

University of Trás-os-Montes e Alto Douro

**Assessment of Adaptil® collar to help handle stress and anxiety in  
puppies during veterinary consultations**

Master Thesis in Veterinary Medicine

Maria Inês Henriques Pinto

Supervisor: Artur Severo Proença Varejão, DMV, PhD

Co-Supervisor: Gonçalo da Graça Pereira, DVM, MsC, PhD, Dip ECAWBM (BM), Dip  
ECAWBM (AWSEL)



Vila Real, 2018



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## Abstract

Puppies' first consultations are extremely important, as they can influence how puppies will react in future veterinary consultations. The veterinarian surgeon plays an essential role in alerting and informing owners, preventing future behavioural problems, explaining how to deal with them and how to prevent consultations from becoming a traumatic experience.

The present study had the objective to evaluate the efficacy of an Adaptil® collar versus a placebo collar in reducing stress and anxiety, during veterinary consultations in puppies. To carry out, a double-blind study was performed in 37 initial puppies, however only 30 met the minimum requirements to be evaluated statistically. As a primary requirement the puppy should be between 2 and 7 months old, have good health condition and have never been previously vaccinated. This way, puppies have no positive or negative association with the veterinary clinic. Depending on their age and their living area if at risk, puppies were vaccinated according to two models: younger puppies would have three vaccinations injections, including, two vaccination boosts. Older puppies would have only two visits, just one boost. At each visit, the observer assessed the anxiety and stress levels according to the "Scale to evaluate stress" and performed a small questionnaire with the owners regarding the puppy' behaviour, 15 minutes after the beginning of consultation. The scale included 10 different parameters, each being assessed on a scale from zero to four. During vaccination boosts, owners were asked if the puppy had shown less / more stress behaviours compared to previous vaccination visits or if there had been no differences.

There were no statistically significant differences between the use of Adaptil® and placebo in reducing stress and anxiety between consultations (Visit 2 / Visit 1,  $p = 0.46551$ ; Visit 3 / Visit 2,  $p = 0.2299$ , Visit 3 / Visit 1,  $p = 0.3818$ ; Wilcoxon's test). However, total puppy scores were low in both groups even at baseline visit, reflecting that puppies were not stressed during consultations. Absolute variation of the total puppy score was equal or inferior to 2 points in the "Scale to evaluate stress", but there were not statistically significant differences between the two groups, regarding all visits. Statistical analysis, revealed that most owners did not observed differences in the behaviour of their puppy, either in the Adaptil® group or placebo one, at visit 2 and at visit 3. Although this study failed to prove the efficacy of using Adaptil® at veterinary consultations in puppies, it served to alert owners about potential behavioural problems and how pheromones can support their resolution and prevention.

Keywords: puppy, pheromones, Adaptil®, stress, anxiety, veterinary consultation





## Resumo

As primeiras consultas de um cão jovem têm extrema importância, pois podem condicionar o modo como o cachorro reagirá futuramente nas consultas médico-veterinárias. O médico veterinário tem um papel essencial, no sentido de alertar e informar os tutores para a prevenção de futuros problemas comportamentais, como lidar com estes e como evitar que as consultas se tornem numa experiência traumatizante para o seu cachorro.

O presente estudo teve como principal objetivo avaliar a eficácia de uma coleira Adaptil® *versus* uma coleira placebo em reduzir o stresse e ansiedade, nas consultas médico-veterinárias em cachorros. Nesse sentido foi realizado um estudo duplamente cego, em 37 cachorros iniciais, sendo que apenas 30 preencheram os requisitos mínimos para serem avaliados estatisticamente. Como requisito principal o cão jovem deveria ter entre 2 e 7 meses, ser saudável e nunca ter sido vacinado anteriormente. Deste modo, o cachorro não possui nenhuma associação positiva ou negativa com a consulta médico veterinária. Os cães jovens, dependendo da sua idade e residência em área de risco, eram vacinados segundo dois protocolos: cachorros jovens teriam três consultas de vacinação, ou seja, dois reforços vacinais. Cachorros mais velhos teriam duas visitas, apenas um reforço vacinal. Em todas as consultas, o observador preenchia a tabela “*Scale to evaluate stress*” relativa aos níveis de stresse e ansiedade e realizava um curto questionário aos tutores relativamente ao comportamento do cachorro, 15 minutos após o início da consulta. A escala contempla 10 parâmetros diferentes, divididos numa escala de zero a quatro. Nos reforços vacinais, era questionado aos tutores se o cachorro se tinha mostrado menos/mais stressado comparando com consultas anteriores ou se não tinham ocorrido diferenças.

Não houve diferenças estatisticamente significativas entre o uso de Adaptil® e placebo em reduzir o stresse e ansiedade entre consultas (Visita 2/Visita 1,  $p=0,46551$ ; Visita 3/Visita 2,  $p=0,2299$ , Visita 3/Visita 1,  $p=0,3818$ ; teste de Wilcoxon). No entanto, as pontuações totais dos cachorros eram baixas em ambos os grupos, mesmo na primeira consulta, sugerindo que os cachorros não se encontravam stressados durante as consultas. A variação absoluta da pontuação total de cada cachorro foi igual ou inferior a dois pontos na “*Scale to evaluate stress*” e não houve diferenças estatisticamente significativas entre os dois grupos, em relação a todas as consultas. Após análise estatística, revelou-se que a maioria dos tutores não observou diferenças no comportamento do seu cachorro, tanto em cachorros do grupo Adaptil® como do grupo de placebo, na visita 2 e na visita 3.

Apesar deste estudo não ter conseguido comprovar a eficácia do uso de Adaptil® nas consultas médico-veterinárias em cachorros, serviu para alertar os tutores sobre a temática dos problemas comportamentais e como as feromonas podem auxiliar na resolução e prevenção dos mesmos.

Palavras-chave: cachorro, feromonas, Adaptil®, stresse, ansiedade, consulta médico-veterinária

## Table of contents

Acknowledgements .....	I
Abstract .....	III
Resumo .....	IV
Table of contents .....	VI
Index of tables .....	VIII
Index of charts .....	IX
Index of figures .....	X
Index of abbreviations and symbols .....	XI
Introduction .....	1
Chapter I – Literature review .....	3
1 - Origins and domestication .....	3
2 - Social organization and relationships .....	6
3 - Communication .....	10
3.1 - Auditory communication .....	10
3.2 - Visual communication .....	12
3.3 - Tactile communication .....	14
3.4 - Olfactory communication .....	14
4 - Development periods .....	16
4.1 - Neonatal period .....	17
4.2 - Transition period .....	18
4.3 - Socialization period .....	19
4.4 - Juvenile period/ adulthood .....	21
5 - Behavioural problems in puppies .....	22
5.1 - Elimination problems .....	23
5.2 - Separation-anxiety .....	24
5.3 - Destructive behaviour .....	25
5.4 - Fear-aggression .....	26
6 - Pharmacologic treatment .....	29
7 - How to decrease stress and anxiety in puppies .....	31
7.1 – Socialization .....	33
7.2 - At home .....	34
7.3 - Veterinary clinic .....	35
7.4 - Puppy classes and puppy parties .....	37

8 - Pheromonotherapy in dogs .....	40
8.1 - Dog-appeasing Pheromone.....	47
8.2 - Applications of Dog-appeasing Pheromone.....	48
Chapter II - Assessment of Adaptil® collar to help handle stress and anxiety in puppies during veterinary consultations .....	51
1 - Objectives.....	51
2 - Material and Methods .....	51
3 - Results .....	61
3.1 - Analysis of the population.....	61
3.2 - Analysis of the demographic data.....	62
3.3 - Waiting time .....	67
3.4 - Additional medical procedures.....	69
3.5 - Efficacy: primary outcome .....	71
3.6 - Efficacy: secondary outcome - relative variation between visit 1 and visit 3, visit 2 and visit 3.....	72
3.7 - Efficacy: secondary outcome - total puppy score.....	75
3.8 - Secondary outcome: sub-scores .....	76
3.9 - Secondary outcome: owner assessment at visit 2 .....	78
3.10 - Secondary outcome: owner assessment at visit 3 .....	79
4 - Discussion .....	80
5 - Conclusion.....	84
6 – Bibliography .....	85

## Index of tables

Table 1- Collars were given according to a randomization list .....	58
Table 2 - Scale to evaluate stress at the veterinary clinic .....	59
Table 3 – Allocation of subjects between Adaptil® and Placebo.....	61
Table 4 – Allocation of subjects in the FAS population .....	61
Table 5 – Allocation of subjects in the PP population.....	62
Table 6 – Puppies gender by collar in the FAS population.....	62
Table 7 – Comparison of age in months between puppies tested with Adaptil® and Placebo .....	63
Table 8 – Puppies breed by collar in the FAS population.....	64
Table 9 - Puppies living place by collar in the FAS population .....	65
Table 10 - Owners having other pets in the FAS population .....	65
Table 11 – Other pets according to owners the FAS population .....	66
Table 12 – Puppy being the owners' first puppy in the FAS population.....	66
Table 13 – Place where puppy was obtained in the FAS population.....	67
Table 14 - Comparison of waiting time in minutes between Adaptil® and Placebo group .....	68
Table 15 – Medical procedures for visit 1 for FAS population .....	69
Table 16 – Other medical procedures for visit 1 for FAS population .....	69
Table 17 - Medical procedures for visit 2 for FAS population .....	70
Table 18 - Other medical procedures for visit 2 for FAS population .....	70
Table 19 - Medical procedures for visit 3 for FAS population .....	70
Table 20 - Other medical procedures for visit 3 for FAS population .....	70
Table 21 - Comparison of the relative variation of total score between the first visit and second visit for Adaptil® and Placebo group .....	72
Table 22 – Comparison of the relative variation of total score between the second visit and third visit for Adaptil® and Placebo group .....	73
Table 23 - Comparison of the relative variation of total score between the first visit and third visit for Adaptil® and Placebo group.....	73
Table 24 - Absolute variation of the total puppy score “under or equal 2 points” and “scores above 2 points” between visits of puppies tested with Adaptil® and Placebo.....	74
Table 25 - Comparison between Adaptil® group and Placebo group for mean total puppy score .....	75
Table 26 – Owner assessment at visit 2 .....	78
Table 27 – Owner assessment at visit 3 .....	79



## Index of charts

Chart 1 – Puppies gender by collar in the FAS population .....	62
Chart 2 – Comparison of age in months between puppies tested with Adaptil® and Placebo .....	63
Chart 3 - Puppies breed by collar in the FAS population.....	64
Chart 4 – Puppies living place by collar in the FAS population .....	65
Chart 5 - Owners having other pets in the FAS population .....	65
Chart 6 - Puppy being the owners' first puppy in the FAS population.....	66
Chart 7 – Place where puppy was obtained in the FAS population .....	67
Chart 8 - Comparison of waiting time in minutes between Adaptil® and Placebo group .....	68
Chart 9 - Comparison of the relative variation of total score between the first visit and second visit for Adaptil® and Placebo group.....	72
Chart 10 - Comparison of the relative variation of total score between the first visit and third visit, second visit and third visit, for Adaptil® and Placebo group.....	74
Chart 11 – Comparison between Adaptil® group and Placebo group for mean total puppy score .....	76
Chart 12 – Sub-scores at visit 1 for Adaptil® group and Placebo group.....	77
Chart 13 – Sub-scores at visit 2 for Adaptil® group and Placebo group.....	77
Chart 14 – Sub-scores at visit 3 for Adaptil® group and Placebo group.....	78
Chart 15 – Owner assessment by collar at visit 2 .....	79
Chart 16 – Owner assessment by collar at visit 3 .....	79





## Index of figures

Figure 1 – Wolves started to live peacefully within the outskirts of villages, becoming naturally tamed over time. ....	4
Figure 2 - Comparing wolves and dogs .....	5
Figure 3 – Body postures in dogs .....	13
Figure 4 – Development periods for dogs. ....	16
Figure 5 – Representation of attraction and fear in puppies during the “sensitive period”. ....	20
Figure 6 – Canine ladder of aggression. ....	28
Figure 7 - Socialization program for a puppy. ....	34
Figure 8 – During consultations, puppies should be rewarded with toys or treats. ....	36
Figure 9 – Vomeronasal location and structure in dogs. ....	43
Figure 10 - VNO pathway bypasses higher cognitive structures, resulting in innate and stereotyped behavioural and neuroendocrine responses. ....	44
Figure 11 - Primary pheromone-secreting glands in dogs are labial, auricular, perianal, genital, interdigital (pedal) and mammary complex .....	45
Figure 12 – Systematic protocol for puppies’ vaccination followed up during the study.....	52
Figure 13 – Form for the first visit (core vaccination) .....	54
Figure 14 – Form for the second and third visit (boost vaccination) .....	55
Figure 15 – Puppy wearing a collar during the veterinary consultation. ....	57



## Index of abbreviations and symbols

% - percentage

°C – Degrees Celsius

AOB – Accessory olfactory bulb

CNS – Central nervous system

DAP – Dog-appeasing pheromone

FAS – Full analysis set

GABA – Gamma-aminobutyric acid

MAB – Main olfactory bulb

OE – Olfactory epithelium

PP – Per protocol

VNO – Vomeronasal organ



## Introduction

The present Master Thesis is divided into two parts. The first part will introduce some basic concepts and understanding about dogs' behaviour and how they communicate. It also covers the different development periods a dog goes through and methods to deal with stress and anxiety, such as puppy parties for adequate socialization, correct management at the veterinary clinic and at home. In addition to this, it also covers some essential aspects regarding dogs' pheromones. The second part consists in a study conducted to determinate if Adaptil® collar reduces stress and anxiety in puppies during the veterinary consultation.

Humans and dogs evolved together through the years. The process of domestication changed the dog's ancestral, the wolf, into the dog we know nowadays (Scott, 1958). Dogs are present all around the world and inserted in many cultures, most of them being companion animals (Miklósi, 2009). Dogs evolved their social skills into a better understanding of our way of living, along with innovative ways of inter-species communication. It is a veterinarian's role to educate owners on how to better understand their dogs' behaviour, because inconsistent communication may cause misunderstandings and restrains in the human-dog bond (Horwitz, Mills, & Heath, 2005). Lack of effective communication and welfare knowledge, between owner and dog, can evolve to a behavioural disorder, such as elimination at an inappropriate place and at last instance relinquishment and euthanasia (Beaver, 2009).

Fear and anxiety-related behaviours are a common problem in the veterinary clinic (Döring, Roscher, Scheipl, Küchenhoff, & Erhard, 2009; Landsberg, Hunthausen, & Ackerman, 2013). It is vital to carefully address this problem, because it affects the animal's welfare, as well as quality of treatment and consultation. Handling problematic dogs is not only harmful to the own animal, but also to his owner and veterinary staff. Prevention should begin as early as the first consultation and owners should be advised and informed properly (Moffat, 2008). Unpleasant previous experiences will condition future reactions. Along with a thoughtful management of stress at the veterinary clinic, prevention should start at home with appropriate training. If possible, puppies should attend puppy classes or parties, to maximize their socialization. This allows the puppy to grow into being a social and well-balanced dog (Case, 2010; Seksel, Mazurski, & Taylor, 1999). Adopting a puppy is an exciting moment for the whole family, however, moving into a new environment and being separate from littermates can be stressful and traumatic (Case, 2010). Stress affects not only physical health, but also puppy's emotional state (Mills, Karagiannis, & Zulch, 2014). Owning a puppy should be a thought and informed decision (Hargrave, 2012). Puppies aging in different periods of development react differently to novelty and distress situations (Lindsay, 2000a). During the socialization period,

going from 3 to 12 weeks old, puppies should be stimulated with different objects and get used to unknown people and places (Yin, 2009).

Pheromones are natural chemicals released by different animal species and play a major role in the olfactory world (Horwitz, Ciribassi, & Dale, 2014). Adaptil® is a synthetic analogue of the dog-appeasing pheromone (DAP). This compound is secreted by the females' dog sebaceous glands located in the intermammary sulcus, 3 to 4 days after giving birth and remains until 2 to 5 days after weaning (Pageat & Gaultier, 2003). The commercial product is a synthetic mixture of fatty acids with an appeasing effect that help dogs to cope with mild-stress factors, available as a spray, impregnated collar and plug-in diffuser (Mills, Dube, & Zulch, 2012; Pageat & Gaultier, 2003). Adaptil® is a complement for behavioural problems, its use is recommended to prevent, control and manage stress and fear-related disorders (Landsberg, Hunthausen, & Ackerman, 2013). The efficacy of dog-appeasing pheromone has been described in multiple studies and diverse contexts such as anxiety behaviours' (Gaultier, Bonnafous, Bougrat, Lafont, & Pageat, 2005), distress related behaviours in the veterinary clinic (Mills, Ramos, Estelles, & Hargrave, 2006), animals' hospitalization (Kim et al., 2010; Siracusa et al., 2010), sound aversion (Landsberg et al., 2015; Levine et al., 2007), car travelling (Gandia Estellés & Mills, 2006) and kennel/shelter facilities (Grigg & Piehler, 2015; Tod, Brander, & Waran, 2005). Furthermore, other studies were made in puppies, to evaluate the consequences of dog appeasing pheromone in puppy classes and socialization/training (Denenberg & Landsberg, 2008; Graham, Mills, & Bailey, 2007), disturbance at night and house soiling (Taylor & Mills, 2007) and how newly-adopted puppies deal with unfamiliar people and surroundings (Gaultier et al., 2009).

Research in the field of pheromotherapy is increasing as it offers promising results, without causing any secondary effects and ease of treatment. It helps to create a sense of a safer environment, helping animals to cope better with adversity (Swaney & Keverne, 2009). As behaviour problems are one of the main reasons for abandonment, the use of pheromones can become a revolutionary method to prevent this reality.

## Chapter I – Literature review

### 1 - Origins and domestication

Since ancient times, dogs have been around humans, building relationships and evolving together. We have to go way back to their ancestral, the wolf (*Canis lupus*), to better understand how domestication occurred and how we came to know the current dog, known as our best friend. It is believed that the first dogs were originated in Eastern Asia around 12.000 years ago (Case, 2010) as proven by geneticists and archaeological findings (Morey, 1994). Although both wolf (*Canis lupus*) and dog (*Canis familiaris*) are phylogenetic similar, sharing the same family, Canidae, being carnivore, with small, sharp incisors at the front of the mouth for hunting and defence, in reality they are very different (Appleby, 2017; Case, 2010; Luescher & Reisner, 2008; Miklósi, 2009). Due to domestication, dogs are no longer exclusively carnivore and became smaller, with distinctive fur coloration and less alert towards the environment (Beaver, 2009; Case, 2010). Extrapolation from wolves' behaviour to our dogs is inaccurate (Luescher & Reisner, 2008).

Domestication was not a deliberated process and there is a lack of consensus regarding the theory of how it really occurred (Horwitz et al., 2005; Landsberg et al., 2013). It is thought that domestication may have begun when humans left the nomad life and started living in permanent villages. The basis of the first theory was the assumption that hunters co-existed with wild wolves and competed for the same preys. Assuming this theory, hunters acknowledged the excellent wolves' hunting skills and then captured, raised and tamed wolf cubs. When this process was complete, they were used as an auxiliary way for hunting (Appleby, 2017; Beaver, 2009; Case, 2010). Although this theory had gained followers, recently was put aside. The new theory defends that, domestication was an accidental process and resulted from a natural selection in a new ecological niche, the village dump. Outskirts of the villages provided food and were relatively safe from other predators and threats. Selective pressure favoured less timid wolves, thus with higher tolerance towards human proximity - less fear, meant more food and shelter. As time passed by, new wolf cubs were raised among the villagers, becoming 'naturally' tame (Figure 1) (Appleby, 2017; Beaver, 2009; Case, 2010). The wolves who cooperated with humans and showed high levels of inhibited behaviour were the ones who survived, while others that showed aggressive behaviours were killed (Appleby, 2017). Over the years, it has been compiled different behavioural elements to characterise wolves in an ethogram (Miklósi, 2009).



Figure 1 – Wolves started to live peacefully within the outskirts of villages, becoming naturally tamed over time.

(Adapted from <http://www.newtonsapple.org.uk/evolution-from-wolf-to-domestic-dog/>)

Evolution is a very time-consuming process and it is mediated by natural selection. However, when talking about dogs, not only the natural selection took part, but also human pressure (Appleby, 2017). Persistency of physiological and behavioural juvenile traits in adult dogs, gives place to animals who can co-exist with humans and become more dependent on them (Case, 2010; Horwitz et al., 2005). The key for dogs' high popularity may reside in the fact that they build strong attachments with their owners. *Paedomorphism* is a process that involves changes in the rate of development in young animals. In other words, behaviours only presented by young individuals are preserved until adulthood. A subtype of this process, is *neoteny*, where there is a reduction in the development rate resulting in the persistence of juvenile traits (Case, 2010; Luescher & Reisner, 2008; Morey, 1994) (Figure 2).



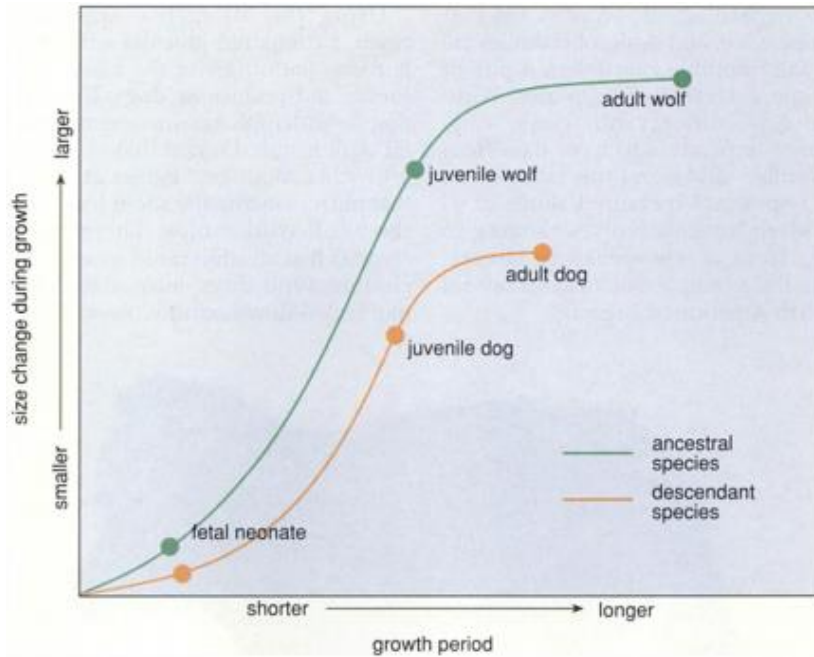


Figure 2 - Comparing wolves and dogs, the last ones are smaller and preserved juvenile features from their ancestral. Absence of fear of humans, higher adaptation ability, acceptance of handling and control, higher sociability with humans and other species, submissive food begging, care-soliciting behaviours, playfulness, different timing of activity periods, lessened territoriality and increasing social contacts were some features that were preferable and kept during reproductive selection.

(Adapted from Morey, 1994)

There is a lot of speculation on how we have such different breeds. Most people believe that *artic breeds* were the first ones, because they are similar to wolves. Others believe that *village dogs* came first, for example, working dogs. Dogs' first roles were guarding livestock and accompany during hunts (Beaver, 2009). Geographic location could have influenced subspecies of wolves, in other words, besides domestication, geographic pressure also had a significant role in creating the wide variety of dogs we know currently (Morey, 1994). Humans expanded their horizons and started to teach more complex functions. Nowadays, we have police dogs, dogs for blinds and even dog assisted therapy (Beaver, 2009). Previous working dogs are now used as companion animals. Working breeds are easily trained, show more interest in engaging in a play and have less fear. On the other hand, fearful dogs tend to be more aggressive. It was found a connection between the relationship with owners, training and playfulness (Eken Asp, Fikse, Nilsson, & Strandberg, 2015).



## 2 - Social organization and relationships

In this chapter, it will be considered as social behaviour only the interactions amongst members of the same species. By considering the process of domestication, we can suppose some essential aspects of how dogs behave socially and how they establish relationships with conspecifics and other species. Dogs constitute social groups based on previous experiences and individual motivation (Landsberg et al., 2013). A relationship between two dogs is not based on a hierarchical rank, but on complex interactions, such as learning and resource-holding potential (Appleby, 2017; Landsberg et al., 2013). Genetic, socialization, age, availability of resources and problem-solving skills also have a significant role (Landsberg et al., 2013).

There are different theories, but the main ones consider two sources of information: wild packs and captive packs. The first studies had captive packs as subjects. These subjects often showed higher aggressive levels than in comparison to wild wolves. Recent studies stated that wolves live within a familiar structure, having two main progenitors (alpha male and alpha female) and their cubs for successive breeding seasons. The model parent-family is the one which better describes wolves' behaviour rather than a social hierarchy, more common in captive packs. In wild wolves, aggression is rare, because when they come across a conflict they tend to walk away or appease the other individual. Wolves are group-living social hunters, who cooperate to hunt large preys (Appleby, 2017; Landsberg et al., 2013).

In the wild, wolves' packs consist in small groups of related individuals, who stick together to hunt, reproduce and protect a common territory. Social organization is maintained by using ritualized behaviours that indicate the individuals' intention. Ritualized signs, from dominant or submissive body postures to appeasement improve the groups' relationships and decreases aggressive interactions (Case, 2010). Wolves can form other social groups considering the resources available and territory. This said, they can form a large group or smaller ones only with two progenitors (Bowen & Heath, 2005; Case, 2010). Individuals only stay in the pack when it is advantageous for the main group; when there are fewer resources, the group disperses reuniting only to hunt larger preys (Bowen & Heath, 2005).

Only recently researchers started thinking about dogs in terms of population. We have working, family or free-ranging dogs, all of them form small sub-populations. In addition to affective bonds with group members, they have also the ability to develop new relationships quickly and be socially tolerant (Miklósi, 2009). Free-ranging dogs are solitary or establish groups with one or two members. Larger group formation is occasional and only occur to explore certain resources. These dogs do not show signs of territoriality and agonistic

behaviour is rare (Case, 2010). Feral dogs are a small percentage of the free-ranging population. Feral dogs generally are solitary scavengers, but participate in groups only for brief periods of time. They can be tamed individually, but when in group, they can become very aggressive when forced to interact with humans (Beaver, 2009). Some authors believed these groups have a system of metacommunication, where each member has its role and knows his place (Fox, Beck, & Blackman, 1975). Stray dogs are unowned dogs, that have plasticity regarding pack behaviour. These groups are smaller than the previous ones. Frequently, they are solitary, but can form small groups. To sum up, dogs can adapt to different environments, different resources and establish social relations based on them (Case, 2010).

Nowadays, dogs can live peacefully with humans and even form affection to other species. Bearing in mind social relationships of dogs, we can consider two social plans: with their owners and with other animals (Case, 2010). As dogs and cats are popular companion animals, it is usual that they share the same family and household. Alike dogs, cats also suffered major transformations due to domestication. Though there are many studies concerning human-dog bond, literature has a lack of information about cat-dog bond. Sometimes communication can be tough as it is based on different patterns. Nevertheless, a study by Feuerstein and Terkel (2008) proved that cats and dogs can share a mutual amicability, including play, nose-to-nose greeting and understanding of each other body postures. Research has found that, this type of bond depends on previous experiences specially in an early age (Feuerstein & Terkel, 2008).

In most houses, ranking relationships are less insightful and occur only in specific contexts. For example, a dog may try to protect his food or his favourite toy by making a statement before the other dog. Despite of this, in multiple-dog houses, dogs tend to show less or none competition over resources (Case, 2010). According to Case (2010) it is inappropriate to designate a dog dominant or submissive. Relationships between conspecifics are similar to a social group trying to live in harmony rather than a hierarchy pack. Some breeds suffered more modifications with domestication than others, hence some may tend to form a more fixed social organization while others do not. For example, beagles were selected to work cooperatively and for that reason, they feel less need of a social hierarchy (Case, 2010). On the other hand, working breeds, developed for protecting and guarding livestock, show a natural interest in a social ranking and are less tolerable towards offensive threats and aggression (Case, 2010). However, every individual is unique and a breed does not define the way he inserts himself in a social context (Case, 2010).

In the last years, the world faced new theories regarding the relationship between human and dog. And the biggest change regarding past theories is the disbelief of the

dominance myth and aversive methods of training (using choke chains, pinch collars and electronic collars) which accompanied that theory (Appleby, 2017; Yin, 2008). Some trainers use the *alpha-roll* technique movement so that the owner becomes the dominant element, becoming the alpha. In fact, these professionals are following the past theory models and when using this technique, they are creating a direct confront with the animal (Landsberg et al., 2013). This conduct is inappropriate, as it may result in fear that commonly leads to a much more aggressive dog. The dog will also learn not to trust the owner, as it does not give him a safety image (Beaver, 2009).

Each specie has its particular way of communication and signals emitted by dogs are “universal”, this means, they use the same pattern for humans, other dogs or individuals from another specie (Landsberg et al., 2013). Along the evolution and shaping of different breeds, dogs have improved some signals, for example, barking. They use signals, given by humans unconsciously, to better adapt - dogs use visual cues and tend to follow the direction of the human vision (Landsberg et al., 2013). As long as humans can comprehend different canine behaviours, they can also comprehend their intentions and mood. A high capacity to adapt is an important characteristic in dogs, especially when they are recently adopted. In addition to this, they learn how to interpret body posture of their owners. Lack of mutual understanding can lead to failures during training and use of punishment excessively. This can create conflict, anxiety and misgivings in the dog. It is important to remember that, dogs live essentially in a world of sounds and odours and their behaviour can be a response to something that humans do not perceive (Landsberg et al., 2013). Dogs quickly learn by making associations between the owners’ response and use that information in future encounters. This presents a problem when talking about children, as they do not understand how dogs behave and can give misgiving answers (Landsberg et al., 2013). When a puppy learns that his actions, such as nipping and biting, are a successful way to get attention or play, he can also learn how to use aggression to get his objectives. This situation is worsen when the owner punishes the dog (Landsberg et al., 2013).

Most people own a dog for companionship. The relationship’s nature between dog and owner affects both in various ways, such as their welfare, so it is crucial to invest more time improving this bond (Meyer & Forkman, 2014). Dogs contribute to the emotional stability of the family and have a positive educational effect on children (Miklósi, 2009). In literature, attachment between companion animals and humans is assessed on the basis of questionnaires, having, that way, a subjective component (Meyer & Forkman, 2014; Miklósi, 2009). Dogs demonstrate specific reactions towards their owners (but not towards strangers), looking for them when they are absence and making contact when they return (Miklósi, 2009).

In 2005, Topál *et al.* used the Strange Situation Test (developed initially to evaluate the relationship mother-infant in humans) to compare attachment levels between two groups: hand-reared puppies and hand-reared cubs (Topál *et al.*, 2005). They wanted to test if 16 weeks puppies showed attachment towards their human caregiver, if intense socialization with humans affected attachment and if evolutionary changes affected the appearance of this type of relationships. In conclusion, puppies showed attachment to their owners, but not to strangers. On the other hand, the results from the cubs did not have the same outcome, wolves do not show attachment behaviours (for example seeking attention and contact). Intensive socialization did not influence attachment levels in both groups. Authors suggest that evolution in dogs lead them to develop certain abilities, such as the capability to form strong bonds with humans (Topál *et al.*, 2005).

Picking the right puppy for each family can be a challenge, because normally, acquisition is based on an impulse and physical attractiveness (Case, 2010; Hargrave, 2012). Behaviour and personality are only considered later, particularly if the puppy does not match the owners' expectation. A shy puppy can feel overwhelmed and oppressed with a very "dominant" owner (Case, 2010). Owners should cover some checkpoints such as age, gender and breed of the animal before adoption. Most abandoned dogs have less than 2 years old, are mixed breed and are obtained at zero or low cost (Case, 2010). At this point, is necessary to point out some guidelines to lead owners to the right choice and this way decrease relinquishment and euthanasia numbers (Beaver, 2009; Marder & Duxbury, 2008). Most behavioural tests are made with puppies aging around 5-7 weeks old (Beaver, 2009). However, some authors defended that behavioural puppy tests should be performed at the end of the socialization period (Beaudet, Chalifoux, & Dallaire, 1994).

Wilsson and Sundgren (1998) conducted a study to discover if it is possible to predict adult dogs' behaviour by assessing 8 weeks old puppies. An association between the tests and the behaviour of adult dogs was insignificant and, for this reason, this type of tests was considered unavailing. Heritability was significant in some behaviour traits and maternal effects were stronger in juvenile individuals than in adults (Wilsson & Sundgren, 1998). Most tests are aimed for working dogs, such as Labrador Retriever and German Shepard Dog (Jones & Gosling, 2005). Nevertheless, temperament tests have lack of scientific basis (Marder & Duxbury, 2008; Taylor & Mills, 2006). Variability regarding the tests, investigation methods, each study objective and evaluated attributes are the main reason for that (Jones & Gosling, 2005).

### 3 - Communication

Effective communication is indispensable for inter-species and intra-species understanding (Case, 2010; Horwitz et al., 2005) and implies at least two individuals (Landsberg et al., 2013). Social communication is a process by which an individual's behaviour affects the behaviour of others. It is necessary to differentiate between observed behaviours and their communicative function. Behaviours that show a "guilty look" or "jealousy" can be misinterpreted by us and have another meaning for our animals (Horwitz et al., 2005). In case of doubt about the motivation of a certain behaviour, it is best to distance oneself and do nothing (Horwitz et al., 2005). Dogs' behaviour is complex. Common myths such as "barking dogs don't bite" and a "wagging tail means a friendly dog" are not always truthful (Landsberg et al., 2013).

Every behaviour can be divided in a series of sequences: initiation phase (starting an action), pause (giving time to answer) and taking action (responding to other individual's response). Additionally, it may include an appeasing and a refractory phase. Some phases may be absent, for example, when a dog bites immediately, giving no prior signs (Landsberg et al., 2013).

Occasionally, dogs can perform contradictory behaviours or behaviours out of context known as conflict behaviours. This type of behaviours results from stress, frustration and motivational conflict. A dog is in a motivational conflict when a single experience offers two distinct motivations, such as approach and withdrawal. It includes yawning, lip-licking, looking away, visually scanning the surroundings, squinting the eyes, licking objects, self-scratching and vocalization. As this disturbance is based on stress, punishment should be avoided as it will only increase it (Luescher & Reisner, 2008).

#### 3.1 - Auditory communication

Repertoire of vocal signals is much larger in dogs than in wolves and it evolved for inter-species and intra-specie communication (Landsberg et al., 2013). Auditory communication is particularly important when visual communication is not possible, either because it is dark or the intermediates are far from each other (Bowen & Heath, 2005).

Humans have the ability to communicate via audition with dogs and to notice differences in their bark. Despite this fact, there can happen interpretation's mistakes, for example when a dog may be trying to invite to a play, but his owners interpret it as an aggression signal. Barking is a skill typical of dogs, that rely on it for many reasons, including

seeking attention or excitement play (Bowen & Heath, 2005; Landsberg et al., 2013). During domestication, barking dogs were favourable, since they warned about potential threats. On the other hand, it can be a *neotenized* behaviour, represented by vocalizations of the juvenile wolves (Case, 2010). Some bark characteristics, like the time interval between sounds, have the potential to affect the way humans perceive dogs (Seong Chan Yeon, 2007). The nature of the bark varies according to the social context and it can be divided into different subtypes (Horwitz et al., 2005; Yin & McCowan, 2004). Dogs can identify different situations and adapt accordingly. Although there are many types of bark, not all have a particular function (Yin & McCowan, 2004).

Dog owners claim that, they can recognize their dog based on his barking. Usually, barking is manifested in chaotic situations and it is difficult to assess it correctly. Molnár et al. (2006) tested if the owners' claims were true (Molnár, Pongrácz, Dóka, & Miklósi, 2006). All in all, most owners did not succeed. The reason for this may be explained by the noisy context (Molnár et al, 2006). Low-pitched barks frequently are heard in aggressive situations, whereas high-pitched are categorized as non-aggressive, representing fear and despair behaviours. A study tested what emotions humans felt by listening to several recorded dog sounds (Pongrácz, Molnár, & Miklósi, 2006). They had to divide the sounds into 5 categories: aggressiveness, fear, despair, playfulness e happiness. Although different humans had different experiences with dogs, the results were similar. Overall, different barks function as an efficient communication system, at least in the relationship between humans and dogs. The pitch, frequency and tonality variation can transmit different emotions. The successful use of vocal signals between man-dog communication can lead to positive evolution and acoustic diversification of this type of communication (Pongrácz et al., 2006).

Other auditory signals are:

- Howling: it can be seen as an eliciting sound. In wolves, it is not certain its real function, but it is believed that it is to maintain the group cohesive. Considering dogs, it may have a similar role. Moreover, some dogs respond to high-frequency sounds this way (Case, 2010; Landsberg et al., 2013; Overall, 2013);
- Whining/whimpering: frequently observed in puppies. It is related to care-seeking and distress (Case, 2010; Landsberg et al., 2013; Overall, 2013; Seong Chan Yeon, 2007);
- Growling: occurs during a play or aggression (Case, 2010; Landsberg et al., 2013; Overall, 2013).



### 3.2 - Visual communication

Visual communication has a significant role when dogs are in the presence of each other and it is crucial to alert others about social and emotional status and aggression control (Landsberg et al., 2013). When talking about visual communication we cannot separate signals given by different areas of the body. We must analyse the context and comprehend the body posture, tail and ear position, gaze and size pupils, lips, piloerection, movement and general attitude as a whole (Figure 3) (Bowen & Heath, 2005; Case, 2010; Landsberg et al., 2013). Different breeds result in different physical conformations which can lead to miscommunication. For example, some dogs have floppy ears, so their ear movement is less perceptible, whereas others lack a tail (Appleby, 2017; Bowen & Heath, 2005).

There are scientific evidences that dogs rely on human visual communicative signals and that humans also understand visual signs emitted by dogs (Miklósi, 2009). Dogs are capable of detecting micro-expressions in humans (Case, 2010). They behave differently according to owners' attention. Hence, when dogs are deprived from visual human cues (for an instance a blind folded human), usually they become hesitant (Miklósi, 2009; Schwab & Huber, 2006). Furthermore, after the command "lay down", dogs tend to stay longer in that position when the owner is around (Schwab & Huber, 2006). Dogs can follow human cues, such as gazing or pointing to find hidden food (Elgier, Jakovcevic, Mustaca, & Bentosela, 2009). We can hide food in opaque recipients and point where the food is that dogs will find them (Hare & Tomasello, 2005). This ability is a domestication consequence, because wolves do not possess it (Elgier et al., 2009).

Eye contact is one of the most important ways for visual communication. When two friendly dogs meet, visual contact is initiated. The more confident dog is the one who starts the contact and holds it for longer periods of time. It can also be used as a form of confrontation, if the opponent does not look away and show appeasement behaviour, the threat can escalate to aggression (Case, 2010). This situation can also occur between a human and a dog. In multiple-dog houses, friendly dogs can share short periods of visual contact (Case, 2010).

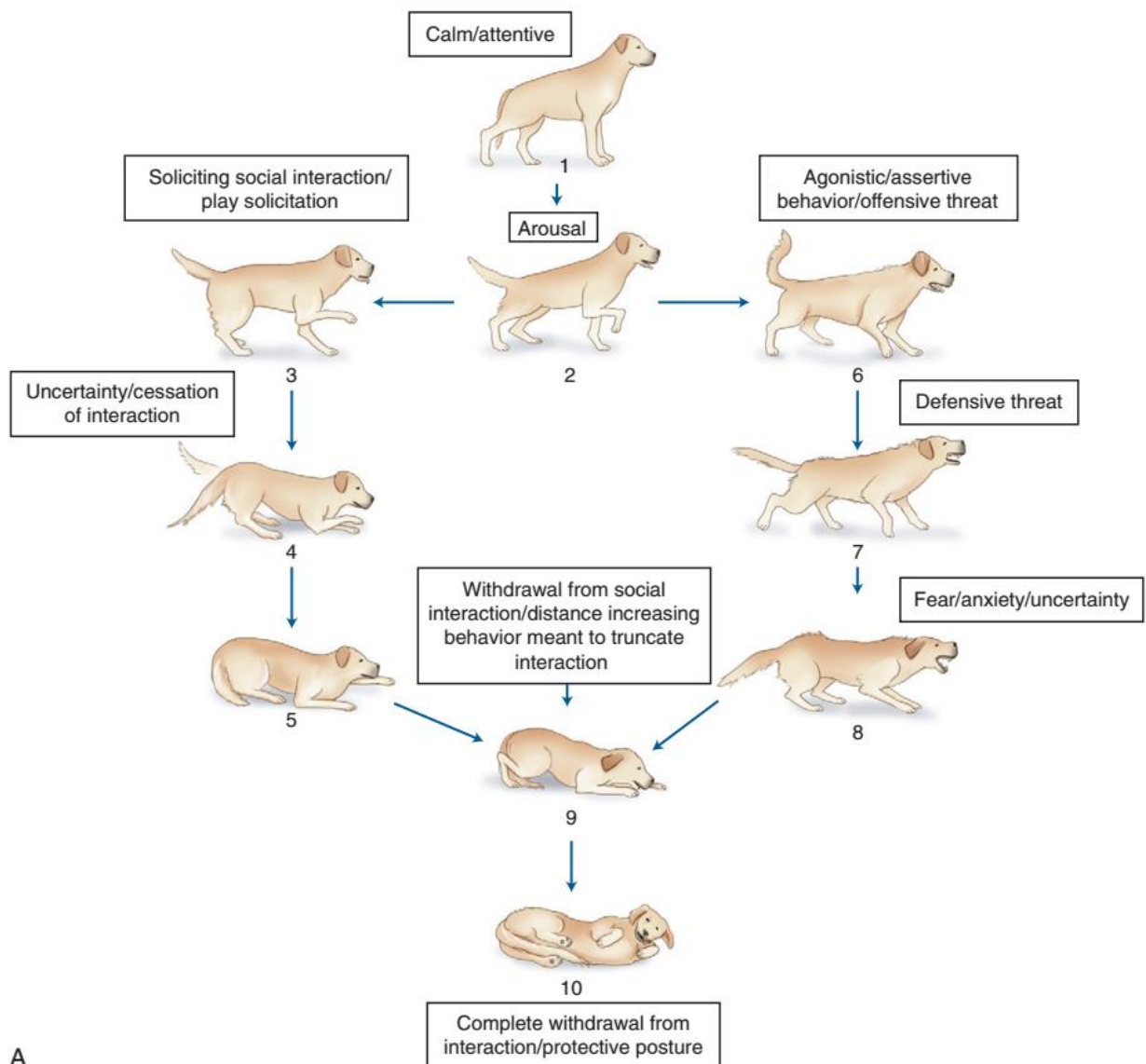


Figure 3 – Body postures in dogs

1. Calm/attentive: head up, ears up and tail down but not between legs;
2. Arousal: tail wagging, ears up and forward, focusing on the person or object longer than usual;
3. Play solicitation: play bow, tail up, mouth open, lips retracted horizontally, tongue lolling out, ears forward and sometimes friendly eye contact;
4. Uncertainty/cessation of interaction
6. Agonistic behaviour/offensive threat: direct visual contact, raising lips with corner of the mouth pulled forward, tail up and wagging at high frequency, ears erect and forward, piloerection, snarling ("C position");
7. Defensive threat: exposure of teeth, ears down, 'whale eye', snarl or snap;
8. Fear/anxiety/uncertainty: avoidance of visual contact, ears down/back, head down, tail down, lowered body posture, closed mouth with retracted lips;
9. Withdrawal, distance increasing, appeasement: blinking eyes, ears down/back, head down, licking lips, yawning, crouching, lying laterally;
10. Complete withdrawal, protective posture: rolling over on back, turning body and face away, licking lips, yawning and averting gaze.

(Adapted from Case, 2010; Landsberg et al., 2013; Overall, 2013)

Although a tail wagging is considered a play signal, in reality, dogs wag their tail in other contexts, some of them non-friendly. Tail wagging occurs in certain situations where the dog is highly aroused and stimulated. Calm and playful dogs have a relaxed and loosely tail. Dogs highly aroused wag their tail excessively. In contrast to this, an anxious dog is going to lower his tail and wag it slowly. If the tail is up and stiff while wagging vigorously, usually means that the dog has not friendly intentions. He can show offensive threat and possible aggression. To sum up, it is necessary to evaluate the general posture and not only the tail's movement (Case, 2010).

### 3.3 - Tactile communication

Tactile communication is the least important way of communication given by owners. However, it is the first sense developed in puppies and tactile stimulation improves neurodevelopment (Overall, 2013). It is important for the bond development and exchange of intimate information (Beaver, 2009). Touch contact decreases heart rate of the dog, blood pressure and respiratory rhythm. Even so, the reaction depends on the human with whom they are in contact with and general environment. That is why petting should be performed with long, slow strokes, deep muscle pressure and massage. Certain dogs lean a paw on the owner, when feeling uncertain and fearful (Overall, 2013). Even on hot temperatures, dogs stick together, this means, that contact is not only for temperature regulation, but also to fulfil social needs (Landsberg et al., 2013).

### 3.4 - Olfactory communication

Humans have a hard time understanding why odours are so important for dogs and sometimes they miss the information conveyed by them (Bowen & Heath, 2005). Information from the odours' world is perceived by three main structures: main olfactory epithelium (smell), oral cavity (taste) and the vomeronasal organ (pheromones) (Mills, 2005). Deposition of odours with long duration, such as urine, faeces and anal sac secretion are body odours characteristic to each dog (Bowen & Heath, 2005; Horwitz et al., 2005). Each dog has its own glandular secretions which give them a particular identity (Appleby, 2017; Overall, 2013). Odours convey information about gender, age, social status, emotional state and individual identity. It is especially important for social communication, maternal bonds and reproductive behaviours (Landsberg et al., 2013).

Smells are volatile molecules that are inhaled and detected by specialized cells within the main olfactory epithelium, which send information to the brain. Sniffing is a disruption of normal breathing pattern and it allows odour molecules to stay longer in the nasal cavity. There are dogs that are trained to smell certain molecules like drugs, explosives and even detect cancer through urine samples (Case, 2010; Overall, 2013).

It is important that the veterinarian surgeon knows how to identify scent marking during a behavioural examination and acknowledges the activity of each gland involved (Pageat & Gaultier, 2003). Scent marking is defined as urinating, defecating and rubbing certain body parts in certain objects. This behaviour is triggered by familiar landmarks, new objects or certain odours. In dogs, occurs mainly by two ways, anal sacs secretions and urine. Other odours such as faeces, saliva and sebaceous glands are used to communicate. Scent rubbing seems to reassure and familiarise an animal in an unknown environment. Overtime it became important to encounter sexual partners, as well, distribution and maintenance of territory. We can divide stimulus into two groups: the first group consists in familiar objects, such as a light pole, and the second one concerns novelty (Beaver, 2009). Scent marking can act as: a) substitute to aggression, warning conspecifics that a determinate territory is occupied b) sexual attraction/stimulant, c) maintain a familiar feeling towards a place, d) individual identity and e) alarm signal to conspecifics (Johnston, 1998). A study enrolling male and female dogs tried to differentiate how they would react to different odours, such as urine, vaginal and anal sac secretions. Males showed more interest in female urine than vaginal and anal sac secretions. Males with or without sexual experience had a tendency to be more curious for oestrous females (Doty & Dunbar, 1974).

#### 4 - Development periods

The concept of behaviour is not static, puppies go through a wide range of modifications since the day they are born until their adulthood. Behaviour's development relies in the relation between genetic predisposition, environment and learning experience during the socialization period (Case, 2010; Landsberg et al., 2013; Miklósi, 2009).

Their maturity develops along their growth and life, adapting to different circumstances of the daily life. Most authors defend the classification of behavioural development into four stages: a) neonatal period, b) transitional period, c) socialization period and d) juvenile/adult period (Figure 4) (Case, 2010; Horwitz et al., 2005; Landsberg et al., 2013; Markwell & Thorne, 1987; Miklósi, 2009; Scott & Marston, 1950). Formerly, the term “critical period” was used, but as it was shown to be a gradual change, the term was replaced for “sensitive period”. In a general manner, sensitive period refers to an age period during which an animal responds to socialization, habituation and attachment in a higher level. Talking about our companions this interaction occurs between family humans and other animals (Case, 2010; Markwell & Thorne, 1987; Scott, 1958; Scott & Marston, 1950).

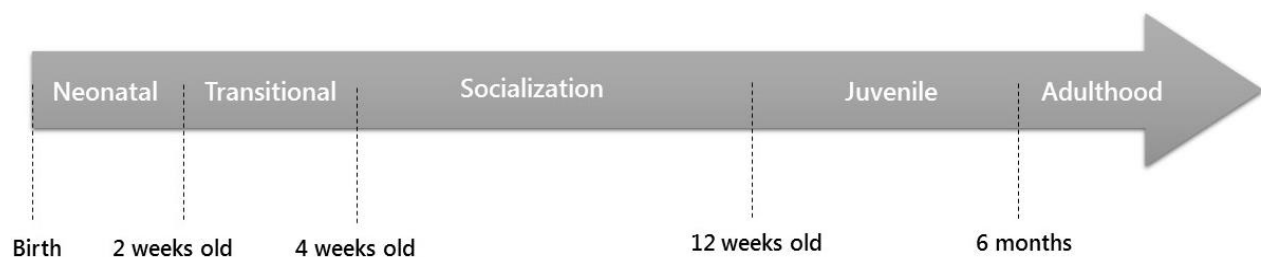


Figure 4 – Development periods for dogs.

(Figure by the author)

Development periods can be approached functionally and mechanically. The functional approach is based in the recognition of the animal's development and the way he adapts to the environment. In this perception, environment is a complex concept, because dogs can relate with other groups besides their mother. Concerning the mechanical approach, it describes the development as a process, during which behaviour and perception abilities' increase over time (Miklósi, 2009).

Still, there is a need to investigate further, most studies are outdated and do not include different breeds (Miklósi, 2009).

#### 4.1 - Neonatal period

Even though influences in behaviour begin before birth, such as, personality and maternal characteristics, this phase comprehends the period since birth until the puppy reaches 2 weeks old (Horwitz et al., 2005; Markwell & Thorne, 1987; Scott, 1958; Scott & Marston, 1950). Attachment and stronger relationships are formed with his mother and not with his littermates. Dogs are born very immature and maternal figure provides sustainability to puppy's basic needs, such as warmth, food and elimination. Stimulation is accomplished, essentially, by touch and smell, as other senses are still not developed completely, for an instance, Scott (1958) stated that puppies avoided the scent of citronella in an early age (Scott, 1958).

The new-born puppies have an undeveloped brain which is few myelinated. The parts myelinated correspond to reflex patterns of behaviour. The general lack of myelination can explain the slow movements and response to stimulus in this early age. In addition to this, at birth, the electroencephalography showed no waves and no differentiation between sleeping and waking states. At 3 weeks old, the brain begins to revert the previous findings and finally at the age of 8 weeks old it is considered an adult (Scott, 1958).

The "rooting reflex" is present since birth. It aids the puppy to find his mother and prevent him to wander around to colder areas. In this phase, puppies cannot regulate their corporal temperature being totally dependent of their mother's warmth. Maternal licking triggers the rooting reflex and puppy starts to show a swimming motion pushing him towards the warmth (Appleby, 2017; Case, 2010; Horwitz et al., 2005). According to Case (2010) puppies also display a particular behaviour named "distress calling". Puppies start whining loudly and in a high-pitch whenever they are cold or hungry. This activates the maternal instinct and these needs are fulfilled rapidly (Case, 2010). Puppies can react to external stimulus, such as pain, but cannot withdraw from it and may take a while to respond to it. Moreover, as they cannot see or hear properly, they are protected against most external stimulus (Case, 2010; Scott, 1958).

Landsberg et al. (2013) took into consideration two studies regarding the benefits of early handling (Landsberg et al., 2013). One study written by Fox (1968) showed that in comparison to the control group, puppies who were handled during short periods of time, from birth to 5 weeks of age, showed more confidence and exploratory behaviour (Fox, 1968). These puppies likewise developed more quickly, in terms of nervous system, hair growth and weight gain (Landsberg et al., 2013). To support this thesis, another study by Gazzano et al. (2008) state that mild stress, such as early handling, may affect the pituitary-adrenocortical system (Gazzano, Mariti, Notari, Sighieri, & McBride, 2008). Thus, puppies can handle better future

stressors in life. This study compared kennel-raised and home-raised dogs and concluded that the first group could handle more efficiently with isolation stress. In a preliminary study with the objective of assessing the effects of early gentle handling and environment in the emotional stability of puppies, 7 litters were evaluated. Half of each one, had their puppies gently handled every day since puppies were 3 days old until they reached 21 days. Results suggest that environment strongly affects the emotional stability of puppies when left in isolation. Handled puppies took more time to start crying and spent more time exploring their surroundings. Moreover, handled puppies had a calmer posture. To summarise, gently and early handling during puppyhood improves puppies welfare and helps them develop emotionally (Gazzano et al., 2008).

#### 4.2 - Transition period

During this phase, drastic physiologic changes occur, allowing the puppy to acquire a larger perception of the world involving him. It lasts around a week. They start by opening both eyes and ears. It is harder to assess the evolution of hearing capability and we must take into account that breeds with dropping ears evolve later in time. Visual and auditory guidance begins to develop around 25 days old. In this period, although puppies have their eyes opened, their retina is not completely developed, and they are only sensible to different shades of light. Furthermore, the first teeth begin to erupt (Case, 2010; Miklósi, 2009; Scott, 1958).

Typical behaviours associated with the previous period begin to vanish, puppies can eliminate for themselves without stimulation from the mother. In the previous stage, they could only crawl, but now they can walk. The “distress calling” also suffers some alterations. Puppies usually cry when they wander away from the mother and their littermates. During this time, the ability to learn starts to develop and they can make simple associations between stimulus and consequences. Learning capability is slow, but puppies should be stimulated both olfactory and audition (Case, 2010; Horwitz et al., 2005; Landsberg et al., 2013; Miklósi, 2009; Scott, 1958). Early exposure to different types of stimuli for short periods enhances physical and mental development (Landsberg et al., 2013). Simple exercises, like walking around different surfaces (various textures and temperatures) can stimulate them. At 20 days old, puppies start to tail wagging, although this depends on the breed, as some do not have a tail (Overall, 2013).

#### 4.3 - Socialization period

Scott and Fuller (1998) took into consideration two periods of socialization (Scott & Fuller, 1998). Primary socialization is when animals learn quickly with a short exposure and the learning process depends only on external exposure, for example, food. Secondary socialization is related to “taming” - when wild animals get accustomed with human presence and show other ways of learning. In domestic dogs these two types of socialization get overlapped (Miklósi, 2009). The socialization period is associated with maturation and myelination of the spinal cord. All sensory systems are developed and their learning ability increased (Horwitz et al., 2005; Scott, 1958). Around 4-5 weeks, puppies' eyes are fully open and gained the ability to process visual information. Regarding the hearing, at the age of 19 days, they can react to a sound stimulus (Scott, 1958).

Weaning begins around 3-4 weeks of age, it is a gradual process and will be complete when puppies reach 7-8 weeks of age. Mother spends longer periods of time away from the nest and this teaches the puppies how to be more independent and confident. Female dogs' ancestors regurgitated the food when the weaning began. Puppies show food-soliciting by submissive posture, whining and licking the mother's mouth. Although puppies keep this behaviour, nowadays the mother does not regurgitate food (Appleby, 2017; Case, 2010). Pierantoni et al. (2011) studied 70 dogs, who as puppies were separated from their mother and littermates for 30-40 days and 70 adults who were not separated until they reached 8 weeks old. The main conclusion was that early separation may result in some predictable behaviours, such as, excessive barking, fearfulness while walking, reactivity to noises, toy possessiveness, food possessiveness and attention-seeking behaviour. Also, the first group had higher risk to show destructive behaviour in the future (Pierantoni, Albertini, & Pirrone, 2011).

Puppies acquire the capability to recognize places, such as, the sleeping area and eating area. Additionally, around this time, social play begins to have a significant role in future communication skills. For example, puppies learn how to inhibit their bite - when playing with a littermate, if the bite is too strong and hurts, the play ends. This way, the puppy learns to control the strength of his biting to continue the play. This self-limit is important for the future leading puppies to bite their designated toys and not humans or other dogs. Besides this, it is essential that each pup learns how to communicate to prevent misleading in communication (Case, 2010).

Between 8-9 weeks puppies leave their nest to eliminate in another area. Furthermore, puppies have more initiative to explore the surroundings, staying away from the mother for



longer periods of time, but closer to their littermates. Older puppies become more curious about the environment and are always ready to go on an adventure, showing little fear or apprehensiveness towards new stimulus (Case, 2010; Markwell & Thorne, 1987).

There is no consensus regarding the period for “fear imprint stage”. For instance, Case (2010) defends it is between 8-10 weeks, but Landsberg et al. (2013) states a more extended period, 5-8 weeks. Beginning at 5 weeks old and a peak at 7-8 weeks, the relaxed posture is lost and replaced by a period of high heart rate and sympathetic dominance (Appleby, 2017) (Figure 5). Despite this, we have to take into consideration that there are individual characteristics. Besides being a period for social development, it is also a “period of sensitivity to psychological stress” (Landsberg et al., 2013). Excessive stress exposure should be avoided, because a chronic excretion of adrenocorticotrophic hormone is related with a decrease of the learning ability (Overall, 2013).

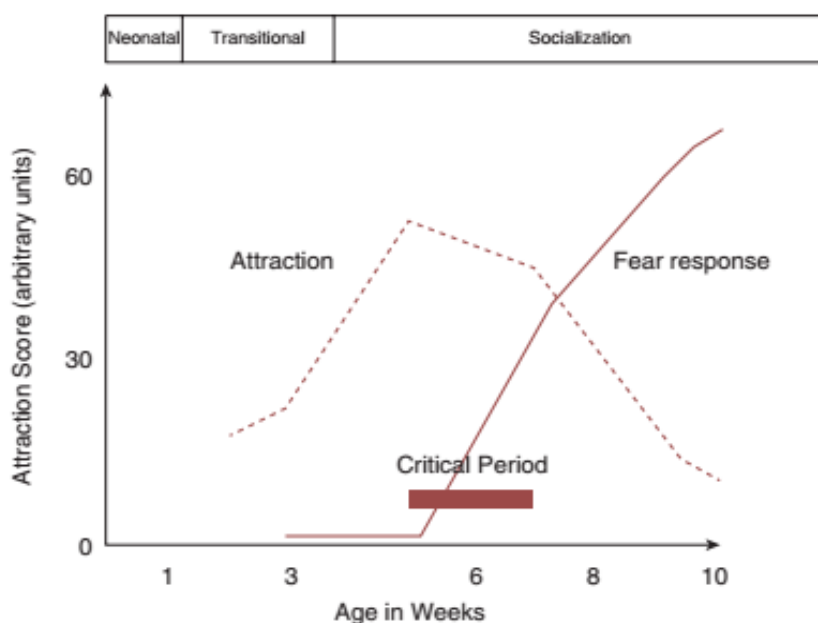


Figure 5 – Representation of attraction and fear in puppies during the “sensitive period”.

(Adapted from England, 2013)

A study by Elliot and Scott (1961), in which the authors intended to investigate how vocalization and activity are altered when puppies are isolated, showed interesting results. Puppies showed more distress (evaluated by vocalization and activity) when isolate from their mother in a strange place. The authors defend that, these reactions are highly influenced by maturation alterations suffered during the sensitive period. The most significant responses were observed in puppies separated to a different location, aging between 6 and 7 weeks old,

period which coincides with the end of primary socialization (Elliot & Scott, 1961). In 1966, Fox and Stelzner, conducted a study to evaluate the effects of different rearing in puppies' behaviour and development (Fox & Stelzner, 1966). They had three groups, a) one control group, b) puppies who were handled and c) puppies who were partially isolated. Results showed that handled puppies had an increased adrenal activity and significant alterations in the electroencephalogram, because they matured quicker. In opposing to this, the partially isolated puppies had lower attachment levels to humans, less explorative behaviours and more tendency to show fear towards new situations (Fox & Stelzner, 1966).

#### 4.4 - Juvenile period/ adulthood

This period starts at the end of socialization period and goes until sexual maturity. Even though it is the longest period, this period has the lowest number of studies (Miklósi, 2009). Learning ability is fully developed, puppies become more independent and explorative as their motor coordination increases (Case, 2010; Landsberg et al., 2013; Scott, 1958). With the onset of puberty, sexual behaviour begins to mature. Dogs reach sexual maturity between 6 and 16 months old depending on breed and size when adults. Frequently females evolve sooner than males (Case, 2010; Landsberg et al., 2013). Despite sexual maturity, social maturity continues to develop until 18 months old. It is during this phase that dominance relationships are formed and dogs show territorial and protective dominance aggression (Case, 2010; Horwitz, Ciribassi, & Dale, 2014).

## 5 - Behavioural problems in puppies

The connection between mental and physical health is being recognized in the veterinary field (Seibert & Landsberg, 2008). Some behaviour problems begin to manifest at an early age, such as, inappropriate elimination, separation-anxiety, destructive behaviour and fear-related aggression. These problems are a strain on the pet-owner bond. During first consultations, like core vaccination, it is essential to instruct the owner about dog's behaviour, communication, training, learning theory, environmental modification and other concepts, in order to minimize negative consequences to dog's welfare and future consultations. Behaviour problems often result in relinquishment to shelters and at a last instance euthanasia (Seibert & Landsberg, 2008). Factors as regular veterinary care and participation in obedience classes reduce the chance of relinquishment (Salman et al., 1998). First time owners have a higher chance to come across with more behaviour problems considering their pets. This fact can be explained by the lack of experience or knowledge to deal with daily life situations (Jagoe & Serpell, 1996).

A study conducted by Clark and Boyer (1993) differentiated between three groups: a) dogs taking obedience training and owners taking counselling classes, b) owners who spent 20 minutes, every day, with their dogs and c) dogs without classes or play-time with the owners. Results showed that dogs included in the first group had a more controlled behaviour and a better relationship with their owners. Contrary to expectations, dogs who spent 20 minutes playing with their owners had equivalent results to the first group. Furthermore, the last group showed more separation-anxiety than the others (Clark & Boyer, 1993).

A study led by Herron et al. (2007) compared two groups of owners, one group received behaviour counselling and the other did not. All dogs, that took training lessons, had a successful house-training, one month after adoption. However, a larger number of dogs that received training were from the first group. Despite this, owners who had taken behaviour counselling, used less verbal punishment and used appropriate cleaning products. A simple and brief explanation during the first consultation raises the house-training's success in newly-adopted puppies from shelters (Herron, Lord, Hill, & Reisner, 2007). This idea is corroborated by Gazzano et al. (2008) who encountered the same conclusions in their study. Counselling is likely to reduce behaviour problems mainly in puppies separated from their mothers with less than 2 months old (Gazzano, Mariti, Alvares, et al., 2008).

### 5.1 - Elimination problems

Most owners have unrealistic expectations when talking about elimination training. When the problem occurs, it can destabilize the family, cause house damage and affect the puppy's welfare. If the problems persist, they can cause dire consequences as relinquishment or euthanasia, as the owners cannot cope with the situation any longer. As a part of the treatment it is important to find the cause. Most common causes are incomplete house-training, marking behaviour, submissive or excitable urination and anxiety due to separation or fear (Case, 2010).

A retrospective study concluded that almost 80 percent of house soiling problems were due to inadequate house-training (Yeon, Erb, & Houpt, 1999). Early house-training is essential to prevent future inappropriate elimination. House-training takes advantage from the puppy's preference to eliminate in a certain location and substrate and its aversion to eliminate in his sleeping or playing area. The objective of the training is for the puppy to eliminate after a command and at the designated location. At 5 weeks old, puppies leave the nest and choose a place to eliminate. If this action is repeated multiple times, puppies can get accustomed to a type of substrate and a certain local to eliminate in the future. If the puppy developed a preference for a substrate and the owner has struggles with the training, he can mimic the same material outdoors (Bowen & Heath, 2005; Case, 2010; Horwitz et al., 2005; Landsberg et al., 2013; Lindsay, 2000c).

A successful training allied to a controlled environment, prevent the puppy to eliminate in the wrong area or inside the house. While young, puppies need regular walks outside, not only to get used to different stimulus, but also to learn how to eliminate outdoors. If the puppy is too young to go outside or he has no vaccines, training can begin indoors, in a newspaper or other appropriate surface. Training must focus in positive rewarding when the puppy eliminates in the correct place and not on punishment. Verbal and physical punishment can cause future aversions, because, puppies can associate the elimination with the owner presence and will start to eliminate in a hidden place. This will cause further struggles, mainly when walking outside, as the puppy will not eliminate outdoors while being observed by the owner, but rather when arriving home. If the owner catches the puppy in the act, he should pick him up and place him in the right place. Usual inappropriate elimination places should be washed with a biological and appropriate product to avoid olfactory stimulation. Puppies should never wander around the house alone and without supervision to avoid elimination accidents or other situation as inappropriate chewing. It is important for the owner to know which periods are more critical for the puppy's elimination. Puppies have a limited control of their bladder,

thus usually eliminate upon waking up, when the play is over, after a meal or a nap and before sleeping. Taking this schedule into account, owners can create a routine to make it easier for the puppy to adapt to good manners (Bowen & Heath, 2005; Case, 2010; Horwitz et al., 2005; Landsberg et al., 2013). Case (2010) wrote that there should be two types of walks, one for leisure and another for elimination. This is justified because some owners end the walk right after the puppy eliminates. This will mean that in future walks the puppy will delay the elimination moment to prolong the walk and leisure time (Case, 2010).

## 5.2 - Separation-anxiety

Isolation can be very stressful for any dog, especially if he did not undertake a gradual adaptation. These cases are more severe when referring to newly-adopted puppies, as they suddenly found themselves in an unfamiliar environment. Distress vocalization and increased activity are normal behaviours when puppies are away from their mother. When puppies are adopted, it is expectable that they will go through mild levels of stress. Puppies must learn that there are play times, but also solitude time, when he can enjoy his toys or rest. Lacking a positive association in such activities, puppies can associate isolation with negative feelings such as discomfort and solitude. Owners must not expect puppies to enjoy their solitude time, they should be taught and receive equal amounts of play, exercise and training time. As in other circumstances, it is crucial to create a routine. Solitude time should be introduced after a long walk or a play session, taking advantage of the puppy's tiredness. Equally important, puppies should have a special toy, reserved for the solitude time. A good example is a toy which can be stuffed with biscuits to keep entertained and interest longer. Solitude time should be increased gradually, in order that puppy can get used to it. Even though training alone does not completely prevent the development of separation anxiety, it is proven to reduce isolation (Case, 2010; Lindsay, 2000b).

Separation-anxiety can occur when owners leave the house or when they are at home but away from the puppy. According to Vilanova (2002) a dog can react in 3 different ways: a) showing no alterations in posture, b) showing low activity, anorexia and some stress and c) high activity, vocalization, inappropriate elimination and high stress levels (Vilanova, 2002). As other studies reported, puppies who were separated from the mother and littermates at a young age have higher chances to develop this problem (Sherman & Mills, 2008; Vilanova, 2002). Dogs with anxiety have a more nervous personality and demonstrate behaviours related with a higher dependency on the owner. Additionally, dogs rescued from shelters also have higher chances to develop separation-anxiety (Case, 2010).

In 2006, Parthasarathy and Crowell-Davis conducted a research to determine to what extent dog-owner relationship affects separation-anxiety. There is some discordance

relatively to the term “hyperattachment” and its influences in this behavioural problem. In this study, authors did not find evidences to correlate anxiety when the owner departs and attachment, however they consider that another attachment concept may be involved (Parthasarathy & Crowell-Davis, 2006). Separation-anxiety can be a disorder from our times, due the fact that owners have less time to spend, most dogs live on apartments with a single-owner (Sherman & Mills, 2008). Dogs who live with only one owner are 2,5 more likely to develop separation-anxiety than in a multiple-owner house. Factors such as spoiling actions, dogs’ gender, presence of another animals were not associated with this disorder. Opposing to the study conducted by Parthasarathy and Crowell-Davis (2006), Flannigan and Dodman (2001) found a significative relation between “hyperattachment” and separation-anxiety. Extreme following of the owner, departure cue anxiety and excessive greeting are taken as examples of “hyperattachment” (Flannigan & Dodman, 2001; Parthasarathy & Crowell-Davis, 2006).

### 5.3 - Destructive behaviour

Destructive behaviour can be due to an exploratory behaviour or as a way to deal with frustration, stress, fear and anxiety. Puppies should have access to a stimulating and interesting environment. When they are not resting or playing, usually they are exploring their surroundings. Often, this behaviour involves chewing. However, they do not always choose the appropriate items. It is the owner’s duty to prevent furniture damage, always supervising the puppy and creating a safe-room. Puppies have stronger drives than adult animals for both social and object play. Owners should create a group of toys and rotate them, to keep novelty present. In addition to this, special toys should be introduced in specific times, in order that training and associative memory can develop easily. Owners should avoid choosing toys similar to personal objects, as slippers, because puppies can easily confuse them. If the puppy is chewing an inappropriate object, then the owner should remove it and give him one of his toys. Punishment in these instances will not solve the problem, as puppies will chew the item away from owners’ control. Along with positive reinforcement, owners can also introduce a verbal command as “leave it” or “give” (Case, 2010; Horwitz et al., 2005; Landsberg et al., 2013; Lindsay, 2000b). Some puppies may have a preference for certain objects because they mimic the “predatory feeding behaviours of evisceration, dissection and gnawing on bones” (Case, 2010).

Nipping and biting are part of a normal behaviour during puppy play. Puppies show these behaviours while playing with their littermates and their owners. The exploratory behaviour is also associated with teething, beginning around 4 months old when permanent

teeth start to erupt (Case, 2010). Play-biting is usually misunderstood, especially by children, as owners see this kind of play as a type of aggression. Puppies can chew unsuitable objects to seek attention from the owner or as a result of boredom. Puppies can learn that if they pick a forbidden object, the owner will give them attention and the puppies will repeat this conduct. This behaviour occurs in certain occasion, for instance when family is reunited. Considering the previous problem, owners should give some toys or food before family times (Bowen & Heath, 2005; Case, 2010; Horwitz et al., 2005).

#### 5.4 - Fear-aggression

Most adopted shelter dogs suffer from behaviour problems and the most common is fearfulness. Most owners referred that their pets demonstrate unpleasant or problematic behaviours and in last instance were retrieved to shelters. Wells and Hepper (2000) declared that puppies had lower behaviour problems than dogs who were adopted as adult or adolescent. This can be related to owners' tolerance, in this case, puppies behaviour were easily accepted when comparing to adult dogs (Wells & Hepper, 2000).

Besides its psychological nature, fear also has a physiological and behaviour component. Fear results in the activation of the hypothalamus-adenohypophysis-adrenal system with a consequent raise of glucocorticoids and the activation of the sympathetic nervous system, resulting in the release of catecholamines. A fear response is controlled by different structures as central nervous system, amygdala, hippocampus and cerebral talus. The neurotransmitter *gamma*-aminobutyric acid (GABA) has an inhibitory effect in fear and stress (Landsberg et al., 2013; Vilanova, 2002).

Fear is a natural response to threatening situations or, at least, situations that are perceived as a threat. The tendency to escape from painful and unknown situations is highly related with the survival of our pets. Nowadays, when expressed excessively in certain circumstances, can cause mixed problems and be the trigger to some behavioural complications, including defensive aggression, separation anxiety and phobias. Stimuli that can trigger a fear response are an extreme novelty, adverse situations and loud noises. While socialization reduces fear-related problem, it is not the only component that should be considered. Fear reaction can be problematic if dog makes negative associations between his family members (Landsberg et al., 2013), health related issues or life quality (Case, 2010; Vilanova, 2002).

Experience obtained by living with the mother and urban environments between the age of 3 and 6 months old are associated with a reduced probability that puppies will show, in

the future, avoidance behaviours and aggression towards unfamiliar people. Regardless, aggression towards familiar people is not related with previous experiences. Puppies who contacted with different people are less likely to display a fearful posture towards a veterinarian. Clinical experiments suggest that dog's early environment influences not only the avoidance and fearful behaviour but also social interactions with other dogs and people (Appleby, Bradshaw, & Casey, 2002). For Overall (2013) there are two types of fearful dogs, dogs who demonstrate fear very soon, around 5-8 weeks old, and dogs that have an increasing level of fear as they gain sexual maturity (Overall, 2013). When confronted with a fear stimulus, animals can react by freezing, fleeing or fighting. Furthermore, animals can also fiddle about, this means, they show a displacement or self-appeasement behaviour (Bowen & Heath, 2005; Lindsay, 2000c).

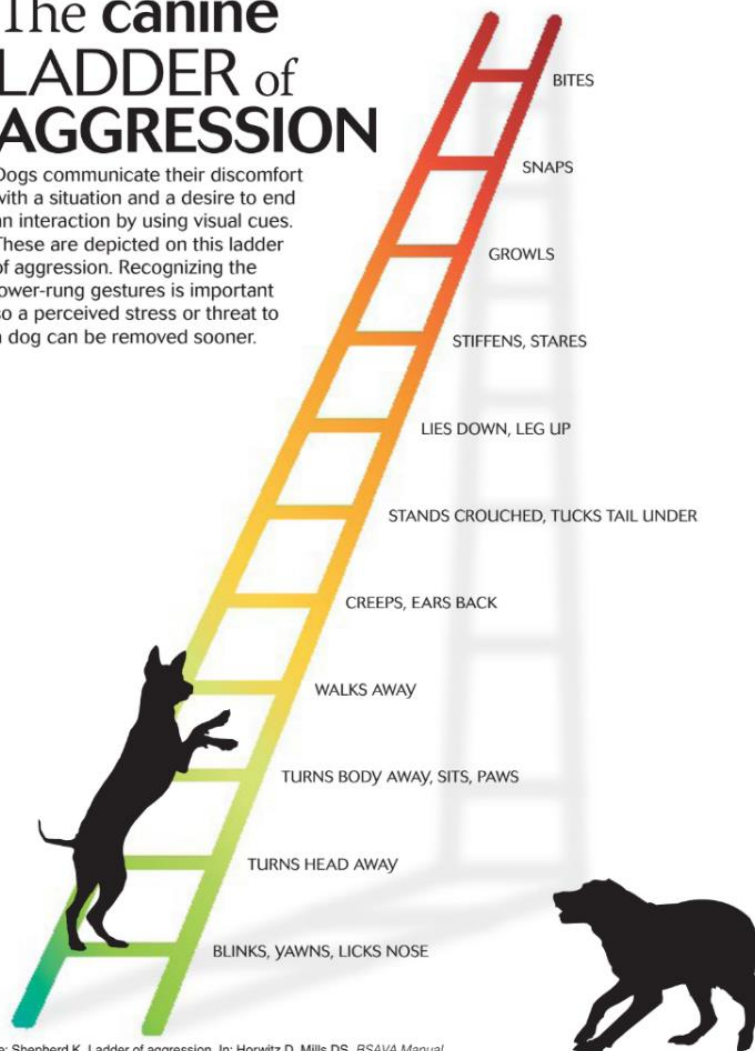
A wide majority of animals only move on to aggression if they fail to flee. Aggression is not a diagnosis, is a term used to describe a group of behaviours in a complex situation (Bowen & Heath, 2005). There are several types of aggression and behaviourists discriminate them taking into consideration trigger, target and motivation. Dogs give signals that they are not comfortable before moving on to aggression.

When a dog snaps or growls and the fear stimuli ends, he learns that this is an effective way to contour the problem. Next time he will pursuit the same behaviour pattern, but will escalate quicker in the aggression levels (Figure 6)(Bowen & Heath, 2005; Case, 2010; Landsberg et al., 2013). Some owners are very surprise because their dog has bitten someone, but the true is that he has given signals but they were misunderstood (Bowen & Heath, 2005). In a clinical environment fear-aggression can become very common if the veterinary staff and owners are not correctly instructed (Overall, 2013).



# The canine LADDER of AGGRESSION

Dogs communicate their discomfort with a situation and a desire to end an interaction by using visual cues. These are depicted on this ladder of aggression. Recognizing the lower-rung gestures is important so a perceived stress or threat to a dog can be removed sooner.



Source: Shepherd K. Ladder of aggression. In: Horwitz D, Mills DS. *BSAVA Manual of Canine and Feline Behavioural Medicine*, 2nd ed. 2009.

Figure 6 –Canine ladder of aggression.

(Adapted from Horwitz & Mills, 2009)



## 6 - Pharmacologic treatment

In certain cases, a behaviour problem is so serious, that it is necessary to resort to pharmacologic methods to deal with the situation as soon as possible, to balance the family and dog's well-being. There are no significant benefits by using excessive force when a patient is not cooperating (Moffat, 2008). Prevention is the best option, but usually that baseline has already been surpassed. Veterinary staff has to understand the background of each case individually, create an exact diagnosis and also know the medical history of the patient, in particular, if he has any concomitant problems that could influence the medication and how it is metabolized, age and other factors (Horwitz et al., 2005; Landsberg et al., 2013). When a puppy comes to his first visit is important to be attentive to any signs of possible behaviour disorder and manage it as soon as possible. It is essential to keep in mind and warn owners that simply adopting a pharmacology therapy is not enough (Bowen & Heath, 2005). Nevertheless, Overall (2013) took into consideration different studies, concluding that the use of psychotherapeutic compounds in combination with behavioural and environmental modification results in better outcomes (Overall, 2013).

According to Landsberg, Hunthausen and Ackerman (2013) there are four situations in which is recommend the implementation of pharmacologic therapy:

- Adjunct to behaviour therapy: serves as a complement to training sessions or behaviour therapy. For instance, during the treatment of separation-anxiety, anxiety or fear commonly it is used anxiolytics drugs. If an animal is in an elevated state of stress, the behaviour modification sessions could be more successful, due its reduction to an acceptable baseline.
- Drug desensitization: this technique is put into practice in cases when the behavioural problem has multiples origins, or it is hard to control them. The main objective is that the animal can cope with the stimulus in a positive way.
- Primary mode of treatment: method used in stereotypical behaviours.
- Underlying pathology present: first it is necessary to discard any medical problem, in other words, endocrine problems can affect behaviour so prescribed medication has to take into account both situations (Landsberg et al., 2013).

To choose the adequate medication it is necessary to understand some aspects concerning: a) neurotransmitters, b) the action in the brain, c) the result of the problem in the central nervous system and d) the secondary behavioural changes (Bowen & Heath, 2005;

Horwitz et al., 2005). Bowen and Heath (2005) consider that serotonin, dopamine, GABA and noradrenaline are the most significant neurotransmitters in behavioural medicine (Bowen & Heath, 2005). The main parts of the brain involved in the process of learning are the frontal cortex and hippocampus. These structures can be affected by learning and behavioural medication, because they both rely on the same molecular alterations and serotonergic neurons (Overall, 2013).

The market offers a wide range of different drugs, each one with its own properties. It is the responsibility of the veterinarian to choose the most suitable. Some examples of psychoactive drugs are neuroleptics, benzodiazepines, tricyclic antidepressants, beta-blockers, antiepileptics, antihistamines among others (Case, 2010; Overall, 2013).

Owners worry about possible adverse effects. Common secondary effects of psychotherapeutic drugs are related with the blockage of the muscarinic acetylcholine receptors (Overall, 2013). Usually, side effects are not very worrying, it may include some gastrointestinal disorder, slight sedation, appetite alterations and modifications in the heart rate. In less severe cases there are different alternatives of treatment, for an instance, nutraceuticals, which are a food product with health and medical benefits (Overall, 2013) and more importantly pheromones as these last ones do not have side effects and are biologically safe (Pageat & Gaultier, 2003).

## 7 - How to decrease stress and anxiety in puppies

Stress has the potential to affect an animals' physical, mental and social health (Mills et al., 2014). Despite our knowledge about the impact of stress in our daily lives, only recently more focus has been taken in the veterinary area (Mills et al., 2014). Practitioners ought to recognize the dimensions of the situation, what causes stress, to what extend and the animal's response (Mills et al., 2014). Overall, stress is defined as a threat to the normal homeostasis.

As mentioned by Mills, Dube and Zulch (2012) the term "stress" is frequently misunderstood and may lead to wrong assumptions. These authors defined the "stress response" to describe how an animal reacts, in terms of behaviour and physiology, to a threat or well-being alteration. The term "stressor" is a trigger of the elements mentioned before (Mills et al., 2012). In order to eliminate any outside variable that could have some influence in stress and animal behaviour evaluation, there are some scales and/or tables that establish standard responses from each individual animal (Mills et al., 2012; Overall, 2013). Stress indicators are important tools to assess an animal's welfare, however there is no direct relationship between stress and welfare (Bodnariu, 2008). Bodnariu (2008) indicated some signals that correlate with distinct categories of stress:

- Physiological indicators of stress
  - hormonal measures (catecholamines, glucocorticoids...)
  - heart rate
  - blood pressure
  - respiratory rate
  - body temperature
  - state of immune system
  - disease incidence
- Behavioural indicators of acute stress
  - avoidance
  - defensive aggression
  - panting
  - salivation
  - vocalization
  - low tail and posture
  - hiding

- Behavioural indicators of chronic stress
  - enhanced locomotion
  - yawning
  - paw-lifting
  - body shaking
  - low posture
  - vocalization

Although there are many stress factors in our environment, that does not necessarily mean that each one of them will be harmful or injurious for an animal. However, its behaviour will respond differently and will adapt to the type of stressor it is dealing with. Therefore, we should not assume that all stressors, or its consequences in the behaviour, are unquestionable evidence of lack of welfare (Mills et al., 2012). Animal welfare should be well-grounded over an evaluation considering every type and number of stressors, intensity, duration, predictability, level of control an animal has over a stressor and previous consequences of the potential stressor applied to each case of study individually (Mills et al., 2012). If the animal cannot cope with the stressor, he experiences distress (Hernander, 2008). Stress may be caused by, both, external factors (such as environment and bad nutrition) and internal factors (such as behaviour changes caused by fear, frustration and anxiety). Chronic frustration may cause meaningful changes in the behaviour if the animal cannot deal with the root of the problem. Lastly it can evolve to a depressed, stereotyped, displacement behaviour. Considering stress as a long-term problem, as it is mediated by the endogenous glucocorticoid system, it can affect the cognitive processing and consequently the animal is more sensitive to aversive situations.

Animals respond to changes in the surround environment using two distinct mechanisms- 1) physiological processes and 2) behavioural processes. In the first one, there are alterations in the physiology, that is, metabolic changes - for example, the temperature rises, and animals' paws start sweating. The central nervous system and endocrine systems are responsible for mediating these mechanisms. Regarding behavioural responses, these are considered a secondary method of response, because it needs more energy and resources (Mills et al., 2012) and a good example is when the exterior temperature rises, the dog starts panting to decrease his body temperature. In addition to this, Mills et al. (2012) also defend that animals have the ability to adapt to a stressor by a cognitive change, such as considering novelty situations as non-threatening (Mills et al., 2012). This last point is essential when we are discussing puppies' behaviour. In conclusion, there are 3 types of changes that an animal

can use to respond to a stressor, a metabolic shift, behavioural change and lastly a psychological adjustment (Mills et al., 2012).

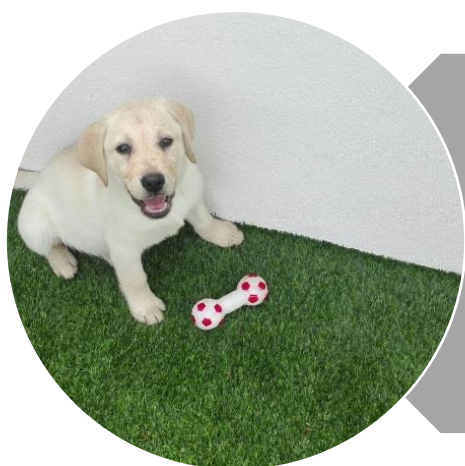
In a clinic environment, animals are exposed to some of these factors meaning that it is required to reduce or prevent them. Usually, a reaction of fear can have two distinctive origins, a social stimulus (unfamiliar people) or a non-social stimulus (loud noises). Different aspects such as breed or gender may influence how a dog reacts to a fearful situation (Hernander, 2008). The study “Indicators of stress and stress assessment in dogs” showed us that rescued dogs suffer more from anxiety and shelter dogs had more stereotypical behaviours (Hernander, 2008). Another point which needs to be taken into consideration is the behaviour of the mother, those who have good maternal behaviour tend to have puppies who grow more confident and less stressed (Landsberg et al., 2013; Levine, 1967). Moreover, maternal deprivation contributes to aggressiveness, fearfulness, anxiety and development of stereotypic behaviours (Landsberg et al., 2013; Latham & Mason, 2008).

## 7.1 – Socialization

Horwitz, Ciribassi and Dale (2014) considered that socialization is a “learning process” which allows dogs to accept coexistence with other individual of the same or different species (Horwitz et al., 2014). During this period is vital to minimize risk, but maximize opportunity (Figure 7) (Mills et al., 2012). Learning process, fear responses, sexual behaviour and preferences can be affected if an animal suffers from social deprivation (Landsberg et al., 2013).

The development of the primary socialization’s period starts while the puppies are still with their mother and littermates, between 3 to 5 weeks old (Horwitz et al., 2014). Therefore, it is vital to promote a social interaction since an early age. Horwitz, Ciribassi and Dale (2014) took into consideration the research done by biologists Scott and Fuller (1965) who defended there is a period during which puppies are more receptive to new stimuli (Horwitz et al., 2014). Moreover, they also stated there is a period named “socialization” period and it comprehends puppies aging between 6 to 12 weeks old (Scott & Fuller, 1965). There is some disagreement regarding the sensitive period, because Landsberg et al. (2013) defend that this period starts at 2.5/3 weeks and declines at 12 weeks of age (Landsberg et al., 2013). Despite the discussion about the range of this period, everyone agrees that during this period the attachment will be made quicker and without a need of counter-conditioning. This way, relationships created in this means will probably last a lifetime (Case, 2010). Other investigations about canine behaviour and socialization have concluded that puppies who are

not introduced to socialization environment with other pets and humans, tend to grow up as disturbed animals possibly becoming defensive or aggressive, inhibited or timid dogs. Environment during the socialization periods influences greatly fear and aggression towards people and another dogs (Kutsumi, Nagasawa, Ohta, & Ohtani, 2013). Regardless of the end of the primary period of socialization (12 weeks old) owners should continue to improve social communication and relationships through the adult life (Horwitz et al., 2014; Yin, 2009). Citing Case (2010) there is evidence suggesting that dogs benefit largely if socialization continues throughout the juvenile period (Case, 2010).



- Controlled exposure to novelty including different people and animals
- Reward positive behaviours with treats/toys
- Do not force any situation in which the puppy feels uncomfortable
- Identification of potential threatening situations and include these situations in the socialization program
- Addressing any problem as early as possible to revert the situation and prevent a future behaviour disorder

Figure 7 - Socialization program for a puppy.

(Adapted from Horwitz, Ciribassi & Dale, 2014; Photograph by the author)

## 7.2 - At home

Boredom, frustration and separation-anxiety are the main causes for behaviour problems and they can be minimized by teaching a puppy that there is a schedule for play, feeding and training (Case, 2010). Generally, most pets are adopted during holidays, sometimes are given as a gift or because people think they have more spare time. However, this is not the best time to bring a pet to an unfamiliar environment. There is a lack of routine, which is essential to prevent future behaviour problems. It is necessary to create a routine that includes our pet, such as feeding time, exercise, play time and training time (Case, 2010). Learning where the toilet is, learn to tolerate how to be alone, learn to inhibit their biting when contacting with people and other dogs and restrict it to proper toys should be part of the training plan and it is easily achievable at home (Mills et al., 2012).

Puppies go through a lot of changes which can cause them distress. During the first days, the puppy should only be allowed to explore one room freely. As time passes by



owners can expand this room to the entire house. While exploring the room (usually where the crate/sleeping area is) introductions should be made. Family members can begin to interact with the new member of the family in a calm environment, giving positive rewards for good manners. Case (2010) suggested that it is best to introduce one person at a time so not to overwhelm the puppy (Case, 2010). Whilst puppies are at a young age regular walks and car trips must occur in a positive way in order to establish a pleasurable routine and habituation through its life. A simple bad car trip can trigger anxiety and fear than can later evolve to a phobia.

### 7.3 - Veterinary clinic

Nowadays, a great percentage of veterinary hospitals and clinics adopt a pet-friendly policy. Some simple aspect such as doorways, noises, pavement, lightning and space need to be reformulated to create a comfortable setting for owners and their companions. In order to avoid an overcrowded waiting room, appointments should be made previously. Overall (2013) recommended that if a patient is distressed is better for him to wait in the car and not in a busy and noisy waiting room (Overall, 2013). If possible there should be separate rooms for dogs and cats. While waiting, dogs should be allowed to sit or lay down comfortably and without being molested by other animals. The study conducted by Hernander (2008) had some interesting conclusions: a) male dogs and females showed the same stress level while in the waiting area, b) dogs accompanied by a single owner were more relaxed and, finally, c) dogs who had been recently at the veterinary clinic had higher stress signs (Hernander, 2008). Furthermore, dogs reacted more anxiously to weighing than waiting for the appointment. All in all, the author suggests weighing the animal after entering the clinic and then let him relax until it is called up. 60% of the dogs who enter in a veterinary clinic show a fearful and submissive behaviour (Beaver, 1999) and 70% of the dogs do not enter voluntarily (Stanford, 1981).

Exam rooms should be large and allow free movement of the veterinary team. Patients feel more secure when a towel or a mat is placed over the examination table. When talking about puppies, they usually come with their own blanket and we can use it for the same propose. It is recommended to have different treats to reward the puppy during the examination (Figure 8). It is preferable to have lots of small biscuits than a large one. Furthermore, we can also reward with verbal appraise and toys. Rubber toys are very useful and easily cleaned. (Moffat, 2008; Yin, 2009). During the physical exam, the veterinarian surgeon should be attentive to any undesirable behaviours such as fear or aggression. Furthermore, should also advise owners how to handle them (Landsberg et al., 2013). Beaver (1999) and Stanford

(1981) defended that fear-related behaviours are very frequent in dogs in veterinary practice (Beaver, 2009; Stanford, 1981).

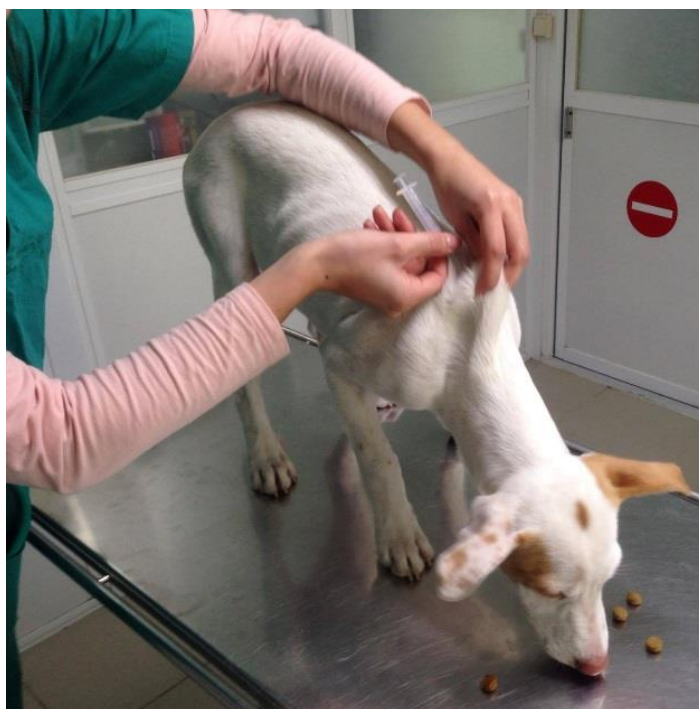


Figure 8 – During consultations, puppies should be rewarded with toys or treats.

(Photograph by the author)

Vaccination is part of a routine veterinary care and a chance to the veterinary staff to work with the owners and their puppy. The patient should be taught to offer a calm and relaxed behaviour as well needed body parts. This consultation should never turn to a bad experience, as the dog needs to get their vaccine at least once a year (Döring et al., 2009). Each negative experience conditions the animal for future consultations, so that next consultations may become more difficult and unsettling (Simpson, 1997). Puppies must be manipulated at home in order to create a habituation to routine procedures. Owners can train their puppy to get used to have their skin pinched and associate that feeling with a reward, this way when the real vaccination occurs the animal will be relaxed, distracted and expecting a treat. (Landsberg et al., 2013; Lindsay, 2000b; Yin, 2009). If the puppy feels insecure we should not force any procedures, instead we should try to calm and relax him. It is useless to use prolonged struggling. While in the examination room, owners have to control the pet's movement and support the animal (Döring et al., 2009; Yin, 2009).

#### 7.4 - Puppy classes and puppy parties

As stated by Mills, Dube and Zulch (2012) experiences during the first months of a puppy's life are crucial in influencing its long-term behaviour. Thus, in the last few years puppy classes and puppy parties increased in popularity. Our society is more conscious about their pets and want to provide them with a better life quality. Puppy classes were originally developed to provide training for puppies and it is different from obedience classes for adult dogs (Kutsumi et al., 2013).

Fundamentally puppy classes or puppy parties promote an exposure, under controlled circumstances, to a wide range of stimulus that otherwise they could not experiment. It is an opportunity for owners to introduce their pet to other puppies and other stimulus, for instance, new sounds, odours, unknown people (Appleby, 2017; Bowen & Heath, 2005; Case, 2010; Horwitz et al., 2014; Kutsumi et al., 2013), among others. Most dogs want to interact with others, but fail to do it properly causing them to see a threat and respond with a fearful or aggressive posture (Yin, 2009). These classes are not aimed exclusively for puppies. Puppy parties should be fun both for owners and puppies. They promote owners' education regarding behaviour, training and how to prevent future behaviour problems. A puppy party should include at a first instance an informal introduction. During this part of the session, owners and puppies get to know each other, always under vigilance of staff members. This first informal introduction is followed by an education section which can comprehend some topics, such as, prevention of common behaviour problems, routine healthcare (parasite control and vaccination), nutrition, neutering. Finally, a practical session involving all puppies and, if possible, the introduction to the consulting room and other facilities as well to staff members. The aim is to create a controlled habituation, in order to create a positive association to the veterinary clinic (Appleby, 2017; Bowen & Heath, 2005; Landsberg et al., 2013). Puppies should also be "taught life skills, in other words, self-control, good manners and frustration tolerance" (Mills et al., 2012).

The preventive medicine is the best approach when we are taking into consideration undesirable behaviours and it is achievable through puppy parties and puppy classes (Bowen & Heath, 2005). The veterinary surgeon is responsible for giving preventive advice to new owners, so that they have real expectations when adopting a puppy (Case, 2010). Bowen and Heath (2005) stated that puppies usually attend parties in between 9 and 12 weeks of age (Bowen & Heath, 2005). This period depends in each case, but it gives a time window to work with. During this period, puppies learn at a rapid rate, so it is important to start obedience training around this period (Case, 2010).

It is important to remember that puppies who attend puppy parties have not finished their vaccination protocol, so all members involved ought to take extra care regarding disinfestation, sanitation as well prohibit sick puppies or ones who have a high health risk to attend these meetings. Also, puppies should not attend a puppy party in the same day as its vaccination (Bowen & Heath, 2005; Horwitz et al., 2014). All puppies should have been dewormed previously and have at least the core vaccination done. The risks of social deprivation should be balanced with the benefits of early socialization and training. The risk level in these controlled facilities is much lower than a walk on the street where stray, sick and unvaccinated dogs wander (Landsberg et al., 2013). According to the American Veterinary Society of Animal Behaviour (2008), the risk of infection is relatively small when compared to rates of relinquishment or euthanasia due to behavioural problems (American Veterinary Society of Animal Behaviour, 2008). A recent study found no association of parvovirus with attendance at puppy classes (Stepita, Bain, & Kass, 2013).

Puppies enrolling this socialization classes should have similar age so to have the same maturity levels (preferably between eight and twelve weeks of age when beginning). A well planned puppy party should have a limited number of puppies, in order to assure a safe and controlled environment (Mills et al., 2012). It is important also to be aware of different sizes, so larger puppies will not be too overwhelming to smaller ones (Horwitz et al., 2014). If a puppy is very shy and shows signs of stress, action should be taken immediately to prevent any further phobias or behavioural problems. A study carried out by Seksel, Mazurski and Taylor (1999), in which the author intended to investigate how a 4-week socialization program affects dogs' behaviour in a short and long term, impelled the owners to teach their puppies basic commands, to take them to walks around the neighbourhood and to interact with different individuals (Seksel, Mazurski, & Taylor, 1999). Participation in puppy classes contributes positively to a response of a dog to strangers and onwards adult dogs tend to be more friendlier to non-family members (Kutsumi et al., 2013). Overall, puppy socialization classes help to create a well-adjusted dog, however the authors noticed a more positive response to command training at the end of each class, which was verified also in another study developed by Kutsumi et al. (2013), in opposing to other factors such as manipulation or response to novelty (Kutsumi, Nagasawa, Ohta, & Ohtani, 2013).

Although puppy parties and puppy classes usually are seen as the same concept, they are different forms of turning a puppy into a healthy and sociable dog. Puppy parties are more casual whereas puppy classes occur in a more often frequency. According to Bowen and Heath (2005) puppy parties usually do not offer a depth-in discussion, on the other hand puppy classes exist to create a course (which spans between 4-6 weeks) and fill in some lacks left

behind (Bowen & Heath, 2005). In puppy classes, puppies can also experience novelty, normally in a greater scale than in puppy parties. During puppy classes, the puppy should be taught how to tolerate a muzzle, grooming, brushing teeth, leash and nail trimming. Overall, exist many benefits for all the parts integrated in this kind of project.

Factors like unrealistic owner expectations, lack of counselling and training, destructive behaviour and house soiling, are known to increase the probability of owner relinquishment (Case, 2010; Coelho, 2013; Landsberg et al., 2013). Owners only feel happy about a new dog when they bring a positive balance to their lives. This is easier achieved with a trained, controlled and happy puppy than with one with fear, anxiety and aggressive reactions. As reviewed by Duxbury et al. (2003) in the study "Evaluation of association between retention in the home and attendance at puppy socialization classes" puppies who had taken socialization classes (puppy classes) tend to remain in their owner's home more often than others that did not attend (Duxbury, Jackson, Line, & Anderson, 2003). In addition to this, puppies that had been more manipulated and who responded to commands had a higher retention at home. To conclude, puppies who had enrolled in early learning and socialization classes had less chance to get abandoned and also helped owners to develop realistic expectations and to create a long-term bond (Case, 2010).



## 8 - Pheromonotheapy in dogs

Carnivora order has developed a greater variety of glands secreting chemical signs. Initially, scientists believed that pheromones were exclusive to invertebrate animals (Pageat & Gaultier, 2003). In 1959, a chemist and his team identified the first silk moth's sex pheromone (Horwitz, Ciribassi, & Dale, 2014; Wyatt, 2014). For mammals, the first pheromone discovered was in boars and the phenomenon was recognised as 'ram effect'. Boars produce an ovulation controlling pheromone in the submaxillary glands. More research was done in this medical area and nowadays it is possible to create synthetic compounds that mimic the effects of some pheromones (Pageat & Gaultier, 2003).

Pheromones are not simple odours, it is important to draw a line between these two concepts. The definition of pheromone has suffered changes throughout the years, the classic definition was made by Karlson and Luescher in 1959 (Mills et al., 2012). Currently, scientists prefer the term semiochemicals to describe a family of chemical mixtures involved in intra and inter-species communication, whereas pheromones are chemicals compounds excreted or segregated for communication between members of the same species (Horwitz et al., 2014).

Pheromones are chemical substances or a mixture of them, shaped by evolution, emitted into the environment by an animal (Pageat & Gaultier, 2003; Wyatt, 2014). These substances produce certain effects on the individual receptor (Landsberg et al., 2013; Pageat & Gaultier, 2003). These type of communication is exclusively for intra-species communication (Overall, 2013; Stowers & Marton, 2005). Semiochemicals used between different species are called allelochemicals (Wyatt, 2014). A special feature of the pheromones is that they are "anonymous", this means that it is the same in males and females and transmits a similar message (Wyatt, 2014).

In mammals, odorant signals transmit cognitive responses and adapted behavioural changes that can be substantially altered by sensory experience. However, pheromones trigger genetically pre-programmed behaviours, such as, mating and defence of the territory, as well as neuroendocrine changes like female oestrous cycle or male testosterone increase (Dulac, 2000). Responses to pheromones are biochemically specific and do not occur in the absence of the compound (Overall, 2013). According to the pheromones' effects, we can divide them in 2 groups:

- Primers: pheromones that produce physiologic alterations in the animal receiving the information, particularly neuroendocrine changes related to reproduction. They do not produce an immediate effect, but once the effect initiates it lasts for a

considerate period of time (Pageat & Gaultier, 2003; Vilanova, 2002). These pheromones have the ability to change an animal's emotional state (Landsberg et al., 2013).

- Releasers: pheromones that originate an immediate and short time reaction. (Pageat & Gaultier, 2003; Vilanova, 2002).

In addition to those types mentioned above, Swaney and Keverne (2009) described another class, the signalers, to describe chemiosignals that transmit information about an individual and induce changes in the behaviour and physiology that depend on the identity of the emitter and receiver animal (Swaney & Keverne, 2009).

There is no consensus if the dog has a flehmen behaviour or not (Lindsay, 2000b; Pageat & Gaultier, 2003). Dogs do not exhibit a true flehmen response or lip curl, they exhibit a similar behaviour called tonguing. Dogs flick their tongue against the incisive papilla, sometimes the teeth chatter and can produce foam that accumulates in the upper lip (Landsberg et al., 2013; Lindsay, 2000b; Pageat & Gaultier, 2003). This movement is typical observed after a dog has licked urine or smelled interesting smells. It is accompanied of several brief sniffs and head' shakes from side to side (Lindsay, 2000b). This behaviour induces the pheromones' aspiration into the vomeronasal organ (VNO), where they are mixed with mucous. The suction occurs as a consequence of the walls' vasoconstriction (Pageat & Gaultier, 2003).

Pheromonotherapy is a method of treatment easily accepted by the owners. Its limitations are related to the lack of precision in this type of communication. It is necessary to choose the right pheromone and the most indicated moment to achieve positive results. The principal action mechanism it is still unknown, but is known that affects the limbic system and the hypothalamus. Depending on the type of the pheromone that we are dealing with, we can induce certain behavioural patterns or inhibit unwanted and unacceptable behaviours. The synthetic analogue of a pheromone is used as a kind of psychotropic that transmits certain messages. This kind of approach has some technical specifications. In natural conditions, pheromones are not expelled alone. The animal, that emits the message, adopts a specific posture, shows a hidden part of the body, modifies the marking location and expels some individual odours. The function of these actions is to stimulate the opening of the VNO which is normally closed. These signals are emphasizing signals as they increase the pheromone emission and the likelihood of it being received. Under clinical conditions this is difficult to achieve whereby the commercial products have a greater amount of pheromone than is necessary in nature. It is necessary to inform the owner beforehand of how the pheromone



works and when it should be applied. It is important in elderly and sick animals or when it is associated with other medications (Pageat & Gaultier, 2003).

Semiochemicals used in pheromone therapy need to have peculiar characteristics (Mills et al., 2012):

- Segregation by specialized sebaceous, sweat or mucous glands;
- Organic molecules with volatile variability (most signals have fatty acids as their base);
- Small quantity production, but when used clinically there is a need for a larger quantity, to maximize the chances to get detected.

Two-hundred years ago, Jacobson described an organ anatomically different in the nasal cavity full of chemoreceptive cells. Erstwhile was referred to 'sexual nose' as it was involved in sexual behaviours (Stowers & Marton, 2005). Some preliminary evidences support the theory that the VNO has a sexual function. They conducted a study in wolves and evaluated their response to a sexual pheromone. The appearance of a certain pheromone matches with the ovary cycle and ovulation. The same compost was later collected in heat female secretions (Lindsay, 2000b).

Pheromones are detected by the VNO or Jacobson's organ which makes part of the accessory olfactory tract (AOT). In most species, this organ is inside a capsule formed by cartilage or the vomer bone. It is located in each side of the nasal septum, in the rostral end of the hard palate and in the dog measures approximately 4 cm (Figure 9) (Landsberg et al., 2013; Lindsay, 2000a; Mills, 2005; Pageat & Gaultier, 2003).

Perception of odours is spontaneous, during breathing they are diverted to the olfactory mucosa. Perception of pheromones is different, and it is not completely understood. The perception of the pheromones is made by a specialized organ the VNO, which is not easily accessible during breathing, it needs to be stimulated first (Miklósi, 2009; Pageat & Gaultier, 2003). Olfactory perception is associative, in other words, dogs learn to associate a smell to a determinate object, food, among others. In addition to this, a behaviour towards a specific stimulus can be altered. The same does not occur with pheromones, we can say that they are in a "hardwire", they have an intrinsic value (Stowers & Marton, 2005). Pheromones detection is more targeted and time-constrained because the VNO needs direct stimulation (Mills, Dube, & Zulch, 2012).

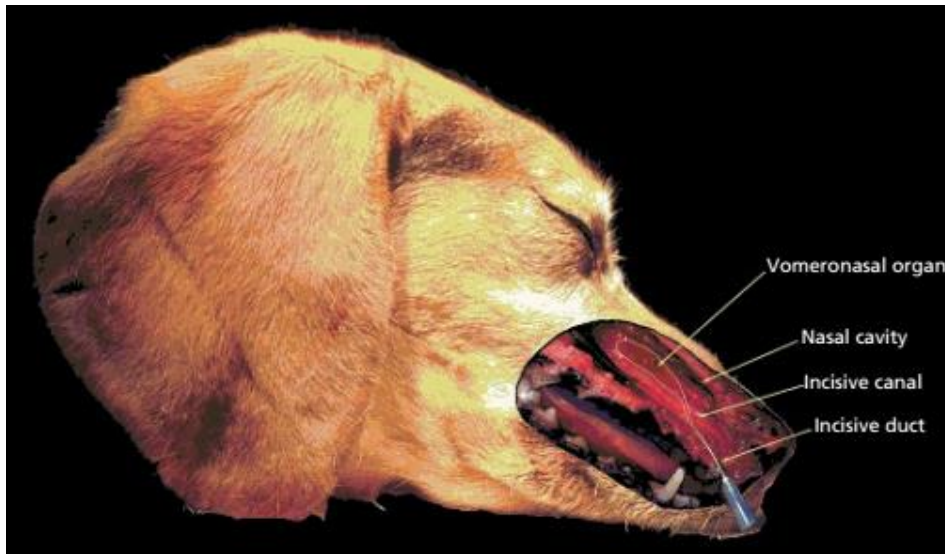


Figure 9 – Vomeronasal location and structure in dogs.

(Adapted from Mills, 2005)

Its ducts open into the mouth, right behind the frontal teeth. It is an elongated pouchlike structure (Lindsay, 2000a) and it has muscle fibres that move in a way that allows the pheromones' suction (Pageat & Gaultier, 2003). The lumen contains a mucous similar to the one present in the respiratory tract, as well as specific lipocaline proteins that capture the pheromones (Pageat & Gaultier, 2003). Pheromones connect to pheromone-binding proteins, each one specific for one type of pheromone (Landsberg et al., 2013).

VNO is more sensible to detection of non-volatile chemical messages, as well as detection and analysis of sexual hormones. Destruction of the VNO results in loss of sexual activities and other functions, such as maternal care in mammals (Lindsay, 2000a). Surgical removal of the VNO in rats showed changes in sexual behaviour, dominance status and individual identity (Dulac, 2000).

Communication by pheromones is a two-component system, it implies signalling pheromones and receiving sensory neurons (Stowers & Marton, 2005). Cells from VNO seem to be more specific to certain pheromones whereas neurons of the olfactory epithelium (OE) seem to be more generalist. Information processing by the VNO and the OE is different, but it can overlap sometimes (Overall, 2013). Another factor to consider is that the nasal cavity has two sectors of independent olfactory neurons (Dulac, 2000). VNO is different when compared to the main olfactory tract - the connections between the sensory cells and the accessory olfactory bulb (AOB) glomeruli and those in the main olfactory bulb (MOB) are completely distinct (Halpern, 1987; Pageat & Gaultier, 2003). In the MOB there are different receptors to

different odours. Each sensory cell is connected to only one glomerulus – this increases the precision and sensitivity. In the AOB, each cell is linked to multiple bulbs, which receive cells' information from different receptors, creating a complex coding with a limited number of receptors (Pageat & Gaultier, 2003).

After a genetic evaluation of the components in the olfactory system, it is possible to affirm that the AOB and the MOB developed independently (Dulac & Axel, 1995). This phenomenon is known as 'differential tuning' and suggest that the receptors of the MOE function to detect environment cues, whereas the receptors of the VNO detect species-specific cues such as pheromones (Swaney & Keverne, 2009). Contrary to the classic olfactory neurons, VNO neurons' do not adapt to a prolonged stimulus exposure (Beaver, 2009; Dulac & Axel, 1995).

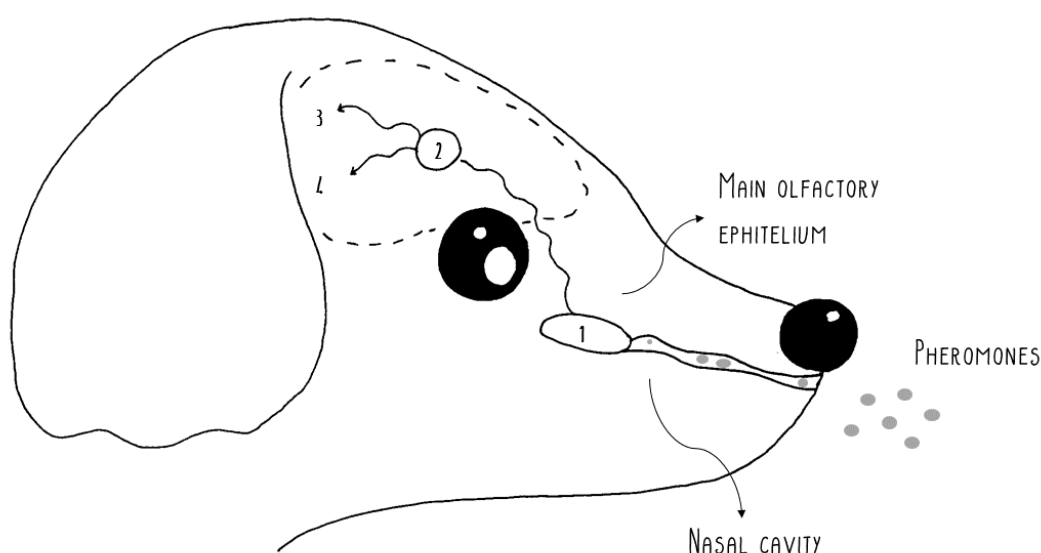


Figure 10 - VNO pathway bypasses higher cognitive structures, resulting in innate and stereotyped behavioural and neuroendocrine responses.

(Adapted from Dulac & Axel, 1995; Figure by the author)

Detection of pheromones is not a spontaneous process - due to the tightness of the nasal ducts, these have to be actively open to allow the entrance of substances (Figure 10). Licking and tongue flicking opens the ducts and allow non-volatile, high-molecular-weight substances and pheromones to access the VNO (1) (Beaver, 2009; Dulac & Axel, 1995). MOE olfactory neurons located in the posterior portion of the nasal cavity detect large amounts of small volatile chemicals that are transported through the air during respiration (Overall, 2013). The VNO is covered in olfactory receptor cells, they are similar to those found in the olfactory mucosa, however they use microvilli instead of cilia (Lindsay, 2000). The VNO neurons' consist

in bipolar neurons that leave the organ and penetrate in the cribriform plate, ending at the glomerular layer of the accessory olfactory bulb (2) (Beaver, 2009). Neurons of the AOB project through the amygdala (3) and follow to the (4) hypothalamus, with responses depending on the region and type of hypothalamic situation (Overall, 2013).

VNO is innervated by 3 nerves (Hewson, 2014; Pageat & Gaultier, 2003):

- Nasopalatine nerve: it is part of the trigeminal nerve and it includes fibres from the parasympathetic and sympathetic nervous system. It controls the vascular activity and mucus secretion by the glands (Miklósi, 2009; Pageat & Gaultier, 2003).
- Vomeronasal nerve: responsible for the transmission of the stimulation initiated by the pheromone. It is connected to the AOB and later to the amygdala and limbic system, (Pageat & Gaultier, 2003) responsible for regulating emotions, mood and memory (Hewson, 2014).
- Nervus terminalis: its actual function is not known, but it travels from nose to the brain (Pageat & Gaultier, 2003).

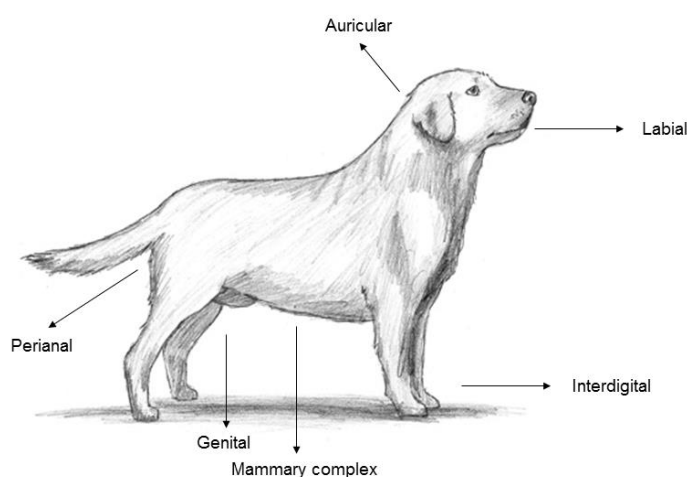


Figure 11 - Primary pheromone-secreting glands in dogs are labial, auricular, perianal, genital, interdigital (pedal) and mammary complex (Adapted from Beaver, 2009)

The main known pheromones and structures secreting pheromones (Figure 11) are:

- **Facial area:** exists in dogs and cats. This area is more important for cats, because it is here that are located their most significant pheromones - F2, F3 and F4. Dogs have another gland, the ear gland, which consists in some ceruminous and sebaceous glands. This area is associated with social relationships - ear gland secretions seem to be involved in the social status. For example, some secretions are only released by dogs with an assertive behaviour (Beaver, 2009; Mills et al., 2012; Pageat & Gaultier, 2003).

- **Pedal complex:** consists in the pedal glands of all extremities. They are diffuse structures present in both plantar pads and interdigital space. It is related to territory marking behaviour and the production of alarm pheromones (Pageat & Gaultier, 2003), therefore fear can be a consequence of alarm pheromones left behind by another individual (Vilanova, 2002). They are associated with a scratching behaviour, usually occurring on the ground. It is believed that dogs act this way to leave a visual mark in addition to the olfactory mark resulted from paws' pheromones (Beaver, 2009).
- **Perianal complex:** consists in the supracaudal glands, circumanal glands and anal sacs. The first ones are more developed in cats. In dogs, they are associated with sebaceous disorders. Circumanal glands include sebaceous glands and modified sweat glands spread around the anus. They are essential to dogs and tend to have a bigger size in males. Secretions play a vital role for social life and the fur colour around this site is also important for the communication signals' perception (Beaver, 2009; Pageat & Gaultier, 2003). Dogs have two symmetrical anal sacs that open into a small ostium between the anus and the skin. Like the previous glands, it is also composed by sebaceous and modified sweat glands. The secretion can vary in colour and it is susceptible to bacterial disease. Consequently, this can affect the chemicals signs emitted and cause social disturbances. This way, it is imperative to consider this gland when talking about behaviour problems, such as aggression in a multiple-dog house (Beaver, 2009; Pageat & Gaultier, 2003). There is some debate regarding its function, even though it is known that it is essential to individual recognition (Bowen & Heath, 2005; Horwitz et al., 2005; Overall, 2013). The secretion is usually released upon elimination (Beaver, 2009), however this can also occur when the dog is afraid or distressed (Case, 2010).
- **Genital complex:** includes the sebaceous glands of the prepuce or vulva and urethral or genital mucous glands together. In dogs, this complex is highly explored during social interactions. Secretions are particularly important for females in heat (Pageat & Gaultier, 2003; Vilanova, 2002). Sexually experienced males are more attracted to urine and vaginal secretions of females in oestrus than females in dioestrus (Beaver, 2009).
- **Urine and faeces:** both are a complex source of pheromones. Urine marking is easily recognized and, although it is observed in both gender, it is more marked in males (Beaver, 2009; Overall, 2013). Males have twice the tendency to use urine to mark territory when compared to females. Raised leg is the most common posture adopted for this behaviour. It is also thought that this posture helps to place the odours at the nose level (Beaver, 2009). It can be associated with ground scratching, especially when

there are females in oestrus in the surroundings (Pageat & Gaultier, 2003). Wolves use their faeces as a way of marking territory (Bowen & Heath, 2005). On one hand, this behaviour is not important for dogs (Pageat & Gaultier, 2003), on the other hand, dogs seem to show interest in other dogs' faeces (Beaver, 2009). Usually, elimination is related to training and its function is to alert to the presence of another individual (Bowen & Heath, 2005). Odour diminishes as time passes by and allows the dog to determinate how long another dog had passed there. It also informs about sex, sexual reception and status (Bowen & Heath, 2005).

- **Mammary complex:** this area was discovered recently and was first isolated from sows. Later, it was recognized in other animals, including dogs. Because these pheromones have an appeasing effect, they are called appeasines. They are segregated by sebaceous glands of the sulcus between the mammary glands. The appeasines of the dog and the cat have the same chemicals structure than other species - 3 fatty acids (oleic acid, palmitic acid and linoleic acid, associated in the same ratio). Furthermore, there are other chemicals specific to each species. These pheromones are not released immediately, only a few days (3-4 days) after the partition and persists 2-5 days after the puppies' weaning (Pageat & Gaultier, 2003).

## 8.1 - Dog-appeasing Pheromone

The appeasing pheromone of the dog was first identified in 1999 by a French veterinarian, Patrick Pageat (Horwitz et al., 2014). During the first life phases of a puppy, smell seems to be an important factor to promote reassurance, secure behaviour and establish bonds with his mother and littermates. In the wild, the dog-appeasing pheromone (DAP) seems to have an insignificant role in adaptation, but artificial selection allowed domestic breeds to gain the ability to retain and to perceive this pheromone. The action mechanism is not fully understood, however its effects may be related to the hormone prolactine, that acts as a neurotransmitter and helps to reduce anxiety (Hewson, 2014).

Adaptil® is a synthetic analogue of the appeasing pheromone produced by lactating female dogs during the first days after labour (Case, 2010; Landsberg et al., 2013; Pageat & Gaultier, 2003). It has beneficial modulatory effects regarding separation distress and it yields a soothing effect on nursing puppies and helps them to guide them in a new environment (Lindsay, 2000b; Mills et al., 2012). Dog-appeasing Pheromone (DAP) is also known to diminish fear and stress. Another use is to facilitate the transition between leaving his mother

and littermates and going to a new home (Lindsay, 2000b). Synthetic compounds of this pheromone seem to have the same effect on puppies and adults (Landsberg et al., 2013).

Currently exist 3 types of synthetic analogues of pheromones on the market, three for cats (F3, Cat Appeasing Pheromone and Feline Interdigital Semiochemical) and one for dogs (DAP) (Bowen & Heath, 2005). DAP is available in different commercial formats, such as a plug-in diffuser, a spray and an impregnated collar (Case, 2010; Landsberg et al., 2013). Effects of DAP seem more subtle than the synthetic analogues for feline pheromones, so it is recommend to use it with a modification behaviour programme (Landsberg et al., 2013; Mills, 2005). It is not clear that current commercial formulas are sufficient so dogs can show “tongue flicking” (Hewson, 2014). Depending on the formulation, pheromones have the potential to be applied on physical objects or directly on the environment (Bowen & Heath, 2005). The spray formulation is usually used to treat punctual and specific problems related to the environment, whereas the plug-in diffuser allows an aerial diffusion (Mills et al., 2006). Synthetic analogues can be used alone, or, since there is no systemic absorption, used safely as a therapeutic complement. DAP is recommended as an adjunct treatment to desensitization and counterconditioning programme in stress situations (Bowen & Heath, 2005). They have no known side effects or toxicity (Landsberg et al., 2013). The diffuser is most suited for preparing the arrival of a new dog or puppy, reducing stress in a clinical environment, behaviours related to fear or anxiety of fireworks or being alone. The spray and collar are suited for car travels or to treated aversive stimuli outside (Mills, 2005).

## 8.2 - Applications of Dog-appeasing Pheromone

Pheromones are not drugs, their use in the clinical field is very promising and aids to modify behaviour problems (Vilanova, 2002). It was particular advocated to have a prophylactic role for newly adopted puppies and help them with the transition to a new home and other novelty situations (Bowen & Heath, 2005).

Several studies were made to test the efficacy of DAP in different contexts such as anxiety behaviours (Gaultier, Bonnafous, Bougrat, Lafont, & Pageat, 2005), distress related behaviour in the veterinary clinic (Mills et al., 2006), animals' hospitalization (Kim et al., 2010; Siracusa et al., 2010), sound aversion (Landsberg et al., 2015; Levine et al., 2007), car travelling (Gandia Estellés & Mills, 2006) and kennel/shelter facilities (Grigg & Piehler, 2015; Tod, Brander, & Waran, 2005). Furthermore, other studies were made in puppies, to evaluate the consequences of DAP in puppy classes and socialization/training (Denenberg &

Landsberg, 2008; Graham, Mills, & Bailey, 2007), disturbance at night and house soiling (Taylor & Mills, 2007) and how newly-adopted puppies deal with unfamiliar people and surroundings (Gaultier et al., 2009). Studies have also showed that using DAP combined with a behaviour plan helps to reduce undesirable behaviours related to separation anxiety, such as destruction, vocalization, elimination problems, excessive licking, sleeping and feeding problems (Gaultier, Bonnaïfous, Bougrat, Lafont, & Pageat, 2005).

Several studies were made to assess the effect of DAP in puppies in different situations.

One of those studies investigated to what extent DAP could decrease fear and anxiety in puppies and how affects training and socialization. The authors enrolled 45 puppies, aging between 12 and 15 weeks. There were significative differences between the DAP group and the placebo one in terms of fear and anxiety levels. The DAP group had more positive interactions (were more relaxed and engaged more often in play) and were better socialized, in other words, they could adapt more rapidly to novelty. Learning ability during training or puppy classes is diminished when puppies suffer from high levels of stress, fear, anxiety and arousal, thus the use of pheromones can help to reduce those previous factors. Ability to learn was similar in both groups. Fear and arousal levels were significative lower in the DAP group and, for this reason, they performed better during training. Puppies' owners from the DAP group reported higher levels of satisfaction towards their companions, however the differences were not relevant. This can be explained by the fact that the puppies were obtained to be companion animals and regardless of using a DAP, puppy classes and training help to build a strong bond between dog-owner (Denenberg & Landsberg, 2008). Graham, Mills and Bailey (2007) previous study corroborate the study conducted by Denenberg and Landsberg (2008), claiming that DAP helps to reduce arousal and acoustics signals, therefore having a beneficial effect during puppy classes (Denenberg & Landsberg, 2008; Graham, Mills, & Bailey, 2007).

Puppies vocalize more often around 6-9 weeks and it is around this time that they usually are adopted. An investigation studied how DAP could influence common behaviour problems, such as disturbance at night and house-soiling. The study was conducted using two distinct groups, one with a DAP diffusor and the other not. To have more realistic results, authors considered gender, breed and differences between the previous and current environment - breed or gender did not influence the overall results obtained. Owners gave an overall feedback by filling a questionnaire regarding two parameters: disturbed them at night and gone to toilet. Both parameters are aggravated in the first nights. Puppies who slept with other dogs, had lower tendency to cry, however, another dog presence did not affect house soiling. One reason behind this, may reside in the fact that ear glands seem to have a similar affect to the appeasine, produced by lactating females and can be a signal of reassurance



(Mills et al., 2012). Disturbance at night improved more quickly than house soiling. House soiling was the parameter which suffered more alterations, taking into account the sudden change in the environment, and it is also related to training. In summary, the authors suggest that DAP helps to prevent extended periods of disturbance at night. Even though, sometimes the initial introduction to a new house and the isolation at night can be too overwhelming to DAP produce effect (Taylor & Mills, 2007).

Pursuing this further, another study was conducted in newly adopted puppies to evaluate how DAP affected them regarding fear to unfamiliar people and new surroundings, two situations frequent in this phase of life. Similar to the previous study, the results were based on owners' feedback. There is a lack of studies regarding the effects of novelty in daily situations. This study integrated puppies who were adopted from a pet-shop, meaning that they were already submitted to stressful situations and were more sensible when they were adopted. Puppies wearing a DAP collar after adoption show less avoidance and uneasiness. They also tested how puppies could react to possible new and frightening situation, such as the presence of shopping bags. This situation was only mildly fear-eliciting, so there were not significative differences between the 2 groups. In summary, they suggest that DAP helps puppies to adapt quicker to novelty and helps them to cope with stressful situations. This goes in line with the study from Sheppard and Mills (2003). DAP does not change dogs' awareness of recognition of stimuli, but helps them to not react fearfully. Puppies from the control group showed more signs of fear in two particular situations: strangers without interaction and stranger with interaction. DAP helps puppies to adapt quicker and reduces the number of exposures needed to create habituation towards a situation. The study lasted for 15 days which was sufficient to create changes on puppies behaviour, suggesting that owners should continue to use the collar longer during this vital period (Gaultier et al., 2009).



## Chapter II - Assessment of Adaptil® collar to help handle stress and anxiety in puppies during veterinary consultations

### 1 - Objectives

The main objective of this study was to determinate to what extent the use of an Adaptil® collar (impregnated collar with the analogue of dog-appeasing pheromone) affects and helps to diminish stress and anxiety in veterinary consultations and simultaneously contributes to animal welfare and quality of the consultation.

### 2 - Material and Methods

The clinical trial was designed in a randomized, double-blind and placebo-controlled with the intent to assess comparatively the usage of Adaptil® versus a placebo. The choice of a double-blind study was based on the need for a free judgment of the observer and other participants, from any outer influence other than the conditions of the study itself. Exploiting the fact that all studies involving drugs' administration are quite easy to turn into double-blind studies, this one took advantage of the randomness in this kind of investigations, dodging all risks of having conclusions affected by influenced opinions.

It was carried out at the veterinary clinic Liga Portuguesa dos Direitos do Animal (Carcavelos, Lisbon, Portugal), during the following period: 14 November 2016 - 31 March 2017.

Phase	Period	Phase description
1	14-20 November 2016	Trial preparation. This period served for the observer to be familiarized with the environment and methods used during consultations. There was no data collected during this period.
2	Starting 21 November	Observation/logging with no products being used. This phase corresponds to the first vaccination for all puppies, acting as a control group, because puppies included in the study were not under the effect of any pheromone compounds previously.
3	5 December – 31 March	Observation and registration during the vaccination boost consultation. This phase corresponds to second and/or third consultations for all puppies included in the study.
4	April/May 2017	Statistical analysis based on collected data.

The first phase consisted of a period of a week, during which the observer tested and adapted to the ethogram and registration form. Following, a second phase of the study during which the observer logged the behaviour of each puppy with no product being used (first consultation, core vaccination). During the following phase, the observer logged the puppies' behaviour, being that some had a collar with Adaptil® and others a collar with a placebo component. Throughout this phase, each puppy had at least two consultations, the first one regarding the initial core vaccination, the second one (vaccination boost) and a third one (another vaccination boost) (Figure 12).

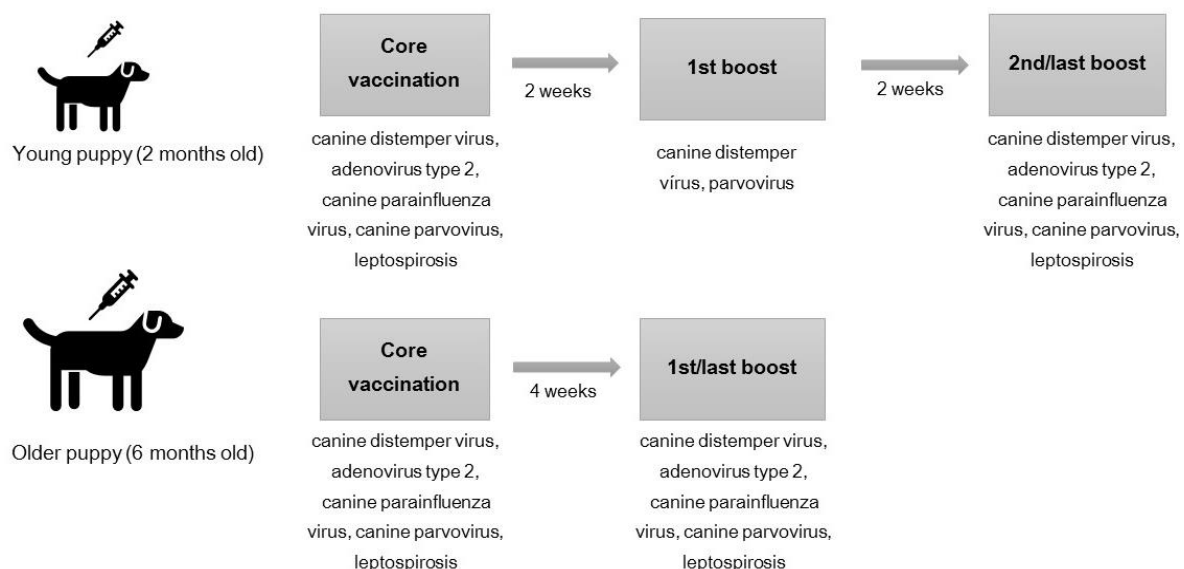


Figure 12 – Systematic protocol for puppies' vaccination followed up during the study.

(Figure by the author)

According to the current guidelines, a vaccination plan should be adapted individually to each puppy and his environment conditions. Puppies immunity is granted in early life by maternal anti-bodies. As they grow up these compounds are reduced, leaving the puppy unprotected. Vaccination should begin ideally at two months old (6-8 weeks old). The periodicity of boosts depends on the age of the puppy, if the initial protocol was strictly completed or even if the puppy lives in a high-risk area. All this is taken into account, so some younger puppies have two vaccination boosts, while older puppies (which already developed some active immunity) only take one vaccination boost, 4 weeks apart from the core vaccination (Day, Horzinek, Schultz, & Squires, 2016; Ford et al., 2017). This protocol is advantageous for the study, because allows a pattern schedule for all enrolled puppies. It was made an appointment with the owners to ensure that every puppy followed their vaccination protocol in time.

Due to the small sample and not to reduce even further the number of subjects, waiting time was not considered a factor of exclusion. Ideally, puppies with a waiting time superior to 25 minutes should have been excluded. Still, in an attempted to overcome the fact that waiting can have a stress effect, owners were asked if there was any incident while in the waiting room that could have altered their puppy emotional state.

Every consultation included an anamnesis, physical exam and vaccination. Additional procedures such as external/internal deworming, microchip placement, ear exam and cleaning, and nail trimming were recorded for each consultation and performed when needed. The puppy was weighed first and then placed in the exam table. Owners were asked some questions regarding their puppy's health state as well information to fill in the formulary (Figure 13, figure 14). Additionally, the veterinary surgeon gave some behaviour counselling to prevent some future behaviour problems. During the second and third visit it was also asked if the owners thought if the collar had affected their puppy's behaviour between consultations (more stressed/less stressed or no difference). The basic physical exam included a mucous membranes examination (oral, ocular), capillary refill time, turgor test, cardiopulmonary auscultation, abdominal palpation and rectal temperature assessment. It was not possible that all consultations were carried out by the same veterinarian surgeon, as the clinic worked based on a shift system, however it was recorded who performed each consultation in the data form and the observer was the same.

**CASE REPORT FORM: "Assessment of Adaptil® collar to help handle stress and anxiety  
in puppies (*Canis lupus familiaris*) during veterinary consultations"**

**CORE VACCINATION**

Form no. \_\_\_\_\_

Puppy's ID \_\_\_\_\_

1. Date (dd/mm/yy) \_\_\_\_/\_\_\_\_/\_\_\_\_
2. Collar A ☐ B ☐ C ☐ D ☐
3. Waiting time (min) \_\_\_\_\_
4. Accidents in waiting room Y ☐ N ☐
5. Puppy's name \_\_\_\_\_
6. Owner's name \_\_\_\_\_
7. M ☐ F ☐ 8. Outdoor ☐ Indoor ☐
9. Age (months) \_\_\_\_\_
10. Breed \_\_\_\_\_
11. Current health condition  
     Good ☐  
     Bad ☐ Reason \_\_\_\_\_ Exclusion ☐
12. Diagnosed disease  
     Yes ☐ Which \_\_\_\_\_ Exclusion ☐  
     No ☐
13. Current medication  
     Yes ☐ Which \_\_\_\_\_ Exclusion ☐  
     No ☐
14. 1<sup>st</sup> puppy obtained Y ☐ N ☐ 15. Other pets Y ☐ N ☐ Which \_\_\_\_\_
16. Place where the puppy was obtained \_\_\_\_\_
17. Attends puppy classes/training Y ☐ N ☐
18. Medical procedures  
     ☐ Vaccination ☐ Microchip placement  
     ☐ External deworming ☐ General physical exam  
     ☐ Internal deworming ☐ Blood sampling  
     ☐ Others \_\_\_\_\_  
     ☐ Exclusion \_\_\_\_\_
19. Consulted by \_\_\_\_\_
20. Total puppy score \_\_\_\_\_
21. Post-inclusion exclusion Y ☐ N ☐ Reason \_\_\_\_\_

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Figure 13 – Form for the first visit (core vaccination)

**CASE REPORT FORM: "Assessment of Adaptil® collar to help handle stress and anxiety in puppies (*Canis lupus familiaris*) during veterinary consultations"**

Form no. \_\_\_\_\_

Puppy's ID \_\_\_\_\_

1. Date (dd/mm/yy) \_\_\_\_/\_\_\_\_/\_\_\_\_

2. Collar A ☐ B ☐ C ☐ D ☐

3. Waiting time(min) \_\_\_\_\_

4. Accidents in waiting room Y ☐ N ☐

5. Outdoor ☐ Indoor ☐

6. Age (months) \_\_\_\_\_

7. 1<sup>st</sup> boost ☐ 2<sup>nd</sup> boost ☐

8. Current health condition

Good ☐

Bad ☐

Reason \_\_\_\_\_

Exclusion ☐

9. Diagnosed disease

Yes ☐

Which \_\_\_\_\_

Exclusion ☐

No ☐

10. Current medication

Yes ☐

Which \_\_\_\_\_

Exclusion ☐

No ☐

11. Attends puppy classes/training Y ☐ N ☐

12. Medical procedures

☐ Vaccination

☐ Microchip placement

☐ External deworming

☐ General physical exam

☐ Internal deworming

☐ Blood sampling

☐ Others \_\_\_\_\_

☐ Exclusion \_\_\_\_\_

13. Consulted by \_\_\_\_\_

14. Total puppy score \_\_\_\_\_

15. Owner's assessment

☐ No difference/same behavior as last vaccination visit

☐ Puppy looked more relaxed/less stressed

☐ Puppy looked more agitated/more stressed

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Figure 14 – Form for the second and third visit (boost vaccination)

Consultations took part in the same room to avoid any external influences. The room temperature and other environment features were considered and regulated as much as possible. For instance, Mills et al. (2012) reported that lightning in the consultation room can influence a dogs' behaviour, in other words, a flicking neon light may seem harmless to us, but can cause visual stress to the animal, increasing its levels of awareness and vigilance (Mills et al., 2012). During the study length, light conditions were controlled to assure homogeneity. Room temperature can have a positive or negative effect. According to specialists, the temperature should be higher than 10°C and lower than 29,5°C, being the ideal temperature between 20°C to 30 °C (thermoneutral zone). When dealing with puppies, there should be extra care, because they cannot fully regulate their temperature (Jordan, Stella, Croney, & College, 2015). This parameter was controlled via an air conditioner and airing the room between consultations.

In veterinary practice, a dog showing fear signals during a consultation can produce alarm pheromones and leave a powerful message for the next patient. The following animal will detect the pheromone and may be feel anxious and distressed (Mills, Dube, & Zulch, 2012). To minimize any chemical traces of stress from previous animals, between consultations the room was aired, by opening the door, and the surfaces where the clinical exam took part were cleaned with disposable paper towels and a cleaning product to denatures alarm chemical signals and disinfect the surface. This was strictly repeated for each consultation to avoid unnecessary variations that could affect the study's results.

Puppies with more than 2 months and less than 7 months were included in this clinical trial. Gender and breed was not a non-inclusion parameter. 37 puppies were included in the study, however only 30 were statistically analysed. As a requisite, the first visit should correspond to the first vaccination (core vaccination) ever made to the puppy and he should be followed in the same veterinary clinic, to follow up until the last boost. This guarantees that the puppy did not had previous traumatic or positive association with the veterinary clinic or clinical procedures for the core vaccination. As soon as the puppy arrived at the veterinary clinic, his arrival time was recorded. Waiting time was registered for every puppy and every visit, but it was not considered an exclusion factor. Even though, to minimize influences in the statistical analysis, owners were asked if there was any incident in the waiting room that could potential have stressed their puppy. All puppies had good health condition and no diagnosed disorders. Even though, one puppy was medicated with Terricil® (oxytetracycline); this medication did not affect the general condition of the animal, so the puppy was not excluded. None of the puppies had attended puppy classes or training, except for one puppy belonging to the placebo group at visit 3. All puppies would be excluded if at the end of the consultation,



they showed any evidence of pathology which might have caused pain, alter their behaviour or even demand much too handling during the veterinary consultation.

Collars were provided by CEVA Santé Animale S.A. free of charge and without restrictions considering the objective of the study. To carry out this study two different collars were used, an Adaptil® collar and a placebo one. CEVA identified four distinct types of collars using capital letters, A, B, C and D. The collar was given and placed by the observer, to assure the correct use according to manufacturer's instructions and decrease the variability of owners' placement. The observer was also responsible for adjusting the collar to the puppy growth in every subsequent consultation, ensuring that each collar was snug enough to skin (Figure 15).



Figure 15 – Puppy wearing a collar during the veterinary consultation.

(Photograph by the author)

The observer also made sure to register what collar each puppy is taking home, A, B, C or D, following the order set out in the randomization (Table 1). Neither the observer nor the veterinary surgeon, study coordinator or any member of the staff involved in the study had knowledge of the code until the statistical analysis stage began. All collars were identical (same color, shape, texture, weight and size), except for their label. The duration of the effect of an Adaptil® collar is reported to be 4 weeks, so in every consultation the collar was changed, assuring that the period of 4 weeks was not exceeded, and all subjects were under the effects of the dog-appeasing pheromone analogue or placebo.

	First consultation	Boost vaccination	Boost vaccination
Puppy 1	Collar A	Collar A	Collar A
Puppy 2	Collar B	Collar B	Collar B
Puppy 3	Collar C	Collar C	Collar C
Puppy 4	Collar D	Collar D	Collar D
Puppy 5	Collar C	Collar C	Collar C
Puppy 6	Collar D	Collar D	Collar D
Puppy 7	Collar B	Collar B	Collar B
Puppy 8	Collar A	Collar A	Collar A

Table 1- Collars were given according to a randomization list

To assess the stress level of each puppy, the observer drawn up an ethogram. Each parameter was assessed using a 5-point score between 0-4. In the end, all scores were sum up so to have an overall look. For example, a totally relaxed puppy has a total score of zero and on the other hand, a fully stressed puppy has a maximum total score of 40. The evaluated parameters were “body posture”, “tail posture”, “ear posture”, “gaze”, “pupils”, “respiration”, “lips”, “activity”, “handling” and “vocalization”. In order to maximize the objective assessment of data and increase validity of results, records regarding the behaviour of puppies were performed fifteen minutes after the beginning of the consultation and this was followed throughout every consultation during the study. At the end of each consultation, owners were asked to answer a questionnaire regarding their puppies behaviour for comparison with previous consultations and monitor their behaviour at home. The questionnaire had three possible answers: “no difference/same behaviour as last vaccination visit”, “puppy looked more relaxed/less stressed”, “puppy looked more agitated/fearful/stressed”.

SCORE POINTS	0	1	2	3	4
BODY POSTURE	Relaxed and moves on own	Tense - can manipulate	Rigid - hard to manipulate and a bit lower	Hunched - hard to see or examine belly and low posture	Curled - completely withdrawn and belly maximally tucked
TAIL POSTURE	At rest for that breed or high	Lower than at rest but not down	Completely down	Tucked between legs	Clamped hard up to belly
EAR POSTURE	High and softly forward	Moving back a bit	Fully back	Ears back and down	As low and back as is possible
GAZE	Will look steadily at vet	Looks only intermittently at vet	Will not look at vet but scans room	Not scanning, looking steadily at distance or owner	Staring fixedly and steadily at immediate fore-distance
PUPILS	Normal response to light	Normal to slight dilated	Dilated, large amount of iris	Dilated, small amount of iris	Completely dilated - no iris
RESPIRATIONS	Normal - jaw relaxed	Normal - jaw tense	Panting - dry	Panting - dripping	Profound panting, salivation, gasping
LIPS	Relaxed	Firm	Licking lips	Yawning and licking	"c" lip position
ACTIVITY	Flexible	Inactive	Paws, flexed, may tremble	Periodic trembling	Uncontrollable trembling
HANDLING	Allows vet to place as needed and remains loose and pliant	Allows vet to place as needed but is stiff	Allows vet to place by stretching out or manipulating areas and is rigid	Avoids manipulations by moving and stiffening, needs some restrain	Not possible to do without stretching and controlling head and legs
VOCALIZATION	None	Whine, cry	Whimper	Snart, snap	Bite

Table 2 - Scale to evaluate stress at the veterinary clinic (Based on Döring et al.,2009; Hernander, 2009)

Every owner gave his written consent to participate in the study after a brief explanation of the clinical trial. The author explained what was the main goal of the study and how the Adaptil® collar works. Owners were instructed to leave the collar on their puppy all time, preventing the puppy from removing it, as the collar releases the DAP analogue when in close contact with skin. This would be an exception when a puppy had to bath, in this case, the collar should be removed for that period, and put on after, for the product not to be washed away.

All collected data was shaped into statistical variables using a SPSS software, in order to evaluate the influence of the dog-appeasing pheromone analogue in stress and anxiety levels of puppies during veterinary consultations. This analysis was done by an independent statistician different from the observer or study coordinator. Different tests were used, based on the analysed parameters, such as Wilcoxon test and Fisher exact test. The normality hypothesis was not respected, so the statistical analysis was based on a non-parametric, 1-sided Wilcoxon test. The Fisher exact test was used due to the small sample and to reduce error occurrence.

The primary endpoint was the relative variation of total score between the first visit and second visit. Each modality of qualitative data is presented with their numbers and percentage. Regarding quantitative data, it will be described in terms of mean, median, minimum, maximum, standard deviation and inter-quartile range. Puppies included in the study were analysed regarding their age, gender, breed, living place, owners having other pets and which, place where puppy was obtained and if the puppy was the first puppy in the family. To achieve a well-balanced group between the puppies with an Adaptil® collar (corresponding to collars coded A and D) and a placebo collar (corresponding to collars coded B and C), efficacy was tested in a full-analysis set (FAS) population. A confidence level of 95% was used in this study, so p values <0,05 were considerate significant.

### 3 - Results

#### 3.1 - Analysis of the population

From the initial 37 initial subjects, 30 puppies were analysed making part of the full analysis set (FAS) population (Table 3). The FAS population included all subjects which were not excluded from the analysis for the first and second visit and two other puppies which only had the first and third visit, but considering this last one as the second one. From the total 37 puppies, 5 were excluded due to having only the first visit, 1 puppy who developed a local allergy to the collar and another puppy in which the collar was not replaced, surpassing the 4-week time (Table 4). The per-protocol (PP) population includes all subjects evaluated in the previous population, who ended the study without any major protocol deviations. This means that the 2 puppies, who had their third consultation analysed as the second one, were excluded from the PP population (Table 5). The FAS population was used for descriptive and efficacy analyses, while the PP was only used for efficacy analyses. The primary efficacy parameter was the total puppy score obtained from the form that evaluated stress levels during consultations.

	Adaptil® N= 18	Placebo N= 19	Total N= 37
<b>Total population</b>	18	19	37
<b>FAS population</b>	15	15	30
<b>PP population</b>	14	14	28

Table 3 – Allocation of subjects between Adaptil® and Placebo

	Adaptil® N= 15	Placebo N=15	Total N= 30
<b>Visit 1</b>	15	15	30
<b>Visit 2</b>	15	15	30
<b>Visit 3</b>	9	13	22

Table 4 – Allocation of subjects in the FAS population

	Adaptil® N= 14	Placebo N= 14	Total N=28
<b>Visit 1</b>	14	14	28
<b>Visit 2</b>	14	14	28
<b>Visit 3</b>	9	13	22

Table 5 – Allocation of subjects in the PP population

### 3.2 - Analysis of the demographic data

#### 3.2.1 - Gender

The descriptive analysis shows that, from all the puppies of the FAS population, 13 (43,3%) were female and 17 (56,7%) were male (Table 6). The distribution regarding gender by collar is shown in chart 1. The number of females is slight higher in the placebo group. On the other hand, the Adaptil® group analysed a higher number of males.

	Adaptil®	Placebo	Total
<b>Female</b>	4 (26,7%)	9 (60%)	13 (43,3%)
<b>Male</b>	11 (73,3%)	6 (40%)	17 (56,7%)

Table 6 – Puppies gender by collar in the FAS population

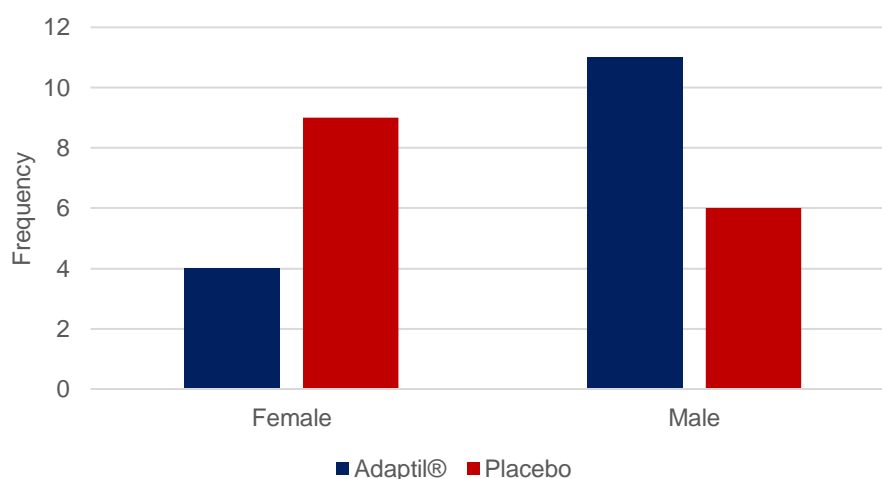


Chart 1 – Puppies gender by collar in the FAS population

### 3.2.2 - Age

The minimum age allowed for this study was 2 months old, around the time for core vaccination. The oldest puppy analysed had 7 months, however, it was excluded at the beginning of the study, as the maximum age for the analysed subjects was 5 months old. The mean age is equal for both collars, there were no major differences between the two groups for this parameter.

	Adaptil®	Placebo	Total
Mean (SD)	2,8 (1,0)	2,8 (1,0)	2,8 (1,0)
Min; Max	[2,0;5,0]	[2,0;5,0]	[2,0;5,0]
Median	2,5	2,5	2,5
Q1;Q3	[2,0;4,0]	[2,0;3,0]	[2,0;3,0]

Table 7 – Comparison of age in months between puppies tested with Adaptil® and Placebo

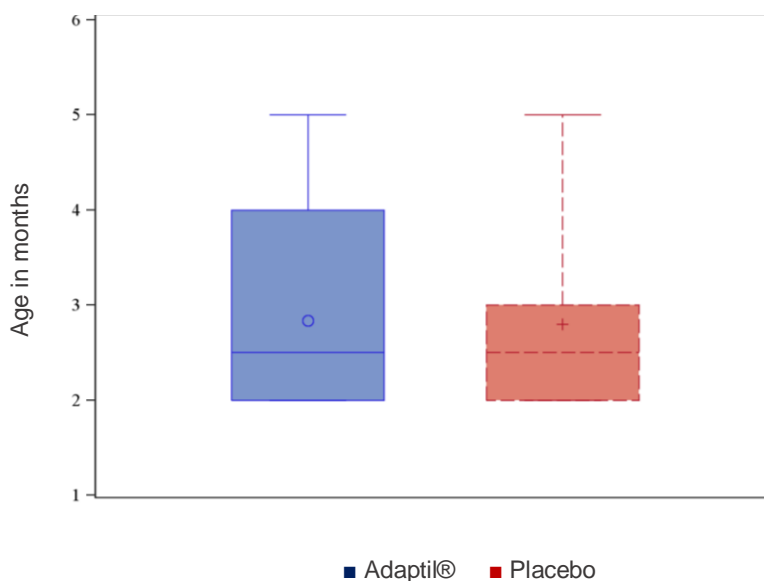


Chart 2 – Comparison of age in months between puppies tested with Adaptil® and Placebo

### 3.2.3 - Breed

A wide range of breeds was covered in this study. Most puppies were mixed breed (13; 43,3%) followed by Poodle (3;10,0%) and Labrador (3;10,0%). Breed distribution by collar is presented in table 8 and chart 3.

	Adaptil®	Placebo	Total
Poodle	2 (13,3%)	1 (6,7%)	3 (10,0%)
Cocker spaniel	1 (6,7%)	0 (0,0%)	1 (3,3%)
Dalmatian	0 (0,0%)	1 (6,7%)	1 (3,3%)
Fila São Miguel	1 (6,7%)	1 (6,7%)	2 (6,7%)
Jack Russel Terrier	0 (0,0%)	1 (6,7%)	1 (3,3%)
Labrador	0 (0,0%)	3 (20,0%)	3 (10,0%)
Golden Retriever	1 (6,7%)	0 (0,0%)	1 (3,3%)
Mixed breed	6 (40,0%)	7 (46,7%)	13 (43,3%)
Pinscher	0 (0,0%)	1 (6,7%)	1 (3,3%)
Pitbull	1 (6,7%)	0 (0,0%)	1 (3,3%)
Podengo mini	1 (6,7%)	0 (0,0%)	1 (3,3%)
Portuguese water dog	1 (6,7%)	0 (0,0%)	1 (3,3%)
Staffordshire terrier	1 (6,7%)	0 (0,0%)	1 (3,3%)

Table 8 – Puppies breed by collar in the FAS population

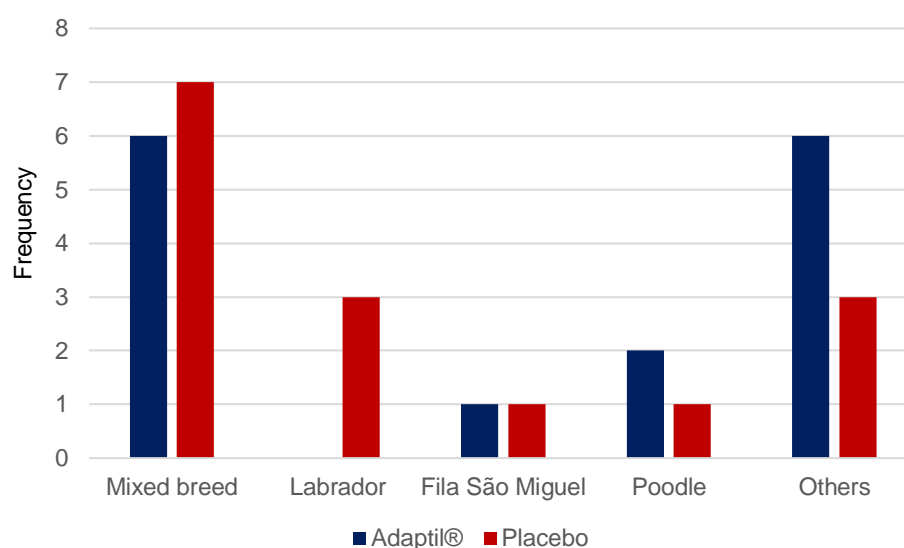


Chart 3 - Puppies breed by collar in the FAS population

### 3.2.4 - Living place and living with other pets

Regarding the living place, most owners answered that their puppies lived most of their time indoors. This tendency was equal for the Adaptil® and placebo collars (Table 9, chart 4). Additionally, owners were asked if they had other pets. Some owners answered yes whereas others responded no, almost at a ratio of 50%/50% (Table 10, chart 5). For those



owners who answered yes, most of them owned another dog, followed by owning a cat and small rodents, such as guinea pig and chinchilla (Table 11).

	Adaptil®	Placebo	Total
Indoor	12 (80%)	14 (93,3%)	26 (86,7%)
Outdoor	3 (20%)	1 (6,7%)	4 (13,3%)

Table 9 - Puppies living place by collar in the FAS population

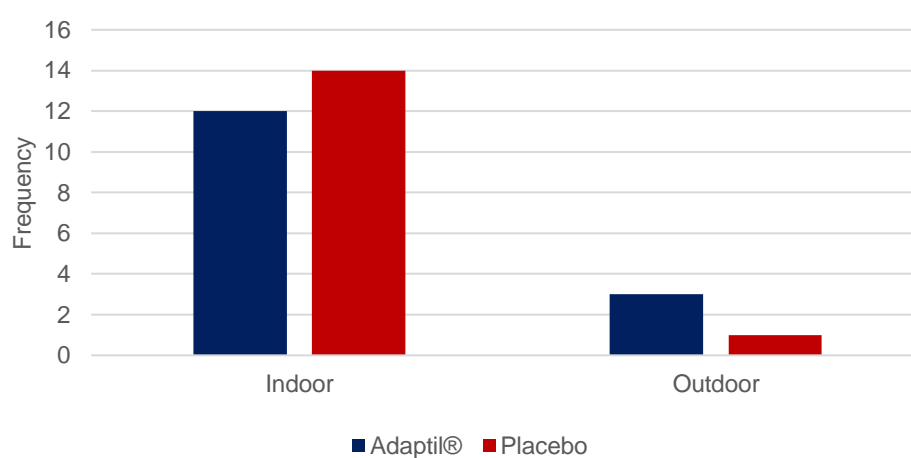


Chart 4 – Puppies living place by collar in the FAS population

	Adaptil®	Placebo	Total
No	9 (60,0%)	8 (53,3%)	17 (56,7%)
Yes	6 (40,0%)	7 (46,7%)	13 (43,3%)

Table 10 - Owners having other pets in the FAS population

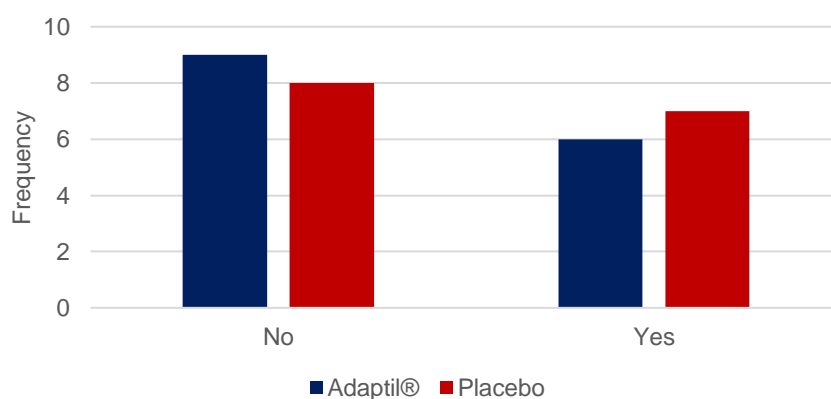


Chart 5 - Owners having other pets in the FAS population

	Adaptil®	Placebo	Total
Cat	2 (33,3%)	0 (0,0%)	2 (15,2 %)
Dog	3 (50,0%)	6 (85,7%)	9 (69,2 %)
Dog, cat and chinchila	0 (0,0%)	1 (14,3%)	1 (7,7 %)
Guinea pig	1 (16,7%)	0 (0,0%)	1 (7,7%)

Table 11 – Other pets according to owners the FAS population

### 3.2.5 - First puppy obtained

Most owners (22;73,3%) already had experience with previous dogs. The previous fact is similar to both collars (table 12, chart 6).

	Adaptil®	Placebo	Total
No	10 (66,7%)	12 (80%)	22 (73,3 %)
Yes	5 (33,3%)	3 (20%)	8 (26,7%)

Table 12 – Puppy being the owners' first puppy in the FAS population

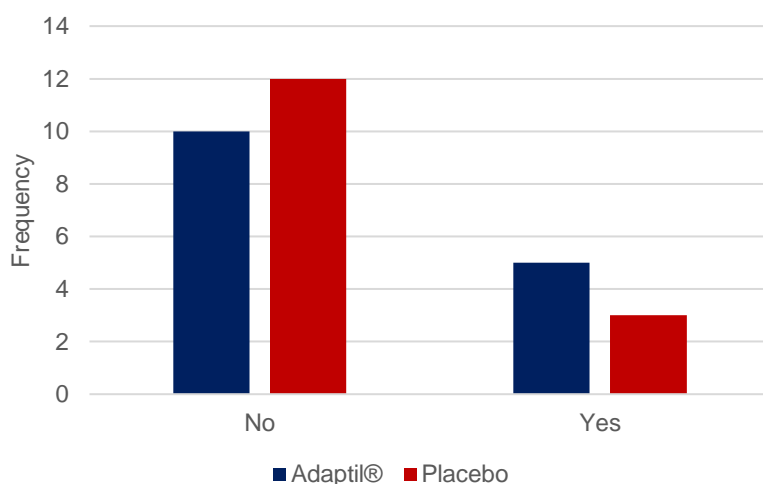


Chart 6 - Puppy being the owners' first puppy in the FAS population

### 3.2.6 - Place where puppy was obtained

As shown in table 13 and chart 7, the number of puppies that were adopted, for Adaptil® collar and placebo collar, was relatively higher than the number of puppies who were bought from a breeder.

	Adaptil®	Placebo	Total
Adoption	9 (60%)	10 (66,7%)	19 (63,3%)
Breeder	6 (40%)	5 (33,3%)	11 (36,7%)

Table 13 – Place where puppy was obtained in the FAS population

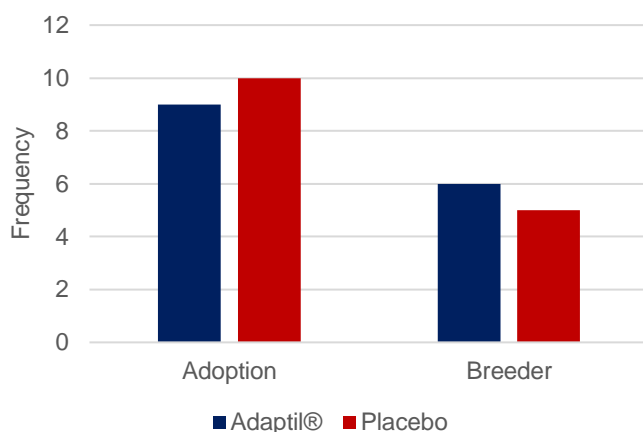


Chart 7 – Place where puppy was obtained in the FAS population

### 3.3 - Waiting time

The descriptive analysis shows that the average waiting time was between 9 and 15 minutes. However, it is important to consider that there are wide intervals of time, for example, for the placebo group, at visit 2, the minimum was 2 minutes, whereas the maximum was 50 minutes. Overall, considering only the average waiting time for both groups they are quite similar (table 14, chart 8).

	Adaptil®	Placebo
<b>Visit 1</b>		
Mean (SD)	11,13 (8,69)	10,4 (7,74)
[Min; Max]	[2;30]	[2;30]
Median	10	10
[Q1;Q3]	[5;15]	[5;15]
<b>Visit 2</b>		
Mean (SD)	11,3 (9,85)	15,9 (13,48)
[Min; Max]	[1;40]	[2;50]
Median	10	15
[Q1;Q3]	[5;15]	[9;20]
<b>Visit 3</b>		
Mean (SD)	9,22 (5,38)	12 (12,36)
[Min; Max]	[3;20]	[2;45]
Median	8	5
[Q1;Q3]	[5;10]	[3;15]

Table 14 - Comparison of waiting time in minutes between Adaptil® and Placebo group

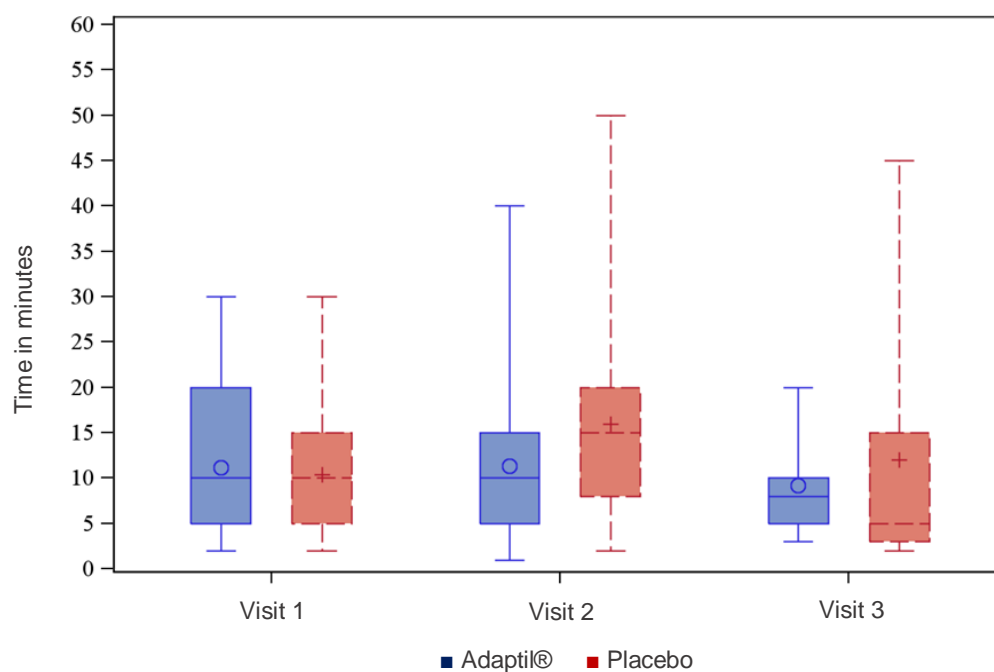


Chart 8 - Comparison of waiting time in minutes between Adaptil® and Placebo group

### 3.4 - Additional medical procedures

Besides being submitted to the normal physical exam and vaccination, some puppies also had to be dewormed externally and/or internally, as well proceed to microchip placement. These three parameters were registered for each puppy and each subsequent consultation. Information corresponding to the core vaccination is shown in table 15 and 16. During the first visit, a lower number of puppies were dewormed externally and internally. Only one puppy was submitted to microchip placement. Regarding other procedures, ear cleaning and nail trimming were the most frequent, whereas other parameters had the same frequency. At the second visit, most dogs were not dewormed externally, contrary to internal deworming. Microchip placement was done in two puppies and only two puppies were submitted to additional medical procedures, being that ear cleaning and nail trimming (table 17 and 18). In the last visit for some puppies, visit 3, the proportion between internal deworming and external is the same. Similar to the core vaccination, only one puppy had his chip placed. Most puppies did not have other medical procedures (table 19 and 20).

	Adaptil®	Placebo	Total
<b>External deworming</b>			
No	13 (86,7%)	13 (86,7%)	26 (86,7%)
Yes	2 (13,3%)	2 (13,3%)	4 (13,3%)
<b>Internal deworming</b>			
No	12 (80,0%)	9 (60,0%)	21 (70,0%)
Yes	3 (20,0%)	6 (40,0%)	9 (30,0%)
<b>Microchip placement</b>			
No	14 (93,3%)	15 (100,0%)	29 (96,7%)
Yes	1 (6,7%)	0 (0,0%)	1 (3,3%)

Table 15 – Medical procedures for visit 1 for FAS population

	Adaptil®	Placebo	Total
<b>Ear cleaning</b>	1 (25,0%)	0 (0,0%)	1 (16,7%)
<b>Ear exam</b>	0 (0,0%)	1 (50,0%)	1 (16,7%)
<b>Ear cleaning and exam</b>	1 (25,0%)	0 (0,0%)	1 (16,7%)
<b>Ear cleaning and nail trimming</b>	2 (50,0%)	1 (50,0%)	3 (50,0%)
<b>None</b>	11	13	24

Table 16 – Other medical procedures for visit 1 for FAS population

	Adaptil®	Placebo	Total
<b>External deworming</b>			
No	11 (73,3%)	14 (93,3%)	25 (83,3%)
Yes	4 (26,7%)	1 (6,7%)	5 (16,7%)
<b>Internal deworming</b>			
No	5 (33,3%)	5 (33,3%)	10 (33,3%)
Yes	10 (66,7%)	10 (66,7%)	20 (66,7%)
<b>Microchip placement</b>			
No	13 (86,7%)	15 (100,0%)	28 (93,3%)
Yes	2 (13,3%)	0 (0,0%)	2 (6,7%)

Table 17 - Medical procedures for visit 2 for FAS population

	Adaptil®	Placebo	Total
<b>Ear cleaning and nail trimming</b>	0 (0,0%)	2 (100,0%)	2 (100,0%)
<b>None</b>	15	13	28

Table 18 - Other medical procedures for visit 2 for FAS population

	Adaptil®	Placebo	Total
<b>External deworming</b>			
No	9 (100,0%)	7 (53,8%)	16 (72,7%)
Yes	0 (0,0%)	6 (46,2%)	6 (27,3%)
<b>Internal deworming</b>			
No	6 (66,7%)	10 (76,9%)	16 (72,7%)
Yes	3 (33,3%)	3 (23,1%)	6 (27,3%)
<b>Microchip placement</b>			
No	9 (100,0%)	12 (93,2%)	21 (95,5%)
Yes	0 (0,0%)	1 (7,7%)	1 (4,5%)

Table 19 - Medical procedures for visit 3 for FAS population

	Adaptil®	Placebo	Total
<b>Ear cleaning</b>	0 (0,0%)	2 (50,0%)	2 (28,6%)
<b>Ear exam</b>	1 (33,3%)	1 (25,0%)	2 (28,6%)
<b>Ear cleaning and exam</b>	1 (33,3%)	0 (0,0%)	1 (14,3%)
<b>Ear cleaning and nail trimming</b>	1 (33,3%)	1 (25,0%)	2 (28,6%)
<b>None</b>	6	9	15

Table 20 - Other medical procedures for visit 3 for FAS population

### 3.5 - Efficacy: primary outcome

The main objective was to assess comparatively the use of an Adaptil® collar versus a placebo collar in puppies. The primary endpoint was the relative variation of total score between the first visit and second visit. To achieve this, total score was calculated by summing up all 10 sub-scores, considering, that each parameter had a score from 0 to 4. This said, the total score could vary between 0 and 40.

The Adaptil® group had a wider interval of relative variation of total score between visit 1 and visit 2, varying between -75 and 400. For the placebo group the interval was narrower (-75 – 150). The wider interval for puppies wearing Adaptil® can explain why this group have a positive mean, but a negative median. With respect to the positive mean, it means that the total score has increased from the first visit to the second one. This is not observed in the placebo group, as it has a negative mean. In other words, most puppies in the placebo group had a decrease in their stress. The missing puppy represented in the table 21 for the Adaptil® subjects, is regarding one puppy that score zero at the core vaccination, making impossible to evaluated if there was a decreased in his stress and anxiety at the following consultation.

The p-value obtained for relative variation of the total puppy score between visit 1 and visit 2 between both groups was 0,4651. As this value is higher than 0,05, we can affirm that there is no significative statistical difference between the Adaptil® group and the placebo group regarding the variation for total score between the first and second visit.

However, the mean total stress scores at visit 1 were respectively 5.7 in the Adaptil® group and 6.7 in the Placebo group. On a total possible range of 0-40, these baseline scores were quite low in both groups, reflecting that puppies were not stressed at their first veterinary visit, whatever their treatment group. Moreover, again, on average, total stress values at visit 2 were respectively 4.9 in the Adaptil® group and 5.5 in the placebo group, thus quite similar to visit 1 values.

	Adaptil®	Placebo	Total
Mean (SD)	25,8 (133,0)	-10,2 (56,3)	7,2 (100,7)
[Min; Max]	[-75,0;400,0]	[-75,0;150,0]	[-75,0;400,0]
Median	-29,20	-5,90	-25,00
[Q1;Q3]	[-55,6;100,0]	[-50,0;0,0]	[-55,6;0,0]
Missing	1,00	0,00	1,00
			p-value: 0.4651

Table 21 - Comparison of the relative variation of total score between the first visit and second visit for Adaptil® and Placebo group

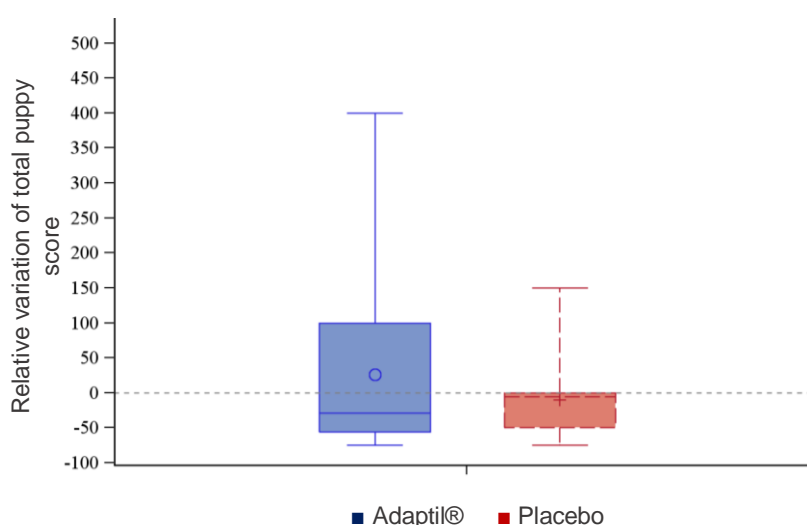


Chart 9 - Comparison of the relative variation of total score between the first visit and second visit for Adaptil® and Placebo group

### 3.6 - Efficacy: secondary outcome - relative variation between visit 1 and visit 3, visit 2 and visit 3

The secondary endpoints were the relative variation of total score between visit 2 and visit 3 and the relative variation of total score between visit 1 and visit 3. In these situations, the missing puppies were 8 puppies that did not attend the third visit, this means, they only had the core vaccination and one boost, so it is impossible to analyse their visit 3. Regarding the analysis of the relative variation between visit 2 and visit 3, the placebo group had a greater interval when compared to the Adaptil® group. Like in the analysis of the efficacy for visit 1 and visit 2, puppies wearing Adaptil® had a positive mean, whereas the placebo group had a negative mean. This is also true for the relative variation between visit 1 and visit 3.



However, between visit 1 and visit 3, it is the Adaptil® group that has a wider range of results, between 71 and 500.

Both descriptive analyses showed that the p-value surpass the significance level, meaning that there was no significant statistical difference between puppies wearing Adaptil and puppies wearing a placebo collar, for relative variation between visit 2 and visit 3 and visit 1 and visit 3 (table 22 and 23).

Furthermore, in order to check if the score evolution between two visits was above 2 points, the absolute variation was calculated. This variation was dichotomised in a first category if the variation was above 2 points and another category if not. Likewise, the primary endpoint, these variables are non-parametric, so a Fisher exact test was used.

In general, most puppies, either wearing a placebo or Adaptil® collar, had a total score variation between visits lower or equal to 2 points in the “Scale for evaluating stress”, on a total interval range of 0-40, this was very limited from a clinical point of view. On top of that, the statistical tests used did not demonstrate any significant differences between the Adaptil® and placebo group (table 24).

	Adaptil®	Placebo	Total
Mean (SD)	9,8 (75,4)	-4,5 (83,1)	1,4 (78,5)
[Min; Max]	[-50,0;200,0]	[-100,0;200,0]	[-100,0;200,0]
Median	0	-25	-10,3
[Q1;Q3]	[-22,2;0,0]	[-50,0;16,7]	[-50,0;16,7]
Missing	6	2	8
			p-value: 0,2299

Table 22 – Comparison of the relative variation of total score between the second visit and third visit for Adaptil® and Placebo group

	Adaptil®	Placebo	Total
Mean (SD)	27,6 (186,7)	-35,3 (40,0)	-9,6 (123,3)
[Min; Max]	[71,4;500,0]	[-100,0;50,0]	[-100,0;500,0]
Median	-44,4	-33,3	-34,5
[Q1;Q3]	[-66,7;-25,0]	[-50,0;-20,0]	[-66,7;-20,0]
Missing	6	2	8
			p-value: 0.3818

Table 23 - Comparison of the relative variation of total score between the first visit and third visit for Adaptil® and Placebo group

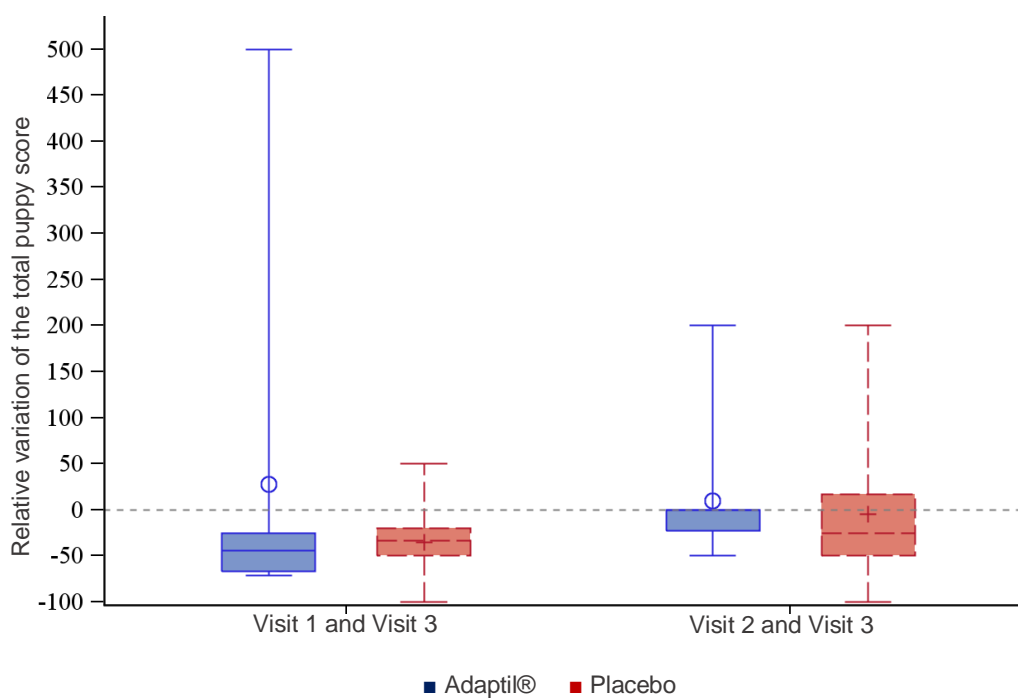


Chart 10 - Comparison of the relative variation of total score between the first visit and third visit, second visit and third visit, for Adaptil® and Placebo group

	Adaptil®	Placebo	Total
<b>V1, V2</b>			
≤ 2	12 (80,0%)	14 (93,3%)	26 (86,7%)
> 2	3 (20,0%)	1 (6,7%)	4 (13,3%)
			p-value 0,59770
<b>V1, V3</b>			
≤ 2	7 (77,8%)	13 (100,0%)	20 (90,9%)
> 2	2 (22,2%)	0 (0,0%)	2 (9,1%)
			p-value 0,15584
<b>V2, V3</b>			
≤ 2	8 (88,9%)	12 (92,3%)	20 (90,9%)
> 2	1 (11,1%)	1 (7,7%)	2 (9,1%)
			p-value 1,00000

Table 24 - Absolute variation of the total puppy score “under or equal 2 points” and “scores above 2 points” between visits of puppies tested with Adaptil® and Placebo

### 3.7 - Efficacy: secondary outcome - total puppy score

Mean of total puppy score is slightly different when comparing puppies from the Adaptil group with the placebo one. The placebo group had a slightly higher total score mean at the core vaccination. For the Adaptil® subjects, between visit 1 to visit 2, and visit 1 to visit 3 there was a decrease in the mean, however, this did not occur between the visit 2 and 3. Regarding the placebo puppies, between every consultation there was a decrease in mean total score. However, from a clinical point of view, these differences were very limited, considering a total 40-point interval.

	Adaptil®	Placebo
<b>Visit 1</b>		
Mean (SD)	5,7 (4,68)	6,73 (4,35)
[Min; Max]	[0;18]	[1;17]
Median	5	6
[Q1;Q3]	[3;7,5]	[4;8,5]
<b>Visit 2</b>		
Mean (SD)	4,87 (3,56)	5,47 (3,91)
[Min; Max]	[1;11]	[1;16]
Median	4	6
[Q1;Q3]	[2;7,5]	[2;7]
<b>Visit 3</b>		
Mean (SD)	5,22 (3,38)	4,61 (4,01)
[Min; Max]	[1;11]	[0;15]
Median	6	4
[Q1;Q3]	[2;7]	[3;6]

Table 25 - Comparison between Adaptil® group and Placebo group for mean total puppy score

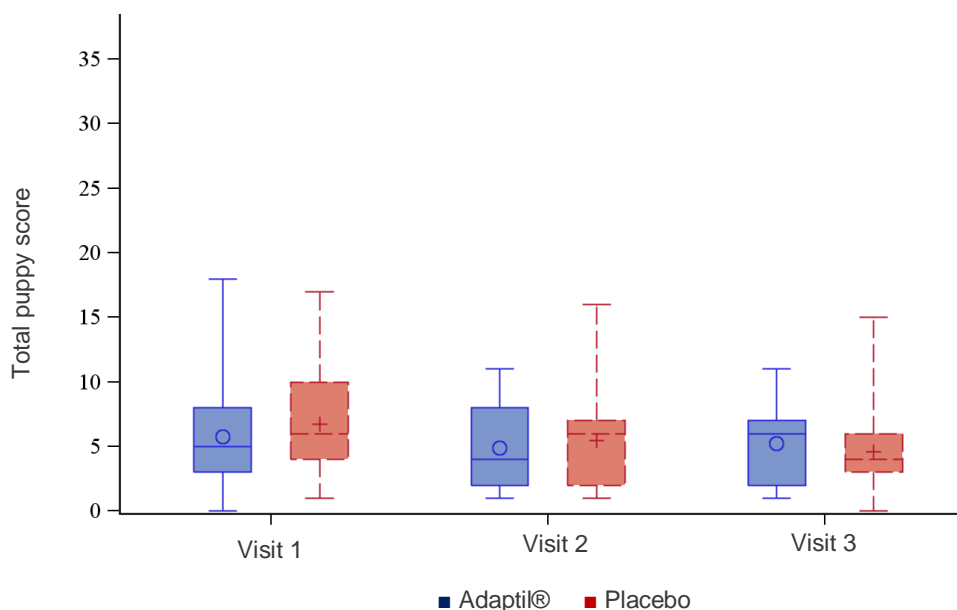


Chart 11 – Comparison between Adaptil® group and Placebo group for mean total puppy score

### 3.8 - Secondary outcome: sub-scores

To evaluate each parameter individually, it was created a dispersion graphic for each consultation and comparing both collars. For visit 1, the parameters with the lowest mean score, for both collars, were pupils, respiration and lips. Additionally, gaze was the parameter with the highest score for all consultations. For visit 1 and 2, puppies from the Adaptil® group tended to score lower in general. This is inverted at visit 3, where the placebo group seems to have lower scores. At visit 1, tail posture and ear posture had the same mean score for both groups. At visit 2, ear posture, pupils, respiration and lips also had the same mean score. This is not seen at visit 3, where there is no parameter equal for both collars.

The difference for each parameter between the two treatment groups at each time point was around 0.2 or less, on a maximum total score of 4, which was negligible.

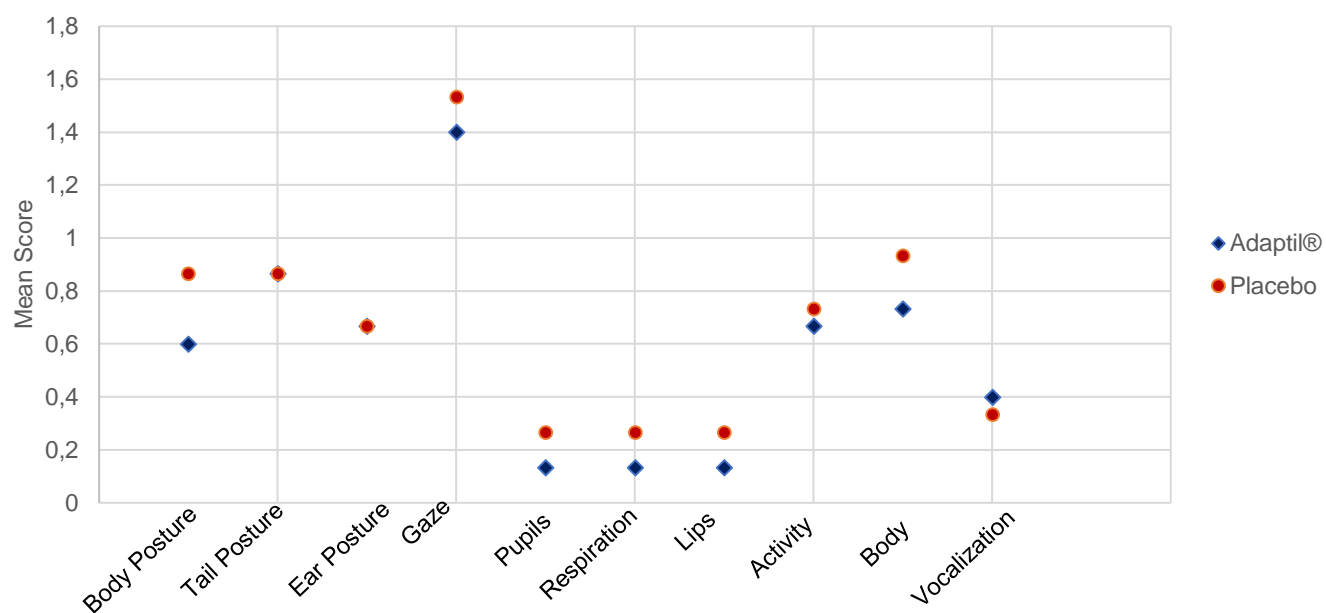


Chart 12 – Sub-scores at visit 1 for Adaptil® group and Placebo group

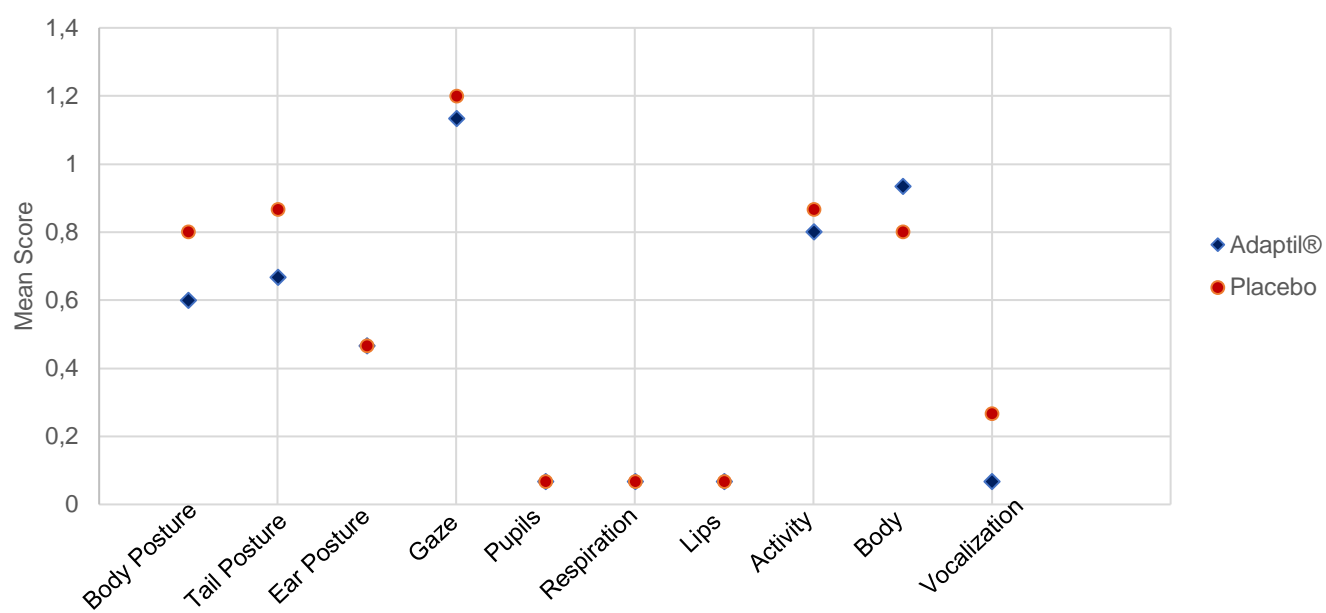


Chart 13 – Sub-scores at visit 2 for Adaptil® group and Placebo group

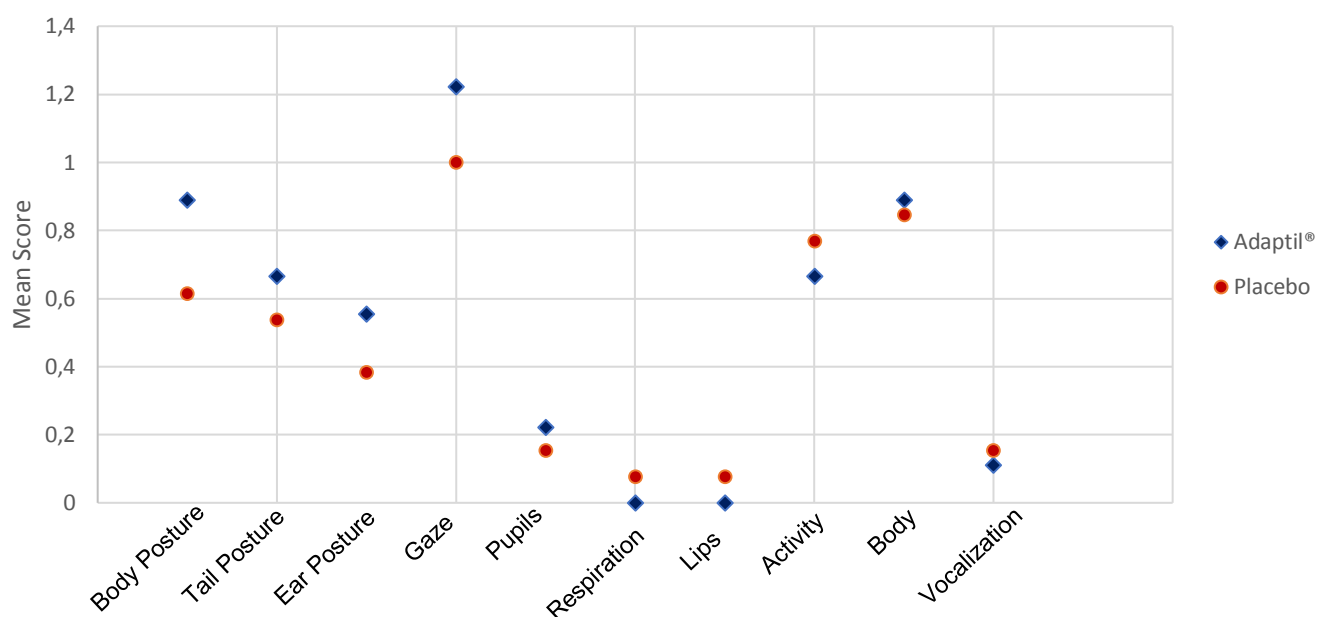


Chart 14 – Sub-scores at visit 3 for Adaptil® group and Placebo group

### 3.9 - Secondary outcome: owner assessment at visit 2

At the second visit owners were asked if their puppy was less stressed, more stressed or had no difference in their behaviour. Overall, owners did not notice any difference, and this goes for both collars. Regarding the Adaptil® group, there were more puppies stressed than relaxed. For the placebo group, the data showed the inverse results, most puppies tended to be less stressed.

	Adaptil®	Placebo	Total
Less stressed	2 (13,3%)	5 (33,3%)	7 (23,3%)
More stressed	3 (20,0%)	2 (13,3%)	5 (16,7%)
No difference	10 (66,7%)	8 (53,3%)	18 (60,0%)

Table 26 – Owner assessment at visit 2

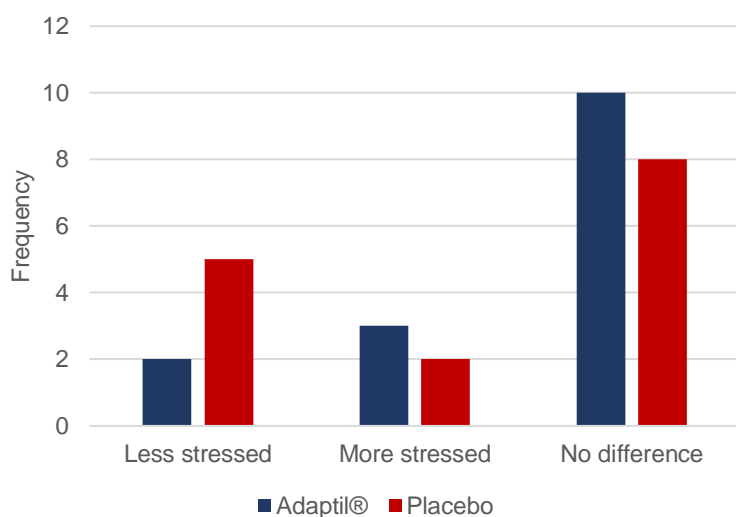


Chart 15 – Owner assessment by collar at visit 2

### 3.10 - Secondary outcome: owner assessment at visit 3

Just as the previous consultation, owners were asked if they noticed any changes on their puppy behaviour. In general, at the third visit, puppies did not change their behaviour. None of the puppies of the Adaptil® group showed signs of being more stressed. The group exposed to the placebo also showed less stressed behaviours.

	Adaptil®	Placebo	Total
Less stressed	3 (33,3%)	6 (46,2%)	9 (40,9%)
More stressed	0 (0,0%)	3 (23,1%)	3 (13,6%)
No difference	6 (66,7%)	4 (30,8%)	10 (45,5%)

Table 27 – Owner assessment at visit 3

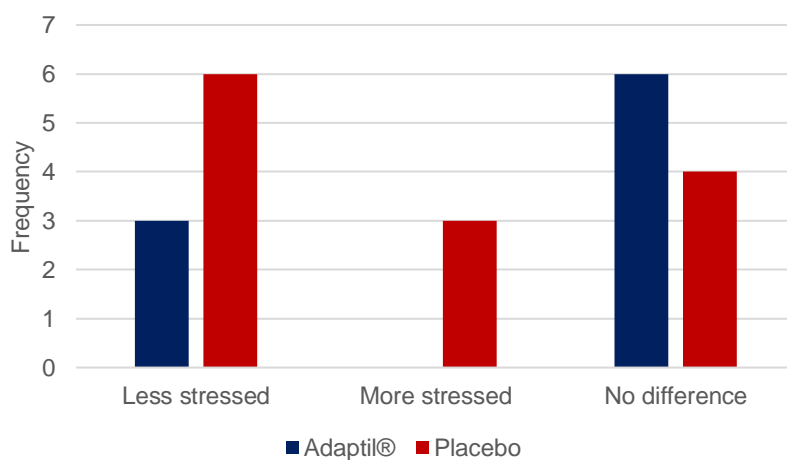


Chart 16 – Owner assessment by collar at visit 3





#### 4 - Discussion

The study sought to demonstrate Adaptil®'s application in daily life of puppies and first visits to the veterinary clinic. A puppy who is relaxed and calm is easier to handle and to perform treatment if needed. Consultation is easier to conduct by respecting the animals' welfare. By avoiding excessive restrain methods and working in a base of rewarding good behaviours, we can prevent future aversion to the veterinary clinic environment. Döring et al. (2009) reported that most times going to the veterinary clinic is a traumatic experience and it is highly influenced by previous experiences (Döring et al., 2009). Prevention starting at an early age is the best method to raise a well-balanced puppy (Beaver, 2009). Indicators of stress, such as postures and signals shown by puppies during consultations and contemplated in the table "Evaluation of stress", are powerful tools to assess animals' welfare, even though there is no direct connection between stress and welfare (Bodnariu, 2008). Welfare assessment is not only advantageous for medical issues, but also to behavioural issues. It provides precious information, such as prognostic or progression of a certain disorder and provides the veterinary surgeon with tools to lessen the harm caused to the animal (Yeates, 2013).

No statistical evaluation was performed regarding gender, age, breed, living place, owners having other pets and being the first puppy between the Adaptil® group and the placebo group. Differences between groups were not clinically relevant, thus no p-values were required. Overall, the study relied in a well-balanced division between groups.

One of the main difficulties faced was for owners to respect the vaccination protocol, to assure that there was no major deviation from the two weeks or one-month interval. In some cases, this was not possible, as the owners did not respect the appointment or forgot to follow the initial plan. This led to high variability in intervals between consultations and puppies being excluded from the study. Consequently, this resulted in a small number of puppies being tested.

Waiting time was recorded for all puppies participating in the clinical trial, but it was not considered in the analysis. None of the owners reported any type of incident while waiting for consultation. According to some authors, waiting time can be beneficial, as some dogs that were excited from the journey, have time to relax and to get accustomed to the environment (Hemander, 2008). There is a lack of consensus in this matter, as Mills (2012) states that most dogs and all cats associate a longer waiting time with increased levels of

stress (Mills et al., 2012). Other stress factors at the waiting room, such as, presence of other dogs, cats or room temperature were not controlled or assessed in this clinical trial.

According to most owners, they did not notice any behavioural changes in their puppy during the study length. For visit 2, most owners (10) from the Adaptil® group did not noticed any behavioural differences, whereas 8 owners stated the same for the placebo group. Furthermore, in the placebo group, 5 puppies were “less stress” when compared to 2 puppies from the Adaptil® group. As for the visit 3, the placebo group had a higher number of “more stress” puppies (3), whereas none of the puppies from the Adaptil® group showed higher levels of stress. Most owners regarding the Adaptil® group did not observed differences and the remaining stated that their puppies were less stressed. It would be expected that puppies from the Adaptil® group had shown a decrease in their stress levels between consultations, yet we must consider that this parameter was highly subjective, as different owners have distinctive perspectives of what is a distressed behaviour or not. In other words, many owners associated some behaviours with the normal puppy conduct. It would be enriching to create a more precise questionnaire to verify if the puppy was really under a considerate level of anxiety or fear. Owners may not be fully informed of how a dog communicates and that can cause some misunderstandings. In addition to this, owners reported that some puppies felt a little uncomfortable with the collar and associated that with higher levels of stress. As puppies have never worn a collar, it is expectable that some may adapt better than others. For extremes cases, for example a puppy that tries to chew his collar constantly, owners can choose to use a plug-in diffuser instead.

In this study, the authors did not consider if the presence of another animal had any effect in the puppy's emotional state. Most puppies in the placebo group lived with another dog. Puppies, who sleep with another dog, have reduced frequency of crying during the night (Taylor & Mills, 2007). Regarding the living place and place where puppy was obtained, both parameters were not associated directly with the efficacy of Adaptil®. Considering both groups, most puppies were adopted. Taylor and Mills (2007) refer that puppies coming from a domestic maternal environment have fewer episodes of disturbance (Taylor & Mills, 2007), consequently they are calmer and more relaxed. Also, rescued dogs tend to show more physiological stress signals (Bodnariu, 2008). First time owners have more chances to face behaviour problems. In this study most owners, for both groups, were not dealing with a puppy for the first time (Jagoe & Serpell, 1996).

Puppies' behaviour can also be influenced by their age. For further studies, authors could create groups by different periods of development and associate them with stress levels shown by puppies. Puppies, in the sensitive period, adapt more quickly to adversities,

thus having low stress levels, as they can cope with unknown situations. Puppies, who are adopted before completing 12 weeks old, are in the sensitive stage for both socialization and adaption to novelty (Denenberg & Landsberg, 2008). First consultations occur during a period in which puppies are in full development and changing quickly. At 8 weeks old, normally when the core vaccination occurs there is a high variability of behaviours, however there are no sufficient literature describing it in puppies during veterinary consultations (Godbout, Palestrini, Beauchamp, & Frank, 2007). All in all, owners feedback about their puppy's behaviour had great variation, due to different individual opinions and puppy's background.

There was a lack of control of stress conditions at home and during the travel until arriving to the veterinary clinic. A simple car travel can trigger anxiety and fear, so prevention should begin at home. Owners are responsible to assure that journeys to the veterinary clinic are stress-free (Mills et al., 2012). It was already proven that DAP has beneficial effects in dogs who suffer from car travels, so it would be interesting to prove it in puppies, since they have no previous experiences (Gandia Estellés & Mills, 2006).

According to a systematic review of the use of pheromones to treat behavioural problems, less subjective studies are required. In others words, most dog-appeasing pheromone related studies rely in owners' feedback and observer's interpretation (Frank, Beauchamp, & Palestrini, 2010). To avoid these incongruities and obtain more precise, rigorous, quantitative and impartial results, studies could contemplate two observers (who would cross over information) and use video recordings. In the present study, consultations were carried out by different veterinarian surgeons, although the observer was the same. To avoid possible influences of how the veterinarian surgeon proceeds, future studies should evaluate consultations only performed by the same person. It would also be enriching to investigate to what extend different veterinarians can influence how a puppy reacts during consultations.

Another hindrance was the low base of the total puppy score, that is, most puppies did not escalate to elevated levels of anxiety during first consultations. This can be due to the informed staff working in the veterinary clinic and because puppies have no previous negative experiences and want to explore new environments. There are several published studies that evaluated how dogs reacts entering a veterinary clinic and during consultations, however there is a lack of information regarding puppies (Stanford, 1981). Older dogs tend to have previous traumatic experiences that condition their emotional state. Regarding puppies, as it is their first impact, we must assure that the consultation goes without major problems while rewarding good behaviours.

Also, owners are conscious about mental and behavioural health and seek knowledge in this area. In the veterinary clinic where the study took part, there is a special attention to behaviour problems, so the veterinary surgeon ensures that in the first consultations some basic concepts are covered, which may have represented a bias in this study, contributing to low stress scores in these puppies. Elimination training, how to train with positive reinforcement and avoidance of punishing methods. Moreover, the veterinary surgeon also advised how to make the adoption less traumatic and adaptation to a new home more uncomplicated. Counselling about food, toys, prophylactic plan, deworming plan among others, were also covered. Each puppy had his own personality and behaviour issues that were not taken into account while analysing the information collected.

Overall, the present study did not show major differences between the two groups, this means, that there were no statistically significant differences regarding the Adaptil® group and the placebo group in reducing distressed behaviours. This is verified by the high p-value ( $p=0.4651$ ) obtained for the primary efficacy analysis. Likewise, p-values for secondary outcomes, were equally high (Visit 2 / Visit 3,  $p = 0.2299$ , Visit 1 / Visit 3,  $p = 0.3818$ ). One reason behind these results is the small number of puppies, that was expressed in a low statistical power. Looking closer to the total score of each puppy, we can assume that puppies were globally not stressed at baseline. The mean total stress scores for the core vaccination is lower in the Adaptil® group (5.7) than in the placebo group (6.7). This situation is similar to the second visit, where total stress values were also low, 4.9 for the Adaptil® group and 5.5 for the placebo one. Pursuing this further, at visit 1 there was a puppy that had a zero score, it was totally comfortable and relaxed. Hence, it could not be assessed for further consultations, because the score was the lowest possible. The low baseline scores can be explained by the fact that young puppies have never experienced traumatic events at the veterinary clinic and do not associate it with stress or anxiety postures.

## 5 - Conclusion

The importance of this study lied in the attempt to handle and decrease stress and anxiety in puppies during the veterinary consultation. A relaxed behaviour contributes positively to handling and course of the consultation, but also to the animal welfare.

There are several published studies verifying the efficacy of the dog-appeasing pheromone in different contexts, including helping young puppies to cope with stressful situations such as adoption and veterinary consultations (Denenberg & Landsberg, 2008; Gaultier et al., 2009; Graham, Mills, & Bailey, 2007; Taylor & Mills, 2007). Adaptil®, the synthetic analogue of dog-appeasing pheromone is an alternative for treatment and prevention of behaviour problems. This therapeutic approach is less invasive, safer and results in reduced secondary effects. To achieve a behavioural improvement is recommend using Adaptil® along with a behavioural therapy.

The study's main limitation was working with a small sample, which resulted in less significative and more variable results. Most owners did not notice changes in their puppies' behaviour between visits and there were not statistically significative differences between using a placebo collar or an Adaptil® collar, in reducing stress and anxiety during the vaccination protocol. Although it was not possible to prove the efficacy of Adaptil® in reducing fear and anxiety in puppies, during veterinary consultations, this study serves as a guide for potential future studies in the pheromonotherapy area. It would also be positive to make associations between age, gender, living place, among other parameters, with stress levels manifested by puppies during the consultation. Other similar study could also be carried out, but investigating further welfare assessments and for a longer period. For the present study, it would also be gratifying, if it was made a more extensive questionnaire to owners regarding their puppy's behaviour at home. Elimination at night or crying could be some of the parameters analyzed. Assessing if the continuous exposure to DAP in puppies until they reach social maturity has a cumulative effect or if it acts punctually, could also be another interesting study to conduct.

Moreover, the study served to brought attention to owners concerning behavioural issues and how the use of pheromones, namely Adaptil®, can aid to treat and prevent them.



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