril (Ei1 and Ei2) (see Figure 6).

To delimit the area of the nasal passages to be measured, a horizontal line was drawn from one caruncle to the other (Li3), followed by a line parallel to Li3 at the top of the muzzle (Li4) (see Figure 6). Next, two vertical lines (Li5 and Li6) were drawn from the centre of the nostrils (Ei1 and Ei2) towards Li3. Then, a vertical line (Li7) was drawn between Li5 and Li6, from Li4 to the top of the head. Finally, the areas encompassing pixels in the nasal passage used to measure nasal temperature were defined by two narrow vertical lines (Li8 and Li9) in the spaces between Li5 and Li7, and Li6 and Li7. Those lines extended up from Li4 and were half the length of Li5 and Li6.

Pixels for measuring muzzle temperatures (Li10) lay within a vertical line from Li4 to the bottom of the muzzle, centrally located between the two nostrils (an extension of Li7) (see Figure 6).

The Hair whorl is the hairless area in between the eyes (observed as the warmest point in the forehead), measured within the Pixels contained in a circle (Ei3) with a radius extending from the centre of the whorl to its furthest visible point (hairless area) (see Figure 6).

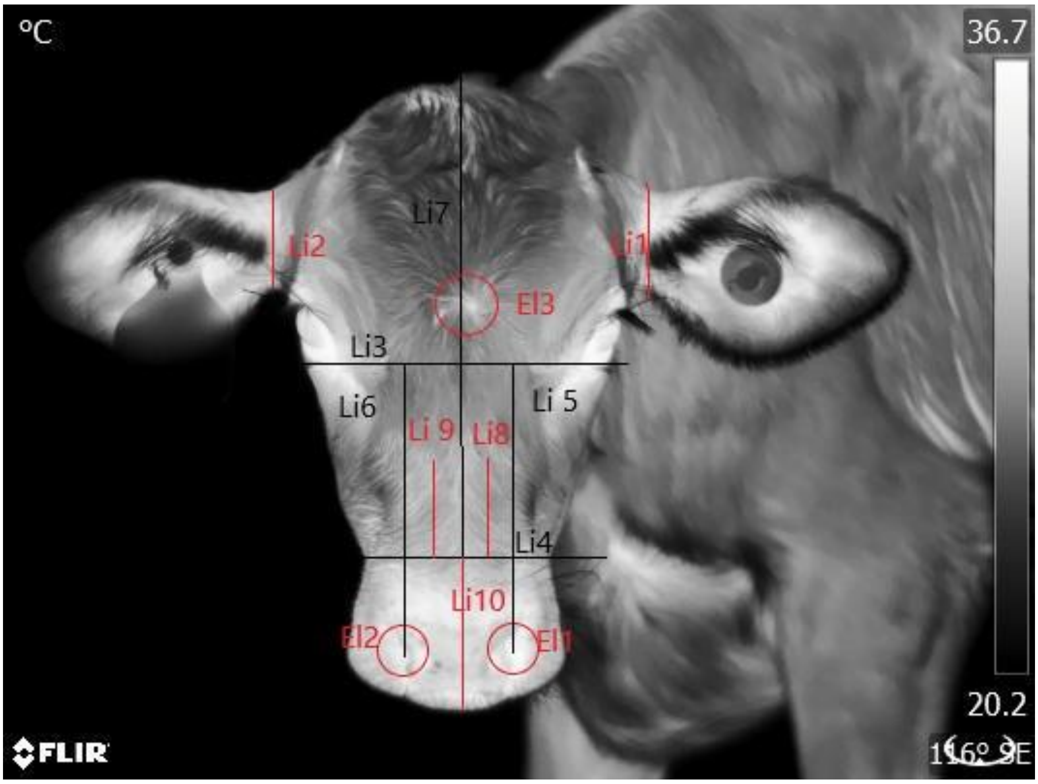


Figure 6. Delimitation of the ear (Li1 & Li2), nostrils (El1 & El2), nasal passages (Li8 & Li9), hair whorl (El3) and muzzle areas (Li10) from an image taken from the front of a calf.

2.3.1.3. Left and Right views: Inner corner, eye and surrounding areas

The area of the eyeball was delimited drawing a circle (Ei1) with a radius extending from the centre of each eyeball to the edges of the iris. The inner corner of the eye was delimited by a circle (Ei2) half the diameter of Ei1 centred on the inner canthus of the eye (see Fig 7).

To measure the rostral eye surrounding area, a line (Li1) from the inner canthus to the outer canthus of the eye, which was 5 cm wider than the eye on both sides, was drawn. A circle (Ei3) with a radius equivalent to the distance from the centre of the iris to the inner corner was drawn. Finally, the centre of Ei3 was moved along Li1 to a length equivalent to the radius of E1 in front of the eye. This defined the rostral eye surrounding area (see Fig 7).

The caudal eye surrounding area was a circle (Ei4) equivalent in size to Ei3 with its centre located on Li1 at a distance equal to the radius of E1 from the eyelashes of the calf (see Fig 7).

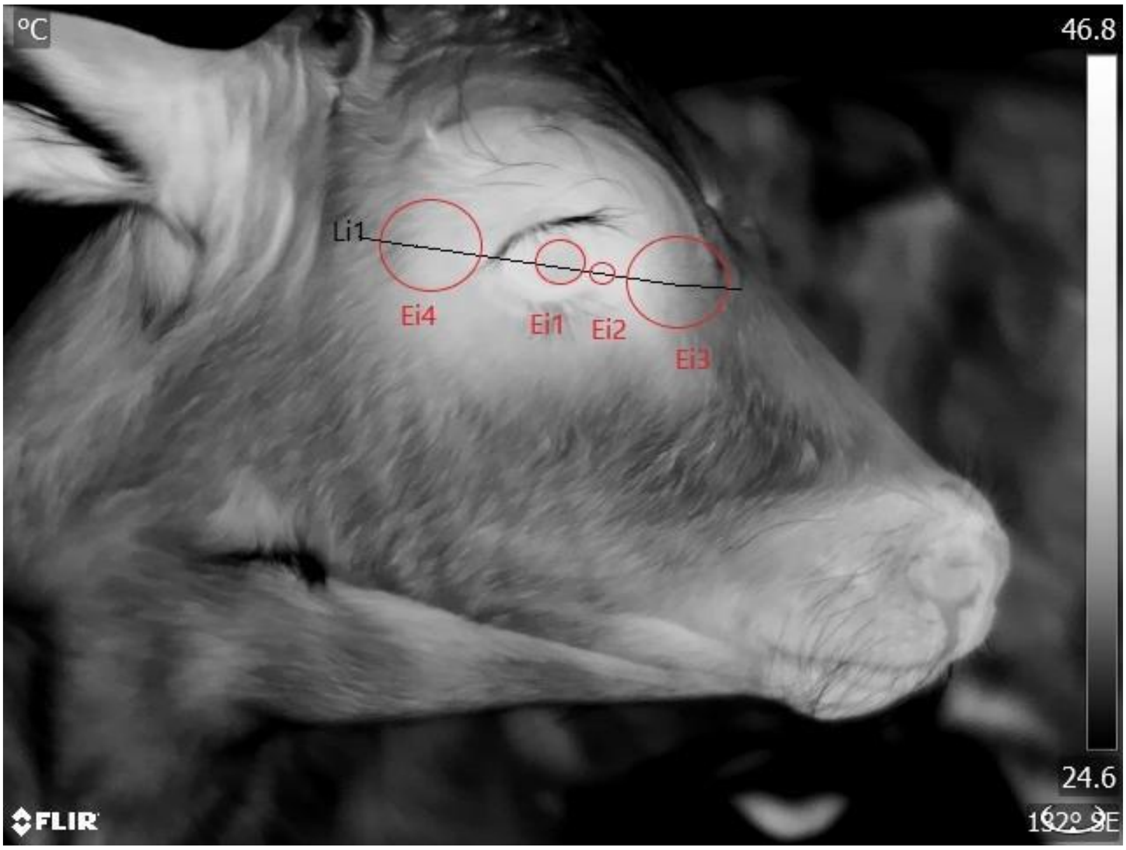


Figure 7. Eyeball (Ei1), inner corner (Ei2), rostral eye surrounding (Ei3) and caudal eye surrounding (Ei4) from an image taken from the right view of a calf.

2.3.2. Modifications for chapter 6

Due to the design of our study in chapter 6 and the limitations of moving around horses in a small space, only images from the front view were collected. The ROIs used in these images were the same mentioned in point 2.3.1.2, with the addition of two circles (Ei4 and Ei5) to measure the maximum temperature of the inner corner of the eye; such circles had a radius half the size of Ei1 and were allocated on top of Li3 at the level of the inner canthi of the eye (full details in chapter 6)

2.4. Statistical analysis

Once the lines and circles delimiting the ROIs for the studies using FLIR Tools software, the maximum and average temperatures were transferred to an excel spreadsheet, were the asymmetries of the bilateral ROIs were calculated by subtracting the value from the ROI on the right side from the ROI in the left side (L-R) giving positive values when the temperatures on the left side were higher and negatives when temperatures of the right side were higher. In the case of non-bilateral ROIs (i.e. hair whorl and muzzle), only the raw values for maximum and/or average temperatures were analysed (Only for Chapter 3, raw temperatures for the bilateral areas were used for analysis). All studies involved the use of repeated measurements and the analysis of multiple factors (depending on the aim of the study) through regression models for multilevel structures carried on using the MLwiN software to determine the effect of the different variables.

The data analysed were categorised as either arousal or emotional valence, based on the bi-dimensional approach proposed in the core affect theory (Russell and Barrett, 1999). The temperatures obtained from the muzzle and hair whorl areas were regarded as potential thermal indicators of arousal, whilst temperature asymmetries calculated from bilateral areas (left-right temperatures) were explored as possible thermal indicators of emotional valence i.e. ears, eyes, nostrils and nasal airways.

In all cases, the relationship between different variables (stated in each of the experimental chapters) and proposed thermal indicators of arousal and valence were analysed using the multilevel modelling software MLwiN V3.00 (http://www.bristol.ac.uk/cmm/software/mlwin/).

Multilevel models were created using a random effect structure with individuals nested within the repeated measurements, (e.g. calves within recording). Stepwise regression with a forward selection of the most significant variables was used to create the final models in each study. For this process, an initial association of each individual variable with the parameter in question was made. Then, the variable that improved the model the most, based on p- values calculated from the likelihood ratio test (taking into account the degrees of freedom), was incorporated into the model; when continuous variables were included in the analysis, quadratic and cubic relationships were explored. Once the first variable was incorporated into the model, the process was repeated until no further improvement of the model was found (based on p-values), e.g. eyeball eye temperature (L-R) = β_{oji} *constant* + *Variable* A_{ij} + Variable B_{ij} + Variable C_{ij} +Variable D_{ij} …".

Once the models were fitted, tables and figures were created to have a better understanding of the associations between the variables analysed and the head temperatures, particularly for variables showing quadratic and cubic relationships.

To control for multiple hypotheses, the Benjamin-Hochberg procedure to control for a False Discovery Rate of 0.2 was carried out using all the p-values calculated from the likelihood-ratio tests between the base model (without variables) and the models including the single variables.

3. “ASYMMETRIC DIFFERENCES IN SKIN TEMPERATURE AS INDICATORS OF AFFECTIVE STATES IN CALVES EXPERIENCING OR OBSERVING DISBUDDING”

Abstract

Emotions are short-term affective states that can be assessed in terms of valence (positive or negative) and arousal (low or high). Previous studies have found asymmetries in brain processes linked to emotions, including increased left-hemisphere activation during positive situations and increased right-hemisphere activity during negative and novel situations, in line with the emotional valence lateralisation hypothesis. Whether infrared thermography can detect measurable asymmetries in temperatures on the face and head of calves that may reflect physiological asymmetries related to lateralised brain function was investigated under this hypothesis. For this purpose, thermal imaging was recorded whilst animals were restraint during routine hot-iron disbudding, which is considered to induce a negative state. It was also studied whether the previous experience of being disbudded or not can influence a calf's thermal response to another calf being disbudded. Since affective arousal is linked to sympathetic activation and associated peripheral vasoconstriction, non-asymmetric skin temperature measures were used to assess arousal. Thirty-six dairy calves were assigned to three groups: calves that were disbudded (Disbudded); calves observing disbudding that had been disbudded previously (ExpObs); calves observing disbudding that had not experienced disbudding (InexObs). Thermal videos for each calf were recorded on three different days: Baseline (D1), Disbudding day (D2) and Day after disbudding (D3), and at two different times on each day: Disbudding time (T1) and Afternoon recording (T2). Data were analysed using multilevel and regression models. Calves from all groups had higher left relative to right temperatures at the base of the ears during the disbudding session (D2xT1) than on the day before (D1xT1), which may indicate higher right hemisphere activity of the calves on the disbudding session (P=0.005) . ExpObs calves had higher left than right eye temperatures (p=0.024) when observing a conspecific being disbudded (D2xT1) than InexObs calves. However, on the day after disbudding (D3xT1), InexObs calves had higher left than right eye temperatures compared to Disbudded (p=0.020) and ExpObs (p=0.025) calves. Calves from all groups had higher muzzle temperatures on D3 than on the disbudding day (D2: p=0.013) and the day before disbudding (D1: p=0.016), suggesting higher arousal on day 2. InexObs calves had lower muzzle and whorl temperatures at the time of the disbudding event (D2xT1) than both disbudded (p=0.001) and experienced observer calves (p=0.001). This study provides initial evidence of temperature asymmetries in the skin of dairy calves when observing or directly experiencing a negative event and highlights the potential for future development of methods using infrared thermography as a proxy measure of affective valence and other processes that are linked to asymmetric functioning of the brain. It also indicates that experience may substantially affect how animals perceive a situation and the resulting affective changes. Further research is required to fully understand the mechanisms underlying the skin asymmetries found in this study and to isolate those related to affective as opposed to other brain processes.

3.1. Introduction

This study explores the effect of exposure to a situation likely to cause pain and therefore elicit a negative emotional state in dairy calves (hot-iron disbudding) on asymmetries in skin temperatures as a potential indicator of such states. Hot-iron disbudding is a common practice for dairy calves that facilitates safer handling of adult animals and protects conspecifics and farmers against injuries caused by the horns of the cows. The procedure consists of applying a hot iron to the '"bud' tissue that generates the horns of adult cows at temperatures above 500ºC which may cause secondary damage to the surrounding tissues (Adcock and Tucker, 2018). The use of local anaesthesia and analgesics for this procedure reduces the sensitivity of the area and expression of behaviours considered to indicate pain, such as restlessness, ear flicking, head shaking, scratching and rubbing (Morisse et al., 1995; Graf and Senn, 1999). However, there is some evidence that other behavioural and physiological indicators of pain/discomfort last for several days after the procedure, suggesting that anaesthetic and analgesic protocols do not completely prevent disbudding from inducing a negative emotional state in calves (Heinrich et al., 2010a; Adcock and Tucker, 2018; Casoni et al., 2019).

Infrared thermography is a technology that allows the measurement of surface temperatures of tissues or objects in real-time with high accuracy, making it a potential tool for the study of thermal patterns associated with physiological changes occurring during the processing of emotions.

It is likely that temperature asymmetries in different areas of the face may reflect asymmetries in physiological function related to lateralised brain activity. Brain lateralisation ideas posit that different types of stimuli are processed predominantly by different hemispheres of the brain. For example, mammals process spatial cues (King and Corwin, 1992; Cowell et al., 1997), facial recognition (Hamilton and Vermeire, 1988; Peirce et al., 2000; Guo et al., 2009) and novelty (Larose et al., 2006; Siniscalchi et al., 2011; Phillips et al., 2015) predominantly in the right hemisphere, whilst processing familiarity (Basile et al., 2009; Phillips et al., 2015) and recognition of conspecific vocalisation (Poremba et al., 2004; Basile et al., 2009) predominantly in the left hemisphere.

From an evolutionary point of view, it has been proposed that a lateralised processing of emotions in the brain is likely to increase survival rate, as it allows a faster assessment of situations that could represent an imminent threat (Vallortigara et al., 1999; Vallortigara, 2006). There are three main hypotheses on how brain lateralisation could function in this context. The approach-withdrawal hypothesis states that emotions characterised by an active approach towards the stimuli, e.g. reward acquisition system are processed in the left-hemisphere; and emotions that result in behavioural avoidance by the individual, i.e. punishment avoidance system, are processed in the right hemisphere (Davidson et al., 1990; Sobotka et al., 1992b). A variant of the above, the emotional-valence hypothesis, suggests that negative emotions are processed more intensively in the right hemisphere and that the left hemisphere has more involvement in the processing of positive ones (Silberman and Weingartner, 1986; Cameron and Rogers, 1999; Lee et al., 2004; Leliveld et al., 2013). The second hypothesis posits that the right hemisphere processes new and unexpected situations, while the left hemisphere processes familiar situations and actions (MacNeilage et al., 2009). Finally, the right hemisphere hypothesis states that the majority of emotion processing takes place in the right hemisphere regardless of valence (Gainotti, 1972).

It is possible that temperature asymmetries in different areas of the face reflect lateralised processing of emotions. It was speculated that some contralateral changes in the skin temperature at the base of the ears could be found as a result of increased muscular activity: increased temperatures on the left ear could indicate a higher activation of the right hemisphere and vice versa. Studies have shown that direct stimulation of the frontal cortex of the brain hemispheres of macaque monkeys results in contralateral movements of the ears and eyes (Bon and Lucchetti, 1994), and there is evidence that horses preferentially move their right ears (left hemisphere) when listening to conspecific vocalisations (Basile et al., 2009).

Olfactory nerves communicate to the ipsilateral brain hemisphere. There is evidence that dogs show a preference for using their left nostril in response to cotton swabs impregnated with attractive odours such as food smell or vaginal secretions from conspecifics and a preference for using their right nostril in response to cotton swabs impregnated with putatively aversive odours such as adrenaline or familiar vet smell (Siniscalchi et al., 2011). Similarly, there is some evidence that forced unilateral breathing improves the performance of cognitive tasks processed in the ipsilateral hemisphere of the brain in humans, e.g. verbal performance is improved when using the left nostril and spatial performance when using the right one (Block et al., 1989), although findings are inconsistent (Shannahoff-Khalsa et al., 1991; Telles et al., 2017). It is thus possible that asymmetries in the nostril and nasal airway temperatures may be indicative of activation of the ipsilateral hemisphere and associated movement of and processing in the nostrils and nasal cavity.

Eyes are located near the brain, and their temperature may be affected by heat convection from the ipsilateral hemisphere. However, in cattle, most of their input is processed by the contralateral hemisphere as the optic nerves decussate. There is evidence of contralateral activation of the brain associated with gaze preferences in the perception of a novel, fearful or familiar stimulus (Rogers, 2000; Robins and Phillips, 2010; de Lussanet and Osse, 2012); suggesting that asymmetries in the ocular area due to increased blood flow, might reflect a higher activation of the contralateral hemisphere.

The relationship between asymmetric changes in skin temperature and brain lateralisation has to our knowledge only been tested in animals by Riemer et al. (2016). They explored asymmetric changes in ear pinnae temperatures using infrared thermography in dogs subjected to a separation test and found a general decrease in temperatures during social isolation, but without significant asymmetries between the right and left ear pinnae, possibly because averaging the temperature readings along the entire pinna may have masked any asymmetries that may have only occurred in those parts of the ears that are in closer proximity to the respective brain hemispheres.

The current study examines asymmetries in skin temperatures on the areas of calves' faces discussed above: eyes, nostrils, nasal passages and in the ear bases (Regions of Interest: ROIs), as these are likely to be influenced by their proximity to the brain and/or by the muscular activity and blood flow generated by brain changes. Our study aims to explore lateralised changes in skin temperature of different ROIs in calves exposed to a negative experience, i.e. restraint and hot-iron disbudding. I was also interested in examining how calves responded to other animals being disbudded – whether there was any evidence of 'emotional contagion' (Reimert et al., 2013; Hernandez Lallement et al., 2020) – and whether this was affected by the individual's own previous experience of being disbudded or not, as found by Goumon and Špinka (2016) in piglets that had experienced restraint vs naïve piglets while observing conspecifics being restrained. To test the effect of experience, face temperatures of calves observing a conspecific being disbudded were recorded: the temperatures of those that had been disbudded in a previous session (ExpObs) were compared with those of calves that had never experienced a disbudding procedure (InexObs).

Arousal was estimated using non-lateralised changes in temperature in an area previously studied (muzzle), and the potential of hair whorl temperature as an additional indicator of arousal was explored; as the centre of the hair whorl on cattle forehead is the only part of the face in addition to the muzzle, where there is bare skin not covered by hair, the insulating effect of which influences thermal readouts.

In line with the emotional valence hypothesis, it is expected to find changes associated with right hemisphere activity during situations inducing a negative affective state, i.e. activation of the right hemisphere in the disbudded calves while restrained before the administration of a local analgesic and after the analgesia had worn off on the day after. Similarly, it was predicted that calves who had experienced disbudding before would show right hemisphere activation while watching other calves being disbudded (see Table1 for the predictions summary) and that this would be less clear in naïve calves who had not experienced disbudding when they observed their conspecifics receiving the treatment.

I also investigated the relationship between temperature asymmetries and behavioural responses of calves during thermal recording that could also reflect affective state, focusing on whether calves approached, did not move, or moved away from the researcher.

Recording session /day	**Differences in Non-lateralised temperatures**	**Differences in lateralised temperatures**
Day before disbudding / Baseline (D1)	No differences between groups expected	No differences between groups expected
Disbudding session (D2 x T1)	Lower muzzle and whorl temperatures compared with baseline and the day after (All groups)	Higher temperatures on sides of the face that suggest right-hemisphere activation (All groups)
	Lower muzzle/hair whorl temperatures in the disbudded calves and ExpObs	ExpObs and Disbudded calves expected to have Higher temperatures in areas suggesting right-hemisphere activation than InexObs

Day after (D3)	Higher whorl and muzzle temperatures than on the disbudding day (All groups)	Disbudded calves to have asymmetries predicted to indicate higher activity of the right hemisphere than InexObs

Table 1. Predictions of non-lateralised and lateralised changes in temperature for the different recording days (ExpObs = experienced observers, InexObs = inexperienced observers).

Region of interest /maximum or average temperatures	**Association with brain hemisphere activity (predicted)**	**Mechanisms that can be involved in the asymmetries**
Back of the ear (average)	Contralateral * or ipsilateral	Muscular activity (contralateral changes expected) or heat convection from the brain (ipsilateral changes expected)
Front of the ear (average)	Contralateral * or ipsilateral	Muscular activity (contralateral changes expected) or heat convection from the brain (ipsilateral changes expected)
Nasal airway (maximum)	Ipsilateral	Muscular activity and / or changes in blood flow
Nostril (average)	Ipsilateral	Muscular activity and / or changes in blood flow
Eyeball (average) and inner corner of the eye (maximum)	Contralateral * or ipsilateral	Increased blood Flow (contralateral changes expected) or heat convection from the brain (ipsilateral changes expected)
Caudal eye surrounding (maximum)	Contralateral	Increased blood Flow

Table 2. Hypothesised association between temperature asymmetries and brain hemisphere activity. *most likely association according to our hypothesis.

3.2. Methods

3.2.1. Animals and treatments / (Subjects and husbandry)

Thirty-six Holstein Friesian heifer calves aged between 20 and 111 days from the University of Bristol's dairy farm were used for this study. To avoid using sick calves, only individuals that were eating and lying in a normal position and responsive to their surroundings (moving the ears to stimuli such as vehicles) were selected on the day before the recordings began.

Routine farm practices involved calves being separated from their mother between one and two days after birth, after which they were moved to a semi-open calf shed and kept for two weeks in individual pens (1.0 m x 1.5 m), allowing visual and tactile contact with other calves, and then group-housed in straw-bedded pens (4.8 m x 4.1 m) of 6 to 8 calves. Replacement milk was provided for the calves twice a day at 9 am and 4 pm. All husbandry procedures complied with UK regulations stated in "The Welfare of Farmed Animals (England) Regulations 2007" (S.I. 2007 No. 2078).

Seven disbudding sessions were recorded between January and June 2018 at the University farm. These all occurred as part of the farm's general management procedures (see Ethical Statement), and the researcher (MAR) worked opportunistically to record thermal data during the sessions. Prior to each disbudding session, the researcher accessed details of the calves to be disbudded and used these to allocate each calf into one of three study groups: 'disbudded', 'inexperienced observers' and 'experienced observers' (n=12 per group): Disbudded calves were those to be disbudded in the disbudding session (age range 27 to 52 days old), Inexperienced Observers (InexObs) were calves that had not been disbudded but were present when others were disbudded during the session (20 to 64 days old), and Experienced Observers (ExpObs) were calves that had been disbudded before the disbudding session and were present when others were disbudded during the study (48 to 111 days old). To reduce the age variability between groups, in each block, the oldest available calves that had not yet been disbudded were selected as InexObs, and the youngest available calves that had been disbudded were selected as ExpObs.

The hot-iron disbudding sessions were performed between 2:00 pm and 3:00 pm by resident veterinary surgeons accompanied by veterinary students who observed the procedure and assisted with calf restraint. Local anaesthesia via cornual nerve block with procaine (5ml per bud) and a single intramuscular injection of meloxicam (0.5mg/kg) was given five minutes prior to disbudding.

Data were collected for all 36 study calves at two different times: between 2:00 pm and 3:00 pm (T1) and between 5:00 pm and 6:00 pm (T2) on three consecutive days: the day before disbudding (D1), the disbudding day (D2) and the day after disbudding (D3); giving a total of six recordings per calf. The data collected at T1 and T2 on D1 were considered the baselines. T1 was selected as it was the time that disbudding sessions were carried out, and T2 was selected as the time when the local anaesthetic would have worn off on the disbudding day, which previous research suggests will take place within 3 hours (Herskin and Nielsen, 2018; Winder et al., 2018).

To analyse the effect of different parameters in the temperatures observed, the following information was collected: ambient temperature (°C) and humidity (%), age of the calf (days), calf's response to the camera during the recording (approaching, moving away, not moving and restricted), whether calves received exclusively solid feed in their diet (Y/N), had diarrhoea during the study (Y/N), cough during the study (Y/N) and whether calves having received analgesic or antibiotic treatment in the two weeks before recording (Y/N).

3.2.2. Thermal video data

Thermal videos were recorded for 5 minutes ± 2 minutes for each calf using a FLIR T660™ thermal camera (FLIR Systems, Inc., USA), with a resolution of 640 x 480 pixels, 30 frames per second, sensitivity 0.02 ºC and accuracy ± 1%, at the six different occasions, focusing on the calf's head. To extract the temperature data from the ROIs (i.e. eyes, nostrils, nasal passages and ears), each video aimed to contain at least one image from each of the four views selected for this study, which were the front, back, left and right sides of the head. The emissivity of the calves' skin was fixed at 0.98 as stated in previous studies (Montanholi et al., 2009; Hoffmann et al., 2013; Talukder et al., 2014) with the ambient temperature and humidity recorded and updated on the camera every 15 minutes.

All the videos were recorded while the calves moved freely in the pen, except for the recordings of the calves in the disbudding group on the disbudding day (D2 x T1). The latter recordings lasted around 2 minutes, and the subjects were restrained in their pen. The experimenter operating the thermal camera was at a distance of 1m to 2m during all recordings.

One day before the data collection (D0), the experimenter visited the pens to select the calves for the experiment and to habituate the calves to the thermal camera by standing in one corner of the pen for 10 minutes and then moving slowly around the pens pointing the camera towards each calf for 2 minutes.

For every recording session (D1, D2, D3), the camera was turned on half an hour prior to data collection, and the experimenter entered the pens and walked slowly around before pointing the camera towards the calves and starting the actual recordings, in line with the habituation process.

On the first recording session (D1 x T1), the experimenter entered the first pen and pointed the camera towards the first calf two minutes prior to starting the recording. The recording was stopped when he considered there were between three and four suitable quality frames from each view, i.e., for the side views, frames with calves having their eyes wide open and at an angle of less than 30° from the camera lens surface; for the front and back views, frames where the calves had their ears spread in a symmetrical position, with the head at camera level and less than 30° deviation between the face/head and the camera lens surface. The experimenter then pointed the camera towards the next calf and repeated the procedure until all the calves selected from that pen were recorded. The same recording order was followed through the recording sessions, except on the disbudding session (D2 x T1), in which the calves to be disbudded were recorded in the order that the veterinary surgeons and students selected and restrained them.

The thermal videos from the disbudded calves during the disbudding session (D2 x T1) were collected during a standardised restraining process lasting about two minutes before the cornual blockage. Care was taken to restrain only the bodies of the calves with two students gently pushing the calf against a fence with their bodies, limiting the contact of their hands to the calf's shoulders and legs and avoiding any contact with the head that could affect the temperature of the areas of interest. Recording the reaction of disbudded calves to restraint before disbudding, instead of recording during disbudding itself, was chosen because of the use of local anaesthetics and contact with the hot iron and/or radiated heat from the hot iron to other parts of the surface of the head, would affect the temperatures recorded (Stewart et al., 2008b).

3.2.3. Selection of thermal images for analysis

The thermal videos were examined by the researcher using FLIR Tools (6.4.18039.1003) software (FLIR Systems, USA), and frames considered to be good quality exemplars of each view were extracted (as explained in the previous chapter). This was followed by the selection of the best frame for each direction of view in each thermal video, according to the following criteria: for the front and back views, images with the calf's ears fully extended and in the most symmetrical position were selected; for the left and right views, the most identical images judged by eye from both sides of the head in terms of eye position (eye opening and iris location) and camera position (distance and angle from the camera) were selected. In all cases, images where the plane of the surface of interest was oriented ≥30^{o} away from the surface of the camera lens and images collected immediately after drinking or external disturbances (e.g. vehicles moving inside the building or staff moving around) were not used.

Once selected, the file names of the images were replaced by randomised numbers to blind researchers to their identity during the measuring process. For each recording, the calf's behavioural response to the camera was also scored according to the following criteria: if calves moved towards the camera more than away from it, the recording was scored as 'following'; if calves moved away from the camera more than they approached it, the recording was scored as 'moving away' and if they did not exhibit any locomotion in response to the camera, the recording was scored as 'not moving'; calves disbudded during the disbudding session (D2 x T1) were scored as 'restrained'. Table 3 summarises the temperatures extracted from the different regions of interest (ROIs). Figures 1, 2 and 3 illustrate the ROIs.

View	**ROIs**	**Temperatures °C extracted**	**Data used in the analysis:**
Back	Ear base	Maximum and average	Asymmetry
Front	Ear base	Maximum and average	Asymmetry
	Nostrils	Maximum and average	Asymmetry
	Nasal airways	Maximum and average	Asymmetry
	Muzzle	Maximum and average	Raw data
	Hair whorl	Maximum	Raw data

Left / right	Eyeball	Maximum and average	Asymmetry
	Inner corner	Maximum and average	Asymmetry
	Caudal eye surrounding	Maximum and average	Asymmetry
	Rostral eye surrounding	Maximum and average	Asymmetry

Table 3. Regions of Interest (ROI) for each of the views and types of temperature data collected. 'Maximum' refers to the pixel with the highest surface temperature within the ROI, and 'average' refers to the average surface temperature of the ROI. Data points were of two types: either raw temperature data extracted from the image or the temperature asymmetry between two ROIs expressed as left-side temperature minus right-side temperature.

3.3.4. Measurements of the Regions of Interest (ROI)

The explanation of the measurements of the different ROIs can be found in the general methodology (chapter 2.3)

3.3.5. Statistical Analysis

For the ROIs used to study whether lateral differences in temperature are linked to affective states, the differences between the left and right temperatures (left minus right) were calculated; positive values indicate higher temperatures on the left side and negative values higher temperatures on the right one. Raw data were used to infer arousal levels as they came from non-lateralised, single regions (muzzle and hair whorl).

Partial correlations controlling for the effect of "calf ID" were run to determine correlations between temperatures measured in different ROIs so as to detect areas which provided similar information. To categorise the correlations between ROIs according to Mukaka (2012), R values of between 0.3 and 0.5 were considered low correlations, R values of 0.5 to 0.7 as moderate correlations, R values between 0.7 and 0.9 as high correlations and above 0.9 very high correlations. If temperatures were moderately correlated (*r-value > 0.5*) across two or more areas, one of the areas was selected for further analysis. Where temperatures across two or more bilateral ROIs were highly correlated, only one of the ROIs was selected for further analysis.In this case, the area that was less vulnerable to measurement error was selected (e.g. nostril and passage data were closely correlated, but nostrils were selected because temperatures delimited by circles are more repeatable than those delimited by lines since the average temperatures are measured in a wider area, and maximum temperatures of circles are less likely to be affected by small variations in position than maximum temperatures of lines).

3.3.5.1. Main effects on temperature (all data)

Statistical models for repeated measures were built using the data from all six recording sessions to identify significant associations between temperature (temperature asymmetries in bilateral areas (L-R), or muzzle and whorl temperatures) and potential predictor variables, i.e. ambient temperature and humidity, age of the calves, recording situation (D1xT1, D1xT2, D2xT1, D2xT2, D3xT1, D3xT2), calf group (InexObs, Disbudded, ExpObs), response of the calves to the camera, calves' diet, presence of diarrhoea, calves coughing, and whether calves received analgesic or antibiotic treatment in the two weeks before recording.

Linear multilevel models were created using a random effect structure nesting calves within the situation, setting level 1 (i) as situation, i.e. 1 to 6 and level 2 (j) as calf number, i.e. 1 to 36. The models were created using a stepwise regression with a forward selection of the variables, starting with the associations with each of the predictor variables one at a time. The predictor variable whose addition resulted in the best improvement of the model based on p-values calculated from the likelihood-ratio test of the difference between the single model and the ones incorporating each of the individual variables separately (taking into account the degrees of freedom from the variables) was added to the model, and the procedure was repeated, adding each of the remaining variables until no significant improvements to the model were found. e.g. *"Inner corner max (Left-Right) D2 x* $T1_{ij} = \beta_{oji}$ *constant + Variable* A_{ij} + Variable B_{ij} +…".

3.3.5.2. Differences between groups within situations

To identify ROIs that differed between the three groups at different situations (timepoints), first, the means of asymmetric differences and muzzle and whorl temperatures were calculated and plotted with their lower and upper 95% confidence intervals in Microsoft Excel version 16.0. Then those time points in which the confidence intervals of two or more groups did not overlap were selected by visual inspection for further analysis.

Single-level regression models (with calf-ID as the level) were then built for each of the areas identified (Calf ID as a single level) using data from the specified situation and adding variables in a stepwise regression (as in 2.5.1 but excluding 'recording situation' as a variable), e.g. *"Inner corner max (Left-Right) D2 x* $T1_i = \beta_{oi}$ *constant + Inner corner max (Left-Right) D1xT1*$_i$ + *Variable* A_i + Variable B_i +…".

All the statistical models were carried out using the multilevel modelling software MLwiN v3.00 (http://www.bristol.ac.uk/cmm/software/mlwin/).

To control for multiple hypotheses, the Benjamin-Hochberg procedure to control for a False Discovery Rate of 0.2 was carried out using all the p-values calculated from the likelihood-ratio tests between the base models (models without explanatory variables) and the models including the single variables.

3.3.6. Ethical statement

The research was approved by the University of Bristol Animal Welfare and Ethical Review Body. All the husbandry procedures during the data collection period were performed normally according to the farm practices, complying with the UK legislation stated in "The Welfare of Farmed Animals (England) Regulations 2007" (S.I. 2007 No. 2078). The disbudding procedures on-farm were performed by specialised veterinary staff from Langford Vets together with veterinary students under their supervision. This study did not alter any timings or routines but simply made thermal recordings of the procedures as described above.

3.3. Results

In presenting results, tables of final model coefficients, p-values and directions of effects were provided in the Appendix chapter. An exemplar table is provided for the first set of results presented (Table 4).

3.3.1. Correlations between temperatures in different ROIs

The maximum and average temperatures of the same ROIs were highly correlated ('r' between 0.770 to 0.872, p<0.001). Muzzle and hair whorl areas had high correlation values ('r' between 0.593, and 0.701, p<0.001) and ROI of areas close to each other were moderately correlated ('r' from 0.248 to 0.518, p<0.001), except for caudal eye surrounding with eyeball (p= 0.320), caudal eye surrounding with rostral eye surrounding (p= 0.551) and caudal eye surrounding with inner corner (r=.181,p=0.008). See Table 4 for all significant correlations and the measures that were selected for further statistical analyses (indicated by an asterisk).

AROUSAL INDICATORS		
ROIs		**CORRELATION**
Muzzle (max)	Muzzle (ave) *	r=0.872, p<0.001
Muzzle (ave) *	Whorl (max) *	r=0.701, p<0.001

Muzzle (max)	Whorl (max) *	r= 0.593, p<0.001
EAR BASE		
ROIs		**CORRELATION**
Ear base back (max) L-R	Ear base back (ave) L-R *	r= 0.815, p<0.001
Ear base front (max) L-R *	Ear base front (ave) L-R	r= 0.785, p<0.001
Ear base back (ave) L-R *	Ear base front (max) L-R *	r= 0.436, p<0.001
Ear base back (max) L-R	Ear base front (max) L-R *	r= 0.394, p<0.001
Ear base back (ave) L-R *	Ear base front (ave) L-R	r= 0.321, p<0.001
Ear base back (max) L-R	Ear base front (ave) L-R	r= 0.295, p<0.001
NOSTRILS / NASAL PASSAGES		
ROIs		**CORRELATION**
Nostril (max) L-R *	Nostril (ave) L-R	r= 0.770, p<0.001
Passage (max) L-R	Passage (ave) L-R	r= 0.766, p<0.001
Nostril (ave) L-R	Passage (ave) L-R	r= 0.573, p<0.001
Nostril (ave) L-R	Passage (max) L-R	r= 0.551, p<0.001
Nostril (max) L-R *	Passage (ave) L-R	r= 0.413, p<0.001
Nostril (max) L-R *	Passage (max) L-R	r= 0.352, p<0.001
OCULAR / PERIOCULAR AREAS		
ROIs		**CORRELATION**
Inner corner eye (max) L-R *	Rostral eye surr (max) L-R *	r= 0.518, p<0.001

Inner corner eye (max) L-R *	Eyeball (ave) L-R	r= 0.248, p<0.001
Eyeball (ave) L-R	Rostral eye surr (max) L-R*	r= 0.214, p=0.002
Inner corner eye (max) L-R*	Caudal eye surr (max) L-R*	r=0.181, p=0.008
OTHER CORRELATIONS		
ROIs		**CORRELATION**
Passage (max) L-R	Eyeball (ave) L-R	r= 0.274, p<0.001
Inner corner (max) L-R	Eyeball (ave) L-R	r= 0.248, p<0.001
Caudal eye surr (max) L-R	Ear base back (ave) L-R	r= 0.231, p=0.001
Ear base front (ave) L-R	Passage (ave) L-R	r= 0.229, p=0.001
Eyeball (ave) L-R	Rostral eye surr (max) L-R*	r= 0.214, p=0.002

Table 4. Partial correlation between the different measurements. The abbreviation L-R means the temperature asymmetry is expressed as the left-side temperature minus right-side temperature. The abbreviation (max) means the data point is the highest temperature of any pixel within the delimited region of interest, and (ave) means the data point is the average temperature across the delimited region of interest * denotes measures selected for statistical analysis.

3.3.2. Main effects on facial temperatures (all data)

Results in this chapter are from multilevel models aiming to identify the main effects of predictor variables on selected temperature asymmetries and muzzle and whorl temperatures (indicated by asterisks in Table 4) from all recording sessions.

3.3.2.1. Temperature asymmetries

Asymmetries in the Inner corner of the eye (L-R) were associated with differences in the reaction of the calf towards the camera (χ^2 = 8.727, df=3, p=0.033; Table 5), explaining 4.68% of the total variance on the data (24.81% at level 1; 4.68% at level 2). Right eye temperature was higher than left eye temperature (L-R is negative) in calves that reacted by approaching the camera compared to calves that reacted by moving away from the camera (L-R is positive, Fig. 8a).

Variable	**categories**	**coefficient**	**S.E.**	**p-value**	**Asymmetries towards**	**Active hemisphere (hypothesised)**
Constant	n/a	0.141	0.120	p= 0.239		
Reaction to the camera	Moving away	Reference category				
	Approach	-0.508	0.175	p= 0.004	Right eye	Left hemisphere
	No movement	-0.167	-0.126	p= 0.187		
	Restrained calves on the disbudding session	-0.273	0.208	P=0.190		

Table 5. Final model for asymmetries in Inner corner Max (L-R). Tables for the rest of the models can be found in the supplementary material.

Asymmetries in rostral eye surround (L-R) were associated with whether calves had milk in their diet (χ^2 = 4.59, df=1, p=0.032; table 17), explaining 6.9% of the total variance on the data (0% at level 1; 38.69% at level 2). Rostral eye surround on the left side was warmer than on the right side (L-R is positive) in calves that were on solid food diet compared to calves that received milk in their diet on the day of recording (Fig. 8b).

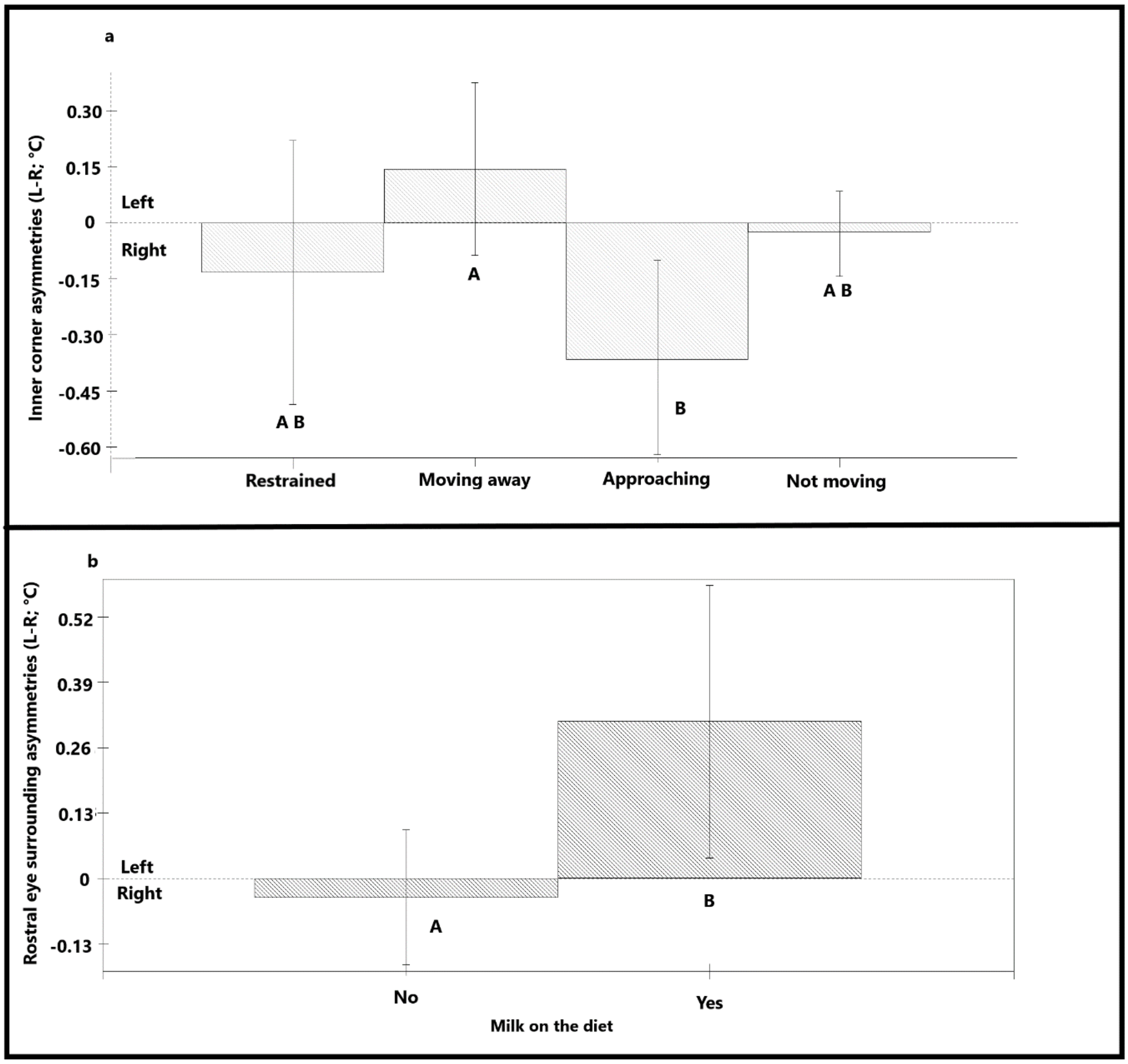


Figure 8. a) inner Corner asymmetries (L-R) by calf response to the camera.
b) rostral eye surrounding asymmetries (L-R) by whether calves receive milk in their diet.
*Bars with different letters are significantly different from each other (p<0.05).

Asymmetries in maximum values of the front ear temperature were associated with the day of recording (χ^2 =7.67, df=2, p=0.021) and had a quadratic relationship with the humidity percentage in the environment during the recording (χ^2 =7.83, df=2, p=0.019; table 18), explaining 6.29% of the total variance on the data (7.73% at level 1; 0% at level 2). On the day of disbudding (D2), temperature asymmetries between the ears increased such that the left ear was even warmer than the right ear in comparison to on the preceding day (D1). At high levels of humidity (>80%), asymmetries shifted such that the right ear was now warmer than the left ear in comparison to middle-level humidity (45 to 70%) (Fig. 9a).

Asymmetries in the Nostril max temperature were significantly associated with whether calves had diarrhoea during the study (χ^2 =6.37, df=1, p=0.011; table 19), explaining 4.68% of the total variance on the data (24.81% at level 1; 4.68% at level 2). Calves that had diarrhoea on the recording day had higher temperatures in the right nostril than the left one (L-R is negative) in comparison to calves not having diarrhoea which did not show asymmetries in temperature (Fig. 9b).

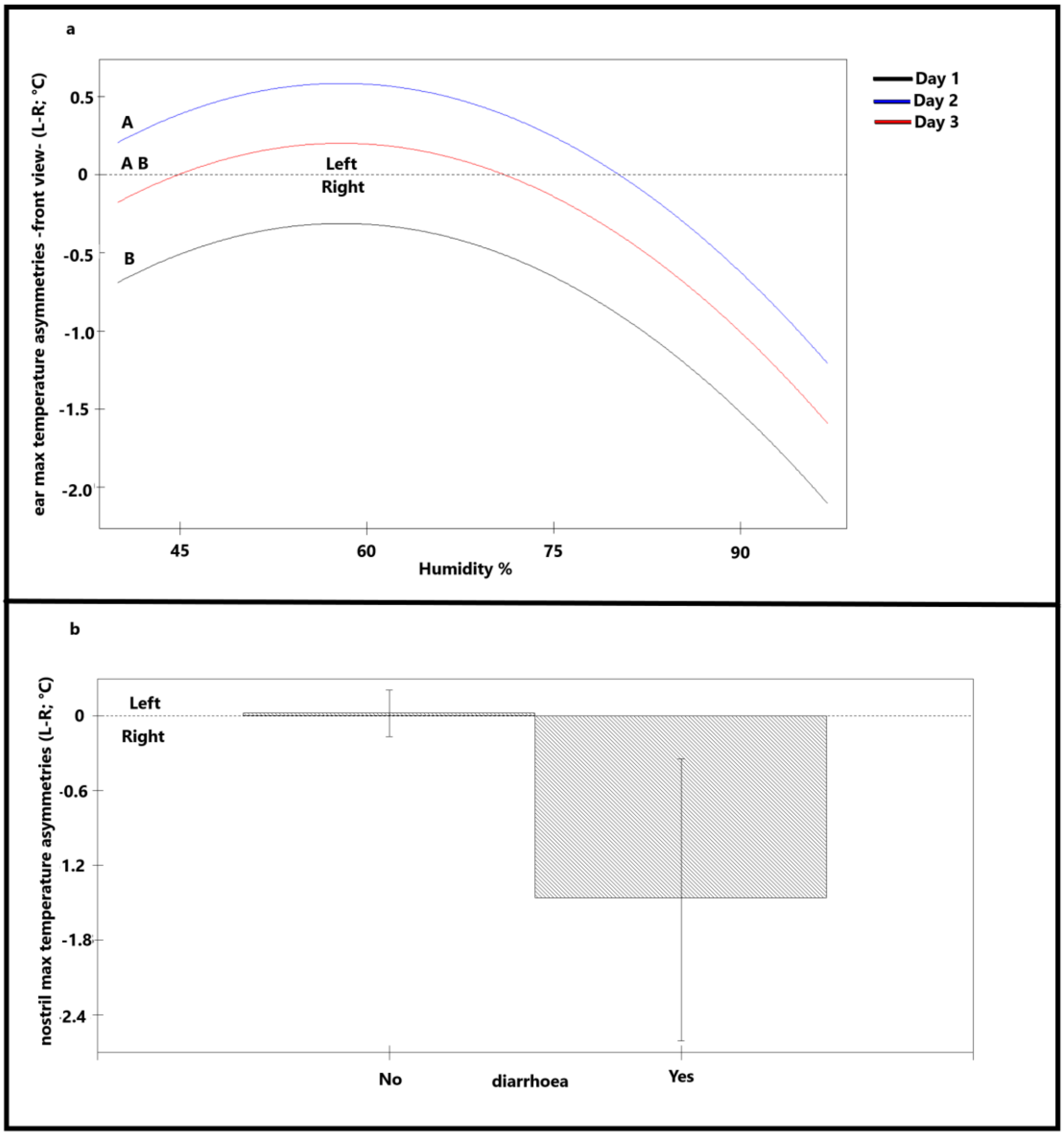


Figure 9. a) front ear asymmetries, day and humidity effect. b) nostril max asymmetries, diarrhoea effect. *Different letters denote significant differences (p<0.05).

3.3.2.2. Hair whorl and muzzle temperatures

The average temperature of the muzzle was associated with day of recording (χ^2 =8.126, df=2, p=0.017), had a quadratic relationship with humidity percentage in the environment (χ^2 =15.086, df=2, p<0.001), and a cubic relationship with the ambient temperature (χ^2 =68.685, df=3, p<0.001; table 20) explaining 49.75% of the total variance on the data (76.59% at level 1; 27.11% at level 2). On the day after the disbudding session (D3) muzzle temperatures were higher than on the disbudding day (D2) and the day before the disbudding session (D1). As the ambient temperature increased from 8^{o}C to 22^{o}C, the muzzle temperatures increased, followed by a decline at higher ambient temperatures (Fig. 10a). There was a small increase in muzzle temperatures as humidity increased from 45% to 60%, followed by a steep decline as humidity increased from that point (Fig. 10b).

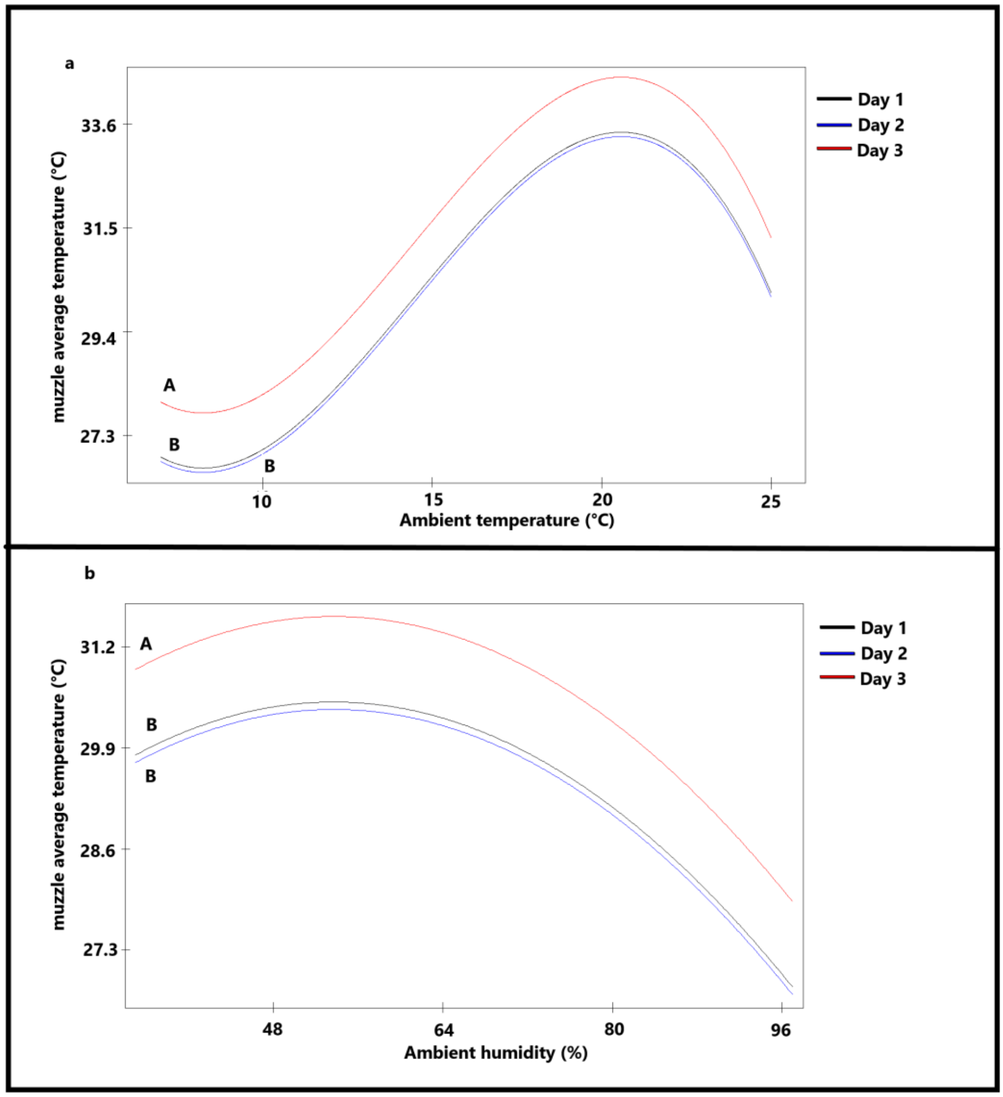


Figure 10. a) muzzle average temperatures, effect of ambient temperature and day. b) muzzle average temperatures, effect of ambient humidity and day. *Different letters denote significant differences between recording days (p<0.05).

The maximum temperature of the hair whorl was associated with antibiotic use in the two weeks prior to the study (χ^2 =6.891, df=1, p=0.009) and, in a cubic relationship with ambient temperature (χ^2 = 68.59, df=3, p<0.001; table 21) explaining 55.25% of the total variance on the data (19.87% at level 1; 78.55% at level 2). Calves that received antibiotics in the two weeks prior to the study had significantly lower hair whorl temperatures than the ones that did not receive treatment prior the study; As the temperature increased from 0^{o}C to 23^{o}C the temperature of the hair whorl increased, followed by a small decline at higher ambient temperatures (Fig. 11).

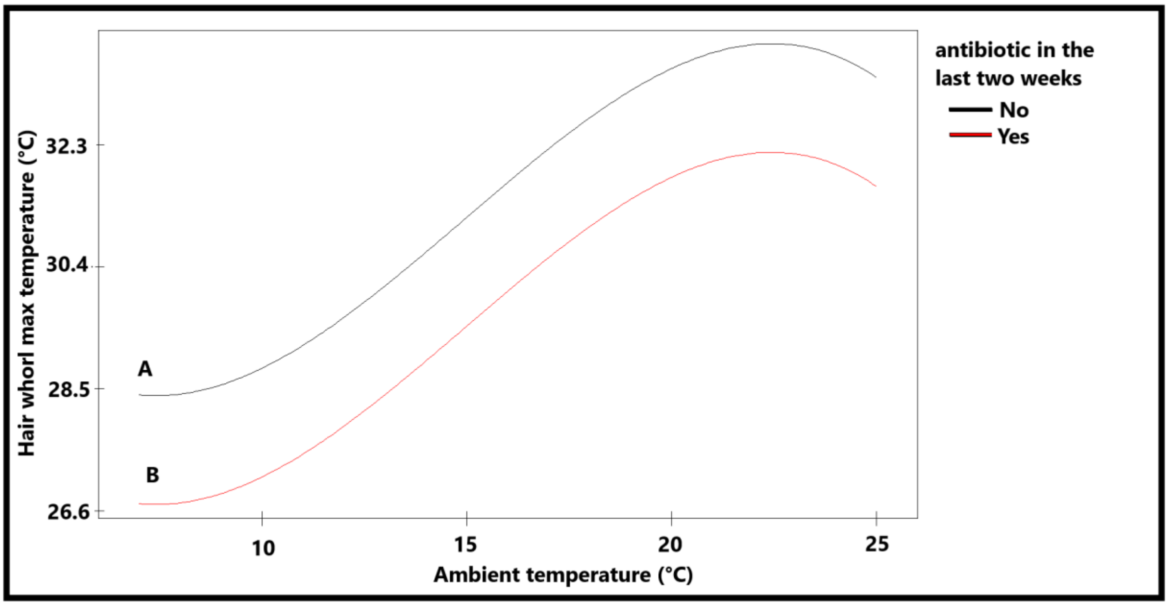


Figure 11. Hair whorl temperature, effect of temperature and antibiotics
*Different letters denote significant differences ($p<0.05$).

3.3.3. Differences between groups within situations (timepoints)

Although calf group (InexObs, Disbudded, ExpObs) was not significant in any of the models above, examination of the means and 95% confidence intervals of data from the disbudding session (D2 x T1) suggested a potential difference between InexObs and ExpObs in asymmetries in the Inner corner of the eye. Therefore, data from the disbudding session were examined in a separate model.

3.3.3.1. Temperature asymmetries

Inner corner eye asymmetries in the Disbudding session (D2 x T1) were associated with receipt of analgesic in the two weeks preceding the study (χ^2 =4.23, df=1, p=0.039) and difference between calf groups (χ^2 =6.82, df=2, p=0.033; table 22), explaining 26.81% of the total variance in the data. Calves that received analgesics in the two weeks prior to the study had higher asymmetries towards the inner corner of the right eye (L-R was negative) than calves not receiving analgesics before the study. Calves in the ExpObs group had significantly higher asymmetries towards the Left eye (L-R was more positive) than Calves in the InexObs group (L-R was negative, Fig. 12).

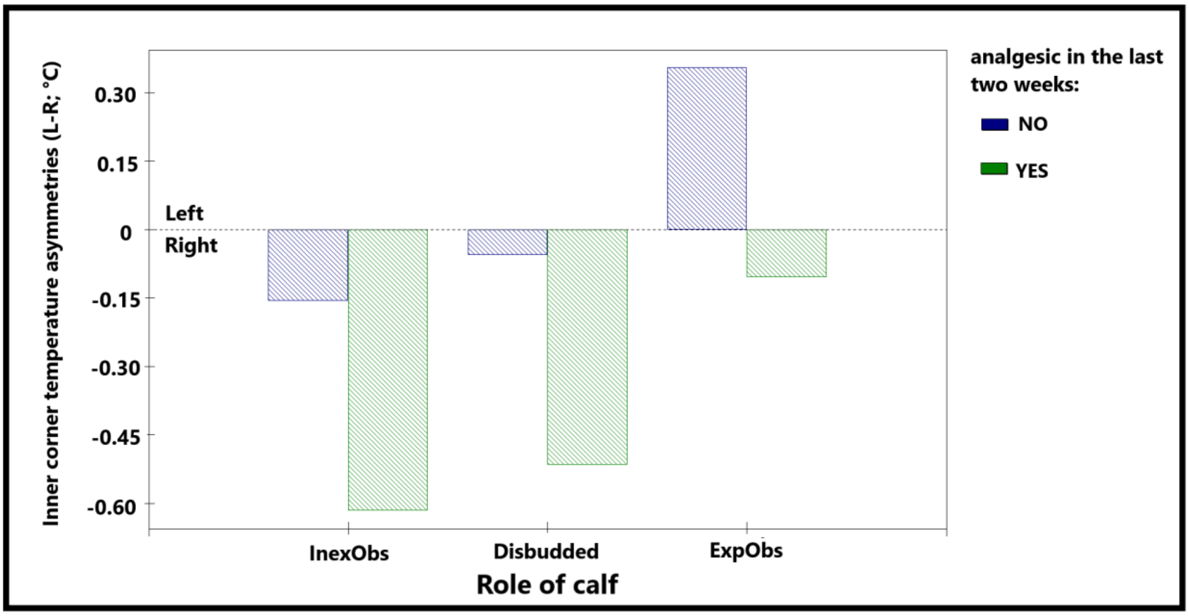


Figure 12. Inner corner temperature asymmetries in the disbudding session (D2 x T1), effect of role and analgesic.

Similarly, examining the means and 95% confidence interval data from the day after disbudding at the disbudding time (D3 x T1) a potential difference between the observer groups was identified in the asymmetries of the inner corner and asymmetries of the Rostral eye surrounding.

Inner corner eye asymmetries on the day after disbudding (D3 x T1) were associated with calf group (χ^2 =9.76, df=2, p=0.007) and the response of calves to the camera (χ^2 =9.095, df=2, p=0.010; table 23) , explaining 39.07% of the total variance in the data. Calves in the InexObs group had temperature asymmetries towards the left inner corner of the eye (L-R is positive) in comparison to calves from the Disbudding and ExpObs group that had asymmetries towards the right eye (L-R is negative, Fig. 13); calves that approached the camera during the recording period had asymmetries towards the right Inner corner (L-R is negative) compared to calves that moved away or did not move in response to the camera who had asymmetries towards the left inner corner (L-R is positive, Fig. 13).

Rostral eye surround asymmetries on the day after disbudding (D3 x T1) were associated with calf group (χ^2 =9.401, df=2, p=0.009; table 24), explaining 23.01% of the total variance in the data. InexObs had asymmetries towards the left rostral eye surround (L-R is positive) compared to calves from the Disbudding and ExpObs group who had asymmetries towards the right eye surround (L-R is negative, Fig. 13).

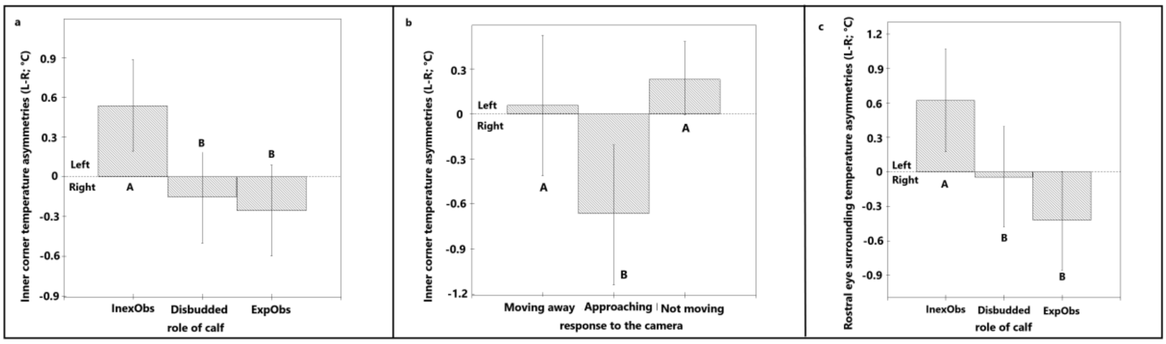


Figure 13. a) inner corner max D3 x T1, effect of calf role. b) inner corner max D3 x T1, effect of response to the camera. c) rostral eye surrounding D3 x T1, effect of calf role *Different letters denotes significant differences (p<0.05).

3.3.3.2. Hair whorl and muzzle temperatures

Examining mean and 95% confidence interval data from the 3 hours after the disbudding session (D2 x T2), potential differences between groups were explored in the muzzle average temperature and the hair whorl max temperature.

Hair whorl maximum temperatures on the day of disbudding (D2 x T2) were associated with the hair whorl maximum temperatures at the same time on the previous day, used as a baseline (D1 x T2) (χ^2 =9.181, df=1, p=0.002) and had a positive relationship with ambient temperature (χ^2 =21.678, df=1, p<0.001; Fig.14; table 25) explaining 45.23% of the total variance in the data.

The muzzle average temperatures (D2 x T2) were associated with the muzzle average temperatures at the same time on the previous day, used as baseline (D1 x T2) (χ^2 =30.6, df=1, p<0.001) , the calf group (χ^2 =16.826, df=2, p<0.001) , the ambient temperature (χ^2 =5.837, df=1, p=0.015) and, in a cubic relationship with humidity (χ^2 =8.57, df=3, p=0.035)(table 26) explaining 64.49% of the total variance in the data. InexObs calves had significantly lower muzzle average temperatures than calves in the disbudding and ExpObs groups; as the ambient temperature increased, muzzle temperature increased as well (Fig. 14), and the relationship between ambient humidity and muzzle temperatures showed different patterns as the humidity increased, e.g. a decrease in muzzle temperature was related with the increase of humidity levels from 40% to 55% and from 80 to 90%, whilst an increase in muzzle temperature was related to an increase in humidity levels from 55% to 80% (Fig.14).

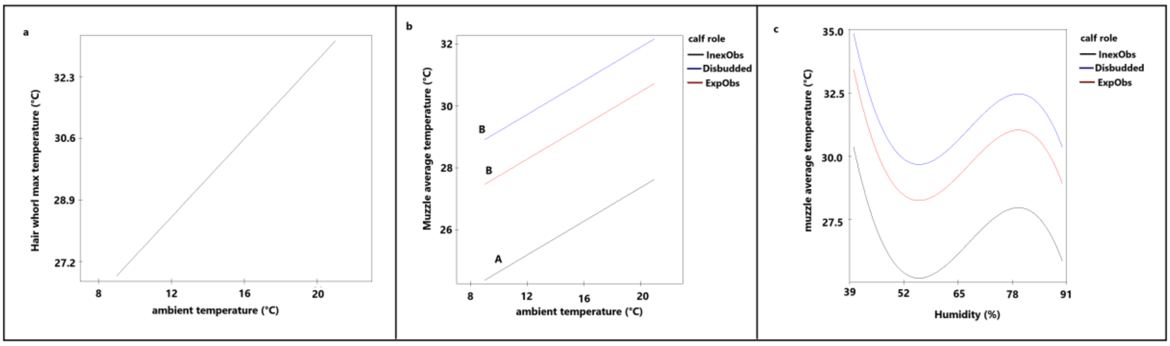


Figure 14. a) whorl maximum temperature D2 x T2, effect of ambient temperature. b) muzzle average D2 x T2, effect of ambient temperature and role. c) muzzle average D2 x T2, effect of humidity and role of the calf. *Different letters denote significant differences (p<0.05).

3.4. Discussion

This study aimed to identify whether asymmetries in calf facial temperature in different regions of interest (ROIs) exist and, if so, whether the direction of those asymmetries differed between a baseline situation in comparison to when calves were subjected to a hot-iron disbudding procedure, as a model of negative emotions.

The differences between calves subjected to the hot-iron disbudding procedure during the study and those who only observed other calves being disbudded were also investigated. For the latter group, whether the prior experience of having been disbudded influenced responses to observing another calf being disbudded was explored. Finally, it was investigated how facial temperature asymmetries were related to approach/withdrawal responses of calves towards the researcher and thermal camera. To gain initial information on potential environmental and physiological confounders to the results, the effects of ambient temperature and humidity, the presence of diarrhoea and recent use of analgesics and antibiotics were also investigated.

Asymmetries in skin temperatures were found, particularly in the ear base (the area where the ear joins the head), inner corner of the eyes and rostral area surrounding the eyes.

In general, innervation of the ears is considered to come from both ipsilateral and contralateral hemispheres, albeit significantly more afferent nerves come from the contralateral hemisphere. Suggesting that temperature changes reflecting blood flow or muscular activity are more likely to reflect higher activation of the contralateral hemisphere.

As predicted, calves from all groups had temperature asymmetries towards the left ear on the disbudding day compared to those observed in the baseline recordings, suggesting a higher activation of the right hemisphere on the disbudding day, which in line with the emotional valence theory might indicate a more negative state on that day than in the baseline recording. It is important to highlight that those temperature asymmetries were observed only in the front images. Some possible explanations are that the delimitation of our measurements from the back ear was not accurate enough or that mechanisms involved in temperature changes between the areas measured in the front and back images differed.

Asymmetries in tympanic membrane temperatures (TMT) suggesting a relationship with the ipsilateral hemisphere have been explored previously, using infrared thermometers, but temperature asymmetries have not been explored through infrared thermography. Asymmetries towards the right TMT have been found in Chimpanzees looking at videos of aggressive conspecifics (Parr and Hopkins, 2000), marmosets being restrained (Tomaz et al., 2003) and cats in response to transport and restraint (Mazzotti and Boere, 2009); whilst asymmetries towards the left TMT associated to allogrooming and affiliative approach, have been found in bushbabies (Mazzotti and Boere, 2009). However, in this study, temperatures in the base of the ear pinnae were used, which are expected to result from muscle-motor activity and thus reflected activity of the contralateral hemisphere.

The muscles in charge of the dilation and contraction of the nostrils are innervated by the buccal branch of the facial nerve, which is considered to be ipsilateral from its origin, reason for which it was hypothesised that asymmetries in nostril temperatures might reflect higher activity of the ipsilateral hemisphere. Having diarrhoea in the recording day was the only parameter associated with nostril temperature asymmetries; calves with diarrhoea had higher temperatures in the right nostril, suggesting a more negative affective state as it could be related to activation of the right hemisphere. To our knowledge, there is not previous evidence on nostril temperature asymmetries; however, a preference to use the right nostril to process novel odours that shifts to the left nostril after several presentations has been observed in dogs (Siniscalchi et al., 2011; Brown and Reimchen, 2020) and horses (McGreevy and Rogers, 2005b), which in line with the hypothesis that processing of novelty occurs predominantly in the right hemisphere and familiarity on the left one, suggests an ipsilateral relationship between nostril preference and brain lateralisation.

Regarding asymmetries in the ocular area, it was considered that inner corner temperatures were more likely to be influenced by heat convection from the brain hemisphere closest to the eye. So, measurements of temperature asymmetries in the areas surrounding the eye were carried on as they might reflect better the activity of the contralateral hemisphere. For the area denominated rostral eye surrounding, temperatures in an area highly irrigated by the angularis oculi vessels were measured, whereas the caudal eye surrounding was allocated in an area irrigated by the lateral palpebral veins and the external ophthalmic artery (Budras and Berg, 2011). The correlation between temperatures in the inner corner of the eye and the rostral eye surrounding area and the similar asymmetric patterns found on the different recording sessions (see Fig. 13) suggest that both measures might reflect the activation of the contralateral hemisphere, as they seem to be influenced by a similar lateralisation process. However, the low correlation between caudal eye surrounding and inner corner, the non-significant correlation between caudal eye surrounding and rostral eye surrounding, and the fact that asymmetries in caudal eye surrounding had no significant association with any of the variables, might suggest that either measurement in the caudal eye surrounding area was not reliable or that temperatures in the area were mediated by a mechanism different to the ones on the other ocular and periocular areas.

The previous experience of the observer groups did influence the direction of lateralised differences in eye temperature during the disbudding session and the day after. On the disbudding day, asymmetries of those observer calves that had not experienced disbudding (InexObs) were towards the right eye/left hemisphere, while the direction of the asymmetries on those observer calves with experience of disbudding (ExpObs) was towards the left eye/right hemisphere. In contrast, asymmetries on the day after disbudding were reversed for both groups, i.e. InexObs towards the left eye/right hemisphere and ExpObs towards the right eye/left hemisphere. Temperature asymmetries in the Disbudded group did not differ significantly from those in the observer groups on the disbudding day.

Asymmetries in rostral eye surround and inner corner of the eye showed higher temperatures in the left eye of experienced observers and right eye of the inexperienced observers during the disbudding session, suggesting the possibility that calves that had experienced disbudding before processed the disbudding of conspecifics as a threatening or negative situation. In contrast, Inexperienced

Observers may have perceived the situation more neutrally. This result is in line with the study of Goumon and Špinka (2016), in which piglets that had had a negative experience (being restrained) behaved more fearfully than inexperienced piglets when observing a conspecific being restrained.

Changes in eye temperature on the day after showed the reverse pattern with Experienced Observers having warmer right eyes and Inexperienced Observers warmer left eyes, suggesting a left hemisphere activation of the former and higher activation of the right hemisphere in the latter. A speculative explanation is that on the day after disbudding, the situation did not resemble the disbudding session for the Experienced Observers since only the experimenter was in the building, other calves were behaving calmly, and olfactory stimuli like gas or burned tissue were absent. On the other hand, the Inexperienced Observers may have perceived the presence of the researcher more negatively as a result of emotional contagion from their mates disbudded on the day before or as a response towards the researcher after the commotion of the day before.

Previous research has shown that cows can associate previous experiences with specific people and specific places or situations (de Passillé et al., 1996; Rushen et al., 1999; Munksgaard et al., 2001; Welp et al., 2004; Ede et al., 2019). For example, de Pasille et al. (1996) found that calves learned to discriminate between an aversive and a positive handler wearing different clothes, showing fewer interactions with the negative handler after repeated exposures and that those differences were more significant in the place where the aversive treatment was carried on (de Passillé et al., 1996). Munksgaard et al. (2001) found that cows observing a handler treating a conspecific adversely learned to avoid the handler without experiencing the negative handling themselves. Similarly, Ede et al. (2019) found that disbudded calves are less likely to lie down and spend less time in the area where the disbudding occurred. Differences between our observer groups could thus also be related to previous experiences as discussed.

Calves that were on a solid diet only (not receiving milk anymore) had asymmetries in the rostral eye surrounding temperature towards the left side / right hemisphere compared to calves receiving milk in their diet. Evidence of the behavioural motivation of calves to suck even in a non-nutritional context (Hammell et al., 1988; Rushen and de Passillé, 1995) as well as of the negative effects on affective states on calves deprived from milk (De Paula Vieira et al., 2008; Jasper et al., 2008; Khan et al., 2011; Eckert et al., 2015), that support the idea that our calves no longer receiving milk could have been in a more negative state.

The asymmetric temperatures in the inner corner of the eye correlating with the calves' response to the camera agreed with our hypothesis and with the approach withdrawal theory, as calves that approached the camera had warmer right eyes suggesting activation of the left hemisphere and the ones that moved away from the camera had warmer left eyes suggesting activation of the right hemisphere.

The research group hypothesised significant differences in eye temperature asymmetries between the Disbudded calves and the observer groups on the disbudding day and on the day after. However, those differences were not observed at any point in the study. During the disbudding session, it was expected to observe changes associated with higher activity of the right hemisphere as a response to physical restraint, but this was not observed, perhaps because calves differed in their behaviour while restrained (personal observation), which could indicate that some find the experience less aversive than others. Differences in responses to handling can be associated with temperament, fearfulness and reactivity to novelty, as well as previous negative or positive experiences (Grandin and Shivley, 2015). Another feasible explanation is that all calves, regardless of their treatment, were similarly distressed by the situation on the disbudding session, and thus no differences were found between observer and disbudded calves. On the day after disbudding, changes suggesting right hemisphere dominance were expected in the disbudded calves as a response to the residual pain caused by the procedure. It is possible that because of the analgesic treatment received after the procedure, calves might not be in enough pain to reflect the asymmetric changes expected. It is not clear to what extent the use of analgesics mitigates the pain caused by hot-iron disbudding; some studies looking at behaviour and cortisol levels have found that meloxicam had a significant effect on the reduction of pain behaviours, and its effect may last up to 5 days after disbudding (Milligan et al., 2004; Stafford and Mellor, 2005; Heinrich et al., 2010a; Theurer et al., 2012); However, other evidence points to long-lasting effects of disbudding even with the use of analgesics (Hokkanen et al., 2010; Mintline et al., 2012a; Adcock and Tucker, 2018; reviewed by Herskin and Nielsen, 2018; Casoni et al., 2019)

In terms of (non-lateralised) arousal indicators, muzzle temperatures differed between days; calves had significantly higher temperatures on the day after disbudding (D3) than on the disbudding day (D2) and the day before (D1). Elevated cortisol levels had been associated with hypertension in humans due to increased cardiac output (Whitworth et al., 2005), which could lead to fluctuations in peripheral temperature. It is possible that elevated levels of cortisol resulting from the commotion of the disbudding session could lead to an increase in peripheral blood flow on the day after disbudding, explaining the significantly higher temperatures in the muzzle area on the day after the disbudding (D3) compared to the ones on the disbudding day (D2) and the day before disbudding (D1). Alternatively, a decrease in arousal due to an altered balance between sympathetic and parasympathetic in favour of the latter could explain the differences between D3 and D2, although differences between D3 and D1 were not expected.

Three hours after the disbudding session (D2 x T2), Experienced Observers and Disbudded calves had higher muzzle and hair whorl temperatures than Inexperienced Observers, perhaps reflecting changes in blood flow mediated by enhanced parasympathetic activity (peripheral vasodilation) as a negative feedback mechanism following sympathetic activation during the disbudding session. For example, calves disbudded without anaesthesia in the study from Stewart et al. (2008) had a significant decrease in eye temperature in the first 5 minutes post-disbudding, followed by temperatures significantly higher than the ones taken at baseline (Stewart et al., 2008b). Similarly, an immediate drop of nasal temperatures in different species has been observed as a response to high arousal emotions from different valences (Kuraoka and Nakamura, 2011; Kano et al., 2016; Proctor and Carder, 2016; Chotard et al., 2018; Ermatinger et al., 2019).

In practice, the muzzle area might not be the most appropriate area to measure temperature, as it can be affected by changes in breathing patterns and is continuously in contact with external factors like food and water. A high correlation between muzzle and whorl temperatures and similar patterns through the different recording sessions were found. Therefore, it can be suggested the use of temperatures on the hair whorl as a reliable alternative to muzzle temperature in dairy cattle.

Factors external to the experimental design, including variation in health status (presence of diarrhoea or coughs, use of medication) and environmental factors (ambient temperature and humidity), significantly influenced some of our results.

Calves that received antibiotics in the two weeks prior to the recordings had significantly lower hair whorl temperatures, suggesting that some degree of fever might be present on untreated calves. Furthermore, asymmetries towards the right nostril /right hemisphere suggest that calves with diarrhoea were in a more negative affective state than healthy calves.

In previous studies, a relationship between skin temperature and ambient temperature has been attributed to thermoregulatory mechanisms involving dynamic changes in peripheral blood flow (Gloster et al., 2011; Fernández-Cuevas et al., 2015; Salles et al., 2016). In the current study, ambient temperatures had a quadratic relationship with muzzle and hair whorl temperatures, whilst humidity only had a relationship with muzzle temperatures, which are likely to be related to thermoregulatory mechanisms. However, it was not expected that those ambient factors would influence temperature asymmetries unless mechanisms other than thermoregulation were involved. It was observed that asymmetries on ear temperature increased towards the right side / left hemisphere as humidity levels increased from 60% onwards, which might suggest the involvement of an affective component in those asymmetries.

To my knowledge, our study is the first showing the existence of asymmetric differences in the ear and eye temperatures on dairy calves; giving support to the idea that asymmetries in skin surface temperatures can be a valuable parameter to consider in developing new measures of animal emotions and highlighting the potential of infrared thermography as a non-invasive tool for monitoring animal welfare.

However, to fully understand the physiological mechanisms underlying asymmetries in skin surface temperature and their relationship with brain lateralisation, a substantial amount of fundamental research and subsequent validation of this method with behavioural and physiological parameters like EEG, HRV and behavioural correlations is required.

4. "ENVIRONMENTAL AND BEHAVIOURAL EFFECTS ON FACIAL TEMPERATURE ASYMMETRIES: SEPARATION FROM THE MOTHER IN DAIRY CALVES AS A MODEL"

Abstract

The emotional valence hypothesis posits that positively valenced emotions (affective states) are mainly processed in the left cerebral hemisphere, whilst negative ones are mainly processed in the right hemisphere. Such lateralisation of processing may be reflected by superficial facial temperature asymmetries. Moreover, non-asymmetric measures of skin temperature can be used as indicators of affective arousal, which are often linked to sympathetic activation and associated peripheral vasoconstriction. This study aimed to investigate whether these indicators of affective valence and arousal could be detected using infrared thermography by studying the separation of dairy calves from their mothers as a model of induced negative affect. Twenty-four dairy calves were separated from their mothers between twelve and twenty-four hours after birth. Eight thermal recordings were collected per calf: 10 minutes before separation, immediately after separation and 10, 20, 30, 40, 50 and 60 minutes after separation. Behavioural observations using one-zero sampling were made 5 minutes before each thermal recording and during each recording, giving a total of 16 observations per calf. Data were analysed using multilevel models for repeated observations. As time from separation elapsed, the right eyeball became increasingly warmer than the left ($p<0.001$), potentially indicating an increase in left hemisphere activity with time. Hair whorl ($p=0.004$) and muzzle ($p=0.030$) temperatures also increased, suggesting a shift to parasympathetically dominated autonomic activity that may follow initial affective arousal and associated sympathetic activation, or a vasodilation effect resulting from elevated levels of cortisol. Left eyeball temperatures were elevated when disturbance/noise scores during separation were higher ($p=0.021$), calves that were standing before thermal recordings had relatively higher temperature asymmetries towards the left caudal eye surrounding ($p=0.004$) and right nasal airway areas ($p<0.001$), and those that were running before recordings had higher temperature asymmetries towards the left eyeball ($p=0.023$). These findings may reflect elevated

right-hemisphere activity given contralateral and ipsilateral brain connections to the eye and nasal areas, respectively. This study provides evidence of temperature asymmetries in different areas of the calf head during a negative experience – separation from the cow – and highlights the potential for developing methods to use infrared thermography as a tool to study animal affective states.

4.1. Introduction

This study aimed to identify whether asymmetries in surface temperatures of dairy calves' faces, measured with infrared thermography, could indicate a negative emotion assumed to occur when calves are separated from their mothers. Whether and how environmental factors and behaviour of calves during the separation process influenced thermal readings was also investigated.

An emotion is a short-lived affective state that can be described in terms of valence (positivity or negativity) and arousal (Russell, 1980; Mendl et al., 2010) and that is based on immediate appraisals of a situation (e.g. its suddenness, predictability, familiarity, pleasantness and agreement with expectations); (Davidson et al., 2003; Paul et al., 2005). Emotions and other affective states such as longer-term ''moods' comprise physiological, behavioural, cognitive and subjective components (Scherer and Ekman, 1984; Kalin et al., 1998; Sanders et al., 2003).

In humans, linguistic self-report is a key measure of subjectively experienced emotion (reviewed by Barrett et al., 2007), but this metric cannot be used in non-human animals (hereafter animals). However, physiological and behavioural changes can be used to study animal affect. Nevertheless, inter and intraspecific differences in behavioural responses to challenges can make it difficult to use behaviour to infer affective states reliably (Austin and Rogers, 2007; Goursot et al., 2018). Furthermore, measurement of physiological parameters may require the use of equipment that itself causes stress, or that can only be used in experimental settings (Zhang et al., 2000; King et al., 2005).

For these reasons, the development of non-invasive methods that can measure physiological changes in real-time is required to increase our understanding of animal emotions. Infrared thermography is a technology that can be utilised in developing such methods, measuring changes in an animal's surface temperature with high accuracy and from a distance (Stewart et al., 2007). This technology has been used in macaques and cows to study correlations between surface temperature distributions and affective states, generating interesting results likely to be associated with arousal levels elicited by changes in the autonomic nervous system (Nakayama et al., 2005; Stewart et al., 2007; Stewart et al., 2008a; Stewart et al., 2010a; Kuraoka and Nakamura, 2011). However, there are only a few studies looking at the valence component of affective states using thermography based on brain laterality theory.

There are two main hypotheses of how brain lateralisation may apply to emotional processing. The emotional valence hypothesis states that positive and negative emotions are processed differently in the brain hemispheres, the former being associated with greater processing in the left hemisphere and the latter in the right (Silberman and Weingartner, 1986; Cameron and Rogers, 1999; Lee et al., 2004). The approach-withdrawal hypothesis states that emotions associated with the approach of the individual towards a stimulus are associated with more processing in the left hemisphere, whereas emotions associated with behavioural withdrawal are processed in the right (Davidson et al., 1990; Sobotka et al., 1992a; Leliveld et al., 2013).

It is feasible that changes in facial skin temperature occur due to differences in blood flow, muscular activity or heat convection from the brain elicited by asymmetric brain activity. Regions of Interest (ROIs) in the facial area are listed in table 6, which also indicates potential mechanisms that may underlie affect-related thermal asymmetries in these areas (for more detailed discussion, see chapter 3). In previous studies from our group, temperature asymmetries in different regions of interest were identified using infrared thermography in animals exposed to a situation likely to induce an affective state. In one study, calves that had experienced disbudding (removal of the horn buds) showed elevated left eye temperatures whilst observing a conspecific being disbudded, indicating higher activation of the right hemisphere (negative emotion processing), in comparison to observer calves that had not been disbudded previously. These findings suggest an association between previous experience of disbudding and temperature asymmetries in the ocular area when observing disbudding (chapter 3).

Region of interest /maximum or average temperatures	**Association with brain hemisphere activity (predicted)**	**Mechanisms that can be involved in the asymmetries**
Back of the ear (average)	Contralateral * or ipsilateral	Muscular activity (contralateral changes expected) or heat convection from the brain (ipsilateral changes expected)
Front of the ear (average)	Contralateral * or ipsilateral	Muscular activity (contralateral changes expected) or heat convection from the brain (ipsilateral changes expected)
Nasal airway (maximum)	Ipsilateral	Muscular activity and / or changes in blood flow
Nostril (average)	Ipsilateral	Muscular activity and / or changes in blood flow
Eyeball (average) and inner corner of the eye (maximum)	Contralateral * or ipsilateral	Increased blood Flow (contralateral changes expected) or heat convection from the brain (ipsilateral changes expected)
Caudal eye surrounding (maximum)	Contralateral	Increased blood Flow

Table 6. Hypothesised association between temperature asymmetries and brain hemisphere activity. *most likely association according to our previous findings.

Here it was investigated whether similar thermal changes are observed when calves are exposed to a different situation likely to induce negative emotion – separation from the mother. Affective states can be defined as those induced by the presence/acquisition of rewards or absence/removal of punishers (positive states), and the absence/loss of rewards or presence/imposition of punishers (negative states), where rewards are things that animals work to access, and punishers are things they work to avoid (Rolls 2005; Mendl & Paul 2020). Given that newborn precocial mammals seek to remain close to their mothers, separation from them can thus be expected to induce a negative state. Indeed, newly separated calves show changes in behaviour and physiology indicative of a negative state during the first hours after separation (Weary and Chua, 2000; Marchant-Forde et al., 2002; Stěhulová et al., 2008b). For example, after separation, calves showed a more pessimistic judgement of ambiguity, indicating negative affect, compared to before being separated from their mothers (Daros et al. 2014). Calves also showed increased heart rates, vocalised more frequently and spent more time standing and moving around immediately after being separated from the mother than before separation, particularly if they were kept near their mother (Stěhulová et al., 2008b).

In the present study, I observed calves separated from the mother at an early age (12 to 24 hours), aiming to identify if separation from the mother elicits temperature asymmetries hypothesised to occur as a result of a negative experience. I looked at whether ambient parameters (i.e. humidity or temperature), environmental factors (i.e. presence of staff, vehicles, noises or conspecifics near the separation pen) and behaviours performed before (BR) and during the recordings influenced the temperature asymmetries observed. Similarly, the influence of the separation itself and the factors mentioned above in non-asymmetric temperature changes associated with elevated arousal was studied, i.e. a reduction in hair whorl and muzzle temperature immediately after exposure to a stimulus eliciting high arousal, followed by an increased temperature of these areas as a result of parasympathetic activity countering the initial sympathetic response (Baker et al., 1976; Merla and Romani, 2007; Proctor and Carder, 2015).

Based on the previous study, it was hypothesised that changes likely to be associated with negative affect (i.e. elevated temperatures in the right nasal airway or the left ear base and eye areas) would occur during and following separation and would also be associated with behaviours indicating a stronger active response to separation (standing, walking or vocalising) and other factors generating increased disturbance such as higher noise levels, vehicles moving around, presence of staff or staff shouting. Furthermore, I predicted that after separation from the mother, thermal indicators of arousal such as decreased hair whorl and muzzle temperatures would become more evident as time passed, as well as temperature asymmetries associated with negative affect (see Table 7).

Calf Behaviour / environmental factors	**Changes in facial temperatures**	**Predictions**
Calf standing, walking or running Calf vocalising Ambient noise levels Vehicles moving (tractor or feeding truck) Presence of staff Staff shouting Adult cows mooing Human approaching from the right side Increasing time after separation.	Temperature asymmetries suggesting right hemisphere activity.	As calf activity, noise levels and other forms of disturbance increase asymmetries are expected to increase as time passes after separation, temperature asymmetries will shift gradually to the sides suggesting right hemisphere activity.
Neighbouring cows or calves present, Milking machinery sound.	Temperature asymmetries suggesting left hemisphere activity.	The sound of milking machinery and the presence of conspecifics in the adjacent pen will influence the asymmetries towards the sides suggesting left hemisphere activity.

Table 7. Asymmetry predictions for the different parameters and behaviours recorded.

4.2. Methods

4.2.1. Animals and treatments / (Subjects and husbandry)

Twenty-four dairy calves (19 females and 5 males) between 12 and 24 hrs of age and of three different breeds, e.g. Holstein, Holstein x Angus and Holstein x Belgian Blue, were used. The study took place at the University of Bristol's farm between October 2017 and October 2018. Only single calves born without difficulties (not considered a result of labour dystocia), that received colostrum within the first 12 hours of life, and that were able to stand for ten seconds and walk five steps in a row during the recording session were included in this study, six of the original thirty calves to be used for this study were deselected as they did not meet the inclusion criteria.

One week prior to calving, cows were allocated to the calving area of the main building, which consisted of three adjacent straw bedded pens (3.16 m x 3.16 m). The front edge of the calving area was adjacent to the feeding passage, and the back of the pen was separated from the milking parlour's exit by a concrete wall.

When the calves were born, they were fed colostrum from the mother within the first six hours and separated from the mother within the first 24 hours after birth, either before the first milking at 4 am or before the 1 pm milking. The herd manager carried out the separation process as per common practice on the farm. The herd manager entered the calving pen and gently herded the mother towards the milking parlour to be milked with the rest of the herd, leaving the calf behind.

4.2.2. Thermal recordings

The researcher quietly entered the pen 30 minutes prior to the separation, turned on the thermal camera and pointed it towards the calf and the mother to habituate them to the researcher and the equipment. 15 minutes prior to the separation, the first behavioural observation started (detailed in 2.3 below), which was then followed by the first thermal recording 10 minutes prior to the separation; all behavioural and thermal data were collected from inside the calving pen by the same researcher.

Thermal video recordings, each lasting five minutes, were made at eight different time points: 10 minutes prior to separation from the mother, immediately after separation (immediately after the exit gate closed behind the leaving mother), and 10, 20, 30, 40, 50 and 60 minutes after the separation (see Fig. 1). Videos were recorded using a FLIR T660™ camera (FLIR Systems, Inc., USA), with a resolution of 640 x 480 pixels, 60 FPS, sensitivity 0.02 ^{o}C, and accuracy ± 1%. All videos were recorded from inside the pen at a distance of between one and two meters from the calf and were aimed to capture the head of the calves from four different views: front, back, right and left sides. To control for potential side bias, half of the calves were recorded starting from the right side and the other half starting from the left side. Once the recording started, the researcher walked around the calf clockwise, recording each view for a period of 30 seconds twice; leaving the remaining minute to record images from any view that was considered to have yielded the fewest images with good enough quality for the study during the previous four minutes. Emissivity was fixed on the camera at 0.98, as suggested in previous studies, to account for the amount of infrared energy emitted by the calves' skin (McCafferty et al., 2011; Salles et al., 2016) .

4.2.3. Behavioural scores

One-zero sampling was used to score the occurrence of behaviours within every five-minute period before and during each of the thermal recordings, giving a total of 16 observations. The behaviours selected were lying, standing, walking, running and vocalising (refer to Table 8 for description). Since these behaviours were not mutually exclusive, calves could be scored as performing several behaviours during the five-minute periods. For example, calves were both standing and lying within the 5 minutes and, therefore, a composite category of lying/standing/ both' was created.

Behaviour	**Definition**	**Modified from**
Lying	Flank is in contact with the ground, and no weight is supported by any of the legs	(Mintline et al., 2012a)
Standing	Body is supported by the four legs, without performing any displacement	(Mølgaard et al., 2012)
Walking	Slow four-beat gain with continuous forward movement. Having two or three of the hooves touching the ground at any time	(Jensen et al., 1998)
Run	Any gait faster than a walk, including trot and gallop	(Mintline et al., 2012a)
Vocalising	The calf grunts or moos with an open or closed mouth	(Mølgaard et al., 2012)

Table 8. Ethogram for behavioural observations.

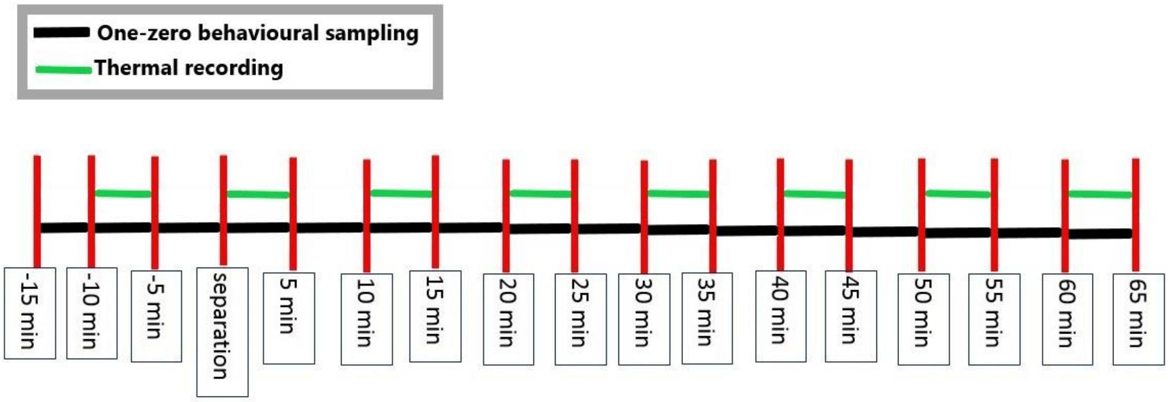


Figure 15. Data collection process. All behavioural and thermal recordings lasted 5 minutes.

4.2.4. Environmental factors

For each calf separated, the presence or absence of cows and calves in the adjacent pens was recorded. The ambient temperature and humidity were recorded every 15 minutes, updated on the camera settings to allow the proper calculations of temperature and to be accounted as a continuous variable in the elaboration of a statistical model to identify possible variations due to physiological responses of the calves to temperature and humidity. To account for side bias during the recording, the side from which the recording started was treated as a categorical variable for statistical modelling.

For each of the eight thermal recordings (5 minute period), the presence of the following environmental factors was given a binary score (0= absent, 1=present): the presence of staff within 5m of the pen other than the researcher), staff shouts (audible in the separation pen), staff whistling (audible in the separation pen), tractor circulating outside the pen, feeding truck outside the pen (larger and louder vehicle than the tractor) and cows mooing (audible in the separation pen), milking machinery noise (audible in the separation pen).

A category “environment disturbance” was created to capture the likely disturbance based on the environmental factors described above. Each recording was scored as either: 1=calm, 2=moderate or 3=noisy. A recording was scored as 1 “calm” when the only disturbance was the milking machinery sound; a score 2 “moderate” was assigned when shouts, whistles, or moos were audible, when a tractor circulated outside the pen or staff were present within 5m of the pen; and a score 3 “noisy” was assigned when the large feeding truck passed outside the pen. To calculate a score for environment disturbance during the separation, the "general environment" was created, adding the individual scores from each of the 8 recordings for each calf.

4.2.5. Selection of thermal images for analysis

Each video was watched, and the best images according to the following criteria were pre-selected. For all views, only images with an angle variation of less than 30° between the camera and the plane of interest were selected. In the case of the front and back views, symmetry between bilateral areas (assessed by eye), e.g. eyes and ears, in which ears were fully extended, were selected. In the case of lateral views (left and right), images perpendicular to the skin surface surrounding the eye were pre-selected, and the most 'alike' images (between left and right views) judged by eye in terms of distance, position and eye opening were paired. After images were pre-selected, only the best image in terms of quality (focus) and position according to the selection criteria above (front, back, left, and right) for each recording was selected for further analysis.

4.2.6. Measurements of the Regions of Interest (ROI)

The explanation of the measurements of the different ROIs can be found in the general methodology (chapter 2.3). For each of the four views, selection of the ROI based on previous research, e.g. inner corner of the eyes, ears and muzzle, and regions considered important for exploration were selected for temperature analysis (See table 9).

Angle	ROI's	Temperatures °C extracted	Asymmetry (Left-Right) or raw temperature
Back	Ear base	Maximum and average	Asymmetry
Front	Ear front	Maximum and average	Asymmetry
	Nostrils	Maximum and average	Asymmetry
	Nasal airways	Maximum and average	Asymmetry
	Muzzle	Maximum and average	Raw data
	Hair whorl	Maximum	Raw data
Left / right	Eyeball	Average	Asymmetry
	Inner corner	Maximum	Asymmetry
	Rostral eye surrounding	Maximum	Asymmetry
	Caudal eye surrounding	Maximum	Asymmetry

Table 9. Regions of Interest (ROI) for each of the views, temperatures used and adjustments.

4.2.7. Statistical Analysis

To determine whether there were any temperature asymmetries in bilateral areas, the difference between left and right temperatures of the bilateral ROIs were measured, e.g. eyes, ears, nostrils and nasal airways. Negative values reflected higher temperatures on the right side and positive ones on the left side. In the case of non- asymmetric muzzle and hair whorl temperatures, the measured values were used for analysis.

To reduce the number of statistical analyses performed, the correlation between the maximum and average temperatures in nostrils, nasal airways and ears, and between ocular areas (inner corner of the eye, eyeball) and periocular areas (rostral and caudal eye surroundings) were calculated and the ones with fewest correlations were selected for further analysis. In the case of muzzle temperature, the measurement (max or average) that has a higher correlation with hair whorl temperature was selected as it was intended to determine the feasibility of using hair whorl as an alternative to muzzle temperatures. The data from all the videos were tested for partial correlations controlling for the effect of 'calf ID' using IBM SPSS Statistics for Windows, version 25.0. To categorise the correlations between ROIs according to Mukaka (2012), R values of between 0.3 and 0.5 were considered low correlations, R values of 0.5 to 0.7 as moderate correlations, R values between 0.7 and 0.9 as high correlations and above 0.9 very high correlations. Where temperatures across two or more bilateral ROIs were highly correlated, only one of the ROIs was selected for further analysis. To illustrate, if eyeball temperature was found to be highly correlated with the temperature of the inner corner of the eye, but the inner corner was highly correlated with rostral eye surrounding, then only eyeball temperature might be selected for further analysis. To determine if maximum whorl temperatures can be used as indicators of arousal, they were analysed alongside with the maximum muzzle temperatures, regardless of their correlation values.

Statistical models for repeated measures were built using the data of all recordings to identify the effect of the 26 predictor variables, including sex, breed, time after separation, environmental factors and behaviours (during each recording "DR" and in the 5 minute period preceding the recordings "BR"), and the chronological changes (i.e. -10, 0, 10, 20, 30,40,50 and 60 minutes after the separation) on the asymmetric (left-right temperatures in the bilateral areas) and non-asymmetric temperatures (whorl and muzzle) measures. For this purpose, the multilevel modelling software MLwiN V3.00 (Charlton et al., 2017) was used to create linear models with a random effect structure nesting calves within the recordings, setting level 1 (i) as recording, i.e. 1 to 8, and level 2 (j) as calf number, i.e. 1 to 24.

The models were created using a stepwise regression with a forward selection of the variables, starting with the effect of each of the variables independently. The predictor variable whose addition resulted in the best improvement of the model was selected for further modelling. Selection was based on the p-values calculated from the likelihood-ratio test of the difference between the single model and the ones incorporating each of the individual variables separately (taking into account the degrees of freedom from the variables). Then the procedure was repeated until no significant improvements to the models were found. So, the final models only included the most significant variable of each of the previous models. To control for testing multiple hypotheses, the Benjamin-Hochberg procedure to control for a False Discovery Rate of 0.2 was carried out using all the p-values calculated from the likelihood-ratio tests between the base models and the models including the single variables (McDonald, 2009).

4.2.8. Ethical statement

The research was approved by the animal ethics committee of the University of Bristol. The separation of the calves from the mother was performed according to the normal practices on the farm.

4.3. Results

In presenting results, a table of final model coefficients, p-values and direction of effects was presented in the Appendix chapter. See table 11 for an exemplar table for the first set of results.

4.3.1. Correlation between the different ROIs

The maximum and average temperatures of each ROI were highly correlated ('r' from 0.727 to 0.872); Hair whorl maximum temperature was highly correlated with muzzle maximum (r=0.815) and with average muzzle temperatures (r=0.760). Bilateral ROIs that showed less correlation with others were selected for further analyses, i.e. nostril (average), front ear base (average), nasal airway (maximum), back ear base (average), eyeball (average) and caudal eye surrounding (maximum). In contrast, muzzle (maximum) and whorl (maximum) temperatures were selected as indicators of arousal, as those were the highest correlated temperatures among those areas (see correlations in Table 10).

	Ear base max	Ear base ave	Front ear base max	Front ear base ave	Nostril max	Nostril ave	Nasal Airway max	Nasal Airway ave	Muzzle max	Muzzle ave	Hair whorl max	Inner corner max	eyeball ave	rostral eyesurrounding	Caudal eyesurrounding
Earbase max	-	0.723	0.046	0.071	0.312	0.315	-0.075	-0.004	-0.180	-0.199	-0.132	0.094	0.110	0.180	-0.016
Earbase ave	0.723	-	0.181	0.161	0.318	0.289	0.044	0.140	-0.231	-0.258	-0.168	0.051	0.025	0.187	0.088
Front earbase max	0.046	0.181	-	0.752	-0.043	0.026	0.088	0.152	-0.088	-0.016	-0.007	-0.007	-0.093	0.038	0.127
Front earbase ave	0.071	0.161	0.752	-	0.052	0.083	0.189	0.274	-0.099	-0.062	-0.031	-0.097	-0.133	-0.092	0.085
Nostril max	0.312	0.318	-0.043	0.052	-	0.772	0.232	0.275	-0.414	-0.537	-0.346	-0.036	0.084	0.077	-0.195
Nostril ave	0.315	0.289	0.026	0.083	0.772	-	0.327	0.344	-0.280	-0.343	-0.252	-0.022	0.082	0.122	-0.104
Nasal Airway max	-0.075	0.044	0.088	0.189	0.232	0.327	-	0.736	-0.122	-0.168	-0.147	-0.194	-0.237	-0.115	-0.066
Nasal Airway ave	-0.004	0.140	0.152	0.274	0.275	0.344	0.736	-	-0.168	-0.190	-0.176	-0.068	-0.105	-0.038	-0.061
Muzzle max	-0.180	-0.231	-0.088	-0.099	-0.414	-0.280	-0.122	-0.168	-	0.872	0.815	0.006	0.014	-0.059	0.003
Muzzle ave	-0.199	-0.258	-0.016	-0.062	-0.537	-0.343	-0.168	-0.190	0.872	-	0.760	0.039	0.063	-0.043	0.095
Hair whorl max	-0.132	-0.168	-0.007	-0.031	-0.346	-0.252	-0.147	-0.176	0.815	0.760	-	0.095	0.101	0.061	0.042
Inner corner max	0.094	0.051	-0.007	-0.097	-0.036	-0.022	-0.194	-0.068	0.006	0.039	0.095	-	0.511	0.500	0.171
eyeball ave	0.110	0.025	-0.093	-0.133	0.084	0.082	-0.237	-0.105	0.014	0.063	0.101	0.511	-	0.350	0.076
rostral eyesurrounding	0.180	0.187	0.038	-0.092	0.077	0.122	-0.115	-0.038	-0.059	-0.043	0.061	0.500	0.350	-	0.152
Caudal eyesurrounding	-0.016	0.088	0.127	0.085	-0.195	-0.104	-0.066	-0.061	0.003	0.095	0.042	0.171	0.076	0.152	-

Table 10. Partial correlation between the different measurements. Green, yellow and red numbers, indicate high, moderate and low R-values respectively. ROIs in bold were selected for further analyses.

4.3.2. Temperature asymmetries (Left-Right temperatures)

Temperature asymmetries in the caudal eye surround (L-R) had a quadratic relationship with humidity % (x^2=9.3453, df=2, p=0.009) and were associated with whether calves were standing or not in the 5 minutes pre-recording (BR) (x^2=7.8311, df=1, p=0.005), and with whether calves vocalised in the 5 minutes pre-recording (BR) (x^2=5.0911, df=1, p=0.024), explaining 14.01% of the total variance (8.43% at level 1; 56.66% at level 2) in the data.

Standing BR influences the asymmetries towards the left eye (right hemisphere / negative valence) and vocalizing BR towards the right eye (left hemisphere / positive valence)(see Table 11 and Figure 16).

Variable	**Categories**	**coefficient**	**S.E.**	**p-value**	**Asymmetries towards**	**Active hemisphere (hypothesised)**
Constant	N/A	-0.317	0.091	0.001		
(Humidity-gm)	N/A	-0.015	0.005	0.003	Right eye	Left hemisphere
(Humidity-gm)^2	N/A	0.0005	0.0002	0.047		
Standing BR	Yes	0.439	0.153	0.004	Left eye	Right hemisphere
	No	Reference category				
Vocalising BR	Yes	-0.543	0.239	0.023	Right eye	Left hemisphere
	No	Reference category				

Table 11. Final model for temperature asymmetries in caudal eye surrounding (L-R).

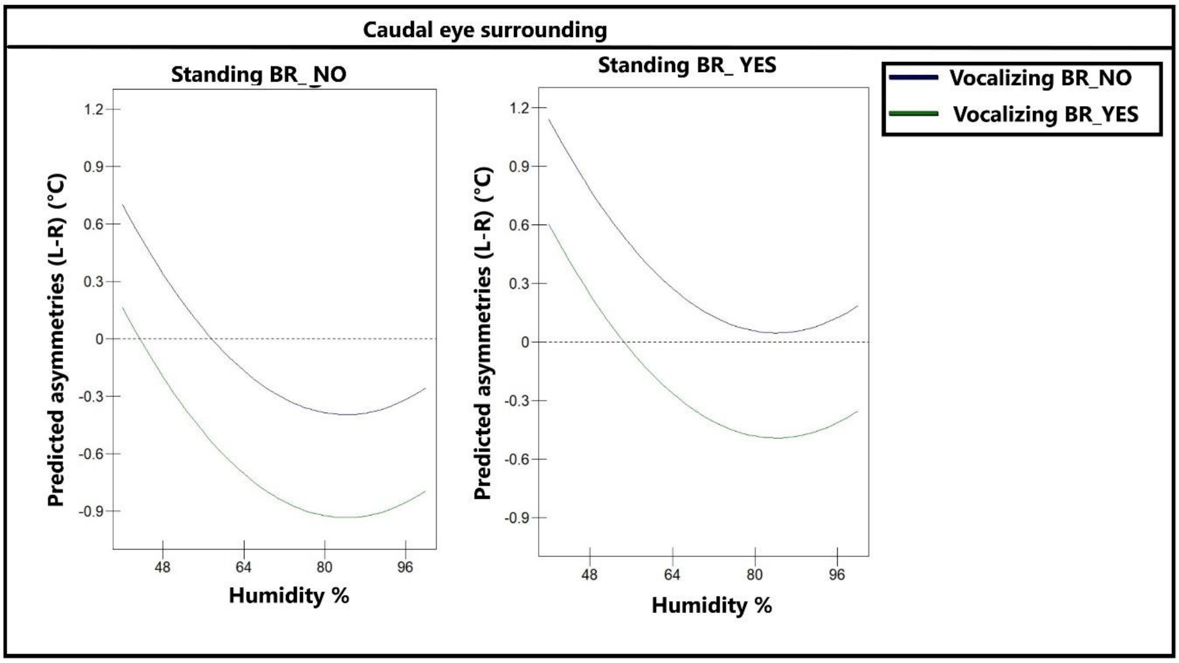


Figure 16. Effect of vocalising BR, Standing BR and humidity % in caudal eye surrounding asymmetries (L-R).

Temperature asymmetries in eyeball average temperature (L-R) were associated with ambient (disturbance) score in a quadratic relationship (x^2=10.268, df=2, p=0.006), Running BR (x^2=5.116, df=1, p=0.027), whether recording of the calf started from the left or the right side (x^2=6.578, df=1, p=0.010), whether milking machinery was audible at the pen (x^2=8.703, df=1, p=0.003), whether cows mooing were heard in the pen (x^2=4.143, df=1, p=0.042), and time elapsed from separation (x^2=10.544, df=1, p=0.001), explaining 24.14% of the total variance (5.48% at level 1; 77.99% at level 2) in the data.

Increased levels of environmental disturbance ("gen_environment”), running BR, approaching from the right, noise from the milking machinery and conspecific vocalisations influenced the asymmetries towards the left eye (right hemisphere / negative valence), whereas the time after separation influenced the asymmetries towards the right eye (left hemisphere / positive valence) (Figure 17; Table 27).

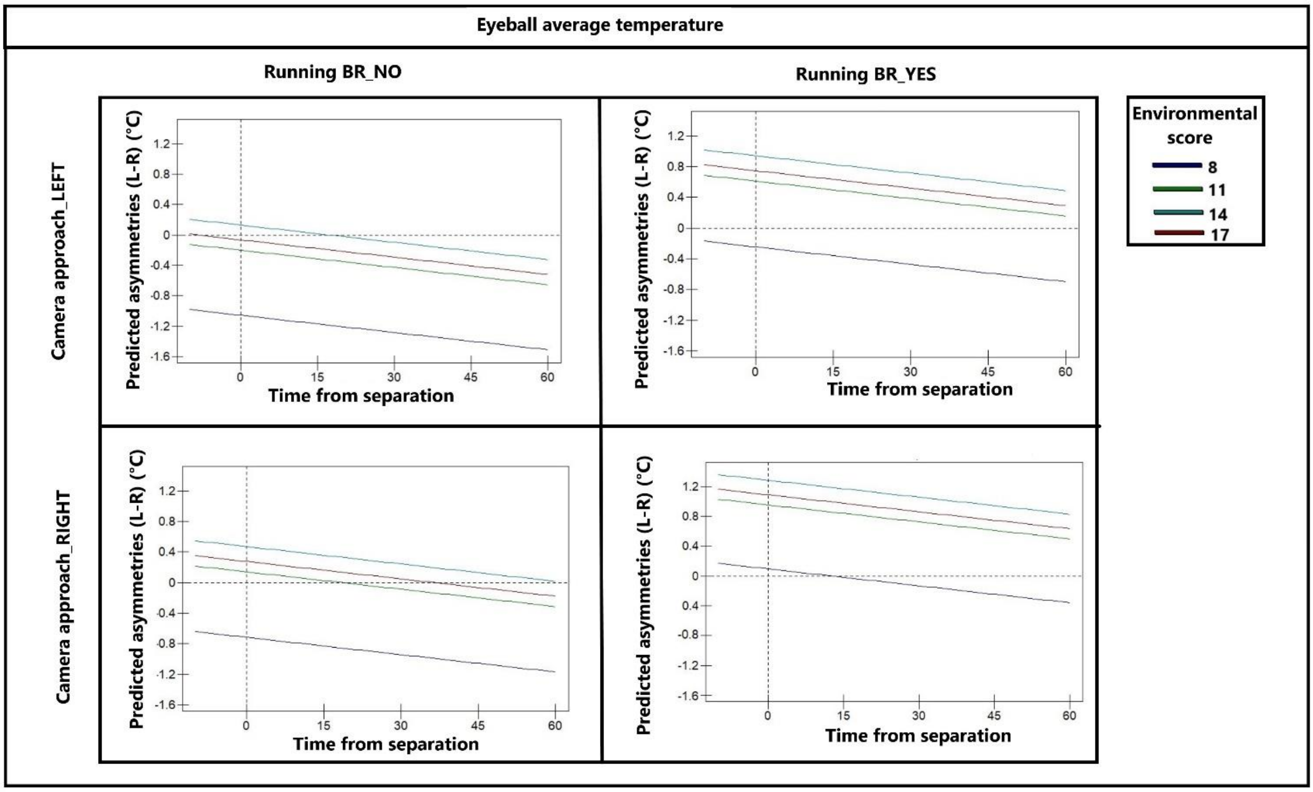


Figure 17. Effect of Running BR, time after separation and general environment score in eyeball temperature asymmetries (L-R)

Asymmetries in the maximum temperature of the nasal airways were associated with the side from which the recording started (x^2=4.058, df=1, p=0.044), whether an adult cow was in the adjacent pen during the separation (x^2=10.187, df=1, p=0.001), whether calf stood before the recording (x^2=15.344, df=1, p<0.001), whether calf walked BR (x^2=7.70, df=1, p=0.005), and in a quadratic relationship with the time from separation (x^2=7.404, df=2, p=0.025), explaining 35.22% of the total variance (7.65% at level 1; 53.79% at level 2) in the data.

Having a cow in the neighbouring pen and walking BR influenced the asymmetries towards the left nasal airway (left hemisphere / positive valence), whereas starting the recording from the right side and standing BR influenced the asymmetries towards the right side (right hemisphere / negative valence). (See Figure 18; Table 28).

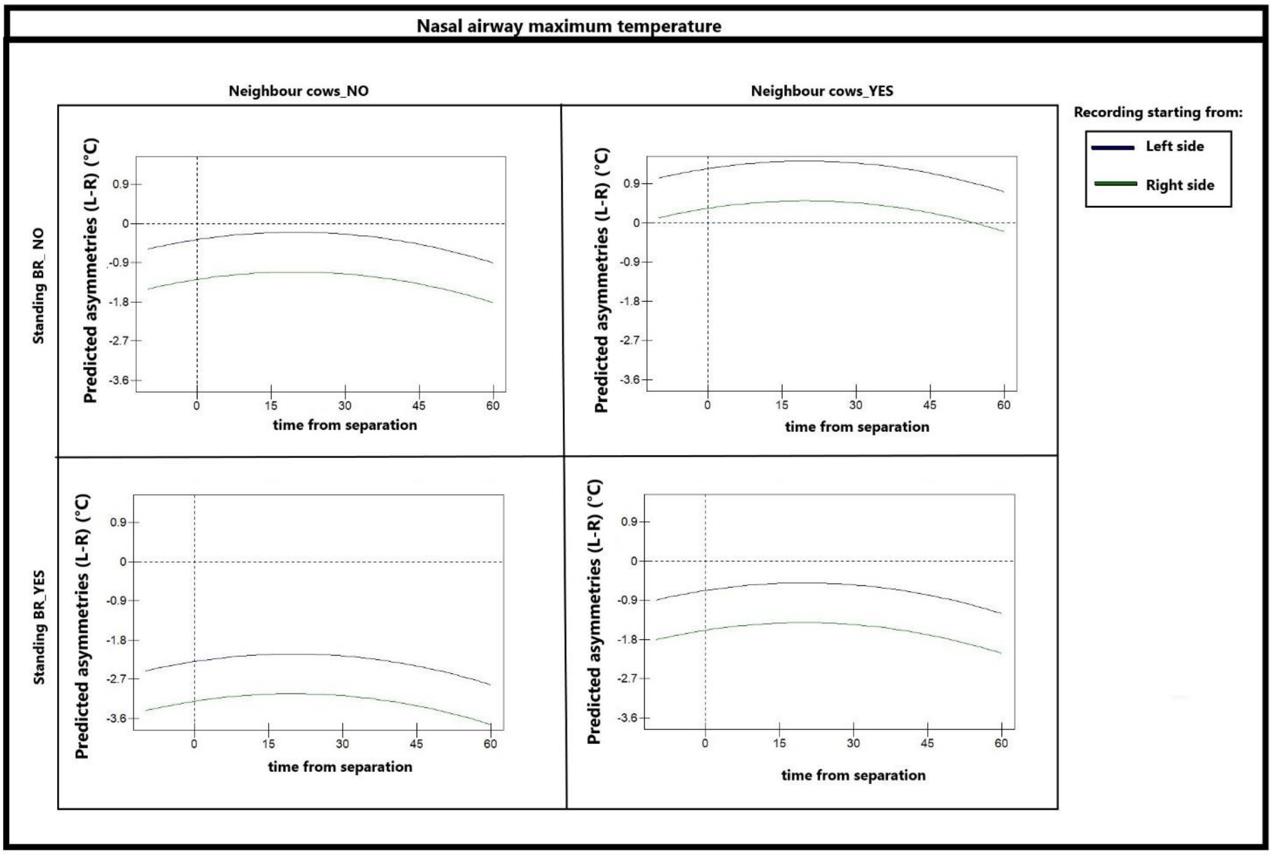


Figure 18. Effect of standing BR, time after separation, having a cow in the neighbour pen and the side from which the recording started in nasal airway maximum temperature asymmetries (L-R).

Temperature asymmetries of the ears were only influenced by single variables (see Table 29), so the only significant effects were calf breed for ear back average temperature asymmetries (x^2=9.431, df=2, p=0.009), explaining 13.68% of the total variance (0% at level 1; 40.46% at level 2) in the data; and for front ear maximum temperature asymmetries (x^2=7.712, df=2, p=0.021), explaining 7.35% of the total variance (0% at level 1; 45.46% at level 2) in the data; whilst having a cow in the adjacent pen was associated with asymmetries in maximum temperatures of the ear (front view), towards the right side (left hemisphere / positive valence) (x^2=4.442, df=1, p=0.035), explaining 4.49% of the total variance (0% at level 1; 27.97% at level 2) in the data.

4.3.3. Hair whorl and muzzle temperatures

Maximum Hair whorl temperature was associated with the time from the separation (x^2=7.614, df=1, p=0.006), whether calves stood before the recording (x^2=18.67, df=1, p<0.001) and interaction between standing BR and time from separation (x^2=5.89, df=1, p=0.015), explaining 30.75% of the total variance (11.77% at level 1; 33.50% at level 2) in the data.

Time after separation increased hair whorl temperature, while standing BR resulted in lower hair whorl temperatures (see Figure 19; Table 30).

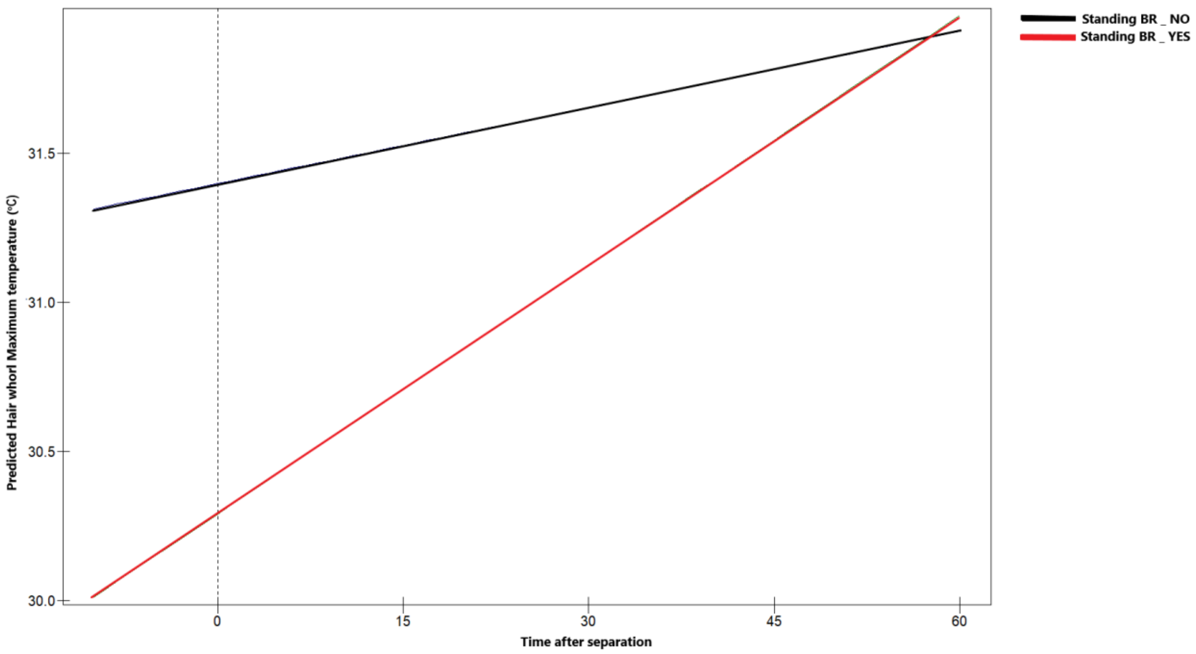


Figure 19. Effect of standing BR and time after separation in hair whorl maximum temperatures.

Muzzle maximum temperature was associated with the time from the separation (x^2=4.636, df=1, p=0.031), whether calves stood before the recording (x^2=10.12, df=1, p=0.001), whether calves stood during the recording (x^2=31.22, df=1, p<0.001) and interaction between standing BR and standing DR (x^2=4.74, df=1, p=0.029), explaining 14.01% of the total variance (28.11% at level 1; 8.89% at level 2) in the data.

As the time from the separation increased, muzzle temperature decreased; both standing DR and standing BR were associated with reduced muzzle temperature, and the interaction between standing DR and standing BR showed that there was not an additive effect if the calf stood before and during the recording (see Figure 20; Table 31).

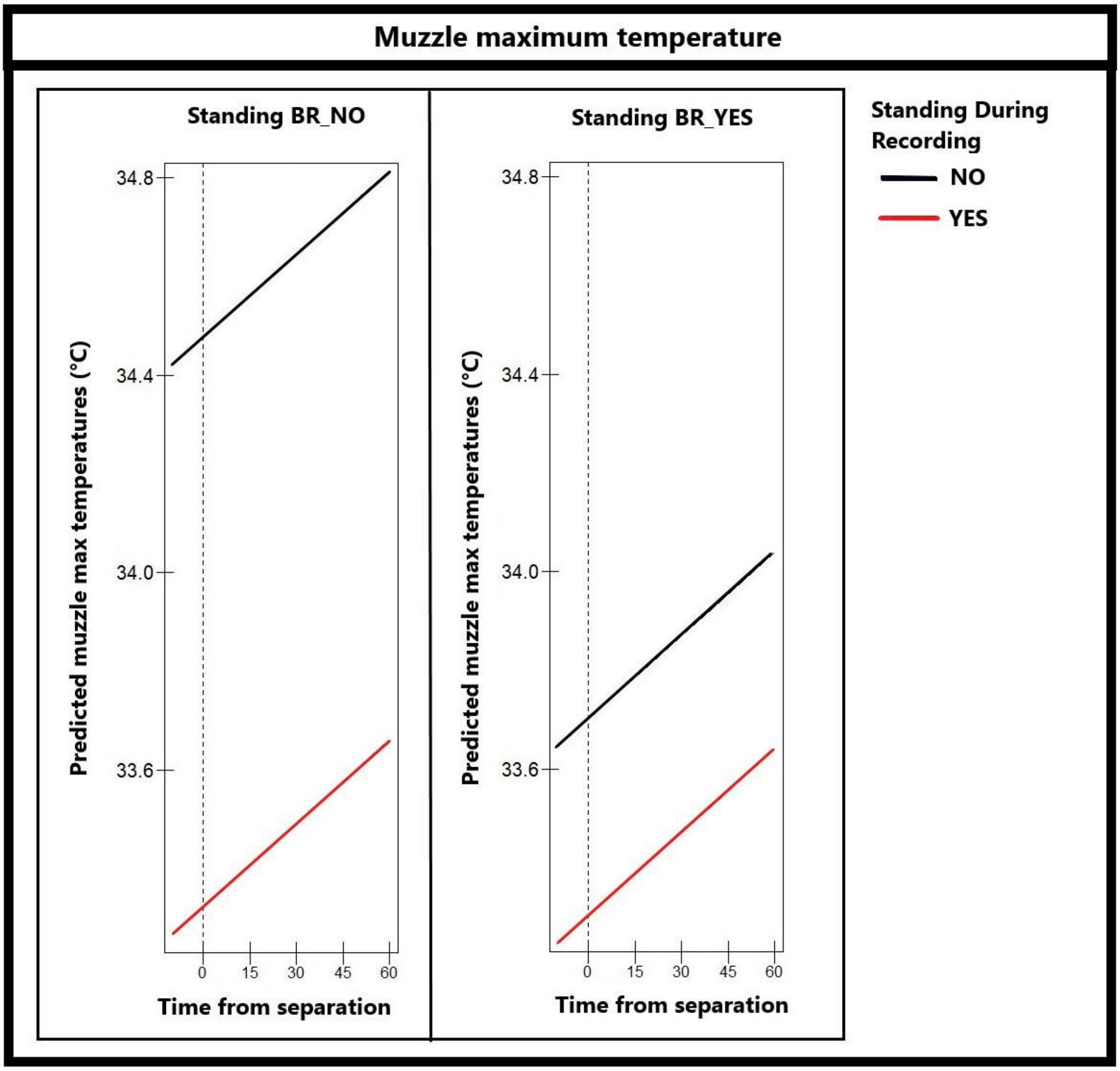


Figure 20. Effect of standing DR, standing BR and time after separation in muzzle max temperatures.

4.4. Discussion

The main aim of this study was to identify if temperature asymmetries in several Regions of Interest (ROIs) hypothesised to indicate a negative affective state could be measured through infrared thermography, using separation of calves from their mothers as a model of negative affect. The effect of environmental factors and behaviours performed by the calves on thermal asymmetries measured were also analysed.

Temperature asymmetries (left-right) during separation from the mother in several ROIs were found, particularly in the caudal eye surrounding (maximum), eyeball (average) and nasal airway (maximum), all of which were also influenced by environmental factors and calf behaviours whilst only eyeball and nasal airway asymmetries changed with time following separation. Our models explained the variance of the asymmetries in a range between 4.49% in the ear area and 35.22 % in the nasal airways, suggesting that several other factors not considered in this study influenced thermal asymmetries.

I hypothesised that asymmetries associated with higher activity of the right hemisphere would increase as the time from removal of the mother passed. The data from eyeball average temperatures (L-R) did not support this hypothesis, as asymmetries increased towards the right eye/left hemisphere as the time from separation elapsed. However, asymmetries in nasal airway temperatures had a quadratic relationship with the time after separation, showing an increased temperature towards the left nasal airway (left hemisphere) during the first 30 minutes post-separation followed by an increase towards the right nasal airway (right hemisphere) in the last 30 minutes of recording. As facultative hider species, it can be expected that cows spend short periods of time separated from their offspring; in semi-wild cattle, it has been observed that mothers stay away from the heard and fed themselves at distances of more than 15 meters from their calves, whilst they hide in the first two to three days after birth, after which cows reunite to the heard and calves start spending more time at higher distances and larger periods from their mothers (Vitale et al., 1986). However, only our results in nasal airways temperatures support this, as asymmetries suggested a negative effect increased during the last 30 minutes of observation.

Both hair whorl and muzzle temperatures increased during the period following separation, which might suggest a higher activation of the parasympathetic activity of the autonomic nervous system, which has been found to occur as a modulatory mechanism following elevated sympathetic activity associated with a high arousal response (Ioannou et al., 2013; Ioannou et al., 2016). However, the initial sympathetic response, which might be reflected as a reduction in peripheral temperature was not observed in the first minutes after separation; one possible explanation for this is that the drop of temperature occurs almost immediately after the high arousal emotion is elicited and our methodology might have failed to detect such sudden variations in temperature. The temperatures of both areas were lower when the calves stood in comparison to when calves were lying down, agreeing with previous research suggesting that more active behaviours are associated with higher levels of sympathetic activity and arousal (Lidfors, 1996; Stěhulová et al., 2008b) and with a decrease in peripheral temperature (Kano et al., 2016; Proctor and Carder, 2016). The interaction between the influence of calves standing before and standing during the recording showed that a reduction in hair whorl temperature in calves standing during the recording only occurred if they were not doing so in the 5 minutes prior to the recording.

Alongside these cross-time changes, several environmental and behavioural factors influenced thermal readings. An increase in humidity between 40% to 80% was associated with an increased temperature in the right caudal eye surround area. Similarly, the presence of neighbour cows during the separation was associated with a higher temperature on the left nasal airway, both findings suggesting a more positive emotional valence.

If neighbouring animals were the calves' mothers, their presence might negatively influence the separation process, as visual and auditory contact between mother and calf increases heart rate, vocalisations and exploratory behaviours (Stěhulová et al., 2008b). However, in our study, neighbouring animals were not the calves' mothers, so it is possible that having a conspecific nearby helped to reduce the stress of the process. This finding is in line with evidence that calves housed in pairs had lower cortisol levels and showed fewer behaviours associated with stress when exposed to novel stimuli (Raussi et al., 2003) and that calves tested in novel settings had lower markers of fear-like states if assessed with a conspecific rather than in isolation (Duve et al., 2012).

On the other hand, approaching from the right side to record the calves seemed to affect them negatively as it was associated with a relative increase in right nasal airway and left eyeball temperature (right hemisphere activity). The noise of the milking machinery, cows mooing, and increases in disturbance levels ("general environment") also shifted the eyeball temperature balance towards the left eye.

Adult cows tend to observe novel or threatening objects with their left eye (Robins and Phillips, 2010). Starting thermal recordings from the right side forced the calves to observe the researcher with their right eye, which is not the eye they would generally use to process a novel situation. This may have led to a more negative response to the camera/researcher. In a recent paper by Robins et al. (2018), similar findings were reported as an increased flight response of adult cattle towards an unfamiliar human when approaching from the right side of the animals. The authors attributed this to a cognitive disparity in the processing of familiar cues in the environment. Alternatively, in our study moving clockwise starting from the right side of the calf put the researcher at the back of the calf, which might be more threatening than if I had started recordings from the left side, in which case the calves would have had more time to look at the researcher before he stood behind them.

Previous studies have shown that calves show more head movements and increased heart rates when listening to adult cow vocalisations after being separated from the mother (Marchant-Forde et al., 2002) and that calves are capable of identifying their mothers' vocalisations (Padilla de la Torre et al., 2016). Increased noise levels have also been previously associated with higher levels of stress in cattle, reflected as increased heart, micturition and defecation rates (Waynert et al., 1999; Arnold et al., 2007; Kauke and Savary, 2010).

In terms of calf behaviour, behaviours performed in the 5-minute periods prior to the recording seemed to have a stronger relationship with subsequent thermal measures than those performed during the recordings themselves. This might reflect that there is a delay between behavioural responses and an associated shift in skin temperature. It was hypothesised that behaviours that were more related to an active response to stressors (walking, running or standing) would reflect negative affect during the separation process, as shown in previous studies (Lidfors, 1996). This idea was partially confirmed as temperature increases towards the left caudal eye surrounding (right hemisphere) and towards the right nasal airway (right hemisphere) were found when the calves had been standing during the five-minute pre-thermal recording period, and an increase towards the left eyeball was found when calves had been running during this period.

Nevertheless, contrary to our predictions, asymmetric changes occurring when calves walked and vocalised prior to thermal recordings seemed to be related to thermal markers of higher activity in the left hemisphere. These contrasting results could suggest that other factors pertinent to the separation context may influence lateralised brain activity. For example, other studies indicate that the left hemisphere may predominate during communication; chimpanzees predominantly use their right hand (left brain hemisphere) when communicating through hand gestures (Hopkins and Leavens, 1998), and male Zebra finches use their right eye (left brain hemisphere) while singing during courtship (George et al., 2006). It is thus possible that left hemisphere activation in the calves was associated with an increase in vocalising or walking behaviours directed at localising or communicating with their mothers.

In conclusion, the findings of this study, alongside our disbudding study (chapter 3), support the idea that temperature asymmetries may be potentially useful markers of emotional valence in animals and highlight the utility of infrared thermography as a tool for the study of affective valence as well as affective arousal. In particular, and as found in our previous study (chapter 3 and 4), relatively warmer left eye temperatures seem to be associated with factors that can be considered negative for calves. The current study also indicates that asymmetries in the nasal area (nasal airway) are worth further exploration. However, it is important to note that no studies that I am aware of have investigated whether and exactly how lateralised brain hemisphere activity may be related to surface temperature asymmetries and that the hypotheses proposed in this study are based on potential anatomical and physiological processes that underlie asymmetries (as discussed in chapter 1). Furthermore, lateralisation of brain activity is not exclusive to processes involving affective states, and therefore temperature asymmetries may reflect other processes as well. Overall, proof of concept is still required, and future studies should explore thermal asymmetries in more depth, including their relationship with positive affective states and the effect of other factors on the temperatures recorded.

5. "EFFECT OF EXTERNAL FACTORS ON TEMPERATURE ASYMMETRIES IN DAIRY CALVES: CAMERA POSITION, AMBIENT PARAMETERS AND TIME BETWEEN THERMOGRAMS"

Abstract

Infrared thermography has been a useful tool for the study of dynamic changes in temperature in real life. This dissertation has been used to identify temperature asymmetries suggesting lateralised processing of emotions and measure temperatures in Regions Of Interest (ROIs) associated with emotional arousal. This technology measures the amount of Infrared emitted by an object. The inbuilt software contains algorithms to calculate the temperature of a surface with high accuracy, taking into account the effect of ambient temperature, humidity, distance from the object, and object emissivity. However, the application of this technology has its limitations as factors like heat reflection, sunlight or other surfaces and wind speed had a strong influence on the temperature of the surfaces measured. Moreover, the impact of environmental factors (temperature, humidity and Temperature-Humidity Index) in the temperature on different animal ROIs due to physiological mechanisms has been explored only in a limited number of tissues (eye temperature); Similarly, the influence of the angle between the camera and animal tissues has only been studied in this area. As discussed in previous chapters, the measurement of temperatures in ROIs other than the inner corner of the eye is essential to identify the temperature asymmetries and arousal indicators of emotion studied in this dissertation. It is known that maximum eye temperature (inner corner) has a good correlation with core temperature and remains relatively stable in variations of camera angle up to 30°. However, it is still unknown to what extent those factors might influence other ROIs. For this reason, the effect of ambient factors (temperature, humidity and Temperature-Humidity Index), camera angle, camera elevation and variation between thermograms (effect of time within recording) was studied using images from baseline recordings from the calves in Chapter 3. Images from 38 calves were selected and classified in terms of camera

angle (front, back. Left and right views) and camera position (front and back views), raw temperatures were extracted from the different ROIs and temperature asymmetries were calculated (same as used in Chapter 3 and 4). Multilevel models with a random structure were elaborated to identify the effect of the environmental factors and variation in camera angle and elevation. The variance in raw temperatures of bilateral areas was influenced in a higher percentage and by more variables than the variance in temperature asymmetries, i.e. the minimum variance explained by the models in raw temperatures was 36.59% in the left nostril maximum temperature and a maximum of 80.22% on the left ear average temperature. In contrast, the minimum variance on temperature asymmetries was 1.54% in L-R nostril maximum temperatures and the maximum of 18.52% in the caudal eye surrounding area, which occurred as the effect of specific parameters like THI and time within recording influenced the temperature on both sides of bilateral regions in a similar way; suggesting that calculation of temperature asymmetries, in general, might be applicable in a wide range of circumstances.

5.1. Introduction

5.1.1. Infrared thermography in animal studies

Infrared thermography is a technology that allows the estimation of the surface temperature of an object or an animal by measuring the amount of infrared radiation emitted by the surface. It is a useful tool, allowing the collection of temperature information from a distance and in real-time, avoiding the stress that can be generated by techniques that require direct contact or attachment of equipment to the subjects (Thompson et al., 2003). In the veterinary and animal welfare sciences, thermography has been used to detect local inflammation (Schwartzkopf-Genswein and Stookey, 1997; Montanholi, 2015; Wood et al., 2015), viral/bacterial infections prior to the onset of clinical signs (Schaefer et al., 2004; Rainwater-Lovett et al., 2009; Schaefer et al., 2012; Digiovani et al., 2016), metabolic changes (Montanholi et al., 2009; McCafferty et al., 2011), reduction and subsequent increase in peripheral temperature during stress (Herborn et al., 2015; Travain et al., 2015; Lecorps et al., 2016; Cannas et al., 2018) and reduction and subsequent increase in peripheral temperature in different areas of the animals' faces in relation to changes in affective states (Primates:Nakayama et al., 2005; Cows, Stewart et al., 2008b; Stewart et al., 2010a; Kuraoka and Nakamura, 2011; Kano et al., 2016; dogs, Travain et al., 2016).

Our research group has recently developed a new method to study animal emotions through evaluating temperature asymmetries between bilateral areas, i.e. ears, eyes, nostrils, and nasal airways (Chapter 3, 4 and 6). It has been hypothesised that the direction of those asymmetries might reflect changes in peripheral blood flow and muscular activity elicited by lateralised processing of emotions in the brain hemispheres. The two main theories for this lateralisation process are the "emotional-valence" theory suggesting that positive emotions are mainly processed in the left hemisphere, and negative ones in the right hemisphere (Silberman and Weingartner, 1986; Lee et al., 2004), and the "approach- withdrawal" theory, suggesting that emotions resulting in a behavioural approach are processed predominantly in the left hemisphere, and the ones resulting in behavioural avoidance are mainly processed in the right hemisphere (Davidson et al., 1990; Sobotka et al., 1992a). However, it is important to bear in mind that other processes can also involve lateralised brain function, such as responses to novelty (Leliveld 2013), and there are some differences between species (Leliveld 2013), which can also affect lateral differences in surface temperature.

In our previous studies, I have identified asymmetries associated with negative states, i.e. hot iron disbudding and separation of calves from their mother; in areas such as the inner corner of the eyes, the rostral area near the eyes, and the external area of the ears (Chapter 3 and chapter 4).

5.1.2. Factors influencing temperature measurements

Temperature readings obtained from infrared thermograms are susceptible to errors due to environmental and methodological factors. The camera software takes the most important factors like the distance between the camera and the object, humidity, ambient temperature and emissivity (relative power of a surface to emit heat by radiation) to make the appropriate calculations of the object/surface temperature. Other factors, like the reflection of sunlight, air currents, presence of liquids and objects that reflect infrared radiation from the environment (i.e. metallic surfaces, walls and heaters), close proximity to objects that are substantially cooler or warmer than the surface being measured, moisture or dust on the surface being measured, as well as thickness of the fur coat of an animal on the area being measured, also influence temperature readings and therefore should be taken into account when planning studies and recording temperatures in situ (Okada et al., 2013; Church et al., 2014; Fernández-Cuevas et al., 2015; Ijichi et al., 2019). For example, a wind speed of 7km/h can decrease eye temperature by 0.43 ± 0.13 °C, and a wind speed of 12km/h by 0.78 ± 0.33 °C and direct sunlight exposure can increase eye temperature by 0.56 ± 0.36°C (Church et al., 2014).

Furthermore, factors such as the shape and position of an object relative to the camera lens can influence temperature readings. In general terms, an increased distance between the camera and the object results in a reduction of the temperature reported; for example, a variation of 0.38°C was found between images taken at 2.0m and 0.5m on an object covered with reflective tape (Okada et al., 2013). Entering the proper distance in the camera settings helps reduce this effect (Church et al., 2014). However, it is still important to use images from similar distances when comparing images to avoid artefacts in the results.

In terms of ambient parameters, a positive correlation between ambient temperature and temperature readings has been reported. In cattle (Okada et al., 2013), eyeball average and maximum temperatures increase by 3.5°C and 2°C respectively when measured at an environmental temperature of 28°C compared to measurements made at 9°C (Okada et al., 2013). Because humidity levels influence the perception of the ambient temperature by the individuals, the calculation of the Temperature Humidity Index ('THI') might be a more adequate measurement of thermal comfort (Scharf et al., 2008; Salles et al., 2016). Salles et al. (2016) reported a positive correlation between THI and forehead temperature (r=0.81) and eye temperature (*r*=0.56) in dairy cattle.

Artefacts in thermal readings associated with ambient temperature and humidity can be reduced simply by entering them into the camera settings to allow the inbuilt software to correct them. However, humans and animals make physiological responses in response to environmental changes to maintain homeostasis, for example, constriction or dilatation of peripheral blood vessels, which will influence skin surface temperature (Tattersall, 2016) in ways that may be unrelated to affective state. This is particularly important when measuring peripheral vasoconstriction as a marker of emotional arousal because peripheral vasoconstriction is also affected by ambient temperature via thermoregulatory processes. Though it has not yet been studied, it is also possible that ambient temperature could affect thermal asymmetries if the extent of those asymmetries varies between, and within-subjects as environmental factors change.

Accuracy of measurement requires that the surface to be measured is as close to parallel to the camera lens as possible. The effect of the camera angle and differences in temperature readings using infrared thermography has been studied in objects which are most likely symmetrical and bidimensional or in areas with a relatively low-temperature variation attributed to this factor as in the maximum temperature of the eyes (inner corner of the eye). Results of those studies had suggested that angles of less than 30° away from a perpendicular position between the camera and the area measured parallel have a negligible effect, and a significant error is introduced when the angle increases between 30 to 60° (Holst, 2000; Fernández-Cuevas et al., 2015). However, curved surfaces such as heads and eyes are more sensitive to this type of error, therefore tolerating less deviation from being parallel with the camera lens. When measuring temperatures on more three-dimensional objects, like in the facial areas of the calves (i.e. ears, nostrils, nasal airways, muzzle, forehead and areas surrounding the eyes), it is not known how angle variation might influence the estimation of temperature or temperature asymmetries. However, it is expected that increases in the camera angle will have a greater influence in temperature readings in bilateral areas with higher separation between them (for example, more variability between ear temperatures than in the nostrils). In terms of elevation It has been proposed that an elevation of 45° upwards or downwards does not have a significant effect when measuring the temperature of a bidimensional object covered with reflective tape, i.e. 0° (21.80±0.16), 45° upward (21.68±0.11) and 45° downward (21.68±0.07) (Okada et al., 2013). However, a specific study of elevation effects on temperature readings from more complex surfaces, such as those of an animal's face, has been limited.

In the case of eye temperatures, Ijichi et al. (2019) highlight that the angles from which to measure eye temperatures in horses have not been standardised and that a perpendicular position between the measured area and the camera is considered the most appropriate, but this requires that the camera be moved during measurement to ensure that it remains perpendicular to each of the areas to be measured, such as the nasal plane, the eye or the sagittal plane on the side of the faces. Ijichi et al. (2019) found that the sagittal plane of the head is the area yielding temperatures most correlated with heart rate, indicating that the camera should be positioned perpendicular to the sagittal plane if it is not possible to take multiple images to attain perpendicular positions relative to each of the areas of interest.

As mentioned earlier, only a few studies have examined the effect of environmental factors and camera lens angle on temperature readings of skin or eyes in dairy cattle. However, it is not known how those factors might influence temperature readings in other regions of the dairy calves’ faces. Therefore, this study aimed to identify how environmental factors, camera position, and time between frames influences temperature readouts and temperature asymmetries in the regions of interest (ROIs) explored in this dissertation enabling to identify which of these regions provide the most repeatable data despite those variations and therefore yielding more reliable data under field situations. For this purpose, images from thermal videos of 36 calves used in an earlier study (Chapter 3) were selected and analysed to assess the effects of the variables of interest on thermal readouts and temperature asymmetries.

5.2. Methods

5.2.1. Animals / (subjects and husbandry)

Data from thirty-six Holstein Friesian heifer calves aged between 20 and 108 days were used. Those calves had been selected for a study on thermal lateralisation and peripheral temperature effects during hot-iron disbudding (Chapter 3).

The calves had been separated from their mothers between one and two days after birth, after which they had been moved to a semi-open calf shed and kept for two weeks in individual pens. After that, they were group-housed in straw-bedded pens (4.8m x4.1m) of 6 to 8 calves. Replacement milk was provided for the calves twice a day at 9 am and 4 pm.

Ethical approval was obtained from the Animal Welfare and Ethics Review Body of the University of Bristol (UIN UB/16/058).

5.2.2. Thermal recordings

Thermal videos with a duration of 5 minutes ± 2 minutes were collected at six different time points for the original study between January and June 2018 at the University of Bristol's dairy farm using a FLIR T660^{TM} camera (resolution 640 x 480 pixels, 30 frames per second, sensitivity 0.02 ^{o}C, accuracy ± 1%). Surface temperature measurement was focussed on the calves' head region to extract data from the ROIs (eyes, nostrils, ears, and nasal airways) from four different directions (front, back, left, and right). The emissivity of the calves' surface was set at 0.98, and the ambient temperature and humidity parameters were updated in the camera settings every 15 minutes. Radiometric thermal videos were collected from a distance of between 1 and 2 meters, with the calves moving freely in the pen.

On the day before recording, the experimenter visited the pens to select the subjects and stood for 10 minutes in the corner of the pen to habituate the calves to the researcher, followed by pointing the camera for 2 minutes to habituate them to the camera. During the recording session, the camera was turned on half an hour prior to the recording, and the experimenter followed the habituation protocol before starting the actual recordings. For this paper, the baseline videos of the calves (the first recording of each calf) were selected for statistical analysis.

5.2.3. Selection of thermal images for analysis

5.2.3.1. Establishing the view angles

As one of the aims of this study was to identify to what extent the angle between the camera lens and the surface to be measured affected the thermal readouts of the images; the first step was to determine how images from different angles for each view looked (front, back, left and right). For this purpose, views were simulated using a protractor to measure different angles from a plastic model of a cow's head. Sample images from each of the variations of camera angle and elevation used for this study were collected. The images do not reflect the same thermal patterns of the calf's head, but they contain enough resolution to classify the angle deviation (from perfectly perpendicular images) in the images extracted from the videos simply and efficiently.

For the front view, a straight line was marked from the middle of the nostril to the thermal camera allocated at 2m using a cotton string to set the 0^0 deviation image; following the same procedure, strings were allocated at 15, 30, and 45 degrees towards to the right and left of the middle of the models' nostril to get sample images representative of each angle (see Figure 21). To calculate the elevation of the camera, a similar procedure was carried out, setting the 0^o image with a straight line from the muzzle of the model parallel to the floor surface. From there, 15, 30 and 45 degrees of elevation upwards and downwards the parallel lines were calculated with the protractor, and the sample images were collected (see Figure 23).

For the Back view, the 0^o line was marked, measuring the distance between the model's ears and locating the middle point; from where the cotton string was stretched 2 meters to allocate the camera. Using the protractor, the strings of 15, 30 and 45 degrees to both sides were allocated, and the pictures were taken with the thermal camera (see Figure 22). For the elevation, a string exactly at the middle of the ears was allocated parallel to the floor surface, and images 15 and 30 degrees upwards and 15 degrees downwards were taken (see Figure 23).

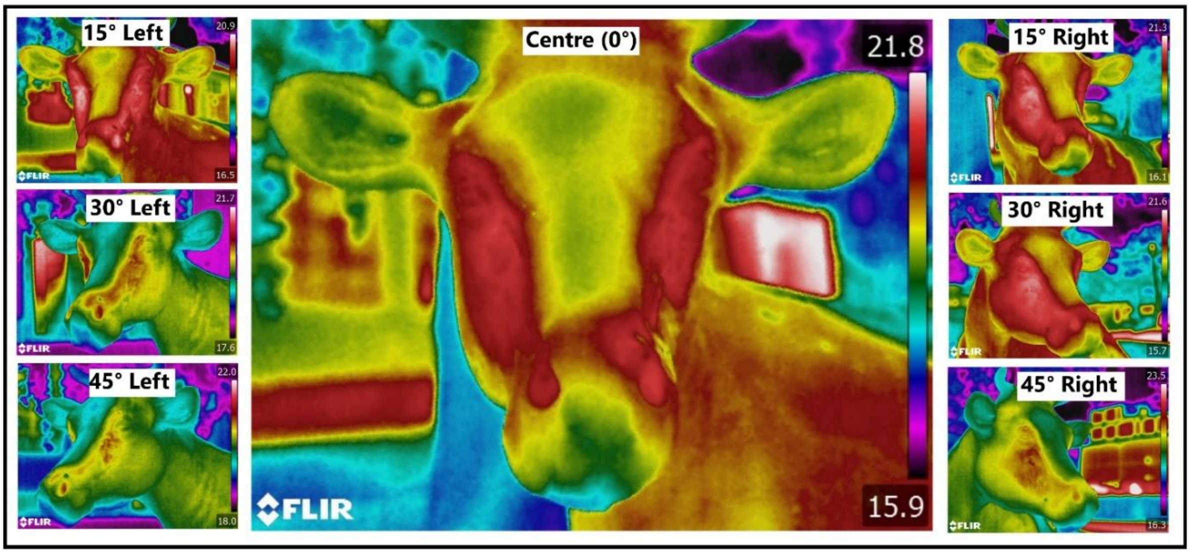


Figure 21. Reference images from a plastic model cow used as a guide to categorise the camera angles from the front view of the real calves thermograms.

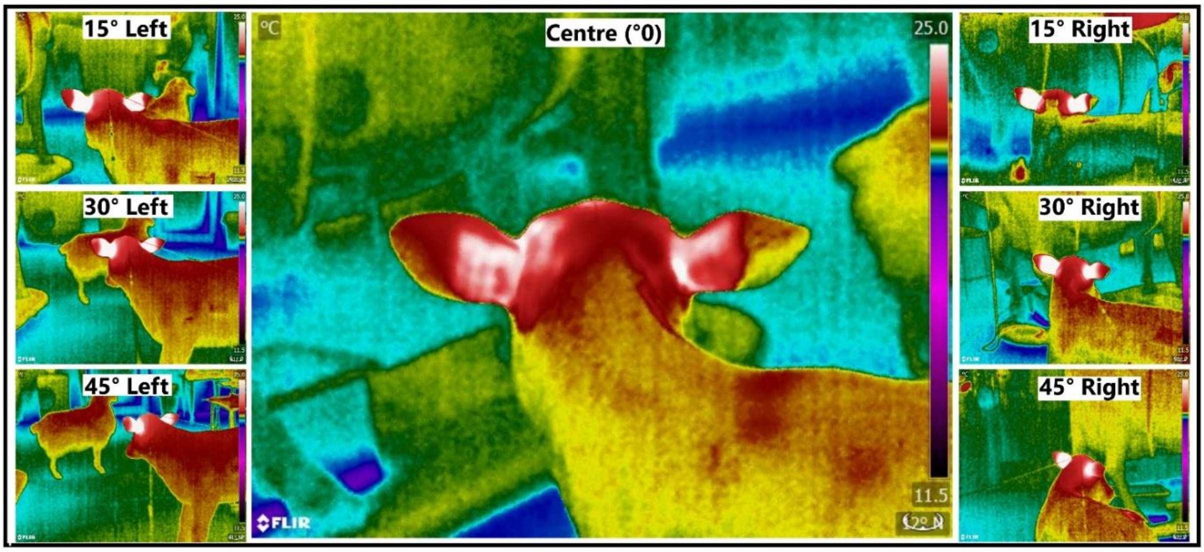


Figure 22. Reference images from a plastic model cow used as a guide to categorise the camera angles from the back view of the real calves thermograms.

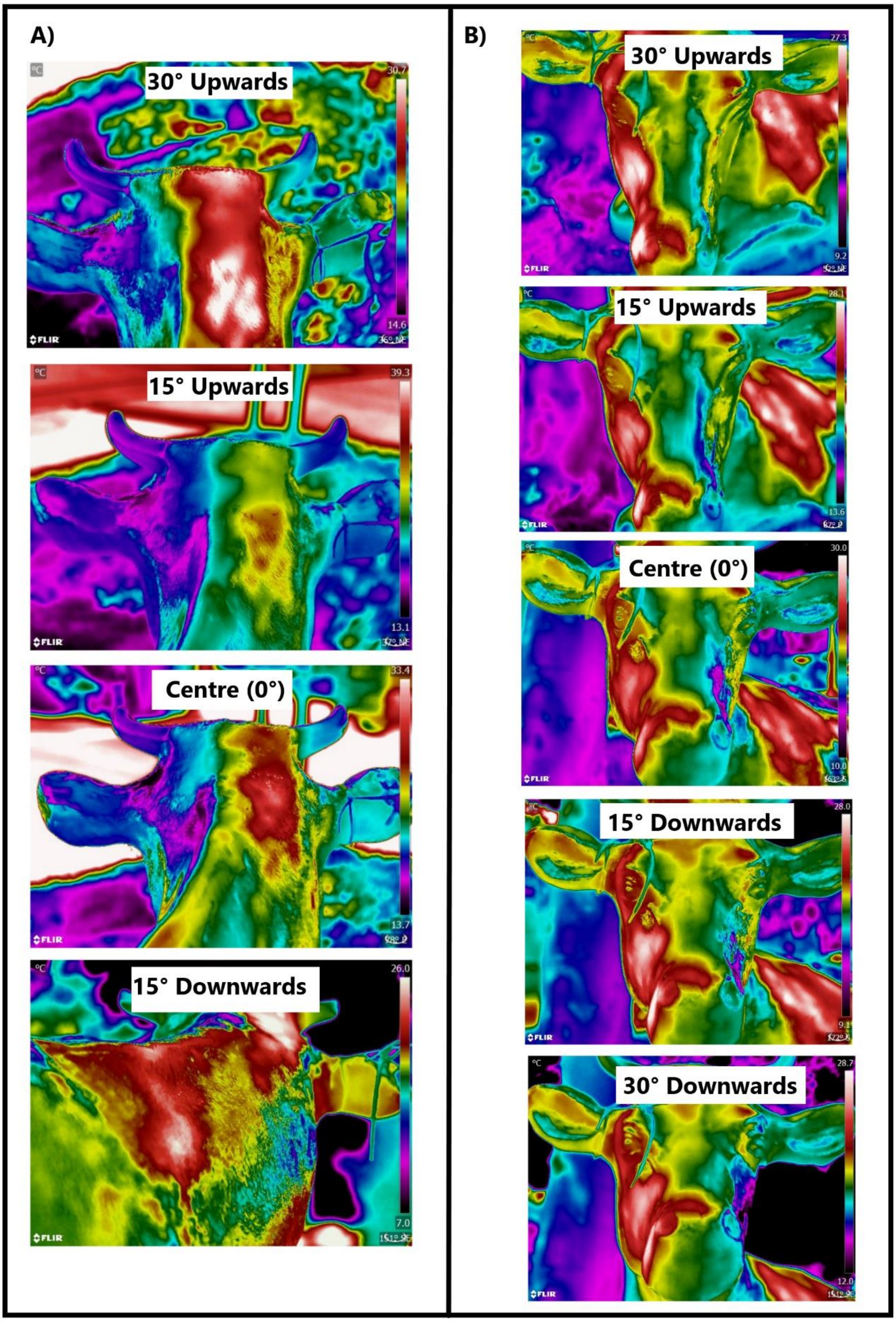


Figure 23. Reference images from a plastic model cow used as a guide to categorise the camera elevation from the back (A) and front (B) views of the real calves thermograms.

For left and right views, only the angle was measured; a perpendicular line to the sagittal plane of the cow's head delimited the 0° category, which was marked with a 2m cotton string from the inner corner of the eye to the camera lens, from there 15,30 and 45-degree lines were calculated towards the front, and 15 and 30 degrees towards the back of the head, and the respective images were collected (see Figure 24).

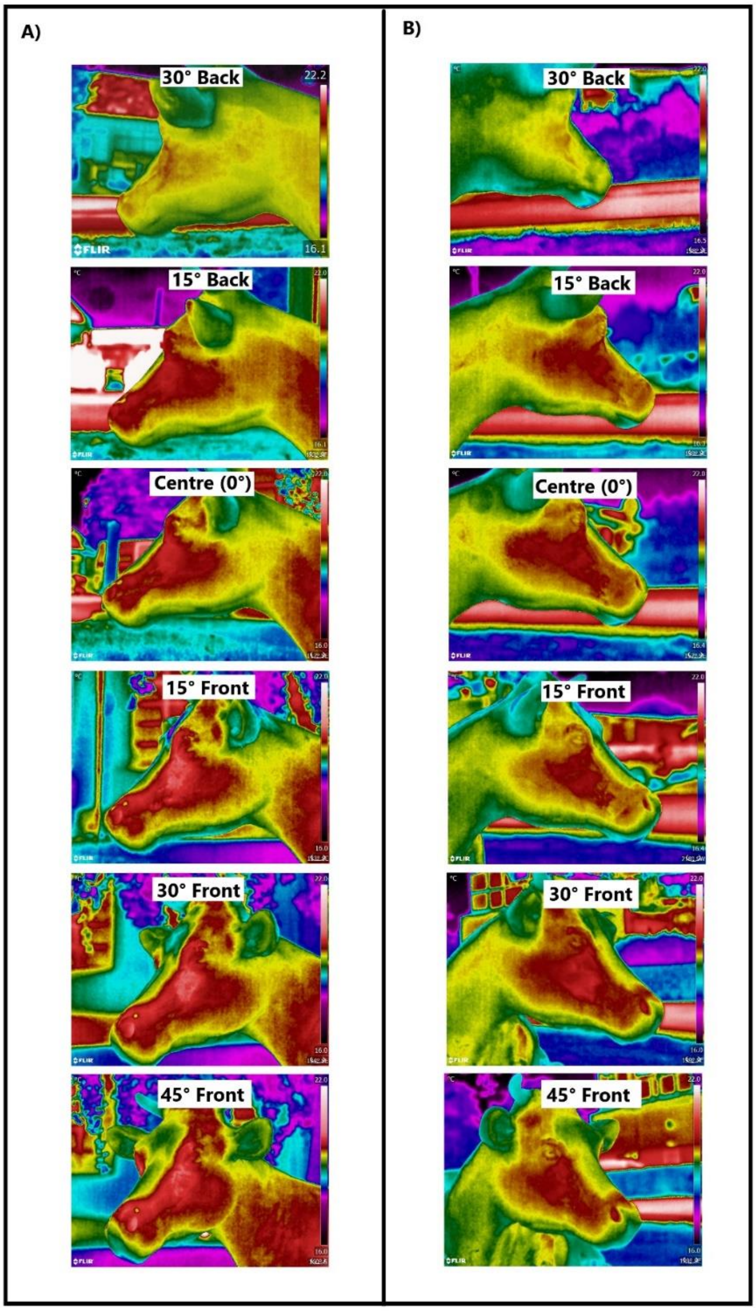


Figure 24. Reference images from a plastic model cow used as a guide to categorise the camera angle from the left (A) and right (B) views of the real calves thermograms.

5.2.3.2. Image selection.

As mentioned earlier, the thermal videos of live calves from the previous study were observed, and as many images as possible for each view (front, back, left and right) that were well in focus (the separation of the hairs clearly visible) and at a distance of around 2 meters were selected. As this study aimed to analyse the effect of camera angle and elevation, images matching changes in camera positions (corresponding with the plastic model explained in Chapter 5.2.3.1.) were selected regardless of the time elapsed between images, when no changes of angle/position occurred, frames were selected with at least 5 seconds of difference between to identify the effect of the time elapsed within the recording. After the images were extracted from the videos, the angle and position were scored, matching them visually with the most similar image from the plastic model. Once classified, images with the same angle/elevation score that were less than 5 seconds apart were eliminated. The remaining images were used for further analysis.

All Thermograms were extracted and measured using the software FLIR Tools v.6.4.18039.1003 (FlirSystems Inc, Oregon, USA).

5.2.4. Selection of Regions of interest ROIs

The temperature measurements were made in each frame using lines and circles delimitating the ROIs. The maximum temperatures were determined by the warmest pixel within an area, whereas average temperatures were calculated as a result of averaging the temperature of all the pixels within the area.

For the front images, the temperatures of the base of the ears (maximum and average), nostrils (maximum and average), the skin covering the nasal airways (maximum and average), muzzle (maximum and average) and hair whorl (maximum) areas were measured following the procedure described in the general methodology chapter (Chapter 2).

For the left and right images, temperatures of the inner corner of the eye (maximum), eyeball (maximum and average), rostral eye surrounding (maximum and average) and caudal eye surrounding areas (maximum and average) were measured following the procedure described in the general methodology chapter (Chapter 2).

For the back images, the maximum and average temperatures from the base of the ears were measured following the procedure described in the general methodology chapter (Chapter 2).

5.2.5. Factors considered for the analysis

Environmental parameters considered for the analysis were: temperature, relative humidity and THI (Temperature Humidity Index) calculated with the formula $THI = 0.8 * T + RH * (T - 14.4) + 46.4$, where T is the ambient temperature in Celsius degrees and RH the humidity expressed in decimals (e.g. 0.58).

Recording parameters considered for the analysis were: angle (e.g. 30),angle direction (left or right) angle and direction (continuous, e.g. -30 towards the left and +30 towards the right), elevation (e.g. 30), elevation direction (e.g. Upwards, Centre or Downwards), elevation and direction (continuous, e.g. -15 downwards and +15 upwards) and time of the image within the video (seconds).

5.2.6. Statistical analysis

5.2.6.1. Single temperatures

To identify the effect of the different variables (chapter 2.5), statistical models for repeated measures were made for each ROIs. Linear multilevel models were created using a random effect structure nesting images within calves, setting image as level 1 (i), i.e. 1 to 448 and calf number as level 2 (j), i.e. 1 to 38. Models were created using a stepwise regression with a forward selection of the variables, starting with the independent effect of each of the predictor variables. The predictor variable whose addition resulted in the best improvement of the model based on p-values calculated from the likelihood-ratio test of the difference between the single model and the ones incorporating each of the individual variables separately (taking into account the degrees of freedom from the variables) was selected. The procedure was repeated, adding each of the remaining variables until no significant improvements to the model were found. e.g. "left eyeball average = B_{oij} constant + Variable A_{ij} + variable B_{ij} + …".

5.2.6.2. Temperature asymmetries

Temperature asymmetries (Left-Right temperatures) of the bilateral areas from the front and back images were calculated for each image as specified in Chapter 2.4. To calculate temperature asymmetries in the ocular and periocular areas, the images from the left and right views were paired, selecting the image from the opposite side that was closest in time and shared the same angle score.

Similarly to the procedure for the individual ROIs, linear multilevel models with a random effect structure were created, nesting images within calves, setting image number as level 1(i) and calf number as level 2 (j). Models were created using stepwise regression with the selection of the most significant variables until none of the remaining variables significantly improved the model. e.g "Eyeball average (L-R) = B_{oij} constant + Variable A_{ij} + variable B_{ij} + …".

As two different observers measured the different ROIs, the Intraclass correlation coefficient (ICC) was calculated to identify the level of agreement between observers. The correlation level of the different ROIs was high, being the less correlated area, the rostral eye surrounding the lower with an ICC of 0.891 and the highest correlation for the Inner Corner of the eye with an ICC of 0.999.

To control for multiple hypotheses, the Benjamin-Hochberg procedure to control for a False Discovery Rate of 0.2 was carried out using all the p-values calculated from the likelihood-ratio tests between the base models and the models, including the single variables.

5.3. Results

For the results chapter, tables showing the final models for each of the ROIs will be presented in the supplementary material; an exemplar of these tables can be found in table 12.

5.3.1. Single temperatures

5.3.1.1. Ears (front view)

In a model of the left ear maximum temperature, 70.01% of the variability was explained by quadratic relationships with THI (X^2=48.70, df=2, p<0.001) and time within recording (X^2=10.60, df=2, p=0.004), and by a linear relationship with camera angle (X^2=15.26, df=1, p<0.001; Figure 25). In the model for the right ear maximum temperature, 65.14% of the variability was explained by a model containing quadratic relationships with THI(X^2=43.97 df=2, p<0.001) and time within recording (X^2=6.85 df=1, p=0.03), and linear relationships with camera angle (X^2=31.53, df=1, p<0.001) and camera elevation (X^2=12.03, df=1, p=0.005; Figure 26). In a model for the left ear average temperature, 80.22% of the variability was explained by quadratic relationships with THI (X^2=71.435, df=2, p<0.001) and camera angle (X^2=53.48.22, df=2, p<0.001) and age (X^2=6.38, df=2, p=0.041), and a linear relationship with camera elevation (X^2=5.96, df=1, p=0.014; Figure 27). In the model for the right ear average temperature, 75.56% of the variability was explained by a quadratic relationship with THI (X^2=61.81, df=2, p<0.001), and linear relationships with angle

(X^2=28.32, df=1, p<0.001) and time within recording (X^2=5.84, df=1, p=0.015; Figure 28).

All ear temperatures showed an increase following a quadratic relationship with THI values. In most of the ear measurements, an increased angle towards one side resulted in higher temperatures on that side and lower temperatures on the opposite side, except for the average temperature on the left ear, in which a quadratic relationship was observed. The elevation of the camera only influenced the right ear maximum and the left ear average temperatures; as the elevation of the camera moves upwards, the temperature on the right ear increased, whereas the left one decreased. Left and right maximum ear temperatures had a quadratic relationship with the time elapsed within the recording, whereas only the average temperature on the right side was influenced negatively as the time within the recording elapsed. Finally, only the average temperature of the left ear was significantly associated with the age of the calves by a quadratic relationship (see Table 12; for average temperatures see Table 32).

Left ear Max (front view)					
Variable	*Details of the variable*	*Coefficient*	SE.	*C.I. (95% confidence)*	p-value
constant		31.6041	0.7110	30.2105. to 32.9977	p <0.001
(THI-gm)	Continuous	0.5294	0.0712	0.3898 to 0.6691	p<0.001
(THI-gm)^2	Continuous, quadratic relation	-0.0584	0.0135	-0.0849 to -0.0319	p<0.001
Angle and direction	Continuous, -ve towards right of the head, +ve towards left of the head	0.0094	0.0024	0.0047 to 0.0142	p<0.001
Time	Continuous	0.0055	0.0017	0.0021 to 0.00880	p=0.001

Time^2	Continuous, quadratic relation	-0.000013	0.000004	-0.000022 to -0.000005	p=0.001
Right ear Max (front view)					
Variable	*Details of the variable*	*Coefficient*	SE.	*C.I. (95% confidence)*	p-value
constant		31.99	0.6835	30.6504 to 33.3297	p<0.001
(THI-gm)	Continuous	0.4813	0.0677	0.3486 to 0.6139	p<0.001
(THI-gm)^2	Continuous, quadratic relation	-0.0446	0.0128	-0.0697 to -0.0196	p<0.001
Angle and direction	Continuous, -ve towards right of the head, +ve towards left of the head	-0.0146	0.0026	-0.0196 to -0.0095	p<0.001
Elevation and direction	Continuous, -ve downwards , +ve upwards	0.0162	0.0047	0.0071 to 0.0253	p<0.001
Time	Continuous	0.0029	0.0019	-0.0007 to 0.0065	p=0.119
Time^2	Continuous, quadratic relation	-0.00001	0.000005	-1.9E-05 to -1E-06	p=0.032

Table 12. Final model for ear maximum temperatures (front view).

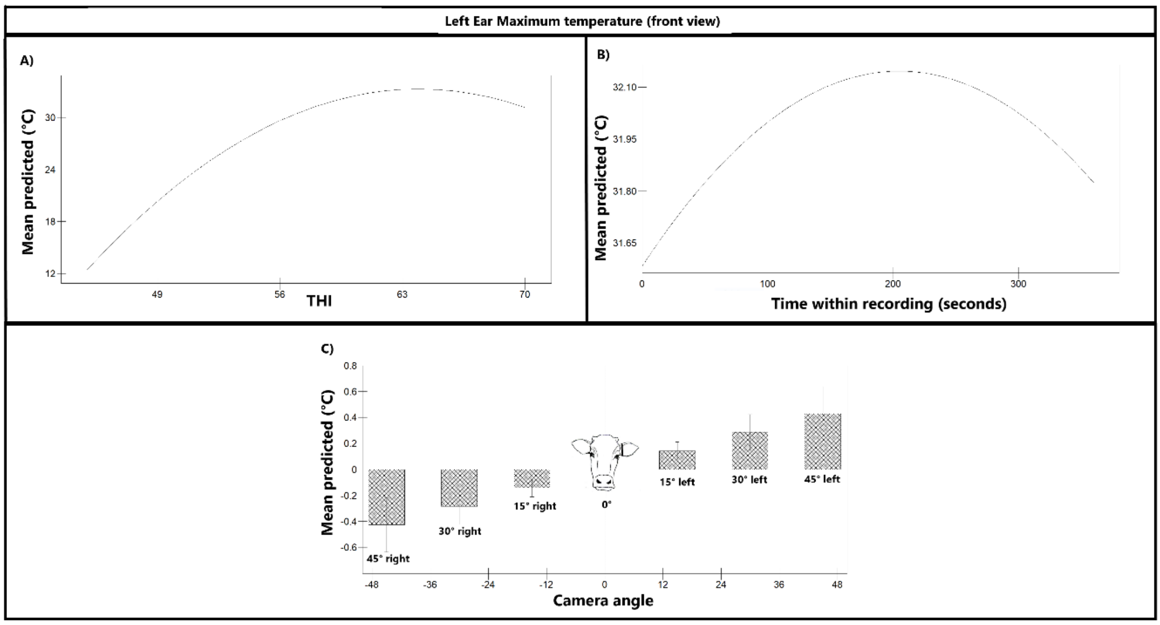


Figure 25. Effect of THI (A), time within recording (B) and camera angle (C) in the left ear maximum temperature (front view).

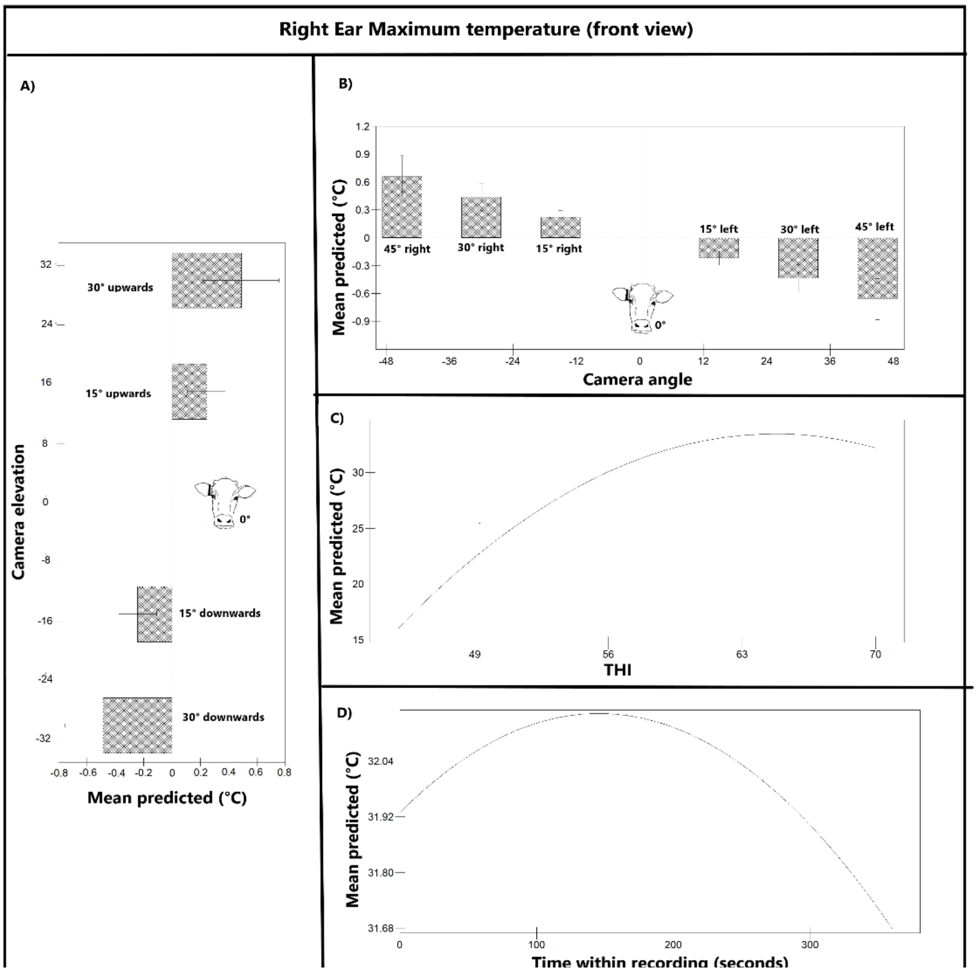


Figure 26. Effect of camera elevation (A), camera angle (B), THI (C) and time within recording (D) in the right ear maximum temperature (front view).

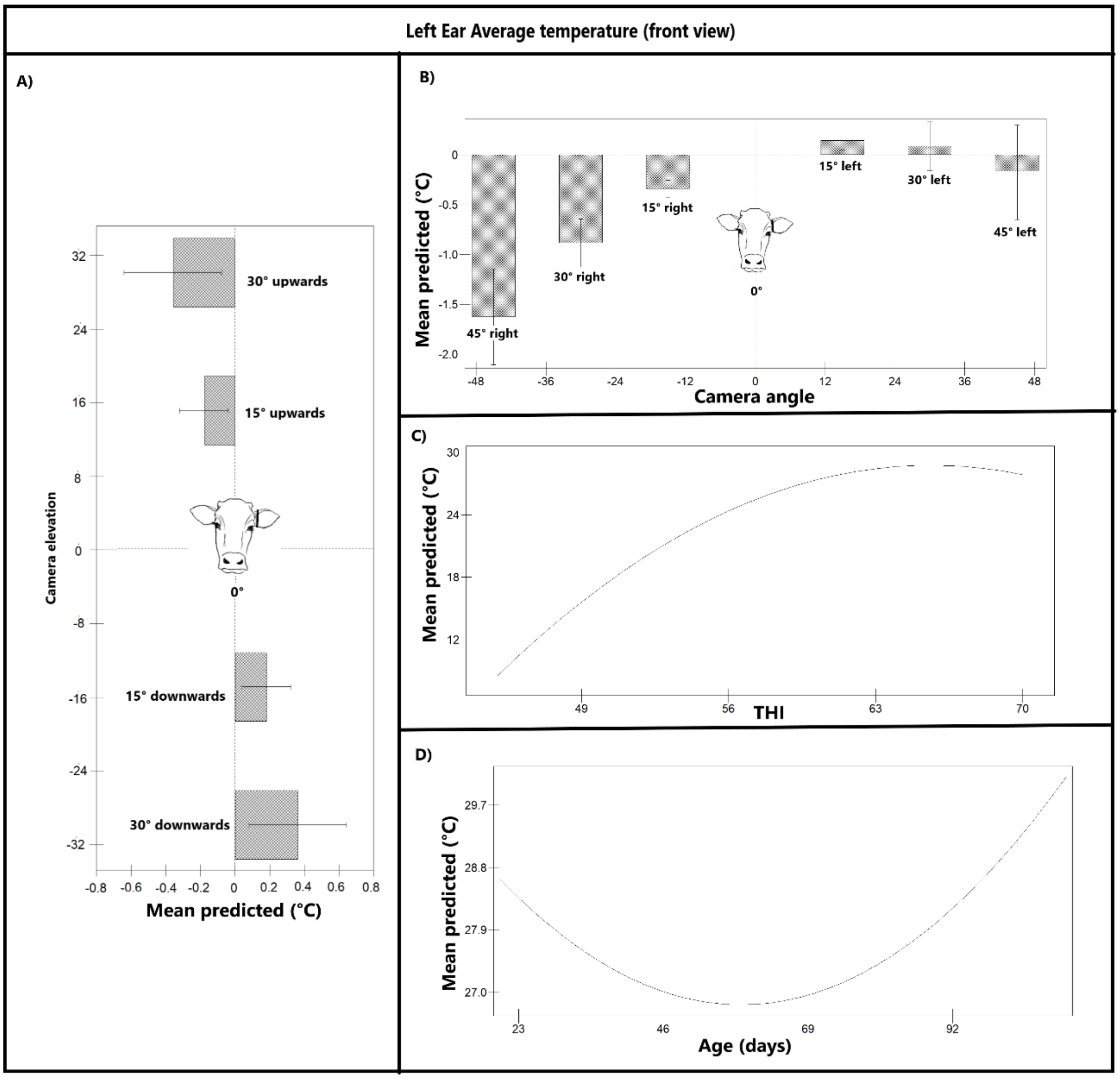


Figure 27. Effect of camera elevation (A), camera angle (B), THI (C) and calves age (D) in left ear average temperature (front view).

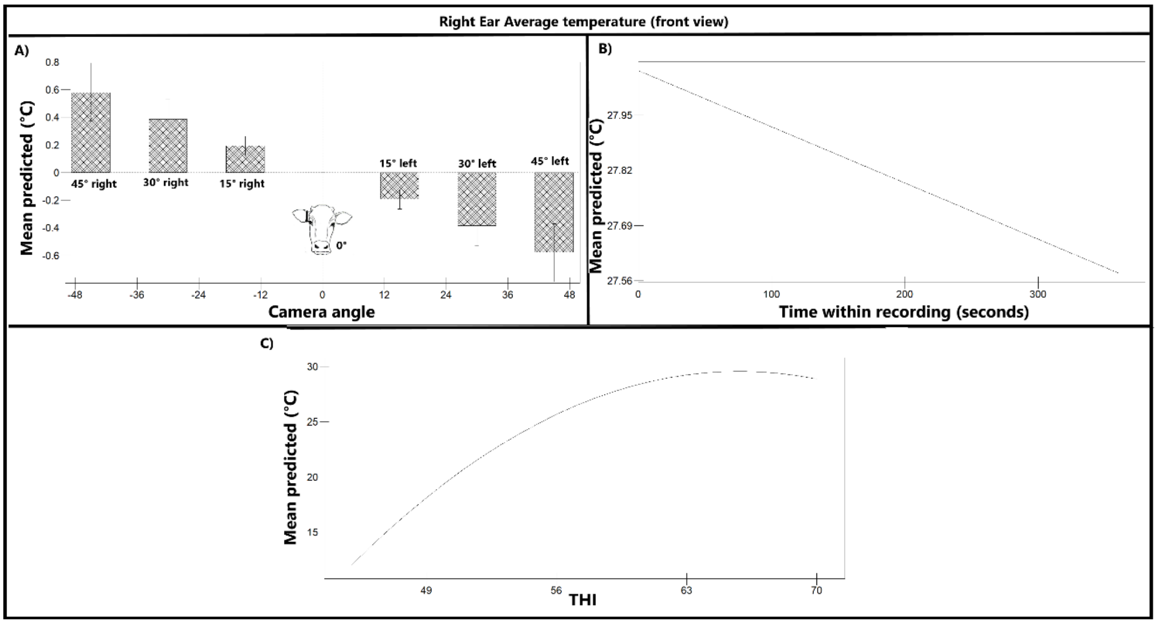


Figure 28. Effect of camera angle (A), time within recording (B) and THI (C) in right ear average temperature (front view).

5.3.1.2. Nostrils

In a model of the left nostril maximum temperature, 36.59% of the variability was explained by a quadratic relationship with time within recording (X^2=14.15 df=2 , p<0.001), and a linear relationship with THI (X^2=17.71 df=1 , p<0.001; Figure 29; Table 33). In the model for right nostril maximum temperature, 40.11% of the variability was explained by a quadratic relationship with time (X^2=9.65 df=2 , p=0.008), and linear relationships with THI (X^2=20.29 df=1 , p<0.001), angle (X^2=11.65 df=1 , p<0.001) and elevation (X^2=19.65 df=1, p<0.001 Figure 30; Table 33). In the model for left nostril average temperature, 47.49% of the variability was explained by quadratic relationships with angle (X^2=41.38, df=2 , p<0.001), time (X^2=7.679, df=2 , p=0.021) and elevation (X^2=6.78 df=2 , p=0.033), and in a linear relationship with THI (X^2=24.99 df=1 , p<0.001; Figure 31; Table 34). In the model for right nostril average temperature, 55.42% of the variability was explained by quadratic relationships with elevation (X^2=25.33 ,df=2 , p<0.001) and THI (X^2=31.11 ,df=2 , p<0.001), and a linear relationship with angle (X^2=14.27 ,df=1 , p<0.001; Figure 32; Table 34).

All the temperature measurements had a positive relationship with THI values. Temperatures influenced by the angle and direction of the camera showed a positive relationship with the angles moving towards the same side and negative with angles moving towards the opposite one. The time within the recording had a quadratic relationship with all the measurements except with the right nostril average, in which had no significant effect. The elevation of the camera had a quadratic relationship for average temperatures on both sides and a positive relationship with the maximum temperature of the right nostril.

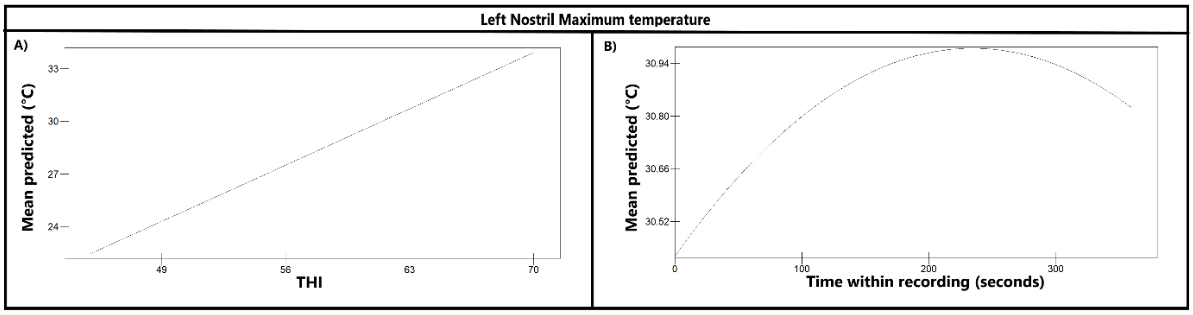


Figure 29. Effect of THI (A) and time within recording (B) in left nostril maximum temperature.

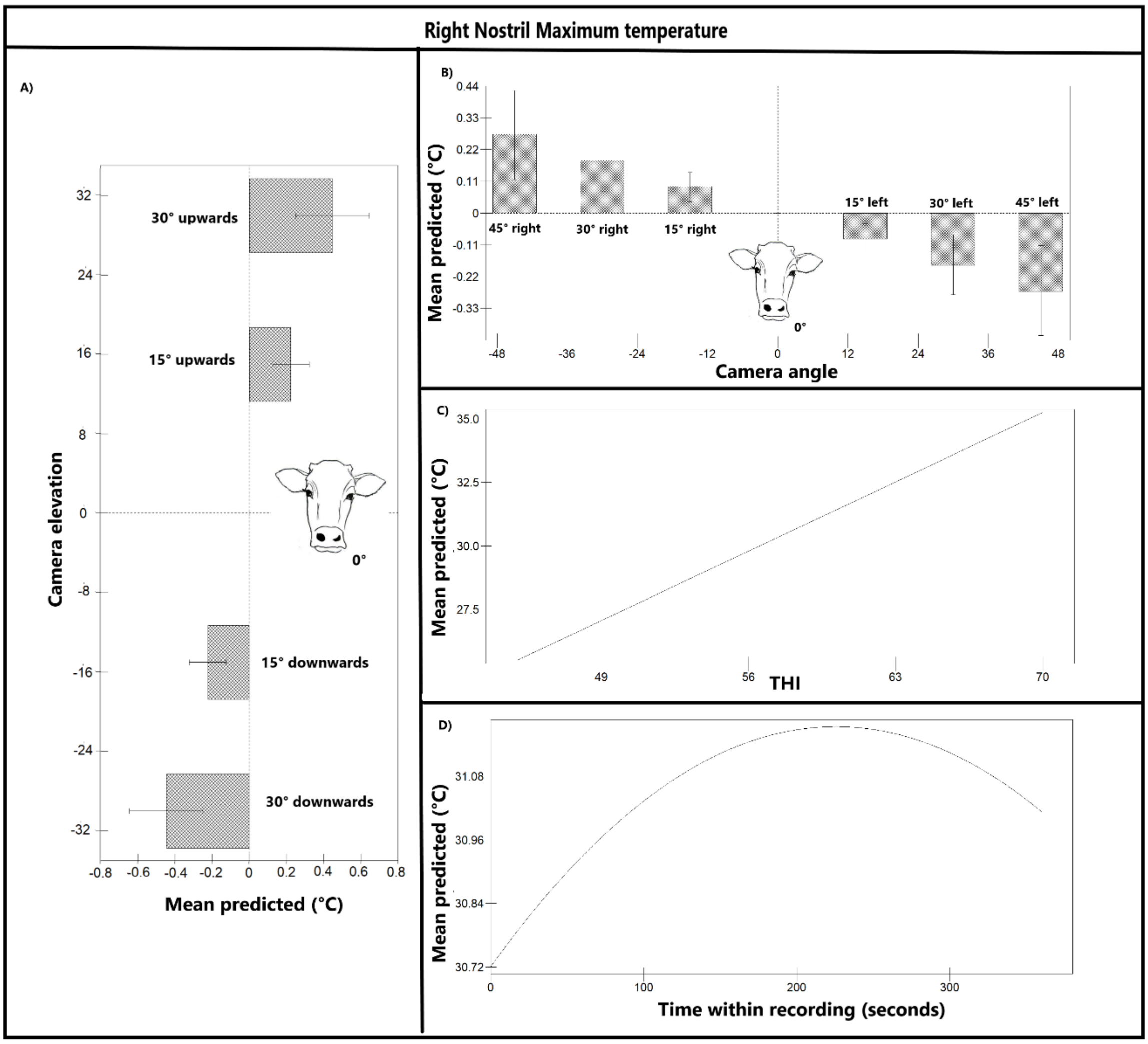


Figure 30. Effect of camera elevation (A), camera angle (B), THI (C) and time within recording (D) in right nostril maximum temperature.

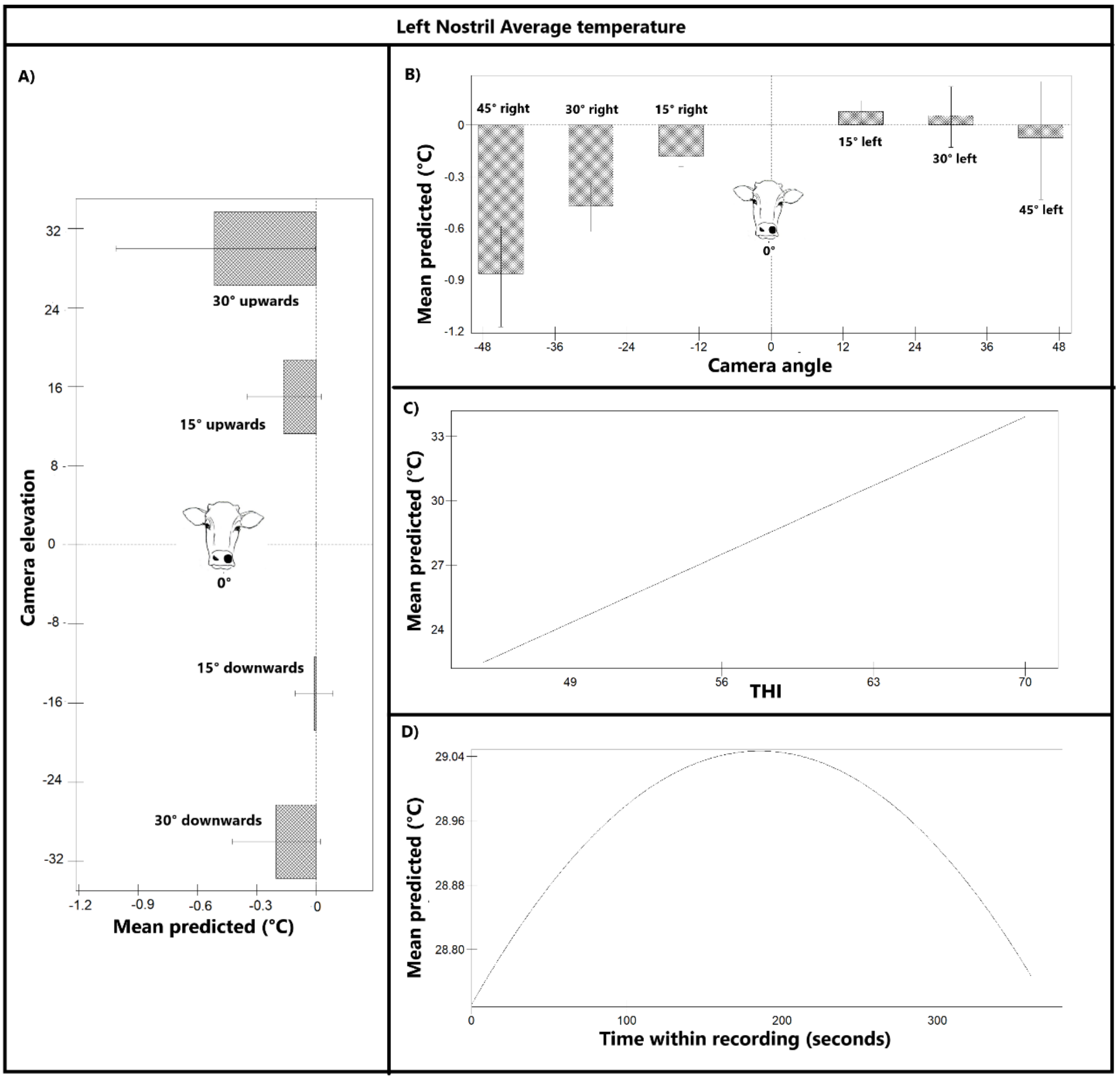


Figure 31. Effect of camera elevation (A), camera angle (B), THI (C) and time within recording (D) in left nostril average temperature.

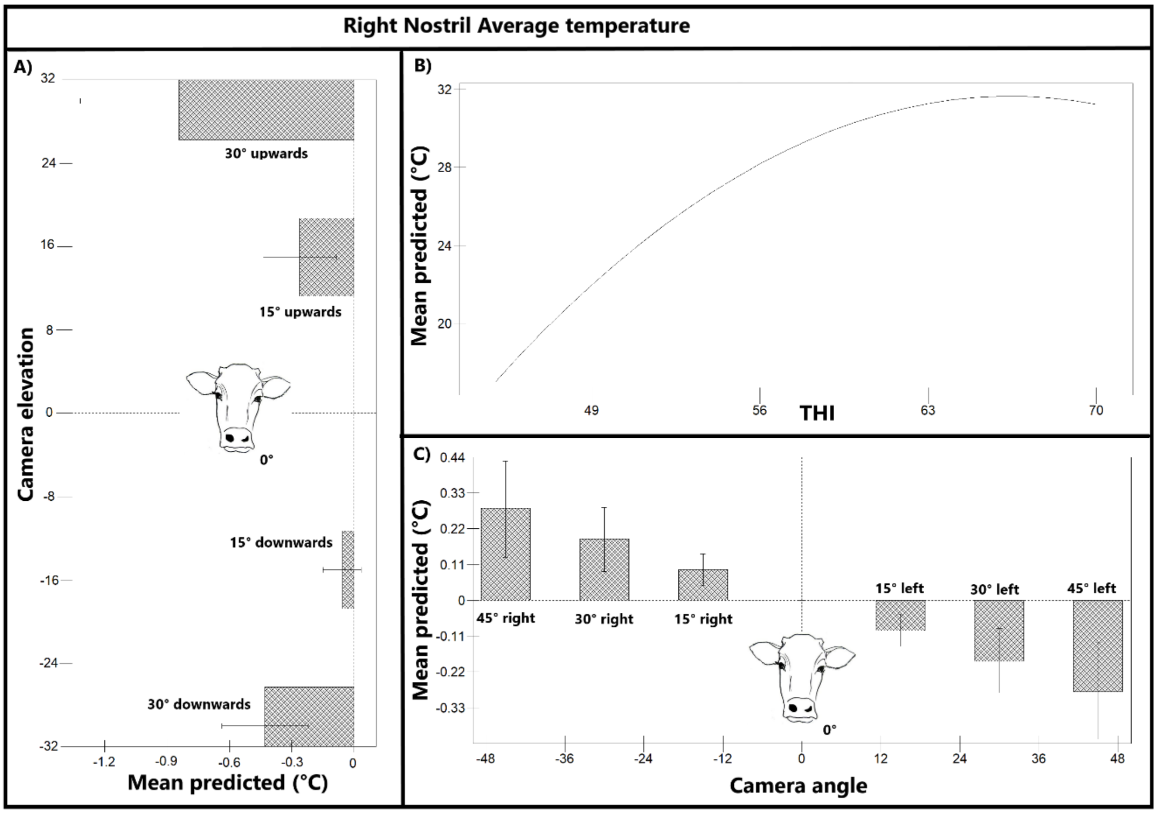


Figure 32. Effect of camera elevation (A), THI (B) and camera angle (C) in right nostril average temperature.

5.3.1.2. Nasal airways

In a model for left nasal airway maximum temperature, 70.02% of the variability was explained by quadratic relationships with THI (X^2=46.43 ,df=2 , p<0.001), time within recording (X^2=25.10 ,df=2 , p<0.001) and camera elevation (X^2=26.09 ,df=2 , p<0.001), and a linear relationship with camera angle (X^2=19.72 ,df=1 , p<0.001; Figure 33; Table 35). In a model for right nasal airway maximum temperature, 65.94% of the variability was explained by quadratic relationships with THI (X^2=41.18 ,df=2 , p<0.001), time within recording (X^2=8.89 ,df=2 , p=0.011), and linear relationships with camera angle (X^2=5.96 ,df=1 , p=0.014), and camera elevation (X^2=28.54 ,df=1 , p<0.001; Figure 34; Table 35). In a model for left nasal airway average temperature, 77.09% of the variability was explained by a cubic relationship with time (X^2=19.58 ,df=3 , p<0.001), a quadratic relationship with THI (X^2=53.56 ,df=2 , p<0.001), and linear relationships with camera angle (X^2=23.50 ,df=1 , p<0.001), camera elevation (X^2=5.41 ,df=1 , p=0.020) and age of the calves (X^2=4.27 ,df=1 , p=0.038; Figure 36; Table 35). In a model for right nasal airway average temperature, 74.42% of the variability was explained by a cubic relationship with time (X^2=27.049, df=3, p<0.001), a quadratic relationship with THI (X^2=49.39, df=2, p<0.001), and linear relationships with camera elevation (X^2=32.57, df=1, p<0.001) and age of the calves (X^2=4.60, df=1 , p=0.031; Figure 36; Table 36).

All the temperatures on the nasal airway area increased in a quadratic relationship with the THI values. The maximum and average temperatures of both nasal airways had cubic and quadratic relationships with the time within recording, respectively. Elevation of the camera influenced the average and maximum temperatures on the right side positively; the maximum temperature on the left side had a quadratic relationship with camera elevation, whereas the average temperature on that side had a negative relationship with the camera elevation. Most of the temperatures were influenced by the camera angle, increasing as the camera angle moved towards the side measured, except for the average temperature of the right side, in which the effect was not significant. Finally, average temperatures on both sides were associated positively with the age of the calves.

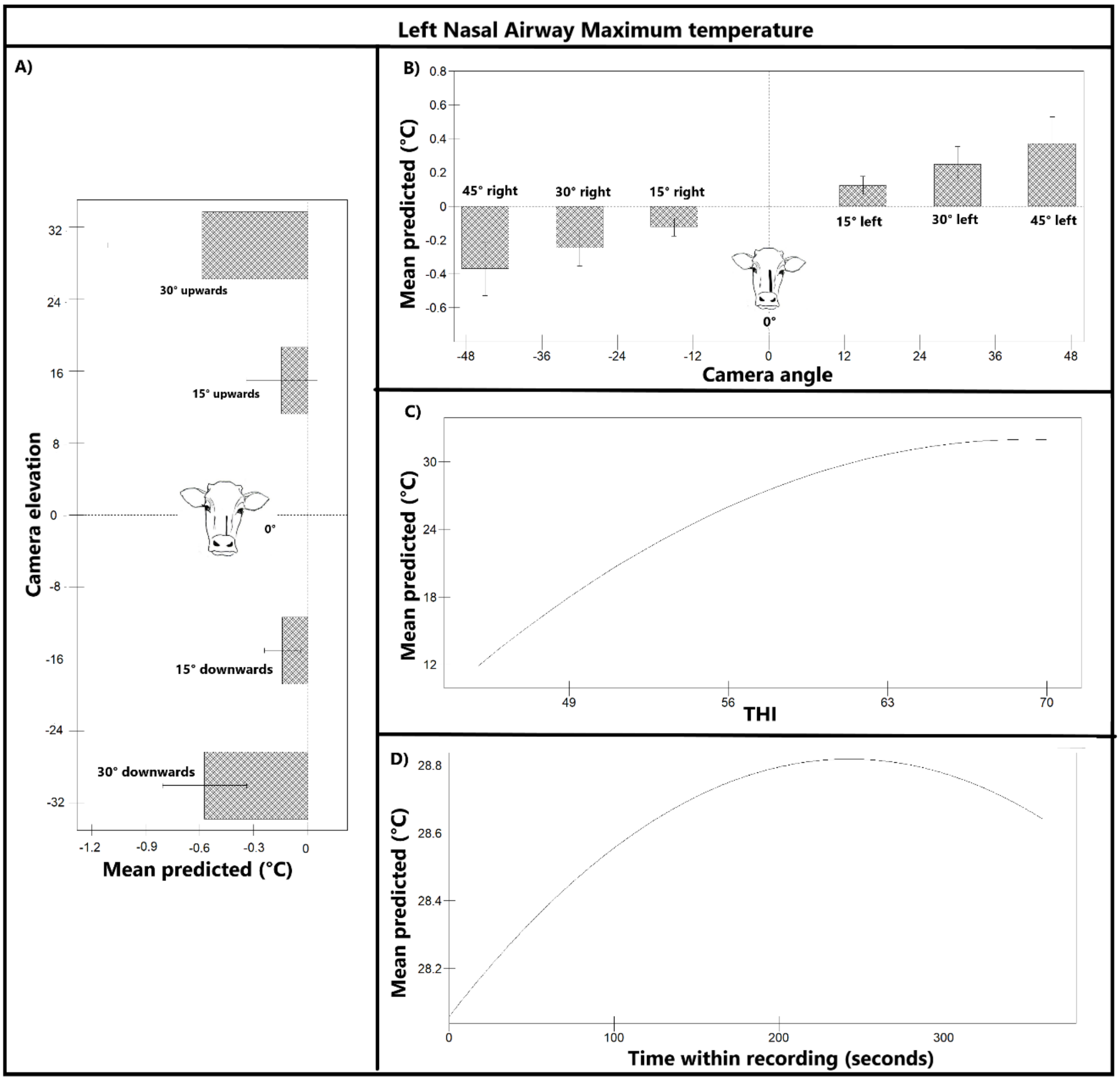


Figure 33. Effect of camera elevation (A), camera angle (B), THI (C) and time within recording (D) in left nasal airway maximum temperature.

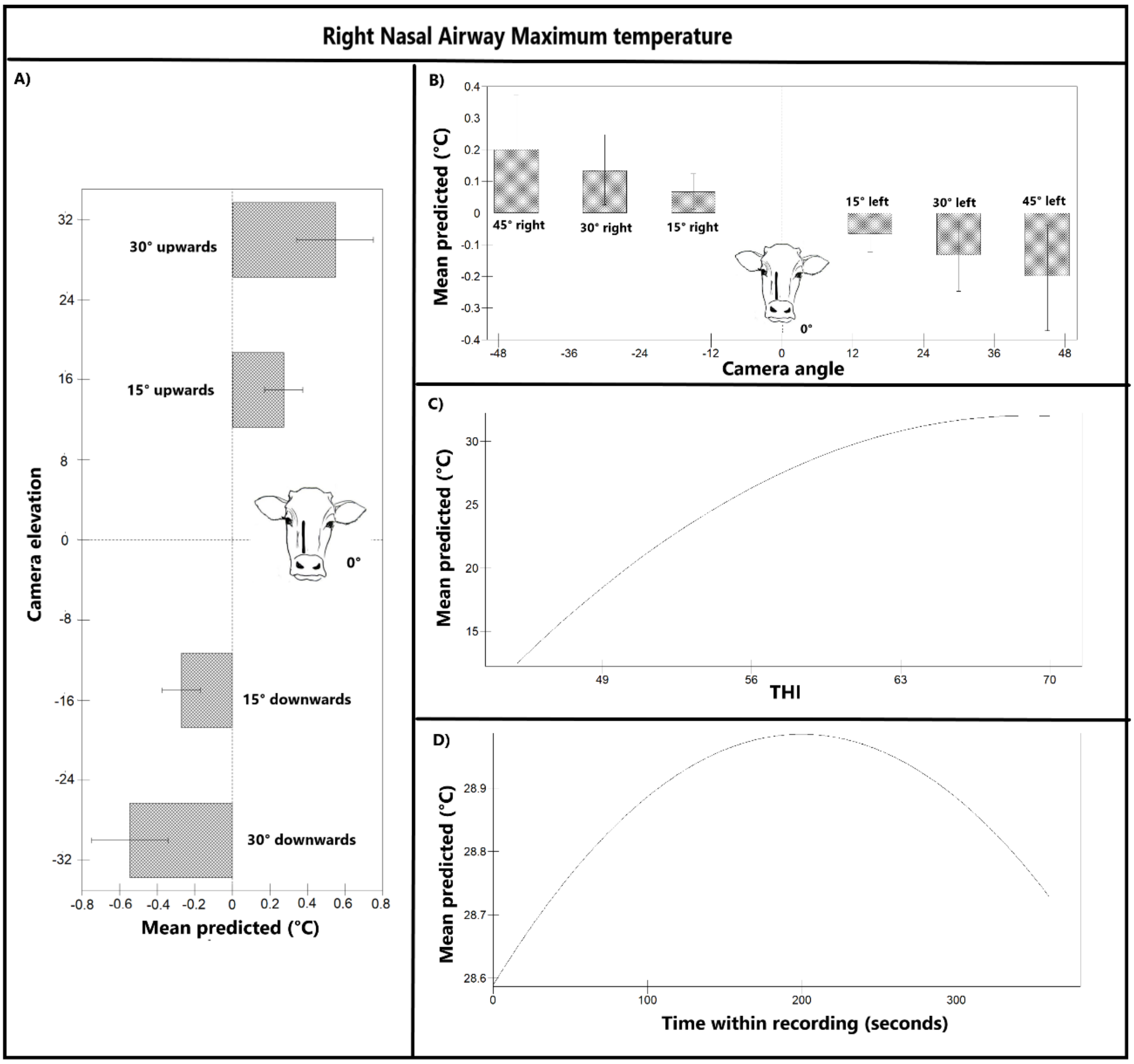


Figure 34. Effect of camera elevation (A), camera angle (B), THI (C) and time within recording (D) in right nasal airway maximum temperature.

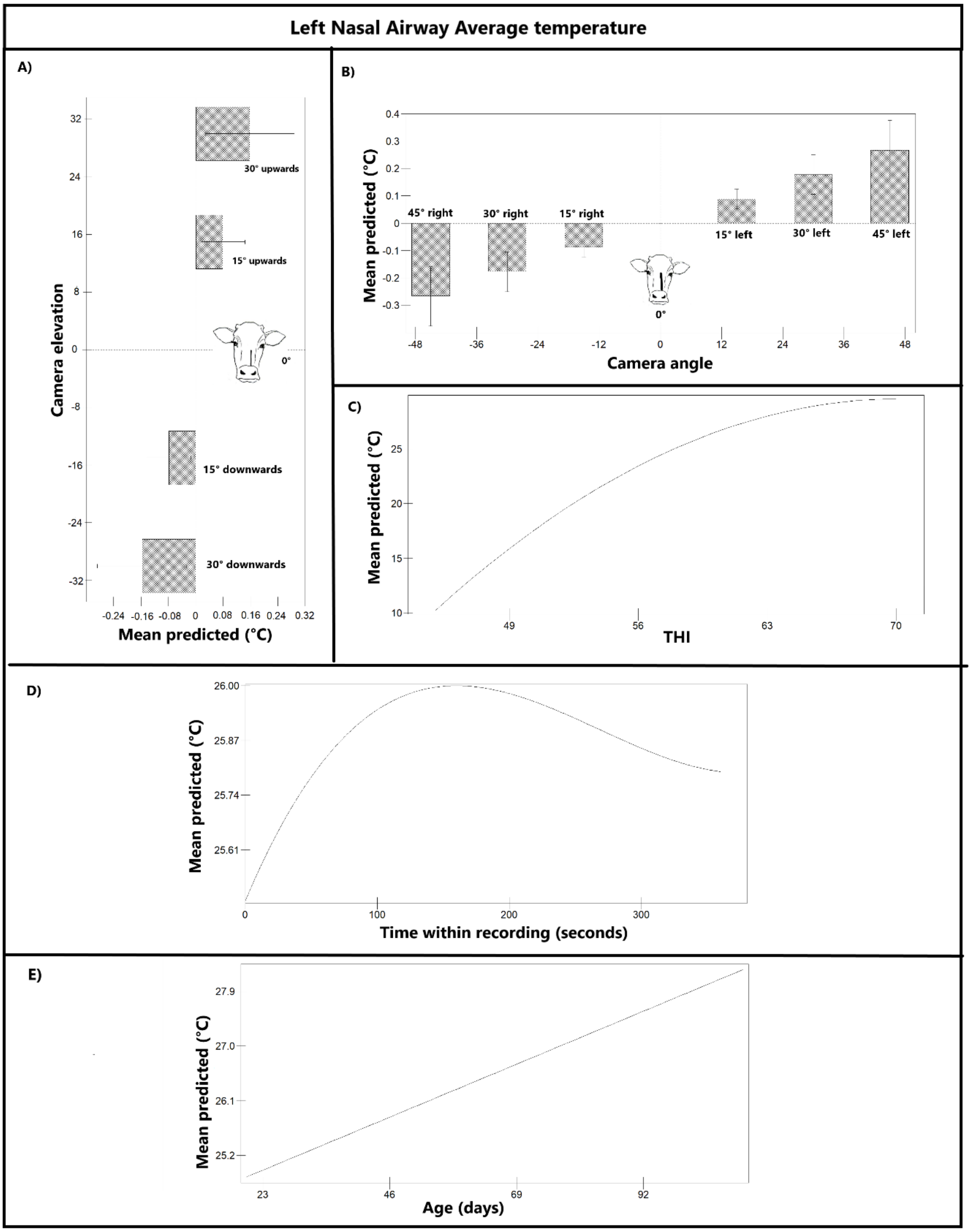


Figure 35. Effect of camera elevation (A), camera angle (B), THI (C), time within recording (D) and calves age (E) in left nasal airway average temperature.

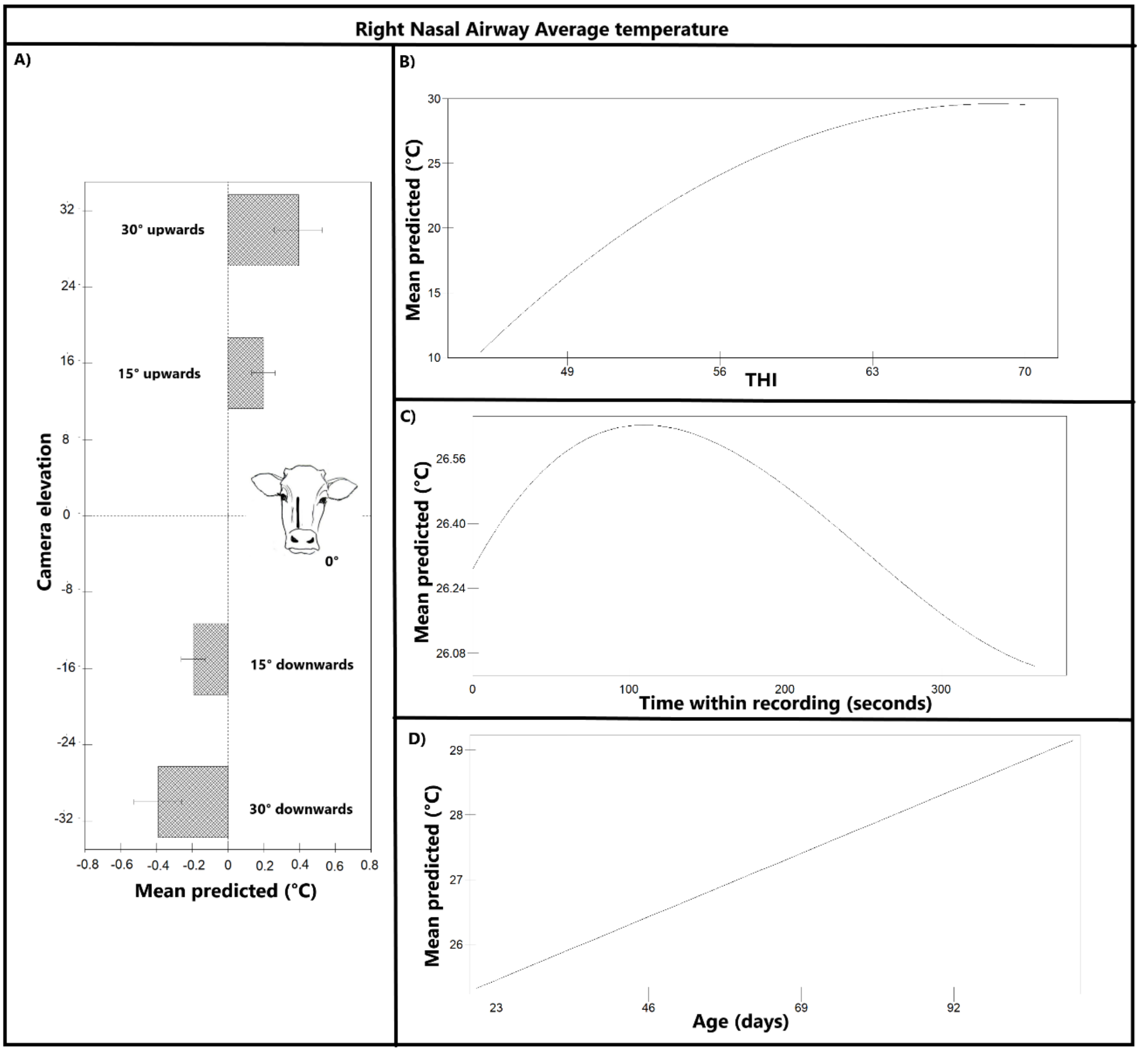


Figure 36. Effect of camera elevation (A), THI (B), time within recording (C) and calves age (D) in right nasal airway average temperature.

5.3.1.3. Muzzle and hair whorl

In a model for muzzle maximum temperature, 46.06% of the variance was explained by a quadratic relationship with THI (X^2=29.36, df=2, p<0.001), and linear relationships with camera angle (regardless direction) (X^2=6.07, df=1, p=0.013) and camera elevation (X^2=29.23, df=1, p<0.001; Figure 37; Table 37). In a model for average muzzle temperature, 63.74% of the variability was explained by a cubic relationship with elevation (X^2=63.06, df=3, p<0.001), and quadratic relationships with THI (X^2=39.16, df=2, p<0.001) and time (X^2=11.84, df=2, p=0.003; Figure 38; Table 37). In a model for hair whorl maximum temperature, 46.14%of the variability was explained by a cubic relationship with camera angle (X^2=10.20, df=3, p=0.017), and quadratic relationships with elevation (X^2=58.98, df=2, p<0.001), time (X^2=30.11, df=2, p<0.001), THI (X^2=22.34, df=2, p<0.001; Figure 39; Table 37).

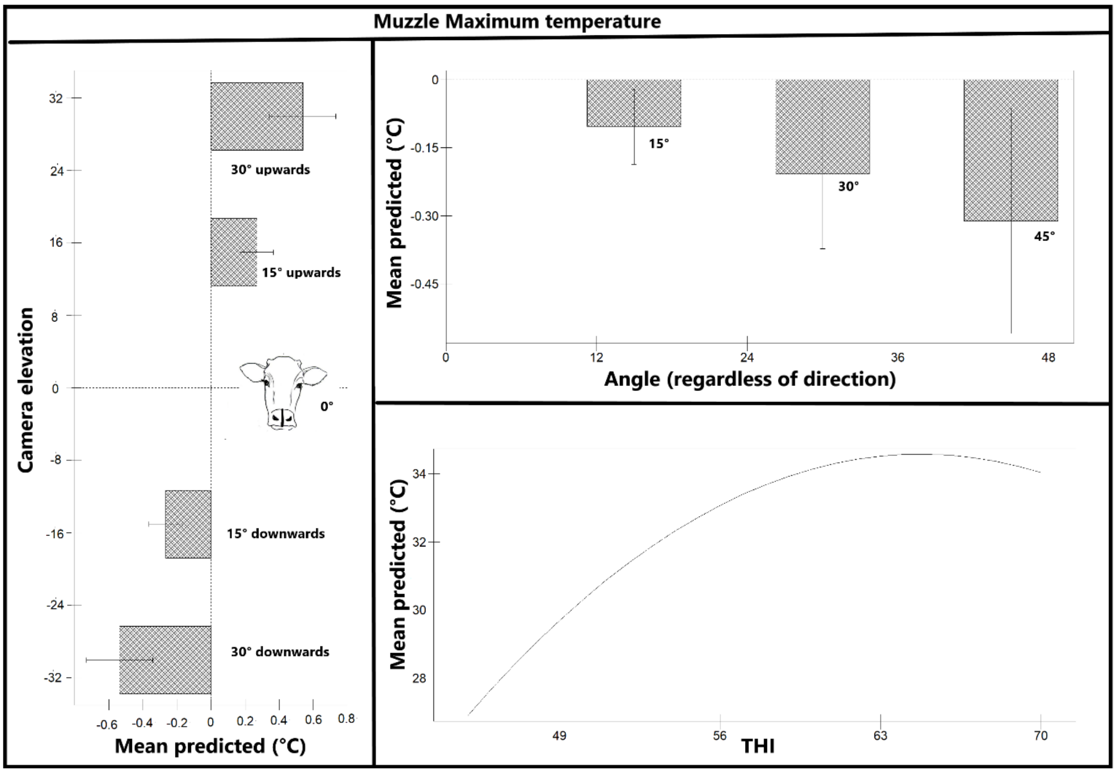


Figure 37. Effect of camera elevation (A), camera angle (B) and THI (C) in muzzle maximum temperature.

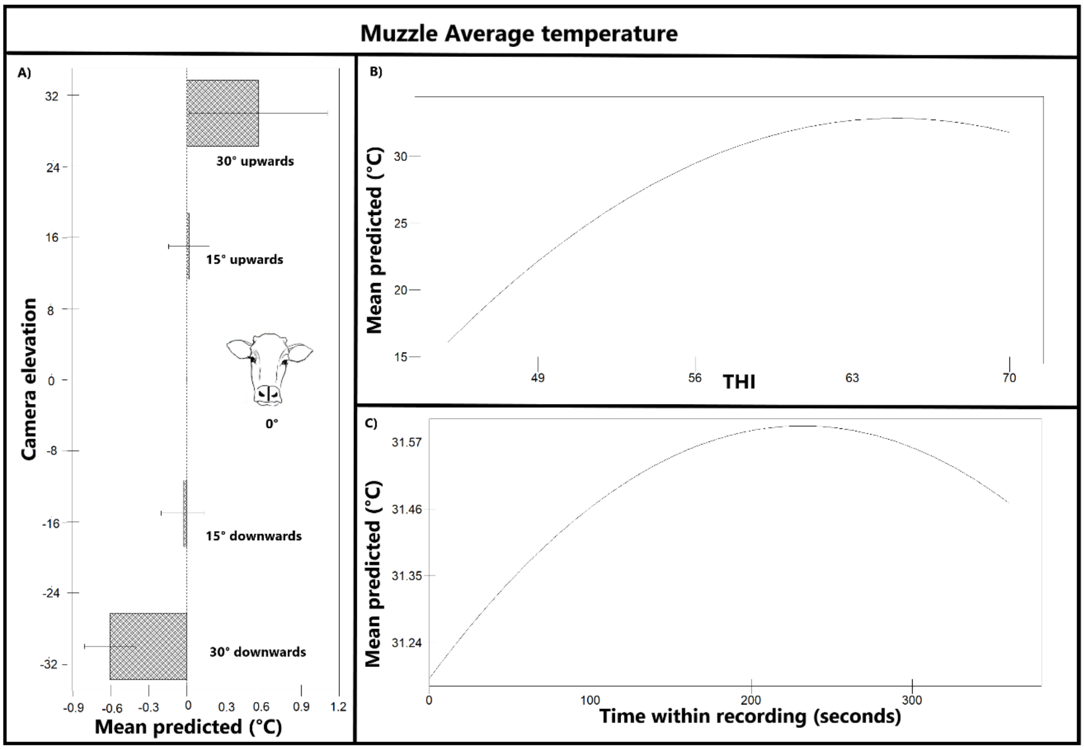


Figure 38. Effect of camera elevation (A), THI (B) and time within recording (C) in average muzzle temperature.

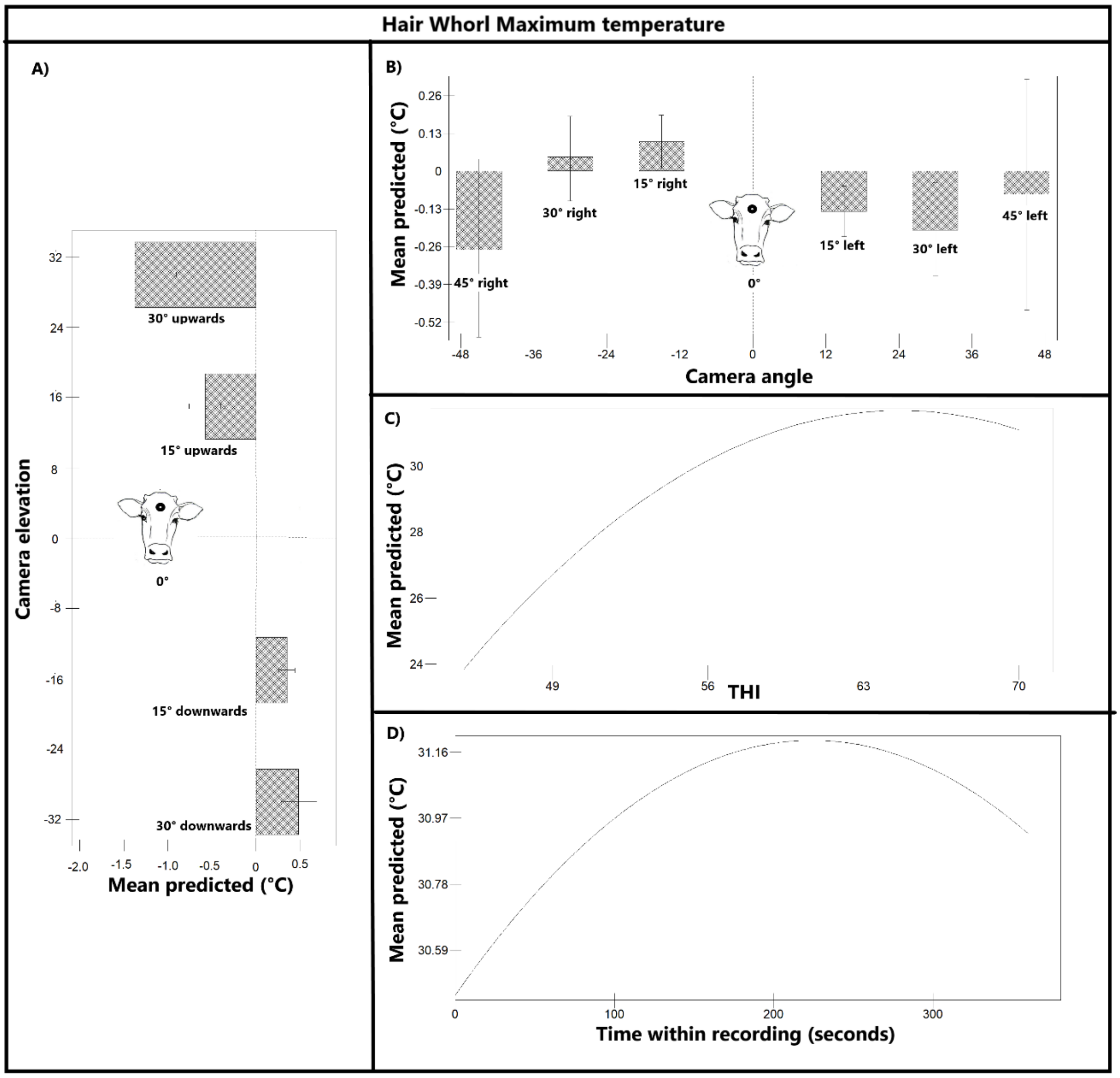


Figure 39. Effect of camera elevation (A), camera angle (B), THI (C) and time within recording (D) in hair whorl maximum temperature.

5.3.1.4. Eye

In a model for left eyeball average temperature, 38.78% of the variance was explained by a cubic relationship with time within recording (X^2=32.29, df=3, p<0.001), a quadratic relationship with camera angle (X^2=43.73, df=2, p<0.001) and a linear relationship with THI (X^2=22.01, df=1, p<0.001; Figure 40; Table 38). In a model for right eyeball average temperature, 33.42% of the variability was explained by a quadratic relationship with camera angle (X^2=46.08, df=2, p<0.001), and linear relationships with THI (X^2=21.41, df=1, p<0.001) and time (X^2=5.24, df=1, p=0.021;

Figure 41; Table 38). In a model for left inner corner max, 54.84% of the variance was explained by a cubic relationship with time (X^2=10.40, df=3, p=0.015), quadratic relationships with THI (X^2=36.19, df=2, p<0.001), camera angle (X^2=174.41, df=1, p<0.001; Figure 42; Table 39). In a model for right inner corner max, 53.13% of the variability was explained by a cubic relationship with time (X^2=30.16, df=3, p<0.001), a quadratic relationship with camera angle (X^2=247.68, df=2, p<0.001) and a linear relationship with THI (X^2=36.12, df=4, p<0.001; Figure 43; Table 39).

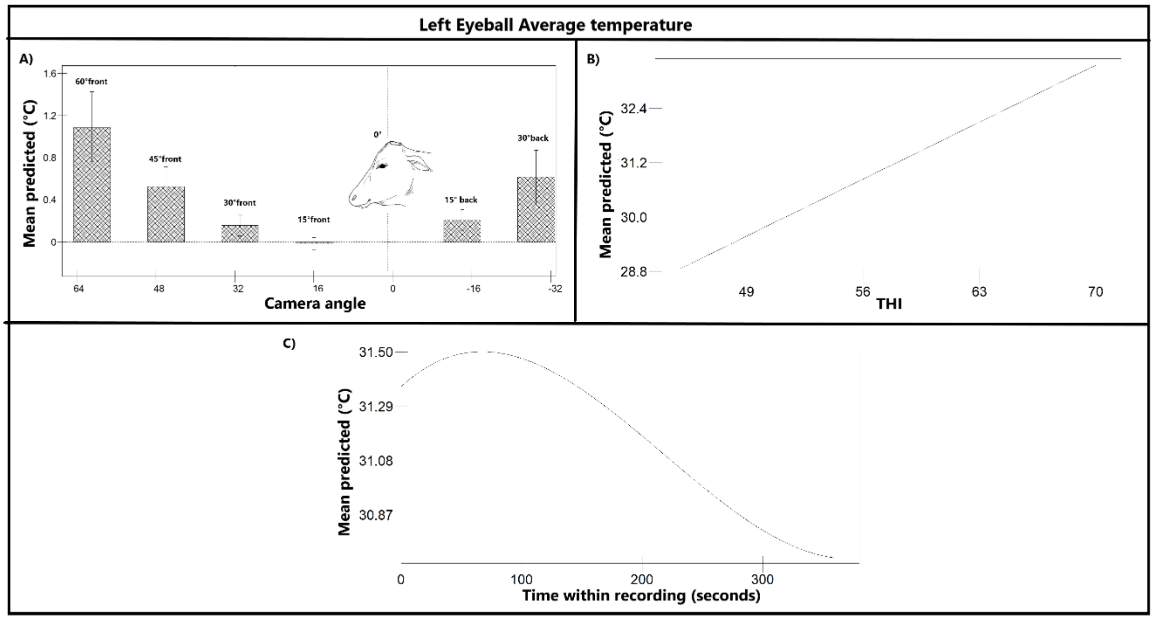


Figure 40. Effect of camera angle (A), THI (B) and time within recording (C) in left eyeball average temperature.

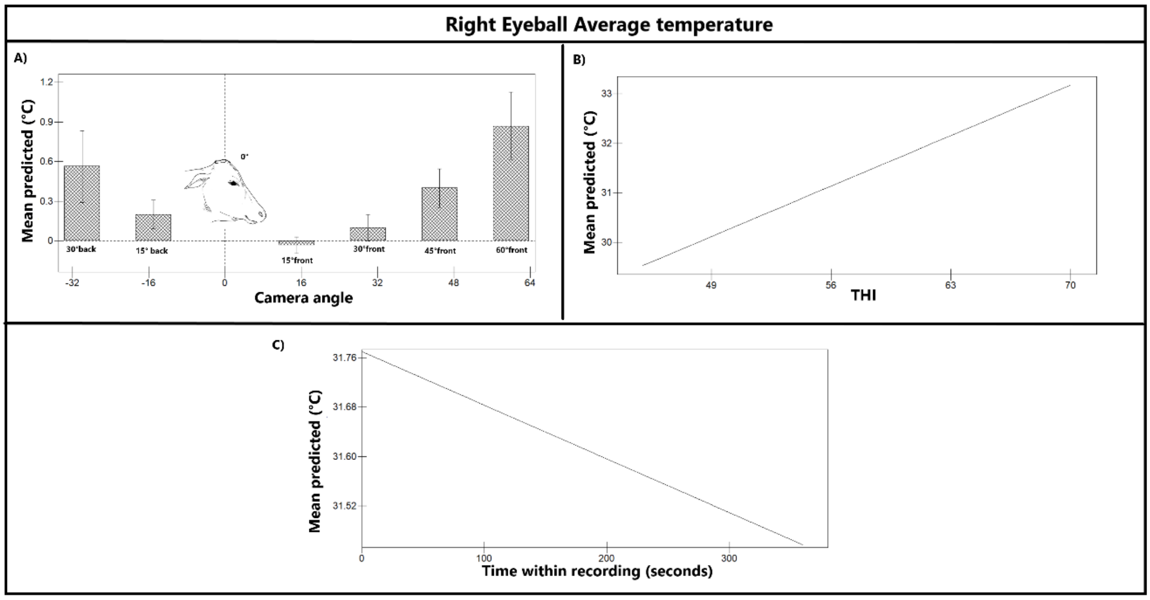


Figure 41. Effect of camera angle (A), THI (B) and time within recording (C) in right eyeball average temperature.

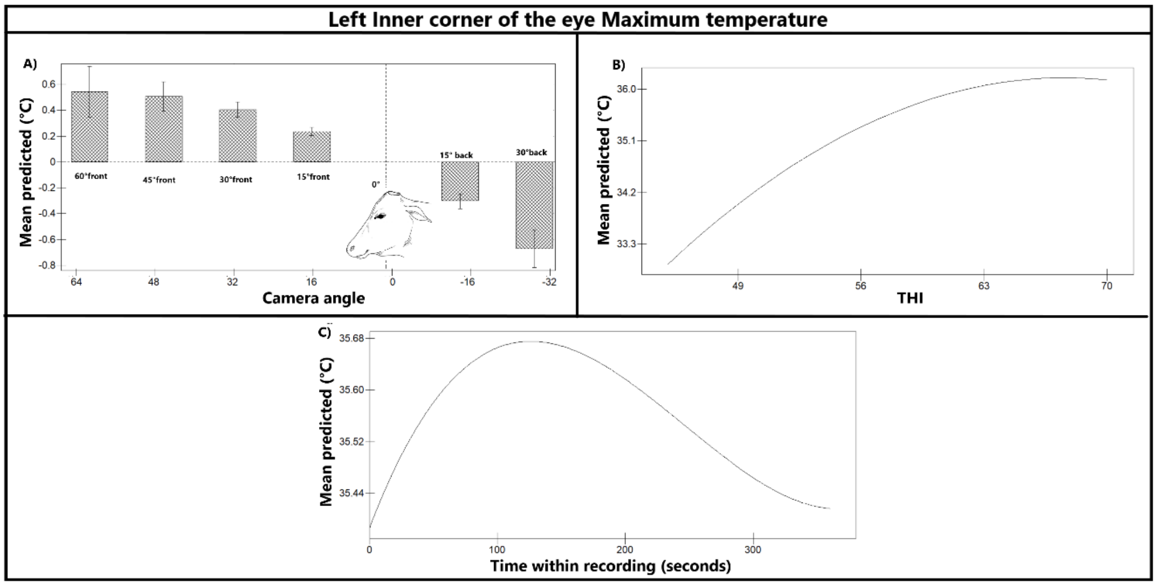


Figure 42. Effect of camera angle (A), THI (B) and time within recording (C) in the left inner corner of the eye temperature.

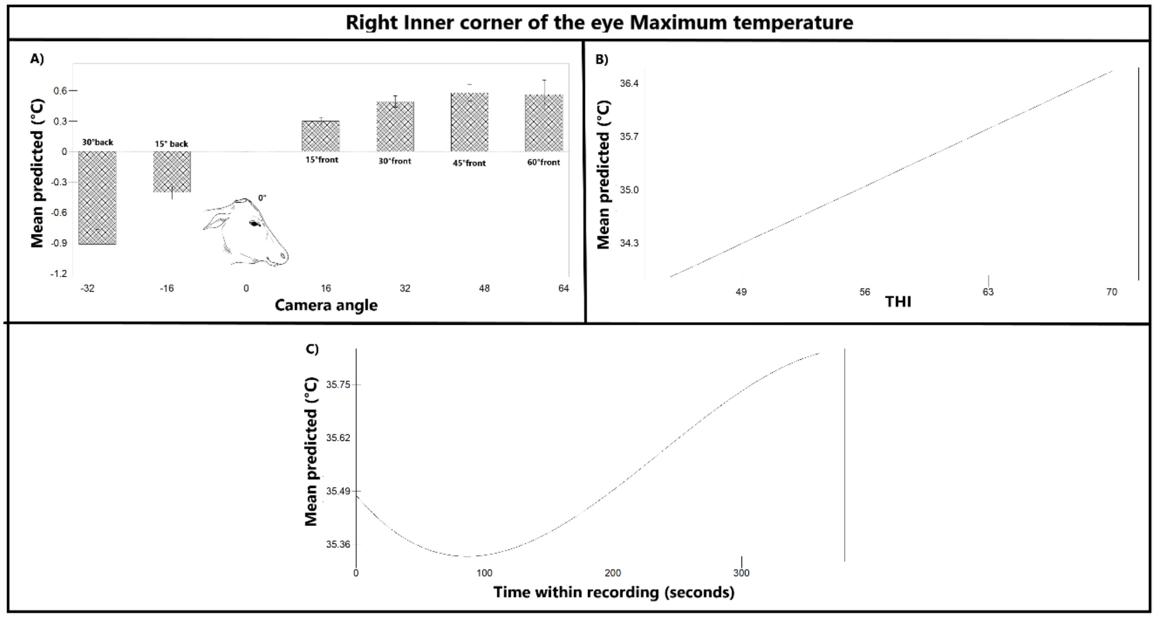


Figure 43. Effect of camera angle (A), THI (B) and time within recording (C) in the right inner corner of the eye maximum temperature.

5.3.1.5. Eye surroundings

In a model for the left rostral eye surrounding maximum temperature, 47.86% of the variance was explained by quadratic relationships with camera angle (X^2=152.72, df=2, p<0.001) and THI (X^2=35.42, df=2, p<0.001; Figure 44; Table 40). In a model for the right rostral eye surrounding maximum temperature, 58.63% of the variance was explained by a cubic relationship with camera angle (X^2=325.11, df=3, p<0.001) and a quadratic relationship with THI (X^2=38.49, df=2, p<0.001; Figure 45; Table 40). In a model for the left caudal eye surrounding maximum temperature, 67.65% of the variance was explained by quadratic relationships with camera angle (X^2=481.64, df=2, p<0.001), THI (X^2=52.748, df=1, p<0.001) and time within recording (X^2=12.27, df=2, p=0.002), and a linear relationship with calf age (X^2=24.20, df=1, p<0.001; Figure 46; Table 41). In a model for the right caudal eye surrounding maximum temperature, 66.69% of the variance was explained by a quadratic relationship with camera angle (X^2=413.98, df=2, p<0.001) and THI (X^2=40.32, df=2, p<0.001), and a linear relationship with calf age (X^2=16.03, df=1, p<0.001; Figure 47; Table 41).

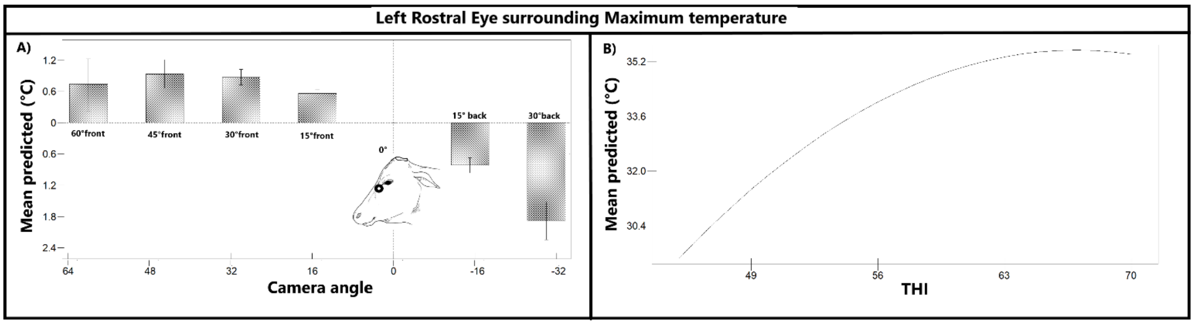


Figure 44. Effect of camera angle (A) and THI (B) in the left rostral eye surrounding maximum temperature.

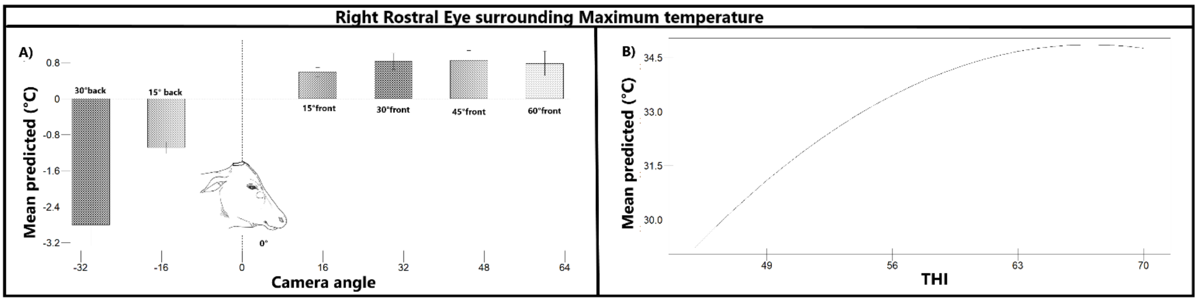


Figure 45. Effect of camera angle (A) and THI (B) in the right rostral eye surrounding maximum temperature.

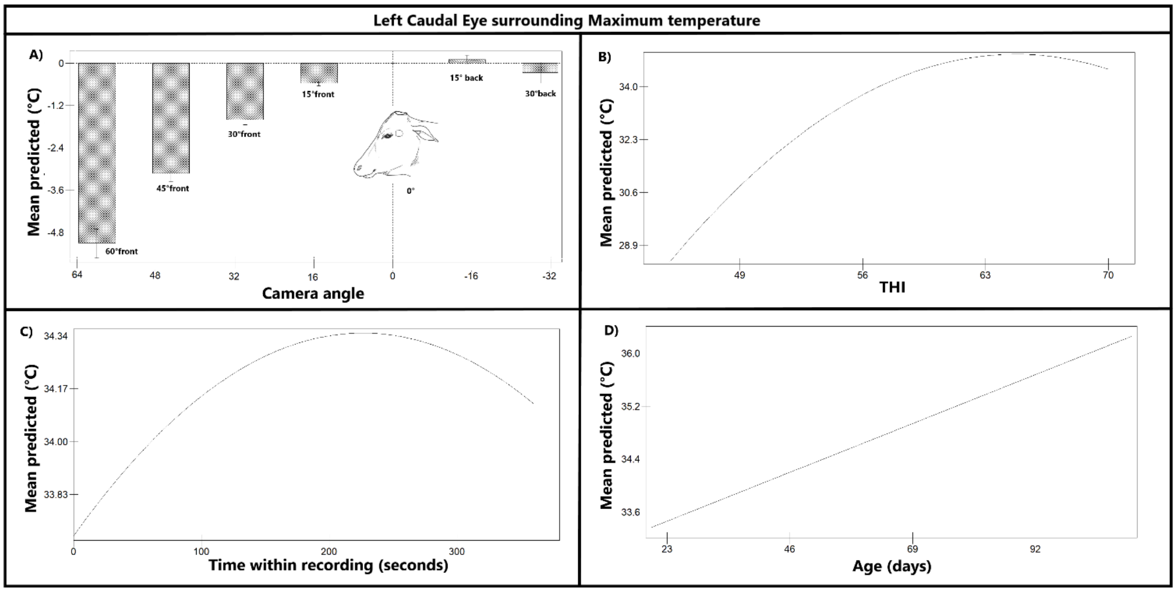


Figure 46. Effect of camera angle (A), THI (B), time within recording (C) and calves age (D) in the left caudal eye surrounding maximum temperature.

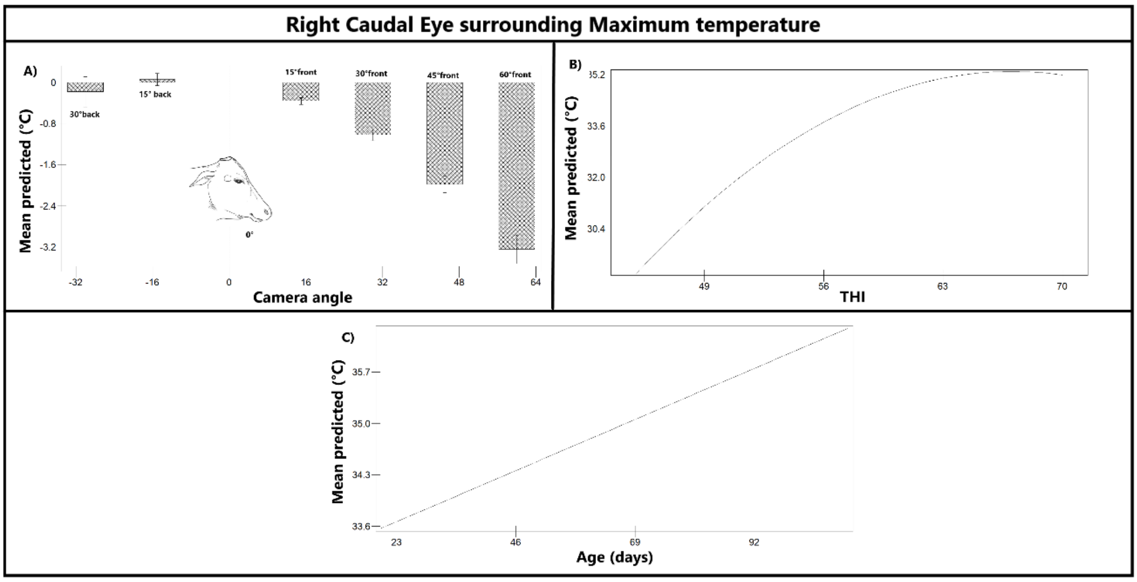


Figure 47. Effect of camera angle (A), THI (B) and calves age (C) in the right caudal eye surrounding maximum temperature.

5.3.1.6. Ears (back view)

In a model for left ear maximum temperature (back view), 17.79% of the variability was explained by a linear relationship with THI (X^2=11.89, df=1, p<0.001; Figure 48; Table 42). In a model for right ear maximum temperature (back view), 28.42% of the variability was explained by linear relationships with THI (X^2=17.35, df=1, p<0.001) and time within recording (X^2=4.69, df=1, p=0.030; Figure 49; Table 41). In a model for left ear average temperature (back view), 21.83% of the variability was explained by linear relationships with THI (X^2=15.27., df=1, p<0.001) and camera elevation (X^2=4.24, df=1, p=0.039; Figure 50; Table 43). In a model for right ear average temperature, 28.38% of the variability was explained by linear relationships with THI (X^2=19.68., df=1, p<0.001) and camera elevation (X^2=5.04, df=1, p=0.025; Figure 51; Table 43).

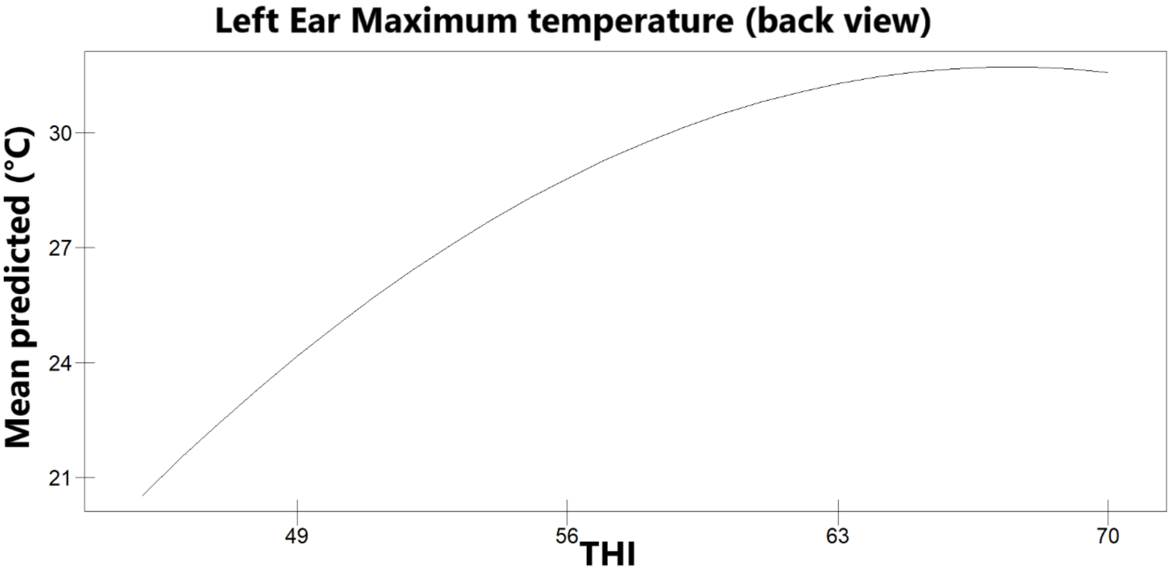


Figure 48. Effect THI (A) in the left ear maximum temperature (back view).

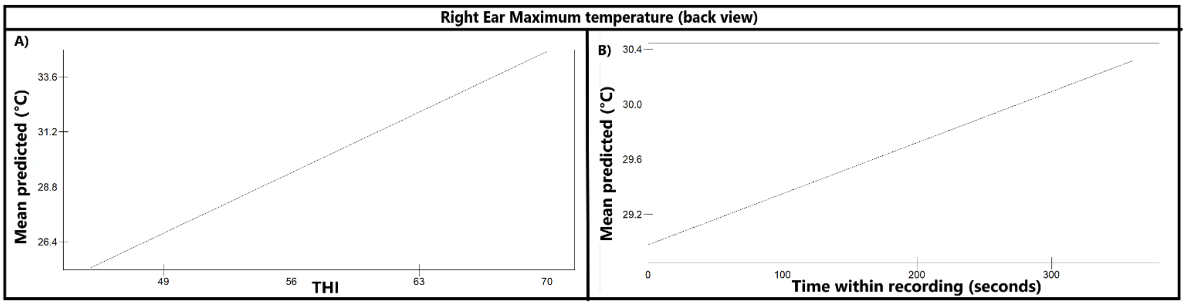


Figure 49. Effect THI (A) and time within recording (B) in the right ear maximum temperature (back view).

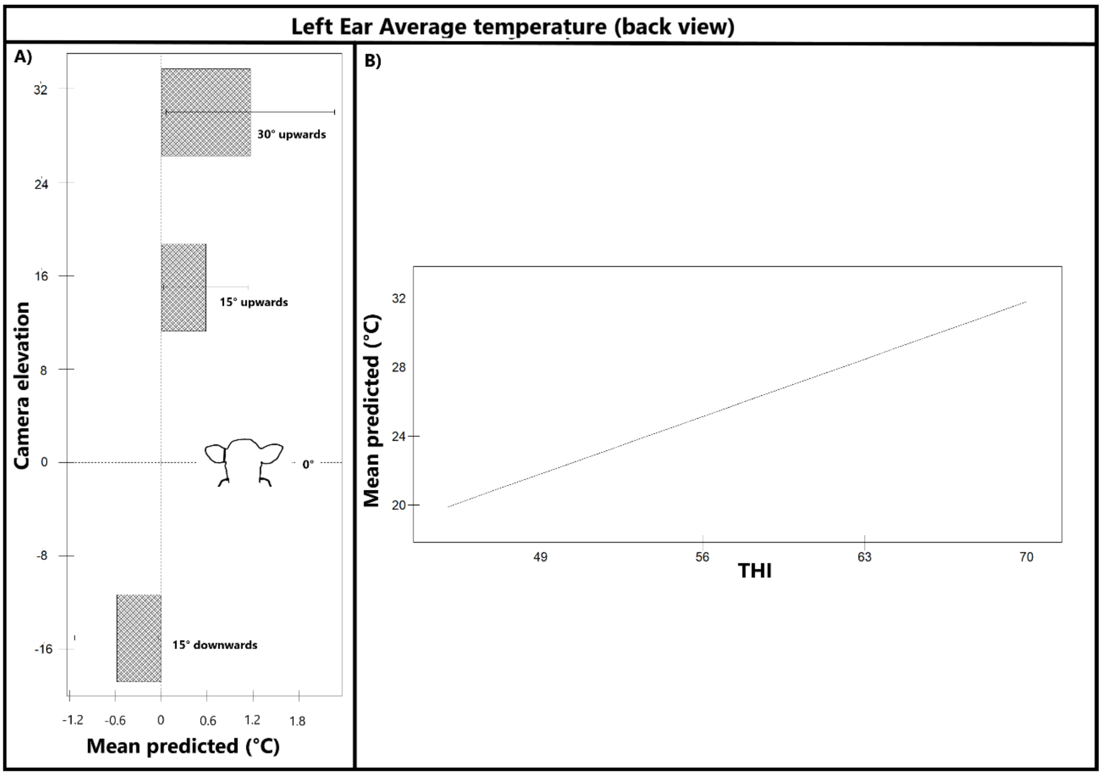


Figure 50. Effect of camera elevation (A) and THI (B) in the left ear average temperature (back view).

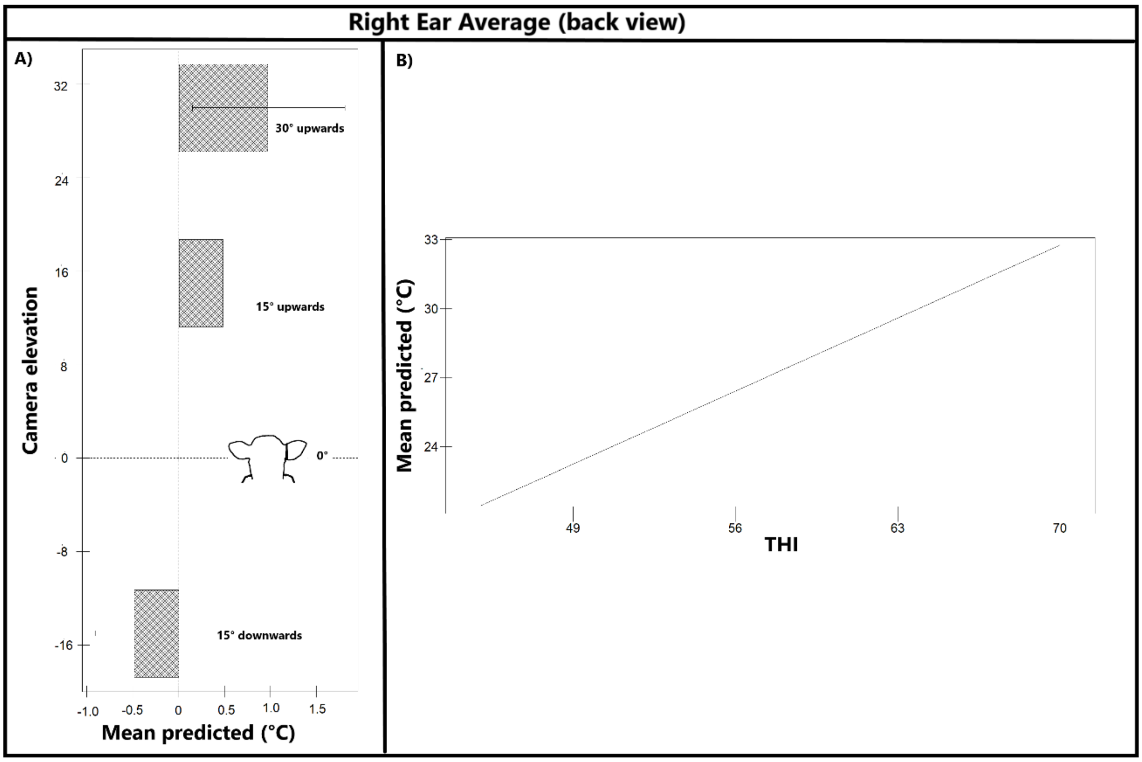


Figure 51. Effect of camera elevation (A) and THI (B) in the right ear average temperature (back view).

5.3.2. Temperature asymmetries (L-R temperatures)

5.3.2.1. Ear (front view)

In a model for asymmetries (L-R) in the maximum ear temperatures, 16.13% of the variability was explained by a quadratic relationship with THI (X^2=7.00, df=2, p=0.030) and linear relationships with camera elevation (X^2=11.53, df=1, p<0.001) and camera angle (X^2=48.74, df=1, p<0.001; Figure 52; Table 44). In a model for asymmetries (L-R) in the average ear temperatures, 15.84% of the variability was explained by a quadratic relationship with camera angle (X^2=72.90, df=2, p<0.001) and linear relationships with camera elevation (X^2=8.45, df=, p<0.001) and THI (X^2=4.77, df=1, p=0.029; Figure 53; Table 44).

A reduction in the asymmetries in average (linear relationship) and maximum (quadratic relationship) temperatures towards the right ear was observed as THI values increased. The angle from which the image was collected influenced the direction of asymmetries in average (linear relationship) and maximum (quadratic relationship) temperatures of the ear; as the angle increased towards one side, the asymmetries shifted towards that side. Asymmetries increased towards the right side when images were collected from an upward position and towards the left side when collected from a downward position.

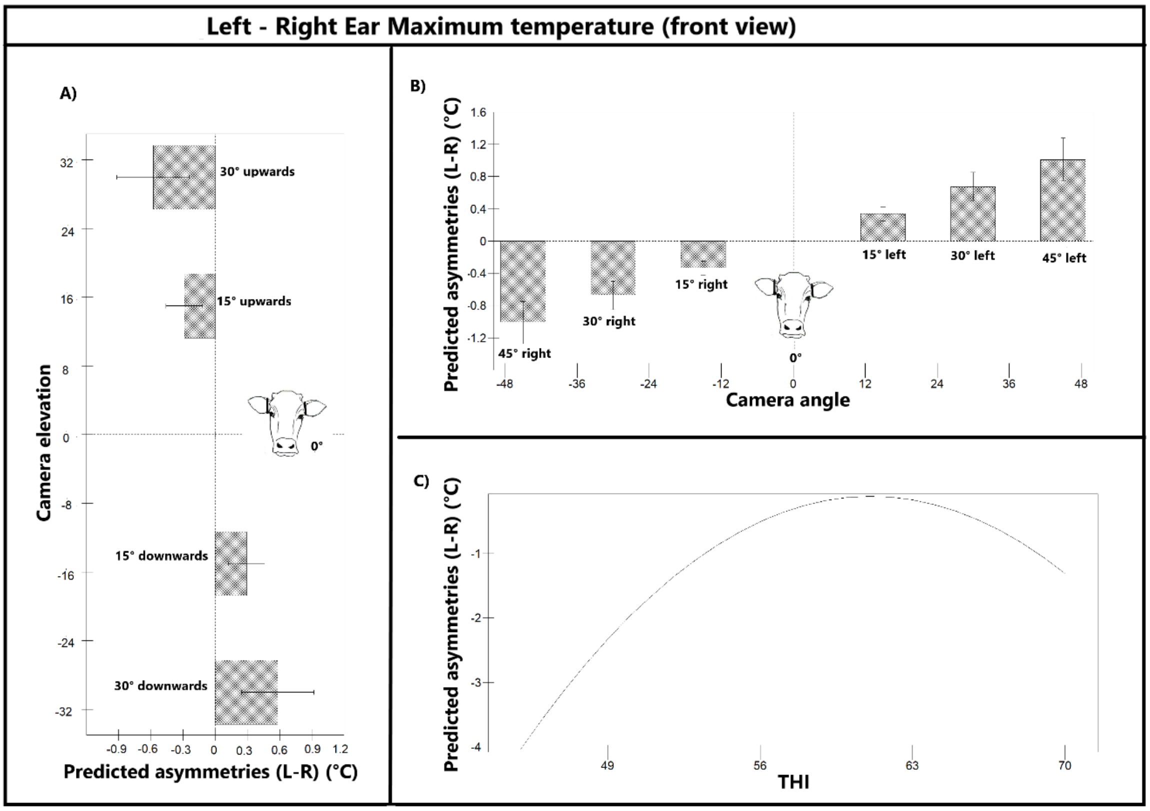


Figure 52. Effect of camera elevation (A), camera angle (B) and THI (C) in L-R asymmetries in ear maximum temperatures (front view).

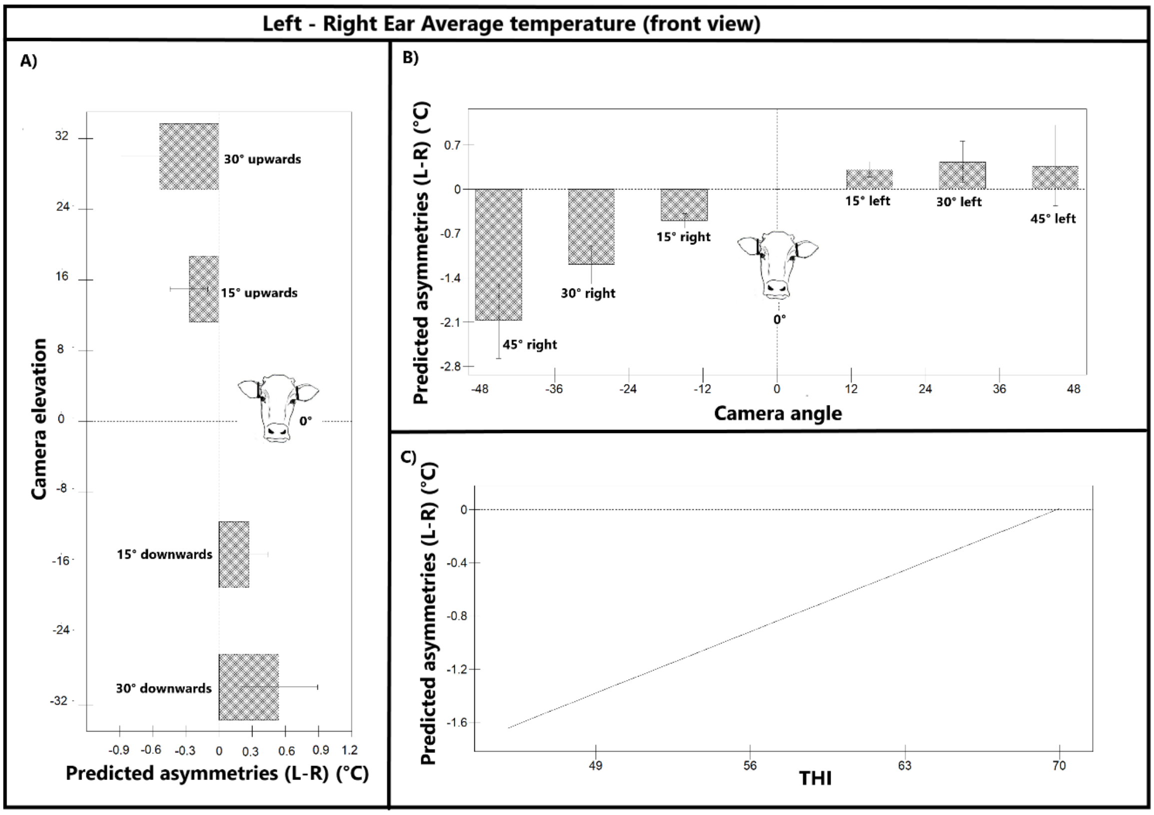


Figure 53. Effect of camera elevation (A), camera angle (B) and THI (C) in L-R asymmetries in ear average temperatures (front view).

5.3.2.2. Nostrils

In a model for asymmetries in nostril maximum temperatures (L-R), 1.54% of the variability was explained by a cubic relationship with camera elevation (X^2=16.592, df=3, p<0.001) and a linear relationship with camera angle (X^2=21.799, df=1, p<0.001; Figure 54; Table 45). In a model for asymmetries in nostril average temperatures (L-R), 6.67% of the variability was explained by a cubic relationship with camera angle (X^2=134.85, df=3, p<0.001) and a linear relationship with camera elevation (regardless of direction) (X^2=5.868, df=1, p=0.015; Figure 55; Table 45).

Asymmetries in average and maximum temperatures were influenced by the angle and direction from which the image was taken, resulting in increased asymmetries towards the left nostril when the image was taken from the left side of the subject and towards the right nostril when taken from the right. Asymmetries on the nostril average temperatures increased towards the left side as a result of a change in camera elevation (regardless of if it was upwards or downwards), whilst asymmetries on the maximum temperatures had a cubic relationship with the elevation and direction of the camera.

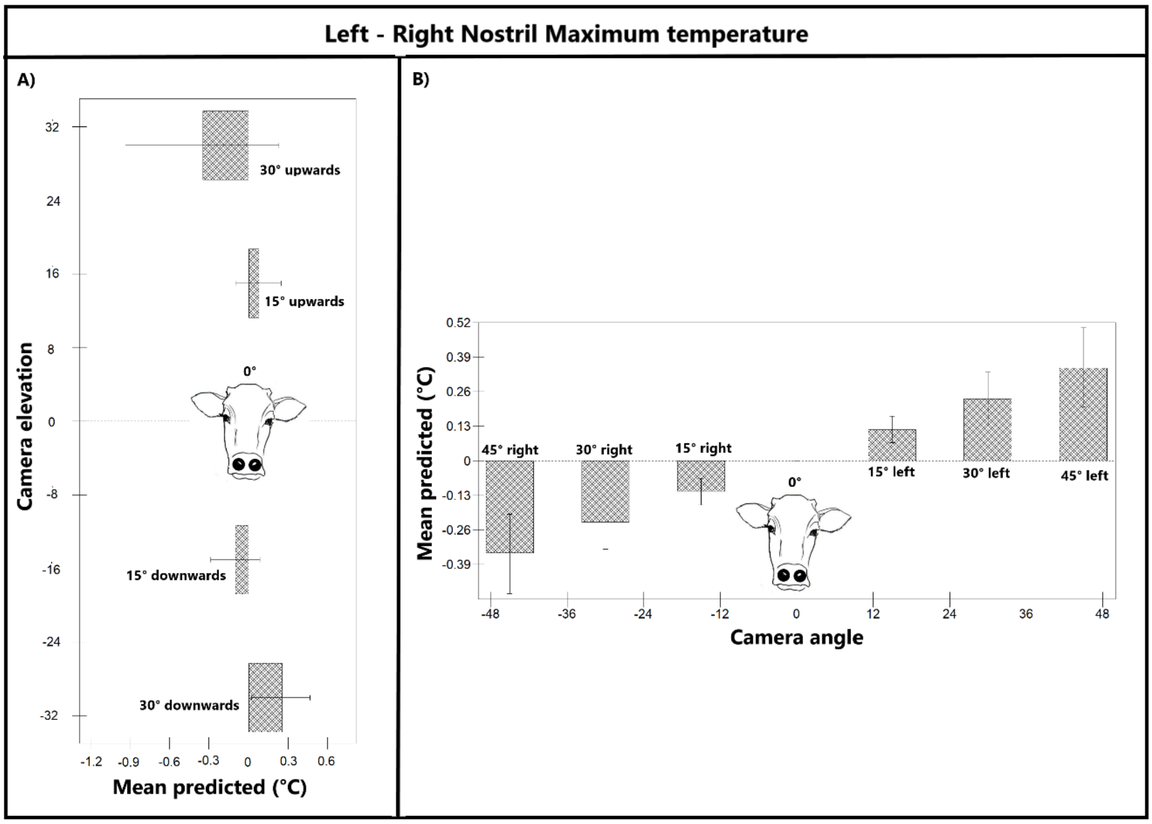


Figure 54. Effect of camera elevation (A) and camera angle (B) in L-R asymmetries in nostril maximum temperatures.

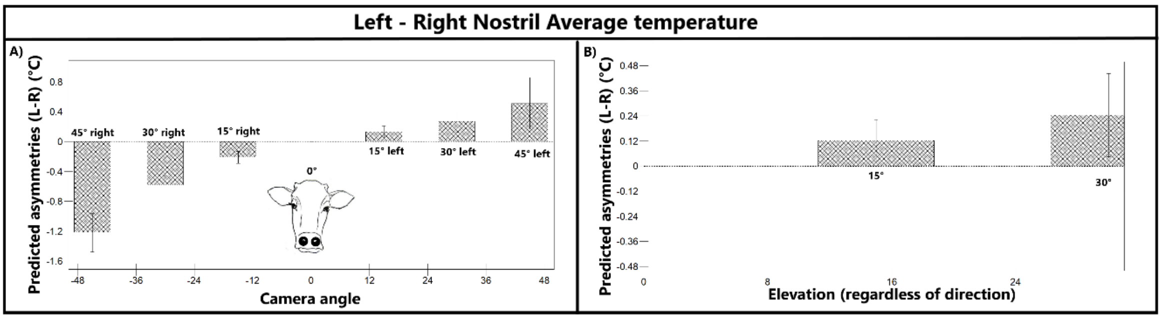


Figure 55. Effect of camera angle (A) and camera elevation (B) in L-R asymmetries in nostril average temperatures.

5.3.2.3. Nasal airways

In a model for asymmetries in nasal airway maximum temperatures (L-R), 6.75% of the variability was explained by linear relationships with camera angle (X^2=59.68, df=1, p<0.001), camera elevation (X^2=4.76, df=1, p=0.029) and time within recording (X^2=8.17, df=1, p=0.004; Figure 56; Table 46). In a model for asymmetries in nasal airway average temperatures (L-R), 2.4% of the variability was explained by a cubic relationship with camera angle (X^2=82.18, df=3, p<0.001), and linear relationships with time within recording (X^2=31.72, df=1, p<0.001) and camera elevation (X^2=14.97, df=1, p=0.001; Figure 57; Table 46).

Asymmetries in maximum and average temperatures of the nasal airways increased towards the side the camera angle shifted. Similarly, asymmetries in the nasal airway temperatures were related positively with camera elevation, shifting towards the right nasal airway as the camera moved upwards and towards the left one as it moved downwards from the perpendicular position (0°). Finally, temperature asymmetries shifted towards the left nasal airway as the time within the recording elapsed.

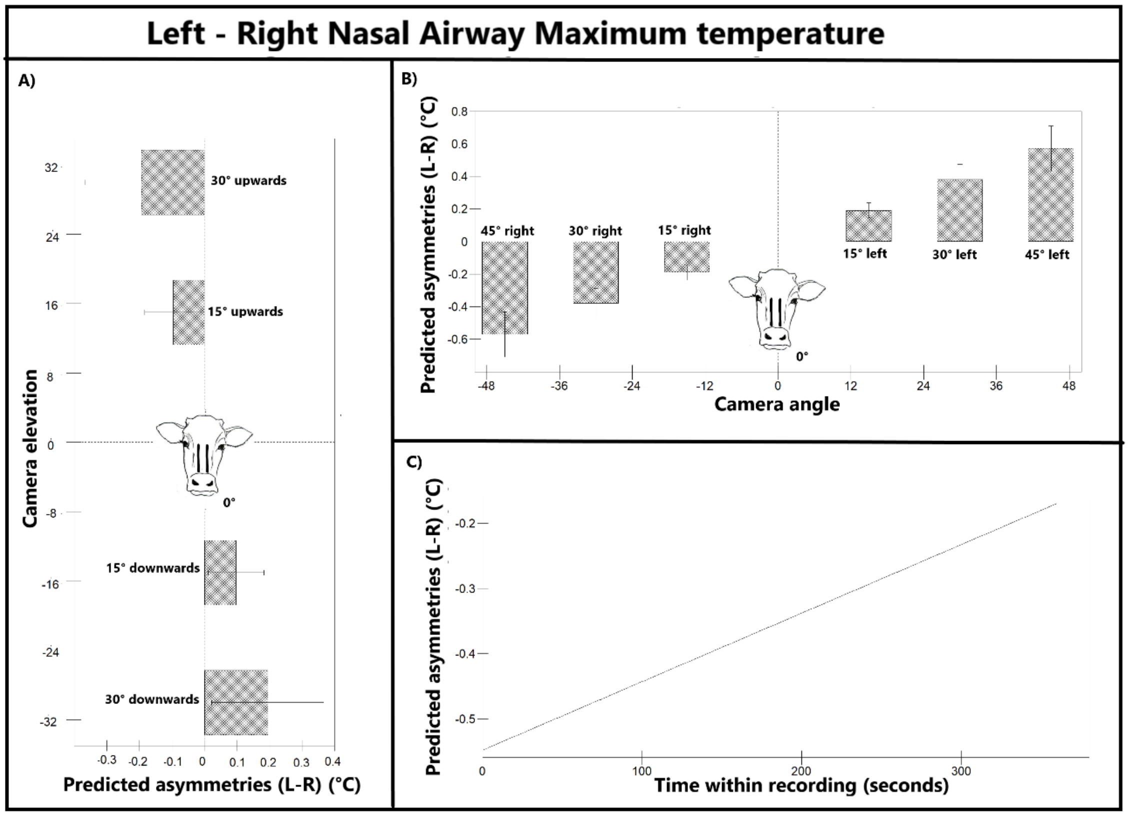


Figure 56. Effect of camera elevation (A), camera angle (B) and time within recording (C) in L-R asymmetries in nasal airway maximum temperatures.

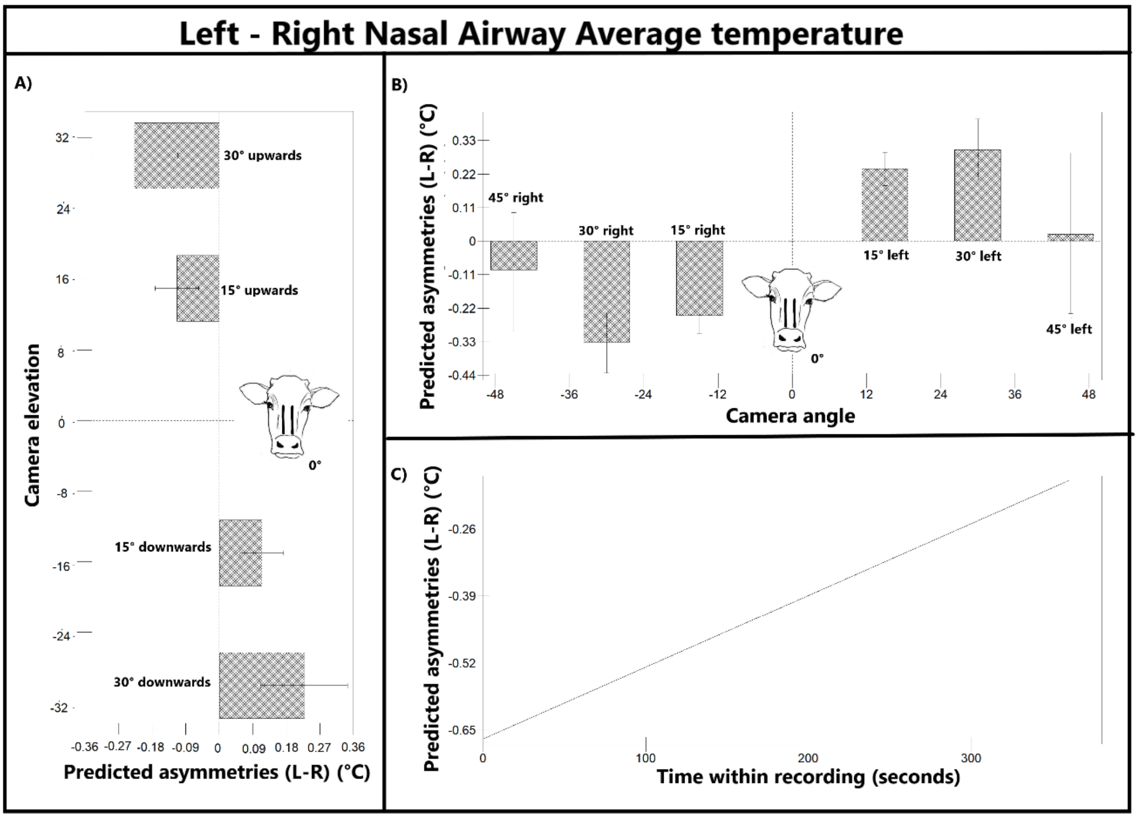


Figure 57. Effect of camera elevation (A), camera angle (B) and time within recording (C) in L-R asymmetries in nasal airway maximum temperatures.

5.3.2.4. Eye

In a model for asymmetries in the inner corner of the eye temperatures, 1.60% of the variability was explained by linear relationships with the time within the video from which the left image was collected (X^2=6.76, df=1, p=0.009) and with the camera angle (X^2=6.43, df=1, p=0.011; Figure 58; Table 47). In a model for asymmetries in eyeball temperatures, 2.39% of the variability was explained by linear relationships with the time within the recording from which the left image was collected (X^2=13.34, df=1, p<0.001) and the time within the recording from which the right image was collected (X^2=8.61, df=1, p=0.003; Figure 59; Table 48).

Inner corner asymmetries were influenced by the angle and direction from which the images were taken, shifting towards the right side when the angle increased towards the front of the head and towards the left side when the angle increased towards the back of the head. Asymmetries in the inner corner of the eye and eyeball shifted towards the right side as the time within the recording in which the left image was selected elapsed, whereas an increase of the time elapsed for the selection of the right image shifted asymmetries in the eyeball towards the left side.

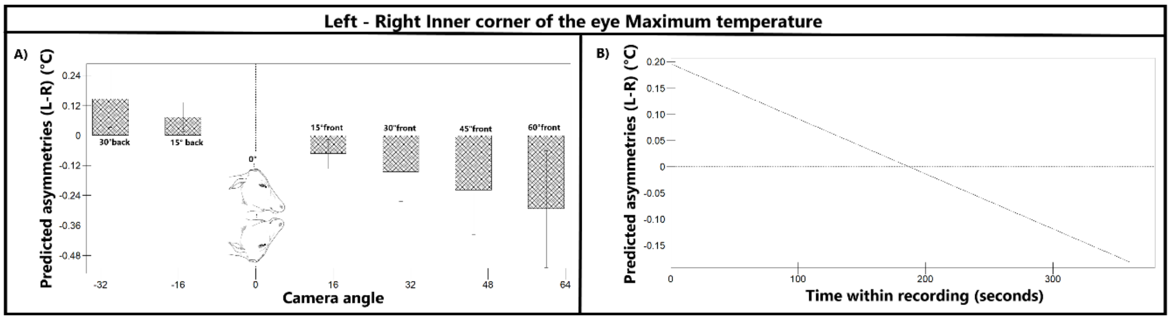


Figure 58. Effect of camera angle (A) and time within recording (B) in L-R asymmetries in the inner Corner maximum temperatures.

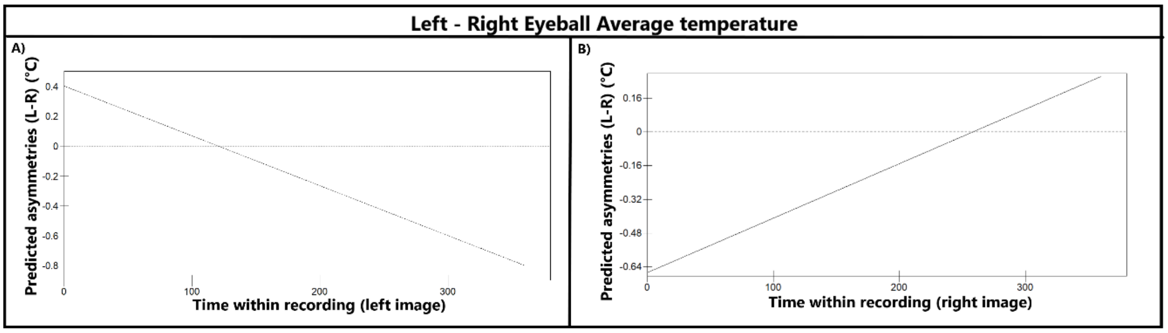


Figure 59. Effect of time within recording of the extraction of the left image (A) and time within recording of the extraction of the right image (B) in L-R asymmetries in the eyeball average temperatures.

5.3.2.5. Eye surroundings

In a model for temperature asymmetries in the rostral eye surrounding area, 3.37% of the variability was explained by a quadratic relationship with camera angle (X^2=18.07, df=2, p<0.001; Figure 60; Table 49). In a model for temperature asymmetries in the caudal eye surrounding area, 18.52% of the variability was explained by quadratic relationships with calves age (X^2=11.77, df=2, p=0.003) and time within the recording from which the right image was collected (X^2=26.74, df=2, p<0.001), and a linear relationship with camera angle (X^2=32.73, df=1, p<0.001; Figure 61; Table 50).

The angle and direction from which the image was taken influenced the asymmetries in rostral and caudal eye surrounding areas. As the angle increases towards the back, the asymmetries increased towards the left side, while they increased towards the right eye when the angle increased towards the front of the head.

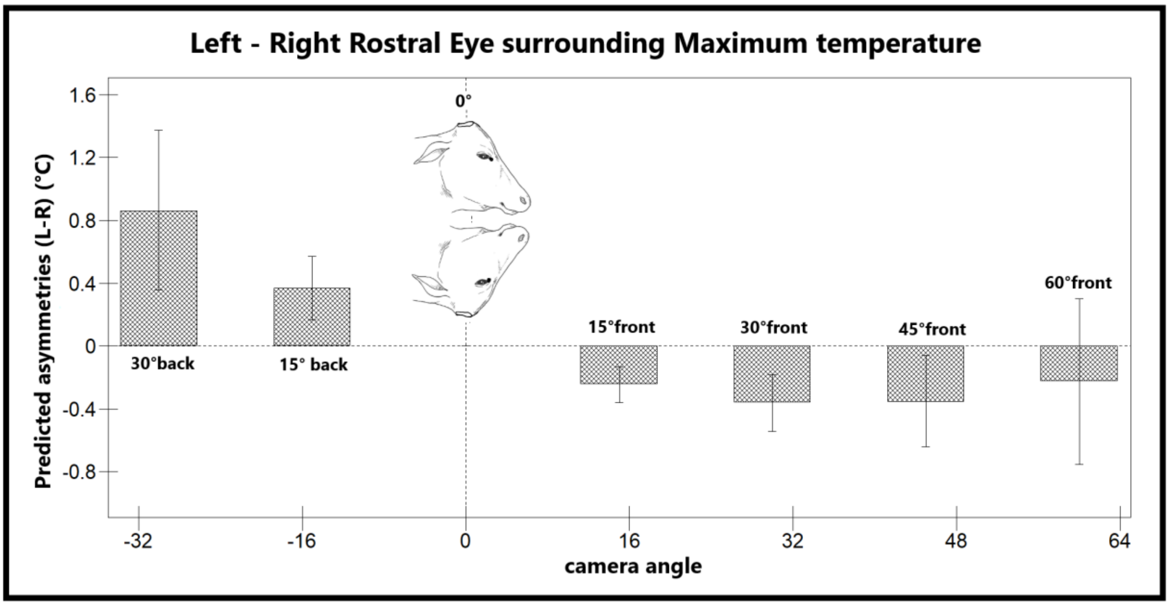


Figure 60. Effect of camera angle in L-R asymmetries in rostral eye surrounding maximum temperatures.

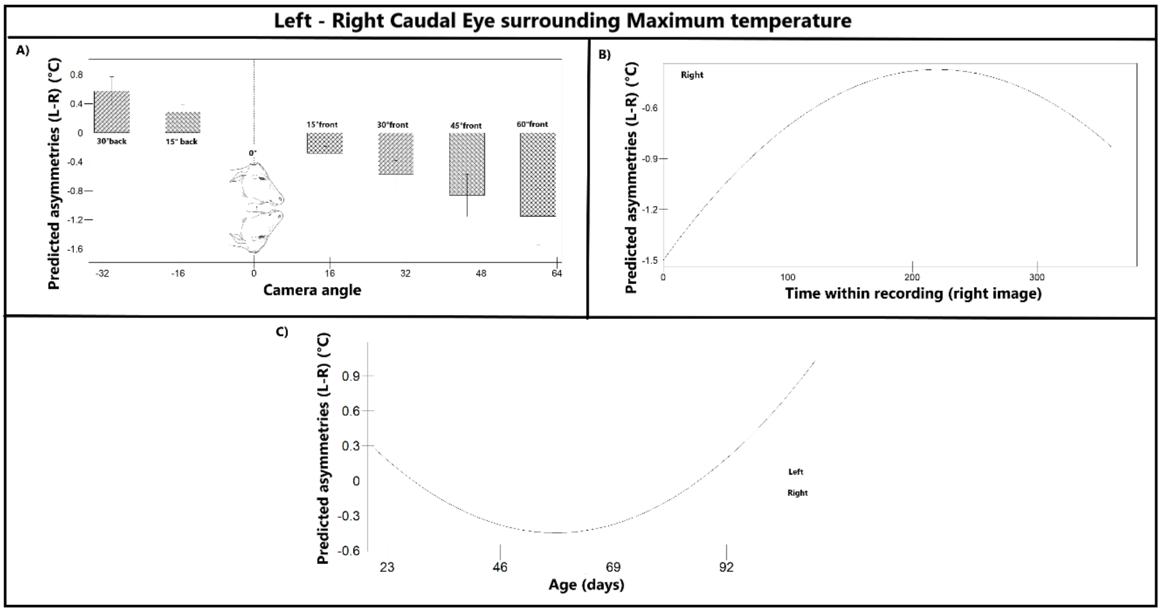


Figure 61. Effect of camera angle (A), time within recording of the extraction of the right image (B) and age of the calves (C) in L-R asymmetries in caudal eye surrounding maximum temperatures.

5.3.2.6. Ears (back view)

Neither asymmetries in maximum nor average temperatures on the ears measured from the back images were influenced significantly by any of the measured parameters.

5.4. Discussion

This study aimed to identify the effect of selected environmental and recording factors in raw temperature measurements and temperature asymmetries in different ROIs and to identify which ROIs were less influenced by variation in the angles between the camera lens and the surface measured and which ambient conditions yielded the least variability in the temperature measurements, therefore providing more reliable results.

As hypothesised, single/raw temperatures from the different ROIs were influenced widely by the factors analysed. However, fewer parameters affected temperature asymmetries (L-R temperatures) in bilateral areas because left and right ROIs were affected in similar ways; for example, 77.9% of the total variance in the average temperatures of the left nasal airway was explained by the time within the recording, THI values, camera angle, camera elevation and age of the calves. Similarly, 74.42% of the total variance in the average temperature of the right nasal airway was explained by the time within recording, THI values, camera elevation and age of the calves. However, asymmetries in average temperature (L-R) in the nasal airway area were not influenced by the THI values nor the age of the calves as the temperature in both sides was influenced similarly by those factors; moreover, only 2.4% of the total variance was explained by the remaining factors (camera elevation, camera angle and time within recording). Results suggesting that measuring temperature asymmetries might be less sensitive to factors like humidity, temperature, time within recording and elevation of the camera, and thus applicable in broader settings. For example, in this study, the variation calculated for nostril maximum temperatures that was attributed to a change in THI of 10 was around 3.5°C for the left nostril and 3.8°C for the right one. Similarly, the variation in nostril maximum temperatures in images collected at 80 seconds and 240 seconds within the recording was around 0.24°C for the left side and 0.18°C for the right side; however, when looking at asymmetries on nostril temperatures, none of the factors discussed above significantly influenced the temperature asymmetries in the area.

5.4.1. Single temperatures

The environmental factors, either THI or ambient temperature, significantly affected all the single temperature measurements. In general, the temperatures of the ROIs increased either in a quadratic or in a linear relationship with these environmental factors, suggesting that higher THI values within the range in our study (49 to 69) resulted in warmer temperatures in the body surface. These results are in line with previous findings by Okada et al. (2013) and Salles et al. (2016), in which a correlation between the forehead and maximum eye temperatures with THI values of around 0.8 and 0.6 were found, respectively. In general, it is considered that THI values under 72 do not represent a thermal challenge as in terms of putting the physiological responses of the individual under physiological pressure to preserve the homeostasis of the individuals; values between 72 and 79 can cause a moderate stress response, values between 80 and 88 elicit moderate stress and values of between 89 and 99 are a cause of severe thermal stress (Armstrong, 1994; Gantner et al., 2011). In our study, the THI values were below 70 and thus are not considered a cause of any heat stress. However, THI values considered to represent the comfort zone in cattle are for adult cows and were calculated based on physiological, reproductive and productive parameters, so it is possible that those ranges might be narrowed if different ages and subtle changes in behaviour are considered.

The ROIs that were influenced less by the ambient parameters were the eyeball and the inner corner of the eye, whereas the most affected were the nasal airways and the ears. For example, the difference between nasal airway average temperatures between 48 and 69 THI was around 12ºC, whilst for the inner corner of the eye was around 1.8ºC. This might be associated with the role of peripheral circulation and respiration as thermoregulatory mechanisms, whereas eye temperatures seem to have a relatively higher correlation with core temperatures. In previous studies with dairy cattle, a correlation between rectal temperatures and maximum muzzle and eye temperatures of an r value 0.48 and 0.58 respectively has been determined (George et al., 2014), which might explain the little variation of temperature in those areas explained by environmental factors and variation in camera angle in the current study.

Bilateral areas from the front images, which were significantly influenced by the angle and direction from which the image was taken, had an increase in temperature towards the side from which the image was taken and a decrease in the opposite side. This was expected because having the ROI turned increasingly towards the camera meant that it was also approaching a parallel position with the camera lens, therefore approaching its true surface temperature value in the thermal readouts. Similarly, turning the other side further away from being parallel to the camera lens meant further increasing the measurement error in the thermal readouts. Muzzle temperature, which was measured at a central location of the muzzle, i.e. it was not a non-bilateral measurement, was influenced by the increased angles regardless of its direction. This was also expected because a muzzle is a slightly curved surface. Therefore, moving away from a parallel position between it and the camera lens causes an increase in measurement error regardless of the direction of movement.

The areas least influenced by the angle of the camera were the muzzle, the eyeball, the nasal airways, and the inner corners of the eyes, whilst the most influenced were the areas surrounding the eye and the ears. For example, maximum temperatures of the inner corner of the eye were on average 0.4 and 0.5 ^{o}C higher in the left and right sides, respectively when taken 30 ^{o} towards the front, compared to images taken perpendicularly to the eye (0^{o}), whereas temperatures in caudal eye surroundings with the same camera angle (30^{o}) were on average 1.4 and 1.2^{o}C lower in the left and right sides respectively in comparison to images taken perpendicular to the eye.

As the elevation of the camera increased upwards or downwards, the average temperatures of the nostrils, the maximum and average nasal airway temperatures and the hair whorl maximum temperatures decreased; which is expected as a deviation from a perpendicular position is expected to reduce the temperature readings of the thermal camera (Church et al., 2014; Fernández-Cuevas et al., 2015; Ijichi et al., 2019). However, the temperature of other ROIs increased if the elevation moved upwards and decreased if moved downwards, e.g. right ear max, right nasal airway max, right nasal airway average, muzzle max, and the average temperature of both ears (back image), with an opposite effect on the left ear average and right nostril maximum temperatures. These differences cannot be directly explained by the physics of infrared radiation, but they might be associated with physiological responses of the calves in response to affective. For example, asymmetries hypothesised to indicate a right hemisphere dominance increased as the elevation of the camera moved upwards, which might reflect a negative affective response towards a threatening stimulus (researcher or object at a position over the head) (Smith, 1998; Forkman et al., 2007). However, the effect of elevation on the temperatures was lower than the one from the angle; for example, the highest variation associated with elevation was for Hair whorl maximum, in which an elevation of 30^{o} upwards reduced the temperature by 1.3^{o}C.

Most of the areas in which time influenced the temperatures showed a quadratic relation characterised by an increase of temperature between the first 3 to 4 minutes of the recording, followed by a gradual reduction in temperature. This might indicate an emotional arousal response, in which a peripherical change in temperature results from a change in the balance between sympathetic and parasympathetic activity.

The age of the calf was related to an increase in the average temperature of the nasal airways and maximum temperature of the caudal eye surrounding and a quadratic relationship with the average temperature of the left ear. The most significant difference was 3.04 and 3.36 ^{o}C in the left and right nasal airways, respectively, between calves aged 20 and 100 days. Those differences might again reflect a change in affective states rather than an age-related physiological change; as older calves had gone through more negative experiences with human handlers than the young ones, i.e. hot-iron disbudding and modifications towards a solid diet.

5.4.2. Temperature asymmetries

Even though environmental factors, like temperature and THI, were significant in all the ROIs analysed; when asymmetries were analysed, only asymmetries in the ear temperatures from the front view were significantly influenced by the THI; as the THI increased, asymmetries shifted from the right to the left side. A possible explanation might be that the lower temperatures were below the optimal temperature range of calves of that age, causing discomfort that could have elicited increased activity in the right brain hemisphere, with the increased blood flow on that side speculatively causing heat convection through tissues to the surface of the base of the ear on that side. The lack of significance in all the other ROIs was mainly because the effect observed in the paired ROIs was very similar for the left and right sides.

The angle and direction from which the image was taken influenced significantly the asymmetries in ears (front view), nostrils, nasal airways, inner corners, rostral and caudal eye surroundings. In bilateral ROIs extracted from the front view (ears, nostrils and nasal airways) the asymmetries shifted towards the side that the angle increased, e.g. as the angle increased towards the left side, the asymmetries shifted towards the left ear, nostril and nasal airway and vice versa. Increased elevation of the thermal camera resulted in an asymmetry towards the left side in the nostril and on the right side of nasal airways, potentially suggesting a complex interplay of the left and right hemispheres in a situation that could have been perceived as an ambiguous potential threat by the calf, but this is very speculative.

In the case of the eyeball, inner corner and surrounding areas, which were extracted from the side views (left and right), it was not expected to find an effect of angle in temperature asymmetries, as images were paired sharing the same angle, e.g. images taken from the left side at 30º towards the back were paired with images from the right side that were collected at 30º towards the back. However, the eyeball was the only area not influenced by the angle of the camera; this might be associated with the shape of the eye, as it is not expected that the angle between the camera and a spheric object influences its temperature.

Inner corner, rostral eye surrounding, and caudal eye surrounding asymmetries increased towards the left side when the angle was towards the back of the calf and shifted towards the right side when the angle increased towards the front. One possible explanation might be associated with the emotional valence theory of brain lateralisation, which states that positive emotions are processed more intensely in the left-brain hemisphere and the negative ones in the right hemisphere. In two of our previous studies (chapter 3 and 4), I used infrared thermography to detect temperature asymmetries associated with changes in affective valence; in those studies, putative negative states were elicited by disbudding dairy calves with a hot-iron and by separating calves from their mothers (as a routine husbandry practice in the farm). Some of the results suggested that asymmetries in the eyes might indicate brain processing from the contralateral hemisphere, e.g. asymmetries towards the left side associated with the processing of negative states by the right hemisphere and vice versa. This hypothesis might explain why asymmetries between ocular and periocular areas were found. It is possible that asymmetries towards the left eye/right hemisphere observed in the images taken from the back of the calves were collected when the animals were looking or moving away from the experimenter, which could be considered a negative response to the situation; whilst asymmetries increased towards the right side/left hemisphere, as the angle from which images were collected increased towards the front of the calves.

Elevation and direction from the camera were not expected to influence temperature asymmetries as a direct effect of the camera position. However, asymmetries in the ears (front view), nostrils and nasal airways showed that as the elevation of the camera increased upwards, the asymmetries shifted towards the right side, whilst images collected from a downwards position showed asymmetries towards the left side. Those asymmetries might be associated with previous findings suggesting correlations between emotional valence and functional lateralisation of the brain. In our previous studies, I have hypothesised that asymmetries in the nasal and auricular areas might reflect the emotional processing of the ipsilateral brain hemisphere. This assumption is based on the speculative mechanism, not yet tested directly, of direct heat convection taking place from the brain hemisphere with increased blood flow, and therefore elevated temperature, through the tissues in the head to the surface in areas where there are openings in the skull for the ears and the eyes, i.e. no layer of bone acting as an insulator hindering heat convection. This could indicate that the asymmetries towards the right hemisphere observed in the images taken from an upwards position were influenced by the emotional state of the animals, as they may perceive the experimenter/ camera in a negative or threatening way when they were above their head level.

As time within the recording goes within 360 seconds, the asymmetries of the nasal airways shifted to the left side up to 0.4 and 0.5 for the maximum and average temperatures, respectively. The ocular and periocular area were influenced by the time within the recording, but as the left and right images were collected at different times, the effect of time was analysed for the left and right images separately. As the time within the video elapsed in which the left or right image were collected, an increase of asymmetries towards the eyeball on the opposite side was found. For asymmetries in the inner corner of the eye, the time within the recording to extract the left image had a cubic relationship with a maximum variation of around +0.14ºC towards the left and +0.30ºC towards the right side. Caudal eye surrounding temperature had a quadratic relationship with the time in which the right image was extracted; the maximum variation was around +0.9ºC towards the right eye. The variation towards the opposite side for eyeball asymmetries could be explained by the reduction of temperature of both eyeballs as time elapsed, suggesting that it is possible to reduce this effect by selecting images from both sides that are closer in time. In the case of asymmetries in the inner corner, it might suggest a lateralised affective response, as discussed in the previous chapter, in which asymmetries towards the left eye/right hemisphere were found at the beginning of the recording and towards the right eye/left hemisphere at the end of the recording.

The age of the calves was only significant for asymmetries in caudal eye surrounding with a quadratic relationship showing increased asymmetries towards the right eye in calves aged from 20 days to 60 days, and then an increase towards the left eye up to 100 days of age. However, there is not a clear explanation for these patterns. One possibility is that older calves might show asymmetries towards the left eye /right brain hemisphere in association with an emotional response. In our study, calves older than 60 days of age were the only ones that had experienced hot-iron disbudding, and at 80 to 90 days old, they were already weaned, which are considered to induce negative affective states.

5.5. Conclusions

This study evidences the effect of different ambient and recording factors in individual and asymmetric temperatures of different ROIs. According to our models, the proportion of variance explained by these factors is high for the individual measurements of the ROIs compared to the variance explained for temperature asymmetries that were very low. This might have a large effect when looking at unilateral ROIs used to measure arousal, like the muzzle and hair whorl areas. Up to date, there are only a few studies looking at temperature asymmetries, and in those studies, the differences observed between groups and/or situations are very small, which makes it essential to consider the effect of the environmental and recording factors regardless of its relatively small effect.

Even though ambient parameters, like temperature and relative humidity, can be set in a thermal camera to correct for variations in temperature readings caused by changes in the way the radiation emitted travels in such circumstances; the effect of those factors in the physiological responses of the calves is high and is the main factor that influences individual temperature measurements of all the ROIs in this study. However, when calculating asymmetries, those factors seem to be unimportant as they have similar effects on the bilateral areas of each ROIs.

Recording factors like angle and elevation of the camera seem to be the most influential parameters on the variance observed at the asymmetries in the different ROIs. In general, the effect of the camera angle is associated with a gradual increase in asymmetries towards the side the camera is allocated. However, the effect of angle direction in the ocular and periocular areas, and the elevation of the camera in asymmetries of different ROIs, suggests that the camera/researcher position might affect the calves' temperature readings. It is likely that having an individual or object moving from the back of the animal or above might be negatively perceived by the calves.

The effect of time within the recording influenced only the asymmetries on the nasal airways, in the inner Corner, eyeball and caudal eye surrounding areas. Even though the experimenter entered the pen and habituated the calves on the day before and the day of recording, the effect of time in those ROIs could suggest that the habituation of the calves to the researcher/camera was not long enough to avoid an effect of its presence in the temperature measurements. Moreover, the need to pair two images to calculate the asymmetries of the ocular and periocular area increases the variability effect of time.

To decide within which temperature range or to what extent variation in camera position is permissible to analyse temperature variations in an animal study will widely vary depending on whether single or asymmetric temperatures will be used, which ROIs will be measured and how much variation between groups or situations it is expected to be found.

However, some recommendations can be drawn from this study. Control the ambient factors as much as possible, unless they are part of the experimental design; this can be achieved by carrying out the study in a space with a controlled temperature or humidity, or that does not have direct wind currents or sunlight; if data collection is required to be made on several occasions. It is recommended to avoid seasonal changes and collect the data at similar times. To reduce the effect of angle and elevation on the measurements, it is recommended to avoid collecting data from the back of the animals, try to keep the camera at the calves' head level; select the images with less variation from a perpendicular view of the ROIs, a variation of less than 15º is the ideal for all the ROIs observed; still, it is likely that a range of ± 30º will be acceptable for most ROIs. However, if images from two sides are used to calculate asymmetries in the ocular or periocular area, it is recommended to pair them from a similar position and as close as possible in time between each other. Finally, the presence of the experimenter may influence the presence of temperature asymmetries, so introducing an adequate habituation protocol will be helpful to reduce unwanted temperature variations due to the novelty of the situation.

6. “TEMPERATURE ASYMMETRIES AS INDICATORS OF POSITIVE AND NEGATIVE AFFECTIVE STATES IN HORSES: A REWARD-FRUSTRATION STUDY”

Abstract

As mentioned in the general introduction (Chapter 1), emotions can be classified in terms of valence (positive or negative) and arousal levels (low or high) according to the core affect theory, which might be processed differently in the brain through lateralised processing. This dissertation aims to identify if temperature asymmetries measured by infrared thermography are likely to be influenced by this lateralisation process; in previous chapters (3 and 4), temperature asymmetries in situations considered to elicit negative affective states had been studied, yielding interesting results. Within those results, a difference in the direction of asymmetries in the Inner corner of the eye was found in calves approaching the researcher during thermal recordings (right eye / left hemisphere) in comparison to calves that moved away or stayed still during the recording (left eye / right hemisphere), which according to the hypothesised relationship between hemisphere activity and emotional valence, suggested that calves approaching the researcher were in a positive affective state whilst calves moving away or standing still perceived the researcher more negatively. To explore further these differences in the direction of the asymmetries, a study looking at contrasting positive and negative affective states was performed. Unfortunately, due to COVID-19 restrictions, working with dairy calves was not possible, so an alternative study was conducted with horses. For this purpose, 24 horses were trained to perform a targeting task (touching a Kong or a teddy ring) in exchange for a reward (a piece of carrot), so they could be tested in a reward / non-reward schedule aiming to identify changes in asymmetries by contrasting a high arousal positive emotion (receiving reward/excitement) with a high arousal negative emotion (not receiving reward/frustration). To account for side bias, the side in which the handler stood during the reward and non-reward trials was balanced. Half of the horses (Group 1) received four rewarded trials in session A, followed by two rewarded and two unrewarded trials in session B, whilst Group 2 performed session B in the first instance, followed by session A. The side in which

the handler stood influenced the asymmetries in maximum eye temperature (X^2=62.588, df=1, p<0.001), maximum nostril temperatures (X^2=6.4544, df=1, p=0.011), nasal airway maximum (X^2=6.64, df=1, p=0.009) and average temperatures (X^2=40.6571, df=1, p<0.001), and ear maximum (X^2=8.796, df=1, p=0.003) and average temperatures (X^2=8.822, df=1, p=0.003); whilst not receiving a reward (negative situation) was associated with asymmetries in nasal airway maximum (X^2=15.188, df=2, p<0.001) and average temperatures (X^2=18.3014, df=2, p<0.001) towards the right nasal airway / right hemisphere, suggesting an association between the direction of the asymmetries and the valence of the emotion elicited.

6.1. Introduction

In previous chapters, a relationship between changes in affective states and temperature asymmetries in different regions of interest (ROIs) in the facial areas of dairy calves has been identified, i.e. asymmetries in nostrils, base of the ears, skin covering the nasal airways, ocular and periocular regions in relation to two situations considered to elicit negative affective states: hot-iron disbudding (chapter 3) and separation from the mother in dairy calves (chapter 4). As discussed in the previous chapters, it has been suggested that those temperature asymmetries might be associated with differences in the processing of information in both hemispheres of the brain (“brain lateralisation”) (Levy, 1977; Rogers, 2000; Daselaar et al., 2006; Hervé et al., 2013). In my previous studies looking at dairy calves, the aim of the methodology has been to identify mainly asymmetries that could be associated with negative affective states. However, some interesting results from the reaction of the calves to the camera (Chapter 3) suggested that opposite directions of the asymmetries in the ocular area (inner corner of the eye) can be found when calves approached the researcher/camera (right eye / left hemisphere dominance) in comparison to when they moved away from him or stayed still during the recordings (left eye / right hemisphere dominance). Therefore designing a study that allowed me to identify differences in the asymmetries associated with contrasting positive and negative ‘emotional responses’ was required to test this hypothesis.

The original idea for the current study was to record thermal videos of calves in contrasting situations likely to elicit emotions from a similar arousal level but contrasting in terms of emotional valence. However, due to health and safety measures associated with the COVID-19 pandemic, access to the University farm was restricted for research purposes. For this reason, the opportunity to work with a colleague (Sarah Kappel; SK) doing related work with horses was taken, being aware that the results from this study might not be fully comparable with the ones from the dairy calf studies.

Similarly to what has been done in the previous chapters, the classification of the emotions was used according to the core affect theory (Russell and Barrett, 1999; Panksepp et al., 2002), as this theory is the one allowing us to classify emotions in a bi-directional axis, constituted by the level of arousal ('X' axis) and emotional valence ('Y' axis) (see fig. 63).

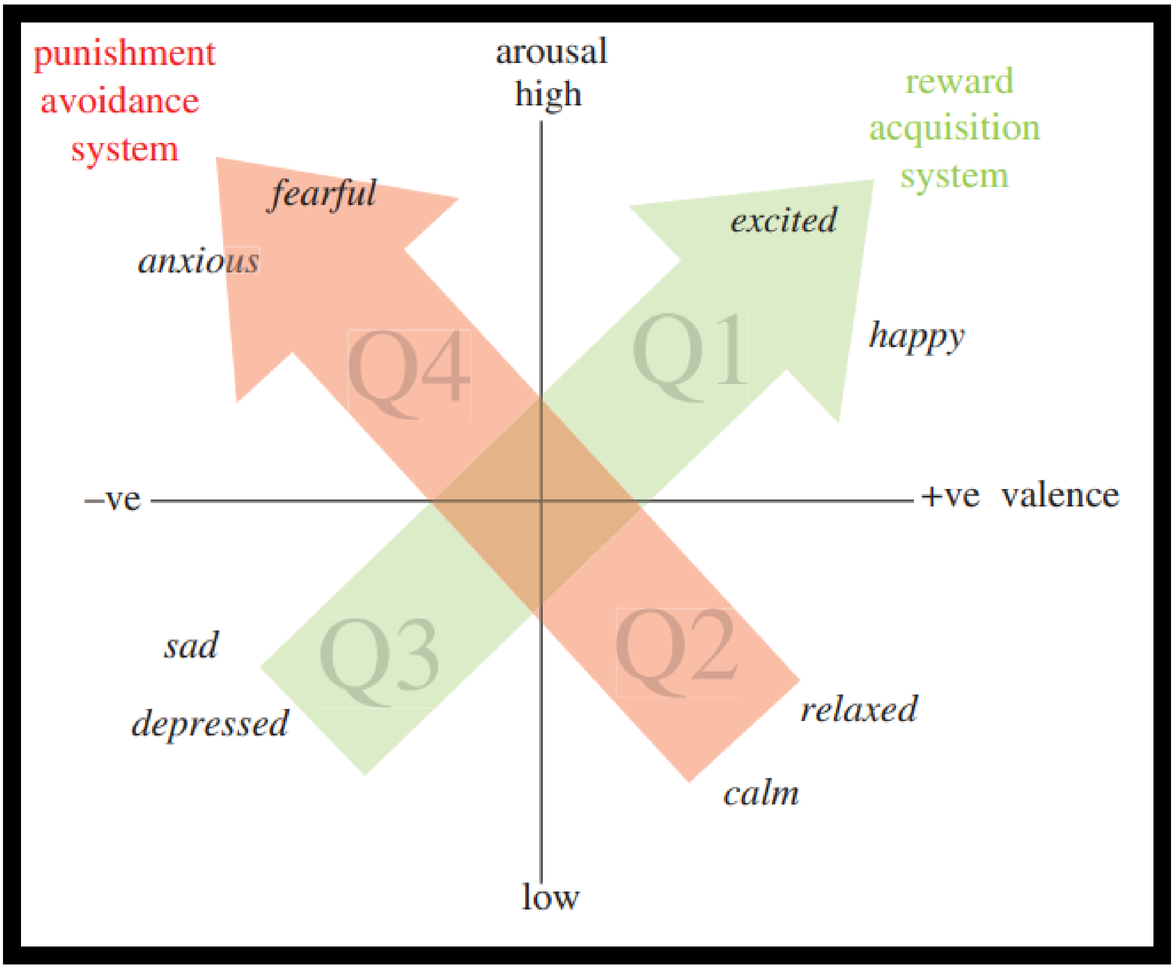


Figure 62. Representation of the two-dimensional space of the Core affect. Q1 and Q2 represent positive affective states with high and low arousal, respectively, and Q3 and Q4 represent negative affective states with high and low arousal. The red arrow represents the punishment avoidance system (from Q2 to Q4), and the green arrow represents the reward acquisition system (from Q3 to Q1). Figure from (2010).

As discussed in the general introduction (Chapter 1) and the experimental chapters (Chapter 3 and 4), predictions in this study are based on the idea that emotions are processed differently in the brain hemispheres through brain lateralisation processes, and therefore it is expected to find temperature asymmetries in different ROIs on the horses' heads by using infrared thermography as a tool. For that purpose, an interpretation of the approach/withdrawal and the emotional valence hypothesis of emotional lateralisation was used; the former suggesting higher processing of emotions resulting in a behavioural approach in the left brain hemisphere and emotions resulting in behavioural withdrawal being mainly processed in the right hemisphere (Davidson et al., 1990; Sobotka et al., 1992a), and the latter hypothesising that positive emotions are mainly processed in the left hemisphere, whereas the negative ones occur mainly in the right hemisphere (Silberman and Weingartner, 1986; Lee et al., 2004).

Most of the work using infrared thermography carried out in animals has focused on non-asymmetrical changes in temperature from a baseline level in specific areas (eyes and muzzle/nose). Changes that have been attributed to differences in the balance between the sympathetic and parasympathetic branches of the ANS. The former mediated by elevated concentrations of noradrenaline in blood, resulting in vasoconstriction at a peripheral level, resulting in a decrease of peripheral temperature and increase in core temperature, and the latter by a decrease in core temperature and an increase in peripheral temperature, mediated by elevated levels of acetylcholine in the circulatory system (Reece et al., 2015). However, those changes in temperature cannot be interpreted reliably in terms of emotional valence but can be considered an accurate indicator of emotional arousal (Merla and Romani, 2007; Ioannou et al., 2014; Ermatinger et al., 2019).

In terms of temperature asymmetries, some ROIs of the face are expected to reflect the activity of the ipsilateral or contralateral hemispheres of the brain depending on the innervation, as differences in local innervation might influence the muscular activity or blood circulation in the ROI; another factor that might influence changes of temperature in certain areas is the proximity of the ROIs with the brain structures. Asymmetries in the ocular area ("inner corner of the eye") are likely to be more influenced by the activity of the contralateral hemisphere as the axons from the optical nerves decussate around 80% in horses (Budras et al., 2007). However as the eye sockets are allocated in close proximity to the brain, it is likely that some heat from the ipsilateral hemisphere might influence the temperature of the eyes. In the case of the nostrils and the skin covering the nasal airways, it has been hypothesised that temperature asymmetries might reflect the activity of the ipsilateral hemisphere, as the dilation and contraction of the nostrils result directly from the innervation of the buccal branch of the facial nerve originating on the same side of the brain (Budras and Berg, 2011; Budras et al., 2012). Finally, as the ear musculature receives innervation from both hemispheres, it is generally considered to have a bilateral innervation, but as significantly more afferent fibres originate from the contralateral hemisphere, it can be considered that asymmetries in the area will be more influenced by the activity of the contralateral hemisphere.

In my previous work with dairy calves looking at putatively negative affective states (chapter 3 and 4), temperature asymmetries hypothesised to be indicative of higher activity of the right hemisphere were found, i.e. Asymmetries in the base of the ears, ocular (inner corner of the eye) and periocular (caudal eye surrounding) areas towards the left side hypothesised reflecting activity of the contralateral hemisphere (right brain hemisphere), and asymmetries in the nostril and skin covering the nasal airway area towards the right side hypothesised to reflect activity of the ipsilateral hemisphere (right brain hemisphere). In those experiments, situations designed to elicit a positive emotion actively were not explored. However, when looking at the response of the calves during the thermal recordings on the disbudding study, calves constantly approaching the researcher, which might be considered as a positive response, had asymmetries towards the right inner corner of the eye (left hemisphere), whereas calves that stayed still or moved away from the researcher had asymmetries towards the left inner corner (right hemisphere), suggesting that the direction of the asymmetries might indeed be associated with the valence of the emotion elicited.

To explore actively if the direction of the asymmetries is associated with the valence of emotions, the present study was developed looking at temperature asymmetries in ROIs in facial areas of horses using infrared thermography. For this purpose, a test aiming to elicit emotions contrasting in valence but with similar arousal levels was designed. Horses trained to perform a targeting task rewarded with a palatable food item were tested in a balanced experiment to elicit an excitement response (high arousal / positive emotion) or a frustration response (high arousal / negative emotion), the former by providing sessions rewarded by the expected item, and the latter by providing unrewarded sessions.

I hypothesised that temperature asymmetries would differ within individuals between the rewarded and unrewarded sessions and that the observed asymmetries would reflect the valence of the affective state elicited; for the rewarded / excitement trials, asymmetries were predicted to indicate higher activity of the left hemisphere, i.e. asymmetries towards the right eye and ear and towards the left nostril and nasal airway, whilst for the unrewarded / frustration trials asymmetries were predicted to indicate higher activity of the right hemisphere, i.e. asymmetries towards the left eye and ear, and towards the right nostril and nasal airway.

6.2. Methods

6.2.1. Animals and treatments / (subjects and husbandry)

Twenty-four horses from various breeds (18 males and six females), between 7 and 22 years old, from two different yards, were used for this study (see table 12). These individuals were used in a study by SK investigating performance in several cognitive and behavioural tests. As part of that study, horses were target trained with either a Kong or a toy (teddy ring) and all horses were used in the current study within seven days of completing SK's study.

variable	Categories	N=
sex	males	16
	females	8
yard	X	8
	Y	16
age	7 years old	4
	9 years old	4
	10 years old	2
	11 years old	1
	12 years old	1
	13 years old	4
	14 years old	3
	15 years old	1
	17 years old	1
	19 years old	1
	20 years old	1
	22 years old	1

Table 13. Summary of the participant details

For this study, data were collected in three different situations: Baseline, session 'A', and session 'B'. The baseline session involved recording a 2-minute thermal video with the horse still in a standing position and the handler standing on either side of the horse (half of the horses were recorded during the baseline with the handler standing on the left side and the other half on the right side), this aiming to control for side bias as the results in dairy calves discussed in chapter 5 suggest this factor as a potential influence in temperature asymmetries. Session "A" consisted of 4 rewarded trials, and session "B" consisted of 2 rewarded trials followed by two unrewarded trials. Rewarded trials lasted 1 minute ± 15 seconds in which the object (either a Kong or a teddy ring) was presented in front of the horse ten times, and a food reward (piece of carrot) provided by the handler after each successful touch. Unrewarded trials had a similar duration (1 minute ± 15 seconds) in which the object was presented ten times, but no food reward was provided after the horse contacted it during the duration of the trial; the side in which the handler stood was switched between each trial to maintain this factor balanced during all the study. All horses were kept individually, and all the recordings were performed at their stall. However, due to the yards' design, some individuals had conspecifics in the adjacent stall.

Horses were randomly allocated in two equal sized groups (12 horses each); group "1" received session "A" followed by session "B", whilst group "2" received session "B" first, followed by session "A", the gap between trials was 2 minutes, whilst a break of 10 minutes was given in between sessions. (see fig. 64).

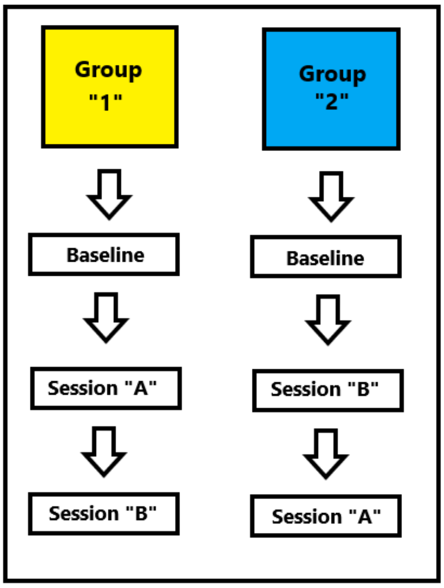


Figure 63. Session differences between groups

6.2.2. Thermal video data

The researchers stayed outside the horse's stable for 10 minutes prior to the baseline recording to habituate the individuals to their presence. Once the habituation period finalised, the handler (SK) entered the stable, placed the halter on the horse's head and stood to one side of the horse whilst the second researcher (MR) started the baseline thermal recording (between 30 and 60 seconds from the halter fitting) lasting 2 minutes ± 20 seconds.

The first rewarded trial (Session A for group 1 or Session B for group 2) started immediately (within 30 seconds) after finishing the baseline recording and lasted 1 minute ± 15 seconds; from there, a 2-minute break between recordings was left between each of the subsequent three trials. After finishing the trials from the first session, a 10 minute break was left before starting the next session (Session B for group 1 of Session A for group 2) with a 2 minute break between the following three trials. During each break, the researchers left the horse stable and moved out of sight from the horse before entering the stable again after each break.

In total, nine thermal videos were recorded per horse (1 baseline and 4 for each session). Videos were recorded using a FLIR T660TM camera (FLIR Systems, Inc., USA), with a resolution of 640 x 480 pixels, 60 FPS, sensitivity 0.02 ^{o}C, and accuracy ± 1%; skin emissivity was fixed at 0.98 as suggested by previous studies (Westermann et al., 2013; Tattersall, 2016; Soroko and Howell, 2018), and environmental factors (temperature and humidity) were updated on the camera before the baseline recording and at the beginning of each session. Each horse was recorded from outside the stable at a distance of 3 ± ½ meters. In contrast to the methodology from previous studies (chapter 3, 4 and 5), horses were only recorded from a front view, as it was not viable for safety and practical reasons to move around the horse while recording the videos.

6.2.3. <u>Selection of thermal images for analysis</u>

Each video was observed using the FLIR Tools v.6.4.18039.1003 software (FlirSystems Inc, Oregon, USA), and images/frames with a sidewise variation of less than 30^{o} between the camera and the horse nostrils were preselected. Following the image pre-selection, only the three best images from each video in terms of symmetry between bilateral areas (judged by eye) according to the criteria mentioned in the general methodology chapter (Chapter 2.2), that were at least 5 seconds apart were selected for analysis, giving a total of 27 images per horse.

6.2.4. Measurement of the Regions of Interest (ROI)

A modification to the method used to delimitate the ROIs stated in the general methods chapter (Chapter 2.3) was made to allow measurement of the maximum temperature of the inner corner of the eyes from the front-facing images (see Figure 62) as the methodology used in this experiment did not record videos from different views as in the previous chapters (chapters 3 to 5).

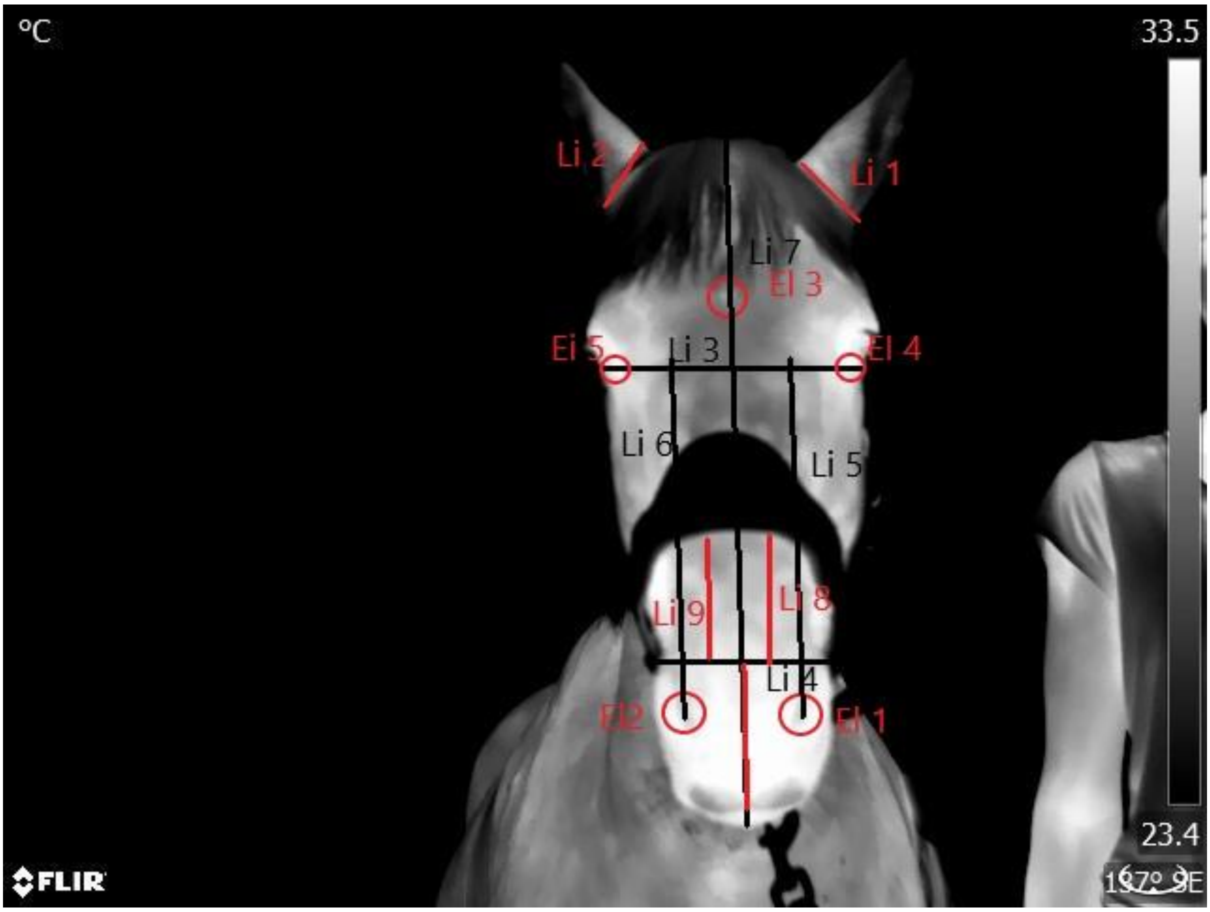


Figure 64. Delimitation of the ear (Li1 & Li2), nostrils (El1 & El2), eye (El4 & El5), nasal passages (Li8 & Li9), hair whorl (El3) and muzzle areas (Li10) from an image taken from the front of a horse.

6.2.5. Statistical Analysis

To analyse temperature asymmetries in the different ROIs, temperature asymmetries of the maximum eye temperature and average temperatures of the base of the ears, nostrils and skin covering the nasal airways were calculated as the differences between left and right side temperatures (Left-Right); positive values indicating higher temperature on the left side and negative values indicating higher temperature on the right one. In addition, raw data from the maximum temperature of the hair whorl and the maximum and average temperatures of the muzzle area were used.

Multilevel models for repeated measures were carried out with the software MLwiN v3.00 (http://www.bristol.ac.uk/cmm/software/mlwin/), using a random effect structure nesting images (repeated measurements) within horses. A stepwise regression method looked at effects of the following variables on temperature measures: horse age (years), sex (male or female), horse yard ('X' or 'Y'), group ('A' or 'B'), trial (baseline, rewarded or unrewarded), session (baseline, 1 or 2), side on which the handler was standing (eft or right) time within recording from which the image was collected (seconds) and THI (Temperature-Humidity Index) as calculated in chapter 5.2.5.

Following the methodology explained in chapter 2, final models for each ROI, an initial association between the temperature asymmetries or the raw temperature (muzzle and hair whorl areas) and the individual variables was made; the predictor variable whose addition resulted in a better improvement of the model, based on p-values calculated from likelihood-ratio tests between the model without variables and the models with each variable incorporated independently (taking into account the degrees of freedom) was added to the model. Once the variable leading to the best improvement of the model was found, it was left in the model, and the rest of the variables were tested for improvement and added to the model. This process was repeated until none of the variables resulted in a significant improvement of the model.

To control for multiple hypotheses, the Benjamin-Hochberg procedure to control for a False Discovery Rate of 0.2 was carried out using all the p-values calculated from the likelihood-ratio tests between the base models and the models, including the single variables.

6.2.6. Ethical statement

This research was approved by the University of Plymouth Local Ethics Committee review body, approval number ETHICS-42-2020.

6.3. Results

6.3.1. Temperature asymmetries

Variable	L-R maximum eye temperature	L-R maximum nostril temperatures	L-R average nostril temperatures	L-R maximum nasal airway temperatures	L-R average nasal airway temperatures	L-R maximum ear temperatures	L-R average ear temperatures
Handler position left vs right	Left side ←	Left side →	NS	Left side ←	Left side ←	Left side →	Left side →
	Right side →	Right side ←	NS	Right side →	Right side →	Right side ←	Right side ←
Session	NS	NS	≠	NS	NS	NS	≠
Reward vs non-reward vs baseline	NS	NS	NS	≠	≠	NS	NS
Group	NS	NS	NS	NS	NS	Group 1 ←	Group 1 ←
	NS	NS	NS	NS	NS	Group 2 →	Group 2 →

Table 14. Table for the effect of the variables in the asymmetries from the different ROIs. Arrows indicate the direction of the asymmetry; 'not equal sign' indicates differences between categories (more than 2), and areas shadowed in grey indicate that the variable is not significant for the respective parameter.

In a model for asymmetries (L-R) in maximum eye temperatures, 7.2% of the variance (9.67% at level 1; 0% at level 2) was explained by the side on which the handler stood during the recording (X^2=62.588, df=1, p<0.001); with asymmetries increasing to the left eye (hypothesised to indicate higher activity of the right brain hemisphere) when the handler stood on the left side of the horse (See figure 65).

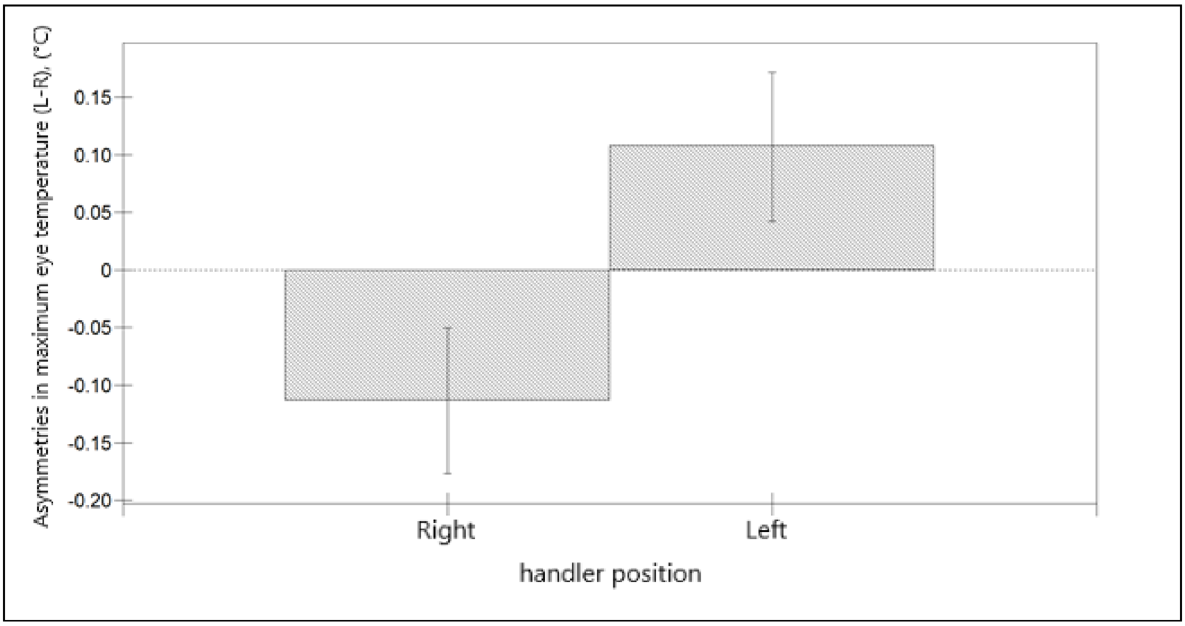


Figure 65. Effect of handler position on maximum eye temperature asymmetries (L-R).

In a model for asymmetries (L-R) in maximum nostril temperatures, 1.81% of the variance was explained (1.14% at level 1; 4.34% at level 2) by the side on which the handler stood during the recording (X^2=6.4544, df=1, p=0.011), with an increase of the asymmetries towards the right nostril (hypothesised to indicate higher activity of the right brain hemisphere) when the handler stood on the left side of the horse (see Figure 66).

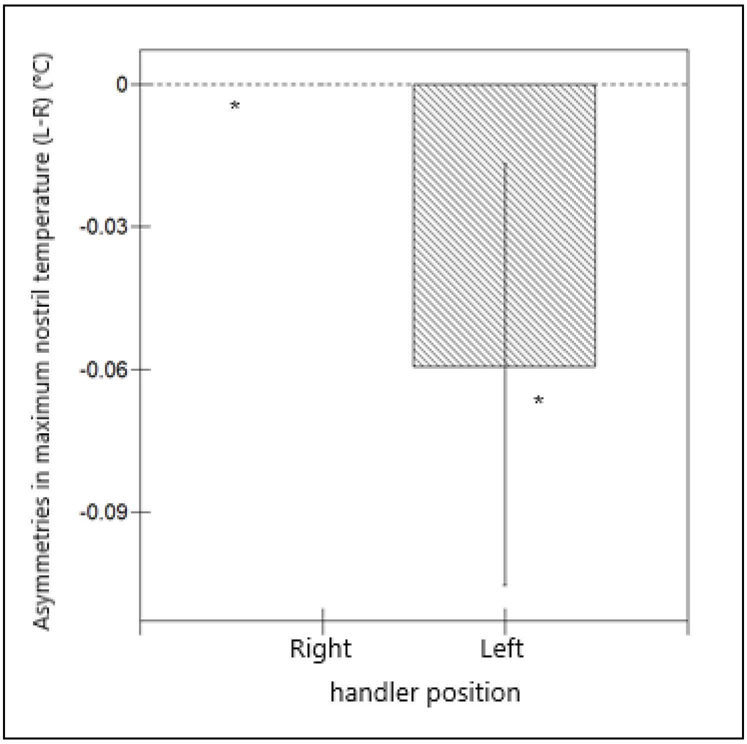


Figure 66. Effect of handler position on maximum nostril temperature asymmetries (L-R).

In a model for asymmetries (L-R) in average nostril temperatures, 1.59% of the variance (1.96% at level 1; 0% at level 2) was explained by the session in which the recording occurred (X^2=12.7253, df=2, p=0.001); with asymmetries differing significantly between the baseline and the second session(p=0.004), increasing towards the right nostril (hypothesised to indicate higher activity of the right brain hemisphere) in session 2 (see Figure 67).

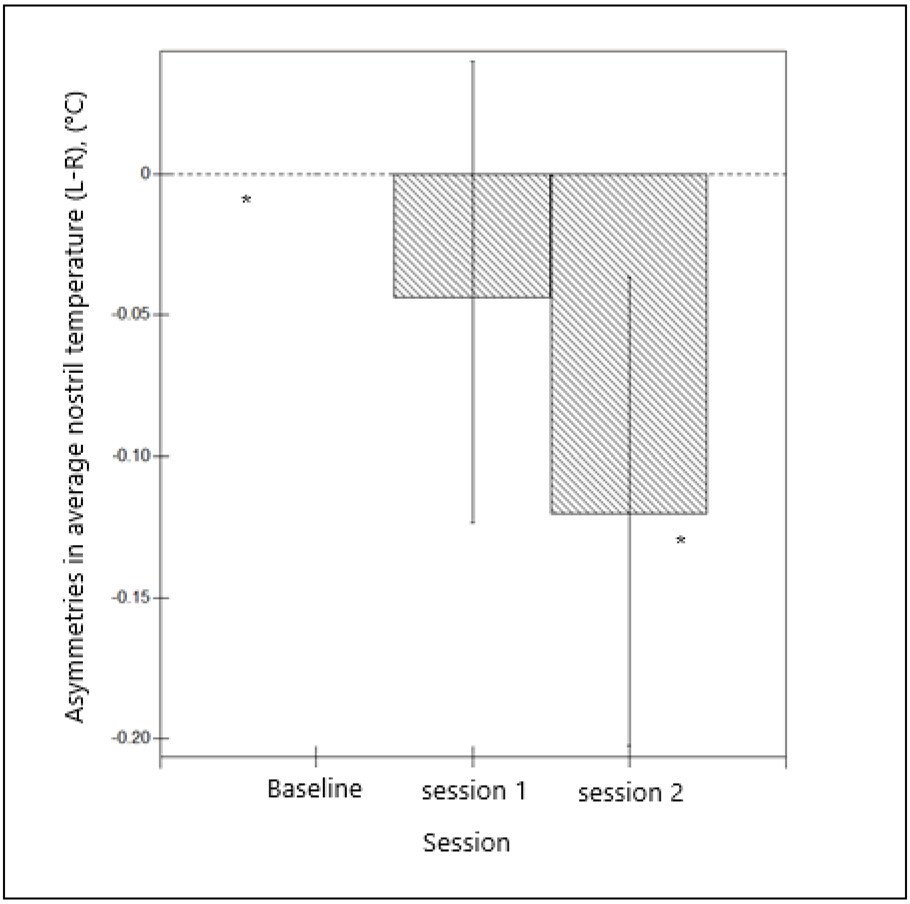


Figure 67. Effect of recording session on average nostril temperature asymmetries (L-R).

In a model for asymmetries (L-R) in maximum nasal airway temperatures, 1.45% of the variance (2.59% at level 1; 0% at level 2) was explained by whether the recording was from a baseline, a rewarded or a non-rewarded trial (X^2=15.188, df=2, p<0.001) and by the side on which the handler stood during the recording (X^2=6.64, df=1, p=0.009). In non-rewarded trials, horses had significantly higher asymmetries towards the right nasal airway (hypothesised to indicate higher activity of the right brain hemisphere) than in rewarded trials (p<0.001) and baseline recordings (p=0.005); in the recordings in which the handler stood on the left side, temperature asymmetries were significantly higher towards the left nasal airway (hypothesised to indicate higher activity of the left brain hemisphere) (see Figure 68).

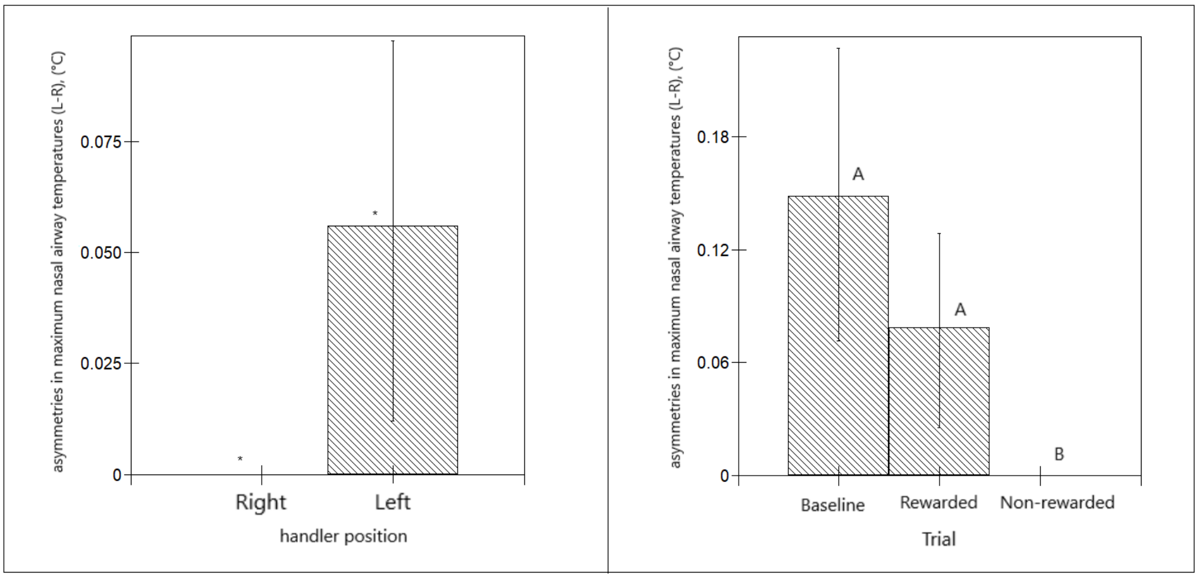


Figure 68. Effect of handler position (A) and trial type (B) on maximum nasal airway temperature asymmetries (L-R).

In a model for asymmetries (L-R) in average nasal airway temperatures, 5.26% of the variance (8.73% at level 1; 0% at level 2) was explained by whether the recording was from a baseline, a rewarded or a non-rewarded trial (X^2=18.3014, df=2, p<0.001) and by the side on which the handler stood during the recording (X^2=40.6571, df=1, p<0.001). In non-rewarded trials, horses had significantly higher asymmetries towards the right nasal airway (hypothesised to indicate higher activity of the right brain hemisphere) than in rewarded trials (p=0.003) and baseline recordings (p<0.001); in the recordings on which the handler stood on the left side, temperature asymmetries were significantly higher towards the left nasal airway (hypothesised to indicate higher activity of the left-brain hemisphere) (see Figure 69).

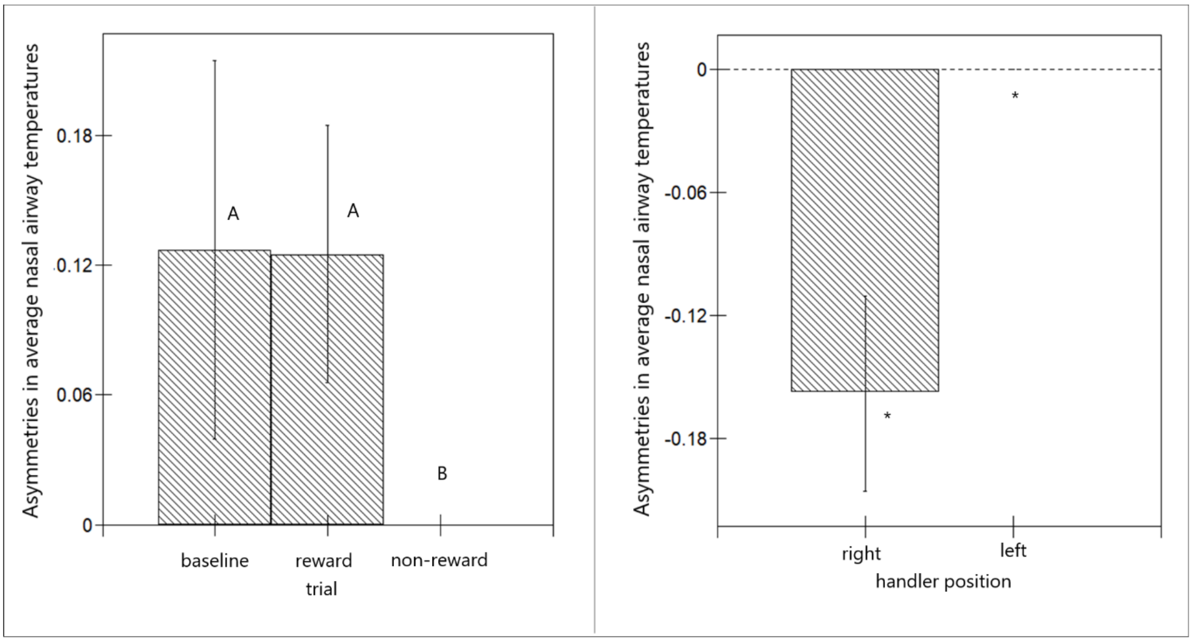


Figure 69. Effect of trial type (A) and handler position (B) on average nasal airway temperature asymmetries (L-R).

In a model for asymmetries (L-R) in maximum ear temperatures, 3.36% of the variance (1.52% at level 1; 15.34% at level 2) was explained by the side on which the handler stood during the recording (X^2=8.796, df=1, p=0.003) and the group assigned to the horses (X^2=4.0147, df=1, p=0.045). In the recordings in which the handler stood on the left side, temperature asymmetries were significantly higher towards the right ear (hypothesised to indicate higher activity of the left brain hemisphere); horses assigned to group 2 (unrewarded trials on first session) had significantly higher asymmetries towards the right ear (hypothesised to indicate higher activity of the left brain hemisphere) (see Figure 70).

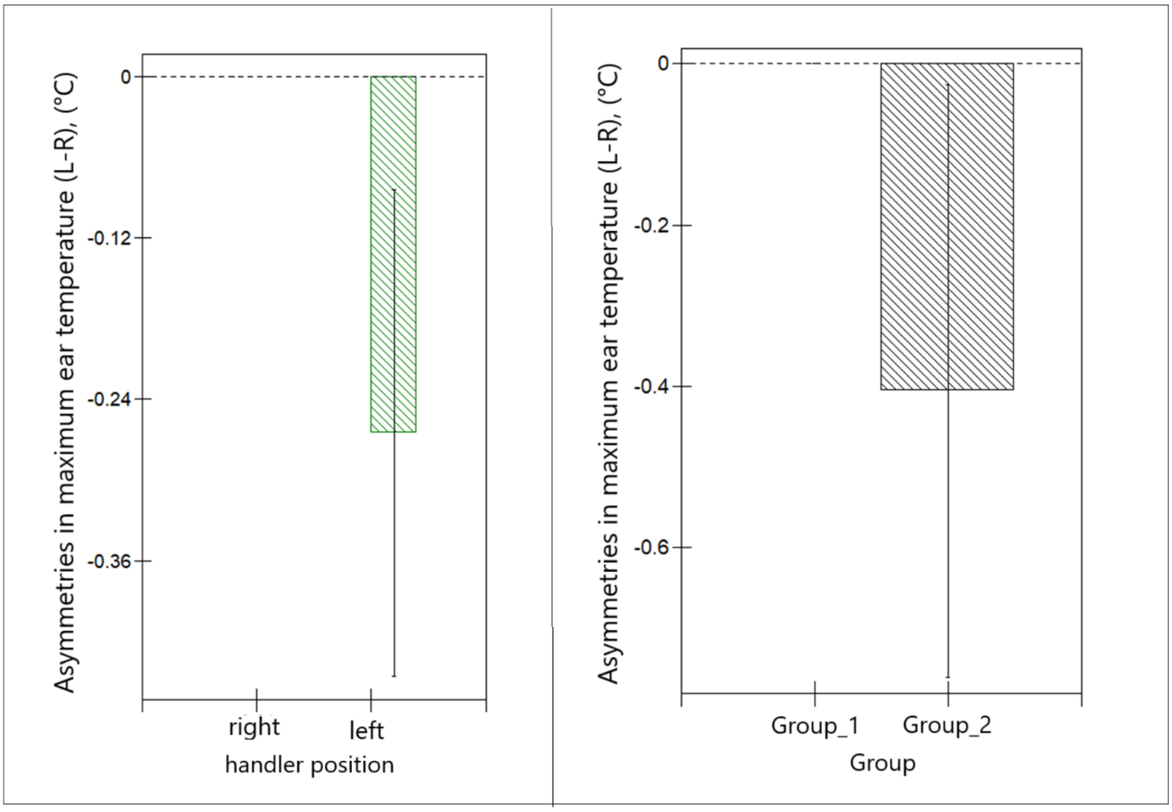


Figure 70. Effect of handler position (A) and horse group (B) on maximum ear temperature asymmetries (L-R).

In a model for asymmetries (L-R) in average ear temperatures, 4.98% of the variance (2.7% at level 1; 16.6% at level 2) was explained by the side on which the handler stood during the recording (X^2=8.822, df=1, p=0.003), the group assigned to the horses (X^2=4.02, df=1, p=0.045) and the session (X^2=8.834, df=2, p=0.012). In the recordings in which the handler stood on the left side, temperature asymmetries were significantly higher towards the right ear (hypothesised to indicate higher activity of the left brain hemisphere); horses assigned to Group 2 (unrewarded trials on first session) had significantly higher asymmetries towards the right ear (hypothesised to indicate higher activity of the left brain hemisphere), and in Session 2 horses had significantly more asymmetries towards the right ear (hypothesised to indicate higher activity of the left brain hemisphere) than in Session 1 (p=0.005) (see Figure 71).

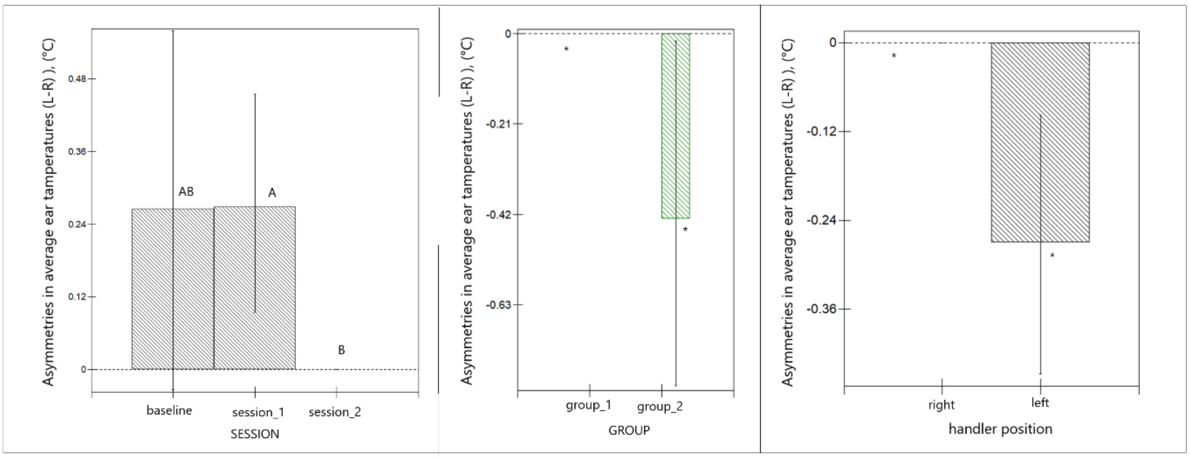


Figure 71. Effect of recording session (A), horse group (B) and handler position (C) on average ear temperature asymmetries (L-R).

6.3.2. Arousal indicators

Variable	Effect in hair whorl maximum temperature	Effect in muzzle maximum temperature	Effect in muzzle average temperature
THI	↑	↑	↑
Session	≠	NS	NS
yard	≠	NS	≠
Reward vs non-reward vs baseline	NS	≠	≠
Time within recording	NS	⤻	⤻

Table 15. Table for the effect of the variables in ROIs hypothesised to indicate arousal levels. Arrows indicate the direction of the effect; 'not equal sign' indicates that there are differences between categories (more than 2), and areas shadowed in grey indicate that the variable is not significant for the respective parameter.

In a model for hair whorl maximum temperature, 46.90% of the Variance (5.59% at level 1; 53.43% at level 2) was explained by the Temperature Humidity Index (X^2=19.549, df=1, p<0.001), session (X^2=19.522, df=2, p<0.001) and yard (X^2=15.105, df=1, p<0.001). As THI values increased, the maximum hair whorl temperatures increased linearly; in session 1, horses had significantly lower maximum hair whorl temperatures than in baseline (p<0.001) and session 2 (p<0.001), horses from Yard “A” had significantly warmer hair whorls than in yard “B” (see Figure 72).

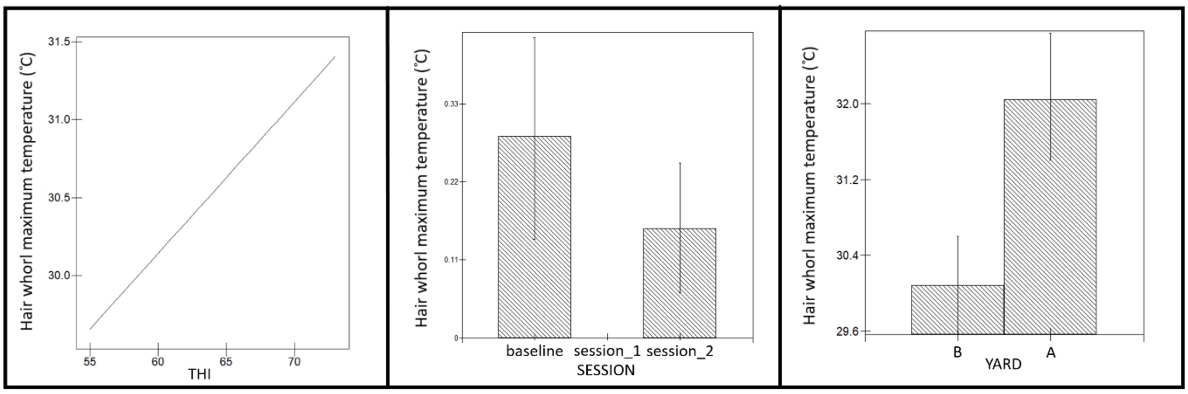


Figure 72. Effect of THI (A), recording session (B) and yard ID (C) on Hair Whorl maximum temperature.

In a model for muzzle maximum temperature, 9.33% of the Variance (21.56% at level 1; 4.79% at level 2) was explained by the Temperature Humidity Index (X^2=4.433, df=1, p=0.035), whether the recording was from a baseline, rewarded or unrewarded trial (X^2=97.143, df=2, p<0.001) and a quadratic relationship with time within recording (X^2=19.51, df=2, p<0.001). As THI values increased, the maximum muzzle temperatures increased linearly; rewarded trials had significantly colder muzzle temperatures than baselines (p<0.001) and non-rewarded (p<0.001) trials. As time within recording elapsed from 0 to 50 seconds, a drop in muzzle temperature was observed, followed by a steep increase until the end of the recording (see Figure 73).

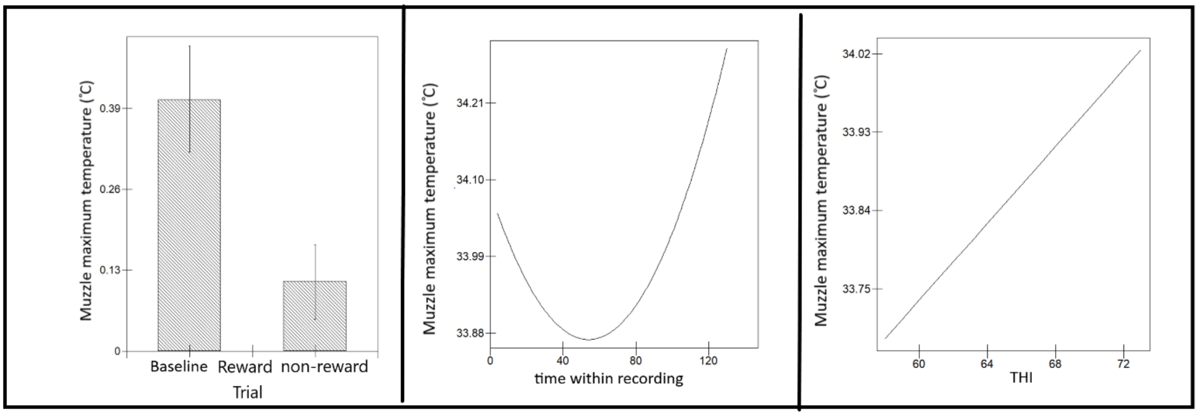


Figure 73. Effect of recording session (A), time within recording (B) and THI (C) on Muzzle maximum temperature.

In a model for average muzzle temperature, 23.73% of the Variance (22.90% at level 1; 24.07% at level 2) was explained by the Temperature Humidity Index (X^2=5.774, df=1, p=0.016), whether the recording was from a baseline, rewarded or unrewarded trial (X^2=84.422, df=2, p<0.001), the horses' yard (X^2=4.616, df=1, p=0.031) and a quadratic relationship with the time within recording (X^2=11.629, df=2, p=0.003). As THI values increased, the maximum muzzle temperatures increased linearly; rewarded trials had significantly colder muzzle temperatures than baseline (p<0.001) and non-rewarded (p<0.001) trials, horses from yard "A" had significantly warmer muzzle temperature than horses from yard "B". As time within recording elapsed from 0 to 50 seconds, a drop in muzzle temperature was observed, followed by a steep increase until the end of the recording (see Figure 74).

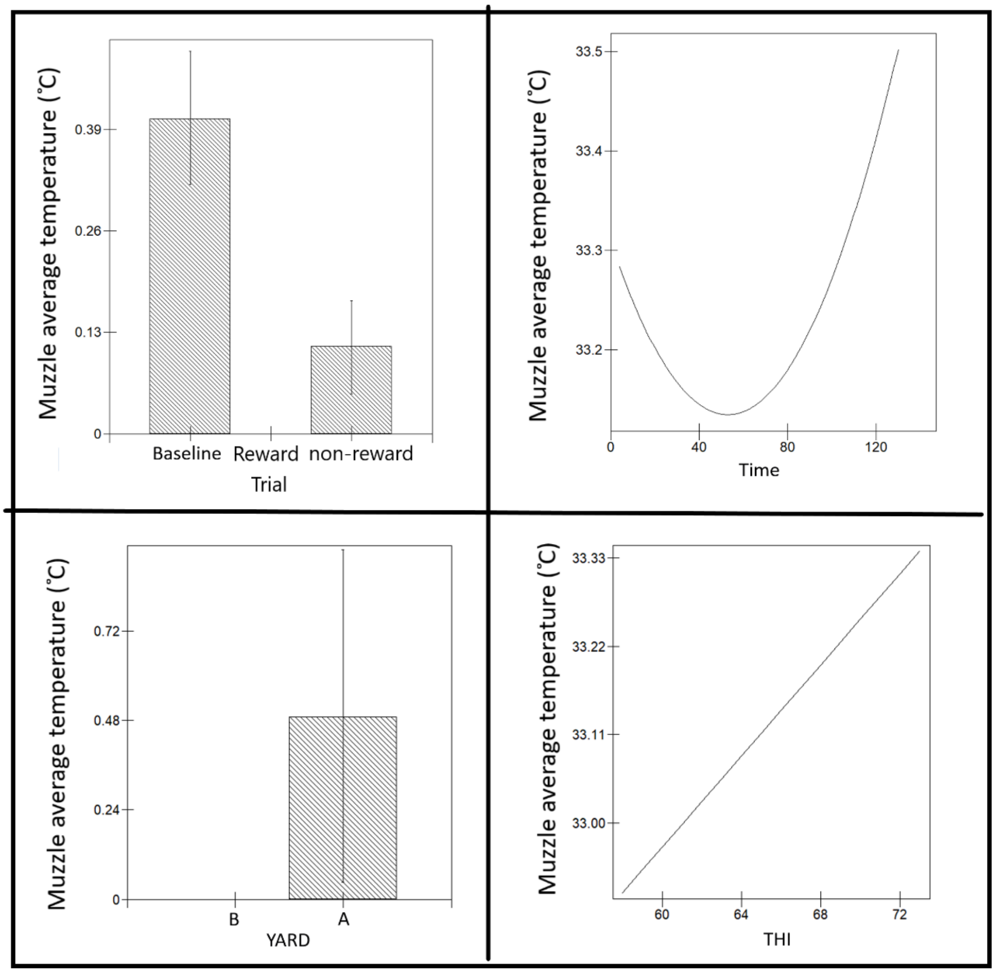


Figure 74. Effect of recording session (A), time within recording (B), yard ID (C) and THI (D) on Muzzle average temperature.

6.4. Discussion

This study aimed to identify if temperature asymmetries in regions of interest in horse faces were measurable using infrared thermography and, if so, whether those asymmetries could be associated with changes in the processing of affective loaded situations in the brain hemispheres according to the brain lateralisation hypothesis. In previous studies, temperature asymmetries in dairy calves associated with putative negative states elicited by common farm practices were identified. As part of those results, some evidence suggesting that emotions of different valence might be reflected as asymmetries towards opposite sides of the ROI was found. To further investigate this hypothesis, temperature asymmetries in horses that distinguished between putatively negative and positive short-term emotions were studied in the current study.

As hypothesised, differences in the direction of temperature asymmetries between what were hypothesised to be positive (rewarded trials) or neutral (baselines) situations and negative situations (non-rewarded trials) were found in the maximum and average temperatures of the nasal airway areas. In non-rewarded trials, asymmetries towards the right nasal airway, hypothesised to be suggestive of the processing of negative affective states / higher activation of the right brain hemisphere, were found. In contrast, asymmetries towards the left nasal airway (suggesting positive affective states / left brain hemisphere) were observed in the baseline and rewarded trials. Results that agreed with those observed in dairy calves in temperature asymmetries in the inner corner of the eye (chapter 3), in association with whether calves approached or moved away from the camera.

When looking at other factors surrounding the experiment, the side on which the horse handler stood during the recording was significantly associated with asymmetries in all the ROIs observed. However, according to the hypothesised predictions, it is not possible to make a straightforward interpretation of this variable. Asymmetries in 2 out of 4 ROIs (maximum eye and nostril temperatures) appear to indicate a higher activation of the right hemisphere when the handler stood on the left side of the horse; whereas asymmetries in the maximum and average temperatures in the nasal airway and ear regions, appear to indicate a higher activity of the left hemisphere when the handler stood on the left-hand side. Those interpretations come from the hypothesised association of ipsilateral relationships between nostril and ear temperatures with brain activation and contralateral relationships between eye and ear temperatures with brain activation. Some studies have found that horses have higher reactivity when approached from the left side (Austin and Rogers, 2007; 2012), which suggest that a higher activity of the right hemisphere occur when a novel or threatening stimulus is presented on the left side. It is important to highlight that even though asymmetries in the ocular and periocular areas were found to be the most significant in the calf studies (chapter 3 to 5), the current study investigated a different species and used a modified methodology, which might have yielded a higher error in measurements of the eye area, as eye temperatures were measured from a front view instead of from the side views.

The group in which the horses were assigned influenced the direction of the asymmetries in the maximum and average temperatures of the ear area, which according to the hypothesised contralateral association with the brain activity, suggests that horses from group 2 were in a more positive state / exhibited higher activity of the left hemisphere than horses from group 1. There is not a clear explanation for this difference between groups. Still, anecdotally researchers observed that horses from group 2 respond in a more excited way in session 2, which might be explained as a rebound effect resulting from receiving rewarded trials after not being rewarded on the last two trials of session 1. Horses in group1 did not experience such a situation and were unlikely to have exhibited a rebound effect. Finally, asymmetries in average temperatures of the nostril and ear areas were associated with the session from which the images were extracted. However, asymmetries in the nostril average suggest higher activation of the right hemisphere in session 2, whereas contradictorily, asymmetries in the average temperatures of the ear seem to suggest a higher activation of the left hemisphere in session 2, making not possible to interpret the effect of session in the affective state of the horses.

Regarding hair whorl and muzzle temperatures, significantly lower temperatures were found in session 1 compared to the baseline and to session 2, which might suggest an increased sympathetic activity when the targeting activity started. Similarly, an initial drop in temperatures, followed by a steep increase, was observed in hair whorl and muzzle regions as the time within recording elapsed. Those changes in peripheral temperature are considered indicators of high arousal, characterised by a peripheral decrease in temperature regulated by the sympathetic activity of the ANS, followed by an increase of peripheral temperature above the initial values in response of the parasympathetic branch of the ANS as a regulatory measure towards the initial response of the ANS (Merla and Romani, 2007; Stewart et al., 2010b; Moe et al., 2012; Ioannou et al., 2014; Ermatinger et al., 2019; Scopa et al., 2020). There was a difference between yards in hair whorl maximum and muzzle average temperatures, indicating higher temperatures in yard A than in yard B, there is no obvious specific reason for this difference, but several variations in the handling, working schedules and practices between yards that were not accounted for this study, might account for this variability in temperatures. Finally, a positive relationship between THI and hair whorl and muzzle temperatures, similarly to the ones in chapter 5 were found in this study, highlighting the relationship between environmental factors and peripheral temperature.

It is essential to highlight that even though all the variables included in the models were significant at a p-value <0.05 and corrections to control for multiple hypothesis testing were in place, models for some of the ROIs explained a very small proportion of the total variance in the asymmetries measured. For example, asymmetries in nasal airway maximum temperature and asymmetries in average nostril temperatures were explained by 1.45% and 1.59% by their respective models. The remaining unexplained variance on the asymmetries in the area is likely to be explained by the complexity of the models, variables not accounted in the models, individual differences and to some extent to differences between measurements, i.e. nasal airway area was measured by a line (1 pixel wide), so it might be expected to find more variability in the measurement of maximum temperatures, whilst nostril temperatures are measured by an ellipse, which might generate less variance in the measurement of maximum temperatures, but a higher variance for average temperatures.

To my knowledge this is the first study proving the existence of temperature asymmetries in horses and the first study showing differences in the direction of temperature asymmetries in situations putatively eliciting responses with opposite emotional valences. It is necessary to explore further the relationships between the valence of affective states and the direction of the temperature asymmetries. In the current study it is likely that horses have made an association between the researchers and receiving a palatable food, which might have elicited a positive affective state even during the baseline recordings. Using a reward / punishment contrast or rutinary situations that might elicit emotions from different valence might help to avoid affective bias towards the researchers in response to the training procedure.

7. GENERAL DISCUSSION

This dissertation aimed to identify if temperature asymmetries in different regions of interest (ROIs) in the head of dairy calves and horses were measurable using infrared thermography, and if so, whether these asymmetries were associated with differences in the valence of affective states elicited in each of the contexts studied in chapters 3 to 6 of this dissertation. For this purpose, it was predicted that the direction of the asymmetries might be linked to lateralised processing of emotions in the brain (reviewed in chapter 1); these predictions had been made based on the anatomic proximity of the different ROIs to the brain and the relationship between the innervation of the ROIs studied and the brain hemispheres (ipsilateral and/or contralateral relationship). In each of the four experimental chapters (Chapters 3 to 6), the presence of asymmetries in the ROIs and changes in temperatures expected to indicate arousal levels were explored.

In the following chapters, the main results from temperature asymmetries in the different chapters that are likely associated with affective states will be explored, followed by a summary of the main results in ROIs associated with emotional arousal. Then, the potential answers to the research questions in this dissertation will be discussed, and finally, the main limitations and further research directions will be addressed.

7.1. Temperature asymmetries

7.1.1. Chapter 3. Hot-iron disbudding

In chapter 3, the routine practice of disbudding calves with a hot-iron (hot-iron disbudding) was used to elicit a negative affective state (short term manipulation) in calves experiencing this procedure. The effect of a previous negative experience (either as an effect of a long-term affective state manipulation or a short-term memory-driven affective response) was studied by looking at temperature asymmetries in individuals previously disbudded while observing conspecifics being disbudded. Asymmetries in response to a human observer were analysed, looking at the reaction of calves (approaching, moving away or standing still) to the human doing the thermal recordings. In this study, significant asymmetries attributed to the variables studied were found in the ear base (front view), nostril, ocular (inner corner of the eye) and periocular area (rostral eye surrounding). The ear base area (from the back view) was the only ROI with no significant asymmetries attributed to the variables studied (see Figure 75).

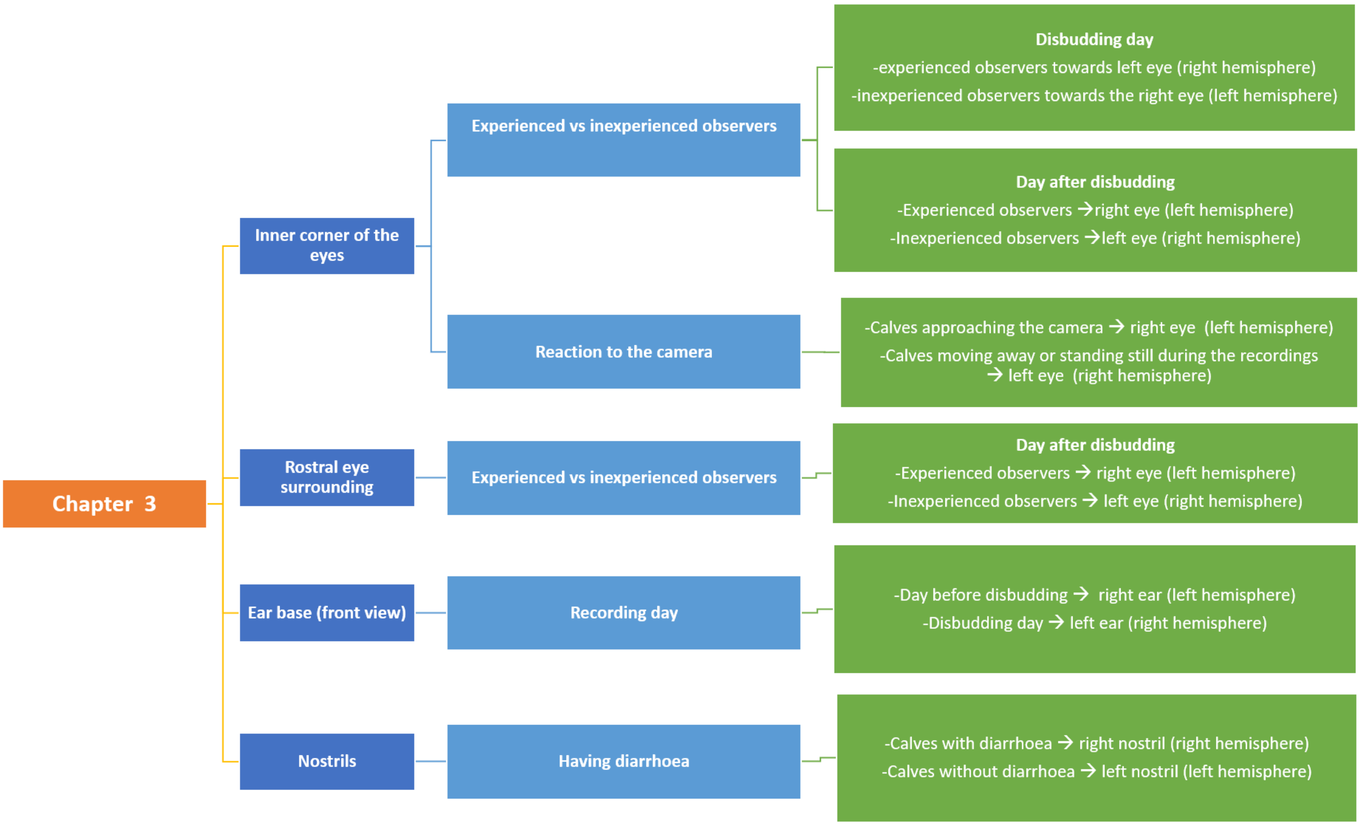


Figure 75. Significant asymmetry results found in chapter 3.

Significant differences in the direction of temperature asymmetries in the inner corner of the eye and rostral eye surrounding were attributable to whether calves had experienced the disbudding procedure by themselves. Observer calves that had previously experienced hot-iron disbudding (experienced observers) in comparison to observer calves that had not experienced the procedure (inexperienced observers) and calves undergoing the procedure at the time of data collection (disbudded calves); calves that had previously experienced disbudding and were now observing a conspecific being disbudded (D2xT1) showed a higher temperature in the left relative to right eye indicating elevated right hemisphere activity and suggesting, according to the emotional valence lateralisation hypothesis, that they perceived the procedure more negatively. Similarly, temperature asymmetries in the inner corner of the eye suggested that calves that approached the observer and camera perceived the researcher more positively (having higher right than left inner corner temperatures indicating greater activation of the left hemisphere) than calves that moved away or stayed still during the recordings.

Asymmetries in the ear base (front view), indicating significantly higher temperatures on the left ear (right hemisphere), were found in calves from all groups in the disbudding day (D2) in comparison to temperature asymmetries on the day before the disbudding session (D1), which suggests that calves might have perceived the disbudding session more negatively. Finally, asymmetries in nostril temperatures indicate higher temperatures towards the right nostril (right hemisphere) in calves that had diarrhoea during the recordings compared to calves that did not present any signs of diarrhoea. However, no evidence of asymmetries suggesting a negative affective state was observed in the recordings of the calves disbudded (disbudded calves) during this study (disbudding session D2 x T1) due to handling pre-disbudding (probably due to gentle handling) or to pain elicited on the day after disbudding (D3) (though it is not clear whether calves were in pain on the day after disbudding as appropriate use of local anaesthetics and analgesics was in place during the procedure) (more detail in chapter 3.3.3.1).

7.1.2. Chapter 4. Separation from the mother

In chapter 4, asymmetries in ROIs were measured during a putatively negative situation in which calves were separated from their mother (as standard practice on the farm) within 48 hours after birth. In this study, thermal indicators of short-term negative affective states were studied, looking at differences in asymmetries in the calves' ROIs before and after being separated from the mother. Links between behaviours performed by the calves during the 60 minutes post-separation and temperature asymmetries and associations between external contextual variables and temperature asymmetries were also analysed. In this study, asymmetries associated with the variables studied were found in the nasal airway, ear (front and back view), ocular (eyeball) and periocular (caudal eye surrounding) areas (see Figure 76).

Figure 76. Significant asymmetry results found in chapter 4.

As time elapsed following separation from the mother, asymmetries towards the right eyeball (left hemisphere) and right nostril (left hemisphere areas suggested that separation from the mother was more negative at the beginning of the process (first half hour) compared to at the end of the recording period (60 minutes from separation). In a similar fashion, active behaviours, like standing or running performed in the 5 minutes pre-recording were associated with asymmetries suggesting a more negative affective state in comparison to individuals lying down during the same period, i.e. asymmetries towards the right nasal airway (right hemisphere) when calves stood before the recording, asymmetries towards the left caudal eye surrounding when calves stood before the recording (right hemisphere), and asymmetries towards the left eyeball (right hemisphere) when calves ran before the thermal recordings. In terms of external variables influencing the direction of the asymmetries, it was found that higher noise scores (described in chapter 4), starting recordings from the right side, and conspecific vocalisations influenced asymmetries suggesting a more negative experience (higher activity of the right hemisphere), whereas having a conspecific in the adjacent pen influenced the asymmetries that suggested a less negative/ more positive experience (higher activity of the left hemisphere).

7.1.3. Chapter 5. Effect of external factors

Chapter 5 focused on analysing the effects of different aspects of the methodology in the direction of the asymmetries in the ROIs, including the effect of camera position (angle and elevation), time within the recording and environmental factors (temperature, humidity and THI). In this chapter, most of the variables analysed yielded results that were expected to influence recorded thermal asymmetries; for example, as the angle of the camera deviated towards one side of the head, the asymmetries measured increased towards the respective side in most of the ROIs, as expected from the physics of infrared radiation (a detailed explanation of this is provided in the discussion section of chapter 5) (see Figure 77).

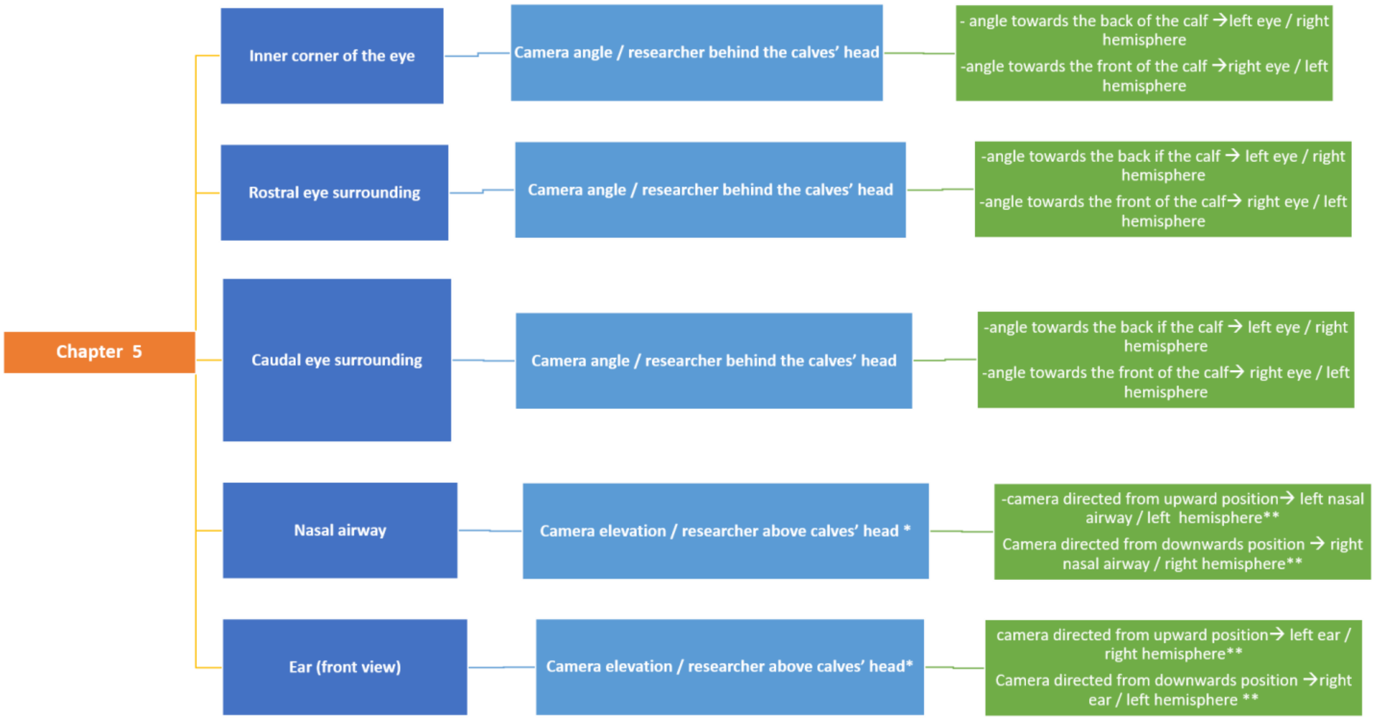


Figure 77. Significant asymmetry results that might be associated with an affective response found in chapter 5. * contradictory results found in different ROIs ** the relationship between ROIs and hemisphere activation was not determined due to confounding results.

However, some results suggested that differences in methodology influencing the temperature asymmetries might rather be explained by the influence of those factors on the affective state of the individuals rather than on the physics influencing the temperature measurements in the camera. For example, it was not expected that camera angle would influence the asymmetries in the ocular (inner corner of the eye) and periocular (caudal and rostral eye surrounding) areas, as the measurement of the asymmetries was made with paired images from both sides in terms of camera angle; the fact that asymmetries on those areas increased towards the left side (right hemisphere) as the angle increased towards the back of the calves' head, suggested that calves might have perceived the presence of the researcher as more threatening or negative as he moved towards the back of the individual. It is not clear why camera elevation also had an effect on asymmetries in nasal airway and ear areas; an upward increase in elevation seemed to influence the direction of the asymmetries towards the right nasal airway (right hemisphere) and towards the right ear (left hemisphere), yielding contradictory interpretations. However, an increased elevation of the camera might reflect a higher position of the researcher and thus might be perceived by the calf as a more threatening stimulus; this is an interesting topic to be explored by itself, but at the moment, it is worth taking this factor into account for further studies, as it is likely to influence the direction of asymmetries measured.

7.1.4. Chapter 6. Positive and negative affective states (horses)

Finally, the aim of chapter 6 was to look at the effect of contrasting short-term affective states of different valence (negative vs positive) on the direction of temperature asymmetries of the ROIs. As discussed in that chapter, this study was planned to be carried out in dairy calves as I had collected relevant data with this species in the previous study. However, due to situations arising from the COVID-19 pandemic, it was not possible to collect data at the farm and thus, I took the opportunity to explore asymmetries in different species (horses), which were accessible for study during the pandemic period. In this study, a positive emotion (receiving a reward for touching an object) was contrasted with the negative emotion of not receiving the expected reward when performing the previously rewarded task. As expected, asymmetries towards different directions in the nasal airway area suggested a positive affective state in the rewarded trials (left nasal airway / left hemisphere), whereas unrewarded trials reflected asymmetries indicating a more negative affective state (right nostril/right hemisphere). Interestingly, asymmetries in all the ROIs (eye, nostrils, nasal airway and ears) were influenced by the side on which the handler stood during the recording; results that suggest a similar influence of the researcher position to the ones found in the calves from chapter 4. However, the effect of this variable is not clear in the horse study as the interpretation from different ROIs is contrary in terms of which hemisphere might be dominant when the handler stands on either side, i.e. standing on the left side was associated with relatively greater temperatures in the left eye and right nostril (right hemisphere dominance), whilst also being associated with relatively higher temperatures in the left nasal airway and the right ear (left hemisphere dominance) (see Figure 78).

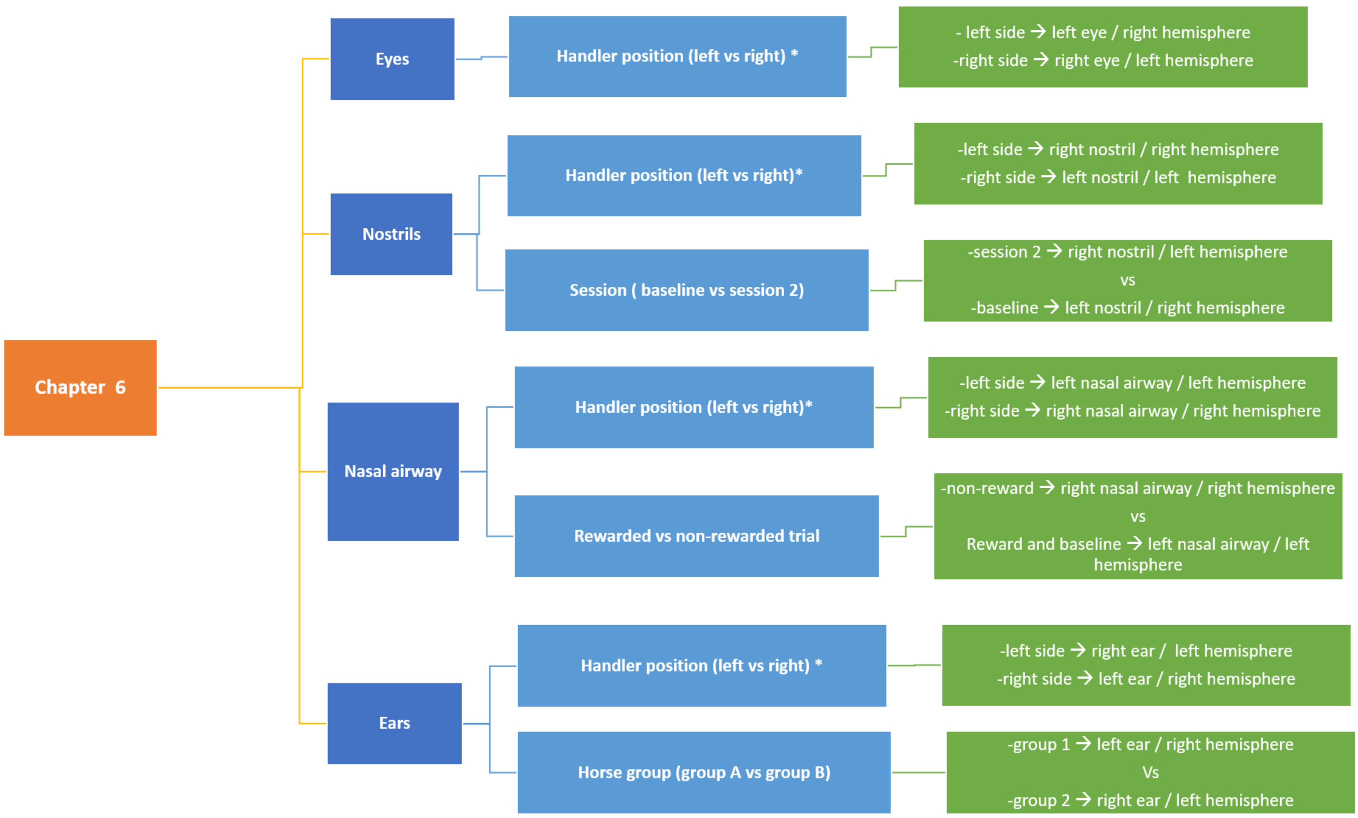


Figure 78. Significant asymmetry results found in chapter 6. * contradictory results found in different ROIs.

7.2. Arousal indicators

Regarding changes in temperature suggesting arousal levels as a result of a balance between the sympathetic and parasympathetic activity of the ANS, significant differences in muzzle temperature were found in all experimental chapters, which, as discussed in the introduction, have been used by several researchers as an indicator of arousal (Zajonc et al., 1989; Nakayama et al., 2005; Proctor and Carder, 2015). However, as the muzzle is an area that is in constant contact with water and food, measurements in this area might change if the area is dirty or if the animal drank water recently; therefore, for my dissertation, changes in the maximum temperature in a hairless area on the forehead of the animals ('hair whorl') were explored as an alternative measure of arousal; a correlation between muzzle maximum temperature and hair whorl maximum temperature was found in the results from Chapter 3 ($r=0.593$, $p<0.001$) and Chapter 4 ($r=0.815$, $p<0.001$), and between average muzzle temperature and hair whorl maximum temperature in

Chapter 3 (r=0.701, p<0.001) and Chapter 4 (r=0.760, p<0.001) and similarities in the results from the different chapters (see Table 15), support the hypothesis that temperatures on this area might be well suited as an alternative to muzzle temperatures.

Chapter	variable	Muzzle average	Muzzle máximum	Hair whorl maximum
3	Day of recording	≠	*	NS
	Ambient temperature	↷	*	↷
	Use of antibiotics	NS	*	↓
4	Time from separation	*	↑	↑
	Standing Before recording	*	↓	↓
5	Camera elevation	if increased upwards** ↑	if increased upwards** ↑	if increased upwards ** ↓
	THI	↑	↑	↑

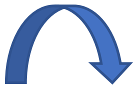
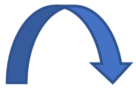
	Time within recording		NS	
6	THI	↑	↑	↑
	Time within recording	NS		
	yard	≠	NS	≠
	Sesión	NS	NS	≠
	Reward vs Non-reward	≠	≠	NS

Table 16. Table for the effect of the variables in ROIs hypothesised to indicate arousal levels. Arrows indicate the direction of the effect; 'not equal sign' indicates that there are differences between categories (more than 2), and areas shadowed in grey indicate that the variable is not significant for the respective parameter. * indicates that the temperature was not selected for analysis in the respective Chapter.

7.3. Research questions addressed

The work in this dissertation aimed to identify if temperature asymmetries in specific areas of interest were measurable through infrared thermography (question 1), whether those asymmetries might be associated with lateralised processing of emotions in the brain (question 2), which of those ROIs might be more reliable indicators for the study of emotions in animals (question 3) and whether those asymmetries might reflect activity of the ipsilateral or contralateral brain hemisphere according to the hypothesis stated in chapter 1.8 (question 4).

Almost all the results from the experimental work suggest the existence of temperature asymmetries in all the ROIs measurable via infrared thermography (question 1). Assuming that the affective states induced by the events/treatments studied in chapters 3 to 6 are correct and that the observed thermal asymmetries reflect asymmetries in brain hemisphere function in line with the emotional valence lateralisation hypothesis and realised through the mechanisms discussed in chapter 1.8, then we can make the following tentative suggestions. Generally, this thesis seem to indicate that the direction of the asymmetries in different ROIs studied might indeed be considered an indicative measurement of emotional valence in dairy calves and horses (question 2). According to results in Chapters 3 and 4, asymmetries in the ocular (inner corner and eyeball), periocular areas (rostral and caudal eye surrounding) and nasal airways were the ROIs more influenced by the affective manipulation on those studies (question 3). However, the variance explained by external factors (Chapter 5) in the ocular and periocular areas was smaller than in the nasal airway area, suggesting that ocular and periocular areas might be more reliable indicators of temperature asymmetries associated to changes in affective states (question 3). On the one hand, dairy calves' asymmetries in ocular and periocular areas appear to indicate higher activity of the contralateral hemisphere as initially hypothesised (not sufficient evidence in horses and the methodology between species to collect data in this area differed), similarly asymmetries in ear temperature measured from the front images seemed to be related to higher activation of the contralateral hemisphere (in dairy calves and horses). On the other hand, the direction of the asymmetries in the nasal airway (dairy calves and horses) and nostril (dairy calves) areas seemed to indicate higher activation of the ipsilateral hemisphere (question 4).

7.4. Limitations and future direction

It is essential to highlight that more research in temperature asymmetries is required to make more precise interpretations of the results and to validate that temperature asymmetries are actually linked to differences in brain activity. This might be achieved through more experiments looking at contrasting affective states with similar arousal levels and different emotional valence alongside other physiological methods like ECG that help identify differences in the brain activity or imaging techniques that allow measuring the activity on different brain regions like fMRI. For example, there have been some successful studies using fMRI to identify changes in the activity of different areas of the brain associated to an affective response of dogs towards pictures of familiar and unfamiliar persons (Karl et al., 2020; Thompkins et al., 2021) or sounds that vary in terms of affective valence (Andics et al., 2014; Gábor et al., 2020); using a similar methodology would make possible to identify the relationship between temperature asymmetries in different ROIs and brain activity. However, it would be required either to train animals in which temperature asymmetries have been tested (dairy calves or horses) or to prove the existence of temperature asymmetries elicited by affective challenges in dogs. In terms of contrasting affective states it might be required to elaborate tests that include the application of minor aversive stimulus (use of air canisters, water sprayers, acoustic or gustative punishments), elicit stronger frustration responses (compared to the ones used in Chapter 6) or that are less predictable for the subjects (it is possible than in the experiment for chapter 6, horses were already in a positive affective state in response to the researchers as they might have already make an association between the researchers and receiving a palatable reward during the training phase of the experiment).

It is important to remember that some of the models elaborated in the experimental chapters explained only a small proportion of the total variance in the data, suggesting that even though the results were significant; other variables not accounted for in the models were important. Furthermore, differences between images and between individuals might have a significant impact in the temperature asymmetries observed. Moreover, an important fact to highlight is that even if temperature asymmetries are associated with lateralised processes in the brain, this physiological processing is not exclusive to the processing of emotions and therefore, it is likely that information not directly related to the affective state of an individual will influence the brain lateralisation process and consequently the direction or magnitude of the temperature asymmetries measured. It is also essential to remember that the situations used to elicit a putatively negative affective response in these studies were only mild stressors and had mitigation measurements in place to reduce the negative impact on the individuals; which might have reduced the magnitude of the asymmetries observed in chapter 3 and 4; i.e. use of local anaesthetic and analgesics for the disbudding procedure reduces the physiological and behavioural signs of pain (discussed in chapter 3) and separating calves at a young age reduces their behavioural reactivity during the process (locomotion and vocalisation) (Lidfors, 1996; Flower and Weary, 2001; Stěhulová et al., 2008b). So, further studies looking at situations associated with more intense affective states might yield more significant results. For example, looking at healthy vs sick animals or differences in long term affective state (moods) between individuals.

Finally, it is crucial to consider several practical limitations for the design of studies using infrared thermography, such as avoiding the effect of wind currents, direct contact with sunlight or reflecting surfaces, and the effect of environmental factors like humidity and ambient temperature in the physiological responses of the individuals (as discussed in Chapter 5). In practice, such issues might have a great impact on the temperature calculations made by thermal cameras in commercial or non-controlled settings. For this reason, I do not recommend the use of infrared thermography to detect small variations in temperature (like the ones measured in this dissertation) under such conditions until further technological development or statistical models accounting for these factors are created.

The results in this dissertation highlight the importance of Infrared thermography as a non-invasive tool that allows us to measure dynamic changes in temperature associated with physiological changes in real-time and raises the potential of measuring temperature asymmetries as an indicator of affective state in animal studies.

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APPENDIX

Chapter 3

Variable	Categories	coefficient	S.E.	p-value	Asymmetries towards	Active hemisphere (hypothesised)
Constant	N/A	-0.039	0.070	p= 0.584		
Weaned	No	Reference category				
	Yes	0.353	0.159	p= 0.027	Left eye	Right hemisphere

Table 17. Final model for asymmetries in Rostral eye surrounding max (L-R).

Variable	Categories	coefficient	S.E.	p-value	Asymmetries towards	Active hemisphere (hypothesised)
Constant	n/a	0.584	0.283	p=0.039		
Recording day	Disbudding day (D2)	Reference category				
	Day before disbudding session (D1)	-0.900	0.321	p=0.005	Right ear	Left hemisphere
	Day after disbudding session (D3)	-0.393	0.316	p=0.214		
(Humidity-gm)	N/A	-0.005	0.011	p=0.645		
(Humidity-gm) ^2 *	N/A	-0.001	0.001	p=0.034		

Table 18. Final model for asymmetries in front ear max (L-R).

Variable	Categories	Coefficient	S.E.	p-value	Asymmetries towards	Active hemisphere (hypothesised)
Constant		0.021	0.095	p=0.822		
diarrhoea	No	Reference category				
	Yes	-1.504	0.570	p=0.008	Right nostril	Right hemisphere

Table 19. Final model for asymmetries in Nostril max (L-R).

Variable	Categories	Mean	S.E.	p-value
Constant		31.536	0.554	p=0.001
Recording Day	Day after disbudding (D3)	Reference category		
	Day before disbudding (D1)	-1.115	0.462	p=0.016

	Disbudding day (D2)	-1.202	0.482	p=0.013
(Humidity-gm)	n/a	-0.026	0.020	p=0.191
(Humidity-gm) ^2	n/a	-0.002	0.001	p=0.029
(Temperature-gm)	n/a	0.822	0.108	P<0.001
(Temperature-gm) ^2	n/a	-0.010	0.013	p=0.449
(Temperature-gm) ^3	n/a	-0.007	0.002	P=0.002

Table 20. Final model for muzzle average temperature.

Variable	Categories	Coefficient	S.E.	p-value
Constant	n/a	31.092	0.378	p<0.001
Receiving antibiotic	No	Reference category		
	Yes	-1.675	0.586	p=0.004
(Temperature-gm)	n/a	0.549	0.086	p<0.001
(Temperature-gm) ^2	n/a	0.001	0.008	p=0.880
(Temperature-gm) ^3	n/a	-0.003	0.002	P=0.042

Table 21. Final model for maximum hair whorl temperature.

Variable	categories	Mean	S.E.	p-value	Asymmetries towards	Active hemisphere (hypothesised)
Constant	n/a	-0.167	0.179	p= 0.350		
Analgesic in the last two weeks	No	Reference category				
	Yes	-0.439	0.207	p= 0.034	Right eye	Left hemisphere
Role of calf	InexObs	Reference category				
	Disbudded	0.110	0.224	p= 0.623		
	ExpObs	0.515	0.227	p= 0.023	Left eye	Right hemisphere

Table 22. Model for Inner Corner L-R in the disbudding session (D2 x T1).

Variable	Categories	coefficient	S.E.	p-value	Asymmetries towards	Active hemisphere (hypothesised)
Constant	n/a	0.534	0.246	p=0.030		
Calf response to the camera	Move away	Reference category				
	Approach	-0.707	0.336	p=0.035	Right eye	Left hemisphere

	Not moving	0.182	0.274	P=0.507		
Role of calf	InexObs	Reference category				
	Disbudded	-0.680	0.246	p= 0.006	Right eye	Left hemisphere
	ExpObs	-0.793	0.260	p= 0.002	Right eye	Left hemisphere

Table 23. Model for Inner Corner L-R on D3 x T1.

Variable	**Category**	**Coefficient**	**S.E.**	**p-value**	**Asymmetries towards**	**Active hemisphere (hypothesised)**
Constant	n/a	0.625	0.219	p= 0.004		
Role of calf	InexObs	Reference category				
	Disbudded	-0.683	0.309	p= 0.027	Right eye	Left hemisphere
	ExpObs	-1.050	0.309	p= 0.001	Right eye	Left hemisphere

Table 24. Model for Rostral eye surrounding L-R on D3 x T1.

Hair whorl max D2 x T2			
Variable	**Mean**	**S.E.**	**p-value**
Constant	21.85	1.494	p<0.001
Hair whorl max (D1xT2)-gm. (covariable)	0.450	0.139	p=0.001
Temperature	0.546	0.100	p<0.001

Table 25. Final model for maximum hair whorl temperature (D2 x T2).

Muzzle ave (D2 x T2)				
Variable	**Categories**	**Mean**	**S.E.**	**p-value**
Constant	n/a	25.917	1.462	p<0.001
(muzzle average (D1 x T2)-gm). (covariable)	n/a	0.748	0.108	p<0.001

Calf role	InexObs	Reference category		
	Disbudded	4.520	0.977	p<0.001
	ExpObs	3.080	0.970	p=0.001
(Temperature-gm)	n/a	-0.272	0.108	p=0.012
(Humidity-gm)	n/a	0.157	0.058	p=0.007
(Humidity-gm) ^2	n/a	0.005	0.002	p=0.043
(Humidity-gm) ^3	n/a	-0.0004	0.0001	p=0.008

Table 26. Final model for muzzle average temperature (D2 x T2).

Chapter 4

Variable	**Categories**	**coefficient**	**S.E.**	**p-value**	**Asymmetries towards**	**Active hemisphere (hypothesised)**
Constant	N/A	- 0.245	0.141	0.081	N/A	N/A

(Ambient score-gm)	N/A	+ 0.073	0.032	0.021	Left eye	Right hemisphere
(Ambient score-gm)^2	N/A	- 0.029	0.013	0.024	N/A	N/A
Running BR	Yes	+ 0.806	0.354	0.023	Left eye	Right hemisphere
	No	Reference category				
Side that the recording started	Right	+ 0.340	0.124	0.006	Left eye	Right hemisphere
	Left	Reference category				
Noise of the milking machinery	Yes	+ 0.384	0.129	0.003	Left eye	Right hemisphere
	No	Reference category				
Cow mooing	Yes	+ 0.245	0.120	0.040	Left eye	Right hemisphere
	No	Reference category				
Time	N/A	- 0.008	0.002	0.001	Right eye	Left hemisphere

Table 27. Final model for eyeball temperature asymmetries.

Variable	Categories	coefficient	S.E.	p-value	Asymmetries towards	Active hemisphere (hypothesised)
Constant	N/A	- 0.607	0.467	0. 194	N/A	N/A
Time	N/A	+ 0.017	0.010	0. 079	N/A	N/A
Time ^2	N/A	- 0.0004	0.0002	0. 016	N/A	N/A
Neighbour cows	Yes	+ 1.626	0.456	< 0.001	Left airway	Left hemisphere
	No	Reference category				

Standing BR	Yes	- 1.908	0.477	< 0.001	Right airway	Right hemisphere
	No	Reference category				
Walking BR	Yes	+ 1.477	0.527	0.005	Left airway	Left hemisphere
	No	Reference category				
Approach side	Right	- 0.904	0.430	p=0.035	Right airway	right hemisphere
	Left	Reference category				

Table 28. Final model for nasal airway asymmetries.

ROI	**Variable**	**coefficient**	**S.E.**	**p-value**	**Asymmetries towards**	**Active hemisphere (hypothesised)**
Back ear average (L-R)	Constant	-0.015	0.142	0.915		
	Angus x Holstein vs Holstein	+ 0.896	0.333	0.007	Left ear	Right hemisphere
	Constant	-.375	0.153	0.014		

Front ear max (L-R)	Belgian Blue x Holstein Vs Holstein	+ 1.088	0.483	0.024	Left ear	Right hemisphere
	Angus x Holstein vs Holstein	+ 0.822	0.358	0.022	Left ear	Right hemisphere
Front ear max (L-R)	Constant	+0.295	0.245	0.229		
	Neighbour cows	-0.664	0.301	0.027	Right ear	Left hemisphere

Table 29. Single models for back and front ear temperature asymmetries.

Variable	**Mean**	**S.E.**	**p-value**
Constant	31.249	0.524	p<0.001
Standing BR	-1.115	0.251	p<0.001
Time	0.009	0.003	p=0.005
Time x standing BR	0.020	0.008	P=0.014

Table 30. Final model for Hair whorl temperature.

Variable	**Coefficient**	**S.E.**	**p-value**
Constant	34.317	0.361	p<0.001
Standing	-1.156	0.197	p<0.001
standing BR	-0.785	0.243	p=0. 001
standing DR x standing BR	0.772	0.352	p=0.028
time	0.006	0.003	p=0.030

Table 31. Final model for muzzle maximum temperature.

Chapter 5

Left ear Average (front view)					
Variable	Details of the variable	Coefficient	S.E.	C.I. (95% confidence)	p-value
constant		31.087	1.743	27.671 to 34.502	p<0.001
(THI-gm)	Continuous	0.596	0.050	0.497 to 0.694	p<0.001
(THI-gm)^2	Continuous, quadratic relation	-0.048	0.010	-0.066 to -0.029	p<0.001

Angle and direction	Continuous, -ve towards right of the head, +ve towards left of the head	0.016	0.003	0.011 to 0.022	p<0.001
Angle and direction^2	Continuous, -ve towards right of the head, +ve towards left of the head	-0.0004	0.0001	-0.0006 to -0.0002	p<0.001
Elevation and direction	Continuous, -ve downwards , +ve upwards	-0.012	0.005	-0.022 to -0.002	p=0.014
age	Continuous	-0.146	0.064	-0.270 to -0.021	p=0.022
Age ^2	Continuous, quadratic relation	0.001	0.0005	0.0003 to 0.0022	p=0.012
Right ear average (front view)					
Variable	Details of the variable	Coefficient	S.E.	C.I. (95% confidence)	p-value
constant		28.075	0.510	27.076 to 29.073	p<0.001
(THI-gm)	Continuous,	0.524	0.051	0.424 to 0.623	p<0.001
(THI-gm)^2	Continuous, quadratic relation	-0.040	0.010	-0.059 to -0.021	p<0.001
Angle and direction	Continuous	-0.013	0.002	-0.018 to -0.008	p<0.001
time	Continuous	-0.0013	0.0005	-0.0024 to -0.0003	p=0.015

Table 32. Final model for Ear average temperatures (front view).

Left nostril Max					
Variable	Details of the variable	Coefficient	S.E.	C.I. (95% confidence)	p-value
constant		30.442	0.487	29.488 to 31.396	p<0.001
(THI-gm)	Continuous	0.352	0.074	0.208 to 0.496	p<0.001
time	Continuous	0.005	0.001	0.002 to 0.007	p<0.001
Time ^2	Continuous , quadratic relationship	-0.000010	0.000003	-1.6E-05 to -4E-06	p=0.002

Right nostril Max					
Variable	Details of the variable	Coefficient	S.E.	C.I. (95% confidence)	p-value
constant		30.737	0.497	29.762 to 31.712	p<0.001
Elevation and direction	Continuous, +ve upwards and -ve downwards	0.0149	0.0033	0.008 to 0.022	p<0.001
(THI-gm)	continuous	0.3887	0.0745	0.243 to 0.535	p<0.001
Angle and direction	Continuous, -ve towards right of the head, +ve towards left of the head	-0.0061	0.0018	-0.010 to -0.003	p=0.001
time	continuous	0.004	0.001	0.001 to 0.007	p=0.002
Time^2	Continuous, quadratic relation	-9E-06	0.000003	-1.5E-05 to -2E-06	p=0.008

Table 33. Final models for nostril maximum temperatures.

Left nostril Ave					
Variable	Details of the variable	Coefficient	S.E.	C.I. (95% confidence)	p-value
constant		28.753	0.511	27.752 to 29.754	p<0.001
Angle and direction	Continuous, -ve towards right of the head, +ve towards left of the head	0.009	0.002	0.005 to 0.012	p<0.001
Angle and direction ^2	Continuous, quadratic relation	-0.0002	0.0001	-0.0004 to -0.0001	p=0.001

(THI-gm)	Continuous	0.458	0.076	0.308 to 0.607	p<0.001
time	continuous	0.003	0.001	0.001 to 0.006	p=0.009
Time^2	Continuous, quadratic relation	-9E-06	0.000003	-1.6E-05 to -3E-06	p=0.005
Elevation and direction	Continuous, +ve upwards and -ve downwards	-0.005	0.005	-0.014 to 0.003	p=0.231
Elevation and direction^2	Continuous, quadratic relation	-0.0004	0.0002	-0.0007 to -0.0001	p=0.012
Right nostril ave					
Variable	Details of the variable	Coefficient	S.E.	C.I. (95% confidence)	p-value
constant		30.085	0.781	28.554 to 31.616	p<0.001
(THI-gm)	Continuous	0.455	0.080	0.299 to 0.610	p<0.001
(THI-gm)^2	Continuous, quadratic relation	-0.032	0.015	-0.061 to -0.002	p=0.035
Elevation and direction	Continuous, +ve upwards and -ve downwards	-0.007	0.004	-0.015 to 0.001	p=0.097
Elevation and direction^2	Continuous, quadratic relation	-0.0007	0.0002	-0.001 to -0.0004	p<0.001
Angle and direction	Continuous, -ve towards right of the head, +ve towards left of the head	-0.006	0.002	-0.009 to -0.003	p<0.001

Table 34. Final models for nostril average temperatures.

Left nasal airway Max					
Variable	Details of the variable	Coefficient	S.E.	C.I. (95% confidence)	p-value
constant		28.078	0.805	26.501 to 29.654	p <0.001
(THI-gm)	Continuous	0.676	0.081	0.517 to 0.835	p <0.001
(THI-gm)^2	Continuous, quadratic relation	-0.034	0.015	-0.064 to -0.004	p=0.025
Time	continuous	0.006	0.001	0.004 to 0.009	p <0.001

Time^2	Continuous, quadratic relation	-1.3E-05	0.000003	-0.00002 to -6E-06	p <0.001
Elevation and direction	Continuous, +ve upwards and -ve downwards	-0.0001	0.005	-0.009 to 0.009	p=0.991
Elevation and direction^2	Continuous, quadratic relation	-0.0006	0.0002	-0.001 to -0.0003	p <0.001
Angle and direction	Continuous, -ve towards right of the head, +ve towards left of the head	0.008	0.002	0.004 to 0.012	p <0.001
Right nasal airway Max					
Variable	Details of the variable	Coefficient	S.E.	C.I. (95% confidence)	p-value
constant		28.613	0.86	26.926 to 30.299	p <0.001
(THI-gm)	Continuous	0.652	0.087	0.482 to 0.822	p <0.001
(THI-gm)^2	Continuous, quadratic relation	-0.035	0.016	-0.067 to -0.003	p=0.033
Elevation and direction	Continuous, +ve upwards and -ve downwards	0.018	0.003	0.012 to 0.025	p <0.001
Time	continuous	0.004	0.001	0.001 to 0.007	p=0.003
Time^2	Continuous, quadratic relation	-0.00001	0.000003	-1.7E-05 to -3E-06	p=0.003
Angle and direction	Continuous, -ve towards right of the head, +ve towards left of the head	-0.004	0.002	-0.008 to -0.001	p=0.014

Table 35. Final models for nasal airway maximum temperatures.

Left nasal airway average					
Variable	Details of the variable	Coefficient	S.E.	C.I. (95% confidence)	p-value
constant		23.613	1.080	21.496 to 25.730	p <0.001
(THI-gm)	Continuous	0.661	0.069	0.526 to 0.796	p <0.001
(THI-gm) ^2	Continuous, quadratic relation	-0.030	0.013	-0.056 to -0.005	p =0.02

Angle and direction	Continuous, -ve towards right of the head, +ve towards left of the head	0.006	0.001	0.004 to 0.008	p <0.001
Time	continuous	0.008	0.002	0.004 to 0.012	p <0.001
Time^2	Continuous, quadratic relation	-3.4E-05	1.1E-05	-5.6E-05 to -1.3E-05	p =0.002
Time^3	Continuous, cubic relation	4E-08	2E-08	1E-08 to 8E-08	p =0.01
Elevation	Continuous, regardless direction	-0.009	0.003	-0.014 to -0.003	p =0.002
Age	Continuous	0.038	0.018	0.003 to 0.074	p =0.032
Right nasal airway average					
Variable	Details of the variable	Coefficient	S.E.	C.I. (95% confidence)	p-value
constant		24.159	1.150	21.905 to 26.413	p <0.001
(THI-gm)	Continuous	0.638	0.073	0.494 to 0.782	p <0.001
(THI-gm)^2	Continuous, quadratic relation	-0.034	0.014	-0.061 to -0.007	p =0.014
Elevation and direction	Continuous, +ve upwards and -ve downwards	0.013	0.002	0.009 to 0.018	p <0.001
Time	continuous	0.007	0.002	0.003 to 0.011	p <0.001
Time^2	Continuous, quadratic relation	-4.2E-05	1.11E-05	-6.4E-05 to -2.1E-05	p <0.001
Time^3	Continuous, cubic relation	6E-08	2E-08	2E-08 to 9E-08	p =0.001
age	continuous	0.042	0.019	0.005 to 0.080	p =0.027

Table 36. Final models for nasal airway average temperatures.

Muzzle Max					
Variable	Details of the variable	Coefficient	S.E.	C.I. (95% confidence)	p-value
constant		34.023	0.410	33.220 to 34.826	p<0.001
Elevation and direction	Continuous, +ve upwards and -ve downwards	0.0180	0.003	0.012 to 0.024	p<0.001

(THI-gm)	Continuous	0.211	0.041	0.131 to 0.292	p<0.001
(THI-gm)^2	Continuous, quadratic relation	-0.020	0.008	-0.035 to -0.004	p=0.012
Angle	Continuous, regardless direction	-0.007	0.003	-0.012 to -0.001	p=0.014
Muzzle ave					
Variable	Details of the variable	Coefficient	S.E.	C.I. (95% confidence)	p-value
constant		31.203	0.717	29.799	p<0.001
Elevation and direction	Continuous, +ve upwards and -ve downwards	-0.005	0.006	-0.017 to 0.008	p=0.481
Elevation and direction^2	Continuous, quadratic relation	-2.5E-05	0.00018	-0.000369 to 0.000318	p=0.885
Elevation and direction^3	Continuous, cubic relation	0.000027	0.000008	0.00001 to 0.000044	p=0.001
(THI-gm)	Continuous	0.468	0.072	0.327 to 0.610	p<0.001
(THI-gm)^2	Continuous, quadratic relation	-0.042	0.014	-0.069 to -0.016	p=0.002
time	continuous	0.004	0.001	0.002 to 0.006	p=0.001
Time^2	Continuous, quadratic relation	-0.000369 to 0.000318	-0.000369 to 0.000318	-0.000013 to -0.000002	p=0.005
Hair whorl max					
Variable	Details of the variable	Coefficient	S.E.	C.I. (95% confidence)	p-value
constant		30.494	0.509	29.496 to 31.492	p<0.001
Elevation and direction	Continuous, +ve upwards and -ve downwards	-0.031	0.004	-0.039 to -0.023	p<0.001
Elevation and direction^2	Continuous, quadratic relation	-0.0005	0.0002	-0.0008 to -0.0002	p=0.001

time	continuous	0.007	0.0012	0.004 to 0.009	p<0.001
Time^2	Continuous, quadratic relation	-1.5E-05	0.000003	-2.1E-05 to -9E-06	p<0.001
(THI-gm)	Continuous	0.216	0.051	0.117 to 0.315	p<0.001
(THI-gm)^2	Continuous, quadratic relation	-0.020	0.010	-0.039 to -0.002	p=0.035
Angle and direction	Continuous, -ve towards right of the head, +ve towards left of the head	-0.009	0.003	-0.0160 to -0.003	p=0.006
Angle and direction^2	Continuous, quadratic relation	-8.8E-05	0.000066	-0.00022 to 0.00004	p=0.177
Angle and direction^3	Continuous, cubic relation	0.000006	0.000003	0.000001 to 0.000011	p=0.026

Table 37. Final models for muzzle average, and muzzle and hair whorl maximum temperatures.

Left eyeball average					
Variable	Details of the variable	Coefficient	S.E.	C.I. (95% confidence)	p-value
constant		31.378	0.263	30.863 to 31.894	p <0.001

Angle and direction	Continuous, -ve towards the back, +ve towards the front	-0.008	0.003	-0.013 to -0.003	p=0.003
Angle and direction ^2	Continuous, quadratic relation	0.0004	0.00006	0.0003 to 0.0006	p <0.001
time	continuous	-0.004	0.001	-0.002 to 0.011	p <0.001
Time ^2	Continuous, quadratic relation	-6.9E-05	-8E-06	-6.9E-5 to -8E-6	p=0.163
Time ^3	Continuous, cubic relation	6E-08	2E-08	2E-08 to 1E-07	p=0.008
(THI-gm)	Continuous	0.180	0.033	0.116 to 0.245	p <0.001
Right eyeball average					
Variable	Details of the variable	Coefficient	S.E.	C.I. (95% confidence)	p-value
constant		31.7795	0.1929	31.402 to 32.158	p <0.001
Angle and direction	Continuous, -ve towards the back, +ve towards the front	-0.0078	0.0028	-0.013 to -0.002	p=0.006
Angle and direction ^2	Continuous, quadratic relation	0.0004	0.0001	0.0002 to 0.0005	p <0.001
(THI-gm)	Continuous	0.146	0.0273	0.093 to 0.200	p <0.001
time	continuous	-0.0009	0.0004	-0.002 to -0.0001	p=0.022

Table 38. Final models for eyeball average temperatures.

Left inner corner max					
Variable	Details of the variable	Coefficient	S.E.	C.I. (95% confidence)	p-value
constant		35.407	0.180	35.055 to 35.760	p <0.001

Angle and direction	Continuous, -ve towards the back, +ve towards the front	0.018	0.002	0.015 to 0.021	p <0.001
Angle and direction ^2	Continuous, quadratic relation	-0.0001	0	-0.0002 to -0.0001	p <0.001
(THI-gm)	Continuous	0.118	0.015	0.089 to 0.148	p <0.001
(THI-gm)^2	Continuous, quadratic relation	-0.006	0.003	-0.012 to 0.0004	p=0.035
time	continuous	0.005	0.002	0.002 to 0.009	p=0.004
Time ^2	Continuous, quadratic relation	-2.8E-05	9.01E-06	-4.5E-05 to -9.9E-06	p=0.002
Time ^3	Continuous, cubic relation	4E-08	1E-08	1E-08 to 6E-08	p=0.003
Right inner corner max					
Variable	Details of the variable	Coefficient	S.E.	C.I. (95% confidence)	p-value
constant		35.498	0.115	35.273 to 35.723	p <0.001
Angle and direction	Continuous, -ve towards the back, +ve towards the front	0.024	0.002	0.020 to 0.027	p <0.001
Angle and direction ^2	Continuous, quadratic relation	-0.0002	3.69E-05	-0.0003 to -0.0002	p <0.001
(THI-gm)	Continuous	0.108	0.014	0.081 to 0.136	p <0.001
Time	continuous	-0.004	0.002	-0.0067 to -0.0007	p=0.015
Time^2	Continuous, quadratic relation	2.63E-05	8.03E-06	1.06E-05 to 4.2E-05	p=0.001
Time^3	Continuous, cubic relation	-4E-08	1E-08	-6E-08 to -2E-08	p=0.001

Table 39. Final models for inner corner max temperatures.

Left rostral eye surrounding					
Variable	Details of the variable	Coefficient	S.E.	C.I. (95% confidence)	p-value
constant		34.110	0.297	33.528 to 34.693	p <0.001

Angle and direction	Continuous, -ve towards the back, +ve towards the front	0.046	0.004	0.039 to 0.053	p <0.001
Angle and direction ^2	Continuous, quadratic relation	-0.0006	0.0001	-0.0008 to -0.0004	p <0.001
(THI-gm)	Continuous	0.221	0.029	0.164 to 0.277	p <0.001
(THI-gm)^2	Continuous, quadratic relation	-0.013	0.006	-0.024 to -0.002	p=0.025
Right rostral eye surrounding					
Variable	Details of the variable	Coefficient	S.E.	C.I. (95% confidence)	p-value
constant		34.203	0.267	33.679 to 34.726	p <0.001
Angle and direction	Continuous, -ve towards the back, +ve towards the front	0.0548	0.0031	0.049 to 0.061	p <0.001
Angle and direction ^2	Continuous, quadratic relation	-0.0011	0.0002	-0.001 to -0.0008	p <0.001
Angle and direction ^3	Continuous, cubic relation	6.7E-6	2.6E-6	1.5E-6 to 1.18E-5	p=0.011
(THI-gm)	Continuous	0.178	0.025	0.129 to 0.227	p <0.001
(THI-gm)^2	Continuous, quadratic relation	-0.0117	0.0048	-0.021 to -0.002	p=0.015

Table 40. Final models for rostral eye surrounding max temperatures.

Left caudal eye surrounding					
Variable	Details of the variable	Coefficient	S.E.	C.I. (95% confidence)	p-value
constant		32.106	0.354	31.413 to 32.799	p<0.001

Angle and direction	Continuous, -ve towards the back, +ve towards the front	-0.022	0.003	-0.029 to -0.016	p<0.001
Angle and direction ^2	Continuous, quadratic relation	-0.001	0.0001	-0.001 to -0.001	p<0.001
(THI-gm)	Continuous	0.220	0.020	0.180 to 0.260	p<0.001
(THI-gm)^2	Continuous, quadratic relation	-0.0171	0.004	-0.025 to -0.009	p<0.001
Age	Continuous	0.032	0.006	0.021 to 0.043	p<0.001
Time	continuous	0.006	0.002	0.003 to 0.009	p<0.001
Time^2	Continuous, quadratic relation	-1.3E-05	0.000004	-0.00002 to -5E-06	p=0.001
Right caudal eye surrounding					
Variable	Details of the variable	Coefficient	S.E.	C.I. (95% confidence)	p-value
constant		32.994	0.428	32.155 to 33.834	p<0.001
Angle and direction	Continuous, -ve towards the back, +ve towards the front	-0.014	0.003	-0.020 to -0.008	p<0.001
Angle and direction ^2	Continuous, quadratic relation	-0.0007	0.0001	-0.0008 to -0.0005	p<0.001
(THI-gm)	Continuous	0.199	0.025	0.150 to 0.248	p<0.001
(THI-gm)^2	Continuous, quadratic relation	-0.013	0.005	-0.023 to -0.004	p=0.007
Age	Continuous	0.030	0.007	0.017 to 0.043	p<0.001

Table 41. Final models for caudal eye surrounding max temperatures.

Left ear Max (back view)					
Variable	Details of the variable	Coefficient	S.E.	C.I. (95% confidence)	p-value

constant		28.405	0.683	27.0661 to 29.7446	p<0.001
(THI-gm)	Continuous	0.388	0.104	0.1837 to 0.5922	p<0.001
Right ear Max (back view)					
Variable	Details of the variable	Coefficient	S.E.	C.I. (95% confidence)	p-value
constant		29.01	0.636	27.764 to 30.256	p<0.001
(THI-gm)	Continuous	0.377	0.081	0.219 to 0.535	p<0.001
Time	Continuous	0.004	0.002	0.0004 to 0.007	p=0.030

Table 42. Final models for ear maximum temperatures (back view).

Left ear Average (back view)					
Variable	Details of the variable	Coefficient	S.E.	C.I. (95% confidence)	p-value
constant		25.590	0.725	24.170 to 27.010	p<0.001
(THI-gm)	Continuous	0.478	0.111	0.261 to 0.695	p<0.001
Elevation and direction	Continuous, +ve upwards and -ve downwards	0.039	0.019	0.002 to 0.076	p=0.039
Right ear Average (back view)					
Variable	Details of the variable	Coefficient	S.E.	C.I. (95% confidence)	p-value
constant		26.870	0.583	25.727 to 28.012	p<0.001
(THI-gm)	Continuous	0.451	0.089	0.276 to 0.625	p<0.001
Elevation and direction	Continuous, +ve upwards and -ve downwards	0.032	0.014	0.004 to 0.061	p=0.024

Table 43. Final models for ear average temperatures (back view).

Left – Right ear Max (front view)					
Variable	Details of the variable	Coefficient	S.E.	C.I. (95% confidence)	p-value

constant		-0.173	0.396	-0.949 to 0.603	p=0.662
Angle and direction	Continuous, -ve towards right of the head, +ve towards left of the head	0.022	0.003	0.016 to 0.029	p<0.001
Elevation and direction	Continuous, -ve downwards , +ve upwards	-0.019	0.006	-0.030to -0.008	p=0.001
(THI-gm)	Continuous	0.052	0.039	-0.024 to 0.128	p=0.180
(THI-gm)^2	Continuous, quadratic relation	-0.015	0.008	-0.030 to -0.0002	p=0.047
Left - Right ear ave (front view)					
Variable	Details of the variable	Coefficient	S.E.	C.I. (95% confidence)	p-value
constant		-0.693	0.228	-1.139 to -0.246	p=0.002
Angle and direction	Continuous, -ve towards right of the head, +ve towards left of the head	0.027	0.004	0.020 to 0.034	p<0.001
Angle and direction ^2	Continuous, quadratic relation	-0.0004	0.0001	-0.0007 to -0.0002	p=0.002
Elevation and direction	Continuous, -ve downwards , +ve upwards	-0.018	0.006	-0.03 to -0.006	p=0.003
(THI-gm)	Continuous	0.066	0.029	0.0085 to 0.124	p=0.025

Table 44. Final models for asymmetries in maximum and average ear temperatures.

Left-Right nostril Maximum					
Variable	Details of the variable	Coefficient	S.E.	C.I. (95% confidence)	p-value

constant		-0.021	0.169	-0.352 to 0.310	p=0.902
Angle and direction	Continuous, -ve towards right of the head, +ve towards left of the head	0.008	0.002	0.005 to 0.011	p<0.001
Elevation and direction	Continuous, +ve upwards and -ve downwards	0.011	0.007	-0.003 to 0.026	p=0.119
Elevation and direction^2	Continuous, quadratic relation	-0.0001	0.0002	-0.0004 to 0.0003	p=0.783
Elevation and direction^3	Continuous, cubic relation	-2.4E-05	0.000009	-0.000042 to -0.000005	p=0.012
Left - Right nostril Average					
Variable	Details of the variable	Coefficient	S.E.	C.I. (95% confidence)	p-value
constant		0.082	0.177	-0.265 to 0.428	p=0.643
Angle and direction	Continuous, -ve towards right of the head, +ve towards left of the head	0.010	0.003	0.005 to 0.016	p<0.001
Angle and direction^2	Continuous, quadratic relation	-0.0002	0.0001	-0.0003 to -0.0001	p=0.002
Angle and direction^3	Continuous, cubic relation	0.000004	0.000002	0 to 0.000009	p=0.047
Elevation	Continuous, regardless of direction	0.008	0.003	0.002 to 0.015	p=0.015

Table 45. Final models for asymmetries in maximum and average nostril temperatures.

Left - Right nasal airway Max					
Variable	*Details of the variable*	*Coefficient*	S.E.	*C.I. (95% confidence)*	p-value

constant		-0.549	0.130	-0.802 to -0.296	p<0.001
Angle and direction	Continuous, -ve towards right of the head, +ve towards left of the head	0.013	0.002	0.010 to 0.016	p<0.001
Time	Continuous	0.001	0.0004	0.0003 to 0.0018	p=0.004
Elevation and direction	Continuous, +ve upwards and -ve downwards	-0.006	0.003	-0.0122 to -0.0007	p=0.029
Left - Right nasal airway Average					
Variable	*Details of the variable*	*Coefficient*	S.E.	*C.I. (95% confidence)*	p-value
constant		-0.671	0.118	-0.903 to -0.439	p<0.001
Angle and direction	Continuous, -ve towards right of the head, +ve towards left of the head	0.018	0.002	0.014 to 0.022	p<0.001
Angle and direction^2	Continuous, quadratic relation	-0.00002	0.000042	-0.000102 to 0.000063	p=0.639
Angle and direction^3	Continuous, cubic relation	-8E-06	0.000002	-0.000011 to -0.000005	p<0.001
Time	categorical	0.0014	0.0002	0.0009 to 0.0019	p<0.001
Elevation and direction	Continuous, +ve upwards and -ve downwards	-0.0076	0.002	-0.0115 to -0.0038	p<0.001

Table 46. Final models for asymmetries in maximum and average nasal airway temperatures.

Left-Right Inner corner Maximum					
Variable	Details of the variable	Coefficient	S.E.	C.I. (95% confidence)	p-value

constant		0.200	0.121	-0.037 to 0.436	p=0.098
Time for left image	continuous	-0.005	0.002	-0.009 to -0.001	p=0.011
Angle and direction	Continuous, -ve towards back of the head, +ve towards the front	-0.001	0.0004	-0.002 to -0.0003	p=0.009

Table 47. Final models for asymmetries in maximum inner corner temperatures.

Left-Right Eyeball Average					
Variable	Details of the variable	Coefficient	S.E.	C.I. (95% confidence)	p-value
constant		-0.027	0.192	-0.404 to 0.350	p=0.889
Time for left image	continuous	-0.003	0.0009	-0.005 to -0.002	p<0.001
Time for right image	Continuous	0.003	0.0009	0.0009 to 0.004	p=0.003

Table 48. Final models for asymmetries in eyeball average temperatures.

Left-Right Rostral eye surrounding Maximum					
Variable	Details of the variable	Coefficient	S.E.	C.I. (95% confidence)	p-value
constant		0.312	0.146	0.026 to 0.599	p=0.033
Angle and direction	Continuous, -ve towards back of the head, +ve towards the front	-0.021	0.005	-0.031 to -0.010	p<0.001
Angle and direction^2	Continuous, quadratic relation	0.0003	0.0001	0 to 0.0005	p=0.027

Table 49. Final models for asymmetries in rostral eye surrounding temperatures.

Left-Right Caudal eye surrounding Maximum					
Variable	Details of the variable	Coefficient	S.E.	C.I. (95% confidence)	p-value

constant		0.231	0.605	-0.954 to 1.416	p=0.703
Angle and direction	Continuous, -ve towards back of the head, +ve towards the front	-0.019	0.003	-0.026 to -0.0129	p<0.001
Time right image	Continuous,	0.010	0.002	0.007 to 0.014	p<0.001
Time right image^2	Continuous, quadratic relation	-2.4E-05	0.000004	-0.000032 to - 0.000015	p<0.001
(age)	Continuous	-0.061	0.021	-0.102 to -0.021	p=0.003
(age) ^2	Continuous, quadratic relation	0.0005	0.0002	0.0002 to 0.0008	p=0.001

Table 50. Final models for asymmetries in caudal eye surrounding temperatures.