

UNIVERSITÀ DEGLI STUDI DI PARMA

DIPARTIMENTO DI NEUROSCIENZE

Dottorato di Ricerca in BIOLOGIA DEL COMPORTAMENTO
XXVIII Ciclo

BEHAVIORAL EXPRESSION OF EMOTIONS AND NON-INVASIVE
ASSESSMENT OF PHYSIOLOGICAL CORRELATES IN DOGS
(*CANIS FAMILIARIS*)

Coordinatore del Dottorato di Ricerca: Prof. STEFANO PARMIGIANI

Relatore:
Prof.ssa PAOLA MARIA VALSECCHI

Dottorando:
TIZIANO TRAVAIN

ABSTRACT	5
1. INTRODUCTION	7
1.1. DOGS. WHEN? WHO?	7
1.1.1. <i>When was the first dog born?</i>	7
1.1.2. <i>Who are the dogs today?</i>	9
1.2. EMOTIONS	14
1.2.1. <i>Role of emotions</i>	15
1.2.2. <i>Neuro-physiological component of emotions</i>	16
1.3. PARAMETERS AND TECHNIQUES.....	17
1.3.1. <i>Infrared thermography</i>	17
1.3.2. <i>Heart rate variability</i>	18
1.3.3. <i>Telemetry</i>	21
1.4. AIMS.....	21
2. HOT DOGS: THERMOGRAPHY IN THE ASSESSMENT OF STRESS IN DOGS (CANIS LUPUS FAMILIARIS) – A PILOT STUDY	23
2.1. ABSTRACT	25
2.2. INTRODUCTION	27
2.3. MATERIALS AND METHODS.....	29
2.3.1. <i>Pre-test</i>	29
2.3.1.1. Subjects	29
2.3.1.2. Procedure	29
2.3.1.3. Data collection.....	30
2.3.1.4. Statistical analysis.....	31
2.3.2. <i>Test</i>	31
2.3.2.1. Subjects	31
2.3.2.2. Procedure	31
2.3.2.3. Data collection.....	32
2.3.2.4. Statistical analysis.....	33
2.4. RESULTS	34
2.4.1. <i>Pre-test</i>	34
2.4.2. <i>Test</i>	34
2.4.2.1. Thermographic data	34
2.4.2.2. Behavioral data	35
2.5. DISCUSSION.....	37
2.6. CONCLUSIONS.....	39
2.7. ETHIC STATEMENT	39

3. HOW GOOD IS THIS FOOD? A STUDY ON DOGS' EMOTIONAL RESPONSES TO A POTENTIALLY PLEASANT EVENT USING INFRARED THERMOGRAPHY	41
3.1. ABSTRACT	43
3.2. INTRODUCTION	45
3.3. MATERIALS AND METHODS.....	46
3.3.1. <i>Subjects</i>	46
3.3.2. <i>Procedure</i>	47
3.3.3. <i>Behavioral measurements</i>	48
3.3.4. <i>Physiological measurements</i>	50
3.3.5. <i>Statistical analysis</i>	51
3.4. RESULTS	52
3.4.1. <i>Behavioral data</i>	52
3.4.1. <i>Physiological data</i>	54
3.4.1.1. Eye temperature.....	54
3.4.1.2. Heart rate and heart rate variability.....	55
3.5. DISCUSSION.....	56
3.6. CONCLUSION	58
3.7. ETHIC STATEMENT	58
4. BEHAVIORAL AND PHYSIOLOGICAL CHANGES IN DOGS DURING A SEPARATION/REUNION PROTOCOL.....	61
4.1. ABSTRACT	63
4.2. INTRODUCTION	65
4.3. MATERIALS AND METHODS.....	66
4.3.1. <i>Subjects</i>	66
4.3.2. <i>Procedure</i>	67
4.3.3. <i>Behavioral measurements</i>	68
4.3.4. <i>Physiological parameters</i>	70
4.3.5. <i>Statistical analysis</i>	71
4.4. RESULTS	72
4.4.1. <i>Behavioral data</i>	72
4.4.2. <i>Physiological data</i>	76
4.5. DISCUSSION.....	77
4.6. CONCLUSION	79
4.7. ETHIC STATEMENT	80

5. ASSESSING BEHAVIORAL AND PHYSIOLOGICAL EFFECTS OF DIFFERENT ENVIRONMENTAL ENRICHMENTS IN ISOLATED SHELTERED MALE DOGS	81
5.1. ABSTRACT	83
5.2. INTRODUCTION	85
5.3. MATERIALS AND METHODS.....	88
5.3.1. <i>Subjects</i>	88
5.3.2. <i>Procedure</i>	88
5.3.3. <i>Telemeter and physiological parameters</i>	89
5.3.4. <i>Behavioral measures</i>	90
5.3.5. <i>Statistical analysis</i>	92
5.3.6. <i>Two different approaches</i>	92
5.3.6.1. Approach 1: Comparing the four weeks.....	92
5.3.6.2. Approach 2: The effect of female presence	92
5.4. RESULTS	93
5.4.1. <i>Approach 1: Comparing the four weeks</i>	93
5.4.1.1. Physiological parameters	93
5.4.1.1.1. Brando	93
5.4.1.1.2. Buck.....	93
5.4.1.1.3. Jack.....	94
5.4.1.2. Behavioral parameters	95
5.4.1.2.1. Brando	96
5.4.1.2.2. Buck.....	97
5.4.1.2.3. Jack.....	98
5.4.2. <i>Approach 2: The effect of female presence</i>	99
5.4.2.1. Brad	99
5.4.2.2. Buck.....	100
5.4.2.3. Jack.....	101
5.4.2.4. Scotty.....	102
5.4.2.5. Sparrow	103
5.5. DISCUSSION.....	104
5.6. CONCLUSION	107
5.7. ETHIC STATEMENT	107
6. GENERAL CONCLUSIONS.....	109
REFERENCES.....	113

Abstract

The aim of this thesis was to validate the use of infrared thermography (IRT) to non-invasively measure emotional reactions to different situations in pet dogs (*Canis familiaris*). A preliminary test, aimed to evaluate the correlation between eye-temperature and rectal temperature in dog, was performed. Then, in three different situations, negative (veterinary visit), positive (palatable food rewards), and mildly stressing followed by mildly positive (separation from and reunion with the owner), variations in heat emitted from lacrimal caruncle (referred to as eye temperature) were measured with an infrared thermographic camera. In addition, heart rate and heart rate variability parameters were collected using a non-invasive heart rate monitor designed for human use and validated on dogs. All experiments were video recorded to allow behavioral coding. During the negative situation dogs' level of activity and stress related behaviors varied across compared to the baseline and dogs showed an increase in eye temperature despite having a significant decrease in the level of activity. The positive situation was characterized by a peak in eye temperature and mean HR and dogs engaged in behaviors indicating a positive arousal, focusing on food treats and tail wagging but there were not variations in HRV during stimulation but only an increment in SDNN immediately after the stimulus. In the separation from and reunion with the owner dogs' eye temperature and mean HR did not vary neither in the stressful nor in the positive situations, RMSSD increased after the positive episode, SDNN dropped during the two stimulations and it increased after the stimulations. During the separation from the owner dogs were mainly directed to the door or to the experimenter while during the reunion with the owner dogs were focused mainly on the owner and on the environment, exhibiting safe base effect.

A different approach was used to assess the welfare of shelter dogs. Dogs were implanted with a telemeter and after implantation dogs were housed in sequence in four different situations lasting 1 week: alone, alone with toys and a stretch cot for sleeping, with an unknown, spayed, female, and alone with a daily 2-hours interaction with an experimenter. Two different approaches were tried: partially random extracted fragments from every week, behaviors from 8 a.m. to 4 p.m. were continuous during baseline and the female situation. Results showed different reactions by dogs to the different situations and interestingly not all enrichments were enjoyed by the dogs improving their welfare.

Overall results suggest that IRT may represent a useful tool to investigate emotional reactions in dogs. Nevertheless, further research is needed to establish the specificity and sensitivity of IRT in this context and to assess how different dogs' characteristics, breed, previous experience and the valence and arousal elicited by the stimulus could influence the magnitude and type of the response. The role

of HRV in understanding emotional valence and the one of telemeters in understanding long-term effects on sheltered dogs' welfare is also discussed.

1. Introduction

1.1. Dogs. When? Who?

Class: Mammalia

Order: Carnivora

Family: Canidae

Genus: *Canis*

Species: *C. lupus familiaris*



Figure 1.1. Blacky, German shepherd.

The domestic dog is a canid which has been domesticated and selectively bred for millennia for various behaviors, sensory capabilities, and physical and working abilities. Even if its origin is debated, dog is recognized as the oldest domesticated animal [1]. Today it is a widespread animal, who share our environment at every latitude and our everyday life. World dog population is estimated at 525 million [2], only 17-24% of them live in a developed country, mostly as pet dogs; others are working dogs owned by a single person, village dogs kept for working purposes owned by a whole community, very common in African rural areas, or dogs that are not used to human presence although living as scavengers in the nearby of human communities [3] often hybridized with other canid species such as coyotes (*Canis latrans*) or different wolf subspecies (*Canis lupus*) [4,5] and in some cultures dogs are bred as source of meat. In our society, dogs play many different roles other than being a pet: they can be protection, military, police, or sled dogs, or help us in hunting, herding, or assisting disabled people (e.g., movement, visual, or hearing impaired, diabetics or epileptics).

Summarizing, in Western civilization, dog-human relationship is a positive relationship both for dogs and for us. Pet dogs are usually considered as family members and dog owners report that they consider dog as confidants, protectors, and friends and how dogs provide them an important emotive support [6]. Some studies have highlighted how dogs can provide even improvements in our health since owning a pet can decrease risk of cardiovascular diseases, heart rate reactivity [7,8], blood pressure, and cholesterol and cortisol concentrations [9] while raising levels of oxytocin, phenylalanine, phenylacetic acid, β -endorphins, and dopamine [9,10].

1.1.1. When was the first dog born?

The origin of the dog is not clear, yet. Despite many years have passed since the image of a prehistoric man stealing a wolf cub from its mother's den [11], and even more years since Darwin proposed that dogs originated from two or more wild *Canis* species due to their enormous phenotypical differences [12], modern discoveries and new techniques as genome sequencing have

generated more questions than answers [1,13,14]. Present data, supported by genetic evidences, suggest that dog, gray wolf and Taymyr wolf (now extinct) shared a common ancestor from whom they diverged roughly together in a time window between 27,000 and 40,000 years before present [15]. Besides, it seems that wolves faced a very severe bottleneck immediately after their divergence from the common ancestor and as a result modern dogs are more closely related to ancient wolf fossils that have been found in Europe than they are to modern gray wolves [16,17]. Therefore, it is likely that all genetic commonalities between dogs and modern gray wolves are due to subsequent admixture [17], while several Arctic dog breeds seem to have commonalities with the Taymyr wolf of northern Asia due to admixture [15]. However, because the fossil remains of this direct ancestor of both dogs and wolves have yet to be found, its existence has not still confirmed.

As for the time, even the area of origin is disputed. In the last years, several independent domestication events have been located thanks to genetic analysis. At present, these areas are located in Africa [18], Arctic north-east Siberia [19], central Asia [20], eastern Asia [21,22], Middle East [23], Europe [16]. Likely, domestication of dog, and thus its origin, effectively happened in different times and areas. A 36,000-year-old skull found in Belgian caves of Goyet was found to be clearly different from recent wolves and was identified as a Paleolithic dog (*Canis cf. familiaris* [24]) after an osteometric analysis [25]. Later, after genetic studies, it was placed, along with other two specimens in an ancient sister-group to all modern dogs and wolves. It is debated if this clade may represent a new distinct population of gray wolf or a domestication episode without descendants [16]. Similarly, a 33,000-year-old skull of a dog-like canid was found in Razboinichya Cave (Altai Mountains, southern Siberia, central Asia) and it has not direct descendants today. Its osteometric analysis showed how the cranium was very close in size and shape to prehistoric Greenland dogs (1,000 years before present) while the lower carnassial tooth was significantly smaller than prehistoric wolves one and slightly smaller than modern European wolves, and the upper carnassial tooth was of a similar size of modern wolves one. This suggested that the skull belonged to a dog in the very early stages of domestication and, because there are not known descendants, probably represents a wolf domestication attempt disrupted by the changes associated with the last glacial maximum [26]. DNA analysis revealed that the specimen is more closely related to modern dogs and prehistoric North American canids than it is to contemporary wolves, supporting the idea of the canid being a precocious dog. Besides, this analysis showed as some dog breeds as Tibetan Mastiff, Newfoundland, Chinese Crested, Cocker Spaniel and Siberian Husky are related to the specimen [27]. A further study analyzing mtDNA suggested a position at the root of a clade including both wolves (two ancient and two modern genomes) and dogs (two genomes of Scandinavian origin) [16].

As previously stated, grey wolf is not the ancestor of modern dog: to identify genetic changes occurred during dog domestication genome sequences of three gray wolves from Croatia (Europe), Israel (Middle East), and China (East/South-East Asia) to represent the regions of Eurasia where domestication likely had taken place, of an Australian dingo and of a Basenji to represent lineages geographically distinct and whose home-range is isolated from wolf were compared. The data provided significant evidence of admixture between the Israeli wolf and both the Basenji and the boxer, and between the Chinese wolf and the Australian dingo. This suggested different admixture episodes; the Australian dingo with the Chinese wolf likely proves an admixture in eastern Asia, while both the Israeli wolf admixtures likely give evidences for the western Eurasia one. Considering that both dingo and basenji have been isolated from wolves in the recent past the gene flow might have been ancestral and has likely affected most dog lineages. This test indicated that dogs and modern wolves form sister clades sharing a common ancestor. Besides, same genome sequences compared with one of a golden jackal (*Canis aureus*) indicated that wolf, dog, and golden jackal shared a common ancestor from whom they divided about 400,000 years before present [17]. Another study evidenced how there was admixture between Taymyr wolf and high latitudes dog breeds. Due to admixture, some modern dog breeds have a closer association with either the gray or Taymyr wolf. While the Saarloos Wolfdog showed more association with the gray wolf due to be an established breed of dog originating hybridizing a gray wolf with a German Shepherd during 1930s, the Siberian Husky, the Greenland Dog, the Shar Pei, and the Finnish Spitz show a greater association with the Taymyr wolf. This indicated admixture between the Taymyr population and the ancestral dog population of these four breeds. Possible explanations of this phenomenon can be the very early presence of dogs in northern Eurasia or the genetic heritage of Taymyr wolves preserved in wolf populations until the arrival of dogs at high latitudes. Besides, it also provides other evidences indicating that modern dogs descends from more than one region [15].

1.1.2. Who are the dogs today?

After millennia of selective breeding modern dog breeds show more variation in size, appearance, and behavior than any other land mammal [28]. Despite being genetically similar [21], dogs can differ greatly in many phenotypic characteristics: height can vary between about 15 centimeters in the Chihuahua to about 76 cm in the Irish Wolfhound; color varies both from white through grays to black, and from light to dark; coats can be short or long, coarse-haired to wool-like, straight, curly, or smooth.

Most modern breeds are recent considering the whole evolutionary history of groups of dog. Besides, even if it would be possible to genetically distinguish among various purebred dogs, the

classification used by kennel clubs is zoological unsystematic. Despite the fact that the Fédération Cynologique Internationale recognizes over 400 pure dog breeds divided in 10 groups based on different parameters as appearance or use, dog genome analysis has revealed that only four major types of dogs can be considered distinct: "old world dogs" (e.g., Malamute and Shar Pei), "Mastiff" (e.g., English Mastiff), "herding" (e.g., Border Collie), and "modern/hunting" (a big group that includes all dog non included in the previous three) [29].

Dogs are animals characterized with highly developed cognitive skills, they are able to retrieve information and keep it as knowledge for applying to solve problems and they have been shown to learn by deduction. A study with Rico, a Border Collie, highlighted that he knew the labels of over 200 different items and that he inferred the names of novel items by exclusion learning and correctly retrieved them immediately and also 4 weeks after the initial training [30]. Dogs are also able to read human body language such as gesturing and pointing [31–33].

As a direct product of intensive selection, dogs have developed the ability to communicate with humans. They are uniquely attuned to our behavior [34] and they own a various set of social-cognitive skills that can be compared to those owned by toddlers [35]. For example, a study indicated that after being trained to solve a simple manipulation task, if dogs are faced with an insoluble version of the same task then they will look at humans seeking for help, while socialized wolves do not express this behavior [36].

Recently, researches are focusing about emotional bond between humans and dogs and results supports the idea that it resembles the one between human infants and their parents. In fact, domestic dogs are entirely dependent on human care in every aspect of everyday life and can develop an attachment bond towards their owners [37–39]. Attachment is defined as a particular form of affectional bond that endures over time and involves a specific individual, which is not interchangeable with anyone else that is called 'attachment figure'; this process is emotionally significant and promotes a series of behaviors aimed to maintain proximity and contact with the attachment figure. Moreover, this figure elicits confident behaviors such as exploring the environment and playing with objects and with unfamiliar persons, the so-called 'secure-base effect' [40].

Attachment was initially studied in human infants, Ainsworth [41] developed a standardized experimental procedure called the 'Strange Situation', that elicits attachment and exploratory behavior under conditions of increasing stress. Toddlers were left in an unfamiliar room, introduced to an unfamiliar adult (the stranger) and subjected to three short episodes of separation from the attachment figure (usually the mother). Successively, several experiments were carried out [37–39] to

test dog-owner dyads in an adapted version of the Strange Situation. Dogs were exposed to an unfamiliar setting (the testing room), a stranger entering the room, and brief separations from the owner, who left the room, followed by reunion with them. These experiments aimed at assessing if dogs exhibited the behavioral components of the attachment system: proximity seeking-maintenance, separation distress and secure-base effect. Collected data showed that the dogs had explorative behaviors and played more in presence of their owners compared to the strangers, greeted their owners more enthusiastically than the strangers during reunion episodes and stood by the door more when separated from their owner than from the stranger.

The crucial role that the relationships with the owner plays for dogs is emphasized in many other studies. Dog's manipulative behavior (e.g., chewing objects) is emphasized when the owner is present, regardless of owner's behavior [42]. Object manipulation is correlated with cognitive abilities and this confirms the remarkable similarity between the secure base effect in dogs and in humans within the cognitive realm. By using the Strange Situation Test on a set of dyads of cohabitants dog, it has been shown as a dog is to another dog a good companion but a human companion remains the figure to which a dog shows its greatest attachment [43]. This study shows indeed that the presence of a cohabitant dog mitigates the distress of being alone at the same extent as an unfamiliar person, but dogs did not show to each other the preference they had in the Strange Situation toward the owner.

The long evolutionary history shared by dogs and humans is responsible for dogs' unique social and cognitive skills. Dogs are able to interpret our referential gestures as pointing with a finger, a hand, an arm or the look to an interesting spot, object or person [31,44]. Most research on dogs' comprehension of referential gestures have a very simple experimental procedure: the dog has to choose between two recipients, only one hiding a food treat. An experimenter sits in proximity of the recipients and indicates one of them by looking, pointing or being oriented towards it. Usually, dogs do not choose randomly which recipient to approach but instead they reach more frequently that indicated by the experimenter, regardless the presence of food. A different study exposed dogs to an experimenter, sit between two recipients, pointing at one of those with its finger alone (by keeping at the same time the arm along the body), hand, arm, elbow or a stick. It resulted that dogs do not respond to any cue, but rather they need to see the arm protruding from the body or to have a signaler oriented to the target with its body. Instead, direction of the movements of the pointing arm did not influence dogs' reaction [44].

It is not clear how the ability to read human cues developed in dogs. However, dogs share this capability with goats [45], wolves [46], foxes [47], different species of primates [48–50], ravens [51],

dolphins [52], and horses [53,54]. It was probably the domestication process that led to the selection of this characteristic in dogs [45,47,55–60]. Supporting this theory, captive foxes selected for their tameness for generations could correctly interpret human referential gestures while non-selected foxes cannot [47]; on the other hand, young wolves raised by hand cannot interpret human referential gestures as dogs do [59]. Against the theory claiming a natural predisposition of dogs, there are studies that have compared the same skills in wolves socialized with humans, owned dogs and rescued dogs hosted in shelters [46,61] with wolves performing better than rescued dogs and as good as owned dogs in following experimenter's clues. Therefore, more than domestication, environment, experiences during ontogeny and socialization with humans could be key factors to the development of the peculiar social cognitive skills of dogs [62].

Nevertheless, dogs are not perfect in understanding our non-verbal communication and there is a limit to their ability to read human cues. In an experiment dogs were tested with a more complicated version of the "object choice task" [63] in which they had to understand indirect pointing towards a recipient (namely, pointing made not directly with the body but instead mediated by an object). An experimenter lifted and shook the target container using a centrally pulled rope in ways that were intended to be intentional or accidental. Results showed that dogs did not understand the experimenter's action during the intentional situation and the ostensive pulling of the rope worsened dogs' performance. This could be because dogs' communicative system is more dependent upon bodily markers, thus dogs need bodily cues.

After evaluating communicative skills of dogs, scientists focused about interpretation of emotions and being able to recognize others' emotions reading facial expressions would have huge advantages in social relationships, allowing adapting one's own behavior to others' emotional state [64]. At present, dogs seems to be able to discriminate different human faces with different expressions [65–69] and using them as a discriminative cue [69]. In this experiment, two groups of dogs were taught to discriminate between happy and angry faces in picture, although for one group only the upper half of the faces were shown and for the other group only the lower half. Successively, dogs faced four different situations: the same half of the faces as in the training but of novel faces, the other half of the faces used in training, the other half of novel faces, and the left half of the faces used in training. Dogs performed significantly above chance in all the tests, suggesting that they were able to transfer their skill from the training session to novel stimuli and dogs for which the happy faces were rewarded during the training learned the discrimination more quickly than dogs for which the angry faces were rewarded during the training and this support the idea that dogs recognize an angry face as an aversive stimulus.

Dogs can use owner's emotion towards an external object to obtain information towards that object. Five dog groups were tested: in the first group, dogs observed the owner having happy or fear reactions towards two identical objects hidden behind a barrier; in the second group, dogs observed the same reaction in the owner but without the object in the room; in the third and in the fourth group dogs observed respectively the owner expressing positive and negative emotions compared to neutral expressions; finally, in the fifth group dogs observed a stranger behaving like the owner of the first group. Results showed that dogs were able to distinguish between the owner's happy and a scared expression and that an object that elicited a positive emotion in the owner is more attractive than one associated with neutral or negative emotions [70]. This study follows another one [71] aimed to investigate social referencing, the use of emotional information provided by a sender about a novel object or stimulus to decide how interacting towards it, in dogs. Adult animals were exposed to four different situations involving an electric fan, with plastic green ribbons attached to it (a potentially scary object). During the experiment, their owner or a stranger acting as the sender delivered either a positive or negative emotional information. Most dogs looked referentially at the sender, regardless of them being the owner or a stranger; however, when the owner delivered a positive message about the fan, dogs changed their behavior, trying more frequently to approach the fan and, similarly, dogs that received a negative message took longer to approach the fan. On the other hand, there have been fewer differences in dogs' behavior when the informant was the stranger.

Empathy is defined as "the naturally occurring subjective experience of similarity between the feelings expressed by self and others without losing sight of whose feelings belong to whom" [72], therefore it is not just recognizing another's emotion. In dogs were found first evidences of interspecific contagious yawning outside primates [73], a reflex elicited by observing another person yawning. Different studies correlated it to the ability of experiencing empathy or emotional contagion [74–76]. Twenty-nine dogs were tested while observing a human yawning or making control mouth movements. In response, twenty-one dogs yawned when they observed a human yawning, but control movements did not elicit yawning. Later, another study [77] exposed dogs to a conversation between the owner and a stranger in which suddenly one of them began to cry or humming without giving any previous clue. Dogs gazed more often to the crying person, being it the owner or the stranger, and they behaved in a way that resembled an emphatic reaction, trying to offer comfort and approaching the person in a submissive, playful, calm or alert way. However, it was not possible to catalogue dogs' reactions as being elicited by empathy or by emotional contagion, a much simpler phenomenon in which a person feels emotions that are similar to those of others and they are influenced by them.

In this context, my research aims to find if it is possible to assess physiological parameters in the study of dog’s emotions and to contribute to provide new evidences about what dogs feel and what they can perceive of our emotions.

1.2. Emotions

“Everyone knows what an emotion is, until they have to define it” [78]. This quote perfectly explains the existing difficulties in assessing human and, above all, animal emotions. In 1872, Darwin published “The expression of the emotion in man and animals” [79] studying and comparing emotions, behavior, postures and expressions of different animal species. This was the first publication focused on animal emotion. Across years and cultures, many different definitions of “emotion” have been considered [80]. From a biological and ethological point of view, emotions are brief in duration and consist of a coordinated set of responses, which may include verbal, physiological, behavioral, and neural mechanisms [81]. An useful approach to investigate emotions is to embed them accordingly to Tinbergen’s four questions [82] (Table 1.1).

Table 1.1. Tinbergen's four questions about emotions.

Proximate explanation: i.e., physiological stimuli and mechanisms	Neurological, hormonal, and cognitive substrate
Ontogeny: i.e., role of experiences and development	Gene interaction, parental care, precocious socialization
Function: i.e., influence on survival and reproduction	Motivate expression of evolutive strategies to optimize fitness
Phylogeny: i.e., evolutionary history of behavior	Unchanged in structure and functions in a very long period along vertebrates’ evolutionary history

Emotions have been divided in two big categories, basic and complex emotions. First ones are universal mimic expressions characterized by a precise physiological activation pattern, while the latter are mostly dependent on culture, social norms, and learning. Different authors proposed a different number of basic emotions, even if at present those proposed by Ekman [83] are the most widely used and they are joy, surprise, sadness, fear, anger, and disgust. These emotions have universal specific traits and commonalities for their expression such as: facial expression, phylogenetic continuity of

expressed behaviors, physiological activation pattern, rapid onset, brief duration, and unconscious stimuli evaluation mechanism [83].

In addition to basal/complex distinction, emotions can also be divided in negative (surprise, sadness, fear, anger, and disgust) and positive. Negative emotions induce specific actions to avoid dangerous situations [84,85], sympathetic nervous system activation and urgency to act [86] while positive emotions are trends of thinking [87] stimulating creativity, curiosity, forming of social bonds, and inducing parasympathetic nervous system activation [87,88]

Moreover, they are characterized by a complex and multi-componential nature [89]. Their principal components are the following five:

1. **Subjective component** – subjective experience and non-verbal emotional experience monitoring;
2. **Neuro-physiological component** – central nervous system (CNS), autonomic nervous system (ANS), endocrine system activation; modification in physiological parameters (e.g., electrodermal, cardiovascular, and muscular activity);
3. **Cognitive component** – conscious or unconscious evaluation of the emotion and/or of the stimulus;
4. **Motivational component** – needs and purposes correlated to the expression of the emotion, concerning preparedness to action;
5. **Expressive-motor component** – observable behavior, verbal and non-verbal (e.g., posture and facial expressions) communication.

These emotional systems, that have a common origin, are widespread across all mammals and, likely, birds [90,91].

Finally, emotions can be defined as composed by two fundamental elements: arousal, the excitement level induced by the stimulus, and valence, evaluating the emotion as positive or negative according to the rewarding or discouraging nature of the stimulus [92].

1.2.1. Role of emotions

Levenson [93] wrote that basic emotions are the time-tested solutions to timeless problems. This means that emotions are responses triggered by difficulties and problems faced during development and lifetime. Therefore, emotions, both in humans and in animals, have a high adaptive value (e.g., relationship with others, avoiding harmful situations, defense of the resources). “If the mind is viewed as an integrated architecture of different special purpose mechanisms, designed to

solve various adaptive problems, a functional description of emotion immediately suggest itself” [84]. Emotions play a significant role in social relationships: abilities to express and understand emotional states and their meaning are essential to have a cohesive group, to adapt own behavior to others [68,79], and to have an efficient way to interact with the environment [94].

Each of these mechanisms can operate in different ways, alone or with other mechanisms. Natural selection had shaped them to work together harmoniously when facing recurrent adaptive situations, generation after generation. Events as fighting, fleeing, finding a suitable mating partner, foraging, and so on, have a very long evolutionary history and they require a certain subset of behaviors and motivations. The characteristic emotion that accompanies each need is the signal which activates the appropriate response to solve that problem [84].

The ability to correctly interpret others’ emotions is called empathy [72] and it is a keystone of “social competence”: understand what others know and being able to predict their behavior is the fundamental and one of the main factor of social intelligence [95]. Therefore, emotionality is extremely important for social societies. More complex the social system is, more important will be the cognitive mechanisms that control not only the pulses coming from the behavioral emotional systems, but also, to a certain extent, the emotional systems themselves [96].

1.2.2. Neuro-physiological component of emotions

As previously stated, my research aims to find if it is possible to assess physiological parameters in the study of dog’s emotions. The neuro-physiological component of emotions includes reactions mediated by central nervous system (CNS), autonomic nervous system (ANS), and endocrine system. These reactions induce modification in physiological parameters and physical responses, such as electrodermal activity, cardiovascular activity, muscular activity, or modifications in blood concentration of hormones that can be measured.

The sympathetic and the parasympathetic nervous systems are the two main components of the autonomic nervous system. Their functions are to regulate the body's unconscious actions. Emotions can heavily modify both sympathetic and parasympathetic (vagal) activity and towards the sinus node, that is subject to both of them, these modifications have effect on the body. Stress and physical exercise are correlated with an augmentation of sympathetic tone, that lead to an accelerated metabolism and stimulate a fight-or-flight response. On the other hand, vagal tone is predominant during both physically and mentally resting conditions, stimulating the rest-and-digest response. Sympathetic and parasympathetic tone are opposite and, in normal conditions, both fluctuate throughout the day. Therefore, the concept of sympathovagal balance has arisen to characterize the

autonomic balanced state resulting from them, describing the effects of the sympathetic and parasympathetic nervous systems on the sinus node [97]. The defensive response against both physical and social stressors due to sympathetic nervous system predominance induces even an increase in heart rate and body temperature known as stress induced hyperthermia (SIH) [98]. It occurs both prior to and during exposure to stress-inducing stimuli, like noise, heat, handling, novelty or pain [99–102], raising core temperature of few Celsius degrees, which might be beneficial to the fight-or-flight reaction warming up muscles and central nervous system [103].

Endocrine system is composed by different glands that secrete hormones directly into the circulatory system. These glands can be activated directly by ANS or by other hormones secreted by a different gland. Glands that activate each other in sequence using hormones are usually referred to as an axis (e.g., hypothalamic-pituitary-adrenal axis, or HPA axis). Different hormones trigger different effects and they are secreted in response to different conditions. For example, oxytocin is a neuropeptide normally produced by hypothalamus and stored in the poster pituitary gland involved in social recognition, maternal behaviors and intraspecific and interspecific pair bonding [104]. It has been reported that urinary oxytocin concentration increases in humans proportionally to the length of their dogs' gaze at them [105], and in both humans and dogs after positive interactions [10]. Its effects are significant even if it is not secreted by hypothalamus but intranasal administrated [106] improving the capability of dogs of following human pointing cues. Another example is given by hypothalamic-pituitary-adrenal axis, a set of direct influences and feedback interactions among these three endocrine glands. It is responsible for the control of reactions to stress and emotions and regulates many other body processes (e.g., digestion and immune system). HPA axis activation start with hypothalamus, that secretes corticotrophin releasing hormone (CRH); it will induce secretion of adrenocorticotrophic hormone (ACTH) from anterior pituitary gland; finally, ACTH will activate adrenal cortex to secrete glucocorticoid hormones (mainly, cortisol). Cortisol is a steroid hormone produced in response, other than to stressful situations, even to low blood glucose concentration, that increases gluconeogenesis and enhances of fat, protein, and carbohydrates providing more available energy; it also has a negative feedback effect on the HPA axis itself. Besides, an increase in oxytocin suppresses HPA axis, counteracting stress and its effects [107].

1.3. Parameters and techniques

1.3.1. Infrared thermography

Infrared thermography (IRT) is an innovative non-invasive method to appraise temperature variations. It has been extensively used in zootechny to investigate inflammatory conditions and

infections [108–111] and, more recently, to assess stressful situations [112–114]. Infrared thermography can detect superficial temperature of a body, creating an accurate graphic representation measuring the electromagnetic energy emitted by every object that has a superficial temperature (theoretically, when its temperature is higher than 0 K). A wavy flux of photons travels at the speed of light from the object to the camera and even small variations of superficial temperature can modify this flux; these alterations can easily be detected by an IRT camera [115,116].

A change in superficial temperature caused by a stressful situation is called stress induced hyperthermia [98], the heat is dissipated through body surface and it is possible to detect a decrement of temperature. In humans, IRT is a common technique used in psychophysiological studies concerning both positive and negative emotions [117–120]. Recently, in animals, Johnson and colleagues [121] have compared rectal and eye temperature in ponies, suggesting that eye temperature could be used to assess core temperature and its variations. Therefore, since there is a close relationship between stress and metabolic system, IRT has been used to assess acute and chronic stress in laboratory [122], farm [123], and sport [124] animals. The available literature concerning the use of IRT in the study of animal emotions points out that surface body temperature can increase/decrease depending on the investigated species, the anatomical areas and the type of stimulus used. Moe and colleagues [125] have used IRT to assess temperature variations in hens' comb when positively stimulated. Food anticipation and consumption elicited a diminution of superficial temperature; however, same response was elicited by a negative stimulation [126,127]. Different studies suggested that when mammals face a stressful or dangerous situation, their core temperature increases and this leads to speculate a correlation between internal temperature and affective states. Rhesus macaques (*Macaca mulatta*) nasal temperature significantly decreases when they are in a negative situation [128], while in cows nasal temperature decreases during positive situation [129]. Among different species, even in dogs a good correlation between eye and core temperature has been found [113].

In dogs, the lacrimal caruncle, a small area around the posterior border of the eyelid, has rich capillary beds and it is innervated by the sympathetic system allowing it to quickly respond to changes in blood flow [130,131]. The lacrimal caruncle is very sensitive to emotional changes and variations of its temperature can be imputed both to sympathetic response of autonomic nervous system and to HPA axis activation [124,131]

1.3.2. Heart rate variability

Heart rate variability (HRV) is the physiological phenomenon of variation in the time interval between heartbeats. It is measured by the variation in the beat-to-beat interval [132]. Its analysis

allows to estimate the activity of cardiac control neural mechanisms. In particular, it has been observed that a high heart rate variability is associated with a good ability of an organism to adapt itself to changes of internal and external conditions and this might indicate a good functioning of neural mechanisms. On the other hand, a low heart rate variability is an indicator of an insufficient adaptability [133].

As stated in paragraph 1.2.2, autonomic nervous system (ANS) is divided in two main branches: sympathetic nervous system (SNS) and parasympathetic nervous system (PSNS). These branches are characterized by a different time kinetics: PSNS can act in milliseconds while SNS requires even seconds to react; consequently, they have a very different potentiality to modulate cardiac activity. The great variability in the beat-to-beat interval is due mainly vagal (parasympathetic) influence [134].

It is possible to evaluate the activity of the ANS using HRV parameters; since the sixties, researches and clinical test to investigate these parameters are common. In addition to clinical application, HRV can be used to explore many physiological processes. For example, body posture changes ANS activity: the erect position is characterized by a sympathetic dominance, while the supine position has a greater vagal dominance [135]. Besides, there are differences among different moments of the day: in particular, early in the morning there is an increment of the sympathetic activity, while the parasympathetic activity is predominant during the night [136]. Negative emotional states can be the substrate for the possible occurrence of a disease [137–140]: it has been hypothesized that this is due to a decrement of the parasympathetic dominance. In fact, a reduction in HRV parameters have been found in depression, anxiety, and post-traumatic stress disorder [141–143].

In the last decade, being a non-invasive method, it has spread in animal studies. It has been used to assess autonomic activity in many different ambits: evaluating farm [144] and laboratory [145] animal welfare; studying autonomic regulation of cardiac activity [146]; evaluating housing conditions [147]; understanding individual characteristics as temperament or coping strategies [148].

Two main categories of analysis methods were individuated: time domain measures and frequency domain measures. Time domain measures are obtained applying statistical transformations that can evaluate the variability in a given electrocardiography registration interval. Most common parameters are:

1. **Mean R-R interval (RR);**
2. **Standard deviation of distribution of R-R intervals (SDNN);**
3. **Standard deviation of distribution of mean R-R interval in a 5-minutes period (SDANN);**

4. **Square root of standard deviation of difference of adjacent R-R intervals (RMSSD);**
5. **Percentage of differences among adjacent R-R intervals above 50 ms (pNN50).**

These indexes provide information about the overall variation caused by both branches of the ANS. Only RMSSD and pNN50 can evaluate the parasympathetic activity and, thus, they can be considered vagal indexes. It is easy to calculate time domain parameters and this makes them widespread, not only to measure vagal activation. A reduction of these indexes might reflect a decrease in the vagal influence and, likely, an increase in the sympathetic activity [149–151].

Frequency domain measures are based on the assumption that every variation happens following a certain pattern characterized by a specific frequency [152]. Different mathematical models are used to analyze frequency domain measures:

1. **Fast Fourier transform (FFT):** it has high computational efficiency, easy applicability, a good graphics resolution, it avoids loss of information but it is limited in resolution of frequency; the maximum frequency limit is imposed by the Nyquist criterion, for which the value of maximum frequency should be half of the sampling frequency [153,154];
2. **Autoregressive model (AR):** time series are regarded as different equations, so that the signal at each time step is expressed as a linear function of its value to a certain number of preceding time steps (J), which represents the order of the parametric model; it is needed to choose a priori J to obtain the best match with the considered data; graphically, obtained spectral components are flatter than FFT ones that are distinct by pre-selected frequency bands; the limit to the application of this model is the adequacy of the choice of the order parametric J [154,155].

In a typical spectrum power density is distributed in three frequency bands determining three fundamental main peaks: very low frequencies (VLF), low frequencies (LF), and high frequencies (HF). In mammals, spectral components have different frequency values in relation to the specific heart rate of the species [153]. Besides, the distribution of power and the value of center frequency in each band can vary according to the changes in the modulation exerted by the ANS on heart rate and blood pressure [156]. Parasympathetic activity is considered to be responsible for HF variability, LF variability is due both to the sympathetic and the parasympathetic activity, and the LF/HF ratio appears to be a good estimator of the sympathovagal balance [157].

However, HRV parameters are often of difficult comprehension, mainly because HRV measurements usually do not have a good correlation with behavioral data [158] and HR, and consequently HRV, are influenced by many different factors like physical activity, posture,

environmental stimuli [159]. Concluding, HRV is simple to use and it allows to have a good quantification of sympathovagal balance but it is difficult to interpret, especially on animals.

1.3.3. Telemetry

Telemetry consists in a surgical operation to implant a transmitter under skin. It is placed in ventral position above the groin. From the transmitter two electrodes depart subcutaneously to reach the cardiac area. Data collected by the transmitter are then sent to a receiver positioned in the nearby. Collected data can be very different, but usually they are heart rate, core temperature, a muscular activity index [160].

Telemetry is an invasive technique, because it requires an initial surgical operation. Nevertheless, it presents some advantages when compared to non-invasive methods: experiments can be performed for prolonged periods, measurements are direct, continuous, and measures are stable for 18 weeks after implant [161,162].

1.4. Aims

The aim of my work was to explore the use of physiological parameters to assess emotions in dogs and correlate them with traditional behavioral analysis testing animals in different situations covering both negative/stressful and positive events.

2. Hot dogs: thermography in the assessment of stress in dogs (*Canis lupus familiaris*) – A pilot study

Tiziano Travain^{1,5}, Elisa Silvia Colombo^{2,5}, Eugenio Heinzl³, Danilo Bellucci⁴, Emanuela Prato-Previde², Paola Valsecchi¹

¹ Dipartimento di Neuroscienze, Unità di Biologia del Comportamento, Università degli Studi di Parma, Parco Area delle Scienze 11/A, 43124 Parma, Italy

² Dipartimento di Fisiopatologia Medico-Chirurgica e dei Trapianti, Sezione di Neuroscienze, Università degli Studi di Milano, via Fratelli Cervi 93, 20090 Segrate (MI), Italy

³ Dipartimento di Scienze Veterinarie e Sanità Pubblica, Università degli Studi di Milano, via Celoria 10, 20133 Milan, Italy

⁴ Studio Medico Veterinario Dr. Danilo Bellucci, via Veglia 40, 20159, Milan, Italy

⁵ These authors contributed equally to the work.

Published in: Journal of Veterinary Behavior, 2015, 10, 17-23

2.1. Abstract

Infrared thermography (IRT) represents a non-invasive method to investigate stress responses in animals. Despite the large existing literature about stress responses in dogs, the potential use of IRT in assessing dogs' stress reactions has not been investigated so far. This study evaluates the usefulness of IRT to assess dogs' emotional responses to an unpleasant and stressful event. Following a preliminary test, aimed to evaluate the correlation between eye-temperature and rectal temperature in dogs in a stressful situation, a sample of 14 adult healthy dogs was observed during a standardized veterinary examination, carried out by an unfamiliar veterinarian in the presence of their owner. Dogs' behavior and eye temperature were recorded before the start of the veterinary visit, during and after the clinical examination. Dogs' level of activity and stress related behaviors varied across the different phases of the visit; interestingly, dogs showed an increase in eye temperature during the examination phase, compared to both pre-examination and post examination phases, despite they had a significant decrease in their level of activity. However, it also emerged that the thermographic camera, although remote and non-invasive, was at some extent disturbing for dogs, as they showed avoidance behaviors, averting gaze and/or turning their head, exclusively when the thermographic camera was oriented to them. Overall results suggest that IRT may represent a useful tool to investigate emotional psychogenic stress in dogs. Nevertheless, further research is needed to establish the specificity and sensitivity of IRT in this context and to assess how different dogs' characteristics, breed, previous experience and the nature and severity of the stressor could influence the magnitude and type of the stress response.

2.2. Introduction

Infrared thermography (IRT) is a passive, remote and non-invasive method that measures surface temperature, detecting infrared radiation emitted by a subject and providing a pictorial representation of body temperature in animals [115,116]. Several studies have shown that IRT provides information on an animal's health detecting inflammatory conditions and infections [108–110]. IRT also represents a useful method to assess acute and chronic stress in laboratory [122], farm [163,164] and sporting animals [123,165] since there is a close relationship between stress and the metabolic system. When an animal becomes stressed the HPA axis is activated and, as a result of increases in catecholamines and cortisol levels as well as blood flow responses, it will produce changes in the animal's heat production and loss [98,166].

The exposure to both physiological and psychological stressors of different intensity activates a defensive response, including an increase in heart rate and body temperature; this relative short-lasting rise in core body temperature induced by stress has been reported across numerous species, such as mice, rats, rabbits, ground squirrels, pigs, silver foxes, baboons and humans and is known as Stress Induced Hyperthermia (SIH) [98]. Many psychological stressors, such as handling, exposure to a novel environment [100] and conditioned fear [101,102], induce an elevation of body temperature in just a few Celsius degrees, which might be beneficial to the “fight or flight” reaction to potential threatening stimuli, through warming up muscles and the central nervous system [103].

Even if it is not yet clear whether SIH is a form of fever (comparable to that produced by exogenous pyrogens like bacteria, through the activation of immune system), these two processes have overlapping properties and both result in a higher body temperature, induced by the activation of some common pathways that likely include neurons in the dorsomedial hypothalamus [103,167].

Recently Johnson and colleagues [121], evaluated whether IRT could be used to detect fever in ponies after vaccination, and they found a good correlation between thermographic eye temperature and rectal temperature, suggesting that eye temperature can be a valid index to measure core body temperature and its variation. Despite the aim of this study was to detect fever, a number of other studies based on IRT has shown that the temperature of the eye is also a good indicator of heat changes in body temperature due to physiological and psychological stress [130,168].

In particular, the temperature of small areas around the posterior border of the eyelid and of the lacrimal caruncle, which have rich capillary beds innervated by the sympathetic system, responds to changes in blood flow [130,131]. The lachrymal caruncle is an anatomical area very sensitive to both pain and stressful events affecting an individual, and changes in its temperature have been attributed

both to the sympathetic response of the autonomic nervous system (ANS) reaction and to HPA activation [124,131]. The sympathetic branch of the ANS responds rapidly, preparing the individual for the “fight or flight” reaction [169,170], whereas the parasympathetic system is predominant during passive reactions such as freezing [171,172]; the HPA axis activation is more delayed and is particularly sensitive to psychogenic stressors not producing physical damage [173–175]. Stewart and colleagues [163,164] found that in cattle, during the first few seconds of a stressor presentation (acute phase), eye temperature dropped rapidly, likely because of a sympathetic response (peripheral vasoconstriction); however, if the stressor persists for a longer time, the HPA axis induces a cortisol release, that can be maintained from minutes to hours (chronic phase), causing several thermogenic reactions in tissue metabolism [124]. Therefore, HPA axis response to stressors, along with peripheral vasodilatation due to the parasympathetic activation that follows the initial sympathetic response, can produce an increase in eye temperature [124].

Dogs are widespread companion animals that highly depend on humans for both health and care, and several studies have investigated stress responses in dogs in different situations, using both behavioral and physiological indices, mainly cortisol sampling and heart rate [102,176–181].

Behavioral parameters are considered an interesting tool to establish stress in dogs easily and non-invasively and a variety of behavioral responses have been reported to occur during acute stress [176]. These behaviors have been used to assess dogs’ welfare in a wide range of situations such as shelter housing [182], separation from the owner [183], agility competition [184] and exposure to novel and startling stimuli [185]. However, these results suggest that behavioral and physiological indicators of stress are not always related, due to a considerable variability in stress-related behaviors: factors such as individual variability (e.g. gender, breed, previous experience) or the type of stimulus involved in the stress situation play a main role in modulating behavioral response [177,181,186]. Therefore, it has been suggested that observation of spontaneous behavior could be useful to facilitate the interpretation of physiological data but not as a welfare indicator per se [187]. In particular, Ogata and colleagues [102] tested a heterogeneous sample of dogs using a Pavlovian fear-conditioning protocol and found that, while behavioral responses had a high individual variability, all subjects had a similar increase in core body temperature (measured with a rectal thermometer), suggesting that this autonomic parameter could be a more reliable and consistent measure of fear in dogs than behavior. As far as we know, IRT has been considered as a diagnostic technique in dogs only recently [188] but so far it has never been used to investigate variation in dogs’ eye temperature due to stress reactions. Only one study has used dogs’ surface temperature (detecting nose temperature by infra-

red thermometer) as a potential index of stress, finding that it seems to be a good indicator of psychological arousal in dogs [181].

The aim of the present study was to evaluate the potentialities of infrared thermography in the investigation of dogs' psychological stress due to veterinary examination. Veterinary examination has been reported to be stressful for most dogs [189], with dogs exhibiting fear reactions especially during the clinical examination, but also showing anticipatory fear reactions prior to entering the veterinary clinic for being examined [190]. A pre-test was carried out on a sample of dogs to evaluate whether the correlation between eye and core body temperature in dogs exists as already shown in other animals [121,168]. Secondly, a different sample of dogs was tested during a standardized veterinary examination, carried out by an unfamiliar veterinarian in the presence of their owner, and their behavior and eye temperature variations were recorded before, during and after the clinical examination. The aim of the examination was to induce a negative psychological state in dogs, and thus it consisted of routine and not painful assessments of dogs' health.

2.3. Materials and methods

2.3.1. Pre-test

2.3.1.1. *Subjects*

The subjects were 20 healthy dogs (8 females, 12 males) of different breeds and body size, whose ages ranged from 7 months to 15 years (mean = 9.00 years, SD = 4.67 years). The sample included 16 pure-breed dogs (1 Pinscher toy size, 1 Pug, 2 Dachshund miniature size, 1 West Highland White Terrier, 2 Jack Russell Terrier, 3 Poodles medium size, 1 Golden Retriever, 1 Czechoslovakian Wolfdog, 1 Italian Pointer, 1 Cane Corso, 1 Spanish Galgo, 1 English Setter) and 4 medium size mixed-breed dogs. All the dogs were kept for companionship and lived within the human household. They were accustomed to being taken to the veterinary; none of them was reported to be aggressive during veterinary examinations. All the owners were informed about the aims of the study and the procedure, and their informed consent was obtained. None of these dogs took part in the subsequent test.

2.3.1.2. *Procedure*

The study was conducted in a veterinary clinic in Milan, Italy (Danilo Bellucci's Veterinary Clinic). The dogs were visited in opening hours, before a routine booster vaccination. The pre-test consisted of a unique phase in which dogs' rectal and eye temperatures were measured.

The owner and the dog went into the examination room, the veterinarian lifted the dog on the examination table and measured rectal temperature. Dogs were not physically restrained, so the

owners assisted the vet holding their dog if necessary, preventing them from jumping down from the table and calming them as needed. The entire examination lasted 2 minutes. The sequence of events was standardized and the examination circumstances (handling, room-features, equipment used) were always the same.

2.3.1.3. Data collection

The thermographic infrared images were captured by a certified technician (E.H.) using a portable IRT camera (AVIO TVS500® camera, NEC, Japan) with standard optic system, and analyzed with IRTAnalyzer Software® (Grayess, FL, USA). To calibrate the camera reflectivity temperature, samples were taken and emissivity was set at 0.97. Several images per dog were collected during the pre-test, to select the images that provided the most optimal operating conditions for analysis (90° angle and 1 m of distance). A total of 62 (per dog: mean \pm SE = 3.10 ± 0.18 ; minimum = 2; maximum = 4) images were analyzed evaluating the emission of eyes lachrymal sites. The maximum temperature for each lachrymal site was determined using an Instantaneous Field of View of 1.68 mm at 1 m of distance, within an oval area traced around the eye, including the eyeball and approximately 1 cm surrounding the outside of the eyelids. Only images perfectly on focus were used (Fig. 2.1). To optimize the accuracy of the thermographic image and to reduce sources of noise, before testing each dog the same image of a Lambert surface was taken to define the radiance emission and to nullify the effect of sunlight or other surface reflections on tested animals, thus controlling for external artefacts [191]. Furthermore, the testing environment was air-conditioned and thus temperature and humidity remained constant during the procedure.

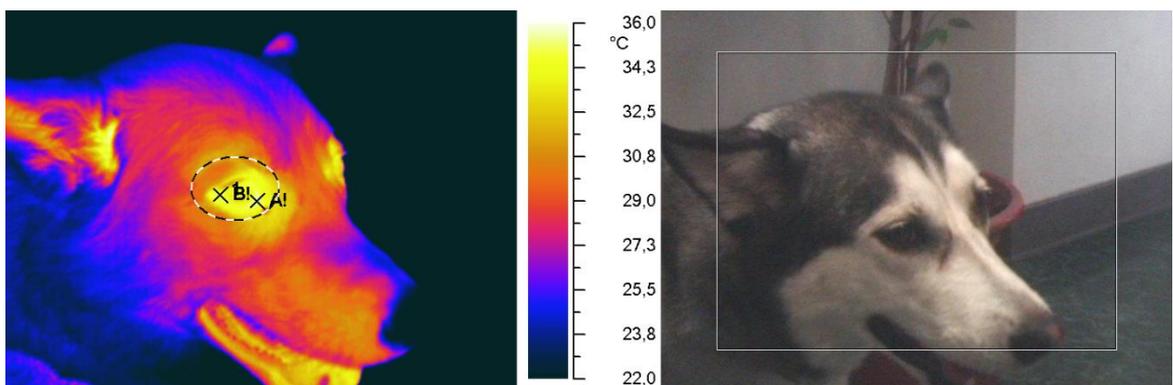


Figure 2.1. Thermographic image and corresponding picture of Tika, Siberian husky mix, in the waiting room (phase 1). A! is the lacrimal caruncle and the hottest spot on the eye. B! is the second hottest spot and it is highlighted for control purposes.

Rectal temperature of all dogs was taken by D.B. with an electronic veterinary rectal thermometer (Solution TD0004, Vega Technology, Taiwan) emitting an acoustic signal when the reached temperature remains stable for more than 15 seconds.

2.3.1.4. *Statistical analysis*

To assess the correlation between mean rectal temperature and mean eye temperature, Pearson correlation was calculated.

2.3.2. Test

2.3.2.1. *Subjects*

The subjects were 14 adult and clinically healthy dogs (9 females, 5 males) of different body size, whose ages ranged from 1.5 to 11 years (mean = 5.80 years, SD = 2.54 years). The sample included 9 pure-breed dogs (1 Poodle toy size, 2 Fox Terrier, 2 Jack Russell Terrier, 1 Dachshund miniature size, 1 Golden Retriever, 1 Bergamasco Shepherd and 1 Great Anglo-French Hound) and 5 mixed-breed (2 small-medium, 1 medium, 1 Alaskan Malamute mix and 1 Siberian Husky mix). All the dogs were kept for companionship and lived within the human household. All dogs had previous experience of being taken to the veterinary and, as reported by their owners, they clearly disliked this kind of situation; however, none of them was reported to be aggressive during veterinary examinations. Furthermore, all dogs had never been to this veterinary clinic before and thus they were completely unfamiliar with the vet and the environment. All the owners were informed about the aims of the study and the procedure, and their informed consent was obtained.

2.3.2.2. *Procedure*

The study was conducted in the same location and under the same conditions as the pre-test. The procedure consisted of three consecutive phases in which dogs' behavior was recorded and eye temperature was measured.

Phase 1, *pre-examination*. After the dog and the owner entered the veterinary clinic waiting room, the owner was asked to sit quietly keeping the dog on leash next to him for 10 minutes, pretending to wait his turn.

Phase 2, *examination*. This phase was a standardized general examination. The owner and the dog went into the examination room, the veterinarian lifted the dog on the examination table and performed the following checks: conjunctiva, ears and oral mucosa, palpation of the dog's abdomen, examination of lymph nodes and heart auscultation with a stethoscope. Dogs were not physically restrained, but the owner assisted the vet holding the dog if necessary, thus preventing his/her dog from jumping down the table and calming it. The entire examination lasted between 4 to 5 minutes (average duration = 262.22 s ± 17.86 SE). During this phase the sequence of events was standardized and the examination circumstances (handling, room-features, equipment used) were always the same.

Phase 3, *post examination*: the veterinarian put the dog down to the ground and sat at his desk with the owner, talking about the dog for 5 minutes. Then the dog and the owner left the examination room and the clinic.

2.3.2.3. *Data collection*

The thermographic infrared images were captured following the same procedures used in the pre-test during the whole time of every phase (Fig. 2.1). A total of 546 (per dog: mean \pm SE = 39.00 \pm 4.20; minimum = 17; maximum = 66) images were analyzed evaluating the emission of eyes lachrymal sites.

Dogs' behavior during the three different phases was recorded using a camcorder (Leica Dicomar, Panasonic, Japan) placed on tripod and behavioral data were scored from videos using Solomon Coder beta[®] 12.09.04 (ELTE TTK, Hungary). The ethogram consisted of two main categories of mutually exclusive behaviors, and in particular we focused on: 1) dogs' level of activity, namely whether the subject was engaged in observable physical activity, like walking, jumping or being agitated while standing on the table (dynamic), or was sitting, standing or lying down and, therefore, movement was almost absent or very limited, i.e. head or ears movements (static); 2) stress/fear related signals which included: shake off, yawning, nose/lip-licking, paw-lift, mouth opening/closing, panting, posture change, freezing and avoidance (see Table 2.1 for a description) [176,177]. In addition, to evaluate whether the mere fact of directing the thermographic camera towards the dogs' muzzle could affect their stress displays, during coding each behavioral element was accompanied by a marker indicating the presence of the thermographic camera. The dogs' behavior was coded from videos by one of the authors (E.S.C) whereas a second independent coder (T.T.) analyzed 20% of the data to assess inter-observer reliability.

Table 2.1. Description and measure of mutually exclusive stress/fear related behaviors.

Behavior	Description	Frequency/Duration
Panting ^a	Rapid breaths in short gasps	D (% on total time)
Freezing ^a	Complete motionless, without noticeable panting	D (% on total time)
Avoidance ^a	Lateral movement of the head and gaze averting	F (event/min)
Nose/lip licking	Rapid extension and flicking of the tongue on the nose or between the lips	F (event/min)
Paw lift	Raise a paw at a time when it is standing or sitting still	F (event/min)
Posture change	Change posture, from lying on the ground to sitting or standing and vice versa	F (event/min)
Mouth opening/closing	Rapid movements of opening/closing mouth	F (event/min)
Shake off	Rapid movements of body shaking	F (event/min)
Yawning	Involuntary intake of breath through a wide open mouth, not for thermal regulation	F (event/min)

^a Behaviors singly analyzed, all other behaviors were pooled for purpose of analysis.

2.3.2.4. Statistical analysis

Interobserver reliability was assessed using Spearman correlations on the main behaviors (dynamic; freezing; panting; avoidance; stress/fear signals). Differences in dogs' degree of activity, stress/fear related behaviors and eye temperature in the three phases were evaluated using non-parametric and two-tailed statistical tests, setting alpha at 0.05. Stress/fear signals reported in Table 2.1 were pooled due to their low occurrence, with the exception of avoidance, which occurred only

when the thermographic camera was oriented to the dogs' muzzle, and of panting and freezing, which were measured as duration. Friedman's ANOVA for ranks with the minimum difference post hoc tests [192] were run to detect differences among phases in dogs' eye temperature and to evaluate differences among phases in behavior and stress/fear related signals. Wilcoxon rank-sum test was used to evaluate whether the presence of the thermographic camera affected dogs' behavior. All the statistical analyses were carried out with SPSS Statistics 21 (IBM, NY, USA).

2.4. Results

2.4.1. Pre-test

The mean rectal temperature of the sample of dogs was 38.57 °C (SD = 0.43 °C), while the mean eye temperature was 36.18 °C (SD = 0.68 °C). Pearson correlation between eye and rectal temperature was $r = 0.661$, $P = 0.002$.

2.4.2. Test

2.4.2.1. Thermographic data

Dogs' eye temperature increased during the examination phase and decreased to pre-examination values in the post examination phase (Fig. 2.2). The Friedman's ANOVA showed that the variation in eye temperature was significant ($\chi^2 = 8.714$; $df = 2$; $P = 0.013$), and the post-hoc test revealed a significant difference between pre-examination and examination phase (minimum D = 0.726; observed D = 0.929, $P < 0.05$), and between examination and post examination phase (minimum D = 0.968, observed D = 1.000, $P < 0.01$).

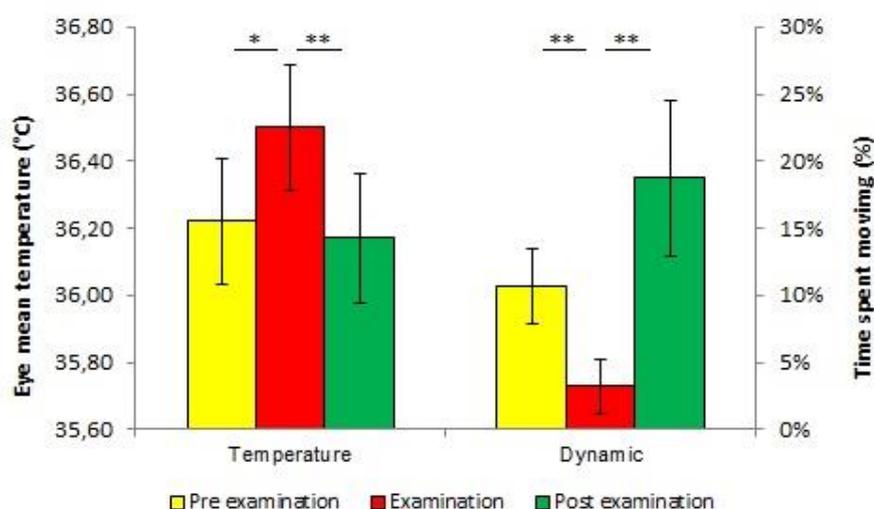


Figure 2.2. Mean eye temperature (°C) and mean percentage of dynamic behavior expressed during the phases of the experiment. Crossing lines represent standard errors. Post hoc results are reported for both temperature and dynamic. * $P < 0.05$ and ** $P < 0.01$.

2.4.2.2. Behavioral data

There was a good inter-observer reliability for the behavioral categories analyzed (dynamic: $r = 1$, $P < 0.001$; freezing: $r = 1$, $P < 0.001$; panting: $r = 1$, $P < 0.001$; avoidance: $r = 0.95$, $P = 0.004$; stress/fear signals: $r = 0.986$, $P < 0.001$).

Considering the level of activity, there was a significant difference among the three different phases in the duration of dynamic behavior (Friedman: $\chi^2 = 16.000$; $df = 2$; $P < 0.001$): in particular, dogs' activity significantly decreased in the examination phase (pre examination vs. examination: minimum $D = 0.968$, observed $D = 1.143$, $P < 0.01$; examination vs. post examination: minimum $D = 0.968$, observed $D = 1.429$, $P < 0.01$; Fig. 2.2). Dogs showed stress/fear signals during the whole test (on average 2.40 stress/fear signal per min), with some signals occurring more than others (Table 2.2).

Table 2.2. Mean and standard error (SE) of stress- or fear-related behaviors in the 3 phases of the experiment (before examination, examination, and after examination) and P value of Friedman T test.

Behavior	Pre examination mean \pm SE	Examination mean \pm SE	Post examination mean \pm SE	P Value
Panting ^a	23.8 \pm 7.1	14.1 \pm 6.9	26.1 \pm 7.7	0.07
Freezing ^a	0.0 \pm 0.0	11.3 \pm 4.4	0.0 \pm 0.0	Not analyzed ^b
Avoidance ^a	0.16 \pm 0.04	0.13 \pm 0.06	0.60 \pm 0.17	0.029
Nose/lips licking	0.68 \pm 0.15	0.38 \pm 0.15	0.63 \pm 0.15	0.135
Paw raising	0.09 \pm 0.04	0.37 \pm 0.18	0.11 \pm 0.09	
Posture change	0.47 \pm 0.08	0.12 \pm 0.07	0.56 \pm 0.16	
Mouth open/close	0.88 \pm 0.29	0.70 \pm 0.22	0.58 \pm 0.22	
Body Shaking	0.09 \pm 0.03	0.00 \pm 0.00	0.19 \pm 0.04	
Yawning	0.14 \pm 0.04	0.00 \pm 0.00	0.11 \pm 0.05	

^a Behaviors singly analyzed, all other behaviors were pooled for purpose of analysis. ^b Freezing behavior was not statistical analyzed since it occurred only in examination phase.

The highest frequency of stress signals, apart from freezing, was observed in Phase 3 (post examination: 2.78 signals/min), whereas the lowest one during Phase 2 (examination phase: 1.68

signals/min). Overall mouth opening/closing was the most frequent signal of stress (0.78 times/min during the whole test), whereas yawning and shaking off were the two less frequent signals (both 0.10 times/min). Differences in stress/fear signals frequency across phases were not statistically significant (Friedman: $\chi^2 = 4.000$; $df = 2$; $P = 0.135$), except for avoidance behavior which varied significantly among phases: it dropped during the examination phase, increasing again in the post examination phase (Friedman: $\chi^2 = 7.091$; $df = 2$; $P = 0.029$). Nine dogs (64.3%) showed panting during the procedure and this behavior occurred for 22.2% of the overall time, being present in Phases 1, 2 and 3 on average for 23.8%, 14.1% and 26.1% of the time respectively. Although panting decreased during the examination phase, differences in this behavior among phases were not significant (Friedman: $\chi^2 = 5.314$; $df = 2$; $P = 0.07$). Freezing behavior was found exclusively during the examination phase, and seven of the 14 dogs (50% of the subjects) exhibited this behavior: this may explain why during the examination phase there was the lowest frequency of stress/fear signals and the lowest duration of panting and dynamic behavior.

It emerged that throughout the test dogs showed avoidance behavior, i.e. turning the head and/or looking away, exclusively when the technician oriented the thermal camera towards the dogs' muzzle to capture images (Fig. 2.3). Thus, a further analysis was carried out to assess differences in the frequency of dogs' other stress/fear signals and the duration of freezing and panting behaviors when the thermographic camera was directed vs. not directed towards the dogs' muzzle. Results revealed a significant difference in the duration of freezing (Wilcoxon: $Z = -2.197$; $P = 0.028$), and no significant difference in frequency of the remaining stress/fear signals (Wilcoxon: $Z = -1.726$; $P = 0.084$) and in the duration of panting (Wilcoxon: $Z = -1.244$; $P = 0.214$).

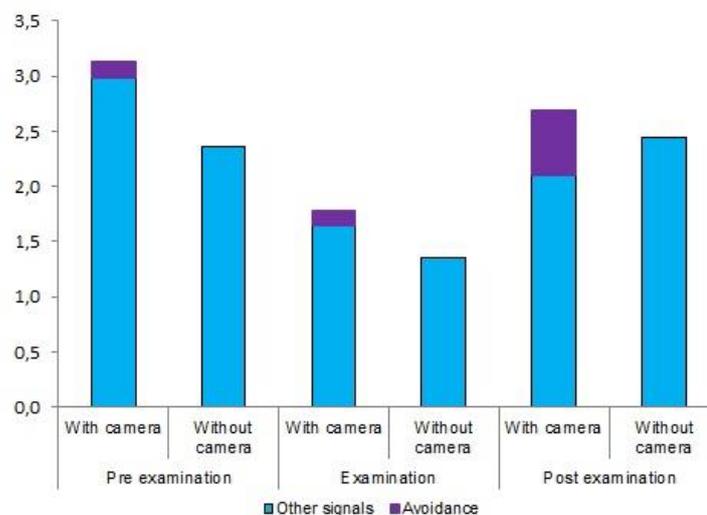


Figure 2.3. Mean frequency of avoidance and other stress or fear signals when the thermographic camera was oriented or not to the dog's muzzle (without camera) in the 3 phases of the experiment.

2.5. Discussion

In the current study, we aimed to evaluate the potentialities of infrared thermography in the investigation of dogs' psychological stress due to an unpleasant situation, i.e. a veterinary visit. In the literature it is well documented that stress can induce an increase in core body temperature [98,100–102] which in turn influences the temperature of a particular area of the eye, the lacrimal caruncle, whose variations can be detected by IRT [124,131,163]. There is also some evidence that eye temperature is particularly sensitive to psychological stress [130].

So far, various studies have focused on stress responses in dogs in a variety of conditions and combining behavioral and physiological measures [102,176–181]: however, to our knowledge this is the first study in which IRT is used with dogs to investigate stress responses.

Initially, in a pre-test conducted on a sample of dogs, we evaluated whether there was a correlation between eye-temperature and rectal temperature in dogs, as already reported for other animal species [121,193]. Results of this pre-test confirm previous findings, revealing that eye temperature can be a good indicator of core body temperature also in dogs.

Given the evidence of a correlation between eye and rectal temperature, a second sample of dogs was tested during a standardized veterinary examination, aimed at inducing a negative psychological state in dogs and carried out by an unfamiliar veterinarian: in this part of the study dogs' behavior and eye temperature were recorded before, during and after the clinical examination.

Thermographic data obtained when the dogs were exposed to the psychologically stressful situation, i.e. the veterinary visit [189], highlighted a peak in dogs' eye temperature during the clinical examination phase. Since at the behavioral level this phase was characterized by a clear drop in dynamic behavior and by the occurrence of freezing behavior in half of the subjects, it is unlikely that the observed increase in eye temperature simply depended on dogs' activity. Rather, it appears that the temperature increase was associated to a condition of emotional stress due to the visit itself. In this respect, our results confirm those by Döring and colleagues [189], who found that veterinary examination is perceived as stressful by dogs. Our procedure did not involve any physical injury to animals, and thus it likely represented a psychogenic stressor due to exposure to a novel (dogs had never been in this veterinary clinic before) and threatening environment, and to a lack of control over external events [174,180]. Probably, when faced with the veterinarian, dogs realized that no active strategy was possible and showed a passive behavior, displaying a more static posture than in the other conditions or even showing freezing. HPA axis is especially sensitive to this kind of psychogenic stressor and its effects on metabolism, along with peripheral vasodilatation due to parasympathetic activation

during freezing response [171,172], may explain the increase in eye temperature detected by thermography when dogs were on the examination table [124]. Moreover, all our dogs had previous experiences with veterinary examinations, so the anticipation of an unpleasant experience could have played a role in the stress response, supporting studies that have linked a cognitive component of stress with the increase in eye temperature [124,130,163]. In particular, our findings are in line with those obtained by Ogata and colleagues [102], who found that dogs' body temperature increased consistently in response to a fear conditioned stimulus, regardless their breed, gender or age. The authors suggested that body temperature response to fear may not be strongly influenced by these variables and could provide an objective index of stress in dogs, particularly when multiple breeds are tested together. A similar result was reported also by Part and colleagues [181], who found no effect of kennel type or experience, sex, neuter status and age on the drop in surface temperature observed following kenneling; thus they suggested that this variable could be an 'easy to measure' indicator of psychological arousal in dogs, even though its emotional valence could not be assessed.

However, this is the first study using IRT to assess dogs' responses to an unpleasant situation and, due to the small and heterogeneous sample tested, further studies should be undertaken to highlight the potential role of individual variables (e.g., age, breed and gender) on eye temperature and other stress responses in dogs since, for instance, these variables were found to be relevant in horses [194]. It would be also interesting to compare dogs with and without previous experience with veterinary examinations to investigate to what extent anticipation of an unpleasant experience could have modulated the dogs' stress response and eye temperature.

It is worth noting that dogs also exhibited an avoidance reaction only when the thermographic camera was directly oriented towards their muzzle. In particular, this reaction decreased during the examination phase and increased again in the post examination phase, exceeding pre examination phase level. Furthermore, freezing and other stress/fear signals increased when the thermographic camera was directed toward the dogs, and this suggests that, although remote and non-invasive, it was to some extent disturbing for dogs and that possibly it determined a short-term sensitization effect. It is also possible that dogs' avoidance response depended also, at least in part, on having an unfamiliar human facing them while holding a strange object. There is evidence that for most non-human species a direct and prolonged duration of gaze is considered as a threatening behavior [195], and this has been reported also for wolves and dogs [196,197]. It has also been shown that, in the absence of other accompanying signals, a direct and prolonged gaze puts dogs in an uncomfortable situation [198–201]. Thus, it is possible that dogs perceived a prolonged photo framing by a static and silent human as a threat and thus reacted with avoidance, averting gaze and turning the head.

2.6. Conclusions

This study represents a first step in the validation of infrared thermography as a method for measuring stress in dogs and our results indicate that the IRT is a useful tool to detect temperature variation due to psychological stress in dogs. Further researches are needed to establish whether sensitivity of IRT is context-dependent, i.e. emotional distress, or is applicable in positive context as well. Both in the field of comparative cognition and animal welfare there is an increasing interest in understanding and assessing animals' positive and negative emotional states, and IRT could be an additional useful instrument to investigate them in dogs. However, the fact that dogs showed avoidance behaviors, looking away or even turning their head, when the thermographic camera was focused on them suggests that it could be a mild stressor per se, and this aspect deserves further investigation. Furthermore, more studies are required to assess whether and in what extent different dogs' characteristics, i.e. breed, age, previous experience, and the nature and severity of the stressor, could influence the magnitude and type of the stress response and consequently the applicability of the IRT. It would be also interesting to compare IRT with other physiological measures of emotional stress, such as heart rate variability, in order to better understand the physiological mechanisms that cause changes in dogs' eye temperature.

2.7. Ethic statement

No special permission for use of animals (dogs) in non-invasive observational studies is required in Italy. The relevant ethical committee is the Ethical Committee of the Università degli Studi di Milano. All dog owners were informed about the nature and scope of the study and their written consent was obtained before the study was initiated.

3. How good is this food? A study on dogs' emotional responses to a potentially pleasant event using infrared thermography

Tiziano Travain¹, Elisa Silvia Colombo², Laura Clara Grandi³, Eugenio Heinzl⁴, Annalisa Pelosi⁵, Emanuela Prato-Previde², Paola Valsecchi¹

¹ Dipartimento di Neuroscienze, Unità di Biologia del Comportamento, Università degli Studi di Parma, Parco Area delle Scienze 11/A, 43124 Parma, Italy

² Dipartimento di Fisiopatologia Medico-Chirurgica e dei Trapianti, Sezione di Neuroscienze, Università degli Studi di Milano, via Fratelli Cervi 93, 20090 Segrate (MI), Italy

³ Dipartimento di Neuroscienze, Unità di Fisiologia, Università degli Studi di Parma, via Volturno 39, 43125 Parma, Italy

⁴ Dipartimento di Scienze Veterinarie e Sanità Pubblica, Università degli Studi di Milano, via Celoria 10, 20133 Milan, Italy

⁵ Dipartimento di Neuroscienze, Unità di Psicologia, Università degli Studi di Parma, via Volturno 39, 43125 Parma, Italy

Currently under review to Physiology & Behavior

3.1. Abstract

Understanding how animals express positive emotions is becoming an interesting and promising area of research in the study of animal emotions and affective experiences. In the present study, we used infrared thermography in combination with behavioral measures, HR and HRV, to investigate dogs' emotional responses to a potentially pleasant event: receiving palatable food from the owner. Nineteen adult pet dogs, 8 females and 11 males, were tested and their eye temperature, heart rate, heart rate variability and behavior were recorded during a 30-minutes test consisting of three 10-minutes consecutive phases: Baseline (Phase 1), positive stimulation through the administration of palatable treats (Feeding, Phase 2) and Post-feeding condition following the positive stimulation (Phase 3). Dogs' eye temperature and mean HR significantly increased during the positive stimulation phase compared with both Baseline and Post-feeding phases. During the positive stimulation with food (Phase 2), dog engaged in behaviors indicating a state of positive arousal, being focused on food treats and increasing tail wagging. However, there was no evidence of an increase in HRV during Phase 2 compared to the Baseline, with SDNN significantly increasing only in Phase 3, after the positive stimulation occurred. Overall results point out that IRT may be a useful tool in assessing emotional states in dogs in terms of arousal but fails to discriminate emotional valence, whose interpretation cannot disregard behavioral indexes. The role of HRV in understanding emotional valence and the actual emotional meaning of food treats are also discussed.

3.2. Introduction

Considerable research showed that besides humans many animal species express emotions through a variety of observable signals [79,202–207]. Emotions can be defined as psychological states occurring when an individual is exposed to specific environmental and/or social stimuli, represent an adaptive interface between the individual and its environment, and guide the selection of appropriate behavioral decisions [208–210]. Psychological research on humans indicates that emotions have a multi-component character and incorporate subjective feelings, physiological activation, motor expressions, cognitive appraisals, and behavioral tendencies [208,211,212]. This complexity of emotional states makes their investigation in non-human animals a challenge. According to one of the current approaches to the study of human emotions (i.e. the dimensional perspective), emotional states are characterized by at least two main dimensions: arousal (low to high activation) and valence (positive to negative) [92,213,214]. Recently, this two-dimensional model has been applied to the understanding of the role that emotions play in animal welfare [215,216]. Most studies focused on emotions induced by distress and negative experiences [146,217,218] but a growing number of researchers pointed out that animal welfare also entails the presence of positive emotional states [145,219,220]. Therefore, the measurement of positive and negative valence of affective states is important to understand and assess animal emotions, and there is agreement that an objective evaluation needs a combination of behavioral, physiological, and cognitive markers. In fact, some parameters can be ambivalent and difficult to interpret when considered separately. For example, HR can increase in both positive and negative emotional states, since it reflects arousal rather than valence [145,178,221]. Imfeld-Mueller [222] reported that in pigs HR and HRV were not differentially influenced by the valence of the test situation consisting of accessing to popcorn (positive situation) and of crossing a black ramp (negative situation). Even behavioral measures are not always easy to interpret [92]: lip licking in dogs has previously been explained as a signal of stress [176,187] but has also been related to an increased arousal determined by the reunion with the owner after a long period of separation [223] and some researchers have considered it as an appeasement signal [224]. Similarly, self-grooming in dogs can be considered as an index of relaxation and appropriate self-maintenance but can also be associated with an attempt to relieve stress or anxiety [225].

While the literature on positive emotions in farm animals has grown [125,129,205,222,226–232], the same topic has received little attention in companion animals [233–236]. Dogs' positive affective states have been investigated in female laboratory beagles tested in three different experimental protocols: Burman and colleagues [233] used the 'cognitive bias test' using a food treat in a bowl; Rehn and colleagues [236] used a separation/reunion to a familiar person paradigm;

McGowan and colleagues [234] used a problem solving operant task comparing different rewards: food, human or dog contact. Kuhne and colleagues [235] evaluated pet dogs' emotional state and behavioral responses to physical human–dog contact by a familiar or unfamiliar person. These studies provided interesting initial evidence that certain circumstances elicit positive emotional states in dogs that can be measured through behavioral (e.g. tail wagging, proximity and contact seeking, gazing, stress signals, vocalizations), physiological (HR and HRV) and endocrine (oxytocin and cortisol) indicators.

The aim of this study was to deepen our comprehension of positive emotions testing a sample of pet dogs receiving food treats from their owners. We combined behavioral and physiological measurements, in particular superficial temperature, heart rate and heart rate variability. Superficial temperature was measured using infrared thermography (IRT), a remote and non-invasive technique that detects changes in peripheral blood flow. This technique has been recently used to explore physiological correlates of stress and emotions in animals [113,124,125,128,163,164,237–240] with only one study on dogs [51], whereas heart rate and heart rate variability are regarded as suitable tools to investigate the role of ANS in the modulation of affect and emotion [146,216,228,241,242].

Given that food is considered a positive reward [92,125,145,230,232,243] we hypothesized that a very palatable food treat received by the owner should attract dogs' attention toward him/her, significantly increasing gazing behavior [244] and should also be a source of positive excitement, determining an increase in HR [201,245]. In addition, if receiving treats caused in dogs a positive emotional state we should observe an increase in tail wagging [234] and in HRV [216].

Making predictions on eye temperature changes is difficult, given that the available evidence is relative to a variety of body surface areas, tools and species tested with opposite results (comb in hens: [125]; nose in cows: [129]; eye in dogs: [113]; eye in horses: [239]; nose in macaques: [128,238,246]). However, in dogs eye temperature correlates with core body temperature [113] and thus it is possible that eliciting an attentional state would result in an increase in eye temperature due to a general state of arousal. To test these predictions, dogs' eye temperature, heart rate, heart rate variability and behavior were recorded prior (Baseline), during (Feeding) and after (Post-feeding) food treats delivery.

3.3. Materials and methods

3.3.1. Subjects

The subjects were 19 healthy dogs (8 females, 11 males) of different breeds and body size, whose ages ranged from 2 to 11 years (mean = 6.36 years, SD = 2.72 years). The sample included 13

pure-breed dogs (1 Jack Russell Terrier, 2 Australian Kelpies, 1 Border Collie, 1 Irish Setter, 1 Irish Red and White Setter, 4 Labrador Retrievers, 2 Golden Retrievers, 1 Newfoundland) and 6 mixed-breed dogs (1 miniature size, 2 small size, 3 medium size). All the dogs were kept for companionship, lived within the human household, were accustomed to share daily activities with their owner (e.g. travel by car, going to unfamiliar places, encountering unfamiliar humans), and were used to wear the harness on daily walking.

3.3.2. Procedure

The study was conducted at the *Canis sapiens* Lab of the University of Milan (Italy). On arrival, the human-dog pairs were escorted to a waiting room where the procedure was briefly described to the owners who were asked to provide their written consent to record behavior and to use the collected data, according to the national Privacy Law 675/96. To apply the heart rate monitor (Polar), the dogs were sheared under the right and left armpits for a surface of approximately 10 cm². Then the Polar was fixed to the dog chest by means of a belt (see data collection paragraph for further details). After this manipulation, dogs were allowed to explore freely the waiting room for an additional period of 5 minutes to relax and to familiarize with the video camera operator and the infrared thermography technician.

The test took place in an unfamiliar adjacent bare room (3.00 x 5.00 m) equipped with one chair, a carpet and a video camera (Leica Dicomar, Panasonic, Japan) mounted on a tripod. During the test the video camera operator (E.S.C.), the infrared thermography technician (E.H.) and the owner were present. To minimize extraneous noise and disturbance, testing was conducted on weekends over a period of four months. The testing environment was air-conditioned and thus temperature (22 °C) and humidity (40%) remained constant during the procedure [113]. Owners were asked not to feed their dogs for at least four hours prior testing.

The test procedure consisted of three consecutive phases:

Phase 1, Baseline: After the dog and the owner entered the experimental room, the Polar was switched on and the owner was asked to sit on the chair while the dog, on a 2 m long leash, could remain close to them or move around for 10 minutes; the aim of this phase was to obtain baseline values for each dog.

Phase 2, Feeding (i.e. positive stimulation): The owner remained seated and attracted dogs' attention by showing the dog treats in her/his hands and gave the dog treats at approximately 20 sec. intervals for a 10-minute period. Treats consisted of 1-gram chicken croquettes (Nature Snack - Mini sandwiches for dog, Ferribiella, Italy). Owner compliance to our instructions resulted in some variation

in the number of treats given to the dogs (mean = 30.9, SD = 13.5) nevertheless the number of croquettes received was not correlated with any physiological parameter measured.

Phase 3, Post-feeding: As in Phase 1, the owners remained seated while the dog, on leash, could remain close to them or move around for 10 minutes.

3.3.3. Behavioral measurements

Behavioral data were scored from videos using Solomon Coder beta® 15.01.13 (ELTE TTK, Hungary). The ethogram consisted of 13 non-mutually exclusive behaviors and of 2 behavioral categories, namely Dynamic (walking, jumping and jumping on the owner) and Static (standing still, sitting, or lying down). These two behavioral categories were mutually exclusive but could co-occur with the other 13 behaviors, thus a dog could sit in front of the owner and gaze at his/her face (static + gazing owner) or could walk while exploring the environment (dynamic + attention). Behaviors and categories are detailed in Table 3.1 and were recorded either as duration or as frequencies.

Only behavioral patterns that were displayed with a frequency ≥ 1 event/min and with a duration $\geq 1\%$ of the time in at least one of the three experimental phases were included in the analysis [247]. Moreover, those behaviors exhibited only in one of the three experimental phases were not statistically analyzed.

Table 3.1. Description and measure of coded behaviors and behavioral categories recorded during the test.

Behavior	Description	Frequency/Duration
Dynamic	Walking, jumping and jumping on the owner	D (% of the total time)
Static	Standing still, sitting, or lying down	D (% of the total time)
Shaking off	Rapid movements of body shaking	F (events/minute)
Yawning	Deeply inhale through wide open mouth	F (events/minute)
Nose/lip licking	Rapid extension and flicking of the tongue on the nose or between the lips	F (events/minute)

Behavior	Description	Frequency/Duration
Panting	Breathing with short, quick breaths	D (% of the total time)
Avoidance	Lateral movement of the head to avoid being focused by thermographic camera	F (events/minute)
Gazing experimenters	Looking at the experimenters	D (% of the total time)
Gazing owner	Looking at the owner	D (% of the total time)
Gazing owner's hand/food	Looking at the owner's hand or at the food held by the owner	D (% of the total time)
Hand sniffing/licking	Sniffing or licking owner's hand, regardless of the presence or absence of the food	D (% of the total time)
Touching with paw	Touching the owner with the paw	D (% of the total time)
Attention	Visual/olfactory exploration of the environment	D (% of the total time)
Tail wagging	Hanging tail in a relaxed manner at half-mast [248] and wagging it	D (% of the total time)

To evaluate whether the thermographic camera directed towards the dogs' muzzle could be perceived as stressing (see [113]), during coding, a marker indicating the presence of the thermographic camera accompanied each behavioral element. The dogs' behavior was coded from videos by one of the authors (T.T.) whereas a second independent coder analyzed 32% of the data to assess inter-observer reliability.

3.3.4. Physiological measurements

The thermographic infrared images were captured by a certified technician (E.H.) using a portable IRT camera (AVIO TVS500® camera, NEC, Japan) with standard optic system, and analyzed with IRT Analyzer Software® (Grayess, FL, USA). To calibrate the camera reflectivity temperature, samples were taken and emissivity was set at 0.97. Several images per dog were collected, to select those images that provided the most optimal operating conditions for analysis (90° angle and 1 m of distance). During the whole study 982 (per dog: mean = 51.68, SD = 10.83; minimum = 31; maximum = 77) images were analyzed to evaluate the emission of eyes lachrymal sites. The maximum temperature for each lachrymal site was determined using an Instantaneous Field of View of 1.68 mm at 1 m of distance, within an oval area traced around the eye, including the eyeball and approximately 1 cm surrounding the outside of the eyelids. Only images perfectly into focus were used (Figure 3.1). To optimize the accuracy of the thermographic image and to reduce sources of noise, before testing each dog the same image of a Lambert surface was taken to define the radiance emission and to nullify the effect of sunlight or other surface reflections on tested animals, thus controlling for external artefacts [191].

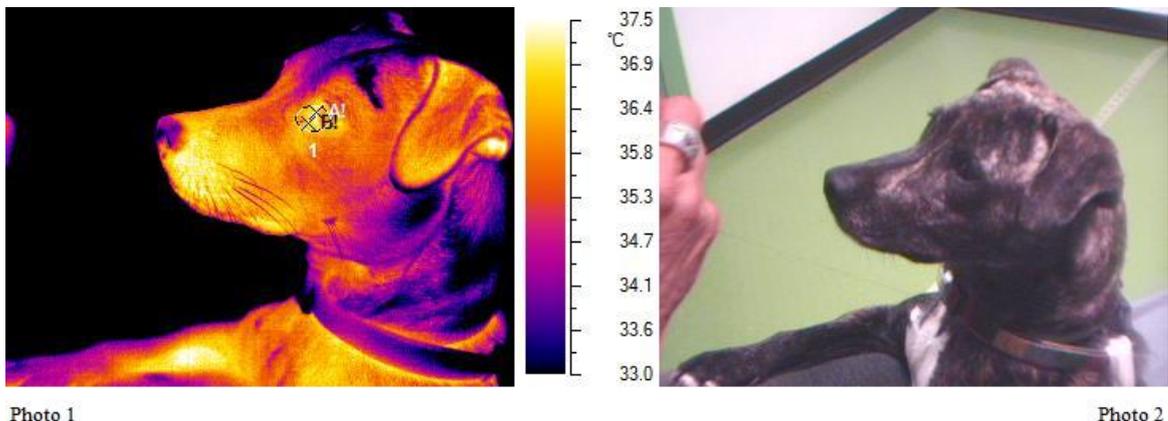


Figure 3.1. Thermographic image (photo 1) and corresponding picture (photo 2) of Easy, Staffordshire bullterrier mix, during Phase 2 (Feeding). A! is the lacrimal caruncle and the hottest spot on the eye. B! is the second hottest spot on the eye and it is highlighted for control purposes. 1 is the marker for the oval area traced around the eye.

Heart rate data were collected using a Polar® RS800CX human HR monitor (Polar® Electro, Finland). The Polar WearLink® strap was positioned around the dog thorax and the size was adjusted to provide a tight but comfortable fit. Farmacare ultrasound transmission gel (Farmacare, Italy) was applied to the 2 electrodes of the Polar WearLink® strap. The electrodes were positioned over the right and left axillary regions. The Polar® watch computer was fixed dorsally to the WearLink strap.

The Polar® was set on the R-R interval-recording mode and data collection lasted for the whole duration of the experiment. R-R interval data were analyzed using Kubios HRV software (Version 2.1

Biosignal Analysis and Medical Imaging Group, Department of Applied Physics, University of Eastern Finland, Kupio, Finland). Prior to analyses, artifacts were removed using Kubios' inbuilt artifact correction feature. The artifact tolerated was not more than 1% on the total length of the recording. Heart rate (HR, beats per minute) and HRV parameters were calculated for each experimental phase. The following time-domain variables were chosen for analysis: mean HR (bpm), root mean square of the standard deviation (RMSSD, ms), standard deviation of R-R intervals (SDNN, ms) and the ratio RMSSD/SDNN.

3.3.5. Statistical analysis

Interobserver reliability was assessed using Spearman correlations and was significant for all the behaviors recorded with r ranging from 0.6 to 1 (Static: $r = 0.604$, $P = 0.008$; Dynamic: $r = 0.880$, $P < 0.001$; Shaking off: $r = 1.000$, $P = 0.000$; Yawning: $r = 0.921$, $P < 0.001$; Nose/lip licking: $r = 0.725$, $P = 0.001$; Panting: $r = 0.720$, $P = 0.001$; Avoidance: $r = 1.000$, $P = 0.000$; Gazing experimenter: $r = 0.922$, $P < 0.001$; Gazing owner: $r = 0.977$, $P < 0.001$; Gazing owner's hand/food: $r = 0.976$, $P < 0.001$; Hand sniffing/licking: $r = 0.835$, $P < 0.001$; Touching with paw: $r = 0.904$, $P < 0.001$; Attention: $r = 0.920$, $P < 0.001$; Tail wagging: $r = 0.600$, $P = 0.008$).

Eye temperature was analyzed using for each dog all the thermographic images taken during each phase and dog's gender as factor.

Behavioral and physiological data were entered in a Generalized Linear Mixed – effect Models Analysis (GLMM). Separate models were set up for each dependent variable measure (behaviors, eye temperature, HR and HRV). All models residuals were checked for normality (Shapiro test) and homoscedasticity (residuals funnel graph). All models included the explanatory variables phase and gender; moreover, Dynamic behavior was also used as co-variate variable for HR and HRV parameters. All models were reduced in a step-wise forward procedure. There was no significant effect of gender for any of the cardiac parameters (HR, RMSSD, SDNN, RMSSD/SDNN) and, when inserted into models, gender determined a significant worsening of their fitting (Akaike Information Criterion, Schwarz's Bayesian Information Criterion and -2 Log-Likelihood variation). Planned contrasts with Bonferroni correction were used for paired comparisons.

All statistical analysis were carried out using R 3.1.3 (R development Core Team) packages: car – Companion of Applied Regression [249] and lme4 – Linear Mixed-Effects Models using Eigen and S4 [250].

3.4. Results

3.4.1. Behavioral data

Dogs' behavioral response changed across the three phases as shown in Tables 3.2 and 3.3. In particular, Hand sniffing/licking and Tail wagging significantly increased between Phase 1 and 2 and decreased between Phase 2 and 3 returning to the baseline values (Hand sniffing/licking: P1 vs P2: $b = 0.247$, $t = 4.985$, $P < 0.001$; P1 vs P3: $b = 0.008$, $t = 0.164$, $P = 0.870$; P2 vs P3: $b = 0.119$, $t = 4.821$, $P < 0.001$; Tail wagging: P1 vs P2: $b = 0.370$, $t = 2.957$, $P = 0.005$; P1 vs P3: $b = -0.020$, $t = -0.168$, $P = 0.868$; P2 vs P3: $b = 0.190$, $t = 3.124$, $P = 0.003$). Conversely, Gaze experimenter and Attention decreased significantly between Phase 1 and 2, whereas they increased between Phase 2 and 3 (Gazing experimenter: P1 vs P2: $b = -0.106$, $t = -2.247$, $P = 0.029$; P1 vs P3: $b = -0.009$, $t = -0.181$, $P = 0.857$; P2 vs P3: $b = -0.400$, $t = -2.066$, $P = 0.044$; Attention: P1 vs P2: $b = -0.040$, $t = -4.822$, $P < 0.001$; P1 vs P3: $b = -0.150$, $t = -1.848$, $P = 0.070$; P2 vs P3: $b = -0.120$, $t = -2.975$, $P = 0.004$). An overall significant difference among the three Phases, but not significant pairwise comparisons, emerged for Static, Gazing owner and Nose/lip licking (Table 3.3). There was no significant effect of gender or a significant gender x phase interaction for all the above mentioned behaviors (Table 3.3).

Panting did not change across the experimental phases whereas gender difference occurred (Table 3.3): males panted longer than females but this difference was significant only in Phase 1 (Phase 1: $b = 177.23$, $t = 2.475$, $P = 0.024$; Phase 2: $b = 125.96$, $t = 1.647$, $P = 0.118$; Phase 3: $b = 143.152$, $t = 1.885$, $P = 0.082$). No other differences emerged.

Table 3.2. Mean and SE of frequency and durations of analyzed behaviors in the three phases of the experiment (Baseline, Feeding and Post-feeding).

Behavior	Baseline	Feeding	Post-feeding
	mean \pm SE	mean \pm SE	mean \pm SE
Dynamic ^a	5.96 \pm 0.95	2.82 \pm 0.91	3.03 \pm 0.74
Static ^a	94.04 \pm 0.95	97.18 \pm 0.91	96.97 \pm 0.74
Shake off ^{b,c}	0.04 \pm 0.02	0.00 \pm 0.00	0.03 \pm 0.01
Yawning ^{b,c}	0.10 \pm 0.03	0.01 \pm 0.01	0.07 \pm 0.02
Nose/lip licking ^b	1.33 \pm 0.22	0.91 \pm 0.22	0.61 \pm 0.12
Panting ^a	♀ 0.56 \pm 0.52	♀ 0.29 \pm 0.27	♀ 5.62 \pm 3.71
	♂ 28.20 \pm 8.93	♂ 7.33 \pm 3.96	♂ 26.52 \pm 9.88
Avoidance ^{b,c}	0.02 \pm 0.01	0.00 \pm 0.00	0.01 \pm 0.00
Gaze owner ^a	10.86 \pm 2.67	5.29 \pm 1.29	16.82 \pm 4.36
Gaze owner's hand/food ^{a,c}	0.00 \pm 0.00	59.13 \pm 4.81	0.00 \pm 0.00
Gaze experimenter ^a	14.56 \pm 2.12	2.87 \pm 0.75	16.99 \pm 2.82
Hand sniffing/licking ^a	1.05 \pm 0.27	20.72 \pm 3.84	1.30 \pm 0.52
Touch with paw ^{a,c}	0.00 \pm 0.00	1.37 \pm 0.51	0.00 \pm 0.00
Attention ^a	53.52 \pm 4.73	11.74 \pm 2.18	34.35 \pm 3.85
Tail wagging ^a	2.61 \pm 0.90	9.09 \pm 4.33	1.82 \pm 0.72

^a Behaviors measured as duration (% of total time). ^b Behaviors measured as frequency (events/min). ^c Behaviors not analyzed because they occurred in only one phase or were rarely displayed.

Table 3.3. Statistical results of the behavioral data.

	Phase		Gender		Phase x Gender	
	F	P	F	P	F	P
Static	3.788	0.029	0.757	0.389	0.381	0.684
Nose/lip licking	3.357	0.043	1.908	0.173	0.636	0.534
Panting	2.188	0.123	10.030	0.003	1.075	0.3488
Gaze owner	3.399	0.041	0.427	0.516	1.260	0.292
Gaze experimenter	12.240	< 0.001	0.360	0.551	0.820	0.446
Hand sniffing/licking	24.632	< 0.001	2.293	0.136	1.069	0.351
Attention	28.158	< 0.001	0.039	0.843	0.153	0.858
Tail wagging	5.500	0.007	0.840	0.364	1.750	0.184

3.4.1. Physiological data

3.4.1.1. Eye temperature

Eye temperature significantly increased from Phase 1 to Phase 2 and decreased from Phase 2 to Phase 3 ($df = 2$, $F = 30.960$, $P < 0.001$; P1 vs P2: $b = 0.224$, $t = 2.140$, $P = 0.032$; P1 vs P3: $b = -0.001$, $t = -0.017$, $P = 0.987$; P2 vs P3: $b = -0.112$, $t = 2.360$, $P = 0.012$). There was no main effect of gender ($df = 1$, $F = 0.170$, $P = 0.682$) while a significant gender x phase interaction emerged ($df = 2$, $F = 4.340$, $P = 0.013$; Figure 3.2) with males showing a greater increment than females between Phase 1 and 2 (M: $b = -0.353$, $t = -2.911$, $P = 0.004$; F: $b = -0.106$, $t = -1.913$, $P = 0.042$). Similarly, males showed a greater decrement than females between Phase 2 and 3 (M: $b = -0.304$, $t = -5.657$, $P < 0.001$; F: $b = -0.320$, $t = -2.832$, $P = 0.035$). Moreover, in males, eye temperature in Phase 3 was significantly higher than that of Phase 1 ($b = 0.250$, $t = 2.427$, $P = 0.004$).

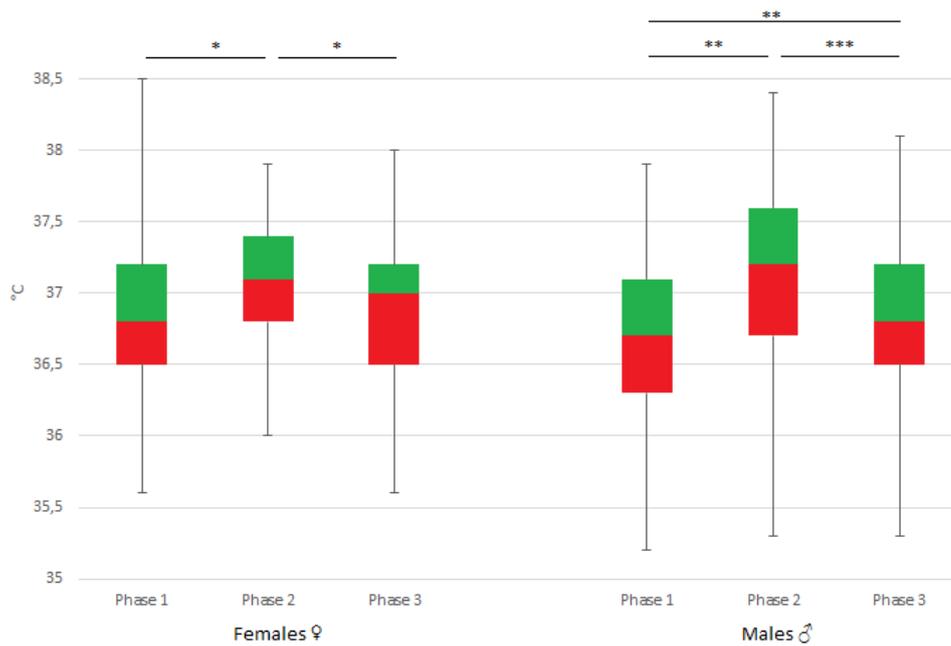


Figure 3.2. Boxplot representing eye temperature ($^{\circ}\text{C}$) exhibited by the male and the female dogs during the three phases of the experiment (Baseline, Feeding, Post-feeding).

3.4.1.2. Heart rate and heart rate variability

There was a positive significant relationship between physical activity (Dynamic) and both HR ($df = 1$, $F = 4.630$, $P = 0.036$) and RMSSD/SDNN ratio ($df = 1$, $F = 6.700$, $P = 0.012$) but neither with RMSSD ($df = 1$, $F = 2.708$, $P = 0.106$) nor with SDNN ($df = 1$, $F = 0.143$, $P = 0.707$). The effect of physical activity was constant across the three phases for all the cardiac parameters (HR: $df = 2$, $F = 0.936$, $P = 0.399$; RMSSD/SDNN: $df = 2$, $F = 1.070$, $P = 0.351$; RMSSD: $df = 2$, $F = 1.558$, $P = 0.220$; SDNN: $df = 2$, $F = 0.248$, $P = 0.781$).

Once controlled for the effect of Dynamic, HR and SDNN changed across phases (HR: $df = 2$, $F = 5.929$, $P = 0.005$; SDNN: $df = 2$, $F = 5.032$, $P = 0.009$). HR increased significantly from Phase 1 to 2 and diminished from Phase 2 to 3 returning to the Baseline values (P1 vs P2: $b = 11.52$, $t = 3.260$, $P = 0.003$. P1 vs P3: $b = -2.097$, $t = -0.623$, $P = 0.537$; P2 vs P3: $b = -6.804$, $t = -4.529$, $P < 0.001$; Figure 3). SDNN decreased from Phase 1 to 2 (P1 vs P2: $b = -22.060$, $t = -2.580$, $P = 0.054$) and significantly increased from Phase 2 to 3 (P2 vs P3: $b = 15.310$, $t = 2.810$, $P = 0.002$), whereas no differences occurred between Phase 1 and 3 (P1 vs P3: $b = 13.056$, $t = 1.538$, $P = 0.133$). On the contrary, RMSSD and RMSSD/SDNN did not significantly differ among phases (RMSSD: $df = 2$, $F = 1.532$, $P = 0.226$; RMSSD/SDNN: $df = 2$, $F = 0.948$, $P = 0.394$; Figure 3.3).

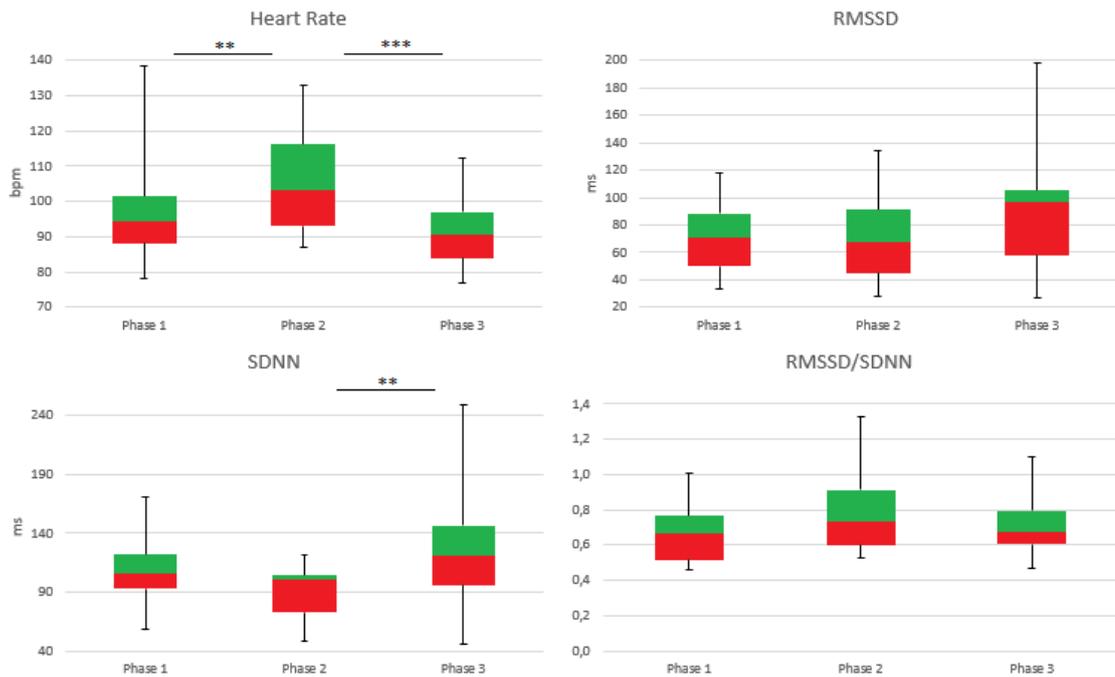


Figure 3.3. Boxplot representing values of heart rate (bpm), RMSSD (ms), SDNN (ms), and RMSSD/SDNN exhibited by the dogs during the three phases of the experiment (Baseline, Feeding, Post-feeding).

3.5. Discussion

The aim of the present study was to investigate eye temperature, detected through IRT, as a potential physiological indicator of emotional states in pet dogs. In addition to eye temperature, cardiac activity (i.e. HR and HRV) was monitored to understand the interplay between the sympathetic and the parasympathetic branch of the ANS when a food stimulus was provided. The analysis of dogs' behavior was used to provide an integrative measure of the response to the stimulation.

Mean eye temperature increased significantly during Phase 2 (Feeding), compared with the mean values of both Phase 1 (Baseline) and 3 (Post-feeding). This result supports our prediction that the arousal determined by the presence of food in the owner hands would have resulted in an increment of eye temperature.

The available literature concerning the use of IRT in the study of animal emotions points out that surface body temperature can increase/decrease depending on the investigated species, the anatomical areas and the type of stimulus used. Nasal temperature decreases in macaques during negative emotional states [128,238,246], and in cows when exposed to a positive stimulus [129]. In hens there is a significant drop in comb surface temperature in response to anticipation and consumption of a palatable food reward [125] but also during exposure to unpleasant events [126]. This contrasting effect on body temperature can be explained in terms of activation of the sympathetic

branch of the ANS which induces an increase in core temperature, reflected in the eye, and a decrease in more peripheral body area, such as nose, face and ears, due to vasoconstriction [101,163,164,251].

Interestingly, we found that in dogs eye temperature increases both when they receive a food treat (positive valence) and when they are stressed by a veterinary visit (negative valence) [113]. In both studies eye temperature showed the same pattern of temporal changes, even though different ranges in the eye temperature emerged possibly due to inter-individual differences. Overall, these evidences suggest that body surface temperature is an optimal index of a general state of arousal but, at this stage of our knowledge on dogs, it does not allow the discrimination of the positive or negative emotional valence of the stimulus itself.

The analysis of behavior provides insight that dogs perceived the present situation as positive; in fact they showed neither fear nor avoidance of the IRT camera during the test, as conversely happened during the veterinary visit [113]. On the contrary, during Feeding dogs remained static most of the time, oriented towards their owner's hands, looking at the food and showing an increased tail wagging. McGowan and colleagues [234] showed that dogs facing a problem-solving task under their control had a more intense emotional response, expressed as frequency of tail wags, when the reward was food rather than contact with a conspecific and suggested that tail wagging can be used as an indicator of a positive affective state in dogs. Quaranta and colleagues [252] showed that dogs wag their tail in an asymmetric manner in response to different emotional stimuli and that the amplitudes of tail wagging also depend on the stimuli, being highest when seeing the owner and lowest when seeing a cat. Being a context dependent behavior, tail wagging could be an interesting index to assess emotional states in dogs, but breed (in our sample the most wagging dogs were 3 Labrador retriever and 1 Golden retriever), temperament and motivation are all factors that may affect its expression and thus further studies would be beneficial. Nose/lip licking has been rarely performed and it occurred mainly during Phase 1 when dogs entered the unfamiliar room for the test. Despite being a signal occurring in different contexts [176,187,223,224], in the present study it could indicate an initial mild discomfort in the new environment. Other behaviors signaling stress were almost completely absent, confirming that the procedure was not stressful as was instead the veterinary visit of the previous study, when dogs showed stress/fear signals in all the three phases of the experiment.

Since cardiac activity can be influenced not only by emotional states but also by physical activity we considered the effect of movement in the statistical analysis and once removed the variance due to the dynamic component of the behavior we found, as expected, a significant rise in dogs' mean HR during Phase 2. On the contrary, a less clear pattern of HRV changes emerged: RMSSD and RMSSD/SDNN remained unvaried across phases, while SDNN decreased during Feeding (even

though not significantly) returning to Baseline values in the Post-feeding. A huge body of evidence in many animal species and in humans demonstrated that HR is linked to emotional arousal during situations of both positive and negative valence for the individual [178,179,253–256], and our result on HR are in line with this well-established knowledge, confirming that receiving treats increased dogs' arousal and activated the sympathetic branch of the ANS [257].

Recent evidence suggested that the analysis of the HRV, and in particular of the cardiac vagal tone, is a potential tool to measure the valence dimension of emotions [145,158,228,242]. We hypothesized that the rewarding nature of the food would have had a hedonic value for dogs and thus consequently receiving food treats would have resulted in an increase in the HRV parameters considered. Overall our results do not support this hypothesis as only the long-term variability measured by the SDNN changed across the three phases. Although in the final phase, when dogs did not receive any more food and relaxed themselves, the lack of a clear HRV pattern suggests that the balance of the branches of autonomic system remained stable. Receiving food treats might be not sufficient to induce a change in adult dogs' autonomic system for different reasons: on one hand they are accustomed to get delicious treats; on the other hand, receiving a reward without the request of performing a specific task could have limited the hedonic value of the context. The evidence available in the canine literature is of limited help in discussing present results due to the heterogeneity of experimental protocols, the variability in the HRV parameters measured and the contrasting results obtained [159,201,258,259].

3.6. Conclusion

Our study is the first assessing eye temperature as a measure of positive emotional states in dogs. Results showed that IRT is suitable to detect in a non-invasive way a state of arousal but not to assess the hedonic values of a positive stimulus, unless combined with behavioral indicators. Thus, the validation of behavioral pattern linked to positive and negative experiences are crucial for understanding dogs' emotional states. The analysis of HRV aimed at providing insight for the evaluation of the valence of emotional states gave ambiguous results, requiring further investigation. The administration of palatable food *per se* might not be the best protocol to elicit an emotion with a positive valence: a paradigm of anticipation and consumption of food reward in a training protocol, successfully used with other species, could be a future development of this research.

3.7. Ethic statement

No special permission for the use of animals (dogs) in non-invasive observational studies is required in Italy. The relevant ethical committee is the Ethical Committee of the Università degli Studi

di Milano. All dog owners were informed about the nature and scope of the study and their written consent was obtained before the study was initiated.

4. Behavioral and physiological changes in dogs during a separation/reunion protocol

Tiziano Travain¹, Laura Clara Grandi², Eugenio Heinzl³, Emanuela Prato-Previde⁴, Paola Valsecchi¹

¹ Dipartimento di Neuroscienze, Unità di Biologia del Comportamento, Università degli Studi di Parma, Parco Area delle Scienze 11/A, 43124 Parma, Italy

² Dipartimento di Neuroscienze, Unità di Fisiologia, Università degli Studi di Parma, via Volturno 39, 43125 Parma, Italy

³ Dipartimento di Scienze Veterinarie e Sanità Pubblica, Università degli Studi di Milano, via Celoria 10, 20133 Milan, Italy

⁴ Dipartimento di Fisiopatologia Medico-Chirurgica e dei Trapianti, Sezione di Neuroscienze, Università degli Studi di Milano, via Fratelli Cervi 93, 20090 Segrate (MI), Italy

Paper in preparation

4.1. Abstract

Assessing animal emotions using non-invasive tools is an interesting and promising area of research in order to better understand animal's perception of different events and to improve their quality of life. In the present study, we used infrared thermography in combination with behavioral measures, HR and HRV, to investigate dogs' emotional responses to a protocol of separation from and reunion with the owner, exposing them a mild stressful situation followed by a pleasant event. Thirty-three adult pet dogs, 17 females and 16 males, were tested and their eye temperature, heart rate, heart rate variability and behavior were recorded during a 27-minutes test consisting of four consecutive phases: Baseline (Phase 1, 10 minutes), Separation from the owner (Phase 2, 3 minutes), Reunion with the owner (Phase 3, 3 minutes), Relaxation, a return to baseline conditions (Phase 4, 7 minutes). Dogs' eye temperature and mean HR did not vary among phases. RMSSD was significantly higher during Phase 4 when compared to all other phases. SDNN dropped during Phase 2 and 3, but it increased during Phase 4. During Phase 2 dogs were mainly involved in behaviors directed to the door or to the experimenter while during Phase 3 they were focused mainly on the owner and they exhibited an increased exploratory behavior, indicating a safe base effect. These data are consistent with previous findings about Ainsworth's Strange Situation. Overall results did not confirm previous findings about whether IRT may be a useful tool in assessing emotional states in dogs both in terms of arousal and of valence and this is discussed, as with the role of HR and HRV in understanding emotional activation.

4.2. Introduction

In the last years an increasing attention has been paid to the study of emotion in animals. Given the intrinsic difficulties of investigating the 'interior world' of animals different non-invasive methodological approaches have been proposed in order to integrate behavioral and physiological parameters related to emotional reactions. One of the most recent non-invasive technique applied to these studies is the Infrared Thermography (IRT) that detects infrared radiation emitted by a subject and provides a pictorial representation [115,116]. In animals, it is widely used to obtain information about health [108–110], to assess acute and chronic stress [113,122,123,126,128,163–165,260] and to investigate emotional responses to positive stimuli [125,145,178,221,222,261]. Recently this technique was employed to investigate emotions in the most popular and ancient domestic animal: the dog. Two studies were conducted to analyze the reactions of dogs in a negative situation of high emotional arousal, i.e. a veterinary visit with an unfamiliar vet, and a positive situation of high emotional arousal, i.e. the delivery of delicious food treats by the owner [261]. In both studies the IRT proved to be a useful technique allowing the detection of T increase in the region of the lacrimal caruncle of the dog's eye associated with the presentation of the both negative and positive stimuli. Nevertheless, IRT did not allow to distinguish between the positive and negative valence of the emotional responses since in both cases the eye temperature increased [113,261]. When the monitoring of the cardiac activity was associated to the measurement of the eye temperature during the positive stimulation with food treats, an increment in HR was found confirming that the situation elicited a high arousal emotional response [261]. On the contrary, the measurement of the heart rate variability HRV (i.e. a non-invasive technique used to investigate the functioning of the autonomic nervous system, especially the balance between sympathetic and vagal activity [146] did not show a clear pattern of vagal activation since only one the HRV parameters measured, the SDNN, increased when dogs relaxation occurred at the end of food delivery. In dogs, HRV is largely used to assess reactions to many different situations. One of HRV parameters, SDNN (standard deviation of the normal to normal intervals) was influenced neither by posture nor by a mild physical activity, while a significant elevation during orientation to a favorite toy was reported [159]. Bergamasco and colleagues [158] compared a group of shelter dogs submitted to a human-interaction program with a control group. Over the course of 3 months, significant differences on some HRV parameters were found suggesting that human interaction supplement sessions have a positive effect and they could affect the physiological indicators of animal welfare. The secure base and safe haven effects of the attachment figure, that are central features of the human attachment theory, have been studied even in dogs. 30 animals were approached by a threatening stranger with or without the owner in the nearby. Comparison of HR and HRV values during the threatening approaches with those before and

after the encounters showed that the threatening approach increased dogs' mean HR, with a parallel decrease in the HRV; besides, the HR increment was significantly less pronounced when dogs faced the stranger in the presence of the owner. Moreover, whether the dog encountered the stranger first with or without its owner, also proved important because HR increment associated with the encounter in separation seemed to be mitigated in those that faced the stranger first with their owner [201]. On the other hand, dogs that were left alone in their home environment did not present any difference in HR and HRV parameters neither in 4 hours of separation nor when the owner came back home despite many behavioral differences [223]. The paradigm of separation and reunion with the owner, eliciting the attachment system, could be an interesting protocol to further investigate the application of IRT to the study of emotions in dogs. Recently, it has been showed that the reunion with a familiar person that greet the dog determined in the animal an increase in oxytocin and a decrease in cortisol concentrations accompanied by sustained physical contact and lip licking [236] indicating that relaxation started upon reunion and lasted for about one hour.

The aim of the present study was to evaluate if a protocol of separation from and reunion to the owner could induce sensible variations in eye temperature and HRV parameters in dogs. A brief separation (i.e. 3 minutes) from the owner occurring in the presence of an unfamiliar person is considered a situation of mild stress [38] and should elicit an emotional state of low arousal and negative valence. On the contrary, the reunion with the owner should elicit an emotional state of high arousal and positive valence. According to this hypotheses eye temperature and HR should increase upon reunion while HRV parameters should decrease during separation and increase upon reunion.

4.3. Materials and methods

4.3.1. Subjects

The subjects were 33 healthy dogs (17 females, 16 males) of different breeds and body size, whose ages ranged from 8 months to 13 years (mean = 4.69 years, SD = 3.35 years). The sample included 16 pure-breed dogs (1 Poodle toy size, 1 Jack Russel Terrier, 1 Miniature Schnauzer, 1 Tibetan Terrier, 1 Beagle, 1 Border Collie, 2 American Pit Bull Terriers, 1 Vizsla, 1 Dalmatian, 2 Labrador Retrievers, 2 Golden Retrievers, 2 American Staffordshire Terriers) and 17 mixed-breed dogs (3 small size, 13 medium size, and 1 big size). All the dogs were kept for companionship, lived within the human household, were accustomed to share daily activities with their owner (e.g., travel by car, going to unfamiliar places, encountering unfamiliar humans), and were used to wear the harness on daily walking. All the owners were informed about the aims of the study and the procedure, and their

informed consent to record behavior and to use the collected data was obtained according to the national Privacy Law 675/96.

4.3.2. Procedure

The study was conducted at Il Cane Lab of the University of Parma (Italy). On arrival, the human-dog pairs were received in the parking lot. Here, the Polar® RS800CX heart monitor was fixed to the dog chest with an elastic belt (see “Physiological parameters” paragraph for further details); if the dog presented a thick hair preventing the correct functioning of the HR monitor, the owner was asked to kindly shorten some hair in the axillary regions. After this manipulation, the owner walked around the department with a relaxed pace for about 10 minutes in order to let the dog relax. Once they came back to the department, they were allowed to enter and escorted to the testing room.

The testing room was an unfamiliar bare room (2.50 x 4.50 m) equipped with an armchair, a carpet, and a video camera (MVX450, Canon, Japan) connected with a computer for instantaneous recording. During the test a video camera operator, the infrared thermography technician (E.H.) and the owner were present. Testing was conducted in May, June, September and October 2015. The testing environment was air-conditioned and thus temperature (24 °C) and humidity (50%) remained constant during the procedure [113].

The test procedure consisted of four consecutive phases:

Phase 1, Baseline: The dog and the owner entered the experimental room, the Polar was switched on and the owner sit on the armchair ignoring their animal while the dog, on a 1.5 m long leash, could remain close to them or move around for 10 minutes; the aim of this phase was to obtain baseline values for each dog.

Phase 2, Separation from the owner (i.e., negative stimulation): The owner unleashed the dog and exit the room. Therefore, the dog was left in the room with the video camera operator and the infrared thermography operator. This phase lasted 3 minutes.

Phase 3, Reunion with the owner (i.e., positive stimulation): The owner come back in the room and greet/pet the dog how they usually do after a separation. This phase lasted 3 minutes.

Phase 4, Relaxation: The owner leashed the dog and sit on the armchair ignoring the dog, as in Phase 1. This phase lasted 7 minutes.

4.3.3. Behavioral measurements

Behavioral data were scored from videos using Solomon Coder beta® 15.11.19 (ELTE TTK, Hungary). The ethogram consisted of 24 behaviors and of 2 behavioral categories, namely Dynamic (walking, jumping and jumping on the owner) and Static (standing still, sitting, or lying down). These two behavioral categories were mutually exclusive but could co-occur with the other 24 behaviors, thus a dog could sit in front of the owner and gaze at their face (Static and Gazing owner) or could walk while exploring the environment (Dynamic and Attention to the environment). Behaviors and categories are detailed in Table 4.1 and were recorded either as duration or as frequencies.

Table 4.1. Description and measure of coded behaviors recorded during the test.

Behavior	Description	Frequency/Duration
Dynamic	Walking, jumping and jumping on the owner	D (% of the total time)
Static	Standing still, sitting, or lying down	D (% of the total time)
Attention to the environment	Visual/olfactory exploration of the environment	D (% of the total time)
Attention to the door	Visual/olfactory exploration of the door	D (% of the total time)
Interactions with the door	Physical interaction with the room door	D (% of the total time)
Exiting from the room	Attempts to exit from the room when the owner enters/exits	D (% of the total time)
Biting carpet	Biting the carpet under the armchair	D (% of the total time)
Biting armchair	Biting the armchair	D (% of the total time)
Approaching armchair without owner	Approaching the armchair while the owner is outside	D (% of the total time)
Gazing owner	Looking at the owner	D (% of the total time)

Behavior	Description	Frequency/Duration
Interactions with the owner	Licking, smelling, greeting, jumping on the owner	D (% of the total time)
Gazing experimenters	Looking at the experimenters	D (% of the total time)
Interactions with experimenter	Licking, smelling, greeting, jumping on the experimenters	D (% of the total time)
Relax	Sleeping or relaxing laying down	D (% of the total time)
Self-grooming	Licking or cleaning itself with teeth	D (% of the total time)
Tail wagging	Hanging tail in a relaxed manner at half-mast [248] and wagging it	D (% of the total time)
Yawning	Deeply inhale through wide open mouth	F (events/minute)
Shaking off	Rapid movements of body shaking	F (events/minute)
Stretching	Straighten the body completely	F (events/minute)
Freezing	Complete motionless, without panting	D (% of the total time)
Avoid IRT camera	Lateral movement of the head to avoid being focused by thermographic camera	F (events/minute)
Vocalization	Barking or whining	F (events/minute)
Avoid owner	Lateral movement of the head to avoid eye contact with the owner	F (events/minute)
Nose/lip licking	Rapid extension and flicking of the tongue on the nose or between the lips	F (events/minute)
Scratching	Scratching itself with paws	D (% of the total time)
Drinking	Drinking from supplied water bowl	D (% of the total time)

Only behavioral patterns that were displayed with a frequency of at least 1 event/min or with a duration over 1% of the time in at least one of the four experimental phases were included in the analysis [61]. Moreover, those behaviors exhibited only in one of the four experimental phases were not statistically analyzed. To evaluate whether the thermographic camera directed towards dogs' muzzle could be perceived as stressing [113] a marker indicating the presence of the thermographic camera accompanied each behavioral element during the coding. Due to technical problems, behavioral data of only 31 dogs were collected.

4.3.4. Physiological parameters

The thermographic infrared images were captured by a certified technician (E.H.) using a portable IRT camera (AVIO TVS500[®] camera, NEC, Japan) with standard optic system, and analyzed with IRTAnalyzer Software[®] (Grayess, FL, USA). To calibrate the camera reflectivity temperature, samples were taken and emissivity was set at 0.97. Several images per dog were collected during the pre-test, to select the images that provided the most optimal operating conditions for analysis (90° angle and 1 m of distance). A total of 2874 (per dog: mean \pm SE = 89.91 \pm 10.34; minimum = 60; maximum = 118) values were analyzed assessing the emission of eyes lachrymal sites. The maximum temperature for each lachrymal site was determined using an Instantaneous Field of View of 1.68 mm at 1 m of distance, within an oval area traced around the eye, including the eyeball and approximately 1 cm surrounding the outside of the eyelids. Only images perfectly on focus were used. To optimize the accuracy of the thermographic image and to reduce sources of noise, before testing each dog the same image of a Lambert surface was taken to define the radiance emission and to nullify the effect of sunlight or other surface reflections on tested animals, thus controlling for external artefacts [191]. Due to a technical problem, IRT data of only 32 dogs were collected.

Heart rate data were collected using a Polar[®] RS800CX human HR monitor (Polar[®] Electro, Finland). The Polar WearLink[®] strap was positioned around the dog thorax and the size was adjusted to provide a tight but comfortable fit. Farmacare ultrasound transmission gel (Farmacare, Italy) was applied to the 2 electrodes of the Polar WearLink[®] strap. The electrodes were positioned over the right and left axillary regions. The Polar[®] watch computer was fixed dorsally to the WearLink strap.

The Polar[®] was set on the R-R interval-recording mode and data collection lasted for the whole duration of the experiment. R-R interval data were analyzed using Kubios HRV software (Version 2.1 Biosignal Analysis and Medical Imaging Group, Department of Applied Physics, University of Eastern Finland, Kupio, Finland). Prior to analyses, artifacts were removed using Kubios' inbuilt artifact correction feature. The artifact tolerated was not more than 1% on the total length of the recording.

Heart rate (HR, beats per minute) and HRV parameters were calculated for each experimental phase. The following variables were chosen for analysis: SDNN (standard deviation of R-R intervals), RMSSD (root mean square of the standard deviation) and SDNN/RMSSD ratio.

4.3.5. Statistical analysis

Interobserver reliability was assessed using Spearman correlations and was significant for all the behaviors recorded with r ranging from 0.6 to 1 (Dynamic: $r = 0.949$, $P < 0.001$; Static: $r = 0.976$, $P < 0.001$; Attention to the environment: $r = 0.968$, $P < 0.001$; Attention to the door: $r = 0.866$, $P < 0.001$; Interactions with the door: $r = 0.861$, $P < 0.001$; Exiting from the room: $r = 0.991$, $P < 0.001$; Biting carpet: $r = 1.000$, $P = 0.000$; Approaching armchair without owner: $r = 0.730$, $P = 0.001$; Gazing owner: $r = 0.973$, $P < 0.001$; Interactions with the owner: $r = 0.793$, $P < 0.001$; Gazing experimenters: $r = 0.676$, $P = 0.004$; Interactions with the experimenters: $r = 0.936$, $P < 0.001$; Relax: $r = 1.000$, $P = 0.000$; Auto-grooming: $r = 0.655$, $P = 0.006$; Tail wagging: $r = 0.924$, $P < 0.001$; Yawning: $r = 0.859$, $P < 0.001$; Shaking off: $r = 0.811$, $P < 0.001$; Stretching: $r = 1.000$, $P = 0.000$; Avoid IRT camera: $r = 0.985$, $P < 0.001$; Vocalization: $r = 0.999$, $P < 0.001$; Scratching: $r = 0.553$, $P = 0.026$; Drinking: $r = 0.633$, $P = 0.008$).

Eye temperature was analyzed using for each dog all the thermographic images taken during each phase and dog's sex as factor.

Behavioral and physiological data were entered in a Generalized Linear Mixed – effect Models Analysis (GLMM). Separate models were set up for each dependent variable measure (behaviors, eye temperature, HR and HRV). All models residuals were checked for normality (Shapiro test) and homoscedasticity (residuals funnel graph). All models included the explanatory variables phase and sex. Moreover, Dynamic behavior was also used as co-variate variable for HR and HRV parameters. All models were reduced in a step-wise forward procedure. There was no significant effect of sex for any of the cardiac parameters (HR, RMSSD, SDNN, SDNN/RMSSD) and, when inserted into models, sex determined a significant worsening of their fitting (Akaike Information Criterion, Schwarz's Bayesian Information Criterion and -2 Log-Likelihood variation). Planned contrasts with Bonferroni correction were used for paired comparisons.

Data from Phase 1 and 4 were analyzed only in the last five and four minutes respectively when absence of any stimulation was consistent.

All statistical analysis were carried out using R 3.1.3 (R development Core Team) packages: car – Companion of Applied Regression [249] and lme4 – Linear Mixed-Effects Models using Eigen and S4 [250].

4.4. Results

4.4.1. Behavioral data

Dogs' behavioral response changed across the three phases as shown in Tables 4.2 and 4.3. In particular, Attention to the door significantly increased during Separation (P1 vs P2, P2 vs P3, P2 vs P4: $P < 0.001$) while during Reunion there were significant increments in Attention to the environment (P1 vs P3: $P = 0.014$; P2 vs P3: $P = 0.001$; P3 vs P4: $P < 0.001$), Gazing owner (P1 vs P3, P1 vs P4, P3 vs P4: $P < 0.001$), Interactions with the owner (3-phases test, it also had Baseline lower than Relaxation, P1 vs P3, P1 vs P4, P3 vs P4: $P < 0.001$), and Tail wagging (P1 vs P3, P2 vs P3, P3 vs P4: $P < 0.001$). Besides, Relax dropped significantly during both Separation and Reunion, being lower during Reunion rather than Separation (P1 vs P2: $P = 0.002$; P1 vs P3: $P < 0.001$; P2 vs P3: $P = 0.040$; P2 vs P4: $P < 0.001$; P3 vs P4: $P < 0.001$). Dynamic significantly increased from Baseline to Separation ($P < 0.001$), it remained constant during Reunion ($P = 0.286$) and during Relaxation it significantly dropped again below Baseline values (P1 vs P4, P2 vs P4, P3 vs P4: $P < 0.001$). Nose/lip licking had a peak during Reunion, compared to Separation and Relaxation (P2 vs P3: $P = 0.033$; P3 vs P4: $P = 0.047$) and it was performed more by males; this is due differences that have been seen in Phase 1 ($P = 0.020$) and, partially, in Phase 3 ($P = 0.061$). An overall significant difference among the four Phases, but not significant pairwise comparisons emerged for Auto-grooming and Interactions with the door (Table 4.2). No other differences emerged, however it might be worth noting that dogs approached armchair without owner only for 0.42% of time during Separation.

Table 4.2. Mean and SE of frequency and durations of analyzed behaviors in the four phases of the experiment.

Behavior	Baseline mean ± SE	Separation mean ± SE	Reunion mean ± SE	Relaxation mean ± SE
Dynamic ^a	5.83 ± 1.10	11.66 ± 2.26	6.77 ± 1.35	1.75 ± 0.61
Static ^a	94.17 ± 1.10	88.34 ± 2.26	93.23 ± 1.35	98.25 ± 0.61
Attention to the environment ^a	44.05 ± 5.68	42.24 ± 4.55	63.36 ± 3.51	36.66 ± 5.60
Attention to the door ^a	2.23 ± 1.83	38.79 ± 5.15	0.42 ± 0.28	0.12 ± 0.12
Interactions with the door ^a	0.00 ± 0.00	2.31 ± 0.90	0.11 ± 0.11	0.00 ± 0.00
Exiting from the room ^{a,c}	0.53 ± 0.33	0.01 ± 0.01	0.39 ± 0.16	0.00 ± 0.00
Biting carpet ^{a,c}	0.59 ± 0.53	0.00 ± 0.00	0.29 ± 0.29	0.10 ± 0.10
Biting armchair ^{a,c}	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Approaching armchair without owner ^{a,c}	0.00 ± 0.00	0.42 ± 0.13	0.00 ± 0.00	0.00 ± 0.00
Gazing owner ^a	9.20 ± 1.86	0.00 ± 0.00	23.34 ± 2.85	3.84 ± 1.19
Interactions with the owner ^a	0.53 ± 0.26	0.00 ± 0.00	8.61 ± 2.57	0.91 ± 0.56
Gazing experimenters ^a	4.50 ± 1.49	5.53 ± 0.81	2.36 ± 0.58	4.91 ± 1.20
Interactions with experimenters ^{a,c}	0.15 ± 0.15	0.34 ± 0.15	0.08 ± 0.06	0.31 ± 0.19

Behavior	Baseline mean ± SE	Separation mean ± SE	Reunion mean ± SE	Relaxation mean ± SE
Relax ^a	35.41 ± 6.38	10.08 ± 3.47	0.54 ± 0.55	52.69 ± 6.54
Auto-grooming ^a	2.81 ± 1.26	0.31 ± 0.22	0.48 ± 0.27	0.46 ± 0.41
Tail wagging ^a	6.20 ± 1.53	10.96 ± 3.67	30.42 ± 5.23	4.47 ± 1.56
Yawning ^{b,c}	0.11 ± 0.09	0.08 ± 0.15	0.20 ± 0.12	0.07 ± 0.12
Shaking off ^{b,c}	0.42 ± 0.04	0.10 ± 0.11	0.39 ± 0.06	0.03 ± 0.24
Stretching ^{b,c}	0.03 ± 0.03	0.09 ± 0.04	0.03 ± 0.11	0.04 ± 0.02
Freezing ^{a,c}	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Avoid IRT camera ^{b,c}	0.02 ± 0.03	0.00 ± 0.04	0.05 ± 0.02	0.00 ± 0.02
Vocalization ^b	0.06 ± 0.04	1.73 ± 0.06	0.06 ± 1.13	0.22 ± 0.06
Avoid owner ^{b,c}	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Nose/lip licking ^b	♀ 0.00 ± 0.00	♀ 0.06 ± 0.03	♀ 0.51 ± 0.24	♀ 0.10 ± 0.06
	♂ 0.42 ± 0.18	♂ 0.20 ± 0.13	♂ 1.83 ± 0.65	♂ 0.22 ± 0.12
Scratching ^{a,c}	0.24 ± 0.17	0.21 ± 0.21	0.16 ± 0.14	0.45 ± 0.31
Drinking ^{a,c}	0.35 ± 0.27	0.00 ± 0.00	0.55 ± 0.24	0.21 ± 0.21

^a Behaviors measured as duration (percentage of total time). ^b Behaviors measured as frequency (events/min). ^c Behaviors not analyzed because they occurred in only one phase or were rarely displayed.

Table 4.3. Statistical results of the behavioral data

	Phase		Sex		Phase x Sex	
	F	P	F	P	F	P
Static	7.660	< 0.001	0.818	0.368	0.353	0.787
Attention to the environment	5.444	0.002	0.206	0.651	0.127	0.944
Attention to the door	47.714	< 0.001	1.565	0.213	0.737	0.532
Interactions with the door	6.048	< 0.001	0.080	0.778	0.028	0.994
Gazing owner	27.805	< 0.001	0.009	0.923	1.384	0.256
Interactions with the owner	37.551	< 0.001	0.357	0.552	0.193	0.825
Gazing experimenters	1.511	0.215	2.650	0.106	0.658	0.579
Relax	23.029	< 0.001	0.513	0.475	0.232	0.874
Auto-grooming	2.918	0.037	0.006	0.939	0.289	0.834
Tail wagging	12.120	< 0.001	0.066	0.798	0.171	0.916
Vocalization	1.849	0.142	1.993	0.161	1.625	0.187
Nose/lip licking	7.483	< 0.001	7.682	0.007	2.415	0.070

4.4.2. Physiological data

Eye temperature did not vary significantly across phases ($df = 3$, $F = 1.455$, $P = 0.230$, Figure 4.1) and no difference emerged comparing females and males ($df = 1$, $F = 0.995$, $P = 0.320$, Figure 4.1).

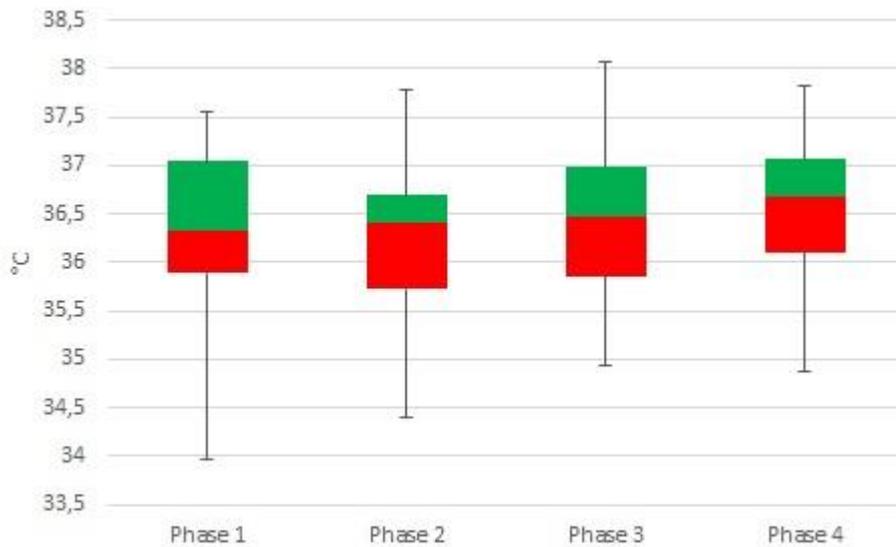


Figure 4.1. Boxplot representing eye temperature ($^{\circ}\text{C}$) exhibited by the dogs during the four phases of the experiment (Baseline, Separation, Reunion, Relaxation)

There was not a significant relationship between physical activity (Dynamic) and any cardiac parameter (HR: $t = 0.683$, $P = 0.170$; SDNN: $t = -1.136$, $P = 0.259$; RMSSD: $T = -0.266$, $P = 0.791$; SDNN/RMSSD ratio: $T = -0.389$, $P = 0.698$). Once controlled for the effect of Dynamic, HR and SDNN/RMSSD ratio did not change across phases (HR: $df = 3$, $F = 2.010$, $P = 0.117$; SDNN/RMSSD: $df = 3$, $F = 1.971$, $P = 0.123$; Figure 4.1). SDNN changed across phases ($df = 3$, $F = 5.661$, $P = 0.001$; Figure 4.1): it dropped significantly from Baseline to Separation ($P = 0.030$), it remained constant between Separation and Reunion ($P = 0.542$) and then it significantly increased during Relaxation ($P < 0.001$). Relaxation values were higher than Baseline ones ($P = 0.044$). An overall effect was found for RMSSD ($df = 3$, $F = 6.976$, $P < 0.001$; Figure 4.1), due to a huge increment during Relaxation (P1 vs P4: $P = 0.001$; P2 vs P4, P3 vs P4: $P < 0.001$).

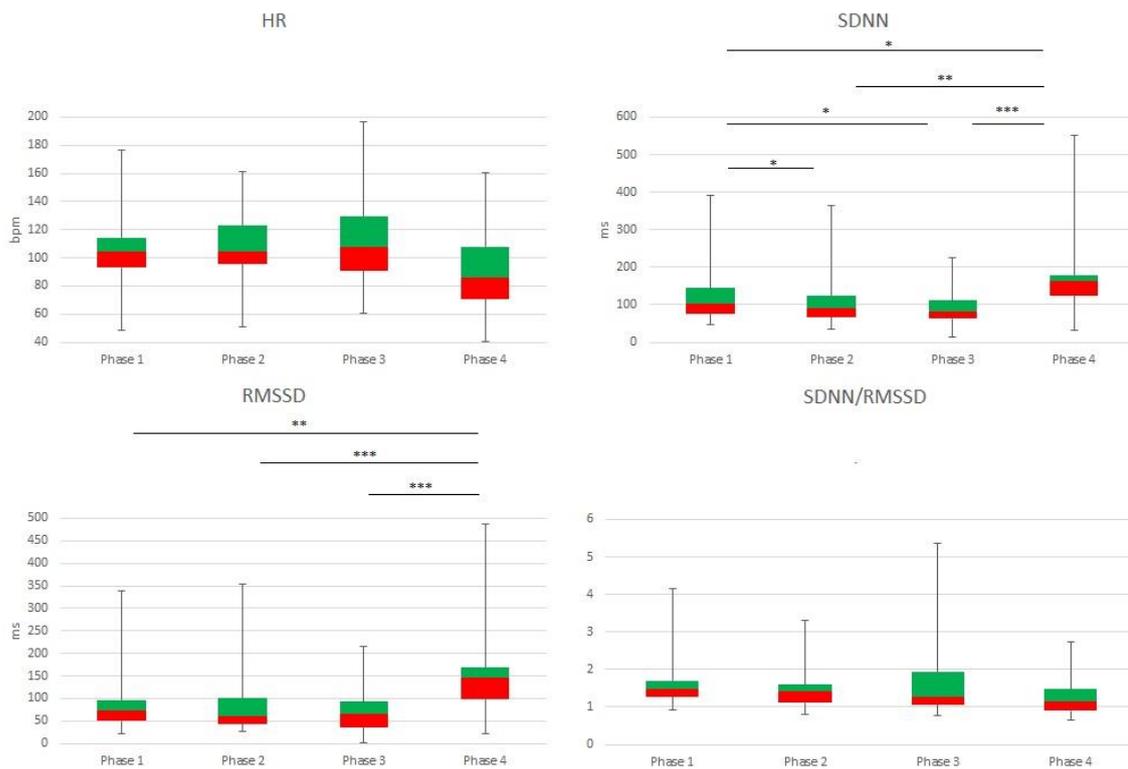


Figure 4.2. Boxplot representing values of heart rate (bpm), RMSSD (ms), SDNN (ms), and SDNN/RMSSD exhibited by the dogs during the four phases of the experiment (Baseline, Separation, Reunion, Relaxation).

4.5. Discussion

The aim of the present study was to investigate eye temperature, detected through IRT, and cardiac activity, detected through an external heart rate monitor, as potential physiological indicators of emotional states in pet dogs during a protocol of separation from and reunion with the owner. The protocol was set up in order to assess possible differences between responses to the negative and to the positive stimulus. In addition, dogs' behavior was used to provide an integrative measure of the response to the positive and negative stimulations.

Contrary to what expected, eye temperature did not increase significantly neither during Phase 2 (Separation) nor during Phase 3 (Reunion), compared with the values of both Phase 1 (Baseline) and 4 (Relaxation). This result did not support our prediction that the arousal determined by both the separation from the owner and the reunion with them would have resulted in an increment of eye temperature.

In dogs, we have found that eye temperature increased both when they received a food treat (positive valence) [261] and when they were stressed by a veterinary visit (negative valence) [113]. In our previous studies, eye temperature showed a very similar pattern of temporal changes, even if different ranges in eye temperature, they could be due to inter-individual differences. Therefore,

according to our previous results and to these evidences, this outcome is unexpected. Separation and reunion protocols, derived by Ainsworth's Strange Situation Test, are known to be effective on dogs, causing separation anxiety related behavior [262,263] and variation in cardiac activity [179] when dogs are left alone with strangers. Both behavioral and cardiac responses are confirmed by our results indicating a state of arousal in dogs, therefore, the lack of modifications in eye temperature is probably due to other factors that need to be further investigated. It could be that stimuli were not intense enough to elicit a response. Besides, different species require different time: cows' nose temperature increased during a positive event in few minutes [129], while macaques' one decreased in not more than 30 seconds [128]; in dogs, this is not being investigated yet and in both our previous studies [113,261] stimulation lasted for longer period.

Because cardiac activity can be influenced not only by emotional states but also by physical activity we considered the effect of movement in the statistical analysis. However, from data emerged an unclear pattern. HR did not vary among phases, confirming what was reported by Maros and colleagues [159], even if it is possible to notice a non-significant drop during Relaxation (Phase 4). However according to existing literature and to our previous work [179,261], we expected an increment in HR during stimuli phases (i.e., Separation and Reunion). Fallani and colleagues [179] reported that during the SST, all dogs showed a HR increase, in particular when they had to play with humans during separation from their caregiver dogs' HR was significantly lower than when the owner or stranger remained in the room. The same finding was reported even by Palestini and colleagues [178]. However, in this study we used Dynamic as a covariate for cardiac data and in the study of Fallani and colleagues heart rate gradually increased when dogs were physically activated; besides, they did not collect heart rate data with a continuous sampling but collecting a datum every 5 seconds making impossible any HRV analysis. As for the HR, SDNN/RMSSD ratio remained unvaried, but SDNN and RMSSD changed across phases. SDNN decreased significantly during Separation and again during Reunion (even though not significantly) and increased in Relaxation, reaching values significantly higher than Baseline; on the other hand, RMSSD remained constant among the first 3 phases but it increased in Relaxation. Analysis of the HRV is a promising tool to assess animal emotions [145,158,228,242]. We hypothesized that the stressful nature of the first stimulus, and on the other hand, the rewarding nature of the second one would have resulted in a decrease in the HRV parameters considered during Phase 2 immediately followed by an increment of the same parameters during Phase 3. Overall results partially support this hypothesis. SDNN significantly decreased during Phase 2, while RMSSD dropped even if not significantly, however, during Phase 3, both SDNN and RMSSD did not significantly changed but the first dropped while the latter increased. Lastly, during

Phase 4, both SDNN and RMSSD greatly increased exceeding Baseline values suggesting that changes occurred in the sympathovagal balance. Even if HRV is not very clear, it is still possible to conclude that a separation and reunion protocol induced emotional changes in dogs. Being separated by the owners seem to be sufficient to induce a change in adult dogs' autonomic system, but being reunited with them and being petted and greeted did not elicit an increment in HRV parameters as stated in available literature [235] while this seems to happen during Phase 4, when dogs are again with their owner but not actively stimulated by them. In the discussion of these data, it has to be noticed that available literature concerning dogs is of limited help due to the great diversity in experimental protocols, HRV parameters used determining a lack of consistency in obtained results [159,201,258,259].

Observed behavioral parameters are consisted with available literature [38,39,178,264,265]. During Separation phase, dogs were mainly engaged in paying attention to the door, to physically interacting with it, scratching and smelling it and, only secondly, to gaze experimenters. Besides, this phase was even characterized by a large amount of barking. However, it may be worth noting as only a small amount of dogs, and for a limited time, approached the armchair while the owner was absent and that stress/fear signals were rarely displayed. During Reunion, dogs, as expected, gazed and interacted with the owner more than other phases; besides they wagged more their tail confirming that tail wagging is signal of an intense emotional response [234,252]. Interestingly, despite the return of their owner, dogs spent a great percentage of time exploring the environment. This increased exploratory behavior coincident with the return of the owner is a typical characteristic of the secure base effect described in humans and in non-human infants. Nose/lip licking has been performed mainly by males and it occurred during Phase 3 when dogs were reunited to their owners. Different authors gave different interpretations of this signal [176,187,223,224], and in the present study, in the context of reciprocal greeting, it could be an appeasement signal toward the owner.

4.6. Conclusion

Our study is the first assessing effects of a separation and reunion protocol on eye temperature. Results do not confirm previous findings showing suitability of IRT as non-invasive method. It is possible that the experimental situation elicits an emotional state of low arousal not detectable by the IRT, thus further studies are needed to compare low and high arousal stimuli. On the other hand, the analysis of HRV aimed at providing insight for the evaluation of the valence of emotional states gave useful indication on emotional regulation: the negative stimulus resulted in a decrease of the HRV, while the delayed increment of HRV expected during the positive stimulation requires further investigation. Behavioral data are consistent with previous findings about Ainsworth's

Strange Situation, confirming it as a good protocol to elicit emotional states of both a positive and a negative valence in dogs.

4.7. Ethic statement

No special permission for the use of animals (dogs) in non-invasive observational studies is required in Italy. The relevant ethical committee is the Ethical Committee of the Università degli Studi di Parma. All dog owners were informed about the nature and scope of the study and their written consent was obtained before the study was initiated.

5. Assessing behavioral and physiological effects of different environmental enrichments in isolated sheltered male dogs

Tiziano Travain¹, Simona Cafazzo², Eugenia Natoli³, Paola Valsecchi¹

¹ Dipartimento di Neuroscienze, Unità di Biologia del Comportamento, Università degli Studi di Parma, Parco Area delle Scienze 11/A, 43124 Parma, Italy

² Wolf Science Center, Dörfles 48, 2115 Ernstbrunn, Austria

³ Azienda Sanitaria Locale Roma D, Ospedale Veterinario, via della Magliana 856, 00148 Rome, Italy

Paper in preparation

5.1. Abstract

The scenery of Italian dog shelters is very diversified. Because of the law 281/1991, the euthanasia of dogs without owner is allowed only for those incurable or dangerous; free-ranging caught dogs, if not immediately returned to an owner, are hosted into long term stay kennels. Health management of these structures can be very difficult considering the high rate of entry, slightly balanced by the number of adoptions. There are kennels in which animals are well treated, they can socialize with other animals and with operators, and overcrowded kennels created and managed by people only interested in obtaining public funding for shelters where animals are kept with no cure, often sick and malnourished; in Italy, these latter cases are pursued by competent authorities. Given that the no-kill policy in Italy increases the need to provide dogs housed in a shelter with an adequate quality of life, the aim of this study was to assess the level of welfare of shelter dogs in different conditions, yielding information on which is less stressful.

The study was conducted in a dog shelter near Rome on 8 adult male mixed breed dogs (apparent age 2-7 years, singly housed, arrived at the shelter since not less than 3 months but not more than 2 years). These dogs are fed and regularly controlled by a veterinarian, but they are socially deprived by both conspecifics and human beings because they are normally kept isolated and they can get in touch with an operator only when they receive food or when the box is cleaned, as they are not carried out of the box for a daily walk.

Our aim was to investigate the effects of exposure to four different situations on heart rate, body temperature, physical activity and behavior. A further aim of the study was to assess whether telemetry (i.e. the continuous record of heart rate, body temperature and physical activity, see below), usually utilized in pharmacological study, could be an appropriate and useful tool to evaluate welfare of sheltered dogs.

The telemetry system consisted of flat transmitters and platform receivers. The dogs were implanted with the radio telemeter and the platform receiver was installed inside the box. After implantation, dogs were allowed 2 weeks for recovery, and then were housed in sequence in each of the following situations: (1) alone in the box (baseline, as previously said this is the normal living situation); (2) alone in a box enriched with toys and a stretch cot for sleeping; (3) in a box with an unknown, spayed, female; (4) alone in the box with a daily 2-hours interaction with an experimenter. Each situation lasted 1 week. Every dog was continuously video-recorded.

A specifically designed ethogram was used for coding, performed with Solomon Coder. Two different approaches were tried: in the first one, 48 fragments of 30 minutes were extracted partially

randomly every week. In the second approach, behaviors from 8 a.m. to 4 p.m. were continuous analyzed during first day of baseline, first, second, and fifth day of the female situation.

5.2. Introduction

In Italy, law 281 enacted in 1991 for the prevention of the phenomenon of stray dogs was a turning point in the relationship between humans and pets. It made illegal suppression of stray dogs and cats except in cases of proven dangerousness or incurable diseases. Before this law, in a definitely different sanitary context because of the presence of rabies, the decree of the President of the Republic 320 enacted in 1954 established that the captured stray dogs were transported to the local rescue shelter where they were kept for a period of three days. After these days, dogs unclaimed by the legitimate owners had to be suppressed with painless methods. Current law states that two distinct structures with different purposes should exist: the sanitary shelter (public) and the rescue shelter (private). The sanitary shelter is a structure hosting captured or abandoned stray dog temporarily; dogs stay there monitored for 10-60 days, available to be claimed by the owner or adopted by a new one. After this period, dog is headed to a rescue shelter. Among other consequences, the application of this law raises the ethical issue of maintaining an adequate level of welfare in shelters. In fact, many of the dogs housed in rescue shelters will spend the rest of their lives there. Today, in Italy, both sanitary and rescue shelters have boxes of 6-7 square meters, allowing little or none social intra- and inter-specific interactions but those provided during some walking in the spaces provided inside shelters themselves. This had led to many different situations of individuals clearly stressed, with different kinds of stereotypes, and abnormal behaviors due to captivity, to the new environment, separation of key figures and exposure to an environment unpredictable and uncontrollable [266].

Dogs are animals that need a complex environment, containing both animate and inanimate objects [267]. Ensuring social contacts, with both other dogs and humans, it is essential and it should be considered the most important enrichment for dogs in captivity [268]. Even the presence of an inanimate but still complex and challenging environment, it is important for dogs living in cages. Introduction of appropriate games, music, odors and accessories might be useful to improve the routine [268]. Besides, the rotation of all these elements is considered particularly important to prevent habituation [269].

It is well known that the dogs forced to live in restricted environments can develop abnormal behaviors [270,271]. To dogs, experience in shelters involves impact on their welfare, but also behavioral changes that can affect the chance of an adoption. In fact, long-term confinement in a cage has been associated with the development of stereotypies in dogs and other species [270–272]. This is supported even by neuroendocrine studies: it was found that dogs entering a rescue shelter have prolonged activation of the HPA axis [273]. Also, according to Voith and Ganster [274], dogs adopted

from shelters appear to have more problems related to play behaviors and to the separation from the owner.

Environmental enrichments are commonly used in applied ethology. They promote animal natural behavior and increase welfare. However, enrichment is a vague notion: even if there is agreement that it means “improvement”, it is often applied to different types of environmental changes (social, physical, sensory, nutritional, etc.) while some authors use it as a synonym for “greater complexity” [275]. Researches based on enrichment aim to reduce negative emotional states. These include fear and stress associated with exposure to new stimuli [276–278], boredom and apathy resulting from lack of stimuli [279], and frustration due to inability to express a wished behavior [280].

Different studies have shown that social and physical enrichments have measurable benefit for welfare. Hubrecht [281] tested four groups of laboratory beagles: a control group, a group that could socialize with conspecifics, a group receiving 30 s per day of intensive handling, and a group provided with three different chew toys suspended in the pen. The study shows that appropriate environmental enrichment changes expressed behavior incrementing its complexity and helps to prevent undesirable behaviors; social interactions changes behavior with conspecifics. Schipper and colleagues [282] investigated the effects of feeding enrichment toys on the behavior of laboratory dogs. These toys enhanced appetitive behaviors, increased their activity time, and lowered barking occurrence. Authors stated that these toys seem to be a useful tool to stimulate appetitive and more variable behavioral patterns, at least in the short-term, and that they may also be useful for dogs in other facilities which lack sufficient stimuli, such as animal shelters. Mehkram [283] evaluated if social interaction with humans could be enhance socialization of pair-housed wolves and wolf–dog crosses hosted in a private sanctuary. Confirming results by Hubrecht [281], human interactions increased occurrence of conspecific-directed affiliative behaviors and reduction of abnormal behaviors. Besides, when caregivers were present an increment in social play were observed supporting the idea that an improvement of the environment might promote conspecifics social interactions. The potential for human interaction to be established as a scientifically validated, cost-effective enrichment strategy is supported by these findings. In large facilities physical enrichment is likely to be the most cost-effective option, but staff should be encouraged to have regular positive socialization sessions with their dogs.

Telemetry consists of miniature subcutaneous sensors and transmitters to detect and broadcast biological signals to a receiver and to a computerized data acquisition system. Currently, telemetry systems can collect blood pressure and flow, heart rate, electrocardiogram, electroencephalogram, electromyogram, respiratory rate, pH, body temperature, and activity indexes [284]. In the last years, cost of telemetry systems is significantly decreased and, consequently, there

has been a significant increase in the use of this technique. In biomedical research, telemetry has several advantages: (1) there is a reduction of distress compared with conventional measurement techniques, allowing monitoring of physiological parameters in conscious, freely moving laboratory animals; (2) there is a reduction of animal use [285]; (3) it allows unrestricted continuous data collection for prolonged periods of time without the need of any special animal care; (4) it is available for use in a wide range of laboratory species (e.g. mice, monkeys, some fishes). These advantages are only partially counterbalanced by some disadvantages: (1) even if purchase costs are much more affordable now than in the past, the cost to acquire the equipment is not negligible; (2) it requires surgery to prepare animals, and thus this requires specialized staff; (3) continuous or regular scheduled sampling generates large amounts of data, which can lead to analysis problems [284]. Data collected with this technique were evaluated in several studies and compared with those collected by conventional methods. Measurements performed with other conventional methods validate the calibration of the telemetry system [162]. In addition, it has been found that the measures of blood pressure, and consequently those of heart rate, were stable up to 18 weeks after implantation [161].

In dogs, it is still a quite novel technique: Klumpp [286] tested the influence of different housing conditions on hemodynamics during cardiovascular general pharmacological studies implanting telemeters on dogs. Two different groups were housed in two different housing situations. In the first one, four cages were placed two on each side of a corridor; in the second, the four cages were positioned in a row and the bordering cages were not separated by a metal plate. The physiological status of the dogs in the different housing models was assessed using average resting heart rate as physiological parameter while both frequency of vocalizations and video monitoring were used to assess behavioral responses. The housing had a remarkable effect on the measured hemodynamics. The physiological parameter was best when the dogs were housed with their usual run mate. Besides, they emitted fewer vocalizations. Authors stated that their study demonstrated that the quality of the acquired cardiovascular data for conscious dogs is dependent on the pen configuration and group make-up during a study.

This study aims to apply telemetry technique to assess welfare levels and, consequently, the stress state of the dogs in rescue shelters. This system has never been previously applied to dogs housed in a rescue shelter. If this technique proves to be effective, it could be used to assess which environmental enrichments (physical and/or social) can improve their quality of life.

5.3. Materials and methods

5.3.1. Subjects

The subjects were 8 healthy mixed breed male dogs, whose apparently ages ranged from 2 to 7 years. The sample included 6 medium size dogs, and 2 big size dogs. These dogs arrived at the shelter since not less than 3 months but not more than 2 years. They were fed and regularly controlled by a veterinarian, they were socially deprived by both conspecifics and human beings because they were normally kept isolated and they could get in touch with an operator only when they received food or when the box was cleaned, as they were not carried out of the box for a daily walk.

5.3.2. Procedure

The study was conducted at rescue shelter “Centro Cinofilo del Lago” in Bracciano (Rome, Italy). It has 1.5 x 5 m boxes arranged in series inside a shed, overlooking all on the same side of a single hallway. Boxes have an inside and an outside part of the same dimension (1.5 x 2.5 m) separated by a wall having a passage. Boxes are divided one by the other by a 1-meter wall, on which is fixed a metal mesh reaching the ceiling. This allows dogs to stand up to look into other boxes. The front and the rear side have only wire mesh. Door is located in the front, inside, side, towards the hallway. The outside part of the box overlooks the countryside. Once a day, in the morning, boxes were cleaned with high pressure water and dogs were fed with dry food. Sample dogs were never taken out of this box, except for rare displacements due to the reorganization of shelter or medical care, but this did not happen during the study. The box used for this experiment was the last of ten of the hallway, so only one side was facing another box. All other boxes were normally occupied by others dogs.

Each dog was operated under general anesthesia to implant subcutaneously the transmitter. After the operation, the recovery period of the dogs was 14 days. Subsequently, the dogs were housed for 4 weeks in 4 different situations, always inside the box containing the receivers. These situations lasted a week each and they were presented one after the other, without any interval and always in the same sequence. The following situations were presented:

1. **Baseline** – first week: the dog is alone in the box without any kind of enrichment; in the box there were only a wooden platform to sleep, a metal bowl for the food and one for the water. This is their standard housing condition.
2. **Environmental enrichment** – second week: the box had been enriched with a cot, a rubber ball, a squeezing toy, a knotted rag, and a big bone.

3. **Social enrichment (female)** – third week: the dog shared the box with an unknown, spayed female; a second wooden platform and a second food bowl were implemented.
4. **Social enrichment (human)** – fourth week: the dog was alone in its box, as it was during the first week; however, once a day, in the morning, after cleaning and feeding, a person visited it spending two hours playing, cuddling, or just keep the dog company if it did not want to physically interact; the same person visited the dog across the week.

Different situations did not overlap, e.g., second week the cot replaced the wooden platform and the toys were removed before adding the female to the scenario, while the wooden platform was introduced again. Data collected during box cleaning and food administration were removed from the analysis.

5.3.3. Telemeter and physiological parameters

Telemeter, Dataquest A.R.T. system (Data Sciences International, MN, USA), consisted of a disk (diameter: 5 cm, thickness: 0.8 cm) from which two electrodes departed. These were two copper wires covered by rubber of 20 cm, the ends were placed near the heart.

Collected data were transmitted via radio waves to receivers placed inside the box. Receivers were antennas contained inside metallic plates. In the box there were four receivers, two inside and two outside, two on the right side and two on the left side; all of them were fixed on the wall about 50 cm over the ground. From the receivers, a cable departed to reach the Data Exchange Matrix connected to a PC. Cables were channeled in a plastic tube exiting the shed and reaching a nearby workstation about 5-6 meters far. The Data Exchange Matrix had the following functions: (1) energizing the receivers; (2) converting and sending to the PC the collected data; (3) calculating the muscular activity index.

Physiological parameters collected were core temperature (°C), heart rate (bpm), muscular activity (measured in movement units). As previously said, the latter is calculated by the Data Exchange Matrix: every time dogs moved, the signal emitted by the transmitter to the receivers varied in strength, orientation and distance from the antennas allowing the software to generate the movement units. Sampling rate allowed to have a data collected every minute. These temperature, heart rate and movement values were the mean values of the minute itself. Data were collected every day, 24 hours/day.

5.3.4. Behavioral measures

Outside of the box, in the hallway, an infrared video camera has been placed on a tripod allowing recording during both daytime and nighttime. The shot included the whole inside part of the box including the entry, and part of outer space. Behaviors were registered every day 24 hours/day. Behavioral data were scored from videos using Solomon Coder beta® 12.09.04 (ELTE TTK, Hungary).

The ethogram consisted of 13 behaviors that were coded for their duration, six that were coded for their occurrence, one marker to indicate if the dog was standing on the wooden platform or on the cot, and one to indicate in the dogs was eating, drinking, urinating, or defecating (Table 5.1). Any occurrence of aggressive behaviors between dogs led to an end of the experiment. Only males' behaviors were coded and analyzed.

The dogs' behavior was coded from videos by one person whereas a second independent coder analyzed 20% of the data to assess inter-observer reliability.

Table 5.1. Males' behaviors and markers coded

Behaviors (duration - % of the total time)	
Abnormal behaviors	Coprophagy, Fly catching
Self-grooming	Dogs licks itself or clean itself with teeth
Attention	Dogs look outside, smell odors, items, or environment
Locomotor stereotypies	Dogs walk back and forth, in a circle, or jump compulsively on the walls
Movement	Running or walking
Oral stereotypies	Dogs lick or bites compulsively objects or bars
Sleep	Sleeping or relaxing laying down with closed eyes
Scratch	Dogs scratch itself

Behaviors (duration - % of the total time)

Playing (only environmental enrichment week)	Dogs play with provided toys
Affiliative behaviors (only social enrichment – female week)	Towards the female, dogs court, lick and clean with the teeth, sleep in contact, have a passive contact, wag
Aggressive behaviors (only social enrichment – female week)	Towards the female, dogs growl, raise hair, curl lips, show teeth, try to attack
Dominance behaviors (only social enrichment – female week)	Towards the female, dogs looks straight into her eyes holding their gaze, puts their head or paw on her back or shoulder (namely, T position), wag their tail high, hold the tail high and still
Submissive behaviors (only social enrichment – female week)	Towards the female, dogs lower their head, hold their tail between legs, lick her muzzle, lay on their back exposing the belly

Behaviors (occurrence – events/minute)

Barking	Dogs bark
Change posture	Dogs change their posture (from/to: laying down, sit, standing)
Nose/lip licking	Dogs lick their nose and/or lip
Shaking	Dogs shake
Stretching	Dogs stretch
Yawning	Dogs yawn

Markers	
Wooden platform/Cot	Dogs are on the wooden platform or, during Environment enrichment week , on the cot
Maintenance behaviors	Dogs are eating, drinking, urinating, or defecating

5.3.5. Statistical analysis

Descriptive statistics was used to present both physiological and behavioral results. Differences in behavioral responses among dogs and for both behavioral and physiological data among different situations were evaluated using non-parametric and two-tailed statistical tests, setting alpha at 0.05. Friedman's ANOVA for ranks and Wilcoxon rank-sum test with Bonferroni correction were run. All the statistical analyses were carried out with SPSS Statistics 23 (IBM, NY, USA).

5.3.6. Two different approaches

Two different approaches were chosen to analyze data: (1) a comparison between all the situations was conducted; (2) a detailed analysis was conducted, but only comparing the baseline with the situation that was hypothesized to be the most impactful for non-socialized isolated dogs. For both approaches physiological and behavioral measurements were considered.

5.3.6.1. *Approach 1: Comparing the four weeks*

In this approach, every day of the week was divided in 48 fragments of 30 minutes. Subsequently, from the 336 fragments composing a week, 48 were partially randomly chosen in order to have the fragments equally distributed among the days of the week (7 segments a day for 6 days and 1 randomly chosen day with only 6) and to do not have segments from the same day consecutive. This was done for every week of every dog. Behavioral data were collected with continuous sampling; physiological data were collected as mean value of every segment. Analysis are still in progress. Data of only 3 dogs are presented: Brando, Buck, and Jack.

5.3.6.2. *Approach 2: The effect of female presence*

In this approach, the first day of the baseline and the first, second, and fifth day of the social enrichment (female) week were chosen to be analyzed. Because during night dog mainly slept, only daylight time, from 8.00 a.m. to 4.00 p.m., was analyzed. Behavioral data were collected with

continuous sampling; physiological data were collected as mean value of every minute. Analysis are still in progress. Data of only 5 dogs are presented: Brad, Buck, Jack, Scotty, Sparrow.

5.4. Results

5.4.1. Approach 1: Comparing the four weeks

5.4.1.1. Physiological parameters

Physiological parameters of the three dogs during the 48 segments of every week were separately analyzed.

5.4.1.1.1. Brando

Neither Brando's index of muscular activity nor heart rate vary among weeks (Muscular Activity: $df = 3$, $\chi^2 = 1.725$, $P = 0.631$; Heart Rate: $df = 3$, $\chi^2 = 4.725$, $P = 0.193$). However, there is a difference in temperature values ($df = 3$, $\chi^2 = 35.425$; $P < 0.001$): this is due to a peak in core temperature during environmental enrichment week comparing it with baseline and female weeks (Baseline vs Enrichment: $Z = -5.057$, $P < 0.001$; Female vs Enrichment: $Z = -2.836$, $P = 0.005$; Figure 5.1).

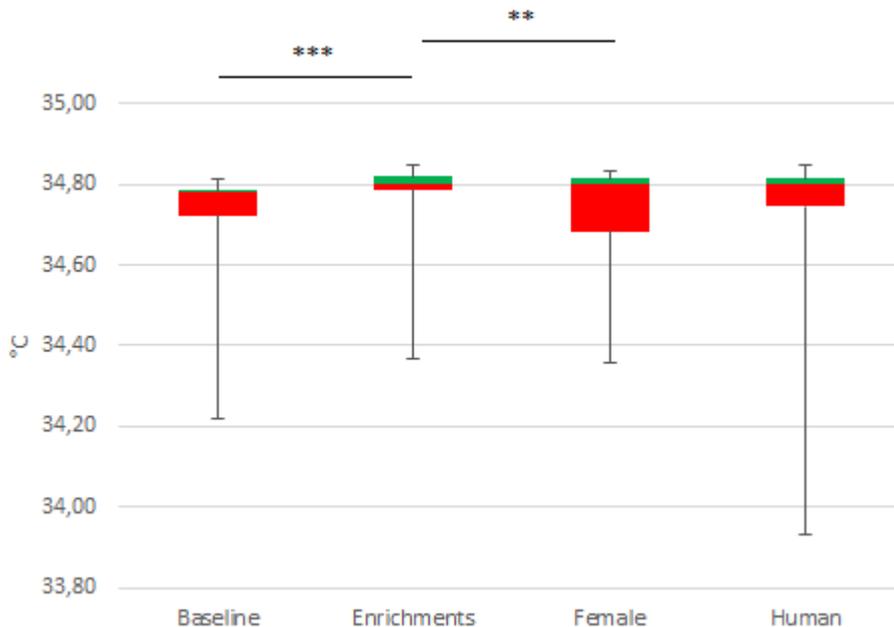


Figure 5.1. Boxplot representing Brando's core temperature (°C) during the four weeks of the experiment.

5.4.1.1.2. Buck

Buck's index of muscular activity does not change among phases ($df = 3$, $\chi^2 = 4.930$, $P = 0.177$). There are differences in both heart rate and temperature values (Heart Rate: $df = 3$, $\chi^2 = 61.772$, $P < 0.001$; Temperature: $df = 3$, $\chi^2 = 37.298$, $P < 0.001$). Heart rate has its maximum peak when the female

was present and a second, but lower, peak was during human social enrichment week (Baseline vs Female: $Z = -5.959$, $P < 0.001$; Baseline vs Human: $Z = -5.086$, $P < 0.001$; Enrichment vs Female: $Z = -5.345$, $P < 0.001$; Enrichment vs Human: $Z = -4.540$, $P < 0.001$; Female vs Human: $Z = -2.912$, $P = 0.004$; Figure 5.2). Temperature has only one peak, during environmental enrichment week (Baseline vs Enrichment: $Z = -4.408$, $P < 0.001$; Enrichment vs Female: $Z = -4.882$, $P < 0.001$; Enrichment vs Human: $Z = -4.226$, $P < 0.001$; Figure 5.2).

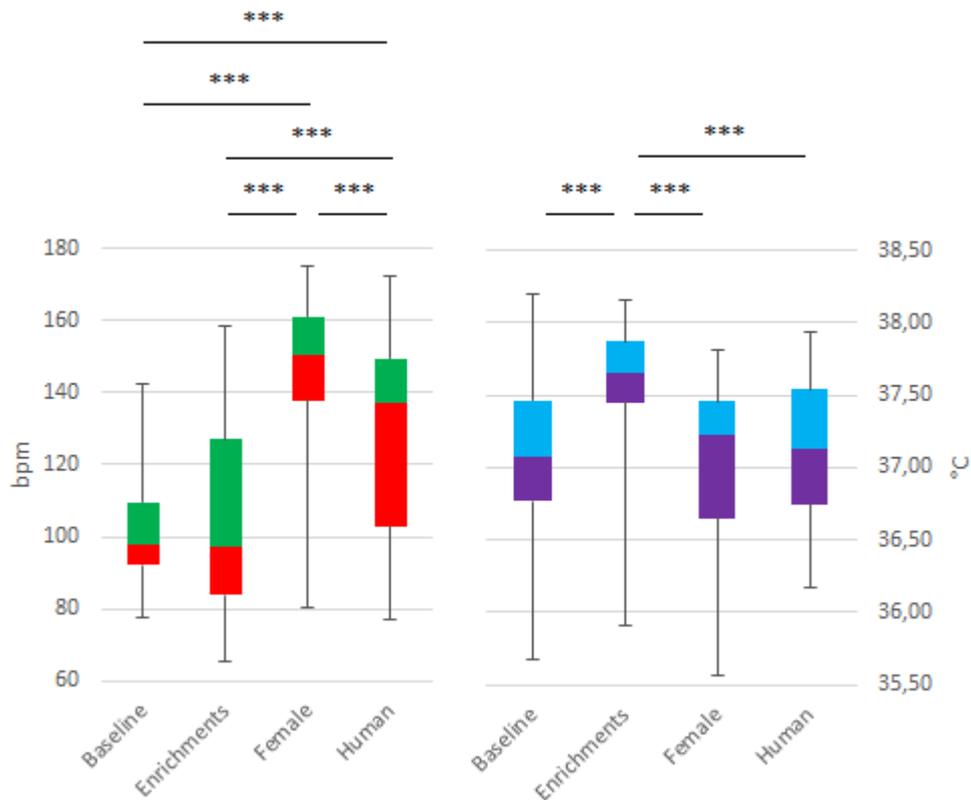


Figure 5.2. Boxplot representing Buck's heart rate (bpm) and core temperature ($^{\circ}\text{C}$) during the four weeks of the experiment.

5.4.1.1.3. Jack

Neither Jack's index of muscular activity nor temperature vary among weeks (Muscular Activity: $df = 3$, $\chi^2 = 6.739$, $P = 0.081$; Temperature: $df = 3$, $\chi^2 = 5.543$, $P = 0.136$). Heart rate presents a significant drop during environmental enrichments week and a significant peak during both female and human weeks ($df = 3$, $\chi^2 = 72.044$, $P < 0.001$; Baseline vs Enrichment: $Z = -4.913$, $P < 0.001$; Baseline vs Female: $Z = -3.528$, $P < 0.001$; Baseline vs Human: $Z = -4.841$, $P < 0.001$; Enrichment vs Female: $Z = -5.651$, $P < 0.001$; Enrichment vs Human: $Z = -5.969$, $P < 0.001$; Figure 5.3).

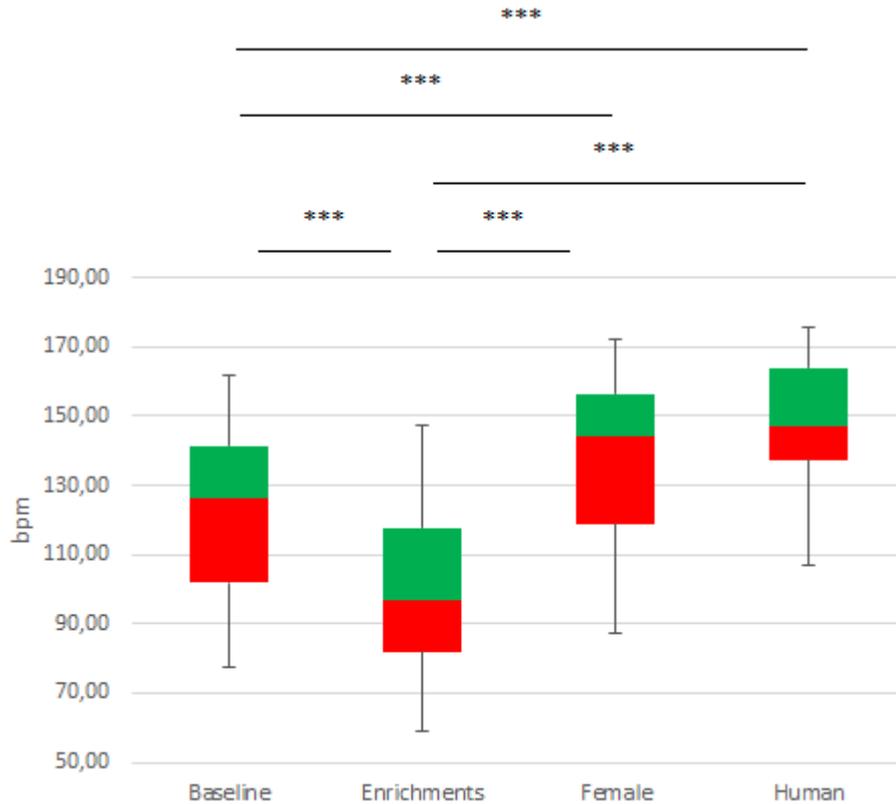


Figure 5.3. Boxplot representing Jack's heart rate (bpm) during the four weeks of the experiment.

5.4.1.2. Behavioral parameters

During baseline week, dogs behaved differently (Figure 5.4). There are significant differences among dogs in percentages of Movement ($df = 2$, $\chi^2 = 9.652$, $P = 0.008$), Attention ($df = 2$, $\chi^2 = 11.411$, $P = 0.003$), Sleep ($df = 2$, $\chi^2 = 9.263$, $P = 0.010$), Scratch ($df = 2$, $\chi^2 = 11.281$, $P = 0.004$), and Locomotor stereotypies ($df = 2$, $\chi^2 = 21.412$, $P < 0.001$), in the frequency of occurrence of Nose/lip licking ($df = 2$, $\chi^2 = 16.390$, $P < 0.001$), Barking ($df = 2$, $\chi^2 = 8.000$, $P = 0.018$), and Change Posture ($df = 2$, $\chi^2 = 8.061$, $P = 0.018$) and in percentages of time spent for the two markers, Maintenance behaviors ($df = 2$, $\chi^2 = 11.437$, $P = 0.003$) and Wooden platform/Cot ($df = 2$, $\chi^2 = 20.689$, $P < 0.001$).

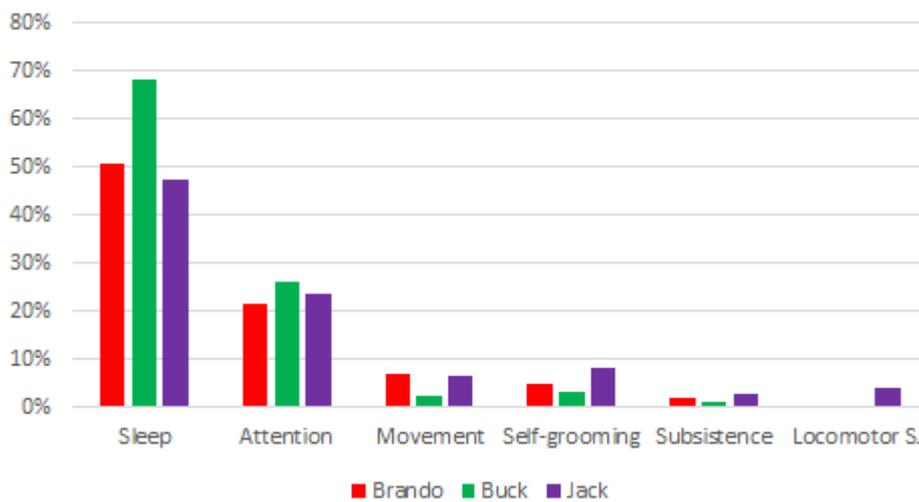


Figure 5.4. Percentage of time spent by dogs engaging in different activities during baseline week.

Given these differences during baseline weeks, subsequently analyses were performed comparing behavioral responses among week for each dog.

5.4.1.2.1. Brando

Brando kept his behavioral pattern constant across the four weeks (Figure 5.5). He spent a very little time (0.61%) interacting with the toys available during the environmental enrichments week and he interacted with the female showing mainly affiliative behaviors for 3.66% of the total time, aggressive behaviors (0.02%), and dominant behaviors (0.05%). During social enriched weeks Movement values were almost doubled compared to baseline and environmental enrichments weeks. Besides, Attention was lower when toys were present and Sleep was lower with the female.

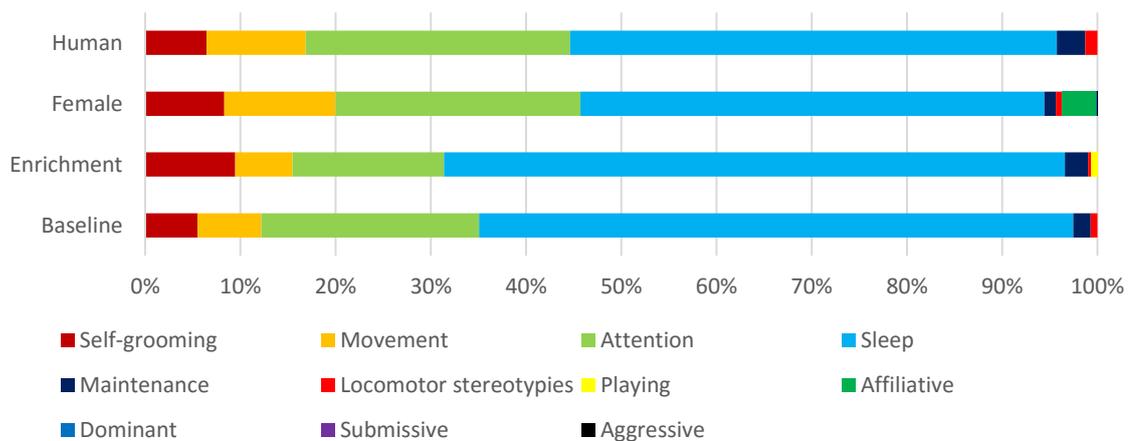


Figure 5.5. Percentage of time spent by Brando in main activities during the four weeks

Time spent on the Wooden platform/Cot changes significantly across weeks ($df = 3, \chi^2 = 18.674, P < 0.001$), and Brando spent less time on the cot than on the wooden platform ($Z = -3.768, P < 0.001$). Furthermore, time spent on the Wooden platform/cot is lower during both the intra- and interspecific social weeks than during the baseline (Baseline vs Female: $Z = -4.626, P < 0.001$; Baseline vs Human: $Z = -3.779, P < 0.001$). Change Posture, Stretching, and Yawning varied significantly across weeks (Change Posture: $df = 3, \chi^2 = 12.806, P = 0.005$; Stretching: $df = 3, \chi^2 = 22.443, P < 0.001$, Yawning: $df = 3, \chi^2 = 11.017, P = 0.012$; Figure 5.6). While differences in Yawning were due to a drop of occurrence during environmental enrichments week compared to baseline ($Z = -3.240, P = 0.001$), Stretching occurred mainly during baseline (Baseline vs Enrichment: $Z = -3.906, P < 0.001$; Baseline vs Female: $Z = -3.474, P = 0.001$, Baseline vs Human: $Z = -3.244, P = 0.001$). Change Posture was showed mostly during baseline and human weeks (Baseline vs Enrichment: $Z = -3.343, P = 0.001$; Baseline vs Female: $Z = -2.932, P = 0.003$, Enrichment vs Human: $Z = -2.852, P = 0.004$). No other statistical difference emerges.

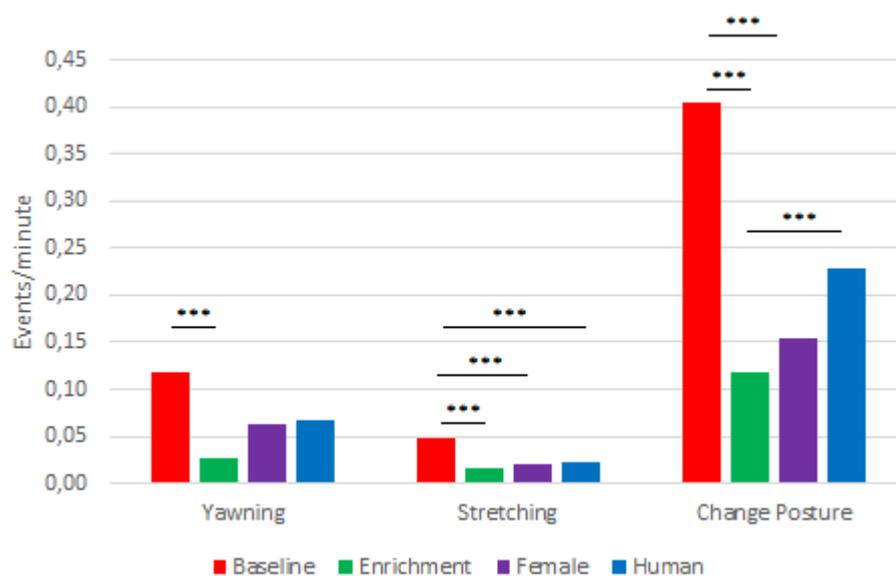


Figure 5.6. Events per minute of Yawning, Stretching and Change Posture performed by Brando during the four weeks.

5.4.1.2.2. Buck

Buck is a very apathetic dog, that spends large part of the time sleeping (range: 75.76% - 81.34%; Figure 5.7). All other behaviors are consequently reduced: Buck ignored almost completely both the female (Affiliative behaviors: 0.04%, Dominance Behaviors: 0.01%) and the toys (0.08%). An interesting result is the change in the use of Wooden platform/Cot ($df = 3, \chi^2 = 18.229, P < 0.001$): the female monopolized the wooden platform reducing its availability for Buck (Baseline vs Female: $Z = -4.639, P < 0.001$; Enrichment vs Female: $Z = -3.372, P < 0.001$; Female vs Human: $Z = -1.892, P = 0.058$).

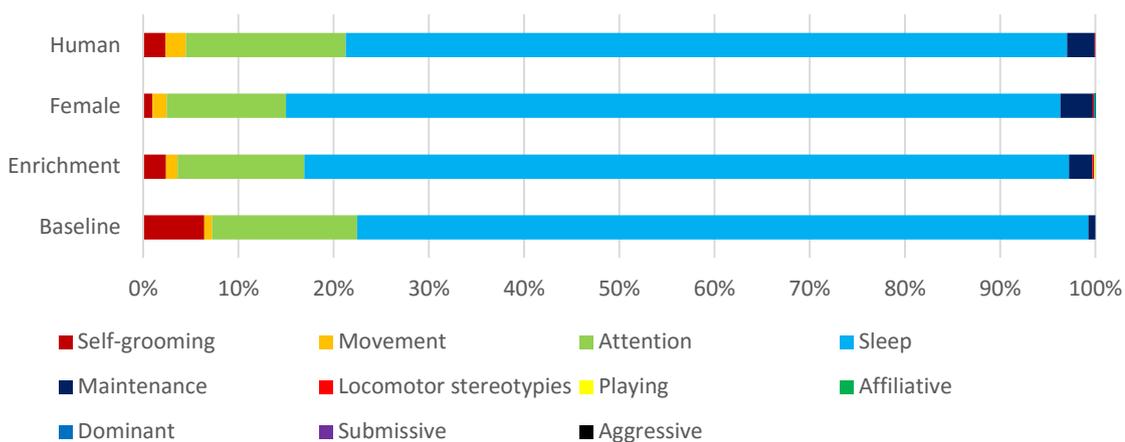


Figure 5.7. Percentage of time spent by Buck in main activities during the four weeks.

5.4.1.2.3. Jack

Opposite of Buck, Jack is a very active dog focused on what happens around him (Attention range: 28.22% - 33.77%). During the environmental enrichment week, he interacted with the toys for a very limited time (0.98%). His relationship with the female was good (4.18% of Affiliative behaviors), performing both Dominance (0.57%) and Submissive behaviors (0.15%) at a very low rate. As for Brando and Buck, even for Jack, time spent over the wooden platform (or the cot) changes among weeks ($df = 3, \chi^2 = 56.894, P < 0.001$). Two clear patterns emerged from post-hoc test: the first one is that the female monopolized the wooden platform (Baseline vs Female: $Z = -5.049, P < 0.001$; Enrichment vs Female: $Z = -3.669, P < 0.001$; Female vs Human: $Z = -4.962, P < 0.001$); the second is that when the wooden platform was available and not occupied by the female, Jack preferred it than the cot (Baseline vs Enrichment: $Z = -4.275, P < 0.001$; Enrichment vs Human: $Z = -3.994, P < 0.001$).

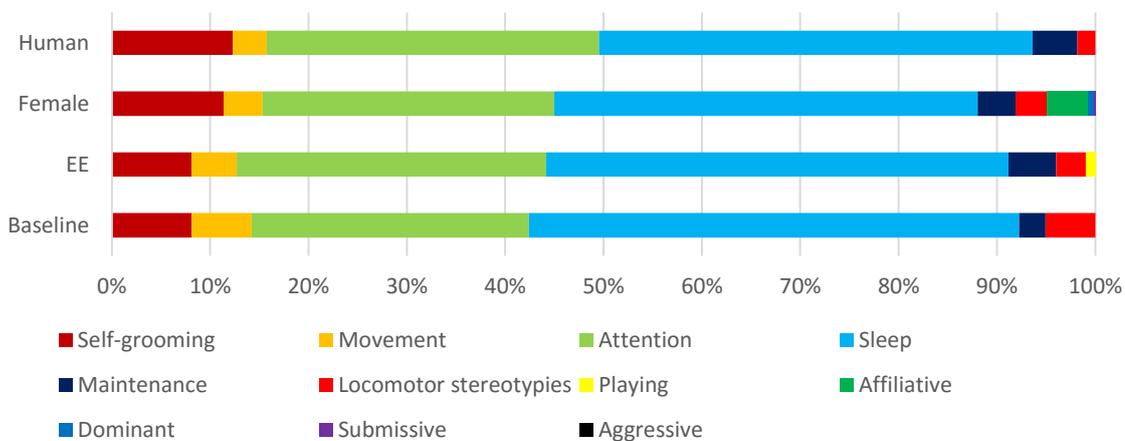


Figure 5.8. Percentage of time spent by Jack in main activities during the four weeks.

5.4.2. Approach 2: The effect of female presence

5.4.2.1. Brad

Brad behavioral pattern among days highlights a strong increment in percentage of Sleep, raising from 30.86% during Baseline to values ranging from 41.85% to 57.28% during experimental days. Consequently, all other behaviors were less expressed with the only exception of Attention during day 2 (48.25% compared to 43.94% of the baseline). It may be worth noticing that Locomotor stereotypies that was performed for 7.24% of the time during baseline day, dropped to 0.56% during day 1 and then disappeared. However, Brad interacted with the female every day for less than 0.2% of the time (Figure 5.9). Among behaviors registered as events per minute, Nose/lip licking dropped from 30.48 to about 18 during the experimental days, while female presence seems to inhibit Barking in Brad (from 1.78 events/minute to about 0.1 events/minute), while.

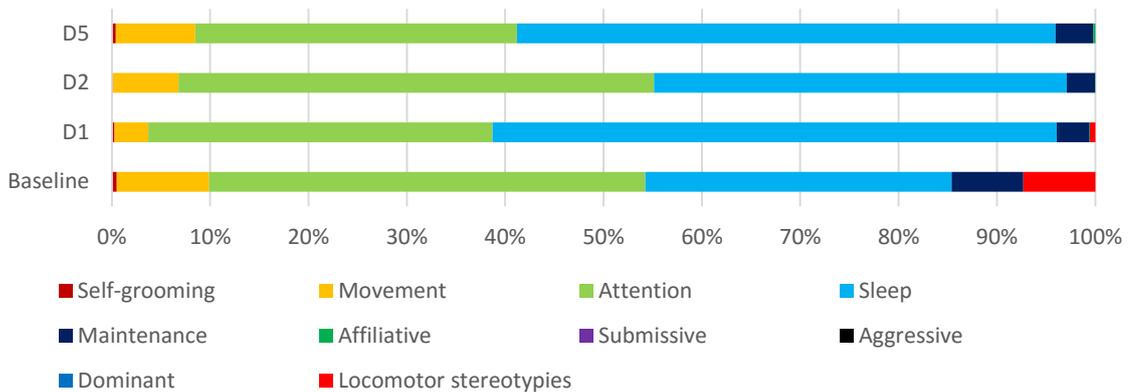


Figure 5.9. Percentage of time spent by Brad in main activities during the four days.

Brad's muscular activity index remained constant among days with a drop during day 1 (Figure 5.10). This reflects the drop in movement of the same day. His heart rate, instead, remained constant at about 100 bpm for the whole experiment (Figure 5.10), so it does not provide any indication about effect of the female on Brad's welfare.

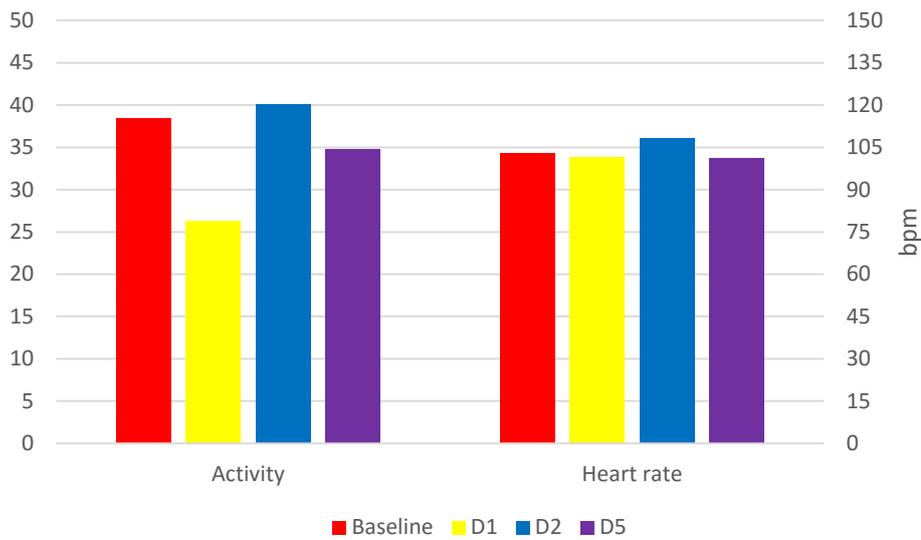


Figure 5.10. Mean activity muscular index and heart rate during the four days.

5.4.2.2. Buck

Buck confirms to be an apathetic dog (Figure 5.11): he increased sleeping time particularly the first day with the female (baseline Sleep: 35.29%, mean value of Sleep during female days 76.06%). Waking time is spent mainly in exploration of the external environment while the female is completely ignored.

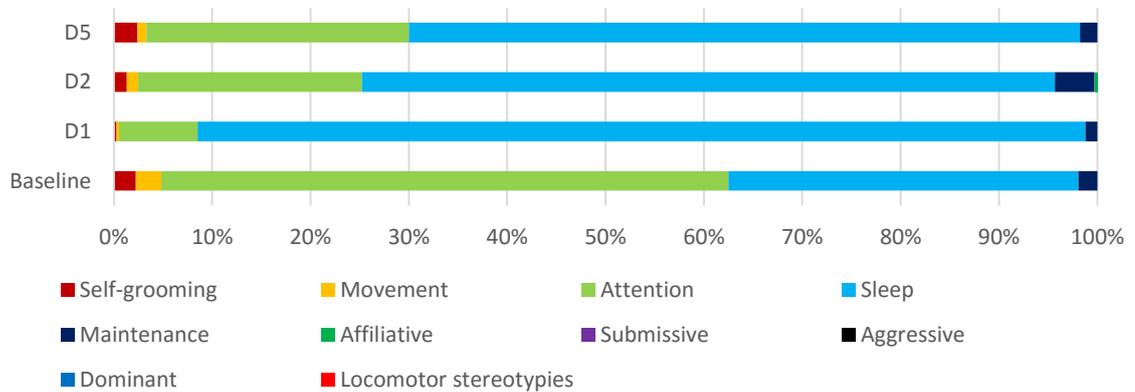


Figure 5.11. Percentage of time spent by Buck in main activities during the four days.

Buck's muscular activity index was consistent on very low values, according to his percentage of Movement (Figure 5.12). Interestingly, despite his percentage of Sleep greatly increased, his heart rate was higher than the baseline during all experimental days (Figure 5.12), suggesting that he did not enjoy female presence and could have been negatively influenced.

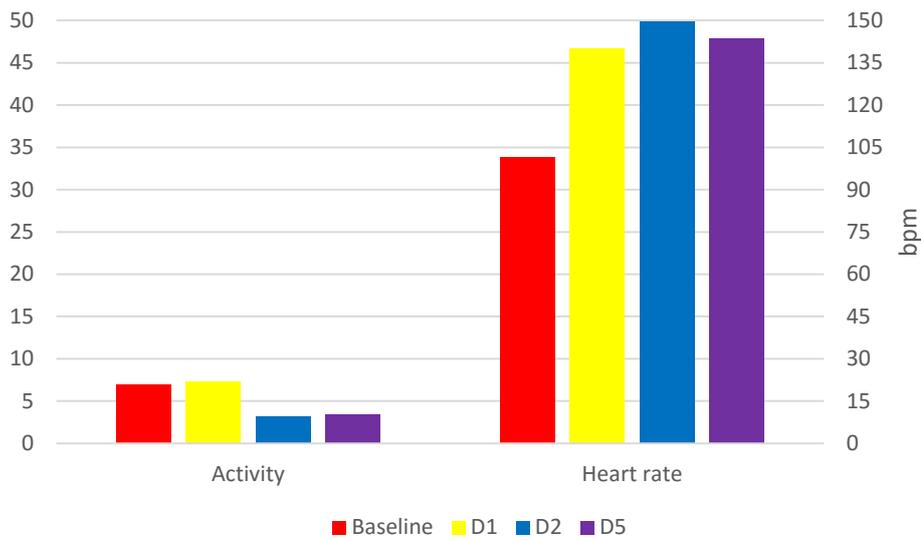


Figure 5.12. Mean activity muscular index and heart rate during the four days.

5.4.2.3. Jack

On the other hand, Jack confirms to be a very active dog focused on what happens around him (Figure 5.13). The interaction with the female determined a reduction of Locomotor stereotypies from 37.48% during baseline to 4% during day 1 and to values very close to 0% in the latter days. At the same time, Attention increases of a comparable amount of time. Besides, Jack was the one who most interact with the female, mainly in an affiliative way (10.81%, 5.24%, and 13.60% in day 1, day 2 and day 5 respectively) with very limited episodes of both dominance and submission towards her.

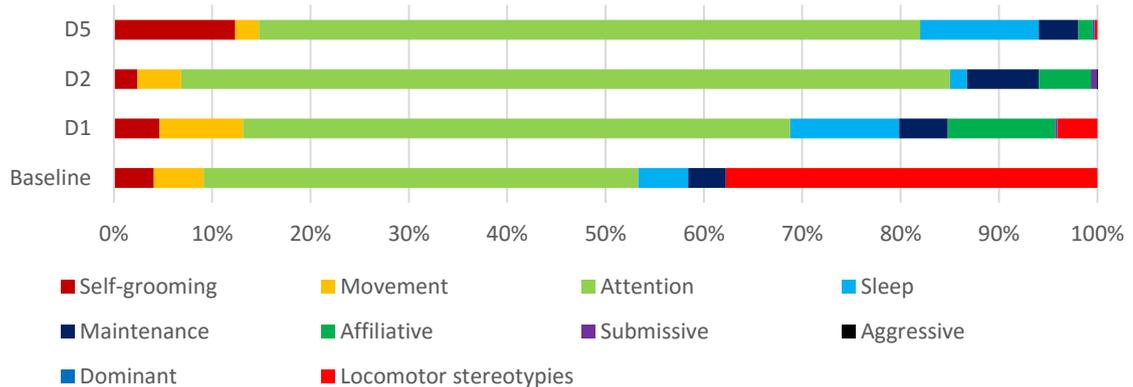


Figure 5.13. Percentage of time spent by Jack in main activities during the four days.

Intuitively, from his percentage of Locomotor stereotypies, Jack's muscular activity index had a great drop during the experimental days (Figure 5.14).

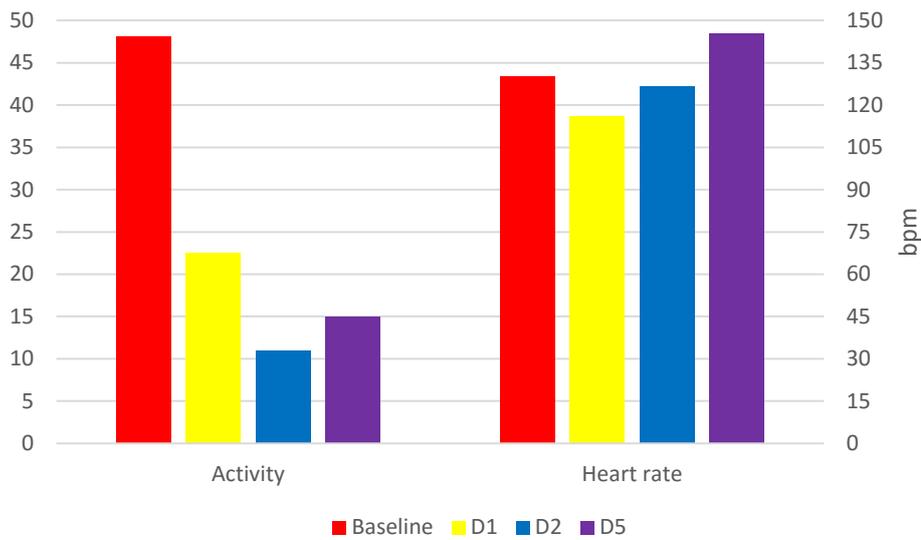


Figure 5.14. Mean activity muscular index and heart rate during the four days.

5.4.2.4. Scotty

As seen in Buck, Scotty seems to react to the presence of the female increasing sleep time from 47.41% to values always over 60% (Figure 5.15). This additional time spent sleeping reduced Self-grooming, Movement and Maintenance behaviors and Locomotor stereotypies (absent during experimental days). His interactions with the female are less than 0.01% of the time, with an episode of Dominance followed by an Aggression without consequences during day 1.

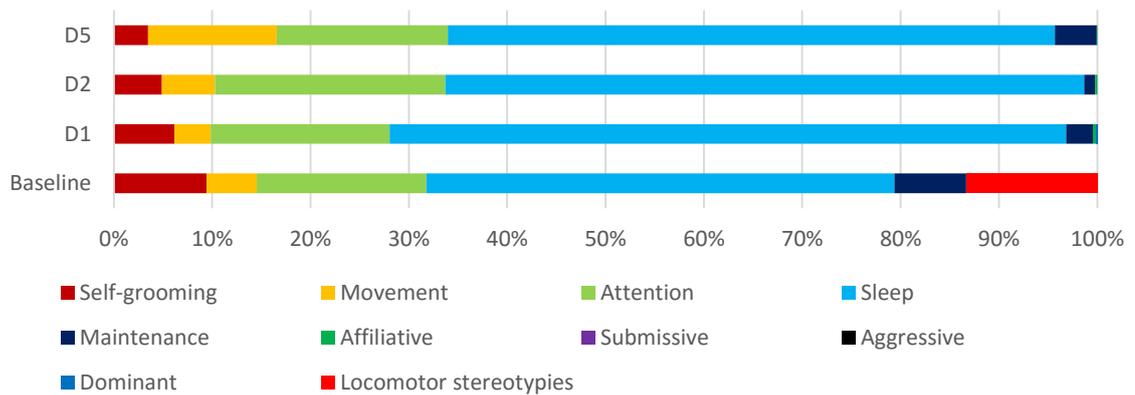


Figure 5.15. Percentage of time spent by Scotty in main activities during the four days.

Scotty's physiological parameters had both a drop during day 1 (Figure 5.16). It could be explained by a drop both in Movement than in Locomotor stereotypies; however, during day 2 and day 5 both muscular activity and heart rate returned to value close to baseline, as Movement did, but with a null percentage of Locomotor stereotypies (Figure 5.15, 5.16).

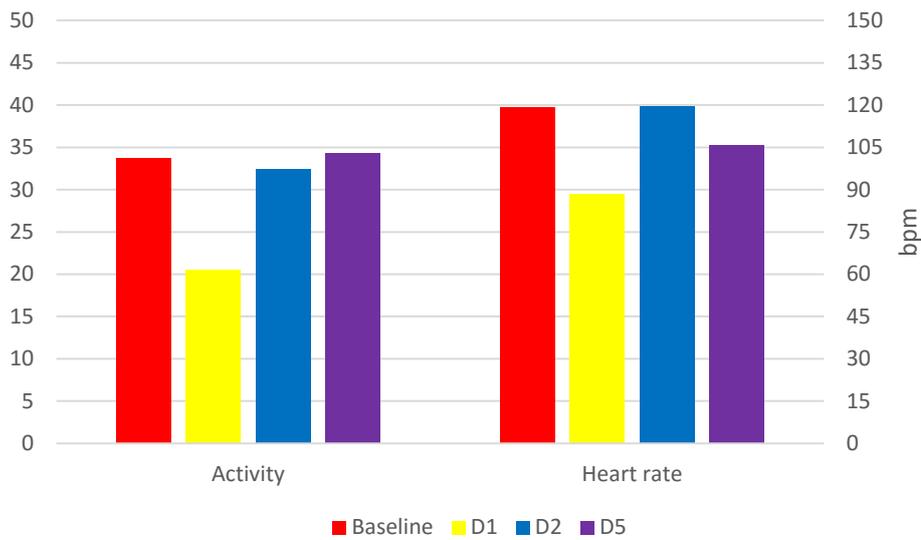


Figure 5.16. Mean activity muscular index and heart rate during the four days.

5.4.2.5. Sparrow

Sparrow seems to be stimulated by female's presence, because there is a clear drop of Sleep percentage from baseline (66.48%) to day 1 (36.66%) and day 2 (48.54%); however, probably due to habituation, day 5 is characterized by a return to baseline value (61.93%; Figure 5.17). This increased waking time is not associated with interactions with the female, that are less than 0.01% of the time during all days. Instead, dog is more focused of what happens in the surrounding environment and as for Sleep, percentage of Attention raise from baseline (26.38%) to day 1 (53.88%) and day 2 (42.83%), while returning to baseline value during day 5 (29.55%). Besides, there is a clear drop in Nose/lip licking, from 0.60 events/minute to 0.19 during day 5 passing through 0.35 in day 1 and 0.25 in day 2.

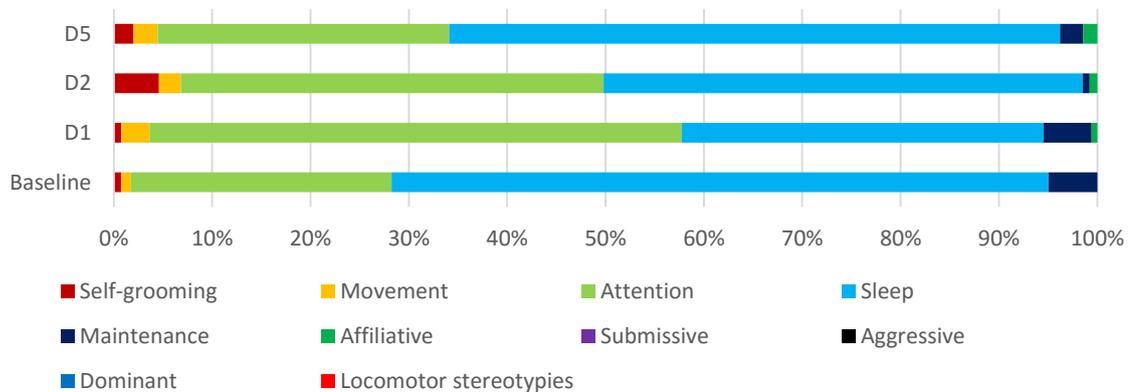


Figure 5.17. Percentage of time spent by Sparrow in main activities during the four days.

While Sparrow’s heart rate was constant among the days, his muscular activity presented two peaks during day 1 and day 2 (Figure 5.18). These peaks were in correspondence of a drop of Sleep percentage, but they are not associated to an increase of Movement. Therefore, these peaks are due to different behaviors, as Self-grooming, that has a peak in day 2 or other minor behaviors (e.g., Scratch).

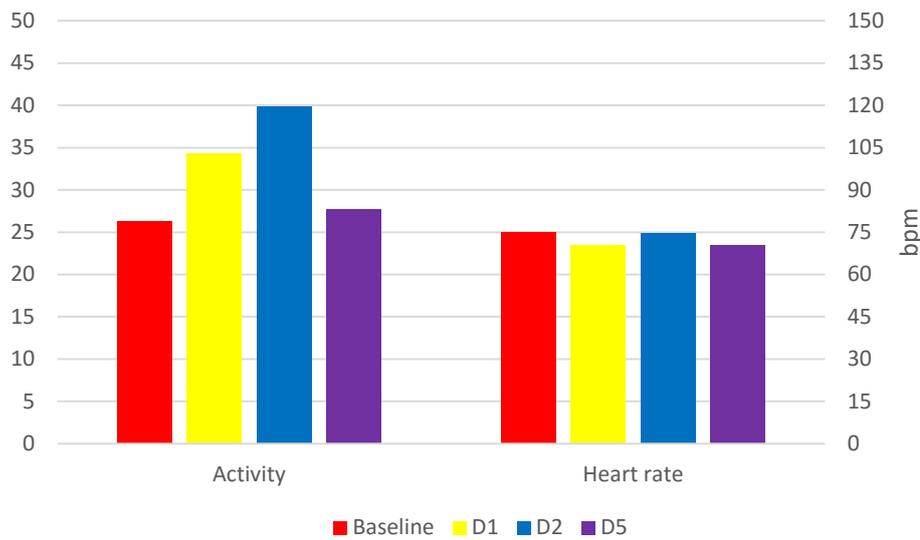


Figure 5.18. Mean activity muscular index and heart rate during the four days.

5.5. Discussion

The aim of the present study was to investigate long term physiological parameters in sheltered dogs when exposed to different enrichments using surgically implanted telemeters. In addition, dogs’ behavior was used to provide an integrative measure of the response to the stimulation.

The technique resulted to be appropriate for the experiment’s purpose. However, some critical issues emerged. First of all, telemeters provided a huge amount of data that have proved to be hard and time consuming to analyze. The way in which physiological data were collected generated 40,320 values for every parameter per dog. Besides, this methodology is invasive and requires monitoring if used on a long period because even a little period of power failure means losing corresponding data. Lastly, even if costs are dropping relatively fast, still it is an expensive technique. On the other hand, telemeters allowed to point out some matters that can be later investigated, such as possible correspondence between muscular activity index and heart rate, which situation had most impact on heart rate and/or temperature, and which parameter is more sensible to changes. These observations support literature findings [284,285].

In normal conditions, dogs need an environment that could be differentiated and both physically and socially stimulating [267–269]. This kind of environment is crucial to avoid development of stereotypies and/or abnormal behaviors [270–272], abnormal HPA axis activation [273], and separation-related anxiety problems after adoption [274]. Besides, enrichments promote animal natural behavior and increase welfare [281–283].

However, findings from this experiment seem to provide contrasting data. After a preliminary analysis of data, it appears that there is not a pattern shared among dogs neither in physiological nor in behavioral data. In the first approach, behavioral data are statistically different among dogs and, even if test were not performed for the second approach, descriptive statistics points out many differences inside the sample.

Physiological data have proven to be hard to interpret. If it could be possible to consider more positive a situation characterized by a lower heart rate, core temperature seems to provide only information about emotional activation [113,261] but not about emotional valence while the muscular activity index cannot be used as a welfare indicator.

Approach 1 points out that dogs reacted differently to the proposed situations. Brando showed a peak in temperature during environmental enrichment week that was not accompanied by a similar raise neither in heart rate nor in muscular activity index suggesting an interest towards the toys that was probably very moderated according to very little time spent interacting with them. On the other hand, behavioral data indicated that Brando had some kind of interest for the female, but this was not supported by physiological data that did not vary compared to the baseline, as happened for the week with the human visitor.

Buck physiological parameters varied among weeks, however their patterns were not overlapping. Between baseline and enrichments week, heart rate remained constant while temperature increased; the next week they had an opposite trend as heart rate increased while temperature decreased and finally in the fourth week heart rate decreased again (remaining higher than baseline values) while temperature remained constant. Behavioral data did not help as Buck slept for the majority of the time ignoring the stimuli during both the female and the environmental enrichments week. Reading together behavioral and heart rate data suggests that Buck was disturbed by social enrichments having a higher heart rate without a detectable change in his behavior. Overall, his welfare does not seem to be improved.

Jack reacted positively to the enrichments proposed even though at a different extent. According to heart rate, while environmental enrichments seem to have a calming effect on him, both

social stimuli raised this parameter. Behavioral data partially confirm these evidences. During the environmental enrichments he interacted for a very little time with the toys (even if time spent on the cot suggests that he did not like it) but he spent a lot of time with the female. Besides, during the whole experiment he performed less stereotypies but this drop did not reach any statistical threshold.

Approach 2 was focused on the male-female relationship because it was hypothesized that it could be the most impactful situation given the importance of socialization and because the female remained continuously in contact with the male and she was not limited to only daily visits. Analyzing descriptive statistics, data collected for Buck and Jack confirmed results of Approach 1.

Brad muscular activity decreased during day 1, due to a strong decrease in Locomotor stereotypies and to the increment in the percentage of time spent sleeping. It returned to baseline values during day 2 and day 5 since he increased Movement, and this pattern suggests that Brad is getting used to the presence of the female. Overall, this pattern of behavior indicates that Brad did not enjoy the presence of the female even though the constant pattern of HR suggests that the disturbance caused is not too stressful.

Scotty is another dog that spends a lot of time sleeping. Muscular activity pattern and heart rate had both a drop during day 1, in correspondence of a drop in Movement and a peak in Sleep, but they quickly recovered to baseline value during day 2 and day 5. Again, behavioral data points out an overwhelming time spent sleeping and almost no interactions between the animals and therefore they did not suggest that inserting the female improved the welfare.

Finally, Sparrow presents constant heart rate and an increased muscular activity during day 1 and day 2 but it was not correlated to either an increment in Movement or to social relationships; instead, it was due self-directed behaviors like Self-grooming or Scratch. However, behavioral data suggested that he was positively influenced by the presence of the female that increased explorative behaviors and elicited even affiliative behaviors; but this effect seems lasting only for the first two days as during the fifth day behaviors returned to baseline value.

Dogs reacted differently to the different situations: some dogs seem to be disturbed by the enrichments and others seem to benefit from them. However, environmental enrichments have elicited mild reactions in all dogs and therefore it is difficult to say if this situation had been perceived as positive or negative. On the other hand, social enrichments elicited stronger reactions, both physiological and behavioral, but very different among dogs. However, it is important to note that background information about these dogs were unknown and the sample included only male socially isolated since a long time; therefore, even if the sample seems very homogeneous, it could be possible

that past experiences play an important role in modulating responses to the enrichments. This lead to think that there is not a single path to improve sheltered dogs' welfare and enrichments should be carefully thought on a case-by-case basis.

Aside from completing physiological and behavioral analysis, future developments should include extensions of this experiment, comparing similar situations with bigger samples. Since it is unlikely that would be possible to use telemeters again, different physiological measurements have to be taken, such as hair, plasmatic or fecal cortisol [177,287,288]. Besides, it will be a paramount issue an accurate as possible analysis of dogs' background and the choice of samples including dogs of different age, sex, and socialization.

5.6. Conclusion

Our study is the first assessing long-term effects of different situations in sheltered dogs using telemeters combined with behavioral data. After a period of baseline, dogs were exposed to three different conditions: an environmental enrichment situation with toys and a cot and two social enrichment situations with a female sharing the cage and with a human experimenter paying daily visits to the dogs. Physiological and behavioral data have proven to be difficult to collect and to interpret due to technical problems and to the huge amount of data collected, but it has been possible to assess a general outcome. These preliminary results suggest that environmental enrichments, both physical and social, affect dogs' behavior differently and not always positively. Thus, modifying life conditions of dogs will have to be carefully projected after a deep analysis of their behaviors and attitudes.

5.7. Ethic statement

This study was conducted in collaboration with veterinary hospital of Local Health Board "Roma D", via della Magliana 856, 00148 Rome, Italy. Proper authorization was asked and obtained by the Italian Ministry of Health, viale Giorgio Ribotta 5, 00144 Rome, Italy.

6. General conclusions

In the course of the last century studies of stress response and of negative emotional states (fear, depression, frustration) related to stress have been extensively carried out in both human and non-human animals. Therefore, there is a wide literature on negative emotions and stress in domestic animals as well. Recently the idea that the absence of negative emotions cannot be considered a sufficient condition to guarantee animal welfare in laboratories, breeding farms, and households was put forward but related studies are scarce and focused mostly of farm animals. There is general agreement among researchers that an objective evaluation of emotional states in animals needs a combination of behavioral, physiological, and cognitive markers. This thesis is the first attempt to validate eye temperature, measured by infrared thermography (IRT), as a non-invasive tool for assessing emotions in dogs. Following the suggestions to integrate different physiological and behavioral measures, thermography data were associated with heart rate and heart rate variability parameters and with behavioral data. Heart rate and heart rate variability are well-established physiological parameters to assess emotional activation; especially heart rate variability has obtained a growing consensus in the last years and an indirect and easy collectible measure of sympathovagal balance. On the other hand, physiological parameters would be much harder to interpret without behavioral analyses. Behaviors are the guideline that allows to discriminate between different situations that have a similar physiological response. Different emotional situations were tested to provide different information about dogs' emotional reactions and their physiological correlates: (1) a very stressful situation (i.e., veterinary visit); (2) a positive stimulation with palatable food; (3) a double valence situation, initially negative (i.e. separation from the owner) and then positive (i.e. reunion to the owner), but with small arousal. In addition, a longitudinal study with telemeters used on sheltered dogs have been performed to test long effects of different environmental enrichments in order to assess possible improvements of their quality of life.

All techniques used in this thesis present both advantages and disadvantages. Thermographic imaging is a relatively new method, non-invasive, very precise, and final data can easily be handled; however, precise thermographic cameras are expensive and they require to be calibrated, a control of environmental temperature and humidity, and even little errors in the procedure can result in artefacts that are very difficult to detect. IRT results seem to be good to detect emotional activations but our results point out as it can detect only high arousal situations and it provides similar results for situations of both positive and negative valences. Finally, it is worth noting that the thermographic camera was at some extent disturbing for dogs during the veterinary visit study (i.e., a stressful situation per se),

as they showed avoidance behaviors, averting gaze and turning their head when the thermographic camera was oriented to them.

On the other hand, heart rate and heart rate variability are reliable parameters, common in studies about emotions and stress, and even very used in biomedical research. The chance offered by external heart monitors that allow to assess HR and HRV parameters in a non-invasive way has greatly increased the number of studies utilizing cardiac activity. However, in non-primate animals, most authors use only HR and the time domain parameters of the HRV (e.g., RMSSD, SDNN) because frequency-domain parameters (e.g., HF, LF) still require validation in animals and they could be species-specific. Consequently, determination of the functional regulatory characteristics of the ANS, and particularly the complex interplay between the sympathetic and the parasympathetic branches is less accurate and detailed. Overall present results suggest that HR detects emotional states characterized by high level of arousal but without defining their valence. However, heart rate variability can provide indications even about valence if the arousal is sufficiently high. Heart rate monitors are designed to be used by anyone who wants to practice sport; therefore, their settings, use, and related software are simple and intuitive and their applicability on animals is simple, requiring little experience and care in order not to collect partial or incorrect data from a very hairy or a very stressed dog. Nevertheless, data manipulation and interpretation can be difficult because different heart rate variability parameters often do not provide consistent information.

Behavioral data can provide precise information about dogs' reactions to given stimuli but even some behaviors can be tricky to interpret and can be performed in response to different situations. Likely, dog is the animal whose behavior is best known by humans, thanks to our long shared evolutionary history and to the huge amount of studies on dog behavior that have been made in the last 30 years. The growing number of scientific papers and publications about dog's behavior, sociality, attitudes and cognitive skills shed light on the extraordinary dog-human relationship. However, in the rush to prove dog's cognitive skills researches have to avoid the risks to trespass in an excessive of anthropomorphism attributing to the dogs' complex cognitive abilities and feelings.

Telemetry is a good approach to the study of prolonged variation in physiological parameters, but it is still expensive in terms of necessary money and time to obtain a significant result. Furthermore, telemetry requires an invasive approach even though superficial and generate a huge database that in the present case is of difficult analysis due to the heterogeneity and size limitation of the sample. The preliminary analysis carried out suggests that the environmental and social enrichments aimed to increase dogs' welfare and quality of life have different impacts on dogs. Understanding which enrichments could have a beneficial effect on dogs' welfare is not an easy task: sheltered dogs are not

laboratory mice and their background is almost always unknown; consequently, before designing environmental or social enrichments it is necessary to assess social competence and attitude of every dog.

Concluding, the most promising path would be the validation of some behavioral pattern to use them as markers for positive and negative emotional states. Validation of behavioral indicators require the integration of cardiac, hormonal and other physiological measures. Emotions are a very complex topic that involves central and peripheral nervous systems, endocrine systems and, therefore, behavior. Their multi-componential-nature allows to analyze every component singly but even to combine two or more components to obtain more precise answers to experimental questions.

References

- [1] R.K. Wayne, C. Vilà, Phylogeny and Origin of the Domestic Dog, in: A. Ruvinsky, J. Sampson (Eds.), *Genet. Dog*, CAB International, Oxon, 2001: pp. 1–14.
- [2] M.W. Gompper, The dog–human–wildlife interface: assessing the scope of the problem, in: M.E. Gompper (Ed.), *Free. Dogs Wildl. Conserv.*, Oxford University Press, Oxford, 2013: pp. 14–54.
- [3] A. Ortolani, H. Vernooij, R. Coppinger, Ethiopian village dogs: Behavioural responses to a stranger’s approach, *Appl. Anim. Behav. Sci.* 119 (2009) 210–218. doi:10.1016/j.applanim.2009.03.011.
- [4] R.M. Mengel, A Study of Dog-Coyote Hybrids and Implications Concerning Hybridization in *Canis*, *J. Mammal.* 52 (1971) 316–336.
- [5] C. Vila, R.K. Wayne, Hybridization between Wolves and Dogs, *Conserv. Biol.* 13 (1999) 195–198. doi:10.1046/j.1523-1739.1999.97425.x.
- [6] L.C. Marston, P.C. Bennett, Reforging the bond - Towards successful canine adoption, *Appl. Anim. Behav. Sci.* 83 (2003) 227–245. doi:10.1016/S0168-1591(03)00135-7.
- [7] E. Friedmann, S.A. Thomas, Pet ownership, social support, and one-year survival after acute myocardial infarction in the cardiac arrhythmia suppression trial (CAST), *Am. J. Cardiol.* 76 (1995) 1213–1217. doi:10.1016/S0002-9149(99)80343-9.
- [8] E. Friedmann, S.A. Thomas, L.K. Cook, C.-C. Tsai, S.J. Picot, A Friendly Dog as Potential Moderator of Cardiovascular Response to Speech in Older Hypertensives, *Anthrozoos.* 20 (2007) 51–63. doi:10.2752/089279307780216605.
- [9] J.S.J. Odendaal, Animal-assisted therapy - Magic or medicine?, *J. Psychosom. Res.* 49 (2000) 275–280. doi:10.1016/S0022-3999(00)00183-5.
- [10] J.S.J. Odendaal, R.A. Meintjes, Neurophysiological correlates of affiliative behaviour between humans and dogs, *Vet. J.* 165 (2003) 296–301. doi:10.1016/S1090-0233(02)00237-X.
- [11] K. Lorenz, M.K. Wilson, *Man Meets Dog*, Houghton Mifflin, Boston, 1954.
- [12] C. Darwin, *The variation of animals and plants under domestication*, John Murray, London, 1868.
- [13] R.K. Wayne, E.A. Ostrander, Origin, genetic diversity, and genome structure of the domestic

- dog., *BioEssays*. 21 (1999) 247–257. doi:10.1002/(SICI)1521-1878(199903).
- [14] G. Larson, E.K. Karlsson, A. Perri, M.T. Webster, S.Y.W. Ho, J. Peters, et al., Rethinking dog domestication by integrating genetics, archeology, and biogeography., *Proc. Natl. Acad. Sci. U. S. A.* 109 (2012) 8878–8883. doi:10.1073/pnas.1203005109.
- [15] P. Skoglund, E. Ersmark, E. Palkopoulou, L. Dalén, Ancient Wolf Genome Reveals an Early Divergence of Domestic Dog Ancestors and Admixture into High-Latitude Breeds, *Curr. Biol.* 25 (2015) 1515–1519. doi:10.1016/j.cub.2015.04.019.
- [16] O. Thalmann, B. Shapiro, P. Cui, V.J. Schuenemann, S.K. Sawyer, D.L. Greenfield, et al., Complete mitochondrial genomes of ancient canids suggest a European origin of domestic dogs., *Science* (80-.). 342 (2013) 871–874. doi:10.1126/science.1243650.
- [17] A.H. Freedman, I. Gronau, R.M. Schweizer, D. Ortega-Del Vecchyo, E. Han, P.M. Silva, et al., Genome Sequencing Highlights the Dynamic Early History of Dogs, *PLoS Genet.* 10 (2014). doi:10.1371/journal.pgen.1004016.
- [18] A.R. Boyko, R.H. Boyko, C.M. Boyko, H.G. Parker, M. Castelhana, L. Corey, et al., Complex population structure in African village dogs and its implications for inferring dog domestication history, *Proc. Natl. Acad. Sci. U. S. A.* 106 (2009) 13903–13908. doi:10.1073/pnas.0902129106.
- [19] E.J. Lee, D.A. Merriwether, A.K. Kasparov, P. a. Nikolskiy, M. V. Sotnikova, E.Y. Pavlova, et al., Ancient DNA Analysis of the Oldest Canid Species from the Siberian Arctic and Genetic Contribution to the Domestic Dog, *PLoS One.* 10 (2015) e0125759. doi:10.1371/journal.pone.0125759.
- [20] L.M. Shannon, R.H. Boyko, M. Castelhana, E. Corey, J.J. Hayward, C. Mclean, et al., Genetic structure in village dogs reveals a Central Asian domestication origin, 112 (2015) 3–8. doi:10.1073/pnas.1516215112.
- [21] P. Savolainen, Y. Zhang, J. Luo, J. Lundeberg, T. Leitner, Genetic evidence for an East Asian origin of domestic dogs., *Science.* 298 (2002) 1610–1613. doi:10.1126/science.1073906.
- [22] J.-F. Pang, C. Kluetsch, X.-J. Zou, A.-B. Zhang, L.-Y. Luo, H. Angleby, et al., mtDNA Data Indicate a Single Origin for Dogs South of Yangtze River, Less Than 16,300 Years Ago, from Numerous Wolves, *Mol. Biol. Evol.* 26 (2009) 2849–2864. doi:10.1093/molbev/msp195.
- [23] B.M. Vonholdt, J.P. Pollinger, K.E. Lohmueller, E. Han, H.G. Parker, P. Quignon, et al., Genome-wide SNP and haplotype analyses reveal a rich history underlying dog domestication., *Nature.*

- 464 (2010) 898–902. doi:10.1038/nature08837.
- [24] H. Bocherens, D.G. Drucker, M. Germonpré, M. Lázničková-Galetová, Y.I. Naito, C. Wissing, et al., Reconstruction of the Gravettian food-web at Předmostí I using multi-isotopic tracking (^{13}C , ^{15}N , ^{34}S) of bone collagen, *Quat. Int.* 359-360 (2015) 211–228. doi:10.1016/j.quaint.2014.09.044.
- [25] M. Germonpré, M. V Sablin, R.E. Stevens, R.E.M. Hedges, M. Hofreiter, M. Stiller, et al., Fossil dogs and wolves from Palaeolithic sites in Belgium, the Ukraine and Russia: osteometry, ancient DNA and stable isotopes, *J. Archaeol. Sci.* 36 (2009) 473–490. doi:10.1016/j.jas.2008.09.033.
- [26] N.D. Ovodov, S.J. Crockford, Y. V Kuzmin, T.F.G. Higham, G.W.L. Hodgins, J. van der Plicht, A 33,000-Year-Old incipient dog from the Altai Mountains of Siberia: Evidence of the earliest domestication disrupted by the last Glacial Maximum, *PLoS One.* 6 (2011) 4–10. doi:10.1371/journal.pone.0022821.
- [27] A.S. Druzhkova, O. Thalmann, V.A. Trifonov, J.A. Leonard, N. V Vorobieva, N.D. Ovodov, et al., Ancient DNA Analysis Affirms the Canid from Altai as a Primitive Dog, *PLoS One.* 8 (2013) e57754. doi:10.1371/journal.pone.0057754.
- [28] T.C. Spady, E. a. Ostrander, Canine Behavioral Genetics: Pointing Out the Phenotypes and Herding up the Genes, *Am. J. Hum. Genet.* 82 (2008) 10–18. doi:10.1016/j.ajhg.2007.12.001.
- [29] H.G. Parker, L. V Kim, N.B. Sutter, S. Carlson, T.D. Lorentzen, T.B. Malek, et al., Genetic structure of the purebred domestic dog., *Science.* 304 (2004) 1160–1164. doi:10.1126/science.1097406.
- [30] J. Kaminski, Word Learning in a Domestic Dog: Evidence for “Fast Mapping,” *Science (80-.).* 304 (2004) 1682–1683. doi:10.1126/science.1097859.
- [31] B.A. Hare, M. Tomasello, Domestic dogs (*Canis familiaris*) use human and conspecific social cues to locate hidden food., *J. Comp. Psychol.* 113 (1999) 173–177. doi:10.1037/0735-7036.113.2.173.
- [32] Á. Miklósi, P. Pongrácz, G. Lakatos, J. Topál, V. Csányi, A Comparative Study of the Use of Visual Communicative Signals in Interactions Between Dogs (*Canis familiaris*) and Humans and Cats (*Felis catus*) and Humans., *J. Comp. Psychol.* 119 (2005) 179–186. doi:10.1037/0735-7036.119.2.179.
- [33] M. Gácsi, B. Györi, Z. Virányi, E. Kubinyi, F. Range, B. Belényi, et al., Explaining dog wolf differences in utilizing human pointing gestures: Selection for synergistic shifts in the

- development of some social skills, *PLoS One*. 4 (2009) 4–9. doi:10.1371/journal.pone.0006584.
- [34] G.S. Berns, A.M. Brooks, M. Spivak, Functional MRI in awake unrestrained dogs, *PLoS One*. 7 (2012). doi:10.1371/journal.pone.0038027.
- [35] M. Tomasello, J. Kaminski, Like Infant, Like Dog, *Science* (80-.). 325 (2009) 1213–1214. doi:10.1126/science.1179670.
- [36] Á. Miklósi, E. Kubinyi, J. Topál, M. Gácsi, Z. Virányi, V. Csányi, A Simple Reason for a Big Difference: Wolves Do Not Look Back at Humans, but Dogs Do, *Curr. Biol.* 13 (2003) 763–766. doi:10.1016/S0960-9822(03)00263-X.
- [37] J. Topál, Á. Miklósi, V. Csányi, A. Dóka, Attachment behavior in dogs (*Canis familiaris*): A new application of Ainsworth’s (1969) Strange Situation Test, *J. Comp. Psychol.* 112 (1998) 219–229. doi:10.1037/0735-7036.112.3.219.
- [38] E. Prato-Previde, D.M. Custance, C. Spiezio, F. Sabatini, Is the dog – human relationship an attachment bond ? An observational study using Ainsworth ’ s strange situation, *Behaviour*. 140 (2003) 225–254. doi:10.1163/156853903321671514.
- [39] R. Palmer, D.M. Custance, A counterbalanced version of Ainsworth’s Strange Situation Procedure reveals secure-base effects in dog-human relationships, *Appl. Anim. Behav. Sci.* 109 (2008) 306–319. doi:10.1016/j.applanim.2007.04.002.
- [40] M.D.S. Ainsworth, Attachments beyond infancy, *Am. Psychol.* 44 (1989) 709–716. doi:10.1037/0003-066X.44.4.709.
- [41] M.D.S. Ainsworth, S.M. Bell, Attachment, Exploration, and Separation: Illustrated by the Behavior of One-Year-Olds in a Strange Situation, *Child Dev.* 41 (1970) 49–67. doi:10.2307/1127388.
- [42] L. Horn, F. Range, L. Huber, Dogs’ attention towards humans depends on their relationship, not only on social familiarity, *Anim. Cogn.* 16 (2013) 435–443. doi:10.1007/s10071-012-0584-9.
- [43] C. Mariti, B. Carlone, E. Ricci, C. Sighieri, A. Gazzano, Intraspecific attachment in adult domestic dogs (*Canis familiaris*): Preliminary results, *Appl. Anim. Behav. Sci.* 152 (2014) 64–72. doi:10.1016/j.applanim.2013.12.002.
- [44] K. Soproni, Á. Miklósi, J. Topál, V. Csányi, Dogs’ (*Canis familiaris*) responsiveness to human pointing gestures, *J. Comp. Psychol.* 116 (2002) 27–34. doi:10.1037//0735-7036.116.1.27.

- [45] J. Kaminski, J. Riedel, J. Call, M. Tomasello, Domestic goats (*Capra hircus*) follow gaze direction and use some social cues in an object choice task, *Anim. Behav.* 69 (2005) 11–18.
- [46] M.A.R. Udell, N.R. Dorey, C.D.L. Wynne, Wolves outperform dogs in following human social cues, *Anim. Behav.* 76 (2008) 1767–1773. doi:10.1016/j.anbehav.2008.07.028.
- [47] B.A. Hare, I. Plyusnina, N. Ignacio, O. Schepina, A. Stepika, R. Wrangham, et al., Social cognitive evolution in captive foxes is a correlated by-product of experimental domestication, *Curr. Biol.* 15 (2005) 226–230. doi:10.1016/j.cub.2005.01.040.
- [48] J.R. Anderson, P. Sallaberry, H. Barbier, Use of experimenter-given cues during object-choice tasks by capuchin monkeys, *Anim. Behav.* 49 (1995) 201–208. doi:10.1016/0003-3472(95)80168-5.
- [49] S. Itakura, M. Tanaka, Use of experimenter-given cues during object-choice tasks by chimpanzees (*Pan troglodytes*), an orangutan (*Pongo pygmaeus*), and human infants (*Homo sapiens*), *J. Comp. Psychol.* 112 (1998) 119–126. doi:10.1037/0735-7036.112.2.119.
- [50] S.-J. Vick, J.R. Anderson, Cue-reading by olive baboons in a competitive task, *J. Comp. Psychol.* 117 (2003) 209–216.
- [51] T. Bugnyar, M. Stöwe, B. Heinrich, Ravens, *Corvus corax*, follow gaze direction of humans around obstacles, *Proc. R. Soc. B Biol. Sci.* 271 (2004) 1331–1336. doi:10.1098/rspb.2004.2738.
- [52] A.A. Pack, L.M. Herman, Bottlenosed Dolphins (*Tursiops truncatus*) Comprehend the Referent of Both Static and Dynamic Human Gazing and Pointing in an Object-Choice Task., *J. Comp. Psychol.* 118 (2004) 160–171. doi:10.1037/0735-7036.118.2.160.
- [53] J. McKinley, T.D. Sambrook, Use of human-given cues by domestic dogs (*Canis familiaris*) and horses (*Equus caballus*), *Anim. Cogn.* 3 (2000) 13–22. doi:10.1007/s100710050046.
- [54] L. Proops, M. Walton, K. McComb, The use of human-given cues by domestic horses, *Equus caballus*, during an object choice task, *Anim. Behav.* 79 (2010) 1205–1209. doi:10.1016/j.anbehav.2010.02.015.
- [55] J. Call, M. Tomasello, Production and comprehension of referential pointing by orangutans (*Pongo pygmaeus*), *J. Comp. Psychol.* 108 (1994) 307–317. doi:10.1037/0735-7036.108.4.307.
- [56] L.M. Herman, S.L. Abichandani, A.N. Elhajj, E.Y.K. Herman, J.L. Sanchez, A.A. Pack, Dolphins (*Tursiops truncatus*) comprehend the referential character of the human pointing gesture, *J.*

- Comp. Psychol. 113 (1999) 347–364. doi:10.1037/0735-7036.113.4.347.
- [57] B.A. Hare, M. Brown, C. Williamson, M. Tomasello, The domestication of social cognition in dogs, *Science*. 298 (2002) 1634–1636. doi:10.1126/science.1072702.
- [58] Á. Miklósi, K. Soproni, A comparative analysis of animals' understanding of the human pointing gesture, *Anim. Cogn.* 1 (2006) 113–121.
- [59] Z. Virányi, M. Gácsi, E. Kubinyi, J. Topál, B. Belényi, D. Ujfalussy, et al., Comprehension of human pointing gestures in young human-reared wolves (*Canis lupus*) and dogs (*Canis familiaris*), *Anim. Cogn.* 11 (2008) 373–387. doi:10.1007/s10071-007-0127-y.
- [60] S. Pika, T. Bugnyar, The use of referential gestures in ravens (*Corvus corax*) in the wild, *Nat. Commun.* 2 (2011) 560. doi:10.1038/ncomms1567.
- [61] M.A.R. Udell, N.R. Dorey, C.D.L. Wynne, What did domestication do to dogs? A new account of dogs' sensitivity to human actions, *Biol. Rev.* 85 (2010) 327–345. doi:10.1111/j.1469-185X.2009.00104.x.
- [62] C.D.L. Wynne, M.A.R. Udell, K.A. Lord, Ontogeny's impacts on human-dog communication, *Anim. Behav.* 76 (2008) 1–4. doi:10.1016/j.anbehav.2008.03.010.
- [63] R. Moore, B. Mueller, J. Kaminski, M. Tomasello, Two-year-old children but not domestic dogs understand communicative intentions without language, gestures, or gaze, *Dev. Sci.* (2014) 1–11. doi:10.1111/desc.12206.
- [64] N. Nelson, J. Russell, Preschoolers' use of dynamical facial, bodily and vocal cues to emotion, *J. Exp. Child Psychol.* 110 (2011) 52–61.
- [65] A. Morisaki, A. Takaoka, K. Fujita, Are dogs sensitive to the emotional state of humans?, *J. Vet. Behav. Clin. Appl. Res.* 4 (2009) 49.
- [66] B.L. Deputte, A. Doll, Do dogs understand human facial expressions?, *J. Vet. Behav. Clin. Appl. Res.* 6 (2011) 78–79. doi:10.1016/j.jveb.2010.09.013.
- [67] M. Nagasawa, K. Murai, K. Mogi, T. Kikusui, Dogs can discriminate smiling faces from blank expressions, *Anim. Cogn.* 14 (2011) 525–533.
- [68] A. Racca, K. Guo, K. Meints, D.S. Mills, Reading faces: Differential lateral gaze bias in processing canine and human facial expressions in dogs and 4-year-old children, *PLoS One*. 7 (2012) 1–10. doi:10.1371/journal.pone.0036076.

- [69] C.A. Müller, K. Schmitt, A.L.A. Barber, L. Huber, Dogs Can Discriminate Emotional Expressions of Human Faces, *Curr. Biol.* 25 (2015) 601–605. doi:10.1016/j.cub.2014.12.055.
- [70] I. Merola, E. Prato-Previde, M. Lazzaroni, S. Marshall-Pescini, Dogs' comprehension of referential emotional expressions: Familiar people and familiar emotions are easier, *Anim. Cogn.* 17 (2014) 373–385. doi:10.1007/s10071-013-0668-1.
- [71] I. Merola, E. Prato-Previde, S. Marshall-Pescini, Dogs' social referencing towards owners and strangers., *PLoS One.* 7 (2012) e47653. doi:10.1371/journal.pone.0047653.
- [72] J. Decety, P.L. Jackson, The functional architecture of human empathy, *Behav. Cogn. Neurosci. Rev.* 3 (2004) 71–100. doi:10.1177/1534582304267187.
- [73] R.M. Joly-Mascheroni, A. Senju, A.J. Shepherd, Dogs catch human yawns., *Biol. Lett.* 4 (2008) 446–448. doi:10.1098/rsbl.2008.0333.
- [74] S.D. Preston, F.B.M. de Waal, The communication of emotions and the possibility of empathy in animals, in: S.G. Post, L.G. Underwood, J.P. Schloss, W.B. Hurlburt (Eds.), *Altruism Altruistic Love Sci. Philos. Relig. Dialogue*, 2002.
- [75] S. Platek, S. Critton, T. Myers, G. Gallup, Contagious yawning: the role of self-awareness and mental state attribution, *Cogn. Brain Res.* 17 (2003) 223–227. doi:10.1016/S0926-6410(03)00109-5.
- [76] J. Porter, S. Platek, In-group/out-group bias in contagious yawning, *PLoS One.* 6 (2012).
- [77] D.M. Custance, J. Mayer, Empathic-like responding by domestic dogs (*Canis familiaris*) to distress in humans: An exploratory study, *Anim. Cogn.* 15 (2012) 851–859. doi:10.1007/s10071-012-0510-1.
- [78] B. Fehr, J.A. Russell, Concept of emotion viewed from a prototype perspective., *J. Exp. Psychol. Gen.* 113 (1984) 464–486. doi:10.1037/0096-3445.113.3.464.
- [79] C. Darwin, *The expression of emotions in man and animals*, Oxford University Press, Oxford, 1872.
- [80] J.A. Russell, Culture and the categorization of emotions., *Psychol. Bull.* 110 (1991) 426–450. doi:10.1037/0033-2909.110.3.426.
- [81] E. Fox, *Emotion Science Cognitive and Neuroscientific Approaches to Understanding Human Emotions*, Palgrave Macmillan, 2008.

- [82] N. Tinbergen, On aims and methods of ethology, *Z. Tierpsychol.* 20 (1963) 410–433.
- [83] P. Ekman, *Emotion in the Human Face*, Cambridge University Press, New York, NY, 1982.
- [84] J. Tooby, L. Cosmides, The past explains the present: Emotional adaptations and the structure of ancestral environments, *Ethol. Sociobiol.* 11 (1990) 375–424. doi:10.1016/0162-3095(90)90017-Z.
- [85] R.W. Levenson, Human emotion: A functionalist view, in: P. Ekman, R.J. Davidson (Eds.), *Nat. Emot. Fundam. Quest.*, Oxford University Press, New York, NY, 1994: pp. 123–126.
- [86] R.S. Lazarus, Cognition and motivation in emotion, *Am. Psychol.* 46 (1991) 352–367. doi:10.1037/0003-066X.46.4.352.
- [87] B.L. Fredrickson, Cultivated Emotions: Parental Socialization of Positive Emotions and Self-Conscious Emotions, *Psychol. Inq.* 9 (1998) 279–281. doi:10.1207/s15327965pli0904_4.
- [88] B.L. Fredrickson, The Role of Positive Emotions in Positive Psychology: The Broaden-and-Build Theory of Positive Emotions, *Am. Psychol.* 56 (2001) 218–226. doi:10.1037/0003-066X.56.3.218.
- [89] K.R. Scherer, Emotion as a multicomponent process: A model and some cross-cultural data., *Rev. Personal. Soc. Psychol.* 5 (1984) 37–63.
- [90] A. Reiner, D.J. Perkel, L.L. Bruce, A.B. Butler, A. Csillag, W. Kuenzel, et al., Revised Nomenclature for Avian Telencephalon and Some Related Brainstem Nuclei, *J. Comp. Neurol.* 473 (2004) 377–414. doi:10.1016/j.drugalcdep.2008.02.002.A.
- [91] E.D. Jarvis, O. Güntürkün, L. Bruce, A. Csillag, H. Karten, W. Kuenzel, et al., Avian brains and a new understanding of vertebrate brain evolution, *Nat. Rev. Neurosci.* 6 (2005) 151–159. doi:10.1038/nrn1606.
- [92] M. Mendl, O.H.P. Burman, E.S. Paul, An integrative and functional framework for the study of animal emotion and mood., *Proc. Biol. Sci.* 277 (2010) 2895–2904. doi:10.1098/rspb.2010.0303.
- [93] R.W. Levenson, Basic Emotion Questions, *Emot. Rev.* 3 (2011) 379–386. doi:10.1177/1754073911410743.
- [94] C.R. Brown, M. Bomberger Brown, Ectoparasitism as a Cost of Coloniality in Cliff Swallows (*Hirundo Pyrrhonota*), *Ecology.* 67 (1986) 1206–1218. doi:10.2307/1938676.
- [95] J.D. Mayer, G. Geher, Emotional intelligence and the identification of emotion, *Intelligence.* 22

- (1996) 89–113. doi:10.1016/S0160-2896(96)90011-2.
- [96] R.S. Lazarus, Thoughts on the relations between emotion and cognition., *Am. Psychol.* 37 (1982) 1019–1024. doi:10.1037/0003-066X.37.9.1019.
- [97] J.J. Goldberger, Sympathovagal balance: how should we measure it?, *Am. J. Physiol.* 276 (1999) H1273–H1280. doi:10.1016/j.ejim.2004.06.016.
- [98] J.A. Bouwknecht, B. Olivier, R.E. Paylor, The stress-induced hyperthermia paradigm as a physiological animal model for anxiety: A review of pharmacological and genetic studies in the mouse, *Neurosci. Biobehav. Rev.* 31 (2007) 41–59. doi:10.1016/j.neubiorev.2006.02.002.
- [99] B. Olivier, T. Zethof, T. Pattij, M. Van Boogaert, R. Van Oorschot, C. Leahy, et al., Stress-induced hyperthermia and anxiety: Pharmacological validation, *Eur. J. Pharmacol.* 463 (2003) 117–132. doi:10.1016/S0014-2999(03)01326-8.
- [100] T. Oka, K. Oka, T. Hori, Mechanisms and mediators of psychological stress-induced rise in core temperature., *Psychosom. Med.* 63 (2001) 476–486. doi:10.1097/00006842-200105000-00018.
- [101] D.M.L. Vianna, P. Carrive, Changes in cutaneous and body temperature during and after conditioned fear to context in the rat, *Eur. J. Neurosci.* 21 (2005) 2505–2512. doi:10.1111/j.1460-9568.2005.04073.x.
- [102] N. Ogata, T. Kikusui, Y. Takeuchi, Y. Mori, Objective measurement of fear-associated learning in dogs, *J. Vet. Behav. Clin. Appl. Res.* 1 (2006) 55–61. doi:10.1016/j.jveb.2006.06.002.
- [103] N. Kataoka, H. Hioki, T. Kaneko, K. Nakamura, Psychological stress activates a dorsomedial hypothalamus-medullary raphe circuit driving brown adipose tissue thermogenesis and hyperthermia, *Cell Metab.* 20 (2014) 346–358. doi:10.1016/j.cmet.2014.05.018.
- [104] H.J. Lee, A.H. Macbeth, J.H. Pagani, W. Scott Young, Oxytocin: The great facilitator of life, *Prog. Neurobiol.* 88 (2009) 127–151. doi:10.1016/j.pneurobio.2009.04.001.
- [105] M. Nagasawa, T. Kikusui, T. Onaka, M. Ohta, Dog's gaze at its owner increases owner's urinary oxytocin during social interaction, *Horm. Behav.* 55 (2009) 434–441. doi:10.1016/j.yhbeh.2008.12.002.
- [106] J.L. Oliva, J.-L. Rault, B. Appleton, A. Lill, Oxytocin enhances the appropriate use of human social cues by the domestic dog (*Canis familiaris*) in an object choice task, *Anim. Cogn.* 18 (2015) 767–775. doi:10.1007/s10071-015-0843-7.

- [107] C.E. Detillion, T.K.S. Craft, E.R. Glasper, B.J. Prendergast, A.C. DeVries, Social facilitation of wound healing, *Psychoneuroendocrinology*. 29 (2004) 1004–1011. doi:10.1016/j.psyneuen.2003.10.003.
- [108] K.S. Schwartzkopf-Genswein, J.M. Stookey, The use of infrared thermography to assess inflammation associated with hot-iron and freeze branding in cattle, *Can. J. Anim. Sci.* 77 (1997) 577–583. doi:10.4141/A97-019.
- [109] R.J. Berry, A.D. Kennedy, S.L. Scott, B.L. Kyle, A.L. Schaefer, Daily variation in the udder surface temperature of dairy cows measured by infrared thermography: Potential for mastitis detection, *Can. J. Anim. Sci.* 83 (2003) 687–693. doi:10.4141/A03-012.
- [110] B.P.A. Da Fonseca, A.L.G. Alves, J.L.M. Nicoletti, A. Thomassian, C.A.L. Hussni, S. Mikail, Thermography and ultrasonography in back pain diagnosis of equine athletes, *J. Equine Vet. Sci.* 26 (2006) 507–516. doi:10.1016/j.jevs.2006.09.007.
- [111] M. Manirul Islam, T.A. Sonia, M. Hong Seok, B.M.R.B. A, K. Yae Jin, Y. Chul Ju, Use of thermal imaging for the early detection of signs of disease in pigs challenged orally with *Salmonella typhimurium* and *Escherichia coli*, *African J. Microbiol. Res.* 9 (2015) 1667–1674. doi:10.5897/AJMR2015.7580.
- [112] P. Jerem, K. Herborn, D. McCafferty, D. McKeegan, R. Nager, Thermal Imaging to Study Stress Non-invasively in Unrestrained Birds, *J. Vis. Exp.* (2015) 1–10. doi:10.3791/53184.
- [113] T. Travain, E.S. Colombo, E. Heinzl, D. Bellucci, E. Prato-Previde, P. Valsecchi, Hot dogs: Thermography in the assessment of stress in dogs (*Canis familiaris*)—A pilot study, *J. Vet. Behav. Clin. Appl. Res.* 10 (2015) 17–23. doi:10.1016/j.jveb.2014.11.003.
- [114] M.J. Sánchez, E. Bartolomé, M. Valera, Genetic study of stress assessed with infrared thermography during dressage competitions in the Pura Raza Español horse, *Appl. Anim. Behav. Sci.* In Press, (2015). doi:10.1016/j.applanim.2015.11.006.
- [115] J.R. Speakman, S. Ward, Infrared thermography: principles and applications, *Zoology*. 101 (1998) 224–232.
- [116] M. Stewart, J.R. Webster, A. Schaefer, N.J. Cook, S.L. Scott, Infrared thermography as a non-invasive tool to study animal welfare, *Anim. Welf.* 14 (2005) 319–325.
- [117] P.R. Abramson, L.B. Perry, T.T. Seeley, D. Masters Seeley, A.B. Rothblatt, Thermographic measurement of sexual arousal: A discriminant validity analysis, *Arch. Sex. Behav.* 10 (1981)

- 171–176. doi:10.1007/BF01542177.
- [118] T.M. Kukkonen, Y.M. Binik, R. Amsel, S. Carrier, Thermography as a physiological measure of sexual arousal in both men and women, *J. Sex. Med.* 4 (2007) 93–105. doi:10.1111/j.1743-6109.2006.00399.x.
- [119] R. Nakanishi, K. Imai-Matsumura, Facial skin temperature decreases in infants with joyful expression, *Infant Behav. Dev.* 31 (2008) 137–144. doi:10.1016/j.infbeh.2007.09.001.
- [120] S. Ioannou, V. Gallese, A. Merla, Thermal infrared imaging in psychophysiology: Potentialities and limits, *Psychophysiology*. 51 (2014) 951–963. doi:10.1111/psyp.12243.
- [121] S.R. Johnson, S. Rao, S.B. Hussey, P.S. Morley, J.L. Traub-Dargatz, Thermographic Eye Temperature as an Index to Body Temperature in Ponies, *J. Equine Vet. Sci.* 31 (2011) 63–66. doi:10.1016/j.jevs.2010.12.004.
- [122] N. Ludwig, M. Gargano, F. Luzi, C. Carezzi, M. Verga, Technical note: Applicability of infrared thermography as a non invasive measurements of stress in rabbit, *World Rabbit Sci.* 15 (1997) 199–205. doi:10.4995/wrs.2007.588.
- [123] K. Yarnell, C. Hall, E. Billett, An assessment of the aversive nature of an animal management procedure (clipping) using behavioral and physiological measures., *Physiol. Behav.* 118 (2013) 32–9. doi:10.1016/j.physbeh.2013.05.013.
- [124] M. Valera, E. Bartolomé, M.J. Sánchez, A. Molina, N. Cook, A. Schaefer, Changes in Eye Temperature and Stress Assessment in Horses During Show Jumping Competitions, *J. Equine Vet. Sci.* 32 (2012) 827–830. doi:10.1016/j.jevs.2012.03.005.
- [125] R.O. Moe, S.M. Stubsjøen, J. Bohlin, A. Flø, M. Bakken, Peripheral temperature drop in response to anticipation and consumption of a signaled palatable reward in laying hens (*Gallus domesticus*), *Physiol. Behav.* 106 (2012) 527–533. doi:10.1016/j.physbeh.2012.03.032.
- [126] M. Cabanac, S. Aizawa, Fever and tachycardia in a bird (*Gallus domesticus*) after simple handling, *Physiol. Behav.* 69 (2000) 541–545. doi:10.1016/S0031-9384(00)00227-4.
- [127] J.L. Edgar, C.J. Nicol, C.A. Pugh, E.S. Paul, Surface temperature changes in response to handling in domestic chickens, *Physiol. Behav.* 119 (2013) 195–200. doi:10.1016/j.physbeh.2013.06.020.
- [128] K. Nakayama, S. Goto, K. Kuraoka, K. Nakamura, Decrease in nasal temperature of rhesus monkeys (*Macaca mulatta*) in negative emotional state, *Physiol. Behav.* 84 (2005) 783–790. doi:10.1016/j.physbeh.2005.03.009.

- [129] H.S. Proctor, G. Carder, Nasal temperatures in dairy cows are influenced by positive emotional state, *Physiol. Behav.* 138 (2015) 340–344. doi:10.1016/j.physbeh.2014.11.011.
- [130] I. Pavlidis, N.L. Eberhardt, J.A. Levine, Seeing through the face of deception., *Nature.* 415 (2002) 35. doi:10.1038/415035a.
- [131] M. Stewart, Non-invasive measurement of stress and pain in cattle using infrared thermography, Massey University, 2008.
- [132] Task Force of the European Society of Cardiology, The North American Society of Pacing and Electrophysiology, Heart Rate Variability: Standards of Measurement, Physiological Interpretation, and Clinical Use, *Eur. Heart J.* 17 (1996) 354–381. doi:10.1161/01.CIR.93.5.1043.
- [133] J. Pumprla, K. Howorka, D. Groves, M. Chester, J. Nolan, Functional assessment of heart rate variability: physiological basis and practical applications, *Int. J. Cardiol.* 84 (2002) 1–14. doi:10.1016/S0167-5273(02)00057-8.
- [134] J.F. Thayer, J.F. Brosschot, Psychosomatics and psychopathology: looking up and down from the brain, *Psychoneuroendocrinology.* 30 (2005) 1050–1058. doi:10.1016/j.psyneuen.2005.04.014.
- [135] A. Malliani, M. Pagani, R. Furlan, S. Guzzetti, D. Lucini, N. Montano, et al., Individual Recognition by Heart Rate Variability of Two Different Autonomic Profiles Related to Posture, *Circ.* 96 (1997) 4143–4145. doi:10.1161/01.CIR.96.12.4143.
- [136] D.J. Ewing, J.M.M. Neilson, C.M. Shapiro, J.A. Stewart, W. Reid, Twenty four hour heart rate variability: effects of posture, sleep, and time of day in healthy controls and comparison with bedside tests of autonomic function in diabetic patients, *Br. Heart J.* 65 (1991) 239–244. doi:10.1136/hrt.65.5.239.
- [137] D.L. Musselman, C.B. Nemeroff, Depression and cardiovascular disease: Increasing evidence of a connection, *Cardiovasc. Rev. Rep.* 19 (1998) 34–42.
- [138] A. Rozanski, J.A. Blumenthal, J. Kaplan, Impact of Psychological Factors on the Pathogenesis of Cardiovascular Disease and Implications for Therapy, *Circulation.* 99 (1999) 2192–2217.
- [139] J.K. Kiecolt-Glaser, L. McGuire, T.F. Robles, R. Glaser, Emotions, morbidity, and mortality: new perspectives from psychoneuroimmunology, *Annu.Rev.Psychol.* 53 (2002) 83–107. doi:10.1146/annurev.psych.53.100901.135217.
- [140] D.S. Krantz, M.K. McCeney, Effects of Psychological and Social Factors on Organic Disease: A

- Critical Assessment of Research on Coronary Heart Disease, *Annu. Rev. Psychol.* 53 (2002) 341–369. doi:10.1146/annurev.psych.53.100901.135208.
- [141] R.M. Carney, R.D. Saunders, K.E. Freedland, P. Stein, M.W. Rich, A.S. Jaffe, Association of depression with reduced heart rate variability in coronary artery disease, *Am. J. Cardiol.* 76 (1995) 562–564. doi:10.1016/S0002-9149(99)80155-6.
- [142] H. Cohen, M. Kotler, M. a. Matar, Z. Kaplan, H. Miodownik, Y. Cassuto, Power spectral analysis of heart rate variability in posttraumatic stress disorder patients, *Biol. Psychiatry.* 41 (1997) 627–629. doi:10.1016/S0006-3223(96)00525-2.
- [143] B.H. Friedman, J.F. Thayer, Autonomic balance revisited: Panic anxiety and heart rate variability, *J. Psychosom. Res.* 44 (1998) 133–151. doi:10.1016/S0022-3999(97)00202-X.
- [144] K. Hagen, J. Langbein, C. Schmied, D. Lexer, S. Waiblinger, Heart rate variability in dairy cows - Influences of breed and milking system, *Physiol. Behav.* 85 (2005) 195–204. doi:10.1016/j.physbeh.2005.03.019.
- [145] A. Boissy, G. Manteuffel, M.B. Jensen, R.O. Moe, B. Spruijt, L.J. Keeling, et al., Assessment of positive emotions in animals to improve their welfare, *Physiol. Behav.* 92 (2007) 375–397. doi:10.1016/j.physbeh.2007.02.003.
- [146] E. von Borell, J. Langbein, G. Després, S. Hansen, C. Leterrier, J. Marchant-Forde, et al., Heart rate variability as a measure of autonomic regulation of cardiac activity for assessing stress and welfare in farm animals - A review, *Physiol. Behav.* 92 (2007) 293–316. doi:10.1016/j.physbeh.2007.01.007.
- [147] M. Kuwahara, Y. Tsujino, H. Tsubone, E. Kumagai, H. Tsutsumi, M. Tanigawa, Effects of pair housing on diurnal rhythms of heart rate and heart rate variability in miniature swine., *Exp. Anim.* 53 (2004) 303–9.
- [148] E.K. Visser, C.G. Van Reenen, J.T.N. Van der Werf, M.B.H. Schilder, J.H. Knaap, a. Barneveld, et al., Heart rate and heart rate variability during a novel object test and a handling test in young horses, *Physiol. Behav.* 76 (2002) 289–296. doi:10.1016/S0031-9384(02)00698-4.
- [149] R.E. Kleiger, J.P. Miller, J.T. Bigger, A.J. Moss, Decreased heart rate variability and its association with increased mortality after acute myocardial infarction, *Am. J. Cardiol.* 59 (1987) 256–262. doi:10.1016/0002-9149(87)90795-8.
- [150] J.T. Bigger, R.E. Kleiger, J.L. Fleiss, L.M. Rolnitzky, R.C. Steinman, J.P. Miller, Components of

- heart rate variability measured during healing of acute myocardial infarction, *Am. J. Cardiol.* 61 (1988) 208–215. doi:10.1016/0002-9149(88)90917-4.
- [151] T.G. Farrell, Y. Bashir, T. Cripps, M. Malik, J. Poloniecki, E.D. Bennett, et al., Risk stratification for arrhythmic events in postinfarction patients based on heart rate variability, ambulatory electrocardiographic variables and the signal-averaged electrocardiogram, *J. Am. Coll. Cardiol.* 18 (1991) 687–697. doi:10.1016/0735-1097(91)90791-7.
- [152] C. Cérutti, M.L. Ducher, P. Lantelme, M.P. Gustin, C.Z. Paultre, Assessment of spontaneous baroreflex sensitivity in rats a new method using the concept of statistical dependence., *Am. J. Physiol.* 268 (1995) R382–R–388.
- [153] A.E. Aubert, D. Ramaekers, F. Beckers, R. Breem, C. Deneff, F. Van de Werf, et al., The analysis of heart rate variability in unrestrained rats. Validation of method and results, *Comput. Methods Programs Biomed.* 60 (1999) 197–213. doi:10.1016/S0169-2607(99)00017-6.
- [154] A.E. Aubert, B. Seps, F. Beckers, Heart Rate Variability in Athletes, *Sport. Med.* 33 (2003) 889–919. doi:10.2165/00007256-200333120-00003.
- [155] R.H. Fagard, K. Pardaens, J.A. Staessen, L. Thijs, Power spectral analysis of heart rate variability by autoregressive modelling and fast Fourier transform: A comparative study, *Acta Cardiol.* 53 (1998) 211–218.
- [156] M. V. Kamath, E.L. Fallen, Power spectral analysis of heart rate variability: A noninvasive signature of cardiac autonomic function, *Crit. Rev. Biomed. Eng.* 21 (1993) 245–311.
- [157] A. Malliani, M. Pagani, F. Lombardi, S. Cerutti, Cardiovascular neural regulation explored in the frequency domain., *Circulation.* 84 (1991) 482–492. doi:10.1161/01.CIR.84.2.482.
- [158] L. Bergamasco, M.C. Osella, P. Savarino, G. Larosa, L. Ozella, M. Manassero, et al., Heart rate variability and saliva cortisol assessment in shelter dog: Human-animal interaction effects, *Appl. Anim. Behav. Sci.* 125 (2010) 56–68. doi:10.1016/j.applanim.2010.03.002.
- [159] K. Maros, A. Dóka, Á. Miklósi, Behavioural correlation of heart rate changes in family dogs, *Appl. Anim. Behav. Sci.* 109 (2008) 329–341. doi:10.1016/j.applanim.2007.03.005.
- [160] C. O'Brien, R.W. Hoyt, M.J. Buller, J.W. Castellani, A.J. Young, Telemetry pill measurement of core temperature in humans during active heating and cooling, *Med. Sci. Sport. Exerc.* 30 (1998) 468–472. doi:10.1097/00005768-199803000-00020.
- [161] A.A. Truett, D.B. West, Validation of a radiotelemetry system for continuous blood pressure and

- heart rate monitoring in dogs., *Lab. Anim. Sci.* 45 (1995) 299–302.
- [162] A.R.M. Gelzer, H.A. Ball, Validation of a Telemetry System for Measurement of Blood Pressure, Electrocardiogram and Locomotor Activity in Beagle Dogs, *Clin. Exp. Hypertens.* 19 (1997) 1135–1160. doi:10.3109/10641969709083209.
- [163] M. Stewart, J.R. Webster, G.A. Verkerk, A.L. Schaefer, J.J. Colyn, K.J. Stafford, Non-invasive measurement of stress in dairy cows using infrared thermography, *Physiol. Behav.* 92 (2007) 520–525. doi:10.1016/j.physbeh.2007.04.034.
- [164] M. Stewart, K.J. Stafford, S.K. Dowling, a. L. Schaefer, J.R. Webster, Eye temperature and heart rate variability of calves disbudded with or without local anaesthetic, *Physiol. Behav.* 93 (2008) 789–797. doi:10.1016/j.physbeh.2007.11.044.
- [165] C. Hall, R. Kay, K. Yarnell, Assessing ridden horse behavior: Professional judgment and physiological measures, *J. Vet. Behav. Clin. Appl. Res.* 9 (2014) 22–29. doi:10.1016/j.jveb.2013.09.005.
- [166] A.L. Schaefer, L.R. Matthews, N.J. Cook, J.R. Webster, S.L. Scott, Novel non-invasive measures of animal welfare, in: *Anim. Welf. Behav. From Sci. to Solut.*, 2002.
- [167] C.H. Vinkers, L. Groenink, M.J. V van Bogaert, K.G.C. Westphal, C.J. Kalkman, R. van Oorschot, et al., Stress-induced hyperthermia and infection-induced fever: Two of a kind?, *Physiol. Behav.* 98 (2009) 37–43. doi:10.1016/j.physbeh.2009.04.004.
- [168] N.J. Cook, A.L. Schaefer, J.S. Church, Nutritional therapy modulates stress responses of elk (*Cervus elaphus Canadensis*) to removal of velvet antler, *Online J. Vet. Res.* 10 (2006) 20–25.
- [169] W.B. Cannon, Bodily changes in pain, hunger, fear, and rage: an account of recent researchers into the function of emotional excitement, D. Appleton and Co., New York, NY, 1916.
- [170] J.M. Koolhaas, S.F. de Boer, C.M. Coppens, B. Buwalda, Neuroendocrinology of coping styles: Towards understanding the biology of individual variation, *Front. Neuroendocrinol.* 31 (2010) 307–321. doi:10.1016/j.yfrne.2010.04.001.
- [171] P.A. Alm, Stuttering, emotions, and heart rate during anticipatory anxiety: a critical review, *J. Fluency Disord.* 29 (2004) 123–33. doi:10.1016/j.jfludis.2004.02.001.
- [172] L.M. Romero, Fight or Flight responses, in: M.D. Breed, J. Moore (Eds.), *Encycl. Anim. Behav.*, Oxford Academy Press, Oxford, 2010: pp. 710–714.

- [173] E.O. Johnson, T.C. Kamilaris, C.S. Carter, A.E. Calogero, P.W. Gold, G.P. Chrousos, The biobehavioral consequences of psychogenic stress in a small, social primate (*Callithrix jacchus jacchus*)., *Biol. Psychiatry*. 40 (1996) 317–37. doi:10.1016/0006-3223(95)00397-5.
- [174] F. Toates, *Biological Psychology: an Integrative Approach*, Pearson Education, Harlow, 2001.
- [175] K.E. Gabry, G.P. Chrousos, P.W. Gold, The hypothalamic-pituitary-adrenal (HPA) axis: a major mediator of the adaptive responses to stress, *NeuroImmune Biol.* 3 (2003) 379–414. doi:10.1016/S1567-7443(03)80055-8.
- [176] B. Beerda, M.B.H. Schilder, J.A.R.A.M. Van Hooff, H.W. De Vries, Manifestations of chronic and acute stress in dogs, *Appl. Anim. Behav. Sci.* 52 (1997) 307–319. doi:10.1016/S0168-1591(96)01131-8.
- [177] B. Beerda, M.B.H. Schilder, J.A.R.A.M. Van Hooff, H.W. De Vries, J.A. Mol, Behavioural, saliva cortisol and heart rate responses to different types of stimuli in dogs, *Appl. Anim. Behav. Sci.* 58 (1998) 365–381. doi:10.1016/S0168-1591(97)00145-7.
- [178] C. Palestini, E.P. Previde, C. Spiezio, M. Verga, Heart rate and behavioural responses of dogs in the Ainsworth's Strange Situation: A pilot study, *Appl. Anim. Behav. Sci.* 94 (2005) 75–88. doi:10.1016/j.applanim.2005.02.005.
- [179] G. Fallani, E. Prato-Previde, P. Valsecchi, Behavioral and physiological responses of guide dogs to a situation of emotional distress, *Physiol. Behav.* 90 (2007) 648–655. doi:10.1016/j.physbeh.2006.12.001.
- [180] M.B. Hennessey, Using hypothalamic-pituitary-adrenal measures for assessing and reducing the stress of dogs in shelters: A review, *Appl. Anim. Behav. Sci.* 149 (2013) 1–12. doi:10.1016/j.applanim.2013.09.004.
- [181] C.E. Part, J.L. Kiddie, W.A. Hayes, D.S. Mills, R.F. Neville, D.B. Morton, et al., Physiological, physical and behavioural changes in dogs (*Canis familiaris*) when kennelled: Testing the validity of stress parameters, *Physiol. Behav.* 133 (2014) 260–271. doi:10.1016/j.physbeh.2014.05.018.
- [182] E. Tod, D. Brander, N. Waran, Efficacy of dog appeasing pheromone in reducing stress and fear related behaviour in shelter dogs, *Appl. Anim. Behav. Sci.* 93 (2005) 295–308. doi:10.1016/j.applanim.2005.01.007.
- [183] V. Konok, A. Dóka, Á. Miklósi, The behavior of the domestic dog (*Canis familiaris*) during separation from and reunion with the owner: A questionnaire and an experimental study, *Appl.*

- Anim. Behav. Sci. 135 (2011) 300–308. doi:10.1016/j.applanim.2011.10.011.
- [184] C. Pastore, F. Pirrone, F. Balzarotti, M. Faustini, L. Pierantoni, M. Albertini, Evaluation of physiological and behavioral stress-dependent parameters in agility dogs, *J. Vet. Behav. Clin. Appl. Res.* 6 (2011) 188–194. doi:10.1016/j.jveb.2011.01.001.
- [185] T. King, P.H. Hemsworth, G.J. Coleman, Fear of novel and startling stimuli in domestic dogs, *Appl. Anim. Behav. Sci.* 82 (2003) 45–64. doi:10.1016/S0168-1591(03)00040-6.
- [186] H.D.C. Denham, J.W.S. Bradshaw, N.J. Rooney, Repetitive behaviour in kennelled domestic dog: Stereotypical or not?, *Physiol. Behav.* 128 (2014) 288–294. doi:10.1016/j.physbeh.2014.01.007.
- [187] B. Beerda, M.B.H. Schilder, J.A.R.A.M. Van Hooff, H.W. De Vries, J.A. Mol, Behavioural and hormonal indicators of enduring environmental stress in dogs, *Anim. Welf.* 9 (2000) 49–62.
- [188] F. Biondi, P.T. Dornbusch, M. Sampaio, F. Montiani-Ferreira, Infrared ocular thermography in dogs with and without keratoconjunctivitis sicca, *Vet. Ophthalmol.* 18 (2015) 28–34. doi:10.1111/vop.12086.
- [189] D. Döring, A. Roscher, F. Scheipl, H. Küchenhoff, M.H. Erhard, Fear-related behaviour of dogs in veterinary practice, *Vet. J.* 182 (2009) 38–43. doi:10.1016/j.tvjl.2008.05.006.
- [190] T.L. Stanford, Behavior of dogs entering a veterinary clinic, *Appl. Anim. Ethol.* 7 (1981) 271–279.
- [191] W. Smith, *Modern Optical Engineering*, 4th ed., McGraw-Hill Education, New York, 2007.
- [192] S. Siegel, N.J. Castellan, *Nonparametric Statistics for The Behavioral Sciences*, 2nd ed., McGraw-Hill Humanities/Social Sciences/Languages, New York, 1988.
- [193] A.L. Schaefer, N.J. Cook, J.S. Church, J.A. Basarab, B.H. Perry, C.C. Miller, et al., The use of infrared thermography as an early indicator of bovine respiratory disease complex in calves, *Res. Vet. Sci.* 83 (2007) 376–384. doi:10.1016/j.rvsc.2007.01.008.
- [194] E. Bartolomé, M.J. Sánchez, A. Molina, A.L. Schaefer, I. Cervantes, M. Valera, Using eye temperature and heart rate for stress assessment in young horses competing in jumping competitions and its possible influence on sport performance, *Animal.* 7 (2013) 2044–53. doi:10.1017/S1751731113001626.
- [195] N.J. Emery, The eyes have it: the neuroethology, function and evolution of social gaze, *Neurosci. Biobehav. Rev.* 24 (2000) 581–604. doi:10.1016/S0149-7634(00)00025-7.
- [196] R. Schenkel, Submission: Its Features and Function in the Wolf and Dog, *Am. Zool.* 7 (1967) 319–

329. doi:10.1093/icb/7.2.319.
- [197] J.W.S. Bradshaw, H.M.R. Nott, Social and communication behavior of companion dogs, in: J.A. Serpell (Ed.), *Domest. Dog Its Evol. Behav. Interact. With People*, Cambridge University Press, Cambridge, 1995: pp. 115–130.
- [198] J. Vas, J. Topál, M. Gácsi, Á. Miklósi, V. Csányi, A friend or an enemy? Dogs' reaction to an unfamiliar person showing behavioural cues of threat and friendliness at different times, *Appl. Anim. Behav. Sci.* 94 (2005) 99–115. doi:10.1016/j.applanim.2005.02.001.
- [199] J. Vas, J. Topál, B. Gyori, Á. Miklósi, Consistency of dogs' reactions to threatening cues of an unfamiliar person, *Appl. Anim. Behav. Sci.* 112 (2008) 331–344. doi:10.1016/j.applanim.2007.09.002.
- [200] A. Hernádi, A. Kis, B. Turcsán, J. Topál, Man's Underground Best Friend: Domestic Ferrets, Unlike the Wild Forms, Show Evidence of Dog-Like Social-Cognitive Skills, *PLoS One.* 7 (2012) e43267. doi:10.1371/journal.pone.0043267.
- [201] M. Gácsi, K. Maros, S. Sernkvist, T. Faragó, Á. Miklósi, Human Analogue Safe Haven Effect of the Owner: Behavioural and Heart Rate Response to Stressful Social Stimuli in Dogs, *PLoS One.* 8 (2013) e58475. doi:10.1371/journal.pone.0058475.
- [202] M.W. Fox, A Comparative Study of the Development of Facial Expressions in Canids; Wolf, Coyote and Foxes, *Behaviour.* 36 (1970) 49–73. doi:10.1163/156853970X00042.
- [203] R.M. Seyfarth, D.L. Cheney, Meaning and emotional in animal vocalizations, *Ann. New York Acad. Sci.* 1000 (2003) 32–55.
- [204] P. Pongrácz, C. Molnár, Á. Miklósi, Acoustic parameters of dog barks carry emotional information for humans, *Appl. Anim. Behav. Sci.* 100 (2006) 228–240. doi:10.1016/j.applanim.2005.12.004.
- [205] N. Reefmann, F. Bütikofer Kaszàs, B. Wechsler, L. Gyax, Ear and tail postures as indicators of emotional valence in sheep, *Appl. Anim. Behav. Sci.* 118 (2009) 199–207. doi:10.1016/j.applanim.2009.02.013.
- [206] E. Palagi, G. Mancini, Playing with the face: playful facial “chattering” and signal modulation in a monkey species (*Theropithecus gelada*), *J. Comp. Psychol.* 125 (2011) 11–21. doi:10.1037/a0020869.
- [207] C. Kemp, G. Kaplan, Facial expressions in common marmosets (*Callithrix jacchus*) and their use

- by conspecifics, *Anim. Cogn.* 16 (2013) 773–788. doi:10.1007/s10071-013-0611-5.
- [208] K.R. Scherer, On the Nature and Function of Emotion: A Component Process Approach, in: K. Scherer, P. Ekman (Eds.), *Approaches to Emot.*, Psychology Press, New York, 1984: pp. 293 – 317.
- [209] R.S. Lazarus, *Emotion and adaptation*, Oxford University Press, New York, 1991.
- [210] M.S. Dawkins, The science of animal suffering, *Ethology*. 114 (2008) 937–945. doi:10.1111/j.1439-0310.2008.01557.x.
- [211] K.R. Scherer, What are emotions? And how can they be measured?, *Soc. Sci. Inf.* 44 (2005) 695–729. doi:10.1177/0539018405058216.
- [212] P.M. Niedenthal, S. Krauth-Gruber, F. Ric, *Psychology of Emotion: Interpersonal, Experiential and Cognitive Approaches*, Psychology Press, New York, 2006.
- [213] J.A. Russell, A circumplex model of affect., *J. Pers. Soc. Psychol.* 39 (1980) 1161–1178. doi:10.1037/h0077714.
- [214] L. Feldman Barrett, J.A. Russell, The Structure of Current Affect: Controversies and Emerging Consensus, *Curr. Dir. Psychol. Sci.* 8 (1999) 10–14. doi:10.1111/1467-8721.00003.
- [215] L. Désiré, A. Boissy, I. Veissier, Emotions in farm animals: A new approach to animal welfare in applied ethology, *Behav. Processes*. 60 (2002) 165–180. doi:10.1016/S0376-6357(02)00081-5.
- [216] A. Boissy, C. Arnould, E. Chaillou, V. Colson, L. Désiré, C. Duvaux-Ponter, et al., Emotion and cognition: a new strategy to achieve animal welfare, *Prod. Anim.* 20 (2007) 17–21.
- [217] E.O. Johnson, T.C. Kamilaris, G.P. Chrousos, P.W. Gold, Mechanisms of stress: A dynamic overview of hormonal and behavioral homeostasis, *Neurosci. Biobehav. Rev.* 16 (1992) 115–130. doi:10.1016/S0149-7634(05)80175-7.
- [218] I. Veissier, A. Boissy, Stress and welfare: Two complementary concepts that are intrinsically related to the animal’s point of view, *Physiol. Behav.* 92 (2007) 429–433. doi:10.1016/j.physbeh.2006.11.008.
- [219] J.W. Yeates, D.C.J. Main, Assessment of positive welfare: A review, *Vet. J.* 175 (2008) 293–300. doi:10.1016/j.tvjl.2007.05.009.
- [220] M. Coulon, R. Nowak, J. Peyrat, H. Chandèze, A. Boissy, X. Boivin, Do Lambs Perceive Regular Human Stroking as Pleasant? Behavior and Heart Rate Variability Analyses, *PLoS One*. 10 (2015)

e0118617. doi:10.1371/journal.pone.0118617.

- [221] M.T. Mendl, O.H.P. Burman, R.M.A. Parker, E.S. Paul, Cognitive bias as an indicator of animal emotion and welfare: Emerging evidence and underlying mechanisms, *Appl. Anim. Behav. Sci.* 118 (2009) 161–181. doi:10.1016/j.applanim.2009.02.023.
- [222] S. Imfeld-Mueller, L. Van Wezemael, M. Stauffacher, L. Gygax, E. Hillmann, Do pigs distinguish between situations of different emotional valences during anticipation?, *Appl. Anim. Behav. Sci.* 131 (2011) 86–93. doi:10.1016/j.applanim.2011.02.009.
- [223] T. Rehn, L.J. Keeling, The effect of time left alone at home on dog welfare, *Appl. Anim. Behav. Sci.* 129 (2011) 129–135. doi:10.1016/j.applanim.2010.11.015.
- [224] F. Kuhne, J.C. Hößler, R. Struwe, Effects of human-dog familiarity on dogs' behavioural responses to petting, *Appl. Anim. Behav. Sci.* 142 (2012) 176–181. doi:10.1016/j.applanim.2012.10.003.
- [225] N. Rooney, S. Gaines, E. Hiby, A practitioner's guide to working dog welfare, *J. Vet. Behav. Clin. Appl. Res.* 4 (2009) 127–134. doi:10.1016/j.jveb.2008.10.037.
- [226] A.I. Sandem, B.O. Braastad, K.E. Bøe, Eye white may indicate emotional state on a frustration-contentedness axis in dairy cows, *Appl. Anim. Behav. Sci.* 79 (2002) 1–10. doi:10.1016/S0168-1591(02)00029-1.
- [227] N. Reefmann, F. Bütikofer Kaszàs, B. Wechsler, L. Gygax, Physiological expression of emotional reactions in sheep, *Physiol. Behav.* 98 (2009) 235–241. doi:10.1016/j.physbeh.2009.05.017.
- [228] N. Reefmann, B. Wechsler, L. Gygax, Behavioural and physiological assessment of positive and negative emotion in sheep, *Anim. Behav.* 78 (2009) 651–659. doi:10.1016/j.anbehav.2009.06.015.
- [229] A. Boissy, C. Lee, How assessing relationships between emotions and cognition can improve farm animal welfare, *OIE Rev. Sci. Tech.* 33 (2014) 103–110.
- [230] E. Verbeek, D. Ferguson, P. Quinquet de Monjour, C. Lee, Generating positive affective states in sheep: The influence of food rewards and opioid administration, *Appl. Anim. Behav. Sci.* 154 (2014) 39–47. doi:10.1016/j.applanim.2014.02.011.
- [231] H.S. Westerath, L. Gygax, E. Hillmann, Are special feed and being brushed judged as positive by calves?, *Appl. Anim. Behav. Sci.* 156 (2014) 12–21. doi:10.1016/j.applanim.2014.04.003.

- [232] E.F. Briefer, F. Tettamanti, A.G. McElligott, Emotions in goats: mapping physiological, behavioural and vocal profiles, *Anim. Behav.* 99 (2015) 131–143. doi:10.1016/j.anbehav.2014.11.002.
- [233] O.H.P. Burman, R.T.S. McGowan, M.T. Mendl, Y. Norling, E.S. Paul, T. Rehn, et al., Using judgement bias to measure positive affective state in dogs, *Appl. Anim. Behav. Sci.* 132 (2011) 160–168. doi:10.1016/j.applanim.2011.04.001.
- [234] R.T.S. McGowan, T. Rehn, Y. Norling, L.J. Keeling, Positive affect and learning: exploring the “Eureka Effect” in dogs., *Anim. Cogn.* 17 (2014) 577–587. doi:10.1007/s10071-013-0688-x.
- [235] F. Kuhne, J.C. Hößler, R. Struwe, Behavioral and cardiac responses by dogs to physical human-dog contact, *J. Vet. Behav. Clin. Appl. Res.* (2014). doi:10.1016/j.jveb.2014.02.006.
- [236] T. Rehn, L. Handlin, K. Uvnäs-Moberg, L.J. Keeling, Dogs’ endocrine and behavioural responses at reunion are affected by how the human initiates contact, *Physiol. Behav.* 124 (2014) 45–53. doi:10.1016/j.physbeh.2013.10.009.
- [237] J.L. Edgar, J.C. Lowe, E.S. Paul, C.J. Nicol, Avian maternal response to chick distress, *Proc. R. Soc. B Biol. Sci.* 278 (2011) 3129–3134. doi:10.1098/rspb.2010.2701.
- [238] K. Kuraoka, K. Nakamura, The use of nasal skin temperature measurements in studying emotion in macaque monkeys, *Physiol. Behav.* 102 (2011) 347–355. doi:10.1016/j.physbeh.2010.11.029.
- [239] F. Dai, N. Cogi, E.U.L. Heinzl, E. Dalla Costa, E. Canali, M. Minero, Validation of a fear test in sport horses using infrared thermography, *J. Vet. Behav. Clin. Appl. Res.* 10 (2015) 128–136. doi:10.1016/j.jveb.2014.12.001.
- [240] L.C. Grandi, H. Ishida, The Physiological Effect of Human Grooming on the Heart Rate and the Heart Rate Variability of Laboratory Non-Human Primates: A Pilot Study in Male Rhesus Monkeys, *Front. Vet. Sci.* 2 (2015) 1–9. doi:10.3389/fvets.2015.00050.
- [241] M. Zebunke, J. Langbein, G. Manteuffel, B. Puppe, Autonomic reactions indicating positive affect during acoustic reward learning in domestic pigs, *Anim. Behav.* 81 (2011) 481–489. doi:10.1016/j.anbehav.2010.11.023.
- [242] M. Zebunke, B. Puppe, J. Langbein, Effects of cognitive enrichment on behavioural and physiological reactions of pigs, *Physiol. Behav.* 118 (2013) 70–79. doi:10.1016/j.physbeh.2013.05.005.
- [243] R.O. Moe, J. Nordgreen, A.M. Janczak, B.M. Spruijt, A.J. Zanella, M. Bakken, Trace classical

- conditioning as an approach to the study of reward-related behaviour in laying hens: A methodological study, *Appl. Anim. Behav. Sci.* 121 (2009) 171–178. doi:10.1016/j.applanim.2009.10.002.
- [244] E. Prato-Previde, S. Marshall-Pescini, Social Looking in the Domestic Dog, in: A. Horowitz (Ed.), *Domest. Dog Cogn. Behav.*, Springer Berlin Heidelberg, Berlin, 2014: pp. 101–131. doi:10.1007/978-3-642-53994-7_5.
- [245] P. Valsecchi, E. Prato Previde, P.A. Accorsi, G. Fallani, Development of the attachment bond in guide dogs, *Appl. Anim. Behav. Sci.* 123 (2010) 43–50. doi:10.1016/j.applanim.2009.12.012.
- [246] S. Ioannou, H. Chotard, M. Davila-Ross, No strings attached: physiological monitoring of rhesus monkeys (*Macaca mulatta*) with thermal imaging, *Front. Behav. Neurosci.* 9 (2015) 1–10. doi:10.3389/fnbeh.2015.00160.
- [247] E.F. Hiby, N.J. Rooney, J.W.S. Bradshaw, Behavioural and physiological responses of dogs entering re-homing kennels, *Physiol. Behav.* 89 (2006) 385–391. doi:10.1016/j.physbeh.2006.07.012.
- [248] M. Siniscalchi, C. Stipo, A. Quaranta, “Like owner, like dog”: correlation between the owner’s attachment profile and the owner-dog bond., *PLoS One.* 8 (2013) e78455. doi:10.1371/journal.pone.0078455.
- [249] J. Fox, S. Weisberg, *An R Companion to Applied Regression*, Second, SAGE Publications, Inc., Thousand Oaks, 2011.
- [250] D. Bates, M. Maechler, B. Bolker, S. Walker, R.H. Bojesen Christensen, H. Singmann, et al., *lme4: Linear Mixed-Effects Models using “Eigen” and S4*, (2015).
- [251] V. Engert, A. Merla, J.A. Grant, D. Cardone, A. Tusche, T. Singer, Exploring the use of thermal infrared imaging in human stress research, *PLoS One.* 9 (2014) 1–11. doi:10.1371/journal.pone.0090782.
- [252] A. Quaranta, M. Siniscalchi, G. Vallortigara, Asymmetric tail-wagging responses by dogs to different emotive stimuli, *Curr. Biol.* 17 (2007) R199–R201.
- [253] H. Selye, *The Stress of Life*, 1st ed., McGraw-Hill, New York, 1976.
- [254] D.C. Randall, T.L. Skinner, G.E. Billman, A comparison of the autonomic nervous control of the heart during classical aversive vs appetitive conditioning in dog, *J. Auton. Nerv. Syst.* 13 (1985) 125–136. doi:10.1016/0165-1838(85)90029-3.

- [255] F. Aureli, S.D. Preston, F.B.M. de Waal, Heart rate responses to social interactions in free-moving rhesus macaques (*Macaca mulatta*): a pilot study., *J. Comp. Psychol.* 113 (1999) 59–65. doi:10.1037/0735-7036.113.1.59.
- [256] C.A.F. Wascher, I.B.R. Scheiber, K. Kotrschal, Heart rate modulation in bystanding geese watching social and non-social events., *Proc. Biol. Sci.* 275 (2008) 1653–1659. doi:10.1098/rspb.2008.0146.
- [257] B.M. Appelhans, L.J. Luecken, Heart rate variability as an index of regulated emotional responding, *Rev. Gen. Psychol.* 10 (2006) 229–240. doi:10.1037/1089-2680.10.3.229.
- [258] F. Kuhne, J.C. Hößler, R. Struwe, Emotions in dogs being petted by a familiar or unfamiliar person: Validating behavioural indicators of emotional states using heart rate variability, *Appl. Anim. Behav. Sci.* 161 (2014) 113–120. doi:10.1016/j.applanim.2014.09.020.
- [259] A. Bowman, Scottish SPCA, F.J. Dowell, N.P. Evans, “Four Seasons” in an animal rescue centre; classical music reduces environmental stress in kennelled dogs, *Physiol. Behav.* 143 (2015) 70–82. doi:10.1016/j.physbeh.2015.02.035.
- [260] H.S. Proctor, G. Carder, Measuring positive emotions in cows: Do visible eye whites tell us anything?, *Physiol. Behav.* 147 (2015) 1–6. doi:10.1016/j.physbeh.2015.04.011.
- [261] T. Travain, E.S. Colombo, L.C. Grandi, E.U.L. Heinzl, A. Pelosi, E. Prato-Previde, et al., How good is this food? A study on dogs’ emotional responses to a potentially pleasant event using infrared thermography, *Submitt. to Physiol. Behav.* (n.d.).
- [262] M. Gácsi, J. Topál, Á. Miklósi, A. Dóka, V. Csányi, Attachment behavior of adult dogs (*Canis familiaris*) living at rescue centers: forming new bonds., *J. Comp. Psychol.* 115 (2001) 423–431. doi:10.1037/0735-7036.115.4.423.
- [263] C. Mariti, E. Ricci, B. Carlone, J.L. Moore, C. Sighieri, A. Gazzano, Dog attachment to man: A comparison between pet and working dogs, *J. Vet. Behav. Clin. Appl. Res.* 8 (2013) 135–145. doi:10.1016/j.jveb.2012.05.006.
- [264] T. Rehn, U. Lindholm, L. Keeling, B. Forkman, I like my dog, does my dog like me?, *Appl. Anim. Behav. Sci.* 150 (2014) 65–73. doi:10.1016/j.applanim.2013.10.008.
- [265] A. Scandurra, A. Alterisio, B. D’Aniello, Behavioural effects of training on water rescue dogs in the Strange Situation Test, *Appl. Anim. Behav. Sci.* 174 (2016) 121–127. doi:10.1016/j.applanim.2015.10.007.

- [266] M.B. Hennessy, V.L. Voith, T.L. Young, J.L. Hawke, J. Centrone, A.L. McDowell, et al., Exploring human interaction and diet effects on the behavior of dogs in a public animal shelter., *J. Appl. Anim. Welf. Sci.* 5 (2002) 253–273. doi:10.1207/S15327604JAWS0504_01.
- [267] D.L. Wells, P.G. Hepper, The influence of environmental change on the behaviour of sheltered dogs, *Appl. Anim. Behav. Sci.* 68 (2000) 151–162. doi:10.1016/S0168-1591(00)00100-3.
- [268] D.L. Wells, A review of environmental enrichment for kennelled dogs, *Canis familiaris*, *Appl. Anim. Behav. Sci.* 85 (2004) 307–317. doi:10.1016/j.applanim.2003.11.005.
- [269] L. Graham, D.L. Wells, P.G. Hepper, The influence of visual stimulation on the behaviour of dogs housed in a rescue shelter, *Anim. Welf.* 14 (2005) 143–148. doi:10.1016/j.applanim.2004.08.024.
- [270] W.R. Thompson, R. Melzack, Early environment., *Sci. Am.* 194 (1956) 38–42. doi:10.1038/scientificamerican0156-38.
- [271] U.A. Luescher, D.B. McKeown, J. Halip, Stereotypic or Obsessive-Compulsive Disorders in Dogs and Cats, *Vet. Clin. North Am. Small Anim. Pract.* 21 (1991) 401–413. doi:10.1016/S0195-5616(91)50041-3.
- [272] P.A. Mertens, J. Unshelm, Effects of Group and Individual Housing on the Behavior of Kennelled Dogs in Animal Shelters, *Anthrozoos.* 9 (1996) 40–51. doi:10.2752/089279396787001662.
- [273] M.B. Hennessy, H.N. Davis, M.T. Williams, C. Mellott, C.W. Douglas, Plasma cortisol levels of dogs at a county animal shelter, *Physiol. Behav.* 62 (1997) 485–490. doi:10.1016/S0031-9384(97)80328-9.
- [274] V.L. Voith, D. Ganster, Separation anxiety: review of 42 cases, *Appl. Anim. Behav. Sci.* 37 (1993) 84–85.
- [275] R.C. Newberry, Environmental enrichment: Increasing the biological relevance of captive environments, *Appl. Anim. Behav. Sci.* 44 (1995) 229–243. doi:10.1016/0168-1591(95)00616-Z.
- [276] R.B. Jones, D. Waddington, Modification of fear in domestic chicks, *Gallus gallus domesticus*, via regular handling and early environmental enrichment, *Anim. Behav.* 43 (1992) 1021–1033. doi:10.1016/S0003-3472(06)80015-1.
- [277] C.J. Nicol, Effects of environmental enrichment and gentle handling on behaviour and fear responses of transported broilers, *Appl. Anim. Behav. Sci.* 33 (1992) 367–380. doi:10.1016/S0168-1591(05)80073-5.

- [278] G.P. Pearce, A.M. Paterson, The effect of space restriction and provision of toys during rearing on the behaviour, productivity and physiology of male pigs, *Appl. Anim. Behav. Sci.* 36 (1993) 11–28. doi:10.1016/0168-1591(93)90095-7.
- [279] D.G.M. Wood-Gush, K. Vestergaard, Exploratory behavior and the welfare of intensively kept animals, *J. Agric. Ethics.* 2 (1989) 161–169. doi:10.1007/BF01826929.
- [280] I. Gilloux, J. Gurnell, D. Shepherdson, An Enrichment Device for Great Apes, *Anim. Welf.* 1 (1992) 279–289.
- [281] R.C. Hubrecht, A comparison of social and environmental enrichment methods for laboratory housed dogs, *Appl. Anim. Behav. Sci.* 37 (1993) 345–361. doi:10.1016/0168-1591(93)90123-7.
- [282] L.L. Schipper, C.M. Vinke, M.B.H. Schilder, B.M. Spruijt, The effect of feeding enrichment toys on the behaviour of kennelled dogs (*Canis familiaris*), *Appl. Anim. Behav. Sci.* 114 (2008) 182–195. doi:10.1016/j.applanim.2008.01.001.
- [283] L.R. Mehrkam, C.D.L. Wynne, Behavioral differences among breeds of domestic dogs (*Canis lupus familiaris*): Current status of the science, *Appl. Anim. Behav. Sci.* 155 (2014) 12–27. doi:10.1016/j.applanim.2014.03.005.
- [284] K. Kramer, L.B. Kinter, Evaluation and applications of radiotelemetry in small laboratory animals., *Physiol. Genomics.* 13 (2003) 197–205. doi:10.1152/physiolgenomics.00164.2002.
- [285] S.A.B.E. van Acker, K. Kramer, E.E. Voest, J.A. Grimbergen, J. Zhang, W.J.F. van der Vijgh, et al., Doxorubicin-induced cardiotoxicity monitored by ECG in freely moving mice A new model to test potential protectors, *Cancer Chemother. Pharmacol.* 38 (1996) 95–101. doi:10.1007/s002800050453.
- [286] A. Klumpp, T. Trautmann, M. Markert, B. Guth, Optimizing the experimental environment for dog telemetry studies, *J. Pharmacol. Toxicol. Methods.* 54 (2006) 141–149. doi:10.1016/j.vascn.2006.03.010.
- [287] S.P. Brummer, E.M. Gese, J.A. Shivik, The effect of enclosure type on the behavior and heart rate of captive coyotes, *Appl. Anim. Behav. Sci.* 125 (2010) 171–180. doi:10.1016/j.applanim.2010.04.012.
- [288] M.D. Shiverdecker, P.A. Schiml, M.B. Hennessy, Human interaction moderates plasma cortisol and behavioral responses of dogs to shelter housing, *Physiol. Behav.* 109 (2013) 75–79. doi:10.1016/j.physbeh.2012.12.002.