# PERIOPERATIVE STRESS IN DOGS UNDERGOING ELECTIVE SURGERY: EVALUATION OF THE DOG APPEASING PHEROMONE (DAP) FOR THE CONTROL OF BEHAVIOURAL, NEUROENDOCRINE, IMMUNE AND ACUTE PHASE STRESS RESPONSES

Carlo Siracusa

Directores:
JOSEP PASTOR MILÁN
SANTIAGO LAVÍN GONZÁLEZ
Departamento de Medicina y Cirugía Animal
Facultad de Veterinaria
Universitat Autònoma de Barcelona
2009

# PERIOPERATIVE STRESS IN DOGS UNDERGOING ELECTIVE SURGERY: EVALUATION OF THE DOG APPEASING PHEROMONE (DAP) FOR THE CONTROL OF BEHAVIOURAL, NEUROENDOCRINE, IMMUNE AND ACUTE PHASE STRESS RESPONSES

Carlo Siracusa

Bellaterra, Barcelona
2009

Esta tesis fue realizada gracias a una beca predoctoral para formación de personal investigador FI de la Generalitat de Catalunya.

Los doctores JOSEP PASTOR MILÁN y SANTIAGO LAVÍN GONZÁLEZ, Profesor Titular y Catedrático de Universidad del Área de Conocimiento de Medicina y Cirugía Animal de la Facultad de Veterinaria de la Universidad Autónoma de Barcelona, respectivamente,

INFORMAN:

Que la memoria titulada "Perioperative stress in dogs undergoing elective surgery: evaluation of the Dog Appeasing Pheromone (DAP) for the control of the behavioural, neuroendocrine, immune and acute phase stress responses", presentada por el licenciado Don CARLO SIRACUSA para la obtención del grado de Doctor en Veterinaria, se ha realizado bajo nuestra dirección y, considerándola satisfactoriamente finalizada, autorizamos su presentación para que sea evaluada por la comisión correspondiente.

Y para que conste a los efectos que sean oportunos, firmamos el presente certificado en Bellaterra, Barcelona, el 20 de Enero de 2009.

Firmado: JOSEP PASTOR MILÁN Firmado: SANTIAGO LAVÍN GONZÁLEZ

# ACKNOWLEDGEMENTS

Thank you to the AGAUR, Generalitat de Catalunya (Barcelona, Spain) and the European Social Fund for their financial support (grant 2007FIC00655); and to VetMovil - Centre d'Acollida d'Animals Domestics de Companya del Vallés Oriental, Granollers (Barcelona, Spain), Centre d'Atenció d'Animals Domèstics de Companyia del Maresme, Argentona (Barcelona, Spain) and Pherosynthese Laboratories, Saint Saturnin les Apt (France) for their collaboration.

A grateful acknowledgement goes to my advisors, Prof. Josep Pastor and Prof. Santiago Lavín, for being both teachers and friends, and to Prof. Xavier Manteca for guiding me step by step throughout this adventure.

I am also gratefully indebted to Prof. Maria Grazia Pennisi, Dr. Jaume Fatjo, Dr. Patrick Pageat, Dr. Emmanuel Gaultier, Prof. Bonne Beerda, Prof. Joanne van Der Borg, Prof. Daniel Mills, Prof. James Serpell and Prof. Ilana Reisner, for teaching me enthusiastically and introducing me to the behavioural science. I hope to be a good disciple.

An irreplaceable contribution to this work was given by some terrific colleagues and loyal friends: Maria (without her many samples would have been lost!), Mar, Aurora, Belen, Cristina and to all the workers of the two shelters. Thank you.

A special thanks goes to all my friends and colleagues of the UAB: Ester, Jorge, Encarna, Mercè, Gregorio, Laura, Oscar, Pancha, Nilsa, Ana Luisa, Ana Cris, Sara, Ignasi, Rafi, Martina, Paolo, Cinzia and Montse. You have been my family in Barcelona!!

Thank you to my friends and colleagues of the Ethology Group, UAB: Marta, Valentina and Sergio. Your enthusiasm and commitment are contagious.

To Paola, Vicente, Miguel, Meri, Fabian, Raul, Lorena and all the other friends that made my life in Barcelona so happy… thank you. To Guillermo, to be always present and patient, no matter how difficult sometime it was.

Thank you to my friends-for-life: Piero, Giuseppe, Peppo, Antonio, Giovanni, Maria Vittoria and all the other funny and honest friends in Italy. Maybe someday I will come back!

Thank you to all the colleagues of the IZSAM: Paolo, Antonio, Luigi, Emanuela, Nazzareno, Michele, Lejla, Barbara and Dr. Del Papa. It was nice coming back to Italy.

To all my colleagues at Penn and to my friends in the US: Meghan, Mary, Kirk, Alison, Kathy, Tony, Jacqui, Oscar, Ella, Esaul, Maria, Manolo and all the vets of my noisy office!! You are my new American family!

Grazie Papá, Mamma, Davide, Agata, Orazio e… Federico!!! Siete stati e sempre sarete l'unica certezza della mia vita. Questo lavoro lo devo soprattutto a voi, che mi avete sempre dato libertá e fiducia, anche quando questo significava stare molto lontani.

I am sure that I am forgetting someone important... so thank you forgotten friend!!

# TABLE OF CONTENTS

# 1. ABSTRACT

# 1. ABSTRACT

This clinical study is composed of two different trials. In the first we investigated and described the perioperative stress response in dogs undergoing elective orchiectomy and ovariohysterectomy. In the second trial we evaluated the efficacy of a commercial dog synthetic appeasing pheromone for the control of the perioperative stress response.

## Trial 1

The aim of this trial is to describe the behavioural, neuroendocrine, immune and acute phase stress responses in dogs undergoing elective surgery in normal clinical practice conditions. Sixteen dogs were submitted to elective orchiectomy or ovariohysterectomy using a standardized surgical protocol. Each animal was confined to the Intensive Care Unit during pre- and post-surgery, and its perioperative behavioural, neuroendocrine, immune and acute phase responses studied. Behavioural categories, cortisol, prolactin, white blood cells, C-reactive protein and haptoglobin variations were evaluated. Values at different times were compared with basal values shown by the dog in its usual environment. Communicative and explorative behaviours showed high pre-surgery occurrence and were inhibited post-surgery. Decreases in post-surgery activity, interactive behaviours and changes in waking/sleeping patterns were observed. Compared to basal values, cortisol showed a significant increase both during pre- and post-surgery confinement in the ICU cage, being the most sensitive marker of psychological stress. Prolactin values were characterised by a significant decrease at early post-surgery time. The immune response was characterised by long-term neutrophilia and monocytosis, but by short-term lymphopenia and eosinopenia, limited to early post-operative period. With regard to the acute phase response, both C-reactive protein and haptoglobin showed a post-surgery long-term increase. Changes in

behavioural, hematological and biochemical markers showed that perioperative stress represents a major challenge for dog welfare.

## Trial 2

Surgery, together with its related perioperative procedures, is known to be a major source of stress for dogs. Both psychological and physical stressors activate the behavioural, neuroendocrine, immune and acute phase responses in dogs undergoing elective surgery. A synthetic dog-appeasing pheromone (DAP) has been marketed to control dog stress response. Its efficacy has been demonstrated recently in many different contexts. We therefore hypothesized that it could also control the perioperative stress response in dogs undergoing elective surgery. For this purpose we studied the behavioural, neuroendocrine, immune and acute phase responses in dogs undergoing elective orchiectomy or ovariohysterectomy. We analyzed the pre- and postoperative variations in behavioural categories, cortisol, glucose, prolactin, white blood cells, haptoglobin and C-reactive protein. A standardised surgery setting was used. Of the results obtained, the behavioural and the prolactin responses showed to be influenced by the DAP treatment. The dogs treated with the synthetic pheromone were more likely to be alert and visually exploring after surgery, and their prolactin response to perioperative stress was significantly lower ($P \leq 0.05$), when compared with the animals receiving the placebo treatment. The HPA axis, immune and acute phase responses were unaffected by the treatment. These results suggest that the dog-appeasing pheromone modifies the behavioural and the neuroendocrine lactotropic perioperative stress responses in dogs undergoing elective surgery. Thus, the use of this product in a clinical setting could improve their recovery and welfare.

# 2. INTRODUCTION

# 2. INTRODUCTION

In the Constitution of the World Health Organization (1948), health is defined as "a state of complete physical, mental, and social well-being and not merely the absence of disease or infirmity". A modern understanding of veterinary medicine should embrace this definition and consider the mental and social welfare of animals as important as the physical health. Although there is a growing interest in animal welfare, this aspect is often not properly considered in daily veterinary practice.

It is commonly accepted that surgery represents one the most challenging medical practices for animals and humans, for the pain inflicted during the surgical intervention and for the risk related with the anaesthesia and analgesia. However, limited information is available about the stress perceived by animals during the surgery itself and related procedures. This is true also for the domestic dog, undoubtedly the protagonist of companion animal veterinary medicine. Conversely, perioperative stress has been deeply investigated in human medicine since it was recognised in the late 1920s.

Therefore, a study investigating the perioperative stress response in dogs can be helpful to understand which different biological responses are activated and in what measure they are sensitive to the different stressors applied during surgery and related procedures. A better understanding of the perioperative stress response in dogs is an extremely valuable resource for the assessment of pharmacological and behavioural treatments potentially useful for the control of this challenge to animal homeostasis.

The use of drugs to decrease the activation of the perioperative stress response is controversial. The stress response is in fact a powerful biological defence against changes in the homeostatic balance.

Therefore, it is still debated in what measure the stress response should be modified or preserved.

The use of behavioural modification or natural chemical signals, such as the appeasing pheromones, to control the stress response could represent a valid aid to control perioperative stress in a safer way.

# 3. OBJECTIVES

# 3. OBJECTIVES

The main general objectives of this study were to describe the perioperative stress response in dogs undergoing elective surgery and to assess the efficacy of a synthetic dog appeasing pheromone to control this stress response. Specific objectives related to these main purposes were:

1. To describe behavioural changes and variations in salivary cortisol, serum glucose, serum prolactin, total white blood cell count, white blood cell differential, neutrophils/lymphocytes ratio, serum haptoglobin and serum C-reactive protein, due to perioperative stress in dogs undergoing elective orchiectomy or ovariohysterectomy.

2. To evaluate the effect of a synthetic dog appeasing pheromone on the perioperative stress response in dogs undergoing elective orchiectomy and ovariohysterectomy by measuring variations in behavioural categories, salivary cortisol, serum glucose, serum prolactin, total white blood cell count, white blood cell differential, neutrophils/lymphocytes ratio, serum haptoglobin and serum C-reactive protein.

3. To assess the different sensitivity of behavioral, hematological and biochemical markers to psychological and physical stressors involved in the perioperative stress response.

# 4. LITERATURE REVIEW

# 4. LITERATURE REVIEW

## 4.1 THE STRESS RESPONSE AND ITS IMPLICATION IN DOG WELFARE: BEHAVIOURAL, NEUROENDOCRINE, IMMUNE AND ACUTE PHASE RESPONSES

The stress response is the normal biological adaptive response of an individual, when an external or internal stimulus is perceived to be a threat to its homeostasis. In this response, the stimulus perceived as a threat represents the *stressor* (Broom and Johnson 1993; Moberg 2000).

Three general phases can be distinguished in the stress response: the recognition of a stressor, the biological defence against the stressor and the consequences of the stress response. This last stage of the response determines whether the animal suffers a *distress* or if it is simply coping with a brief experience that has no significant impact on its future welfare. If the subject can safely increase the distance from the stressor, usually the stress response does not have any long-term effects on his welfare. But if he is not able to cope, the stressor could cause negative effects on animal well-being, generating a *distress* (Moberg 2000).

Four main different components can be distinguished in the stress response: the behavioural, the autonomic nervous system, the neuroendocrine and the immune responses (Moberg 2000). These four different components are strictly correlated and coordinated by the hypothalamus and Corticotropin Releasing Hormone (CRH) (Rushen 2000).

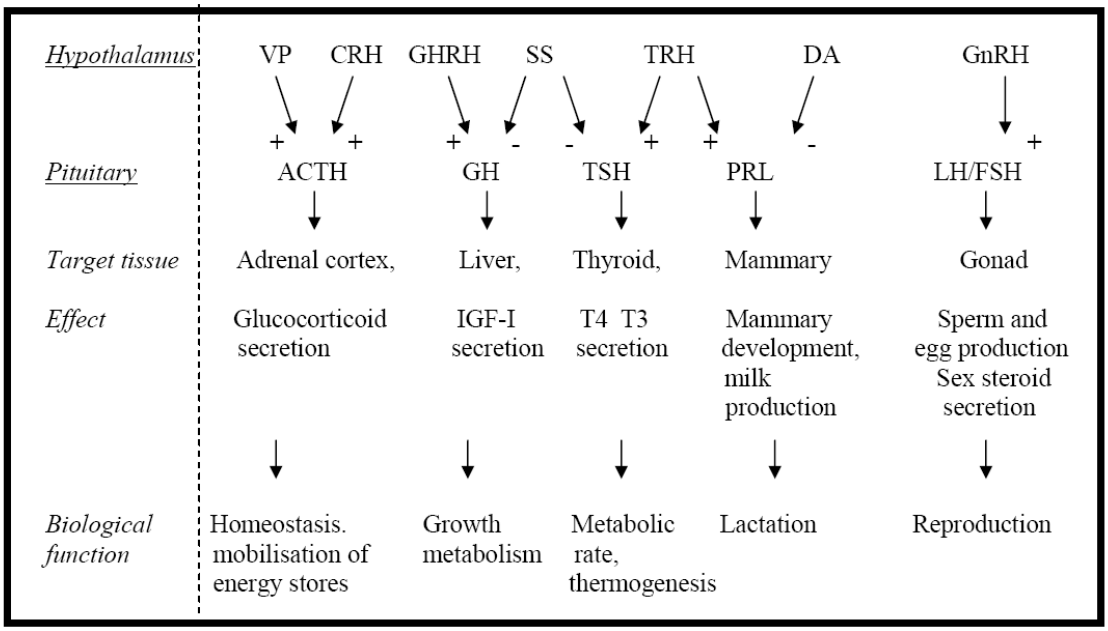
The behavioural response is often the best way for the animal to cope promptly with the stressor. For instance removing itself from the threat could represent a good solution, which is derived from the natural predator avoidance behaviour. However, this is not always possible, particularly for domestic animals that often live in a confined environment. Some behaviour that the animal displays during the

coping response, such as communicative behaviours, may provide some measure of the internal or subjective state of an animal, and so may be useful as measures of stress (Rushen 2000). If the stressor persists the individual can display behaviors that are out of context and non functional. These *displacement behaviours* can help the animal to cope with the psychological aspect of the stressor and in turn alleviate some of the physiological response (Moberg 1985; Moberg 2000; Rushen 2000).

Behaviour analysis for stress evaluation in dogs has been routinely used in the last decades. States of movement, body postures, vocalizations, oral behaviors, communicative and exploratory behaviours, or even displacement behaviors (circling, pacing, tail chasing, excessive licking…) have been used as indicators of welfare conditions (Hubrecht 1995; Hardie *et al* 1997; Beerda *et al* 1997, 1998, 1999; Horvath 2007; Haverbeke 2008). Behaviour evaluation provides an inexpensive and immediate tool for stress assessment, especially useful in those situations where a prompt intervention to control stress is needed, e.g. pain evaluation. Moreover, behavioural analysis allows collection of information simultaneously for a group or population of dogs (Martin and Bateson 1993). It provides a useful tool for those contexts in which population medicine is used for health and welfare control, such as in dog shelters.

The autonomic nervous system response is promptly activated during an acute stress and it usually has a short duration. It is implicated in the *fight or flight* response. The magnitude of the autonomic nervous system response can be easily assessed by measuring the increase of serum catecholamines, serum glucose, heart rate and blood pressure. These variations represent good tools to assess the magnitude of an acute response, but they are not accurate for the evaluation of a long-term one. This aspect, together with the difficulty in assessing parameters like blood pressure or heart rate in the field, make the autonomic nervous system response of less interest in stress assessment (Broom and Johnson 1993; Moberg 2000).

On the contrary, the neuroendocrine response (see Fig. 4.1), derived from the activation of Hypothalamic-Pituitary-Adrenal (HPA) axis, is a long-term response. Therefore, its persistent activation could affect the animal well-being. This neuroendocrine axis holds a great importance in regulating physiologic function like immune competence, reproduction, metabolism and behaviour. Many hormones are involved in the control of the neuroendocrine response.




**CRH** = Corticotropin Releasing Hormone; **DA** = Dopamine; **GHRH** = Growth Hormone – Releasing Hormone; **GnRH** = Gonadotropin – Releasing Hormone; **SS** = Somatostatin; **TRH** = Thyrotropin – Releasing Hormone; **VP** = Vasopressin; **+** = stimulatory hypothalamic factor; **-** = inhibitory hypothalamic factor; **ACTH** = Adrenocorticotropic Hormone; **FSH** = Follicle – Stimulating Hormone; **GH** = Growth Hormone; **LH** = Luteinizing Hormone; **PRL** = Prolactin; **TSH** = Thyroid – Stimulating Hormone; **IGF** = Insulin – like Growth Factor.

**Figure 4.1:** Hypothalamic – Pituitary neuroendocrine axes and its major biological effects (adapted from Matteri et al 2000).

Corticotropin Releasing Hormone (CRH) and Vasopressin (VP), produced in the hypothalamus after the perception of a threat, stimulate the pituitary gland to produce Adrenocorticotropic Hormone (ACTH), which acts on the adrenal cortex, stimulating the production of glucocorticoids (cortisol and corticosterone). Among several different actions, these hormones cause an increase in circulating glucose. ACTH, glucocorticoids and glucose have been proved to be good parameters to

assess stress (Broom and Johnson, 1993; Matteri *et al* 2000; Moberg 2000). Other hormones for which secretion is regulated by the HPA axis, like prolactin (PRL) and growth hormone (GH), are also sensitive to stress (Matteri *et al* 2000; Moberg 2000; Pageat and Gaultier 2003b).

Cortisol is a biomarker commonly used for stress evaluation in dogs (Beerda *et al* 1996, 1997, 1998; Coppola *et al* 2006; Horvath 2007; Haverbeke 2008). It offers the advantage of being a sensitive and universally accepted indicator of stress, easily and inexpensively measurable by commercial kits. Moreover, the ability to use saliva samples for its quantification (Mandel 1990), allows reliable measurement without major influences on dog welfare. Glucose can also be used as a biomarker to assess the HPA axis response to stress. However, because this metabolite is also influenced by the SNA response and other factors, e.g. feeding and starvation, it is not as reliable as cortisol (Matteri *et al* 2000; Mormede *et al* 2008). Nevertheless, its use in conjunction with cortisol determination may lend additional support to the assessment of the HPA axis response to stress.

Although prolactin has been largely used in other animals and humans for stress assessment (Matteri *et al* 2000), few studies have been published about the use of prolactin as stress biomarker in dogs. Dog prolactin is involved in emotional responses and increases during positive interactions with humans (Odendaal and Meintjes 2003; Pageat *et al* 2005). Hyperprolactinemia has been found in dogs with generalised anxiety, but not in dogs with phobias or mild anxiety (Pageat and Gaultier 2003b; Pageat *et al* 2007).

The Acute Phase Response (APR) has also been related to the regulating action of the HPA axis (Thomas 2000; Murata *et al* 2004). The Acute Phase Proteins (APPs) are a group of blood proteins that change in concentration in animals subjected to external or internal challenges, such as infection, inflammation, surgical trauma or stress (Murata *et al* 2004). Some of these proteins decrease in concentration, the negative

APPs, and some others increase, the positive APPs. The latter are glycoproteins synthesised mainly by hepatocytes upon stimulation by pro-inflammatory cytokines and released into the blood stream (Martinez-Subiela *et al* 2001; Murata 2004; Cerón *et al* 2005). The APPs are considered to be non-specific innate immune components involved in the restoration of homeostasis (Murata 2004; Cerón *et al* 2005).

It is widely accepted that, in humans and experimental animals, physical and psychological stress elevates plasma Interleukin 6 (IL6) and APP levels. There is also evidence in cattle that physical stress can induce the APR. Although the mechanism for this is yet to be elucidated, activation of HPA axis by stress signals may be a trigger of systemic or local (intra-pituitary) cytokine production, thereby augmenting hepatic APP synthesis and release into the blood-stream (Murata 2004). Glucorticoid treatment causes an increase in Hp concentrations, while CRP concentrations are not affected (Cerón *et al* 2005).

APPs in dogs can be classified (see Tab. 4.1) by the magnitude of their response to stimuli, i.e. major (10-100 fold increase), moderate (2-10 fold increase) and negative. For major APPs the upper limit of 100 fold seems more appropriate for dogs (Cerón *et al* 2005).

**Table 4.1:** Classification of APPs in dogs by magnitude of their response (adapted from Cerón et al 2005).

| *Major* | *Moderate* | *Negative* |
|---|---|---|
| C-reactive protein (CRP) | Ceruloplasmin (Cp) | Albumin |
| Serum Amyloid A (SAA) | Haptoglobin (Hpt) | |
| | Fibrinogen | |
| | α1-acid glicoprotein (AGP) | |

In general, a significant increase in serum APPs is detectable between 4 and 24 hours after an injury and the peak of maximum concentration is reached between 24 hours and 7 days. In surgical trauma, a significant

increase is detectable between 4-24 hours and a peak is reached in 24 hours-4 days, CRP being the most rapid (Conner *et al* 1988; Jain 1989; Yamamoto *et al* 1993; Thomas 2000).

The immune system response during stress was classically related to the HPA axis response and the increase in circulating glucocorticoids causing suppression in immune competence. However, the immune system is currently considered to have its own primary response to a stressor. Even if neutrophilia, lymphocytopenia and decrease of IL2 have been related to the increase of glucocorticoids, a bi-directional interaction between the nervous and immune systems has also been proven to be mediated in large part by cytokines (Blecha 2000; Moberg 2000; Stockham *et al* 2003).

The magnitude of the neutrophilia associated with glucocorticoids and catecholamines in dogs is limited. In response to catecholamines, neutrophils shift from the marginated to the circulating neutrophil pool. Because the cell concentrations in the two pools are nearly equal in the dog, the canine physiologic neutrophilia is not expected to exceed twice the upper reference limit (URL) of the reference interval. The neutrophilia created by the effects of glucocorticoids also involves shifting of neutrophils from marginal to circulating pools, but this neutrophilia may be enhanced by the release of neutrophils from the marrow storage pool and the decreased emigration of neutrophils to the tissues. As a guideline, a glucocorticoid-associated neutrophilia is typically less than two folds the URL and is not expected to exceed three folds the URL (Stockham *et al* 2003). In response to glucocorticoids, there is a redistribution of lymphocytes from circulating blood to other sites, probably marrow or lymph-nodes (Stockham *et al* 2003). A glucocorticoid-associated monocytosis is typically mild (2xURL) (Stockham *et al* 2003).

The modulation of the stress response is highly variable. In fact, not every stressor activates all the four systems we have seen. Moreover, each system, when activated, could respond to a different degree. Also,

the individual response to the same stressor is greatly variable. Early experience, genetics, age and social relations modify the individual perception of a stimulus as a threat. Although in a laboratory study it is often possible to control these factors, in field studies it is often impossible. Also, manipulation and collection of samples used to assess stress can be perceived by the animal as a threat (Moberg 2000; Rushen 2000).

## 4.2. A COMPLEX STRESS RESPONSE: THE PERIOPERATIVE STRESS

From the first description by Cuthberston in the late 1920s of a generalized metabolic reaction of the body to bone fracture and immobilization to the characterization of the role of the HPA axis by Hune in the early 1050s, surgeons and scientists have been dealing with need to improve surgical recovery by understanding the perioperative stress response (Douglas 2002; Butler 2003). Paradoxically, some of the first classical studies about perioperative stress were realized using dogs as experimental animals (Hume 1953; Egdhal 1959). In these studies the adrenal cortisol response to limb injury in dogs was studied. In animals with an intact sciatic nerve or spinal cord, operative injury or superficial burn caused an immediate and sustained increase of adrenal hormones. If the nerve or cord were transacted, the response was abated. In later studies the role of the hypothalamus and the pituitary gland in this response were clarified (Hume 1953).

Extreme hormonal and metabolic responses to stress are associated with increased morbidity and mortality, and epidural and spinal anaesthesias are known to modulate the stress response (Douglas 2002). Overall mortality is reduced 30% in human patients anaesthetized with neuraxial blockade when compared to general anaesthesia. There is also a reduction in postoperative complications such as deep vein thrombosis, pulmonary embolism, blood loss, pneumonia, respiratory depression, myocardial infarction and renal failure (Rogers *et al* 2000). In another study by Rasmussen *et al* (2005) it was demonstrated that the

alteration of the neuroendocrine system related to the perioperative stress response could be a contributing factor in the development of postoperative cognitive dysfunction in elderly people.

Using neuraxial blockade and β-adrenergic blockade, preventing hypothermia and using appropriate opioid treatment for analgesia have been demonstrated to be effective tools to reduce postoperative stress and to favour a safe and prompt recovery (Douglas 2002). These elements are combined with a minimally invasive operative technique and aggressive postoperative rehabilitation (e.g. enteral nutrition and ambulation) in so called "Fast-track surgery" in humans. This method of care has been shown to reduce the stress response and associated organ dysfunction; it optimizes recovery and prompts early hospital discharges (Brodner *et al* 2001; Kehlet and Wilmore 2002).

In the last decades, surgery and related procedures have also been recognized as major stressors in veterinary medicine (Hansen *et al* 1997; Hardie *et al* 1997; Taylor 1998; Vaisanen *et al* 2005). Surgical procedures represent a major source of stress for the animal, due to the surgery itself and various associated elements, such as pain, analgesia- and anaesthesia-induced dysphoria, human handling and confinement in a hospitalization unit (Hetts *et al* 1992; Hansen *et al* 1997; Hardie *et al* 1997; Mellor *et al* 2000; Wells 2004).

Surgical stress and that associated with related procedures has been evaluated in dogs using different markers. Cortisol and behavioural analysis have often been used (Hetts *et al* 1992; Beerda *et al* 1997; Hansen *et al* 1997; Hardie *et al* 1997; Beerda *et al* 1998; Vaisanen 2002), while more recently the acute phase response has been used to assess the postoperative outcome (Conner *et al* 1988; Yamamoto *et al* 1993; Cerón *et al* 2005).

An assessment based on behavioural evaluation presents some advantages compared with the one based on biochemical evaluation. Observing animal behaviour is a non-invasive method that allows rapid

control of the stressor, when possible. For example, in post-surgery pain evaluation the behavioural assessment makes possible a rapid intervention by analgesic administration while pain perception is increasing. Also, behavioural evaluation has been shown to be a more sensitive tool than physiologic parameters for pain assessment in conscious hospitalised animals (Holton *et al* 2001; Hellyer 2005). On the other hand, the behavioural assessment requires that the observer knows and is trained to observe the normal behaviour of the species, to understand which behaviour could be related with stress and/or pain in the specific context he is analysing (Mellor 2000). Many studies published in the last years in domestic and laboratory animals demonstrate the increasing interest in this issue. They attempted to identify behavioural categories that can be correlated with stress perception in dogs that are caged, hospitalised and/or undergoing surgery. (Hetts *et al* 1992; Beerda *et al* 1997; Hardie *et al* 1997; Beerda *et al* 1998; Mellor 2000; Roughan and Flecknell 2001).

Four different types of scoring system for acute pain are currently used for postoperative behavioural evaluation: the Visual Analogue Scale (VAS), the Simple Descriptive Scale (SDS) and the Numerical Rating Scale (NRS) and composite scales. A SDS has four or five degrees of severity (ex, No evidence of pain, Mild, Moderate, Severe, Very Severe) (Firth and Aldane 1999; Hellyer 2005). The SDS is easy to use but it does not allow small changes in pain response to be assessed. The NRS may be produced by assigning a numeric score to each of the categories of SDS. A NRS may include descriptive definitions of each category of pain (Hardie *et al* 1996) but it often provides no real improvement in accuracy over a SDS; the numeric score simply facilitates tabulation or analysis of results (Firth and Aldane 1999). The VAS is a simple scale, consisting of a straight line (usually 100 mm, horizontal or vertical) on paper, with a description of the limits of the scale written at each end (ex, No pain, Severe Pain). The observer places a mark somewhere along the line to interpret the degree of pain. The VAS is subject to a great degree of observer variation, but because it does not use defined categories, it is often considered to be more sensitive than a NRS or SDS

(Lascelles *et al* 1998; Firth and Aldane 1999). Composite scales have been developed combining some aspects of the different scales (Holton *et al* 2001; Firth and Aldane 1999). They use specific validated behavioural categories and physiological parameters to assess pain. A numerical score can be assigned, but there are no validated criteria to assign a score to a specific category for many of these scales (Holton *et al* 2001; Firth and Aldane 1999; Hellyer 2005).

An exception among composite scales is represented by the Glasgow Pain Scale (see Table 4.2). It is a behaviour-based questionnaire developed and validated to measure acute pain in dogs. The GPS uses well-defined behavioural categories to describe the behaviour of dogs in pain and assigns a scientific-based specific weight to each category for pain scoring (Holton *et al* 2001; Morton *et al* 2005).

As to the Acute Phase Response after surgical trauma, an increase in serum C - reactive protein after surgery in dogs has been described, reaching its maximum peak at 24 hours. It is related to the intensity of surgical trauma, more intense traumas cause a greater increase than low ones (Conner *et al* 1988; Yamamoto *et al* 1993; Cerón *et al* 2005,). By the time of suture removal after a surgery without clinical complications, CRP concentration was markedly decreased in dogs although WBC count was still increased. Therefore, serum CRP determination has been proposed as a more sensible tool than WBC count in post-surgery monitoring (Cerón *et al* 2005). An increase is described for Haptoglobin too, with a peak at 3-4 days after surgery (Cerón *et al* 2005).

**Table 4.2:** GPS questionnaire (Holton et al 2001; Morton et al 2005).

The questionnaire is made up of a number of sections each of which have several possible answers. Please tick the answers that you feel are appropriate to the dog you are assessing. If more than one answer is appropriate then tick all that apply. Approach the kennel and look at the dog's behaviour and reactions. From outside the dog's kennel look at the dog's behaviour and answer the following questions.

Look at the dog's posture, does it seem...

- Rigid **1,20**
- Hunched or tense **1,13**
- Neither of these **0,00**

Does the dog seem to be...

- Restless **1,17**
- Comfortable **0,00**

If the dog is vocalising is it...

- Crying or whimpering **0,83**
- Groaning **0,92**
- Screaming **1,75**
- Not vocalising/none of these **0,00**

If the dog is paying attention to its wound is it...

- Chewing **1,40**
- Licking or looking or rubbing **0,94**
- Ignoring its wound **0,00**

Now approach the kennel door and cal the dog's name. Then open the door and encourage the dog to come to you. From the dog's reaction to you and its behaviour when you were watching it assess its character.
Does the dog seem to be...

- Aggressive **1,22**
- Depressed **1,56**
- Disinterested **1,26**
- Nervous or anxious or fearful **1,13**
- Quiet or indifferent **0,87**
- Happy and content **0,08**
- Happy and bouncy **0,00**

Now look at the dog's response to stimuli. If the mobility assessment is possible then open the kennel and put a lead on the dog. If the animal is sitting down encourage it to stand and then come out of the kennel. Walk slowly up and down the area outside the kennel. If the dog was standing up in the kennel and has undergone a procedure that may be painful in the perianal area, ask the animal to sit down.
During this procedure did the dog seem to be...

- Stiff **1,17**
- Slow or reluctant to rise or sit **0,87**
- Lame **1,46**
- None of these **0,00**

The next procedure is to assess the dog's response to touch. If the animal has a wound, apply gentle pressure to the wound using two fingers in an area approximately 2 inches around it. If the wound is impossible to touch, then apply the pressure to the closest point to the wound. If there is no wound then apply the same pressure to the stifle and surrounding area.

When touched did the dog...

- Cry **1,37**
- Flinch **0,81**
- Snap **1,38**
- Growl or guard wound **1,12**
- None of these **0,00**

The immune response can be assessed in a straightforward and clinically useful fashion by using total and differential white blood cell counts (Blecha 2000; Moberg 2000; Schultze 2000; Stockham *et al* 2003). Neutrophils and lymphocytes have been used in humans and horses to assess postoperative stress, and their ratio has been proposed as a useful and inexpensive indicator of perioperative stress (Zakowsky 1992; Stover *et al* 1998; Tayama *et al* 1999). Changes in neutrophils and lymphocytes are mainly related to the postoperative inflammation and to the activation of the HPA axis during perioperative stress (Blecha 2000; Moberg 2000; Schultze 2000; Stockham *et al* 2003). However, postoperative pain has been also found to be responsible for lymphocyte apoptosis (Delogu *et al* 2001; Alleva *et al* 2003).

Prolactin has been used for perioperative stress assessment in humans (Marrocco-Trischitta *et al* 2004; Elena *et al* 2006; Gauter-Fleckenstein *et al* 2007). But, to our knowledge, it has been never used with this aim in companion animals. Marrocco-Trischitta *et al* 2004 described an increase in serum prolactin level after carotid angioplasty, and the hormone was sensitive enough to detect the effect of two different surgical techniques on the postoperative stress response.

## 4.3. PHEROMONES, ANIMAL BEHAVIOUR AND STRESS

According to Dawkins 1995, "communication occurs when one animal's behaviour can be shown to have an effect on the behaviour of another. *Signals* are the means by which these effects are achieved". Pheromones are a subclass of signals used in chemical communication between animals (and humans), the so called *semiochemicals* (Wyatt 2003).

The word *pheromone* derives from the Greek *pherein*, to carry or transfer, and *hormōn*, to excite or stimulate. Pheromones were originally defined as "substances secreted to the outside by an individual and received by a second individual of the same species in which they release a specific reaction, for instance a definite behaviour (releaser

pheromones)" (Karlson and Lüscher 1959). The action of pheromones between individuals is contrasted with the action of hormones as internal signals within an individual organism.

Pheromones can be classified according to their function. Wyatt 2003 classification includes:

- sex pheromones
- aggregation and host-marking pheromones
- scent marking pheromones
- social pheromones
- recruitment pheromones
- alarm pheromones

Sexual, social and territorial behaviors are under the relevant influence of pheromones, especially for that species living in groups. A scent mark helps to distinguish among individuals of the same group, finding the appropriate partner and learn about his physical and health status, delimitating the territory needed for predatory and sexual activity. Alarm pheromones activate the "flight or fight" response and alert the other individuals of the possible danger. In this case there is a direct activation of the stress response, with a primary importance in predatory behaviour. But also other pheromones can be involved in the activation of the stress response. Social, aggregation or host marking pheromones can trigger the stress response when an animal is separated from his group or when an intruder is present in the group (Wyatt 2003).

Signals sent by chemical messages can be associated with a ritualized behaviour, making them conspicuous and exaggerated (Dawkins 1995). Ritualization could be the evolution of pre-existing chemicals as a pheromone (e.g. body posture associated with marking behaviour in dogs and cats). However, not all signals evolve to be conspicuous. Pheromone signals associated with recognition cues in social insects and mammals may be subtle and complex (Wyatt 2003).

Chemical communication is relevant to the entire animal kingdom, from insects to the most evolved vertebrates. The first pheromone to be isolated in mammals was the boar's pheromone produced by the sub-maxillary glands. It is the responsible for the so called "ram effect", which is the activation of ovulation in the ewes by the ram's skin secretion. The main component of this pheromone is 5 α-androsterone, a steroid with a urine odour (Pageat and Gaultier 2003a; Wyatt 2003).

Pets also use chemical communication. Among many different chemical signals, cats use the cheek and perioral glands during the "rubbing" behaviour, to deposit the F3 facial pheromone, to mark and classify the environment in known and unknown objects. This pheromone increases the control over the environment and has an emotional stabilization function (Pageat and Gaultier 2003a; Wyatt 2003).

As to the dog pheromones, facial pheromones are involved in social communication and seem to be related with social status. Smelling and licking the periauricular area of other dogs, is common in submissive individuals after an aggressive interaction. Glands localized in the interdigital area are responsible for the release of territorial and alarm pheromones, while perianal and genital glands secrete pheromones involved in social and sexual communication. The mammary complex of bitches produces a pheromone with an appeasing effect on puppies, and for this reason called *appeasine*. More details about the dog appeasing pheromone will provided further in these section. Finally, urine and feces contains chemical signals of pimary importance in marking behaviour, as well know by many complaining dog owners (Pageat and Gaultier 2003a).

## 4.4. PRODUCTION OF PHEROMONES

*Carnivora* are the mammalian species with the most complex and varied type of pheromone-secreting glands. Different types of glands and mucous membranes can secrete pheromones. This functional specificity

is not related to a histological common structure, since glands as different as ceruminous, sweat or sebaceous glands are involved in pheromone secretion. Similarly, the chemical structure of pheromones is very diverse. Steroids, fatty acids, aliphatic acids and amines can be pheromones. Moreover, many pheromones in carnivores are produced by the action of bacterial fermentation on the secreted molecule, such as the fatty acids secretion in the anal gland of canids (Pageat and Gaultier 2003a; Wyatt 2003).

The characteristics of pheromones can be related to their signalling function and the signalling environment. Pheromones used in terrestrial environment are usually low weight and volatile molecules. In some mammals, to increase the longevity of small volatile pheromones, they are associated with carrier proteins which release them slowly. On the other hand, in water the main characteristic of pheromones is solubility, rather than volatility (Wyatt 2003).

## 4.5. PERCEPTION OF PHEROMONES

The perception of pheromones is made possible by the vomeronasal organ (VNO, see Figure 4.2). This is a bilateral organ situated at each side of the nasal septum in a small fossa. Each part of the VNO is about 4 cm in the dog. The VNO is surrounded by the vomeronasal cartilage, forming a tube closed at its caudal end. The presence of smooth muscular fibers and elastic fibers allow the suction of pheromones inside the organ. The lumen of the VNO is surrounded medially by nervous epithelium and laterally by respiratory mucous membrane (see Figure 4.3). The respiratory membrane produces mucous containing a particular type of proteins, the pheromone binding proteins (PBPs), with a specific affinity for fatty acids. The axons of the nervous epithelium merge together forming the vomeronasal nerve, which is dedicated to the transmission of the stimulus initiated by the pheromone. This nerve is connected to the accessory olfactory bulb and then to the amygdala through the limbic system. In contrast to the main

olfactory tract, there is no connection between the VNO and the neocortex even through the thalamus (Pageat and Gaultier 2003a; Wyatt 2003).

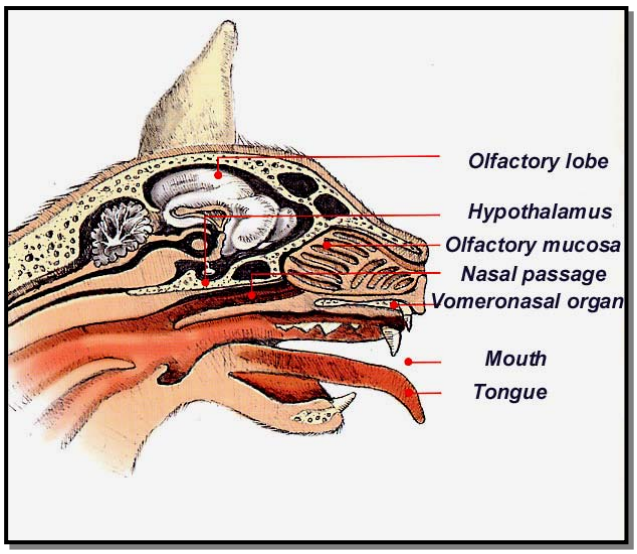



**Figure 4.2: Vomeronasal Olfactory System.** Immediately caudal to the incisor teeth is a papilla onto which open two nasopalatine canals. These canals allow slow passage of odors from the mouth to the vomeronasal organ located within the hard palate. The vomeronasal organ (organ of Jacobson) is lined with olfactory cells, and has central pathways different from those of olfactory epithelium. Impulses first travel to the accessory olfactory bulb and then to areas of the hypothalamus associated with sexual behaviour, feeding behaviour, and, possibly, social interactions (Picture from http://maxshouse.com/vomeronasal-flehmem.htm).

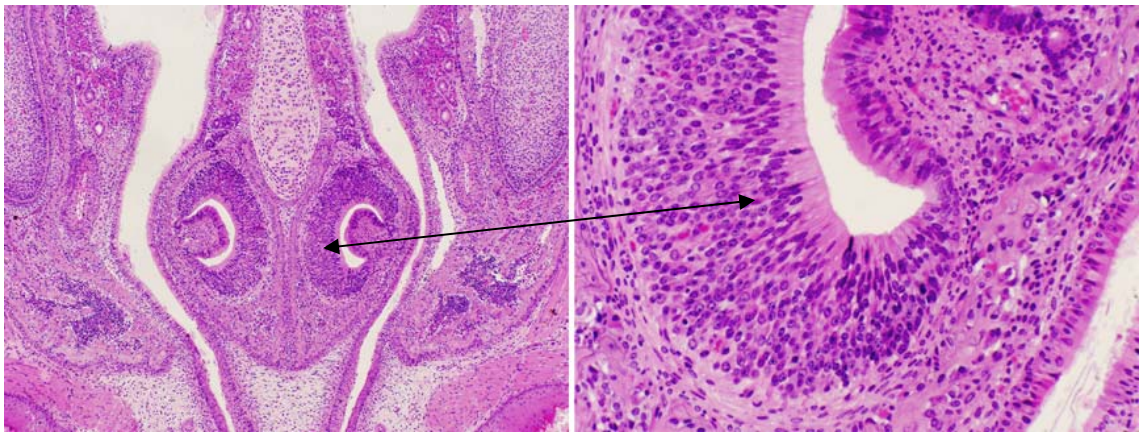


**Figure 4.3: Olfactory epithelium in the vomeronasal organ.** It's a very high pseudostratified columnar type. Three cell types are present, only one of which is chemoreceptive (Photos from education.vetmed.vt.edu/.../labs/Lab25/lab25.htm).

In many species of mammals (e.g. horses and cats) the suction of the pheromones follows a behaviour called *flehmen* (see Figure 4.4). It

consists of raising the upper lip with a partial opening of the mouth, associated with movements of the tongue in dogs and cats. The contraction of the muscle *levator labii maxillaris* is responsible for the movement of the upper lip and for the displacement of the incisive papilla, which allows the opening of the incisive duct. The latter communicates with the vomeronasal duct and then the VNO.

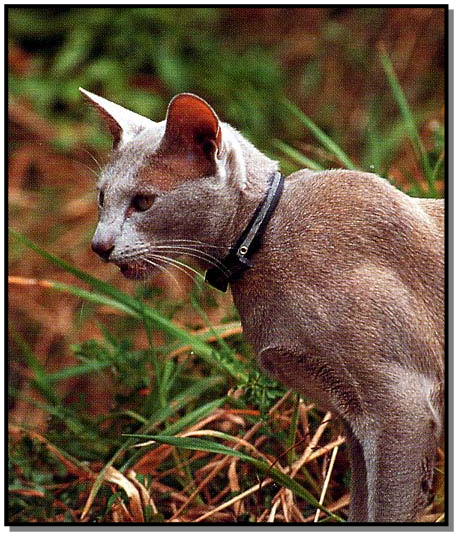


**Figure 4.4: Flehmen** is the behaviour associated with the inhalation of odors into the nasopalatine canals. Beginning as early as 6 weeks, a cat will sniff a particular odor source, such as urine, often touching it with its nose and perhaps its tongue. The head is then raised with the lips drawn back, nose wrinkled, and mouth partially open for inhalation. This behaviour is similar to that seen in ruminants and horses; however, the philtrum of the feline upper lip prevents its complete elevation. Flehmen, also called lip curl or gape, is most frequently displayed by tomcats (Photo: Graham Meadows from http://maxshouse.com/vomeronasal-flehmem.htm).

The occurrence of *flehmen* remains controversial in dogs. We do not observe in fact in dogs the same sequence of behaviors as described before, but the analogous could be represented by the *tonguing* behaviour described in Pageat and Gaultier 2003a. During this behavioural sequence the dog pants, raises the upper lip, creases the nose and rapidly flicks the tongue against the incisive papilla during exploration of faces, urine or proestral blood (see Figure 4.5). The *flehmen* or *tonguing* produces the aspiration of the pheromone that is mixed with the mucus in the VNO. Here the pheromone binds with the PBPs and stimulates the receptors located on the nervous epithelium.

How the brain responds after canine VNO's stimulation, it is still unclear (Pageat and Gaultier 2003a; Wyatt 2003).

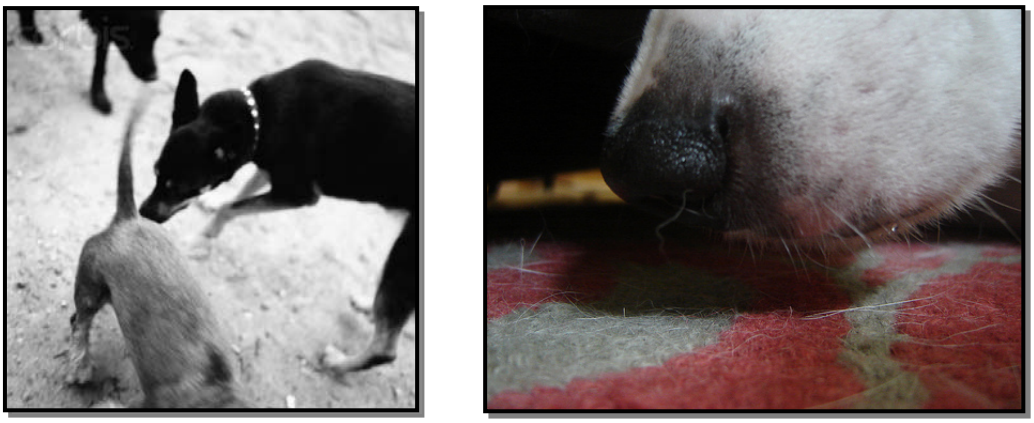


**Figure 4.5: Smelling during social interaction between dogs** is linked to chemical signaling by pheromones´ release and perception (Photos: Ann Giordano from http://pro.corbis.com/ and trends.move.com/help-your-pets-help-your-house/).

## 4.6 APPEASINES

The discovering of the *appeasines* was based on the observation that newborns were calmed down by the proximity to the mammary area. It was supposed that this effect was due to olfactory interaction between the newborn and his mother. This hypothesis was confirmed when the first appeasing pheromone was isolated by the mammary complex of sows (Pageat 2000). Thereafter, the same kind of pheromones was isolated by the sebaceous glands on the inter-mammary sulcus in mares, cows, ewes, queens, does and bitches (Pageat and Gaultier 2003a).

Appeasines are composed by a species specific sequence of fatty acids. The specific sequence of the dog appeasine is: myristic acid, lauric acid, pentadecanoic acid, stearic acid, oleic acid, palmitic acid and linoleic acid. The last three fatty acids are also present in all the appeasing pheromones of the other species. After secretion by sebaceous glands of these acids, that are not too volatile at the normal skin temperature, the saprophytic bacteria transform part of these in the correspondent methyl-esters, which are more volatile. The dog appeasine is secreted

from 3-4 days after parturition and persist 2 to 5 days after the weaving of puppies (Pageat and Gaultier 2003a).

## 4.7. PHEROMONOTHERAPY: THE DOG APPEASING PHEROMONE (DAP)

The use of synthetic pheromones has been proposed as a valid aid to treat behavioural disorders. This therapeutic approach has been defined as *pheromonotherapy* (Pageat and Gaultier 2003a). The use of this therapy is limited to those contexts in which the motivation of the undesired behaviour is related with the pheromone signal. An accurate use of the pheromonotherapy and its prescription in the right context is essential for its success. The right pheromone has to be chosen and emitted at the right time and on the right place so as to obtain the expected result (Pageat and Gaultier 2003a).

The precise mechanism of action of pheromones used in pheromontherapy is still unknown, but they are thought to induce some modifications in both the limbic system and the hypothalamus. In that way, the emotional status and the response of the animal during the behavioural modification program are modified. According to the type of pheromone prescribed, behavioural patterns can be induced (e.g. facial marking in the cat) or inhibited when undesired (e.g. urine marking, fear and anxiety-related behaviors) (Pageat and Gaultier 2003a).

Synthetic pheromones have been created for cats and dogs. Two fractions of the cat facial pheromone (F3 and F4), used during natural facial marking, have been reproduced and marketed. The sequence of the natural dog appeasine has been also reproduced in its synthetic analogous: the Dog Appeasing Pheromone (DAP) (Pageat and Gaultier 2003a). The product has been marketed as a plug-in diffuser, a natural spray and a collar, containing 2% of synthetic appeasing pheromone (see Figure 4.6).


**Figure 4.6:** Marketed DAP products: 2% plug-in diffuser, natural spray and collar.

The DAP has been described as having an appeasing effect in different stressful situations, such as separation-related problems, fear of fireworks, veterinary clinical consultation, house soiling, kenneling and social isolation (Sheppard and Mills 2003; Gaultier *et al* 2005; Tod *et al* 2005; Levine *et al* 2006; Mills *et al* 2006; Taylor and Mills 2006; Gaultier *et al* 2008).

The use of DAP for the treatment of behavioural problems, as well as the use of other synthetic pheromones, has some specific limitations. First, chemical signals are released in the environment often associated with a ritualized behaviour that emphasizes the message sent. The aim of these associated signals is to elicit the receptivity in the other individual, inducing also the opening of the VNO (Pageat and Gaultier 2003a; Wyatt 2003). Chemical appeasing signals could be associated in dogs with oral behaviors, physical contact or vocalizations that contribute to emphasize the calming effect. To increase the probability for the treated animal to smell the odor of the DAP and thus to open the VNO, the synthetic pheromone is administered in a higher concentration than the necessary natural amount. In this way, the same odor of the pheromone is supposed to work as an emphasizing signal and to stimulate the opening of the VNO (Pageat and Gaultier 2003a). However, the absence of the associated ritualized behaviors could compromise the efficacy of the treatment.

Moreover, the DAP can be effective only for those behavioural problems that are stress-, fear- or anxiety-related. An accurate diagnosis

need to precede the prescription of the DAP for behavioural treatment, to avoid a failure in the treatment that could attributed to the inefficacy of the product.

5

# PERIOPERATIVE STRESS RESPONSE IN DOGS UNDERGOING ELECTIVE SURGERY: VARIATIONS IN BEHAVIOURAL, NEUROENDOCRINE, IMMUNE AND ACUTE PHASE RESPONSES

Siracusa C, Manteca X, Cerón J, Martínez-Subiela S, Cuenca R, Lavín S, Garcia F and Pastor J

Published in *Animal Welfare* 2008, 17: 259-273.

## 5.1. INTRODUCTION

The perioperative stress response is a physiological reaction to surgery, and various associated conditions such as pain, analgesia- and anaesthesia-induced dysphoria, human handling and confinement to a hospitalization cage - all elements that may be perceived by the animal as physical and/or psychological threats (Hansen *et al* 1997; Hardie *et al* 1997; Mellor *et al* 2000; Moberg 2000; Väisänen *et al* 2002). Surgery trauma itself elicits a biological stress reaction that has been described in humans, horses and partially in dogs (Hansen *et al* 1997; Hardie et al 1997; Stover *et al* 1988; Taylor 1998; Väisänen *et al* 2002; Marrocco-Trischitta *et al* 2004). Of the factors described as perioperative stressors, those causing psychological stress have not been adequately evaluated. Confinement in an unfamiliar size-restricted environment, usually in the form of an Intensive Care Unit (ICU) cage, together with social isolation and handling by unknown people, represent a major pre and postoperative threat capable of activating the stress response (Hetts *et al* 1992; Wells 2004).

The stress response is a complex phenomenon in which four different components can be distinguished: the behavioural, the neuroendocrine, the immune and the autonomic nervous system responses (Matteri *et al* 2000).

The behavioural response normally represents the initial attempt at stressor control, thus observing animal behaviour may provide a non-invasive tool for identification and rapid control of stressors when possible (Moberg 2000; Rushen 2000). As to the perioperative stress response, many recent studies on domestic and laboratory animals have attempted to identify behaviour correlated with stress perception caused by caging, hospitalisation and/or surgery (Hetts *et al* 1992; Beerda *et al* 1997, 1998, 1999; Hardie *et al* 1997; Mellor *et al* 2000; Roughan & Flecknell 2003; Väisänen 2005). Behavioural changes have also been proposed as good indicators of pain perception during post-surgery stress

response (Morton and Griffiths 1985; Sanford *et al* 1986; Hardie *et al* 1997; Desborough 2000; Mathews 2000; Blackburn-Munro G 2004).

The neuroendocrine stress response involves both the hypothalamic-pituitary-adrenal (HPA) and lactotropic axes (Matteri *et al* 2000). Cortisol and glucose have often been used as markers to assess the HPA axis activation. An increase in serum or salivary cortisol and serum glucose has been described in dogs exposed to several stressors (Hetts *et al* 1992; Beerda *et al* 1996, 1997, 1998; Hansen *et al* 1997; Coppola *et al* 2006). The secretion of pituitary prolactin, used as a marker for lactotropic axis activation, is regulated by the suppressive effect of hypothalamic dopamine and the stimulatory effect of TRH, neurophysin, substance P and other factors (Matteri *et al* 2000). Dog prolactin is known to be involved in the emotional response and increases during positive interaction with humans (Odeendaal and Meintjes 2003; Pageat 2005). Animals with generalised anxiety show hyperprolactinaemia, while dogs with phobias or mild anxiety do not (Pageat and Gaultier 2003; Pageat *et al* 2007).

The immune system response during stress is related mainly to HPA axis activation and an increase in circulating glucocorticoids. It can be assessed in a straightforward fashion by using total and differential white blood cell counts. Neutrophilia, monocytosis, lymphopenia and eosinopenia can thus be observed after exposure to different stressors (Blecha 2000; Moberg 2000; Schultze 2000; Stockham *et al* 2003).

Acute phase proteins may also be elevated in association with physical and psychological stress in humans, cattle, rats and mice (Marrocco-Trischitta *et al* 2004; Murata *et al* 2004; Cerón *et al* 2005). An increase in the hepatic synthesis of acute phase proteins in response to cytokine-mediated HPA axis activation has been proposed as the mechanism involved in the acute phase response to stress (Murata *et al* 2004).

Variations in postoperative stress biomarkers have been widely described in many species. Postoperative increases in cortisol and

glucose (Hansen *et al* 1997; Vaisanen *et al* 2002; Ambrisko *et al* 2005; Devitt *et al* 2005; Sibanda *et al* 2006), changes in immune function and variation of C-reactive protein (CRP) and haptoglobin (Hp) (Taylor 1998; Zahorec 2001; Murata *et al* 2004; Marrocco-Trischitta *et al* 2004; Cerón *et al* 2005) have been described in dogs and humans. Postoperative increases in prolactin have been reported in humans (Marrocco-Trischitta *et al* 2004). However, to our knowledge there has been no description of biochemical and/or hematological changes due to preoperative procedures.

The individual stress response is influenced by many factors such as genetics and early life experiences (Mason 2000, Rushen 2000). In this light, the kind of preoperative response shown by each subject, caused mainly by psychological stressors, should be considered when attempting to evaluate the corresponding post-surgery stress. Though separate studies have been devoted to pre- and post-surgery stress in dogs (Hardie *et al* 1997; Väisänen *et al* 2005), to our knowledge no attempt has been made to date to evaluate pre- and postoperative variations in the same group of animals.

The main aim of the present clinical study is to describe behavioural changes and variations in salivary cortisol, serum glucose, serum prolactin, total white blood cell count, white blood cell differential, neutrophils/lymphocytes ratio, serum haptoglobin and serum C-reactive protein due to perioperative stress in dogs undergoing elective orchiectomy or ovariohysterectomy, while also attempting to assess differing sensitivity to psychological and physical perioperative stressors. Our ultimate goal is to identify useful tools for perioperative stress assessment in clinical conditions.

## 5.2. MATERIALS AND METHODS

### *Animals*

A group of 16 adult dogs (7 females and 9 males, 2.6±1.6 years old, both pure and mixed breed) underwent elective orchiectomy or ovariohysterectomy. All dogs had been kept in a public shelter for a minimum of 20 days, in a 6 $m^2$ pen together with one or two other dogs, according to local by-laws (see Figure 5.1). A thorough physical examination, complete blood cell count, biochemistry panel and leishmania antibodies serum concentration (ELISA test) was performed on all dogs and only healthy animals were included in our study. The reproductive status of each female was determined using a progesterone kit (Active® Progesterone EIA, DSL Inc., Webster, Texas, USA) and pseudo-pregnant, pregnant or lactating females were excluded. In addition, dogs presenting stereotyped behaviour or showing aggressiveness towards humans were not included. The animals enrolled in the study fasted during the surgery-day (at least 18 hours pre-surgery).

**Figure 5.1:** 6 $m^2$ pen representing the usual environment for the sheltered dogs enrolled in the study.

*Sampling procedures*

The timing of sample collection is summarized in Table 5.1. The study began every day between 10 and 11 a.m. local time. The first blood and saliva samples were collected from each dog in its usual environment (T0) the same day as surgery. Samples collected at that time were considered as basal values. The dogs were then transferred by walking to the ICU, located in the same shelter holding facility, and placed in a 110x70x70 cm cage (see Figure 5.2), where behaviour was video-recorded for 30 minutes (T1). The study period ended with a standardised dynamic interaction test for pain evaluation, also video-recorded (The collection of behaviour samples and interaction tests will be described later in this section). After this, further blood and saliva samples were obtained (T1). The dog was afterward transferred to the operating theatre. All surgeries were performed by the same graduate surgeon with the collaboration of different veterinary undergraduates. Surgery was considered to be finalized with extubation of the animal.

**Figure 5.2:** Intensive Care Unit cage.

**Table 5.1:** Sample collection schedule.

| *Time* | *Definition* | *Samples* | *Parameters studied* |
|---|---|---|---|
| **T0 (basal)** | Surgery day<br>Dog in usual environment<br>Pre-surgery | Saliva | *Cortisol.* |
| | | Blood | *Glucose*<br>*PRL*<br>*CRP - Hp*<br>*WBC** |
| **T1** | Surgery day<br>Dog in ICU cage<br>Pre-surgery | Behaviour<br>30 minutes | *Behavioural categories* |
| | | Interaction test | *Behavioural categories*<br>*Pain scoring* |
| | | Saliva | *Cortisol* |
| | | Blood | *Glucose*<br>*PRL*<br>*WBC** |
| **T2** | Surgery day<br>Dog in ICU cage<br>Post-surgery | Behaviour<br>30 minutes | *Behavioural categories* |
| | | Interaction test | *Behavioural categories*<br>*Pain scoring* |
| | | Saliva | *Cortisol* |
| | | Blood | *Glucose*<br>*PRL*<br>*WBC** |
| **T3** | Surgery day<br>Dog in usual environment<br>Post-surgery | Saliva | *Cortisol* |
| **T4** | 24 h post-surgery | Blood | *CRP-Hp*<br>*WBC** |
| **T5** | 48 h post-surgery | Blood | *CRP-Hp*<br>*WBC** |
| **T6** | 8 days post-surgery | Blood | *CRP-Hp*<br>*WBC** |

* Includes total white blood cell count and differential count

A standardised anaesthetic and analgesic protocol was used for the surgical procedure. The dog were pre-medicated with buprenorphine 0,01 mg/Kg IM (Buprex®, Schering-Plough SA, Madrid, Spain), induced with thiobarbital 10 mg/Kg IV (Tiobarbital®, B Braun Medical, Barcelona, Spain) and diazepam 0,5 mg/Kg IV (Valium®, Roche Farma SA, Barcelona, Spain). The anaesthesia was maintained with isofluorane 1-2% (Isoflo®, Abbot Laboratories, Illinois, USA) vaporised in 100% oxygen 0,5-1 L/minute, delivered with a semi-disposable circle circuit

(Burtons Medical Equipment Ltd, Kent, UK). The vaporiser setting was adjusted to maintain a surgical plane of anaesthesia as judged by eye position, jaw tone and lack of response to noxious stimuli. All dogs received intravenous crystalloid solution (Lactato de Ringer Braun, B Braun Medical, Barcelona, Spain) at 5-10 ml mL/Kg/hour. Each dog was treated with an antibiotic, amoxicilline LA 11-22 mg/Kg SC (Bivamox® LA, Boehringer Ingelheim España, Barcelona, Spain) and anti-inflammatory therapy, caprofen 4,4 mg/Kg SC (Rimadyl, Pfizer España, Madrid, Spain) for four days after surgery.

After extubation the dogs were transferred to the ICU cage, where every 30 minutes the degree of sedation was checked. When the animal was able to stand in the ICU cage, its behaviour was video-recorded for 30 minutes, after which the dynamic interaction test for pain evaluation was performed and video-recorded (T2). At the end of this observation, blood and saliva samples (T2) were collected. The dog was afterward transferred to its usual pen where, after 30 minutes, a saliva sample (T3) was taken.

No blood sample was collected at this time (T3) for ethical reasons, i.e. to reduce the risk of post-surgery complications. Blood samples were also obtained at 24 hours (T4), 48 hours (T5) and 8 days (T6) after surgery. No more than two dogs were confined in the ICU at any given time, to minimize the variability of environmental influences on the dog's behaviour.

### *Behavioural data collection and analysis*

All behavioural samples were recorded with a digital video camera (Sony Handycam DCR-HF-40, Sony Corporation, Tokyo, Japan). Videos were always viewed and analyzed on a 21-inch monitor (Sony Corporation, Tokyo, Japan) by the same observer.

Behavioural data were divided into categories, evaluated for frequency of occurrence (*Events,* see Table 5.2) and duration (*States,* see Table 5.3), and collected on a check sheet. Categories evaluated as events were logged by continuous recording and their number of occurrences during the time of observation was considered, while those evaluated as states were logged by instantaneous sampling at 2 minutes intervals (15 instantaneous recording points in 30 minutes) (Martin and Bateson 1993). Behaviour scored in terms of frequency was recorded as occurring once every 5 seconds when the dogs displayed it in a continuous fashion (Beerda 1999).

**Table 5.2:** Behavioural categories evaluated as events studied.

| *Category* | *Definition* |
|---|---|
| BARKING | Low frequency vocalisation, more or less soft or raucous |
| GROWLING | A throaty rumbling vocalisation, usually low in pitch. It may be used in aggressive or defensive interaction. |
| WHINING | Repeated, relatively brief, "exhalation vocalisations" of falling pitch. |
| YELPING | Loud, high pitched vocalisations. |
| MOUTH OPENING | The dog opens and closes the mouth with rapid movements. The tongue is not visible. It could correspond to yawning. |
| AUTOGROOMING | Behaviours directed towards the subject's own body, like scratching, licking and biting-self, to take care of the skin and coat. It includes taking care of wounds. |
| TAIL CHASING | The dog chases its own tail with continuous round movements. |
| CIRCLING | Walking in a circle. |
| PACING | Continuous movements from one extreme to the other of the cage. |
| DIGGING | Scratching the floor with the forepaws in a way that is similar to when dogs are digging holes. |
| BARRIER MANIPULATION | Chewing, touching with legs or licking the enclosure. |
| JUMPING | Springing into the air, either spontaneously or in order to make contact with an object or a person |
| LIP LICKING | The dog licks the lips exhibiting part of the tongue. |
| NOSING | The nose is moved along objects and/or clear sniffing movements are exhibited. |
| PAW LIFTING | A fore paw is lifted into a position of approximately 45º. |
| TAIL WAGGING | Repetitive wagging movements of the tail. |

*(Adapted from Beerda et al 1997; Beerda et al 1998; Goodmann et al., 2002; Hardie et al 1997; Hetts et al 1992; Morton and Griffiths 1985).*

**Table 5.3:** Behavioural categories evaluated as states studied.

| *Category* | *Definition* |
|---|---|
| PANTING | An increased frequency of inhalation and exhalation often in combination with the opening of the mouth |
| VISUAL SCANNING | Visual exploration of the environment through cage's door. |
| AWAKE /ALERT | Dog with opened eyes. |
| REST /SLEEP | Dog inactive and with closed eyes. |
| TREMBLING | Body shaking with little, high frequency, movements. |
| WALKING | Displacement from a point to another, with no clear exploring movements. |
| EXPLORING | The dog moves slowly, sniffing and investigating the environment. |
| LIE ON SIDE | Positioned fully on side, one side of the dog in complete contact with the ground. |
| LIE HALF SIDE/VENTRAL | Positioned on side with body, but not head in complete contact with the ground, or with ventrum and legs in contact with ground. |
| LIE DORSAL | Positioned flat with back in contact with the ground. |
| SITTING | The pads of the front paws are on the ground with the front legs straight and the rump squarely on the ground. |
| STANDING | Positioned with just four paws in contact with the ground, or two with the ground and two with the wall. |
| AGAINST WALL | The dog is against the walls of the enclosure, with the eyes opened or closed. |
| AGAINST DOOR | The dog is against the door of the enclosure, with the eyes opened or closed. |

*(Adapted from Beerda et al 1997; Beerda et al 1998; Goodmann et al., 2002; Hardie et al 1997; Hetts et al 1992; Morton and Griffiths 1985).*

## *Dynamic interaction test for pain evaluation*

The interaction test was performed as follows: an operator knocked at the door of the ICU, opened it and entered. He reached for the cage, opened the door and greeted the dog gently ('Hi, how you doing?'). The operator then withdrew the dog from the cage, patting it gently from the chest to the flank and up to the ventral surgery site.

A single ethologist, familiar with the individual behaviour of the dogs enrolled in the study, carried out and recorded all tests, as well as later analysis and assessment. This strategy was designed to minimise the effect of individual behavioural variability of dogs.

The interactive behaviour of the dogs was analysed by using the Glasgow Pain Scale (GPS) (see Holton *et al* 2001 and Morton *et al* 2005, see Table 4.2). The behavioural categories shown by each dog were recorded. Both the frequency of occurrence of each behavioural category in pre- and post-surgery conditions and the corresponding total scores (Table 5.4) were compared, to obtain a qualitative and quantitative analysis.

### *Blood and saliva samples collection*

Blood samples were taken from the jugular vein using standard procedures. One ml of the sample was stored in an EDTA tube (Tapval Aquisel, Barcelona, Spain) and 3 ml transferred to tubes containing a coagulation activator (Tapval Aquisel, Barcelona, Spain). The samples were refrigerated during transport to the laboratory. After clot formation the serum obtained was transferred to *eppendorf* tubes and stored at –20° C.

Saliva samples were collected by the Salivette system (Sarstedt, Numbrecht, Germany) after salivary flow stimulation with 3% citric acid (Mandel, 1990; Beerda *et al* 1997). Saliva collection always preceded blood sample collection, with the animal never being handled for more than 2 minutes, to avoid direct influence of handling on stress measures (Kobelt *et al* 2003). Tubes were kept refrigerated during transport to the laboratory. The Salivette samples were then centrifuged at 3500rpm for 15 minutes and stored at – 20°.

### *Laboratory analysis*

Saliva cortisol concentration was determined with a commercial human saliva ELISA test (Cortisol Saliva, BLK Diagnostics, Barcelona, Spain) which had been adapted in our laboratory to measure cortisol concentration in canine saliva.

Serum samples were analysed for glucose detection with an enzymatic UV test (hexokinase method) following manufacturer's instructions (Glucose Olympus, Hamburg, Germany). Serum prolactin concentration was measured with a commercial ELISA kit (Milenia® Canine Prolactin, Milenia Biotec, Bad Nauheim, Germany) following manufacturer's instructions.

EDTA blood samples were analyzed within 6 hours of collection with a laser flow-cytometer (ADVIA 120 Haematology System, Bayer, Fernwald, Germany), which provided total white blood cell (TWBC) and differential count (neutrophils, monocytes, lymphocytes and eosinophils).

Serum samples were analysed to measure haptoglobin and C-reactive protein levels. Hp concentration was determined with an automated biochemical assay (Tridelta phase range serum haptoglobin, Tridelta Development, Wicklow, Ireland) following manufacturer's instructions. CRP concentration was measured with a canine CRP-specific solid phase sandwich immunoassay (Tridelta phase range canine CRP kit; Tridelta Development, Wicklow, Ireland).

Immune and acute phase response markers were evaluated on a long-term basis (24, 48 hours and 8 days post-surgery), according to previously published data (Ceron *et al* 2005) and because of the presumed major influence of the inflammatory response caused by tissue damage on marker variation. As glucose, cortisol and prolactin response to stress is known to be rapid (Matteri *et al* 2000), study of the markers was limited to the day of surgery to avoid influence of uncontrolled psychological stressors on response at 24, 48 hours and 8 days post-surgery.

### *Behavioural data statistical analysis*

Intra-observer reliability was determined by analysis of the correlation between two different observations of the same video-recording sample.

Four independent 10-minute samples of different subjects were used to calculate the Spearman Rho coefficient for the following behavioural categories: *nosing*, *lip licking*, *mouth opening*, *visual scanning* and *awake/alert* (Martin and Bateson 1993).

The influence of the type of surgery on behaviour was studied by comparing pre and postoperative values of dogs undergoing orchiectomy with those subjected to ovariohysterectomy -for all behavioural categories and GPS. Comparisons were made with a Mann-Whitney U test for all behavioural categories and GPS score, while the influence of surgery on the frequency of GPS categories was examined by using a $\chi^2$ test. Differences were considered statistically significant when $P \leq 0.01$.

Pre and post surgery differences were studied by using a Wilcoxon test for the occurrence of all behavioural categories analysed and GPS scores, and by using a $\chi^2$ test for the frequency of each GPS category. SPSS® 13.0 software (SPSS Inc., Chicago, USA) was used for the calculations. Differences were considered statistically significant when $P \leq 0.01$.

### *Hematological and biochemical data statistical analysis*

For the statistical analysis, the normal distribution of data was determined with a Shapiro-Wilk test. Data were considered to have a normal distribution when the test showed $P > 0.05$. To study the influence of tissue damage on stress response, basal (T0) and postoperative sampling times (T2, T3, T4, T5 and T6) of dogs undergoing orchiectomy and ovariohysterectomy were compared for all the markers analyzed (e.g. T2 ovariohysterectomy compared with T2 orchiectomy), by using the *t* test for normally distributed data and the Mann-Whitney U test for non-normal data. The values of parameters studied at different times and differences with basal values were analysed by a paired *t* test for data showing a normal distribution, and by a Wilcoxon test for those that did not. Differences were considered significant when $P < 0.05$. SPSS® 13.0 software (SPSS Inc, Chicago, USA) was used for the calculations.

## 5.3. RESULTS

### *Behavioural results*

Twelve (seven females and five males) of the 16 dogs enrolled in the study were suitable for behavioural analysis. Four dogs were excluded (three due to prolonged human presence and/or interaction that disturbed the study conditions; one escaped from its confinement cage).

Intra-observer reliability was high; the correlation, expressed by using a Spearman Rho coefficient, was 0.95 for *nosing*, 1.00 for *lip licking*, 0.95 for *mouth opening*, 0.94 for *visual scanning* and 0.95 for *awake/alert* ($P \leq 0.05$ for all categories).

No differences were found between dogs undergoing either ovariohysterectomy or orchiectomy for both pre and postoperative occurrence of behavioural categories and GPS score (data not shown). Male and female dogs were therefore grouped together for further analysis. The medium length of surgery, considered as the time during which the animal remained intubated, was 58.73 ± 5.04 (SE) minutes.

Behavioural results are shown in Table 5.4. The behavioural evaluation of dogs in the ICU cage showed statistically significant decreases between pre and post surgery frequency of occurrence for the events *mouth opening*, *lip licking* and *nosing*, and between pre and post surgery duration for the states *visual scanning*, *awake/alert* (with a corresponding increase in *rest/sleep*) and *sitting* ($P<0.01$ for all categories).

**Table 5.4:** Pre and post-surgery behavioural evaluation of events (a) and states (b) in dogs in confinement environment (SE= standard error). Categories showing a mean value > 0.00 are represented.

a)

| *EVENTS* | *Pre-surgery* | | *Post-surgery* | | *P* |
|---|---|---|---|---|---|
| | MEAN* | SE | MEAN* | SE | |
| BARKING | 11.09 | 10.89 | 0.09 | 0.09 | 0.180 |
| WHINING | 22.91 | 14.58 | 46.27 | 23.27 | 0.953 |
| OPEN. MOUTH | 3.82 | 1.61 | 0.00 | 0.00 | 0.017 |
| AUTOGROOMING | 1.27 | 0.91 | 1.18 | 0.81 | 0.914 |
| CIRCLING | 8.64 | 4.75 | 1.00 | 0.70 | 0.058 |
| PACING | 0.00 | 0.00 | 0.18 | 0.18 | 0.317 |
| DIGGING | 0.64 | 0.47 | 0.00 | 0.00 | 0.180 |
| BARRIER MANIPULATION | 2.18 | 1.30 | 0.18 | 0.18 | 0.131 |
| LIP LICKING | 29.73 | 11.81 | 0.45 | 0.21 | 0.007 |
| NOSING | 24.09 | 3.71 | 2.82 | 2.53 | 0.004 |
| PAW LIFTING | 3.36 | 2.20 | 0.82 | 0.82 | 0.109 |
| TAIL WAGGING | 10.82 | 10.82 | 0.45 | 0.37 | 1.000 |
| JUMPING | 0.91 | 0.64 | 0.00 | 0.00 | 0.109 |

b)

| *STATES* | *Pre-surgery* | | *Post-surgery* | | *P* |
|---|---|---|---|---|---|
| | MEAN** | SE | MEAN** | SE | |
| PANTING | 3.36 | 1.56 | 1.18 | 1.00 | 0.249 |
| VISUAL SCANNING | 12.18 | 0.84 | 2.64 | 0.79 | 0.003 |
| AWAKE/ALERT | 13.00 | 0.94 | 3.82 | 1.23 | 0.006 |
| REST/SLEEP | 2.00 | 0.94 | 11.18 | 1.23 | 0.006 |
| WALKING | 0.36 | 0.20 | 0.00 | 0.00 | 0.102 |
| EXPLORING | 0.45 | 0.20 | 0.09 | 0.09 | 0.157 |
| LIE ON SIDE | 0.36 | 0.36 | 4.36 | 1.80 | 0.074 |
| LIE HALF SIDE/VENTRAL | 7.00 | 1.89 | 9.09 | 1.84 | 0.285 |
| SITTING | 5.18 | 1.60 | 0.45 | 0.21 | 0.009 |
| STANDING | 1.64 | 0.99 | 1.00 | 0.90 | 0.066 |
| AGAINST WALL | 0.91 | 0.25 | 2.45 | 1.42 | 0.609 |
| AGAINST DOOR | 14.09 | 0.25 | 12.55 | 1.42 | 0.609 |

* Mean value of the total number of occurrences of the behavioural category throughout the 30 minutes of observation.

**Mean value of instantaneous sampling points in which the behaviour was detected during the 30 minutes of observation (15 points with intervals of 2 minutes), considered as an indicator of the duration (Martin and Bateson 1993).

A post-surgery interaction test was performed at a mean time of 92.5 ± 50.29 minutes from end of surgery. No statistically significant difference was observed at post surgery time for the GPS score compared to pre-surgery (1.49±0.42 vs. 1.74±0.43). As to GPS behavioural categories (Table 5.5), a statistically significant decrease of their frequency was seen between pre and postoperative periods for the category *Happy and Bouncy* (tail wagging, jumping in kennel, often vocalising with happy excited noise), while *Happy and Content* (interested in surroundings, positive interaction with observer, responsive and alert) and *Slow or reluctant to rise or sit* (slow to get up or sit down but not stilted in movement) showed a significant increase of frequency ($P \leq 0.01$ for all categories). See Holton *et al* 2001 for definition of GPS behavioural categories.

**Table 5.5:** Pre and post-surgery values on interactive behavioural categories (categories showing a mean value > 0.00 are represented).

| *GPS frequencies of behaviours* | Pre-surgery % | Post-surgery % | *P* |
|---|---|---|---|
| *Crying* | 0 | 8.3 | 0.307 |
| *Not Vocal* | 100 | 91.6 | 0.307 |
| *Depressed* | 0 | 8.3 | 0.307 |
| *Nervous* | 33.3 | 8.3 | 0.132 |
| *Quiet* | 8.3 | 25 | 0.273 |
| *Content* | 8.3 | 58.3 | 0.009 |
| *Bouncy* | 50 | 0 | 0.005 |
| *Stiff* | 33.3 | 16.6 | 0.346 |
| *Reluctant* | 0 | 41.6 | 0.012 |
| *Cry* | 0 | 8.3 | 0.307 |
| *Flinch* | 0 | 8.3 | 0.307 |
| *Growl* | 8.3 | 8.3 | 1.000 |

CRYING= Crying or whimpering; NOT VOCAL: Not vocalising; NERVOUS: Nervous or anxious or fearful; QUIET: Quiet or indifferent; CONTENT: Happy and content; BOUNCY: Happy and bouncy; RELUCTANT: Slow or reluctant to rise or sit; GROWL: Growl or guard wound.

*Haematological and biochemical results*

All the 16 dogs enrolled in the study were suitable for haematological and biochemical analyses. Total white blood cells, neutrophils, monocytes, lymphocytes, eosinophils and glucose showed a normal distribution, so a *t* test was used for statistical analysis. The distribution of all other parameters studied was not normal, so a statistical analysis was carried with Wilcoxon and Mann-Whitney tests.

Dogs undergoing ovariohysterectomy showed a lower lymphocyte value (2.46±0.69 * $10^3$ cells/μL) than those undergoing orchiectomy (3.76±0.76 * $10^3$ cells/μL; $P = 0.01$) 24 hours after surgery (T4) and higher cortisol values (41.04±24.09 ng/mL for ovariohysterectomy vs. 14.80±4.80 ng/mL for orchiectomy; $P < 0.05$) after return to their usual environment (T3). With the exception of these findings, no other differences in basal and postoperative mean values of dogs undergoing orchiectomy versus ovariohysterectomy were observed in any other parameter studied, and for this reason males and females were studied together.

Salivary cortisol concentrations showed significant differences between basal value (T0) and increased pre ($P < 0.05$) and post-surgery ($P = 0.01$) values on the day of surgery (T1 and T2) (see Figure 5.3). A statistically significant difference was seen in serum glucose variation between the basal value (T0) and the increased post-surgery value ($P < 0.01$) on the day of surgery (T2), even though a slight upward tendency began to become apparent before surgery (T1) (see Figure 5.4). For serum prolactin significant differences were detected by comparing the basal value (T0) with the decreased post-surgery value ($P < 0.01$) on day of surgery (T2), although a downward tendency began to be apparent at T1 (see Figure 5.5).

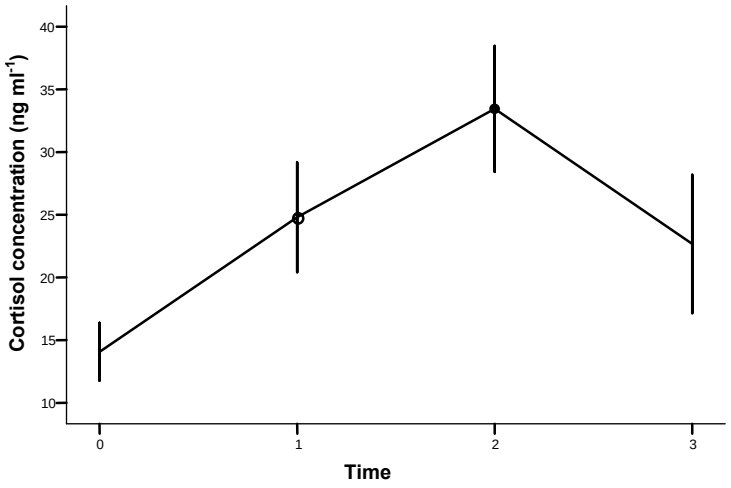



**Figure 5.3:** Variations of salivary cortisol at times T0, T1, T2 and T3 (mean value ± SE). Open marker indicates differences between marked value and T0 with $P < 0.05$; solid marker indicates differences between marked value and T0 with $P \leq 0.01$.

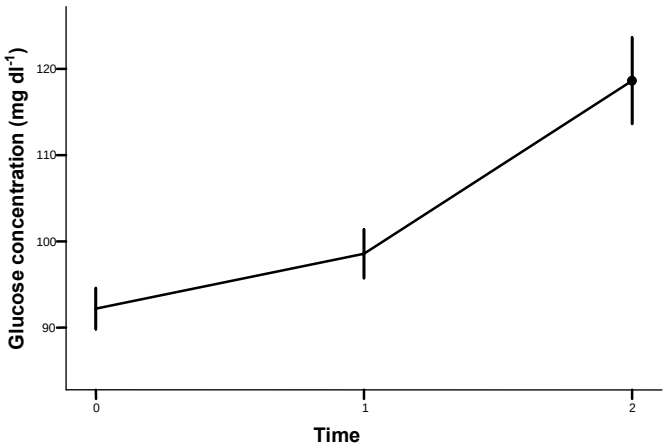



**Figure 5.4:** Variations of serum glucose at times T0, T1 and T2 (mean value ± SE). Open marker indicates differences between marked value and T0 with $P < 0.05$; solid marker indicates differences between marked value and T0 with $P \leq 0.01$.

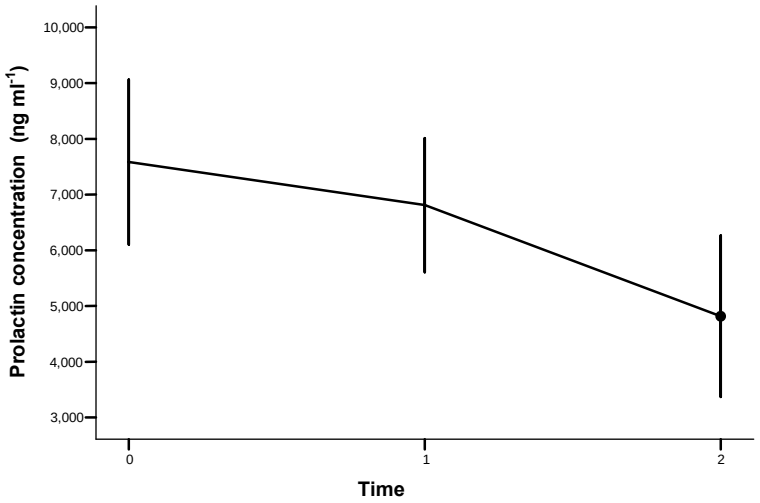



**Figure 5.5:** Variations of serum prolactin at times T0, T1 and T2 (mean value ± SE). Open marker indicates differences between marked value and T0 with $P < 0.05$; solid marker indicates differences between marked value and T0 with $P \leq 0.01$.

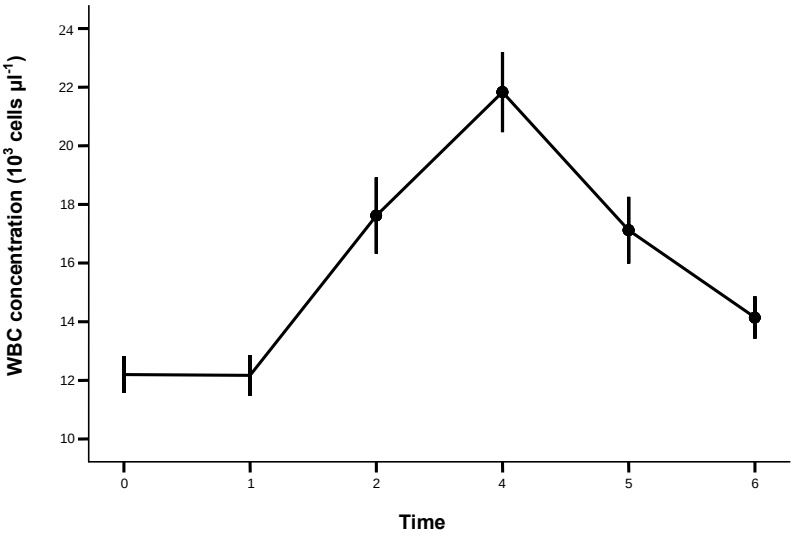



**Figure 5.6:** Variations of total white blood cells at times T0, T1, T2, T4, T5 and T6 (mean value ± SE). Open marker indicates differences between marked value and T0 with $P < 0.05$; solid marker indicates differences between marked value and T0 with $P \leq 0.01$.

Total white blood cells and neutrophils showed a significant increase when basal values (T0) were compared with values on the day of surgery (T2), 24 (T4), 48 hours (T5) and 8 days (T6) post-surgery values ($P < 0.01$ for all these comparisons) (see Figures 5.6 and 5.7). Monocytes showed statistically significant differences between basal value (T0) and increased post-surgery value ($P < 0.01$) on the day of surgery (T2), as well as between basal value and 24 ($P < 0.01$) and 48 ($P < 0.01$) hours post-surgery values (T4 and T5) (see Figure 5.8). Lymphocytes and eosinophils showed statistically significant differences between basal

value (T0) and decreased post surgery value (P < 0.01) on the day of surgery (T2) (see Figures 5.9 and 5.10). The neutrophil/lymphocyte ratio peaked at an early post-surgery time (T2) showing a statistically significant difference (P = 0.001) that was maintained for 8 days after surgery (P < 0.05 for all times) (Figure 5.11).

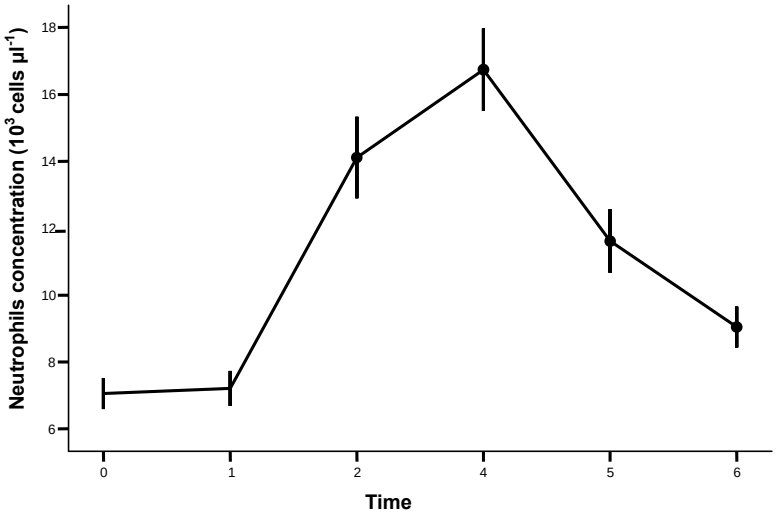



**Figure 5.7:** Variations of neutrophils at times T0, T1, T2, T4, T5 and T6 (mean value ± SE). Open marker indicates differences between marked value and T0 with P < 0.05; solid marker indicates differences between marked value and T0 with P ≤ 0.01.

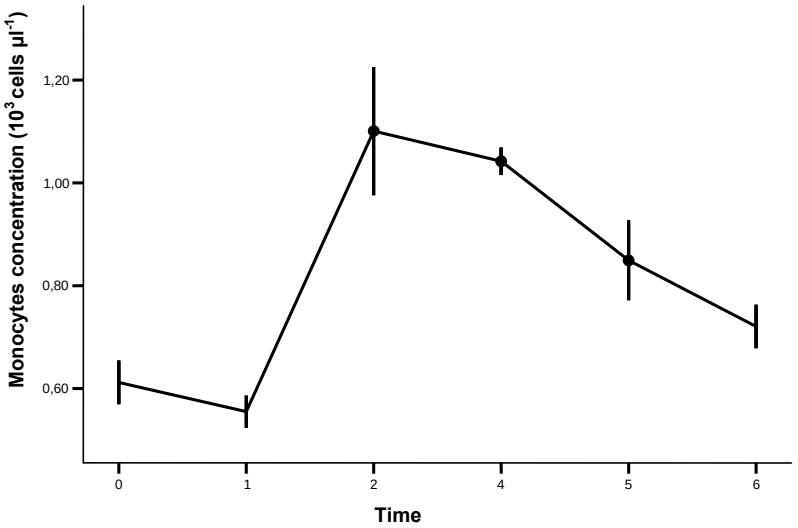



**Figure 5.8:** Variations of monocytes at times T0, T1, T2, T4, T5 and T6 (mean value ± SE). Open marker indicates differences between marked value and T0 with P < 0.05; solid marker indicates differences between marked value and T0 with P ≤ 0.01.

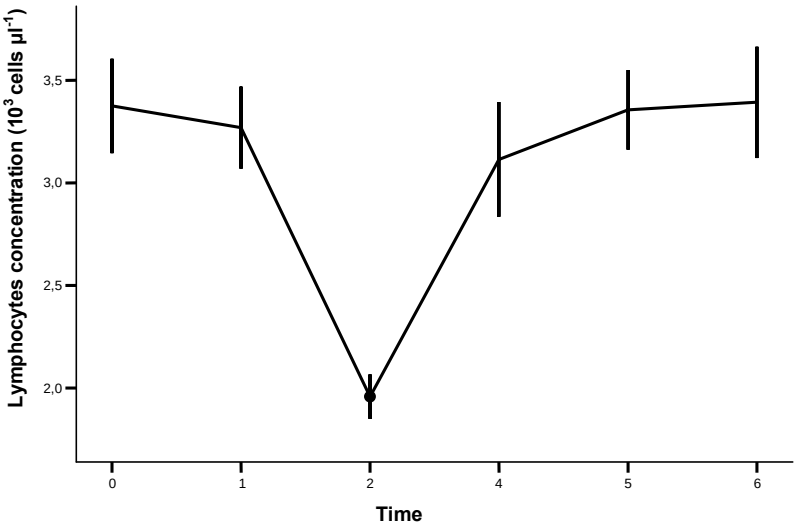



**Figure 5.9:** Variations of lymphocytes at times T0, T1, T2, T4, T5 and T6 (mean value ± SE). Open marker indicates differences between marked value and T0 with $P < 0.05$; solid marker indicates differences between marked value and T0 with $P \leq 0.01$.

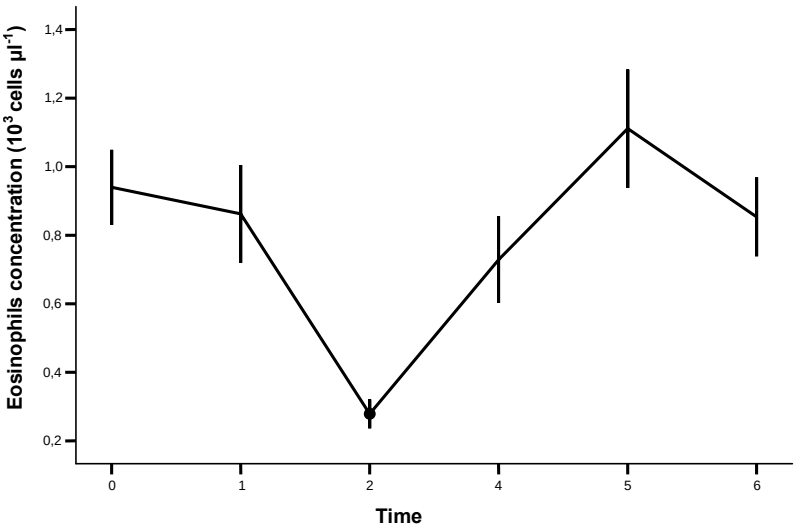



**Figure 5.10:** Variations of eosinophils at times T0, T1, T2, T4, T5 and T6 (mean value ± SE). Open marker indicates differences between marked value and T0 with $P < 0.05$; solid marker indicates differences between marked value and T0 with $P \leq 0.01$.

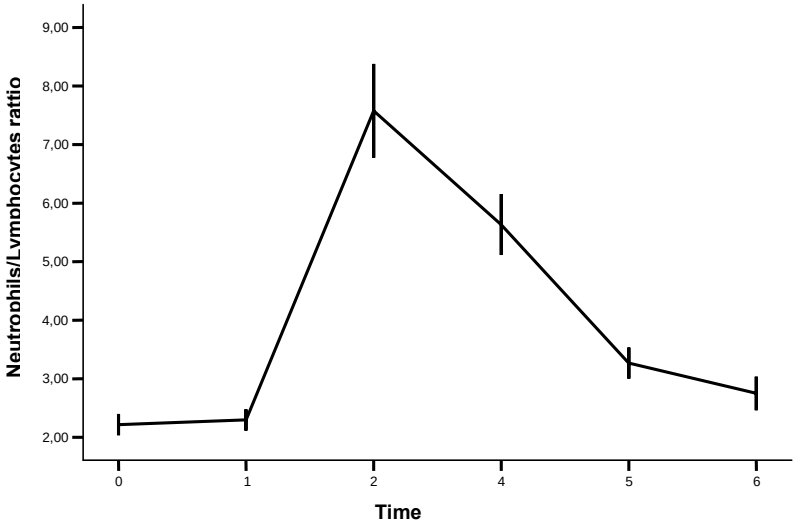



**Figure 5.10:** Variations of neutrophils/lymphocytes ratio at times T0, T1, T2, T4, T5 and T6 (mean value ± SE).

Serum CRP showed a significant increase when the basal value (T0) was compared with values at 24 (T4), 48 (T5) hours and 8 days (T6) after surgery ($P < 0.01$ for all comparisons) (see Figure 5.12). Serum Hp showed statistically significant differences between the basal value (T0) and the increased values (T4 and T5) at 24 ($P < 0.01$) and 48 ($P < 0.01$) hours (see Figure 5.13). Peak values for CRP and Hp were reached at 24 hours and 48 hours post-surgery respectively.

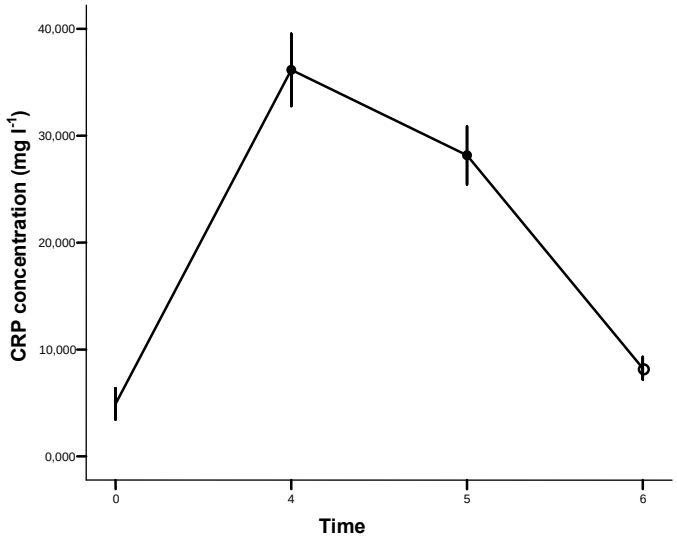



**Figure 5.12:** Variations of serum CRP at times T0, T4, T5 and T6 (mean value ± SE). Open marker indicates differences between marked value and T0 with $P < 0.05$; solid marker indicates differences between marked value and T0 with $P \leq 0.01$.

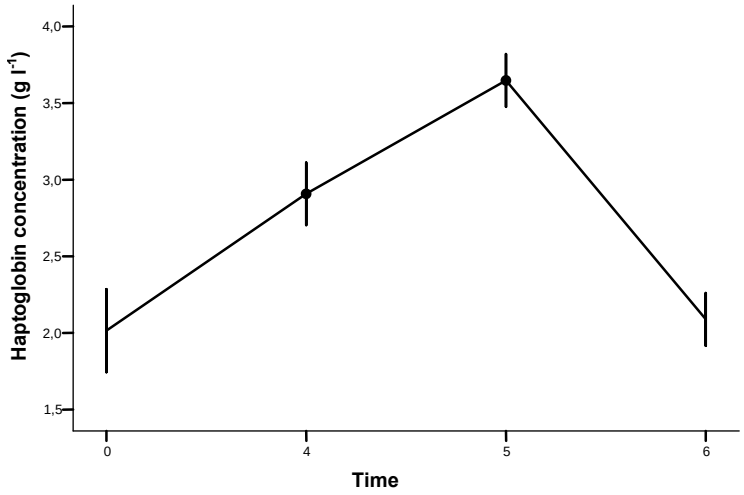



**Figure 5.13:** Variations of serum haptoglobin at times T0, T4, T5 and T6 (mean value ± SE). Open marker indicates differences between marked value and T0 with $P < 0.05$; solid marker indicates differences between marked value and T0 with $P \leq 0.01$.

## 5.4. DISCUSSION

Changes observed in behavioural, neuroendocrine, immune and acute phase responses appear to be good tools for the perioperative stress assessment in dogs undergoing elective surgery. With regard to the behavioural response, explorative (*nosing* and *visual scanning*) and communicative (*mouth opening* and *lip licking*) behaviours were the categories most affected by postoperative stress, as they showed a high frequency of occurrence and duration in pre-surgery (T1) compared with post-surgery (T2). According to Hardie *et al* 1997 postoperative pain can create a decrease in communicative and explorative behaviours, but the use of opioids also played a role in altering communicative abilities.

We observed also an increase in the duration of *rest/sleep* and a decrease in the duration of *sitting* position after surgery (T2) as compared to the preoperative period (T1). These changes may be related to the residual sedative effect of analgesia and anaesthesia and to the pain perceived (Hardie *et al* 1997; Roughan and Flecknell 2002).

By analysing the postoperative (T2) frequency of interactive behavioural categories (GPS) we observed a diminished tendency of the animal to move and interact actively with the handler, even though a positive attitude towards the handler persisted. The categories *Happy and Content*, implying a positive attitude but without active handler interaction, and *Slow or Reluctant to Rise or Sit* increased, while *Happy and Bouncy*, which implies active interaction such as jumping, decreased. Postoperative pain and opioids administration were described as causes for similar changes, while thiobarbiturates and diazepam use did not alter the dog's capacity to move and actively interact according to previous findings (Hart 1985; Hardie *et al* 1997; Pageat 1998; Thompson 1998; Crowell-Davis and Murray 2006).

The kinds of postoperative behavioural changes observed in this study have been described as pain-related in previous literature (Hansen *et al* 1997; Hardie *et al* 1997). Our haematological and biochemical results are also consistent with the effects of significant post-surgical pain. Although the GPS score obtained suggest that post-surgery pain was being controlled by analgesia, the pain scale is based on the subjective impressions of pain-related behaviour by veterinary surgeons (Morton *et al* 2005), and not on direct observational studies of dog behaviour. The absence of significant differences between pre and post-surgery for the GPS scores in this study could be related to high frequency pre and post-surgery behaviours (see Table 5.5) such as *Nervous or Anxious or Fearful* and *Stiff*, which supposedly have a high pain-related weight (1.13 and 1.17 respectively), but were not highly specific for low pain evaluation. Indeed, they were also displayed in cases of psychological stressors acting alone (T1). Moreover, administration of analgesia does not always result in a complete elimination of pain, and low pain perception may be undiagnosed using indirect pain assessment tools (Vetter and Heiner 1996).

Focusing our attention on the neuroendocrine response, cortisol was the most sensitive marker for psychological stress acting without any physical damage and/or pain, since it was the only one to show

significant changes before surgery (T1). This increase in cortisol together with the simultaneous *upward tendency of glucose suggests an activation of the HPA axis (Matteri et al* 2000). This indicates that confinement in an unfamiliar and uncontrolled environment together with handling by unknown people represents a substantial source of stress for the animal that can be detected as early as 30 minutes after caging. The slight reduction in prolactin during preoperative time (T1), presumably related to activation of the dopaminergic system (Matteri *et al* 2000), can also be considered a sign of neuroendocrine sensitivity to preoperative stress. Since previous results have shown that the magnitude of the perioperative neuroendocrine response is directly proportional to postoperative analgesic requirements and morbidity in humans following major surgery (Anand *et al* 1992; Giesecke *et al* 1998), cortisol, glucose and prolactin could be useful biochemical markers in assessing psychological stress so as to guarantee animal welfare and rapid recovery.

After surgery (T2), both the HPA and lactotropic axes were substantially affected with significant increases in cortisol and glucose coupled with a significant decrease in prolactin. Post-surgery cortisol and glucose increases in dogs have been reported previously (Hansen *et al* 1996; Fox *et al* 1998; Matteri *et al* 2000; Väisänen *et al* 2002; Devitt *et al* 2005). The high post-surgery peak reached by cortisol (T2), followed by a rapid return to basal values (T3), confirms its sensitivity to psychological stress, but also shows that major intra and postoperative stressors, such as pain, are acting to heighten the stress response. The significant decrease in prolactin (T3) provides evidence of the major involvement of the lactotropic axis in postoperative stress and the presumed related activation of the dopaminergic axis. A postoperative increase in PRL has been reported in humans (Marrocco-Trischitta *et al* 2004), but to our knowledge, this is the first description of prolactin response as a perioperative stress marker in dogs. The divergent postoperative prolactin response between dogs and humans could be related to the different activation of prolactin feed-back regulatory systems. Considering that the increase in dopamine during an emotional

challenge has an inhibitory effect on prolactin secretion (Matteri *et al* 2000), and that previous exposure to chronic stress increases dopamine release in response to acute stress (Cuadra *et al* 1999), the chronic stress experienced by the sheltered dogs enrolled in this study, kenneled in small structures with limited social contact (Hubrecht *et al* 1992, Beerda *et al* 1996), could explain the prolactin decrease observed.

Regarding the immune response, no significant changes were detected before surgery (T1). Exposure to psychological stressors does not always result in alterations of immune functions, and, when the immune response is activated, its magnitude is normally mild in dogs (Dantzer and Mormede 1995; Stockham *et al* 2003). Furthermore, the hypothesis of an undetected long-term response of the white blood cells to psychological stress should be considered, similarly to what is seen for the acute phase response. On the other hand, postoperative variations proved to be remarkable. Increases in total white blood cells, neutrophils and monocytes were evident soon after post-surgery (T2) and persisted for several days, while the declines in lymphocytes and eosinophils were limited strictly to immediate postoperative time (T2) returning rapidly to basal values (T4). As previously reported (Schultze 2000), in a pure psychological stress response without tissue lesions, neutrophilia usually resolves prior to lymphopenia. In our study, postoperative psychological stress and tissue damage were acting simultaneously, and the long-term neutrophil and monocyte responses detected compared with the extremely short lymphocyte and eosinophil responses, suggests that these cell types have different sensitivity to postoperative stressors. While the lymphocyte and eosinophil responses appear to be related to glucocorticoid increase rather than tissue damage (Schultze 2000; Stockham *et al* 2003), the persistent neutrophilia and monocytosis could be more indicative of the tissue inflammatory stimulus. Concurring with these findings, a redistribution of lymphocytes from circulating blood to marrow or lymph nodes in response to glucocorticoids has been described (Stockham *et al* 2003), as well as lymphocyte apoptosis after perioperative stress (Delogu *et al* 2001; Alleva *et al* 2003). The effect of pain on leukocyte response has

also been reported (Griffis *et al* 2006). Neutrophilia and lymphopenia following anaesthesia and surgery have been described in horses (Stover *et al* 1998). The neutrophil/lymphocyte ratio has been proposed as a useful and inexpensive indicator of sensitivity to postoperative stress in humans (Zakowsky 1992, Tayama *et al* 1999). The results obtained in this study suggest that similar conclusions are also applicable to dogs. As the ratio increases, so does the stress experienced by the animal. In our study, the neutrophil/lymphocyte ratio is about four times the basal value (T0) at the moment when the animal supposedly experiences the greatest challenge due to inflammatory and psychological postoperative stress (T2, see Figure 5.11).

The CRP and Hp tendencies seen in our study agree with results reported in previous work (Yamamoto *et al* 1993; Conner *et al* 1988; Cerón *et al* 2005). Sensitivity to physical and psychological stress of acute phase response has been reported in humans and cattle (Murata *et al* 2004). An Hp increase following glucocorticoid treatment has been observed in dogs, while CRP appeared to be unaffected (Cerón *et al* 2005). Unfortunately, our study design could not usefully differentiate between the psychological and inflammatory perioperative response of acute phase proteins. The long-term nature of this response (Conner *et al* 1988; Yamamoto *et al* 1993; Cerón *et al* 2005) made it impossible to detect changes due to preoperative stress (T1) before the postoperative elements were added (T2). Nevertheless, the acute phase proteins showed a similar response to neutrophils and monocytes, suggesting that they could be a more reliable marker of inflammatory postoperative stress than a psychological one. In this case, they could be regarded as a valid alternative to WBC count, while offering the added advantage of being more stable for sample storage and analysis (Cerón *et al* 2005).

The difference in the amount of tissue damage between dogs undergoing orchiectomy versus ovariohysterectomy did not have any substantial influence on the inflammatory response observed in this study, since no significant post-surgery differences were observed in WBC, neutrophils, monocytes, CRP and Hp of the two groups of animals. Nevertheless, the

dogs undergoing ovariohysterectomy tended to maintain more increased cortisol and decreased lymphocyte values after surgery than those undergoing orchiectomy, showing that ovariohysterectomy is a more stressful and painful experience than orchiectomy, but is not associated with a significant difference in the inflammatory response. Intra-operative pain experience could be related to a different activation of cortisol and lymphocytes responses, even if post-operative pain perception appeared to be the same for both groups.

Although our results need to be considered with due caution in view of the limited number of animals involved, the simultaneous study of several behavioural, haematological and biochemical stress markers provides an extensive description of perioperative stress in dogs undergoing elective surgery. Moreover, our behavioural analysis, carried out by using continuous observation and high intra-observer correlation, contributed in part to increased study reliability.

## 5.5 CONCLUSIONS

Behavioural changes together with haematological and biochemical markers of the neuroendocrine, immune and acute phase stress responses were shown to be sensitive tools for assessing perioperative stress in dogs undergoing elective orchiectomy and ovariohysterectomy. Changes in explorative and communicative behaviours, as well as alterations in waking/sleeping pattern, activity and active interaction with a handler appeared to be the most relevant postoperative behavioural variations. Although, behavioural changes did not allow the effect of psychological stress to be separated from changes due to postoperative pain. Further studies comparing behaviours in usual environment with ICU cage environment are required to quantify the effect of psychological stressors.

Cortisol was the most useful tool for psychological stress assessment, as it was the only marker to show a significant preoperative change.

Although changes observed during preoperative times were not always substantial, all biomarkers studied showed significant variations after surgery. Cortisol response suggests an important (but time-limited) activation of HPA axis, while prolactin decline indicates activation of the dopaminergic-lactotropic system. Neutrophils, monocytes and the acute phase response proved to be good markers for inflammation, while lymphocytes and eosinophils showed greater sensitivity to early postoperative psychological stress. Neutrophil/lymphocyte ratio represents a useful and inexpensive tool for postoperative stress assessment. Changes observed showed that pain, analgesia- and anaesthesia-induced dysphoria, tissue damage, together with persistent psychological stressors, represented a major challenge for the animals' homeostatic balance.

Both preoperative psychological and postoperative multifactorial stresses appear to be involved in perioperative response, confirming the importance of giving adequate consideration to all these factors from the perspective of animal welfare and recovery. We believe that the role of animal psychological stress in daily veterinary practice is not always properly evaluated, probably because it is characterised by subtle signs, particularly when the animal is adopting a passive coping strategy. Choosing the best caging conditions, taking care in handling, using adequate anaesthetic and analgesic drugs to alleviate pain and to reduce dysphoria as much as possible, are some of the critical control points that can be easily managed to improve welfare in dogs undergoing elective surgery.

6

# EFFECT OF A SYNTHETIC APPEASING PHEROMONE ON BEHAVIOURAL, NEUROENDOCRINE, IMMUNE AND ACUTE PHASE PERIOPERATIVE STRESS RESPONSES IN DOGS

Siracusa C, Manteca X, Cuenca R, Del Mar Alcalá M, Alba A, Lavín S and Pastor J

Submitted to the Journal of the American Veterinary Medical Association

## 6.1. INTRODUCTION

The perioperative stress response is a physiological reaction to surgery and various associated conditions such as pain, analgesia- and anaesthesia-induced dysphoria, human handling and confinement to a hospitalization cage - all elements that may be perceived by the animal as being physical and/or psychological threats (Hansen *et al* 1996; Hardie *et al* 1997; Mellor *et al* 2000; Moberg 2000; Väisänen et al 2002).

In Siracusa *et al* 2008 the activation of the behavioural, neuroendocrine, immune and acute phase responses following stress in dogs undergoing elective orchiectomy and ovariohysterectomy has been described. Communicative and explorative behaviors showed high pre-surgery occurrence and were inhibited post-surgery. Decreases in post-surgery activity, interactive behaviors and changes in waking/sleeping patterns were also observed. Cortisol proved to be a useful tool for pre- and postoperative stress assessment, while glucose, prolactin, white blood cells and acute phase proteins showed significant variations after surgery. Cortisol and glucose responses suggested an important activation of the HPA axis, while prolactin reduction indicated activation of the dopaminergic-lactotropic system. Neutrophils, monocytes and the acute phase response proved to be good markers for inflammation, while lymphocytes and eosinophils showed greater sensitivity to early postoperative psychological stress.

A synthetic dog-appeasing pheromone (DAP, CEVA Sante Animale, Libourne Cedex, France) has been marketed to reduce stress in dogs; it is a synthetic mixture of fatty acids reproducing fractions of a natural pheromone identified in sebaceous gland secretions from the inter-mammary sulcus of bitches. This secretion can be isolated from 3-4 days after parturition to 2-5 days after weaning (Pageat and Gaultier 2003a). Although the action mechanism of this pheromone is not well understood (Pageat and Gaultier 2003a), the DAP has been described as having a calming effect in different stressful situations, such as separation-related problems, fear of fireworks, veterinary clinical

consultation, house soiling and kenneling (Sheppard and Mills 2003; Gaultier *et al* 2005; Tod *et al* 2005; Levine *et al* 2006; Mills *et al* 2006; Taylor and Mills 2006; Gaultier *et al* 2008).

Our hypothesis is that this synthetic pheromone can have an effect in controlling a multifactorial perioperative stress response. Thus, the main objective of the present study is to evaluate the effect of DAP treatment on the perioperative stress response in dogs undergoing elective orchiectomy and ovariohysterectomy by measuring variations in behavioural categories, salivary cortisol, serum glucose, serum prolactin, total white blood cell count, white blood cell differential, neutrophils/lymphocytes ratio, serum haptoglobin and serum C-reactive protein.

## 6.2. MATERIALS AND METHODS

### *Animals*

A group of 46 adult dogs, 23 females and 23 males, 29.11±3.11 (mean ± SEM) months old, both pure and mixed breed, with a mean weight of 20.67±1.25 kg (mean ± SEM), underwent elective orchiectomy or ovariohysterectomy. All dogs had been kept in a public shelter for a minimum of 20 days, in a 6 $m^2$ pen (named "usual environment"; see Figure 5.1) together with one or two other dogs, according to local by-laws. Mean environmental temperature was 21.6±0.40 °C (mean ± SEM) during the period in which the study was carried out (Servei Metereologic de Catalunya 2006).

A thorough physical examination, complete blood cell count, biochemistry panel and serum leishmania antibodies concentration (ELISA test) was performed on all dogs, only healthy animals being included in our study. The reproductive status of each female animal was studied using a progesterone kit (Progesterone ELISA, DRG Inc, Marburg, Germany) and pseudo-pregnant, pregnant or lactating females

were excluded. Additionally, dogs presenting stereotyped behaviour or aggression against humans were not included in the trial. Animals eventually enrolled in the study fasted at least 18 hours prior to surgery.

### *Sampling procedures*

Table 6.1 shows times of sample collection. The study began every day between 10 and 11 a.m. local time, when dogs' behaviour was video-recorded during 30 minutes in their usual environment (T0), the same day as surgery. After this, the first blood and saliva samples were collected from each dog in its usual environment (T0). Samples collected at that time were considered as basal values. The dogs were then transferred by walking to the ICU, located in the same shelter holding facility, and placed in a 110x70x70 cm cage (named "ICU cage"; Figure 5.2), where behaviour was video-recorded for 30 minutes (T1). The study period ended with a standardised dynamic interaction test for pain evaluation, also video-recorded (The collection of behaviour samples and interaction tests will be described later in this section). After the interaction test, further blood and saliva samples were obtained (T1). The dogs were later transferred to the operating theatre. All surgeries were performed by the same graduate surgeon with the help of different veterinary undergraduates. Surgery was considered to be finalized with the extubation of the animal.

A standardised anaesthetic and analgesic protocol was used for the surgical procedure. Each dog was pre-medicated with morphine (MORFINA BRAUN® 1%, BBraun España, Barcelona, Spain) 0.1 ml/10 kg IM and medetomidine (DOMITOR®, Pfizer Salud Animal SA, Madrid, Spain) 0.05 ml/10 kg IM, induced with thiobarbital (TIOBARBITAL®, B Braun Medical, Barcelona, Spain) 10 mg/kg IV and diazepam (VALIUM®, Roche Farma SA, Barcelona, Spain) 0.5 mg/kg IV. Anaesthesia was maintained with 1-2% isofluorane (ISOFLO®, Abbot Laboratories, Illinois, USA) vaporised in 100% oxygen 0.5-1 l/min., delivered with a semi-disposable circle circuit (Burtons Medical Equipment Ltd, Kent, UK). The vaporiser setting was adjusted to

maintain a surgical plane of anaesthesia as judged by eye position, jaw tone and lack of response to noxious stimuli. All dogs received intravenous crystalloid solution (LACTATO DE RINGER BRAUN®, B Braun Medical, Barcelona, Spain) at 5-10 ml/kg/hour. Each dog was treated with an antibiotic, amoxicillin LA 11-22 mg/kg SC (BIVAMOX® LA, Boehringer Ingelheim España, Barcelona, Spain) and anti-inflammatory therapy, meloxicam 0.2 mg/kg SC (METACAM®, Boehringer Ingelheim España, Barcelona, Spain) for four days after surgery. After extubation the dogs were transferred to the ICU cage, where every 30 minutes the degree of sedation was checked. When the animal was able to stand in the ICU cage (end of sedation), its behaviour was video-recorded for 30 minutes, after which the dynamic interaction test for pain evaluation was performed and video-recorded (T2). At the end of this observation, blood and saliva samples (T2) were collected. The dog was later transferred to its usual environment where once more the behaviour was video-recorded for 30 minutes, and, at the end of this surgery day, a saliva sample was taken (T3). No blood sample was collected at this time (T3) for ethical reasons, i.e. to reduce the loss of blood volume and minimise the risk of post-surgery complications. Blood samples were also obtained at 24 hours (T4), 48 hours (T5) and 8 days (T6) after surgery. No more than two dogs were confined in the ICU at any given time, to minimize the variability of environmental influences on the dogs' behaviour.

### *Behavioural data collection*

All behavioural samples were recorded with a digital video camera (Sony Handycam DCR-HF-40, Sony Corporation, Tokyo, Japan). Videos were always viewed and analyzed on a 21-inch monitor (Sony Corporation, Tokyo, Japan) by the same observer.

Behavioural data were divided into categories, evaluated for frequency of occurrence (*Events,* see Table 6.2) and duration (*States,* see Table 6.3), and collected on a check sheet. Categories evaluated as events were logged by continuous recording and their number of occurrences during

the time of observation was considered, while those evaluated as states were logged by instantaneous sampling at 2 minutes intervals (15 instantaneous recording points in 30 minutes) (Martin and Bateson 1993). Behaviour scored in terms of frequency was recorded as occurring once every 5 seconds when the dogs displayed it in a continuous fashion (Beerda *et al* 1999).

**Table 6.1:** Sample collection schedule.

| *Time* | *Definition* | *Samples* | *Parameters studied* |
|---|---|---|---|
| **T0 (basal)** | Surgery day<br>Dog in usual environment<br>Pre-surgery | Behaviour<br>30 minutes | *Behavioural categories* |
| | | Saliva | *Cortisol* |
| | | Blood | *Glucose*<br>*PRL*<br>*CRP - Hp*<br>*WBC** |
| **T1** | Surgery day<br>Dog in ICU cage<br>Pre-surgery | Behaviour<br>30 minutes | *Behavioural categories* |
| | | Interaction test | *Behavioural categories*<br>*Pain scoring* |
| | DAP<br>administration | Saliva | *Cortisol* |
| | | Blood | *Glucose*<br>*PRL*<br>*WBC** |
| **T2** | Surgery day<br>Dog in ICU cage<br>Post-surgery | Behaviour<br>30 minutes | *Behavioural categories* |
| | | Interaction test | *Behavioural categories*<br>*Pain scoring* |
| | DAP<br>administration | Saliva | *Cortisol* |
| | | Blood | *Glucose*<br>*PRL*<br>*WBC** |
| **T3** | Surgery day<br>Dog in usual environment<br>Post-surgery | Behaviour<br>30 minutes | *Behavioural categories* |
| | | Saliva | *Cortisol* |
| **T4** | 24 h post-surgery | Blood | *CRP-Hp*<br>*WBC** |
| **T5** | 48 h post-surgery | Blood | *CRP-Hp*<br>*WBC** |
| **T6** | 8 days post-surgery | Blood | *CRP-Hp*<br>*WBC** |

* Includes total white blood cell count and differential count

**Table 6.2:** Behavioural categories evaluated as states studied and environmental condition in which they where analysed.

| *Category* | *Definition* | *Usual Environment T0-T3* | *ICU Cage T1-T2* |
|---|---|---|---|
| **PANTING** | An increased frequency of inhalation and exhalation often in combination with the opening of the mouth | | X |
| **VISUAL SCANNING** | Visual exploration of the environment through cage's door. | X | X |
| **AWAKE /ALERT** | Dog with opened eyes. | X | X |
| **REST /SLEEP** | Dog inactive and with closed eyes. | X | X |
| **TREMBLING** | Body shaking with little, high frequency, movements. | | X |
| **WALKING** | Displacement from a point to another, with no clear exploring movements. | X | X |
| **EXPLORING** | The dog moves slowly, sniffing and investigating the environment. | X | X |
| **LYING** | Positioned fully on side, one side of the dog in complete contact with the ground. - Positioned on side with body, but not head in complete contact with the ground, or with ventrum and legs in contact with ground. - Positioned flat with back in contact with the ground. | X | X |
| **SITTING** | The pads of the front paws are on the ground with the front legs straight and the rump squarely on the ground. | X | X |
| **STANDING** | Positioned with just four paws in contact with the ground, or two with the ground and two with the wall. | X | X |
| **AGAINST WALL** | The dog is against the walls of the enclosure, with the eyes opened or closed. | X | X |
| **AGAINST DOOR** | The dog is against the door of the enclosure, with the eyes opened or closed. | X | X |
| **HIDE** | the entire body or the cranial half of the dog are not visible. | X | |
| **CHANGING STATE** | Changing from one of the following states of locomotion to another: walking, lying, sitting, standing. | X | X |

*(Adapted from Beerda et al 1997; Beerda et al 1998; Goodmann et al., 2002; Hardie et al 1997; Hetts et al 1992; Morton and Griffiths 1985).*

**Table 6.3:** Behavioural categories evaluated as events studied and environmental condition in which they where analysed.

| *Category* | *Definition* | *Usual Environment T0-T3* | *ICU cage T1-T2* |
|---|---|---|---|
| **BARKING** | Low frequency vocalisation, more or less soft or raucous | | X |
| **GROWLING** | A throaty rumbling vocalisation, usually low in pitch. It may be used in aggressive or defensive interaction. | | X |
| **WHINING** | Repeated, relatively brief, "exhalation vocalisations" of falling pitch. | | X |
| **YELPING** | Loud, high pitched vocalisations. | | X |
| **MOUTH OPENING** | The dog opens and closes the mouth with rapid movements. The tongue is not visible. It could correspond to yawning. | | X |
| **LIP LICKING** | The dog licks the lips exhibiting part of the tongue. | | X |
| **AUTOGROOMING** | Behaviours directed towards the subject's own body, like scratching, licking and biting-self, to take care of the skin and coat. It includes taking care of wounds. | X | X |
| **TAIL CHASING** | The dog chases its own tail with continuous round movements. | X | X |
| **CIRCLING** | Walking in a circle. | X | X |
| **PACING** | Continuous movements from one extreme to the other of the cage. | X | X |
| **DIGGING** | Scratching the floor with the forepaws in a way that is similar to when dogs are digging holes. | X | X |
| **BARRIER MANIPULATION** | Chewing, touching with legs or licking the enclosure. | X | X |
| **JUMPING** | Springing into the air, either spontaneously or in order to make contact with an object or a person | X | X |
| **NOSING** | The nose is moved along objects and/or clear sniffing movements are exhibited. | | X |
| **PAW LIFTING** | A fore paw is lifted into a position of approximately 45º. | | X |
| **TAIL WAGGING** | Repetitive wagging movements of the tail. | | X |
| **DOG INTERACTION** | Agonistic or antagonistic interaction with another dog of the same group. | X | |

*(Adapted from Beerda et al 1997; Beerda et al 1998; Goodmann et al., 2002; Hardie et al 1997; Hetts et al 1992; Morton and Griffiths 1985).*

Although many behaviors were analyzed both in the usual environment and in the ICU cage, this was not possible for all the behavioural categories studied. This was mainly due to environmental differences between the usual pens and the ICU cage that influenced the accuracy of video-recording (e.g. single or multiple dogs housed, presence of hiding places, distance of the video-camera from the animals studied; see Table 6.2 and 6.3).

### *Dynamic interaction test for pain evaluation*

The interaction test was performed as follows: an operator knocked at the door of the ICU, opened it and entered. He reached for the cage, opened the door and greeted the dog gently ('Hi, how you doing?'). The operator then withdrew the dog from the cage, patting it gently from the chest to the flank and up to the ventral surgery site.

A single ethologist, familiar with the individual behaviour of the dogs enrolled in the study, carried out and recorded all tests, as well as later analysis and assessment. This strategy was designed to minimise the effect of individual behavioural variability of dogs.

The interactive behaviour of the dogs was analysed by using the Glasgow Pain Scale (GPS) (Holton *et al* 2001; Morton *et al* 2005, see Table 4.2). The GPS behavioural categories shown by each dog were recorded. Both the frequency of occurrence of each behavioural category during pre- and post-surgery and the corresponding total scores were studied, to obtain a qualitative and quantitative analysis. In Siracusa *et al* 2008 the frequency of GPS behavioural categories was shown to be a most sensitive marker of low-intensity pain, when compared to the GPS score.

### *Blood and saliva samples collection*

Blood samples were taken from the jugular vein using standard procedures. One ml of the sample was stored in an EDTA tube

(TAPVAL AQUISEL®, Barcelona, Spain) and 3 ml transferred to tubes containing a coagulation activator (TAPVAL AQUISEL®, Barcelona, Spain). The samples were refrigerated during transport to the laboratory. After clot formation, the serum obtained was transferred to eppendorf tubes and stored at –80° C.

Saliva samples were collected by the SALIVETTE® system (Sarstedt, Numbrecht, Germany) after salivary flow stimulation with 3% citric acid (Mandel 1990; Beerda *et al* 1997). Saliva collection always preceded blood sample collection, the animal never being handled longer than 2 minutes, to avoid influence of handling on stress measures (Kobelt *et al* 2003). Tubes were kept refrigerated during transport to the laboratory. Saliva samples were then centrifuged at 3500rpm for 15 minutes and stored at – 80° C.

### *Allocation of treatment*

The study was conducted as a double-blind, placebo-controlled, longitudinal study. The dogs were randomly assigned to the DAP treatment (verum) or placebo group. Fifty natural spray bottles were made available for the study (DAP®, CEVA Sante Animale, Libourne Cedex, France, see Figure 6.1). Twenty five of these bottles contained 2% of fatty acid methyl esters (FAME) in ethanol, representing the DAP treatment, and 25 contained ethanol only, used as placebo. Each bottle was labeled with an identification code. A random code list was created; neither researchers nor trial supervisor were provided with the codes until all statistical analysis had been completed.

For each dog a bottle was randomly chosen and used for spraying the ICU cage where the animal was allocated. The floor (covered with an absorbent cotton towel) and the corners of the cage were sprayed pre- and postoperatively 20 minutes before the dog was placed in it. Each spray bottle was used for a single dog. Thus, each dog came into contact with either the verum or the placebo for 30 minutes before and after surgery. Prior to treatment, the cage was always thoroughly cleaned

with a detergent containing a non-ionic fraction (EXTRAN® MA 01 Detergent, Merck KGaA, Darmstadt, Germany) to control environmental pollution by natural pheromones.

**Figure 6.1:** natural spray containing DAP or placebo labeled with an identification code.

### *Laboratory analysis*

Saliva cortisol concentration was determined with a commercial human saliva ELISA test (CORTISOL SALIVA®, BLK Diagnostics, Barcelona, Spain) which had been adapted in our laboratory to measure cortisol concentration in canine saliva.

Serum samples were analysed for glucose detection with an enzymatic UV test (hexokinase method) following manufacturer's instructions (GLUCOSE OLYMPUS®, Hamburg, Germany). Serum prolactin concentration was measured with a commercial ELISA kit (MILENIA® CANINE PROLACTIN, Milenia Biotec, Bad Nauheim, Germany) following manufacturer's instructions.

EDTA blood samples were analyzed within 6 hours of collection with a laser flow-cytometer (ADVIA® 120 Haematology System, Bayer,

Fernwald, Germany), which provided total white blood cell (TWBC) and differential count (neutrophils, monocytes, lymphocytes and eosinophils).

Serum samples were analyzed to measure haptoglobin and C-reactive protein levels. Hp concentration was determined with an automated biochemical assay (TRIDELTA PHASE RANGE SERUM HAPTOGLOBIN®, Tridelta Development, Wicklow, Ireland) following manufacturer's instructions. CRP concentration was measured with a canine CRP-specific solid phase sandwich immunoassay (TRIDELTA PHASE RANGE CANINE CRP KIT®; Tridelta Development, Wicklow, Ireland).

Immune and acute phase response markers were evaluated on a long-term basis (24, 48 hours and 8 days post-surgery), according to previously published data (Ceròn *et al* 2005) and because of the presumed major influence of the inflammatory response caused by tissue damage on marker variation. As glucose, cortisol and prolactin response to stress is known to be rapid (Matteri *et al* 2000), study of the markers was limited to the day of surgery to avoid influence of uncontrolled psychological stressors on response at 24, 48 hours and 8 days post-surgery.

### *Statistical analysis*

For statistical analysis, the normal distribution of data was determined with a Shapiro-Wilk test. Data were considered to have a normal distribution when the test showed $P > 0.05$. Differences in sex of individuals in the verum and placebo groups were checked by using the $\chi^2$ test, while differences in age and weight were studied by using the *t* test. A Mann-Whitney test was used to analyze differences in environmental temperature recorded on the days when dogs from the verum or placebo group were studied, as well as differences in length of surgery and sedation (from the end of surgery to the time when the animal was able to stand) between the two groups.

*Behavioural data*

Intra-observer reliability was determined by analysis of the correlation between two different observations of the same video-recording sample. Nine independent 10-minute samples of different subjects were used to calculate the Spearman Rank correlation coefficient for the following behavioural categories: nosing, lip licking, mouth opening, visual scanning and awake/alert (Martin and Bateson 1993). SPSS® 15.0 software (SPSS Inc., Chicago, USA) was used for calculations.

Variations of behavioural categories were studied for every behavioural observation period (from T0 to T3) for those categories for which samples were collected both in the usual environment and then ICU cage; while for the other categories comparisons were limited to the times related to the environment where samples were collected (T0 vs. T3 for behaviors studied only in the usual environment; T1 vs. T2 for categories studied only in the ICU cage; see Tables 6.2 and 6.3). To determine whether the DAP influenced the perioperative change observed in behaviour, the effect of the DAP treatment on the variation of behavioural categories over time, on the variation of GPS behaviour frequencies, and on the GPS score was analyzed by a Generalized Linear Model for repeated measures. The Bonferroni correction was applied to the level of significance for pairwise comparison of different times studied. Differences were considered statistically significant when $P \leq 0.05$. The GEE module of SPSS® 15.0 software (SPSS Inc., Chicago, USA) was used for calculations.

*Hematological and biochemical data*

Not normal data were logarithmically transformed to achieve normality. The effect of DAP treatment on the response of the biomarkers over time studied was analyzed by using an analysis of variance (ANOVA) for repeated measurements. For those parameters showing a significant effect of treatment, the interaction among treatment and sex was also studied, as well as the differences between basal values and other values

at different times were analyzed by post-hoc pairwise comparisons. Univariate F-statistics were corrected with the Huynh-Feldt adjusted degree of freedom, when data sets deviated from the sphericity assumption. The Bonferroni correction was applied to the level of significance for pairwise comparison of different times studied. Differences were considered to be significant when $P \leq 0.05$. The GLM module of SPSS® 15.0 software (SPSS Inc., Chicago, USA) was used for calculations.

## 6.3. RESULTS

All the results are presented as the mean ± SEM. Twenty-four and 22 dogs belonged to verum and placebo groups respectively. They were no different in groups composition considering the sex (12 males and 12 females in the verum group vs. 11 males and 11 females in the placebo group; P = 1.000), the age (mean age 28.50 ± 4.07 months for the verum group vs. 29.77 ± 4.87 months for the placebo group; P = 0.968) and the weight (mean weight 20.80 ± 1.42 Kg vs. 20.53±2.14 Kg for the placebo group; P = 0.917) of animals. The mean environmental temperature of those days in which the DAP group was studied did not differ significantly from the environmental temperature of the placebo group study days (mean temperature 21.64 ± 0.59 °C for the verum group vs. 21.54 ± 0.54 °C for the placebo group; P = 0.858). The mean length of surgery was 40.62 ± 2.75 minutes for the DAP group and 35.95 ± 2.39 minutes for the placebo group. While the mean length of sedation was 72.50 ± 8.45 minutes for the pheromone group and 65.45 ± 6.13 minutes for the placebo group. No statistical significant difference in length of surgery (P = 0.307) and sedation (P = 0.784) between the two groups was seen.

### *Behavioural data*

The intra-observer reliability, expressed by using a Spearman Rank correlation coefficient, was 0.95 for *nosing*, 1.00 for *lip licking*, 0.96 for

*mouth opening*, 0.93 for *visual scanning* and 1.00 for *awake/alert* (P < 0.05 for all categories).

As to the effect of the DAP treatment on the behavioural categories studied, variation of *visual scanning* behaviour along times studied showed to be significantly influenced (Wald chi-square = 6.156; P = 0.012), as well as variation of *awake/alert* (Wald chi-square = 5.318; P = 0.020). Dogs treated with the synthetic pheromone experienced a less important postoperative decrease of these behaviors, compared to dogs of the placebo group (see Figures 6.2 and 6.3). No significant difference between male and females response to DAP treatment, limited to these two behavioural categories, was found (Wald chi-square = 3.396; P = 0,065 for *visual scanning*. Wald chi-square = 0.006; P = 0,938 for *awake/alert*). The other behaviors studied were not significantly influenced by the treatment.

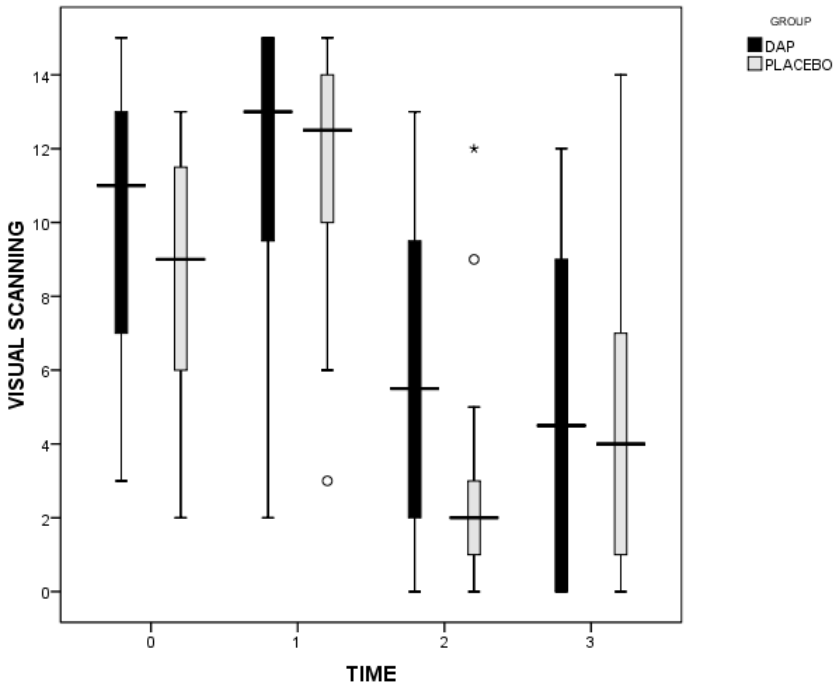



**Figure 6.2:** Variations of visual scanning over time for DAP and Placebo groups. A marked postoperative decrease is visible at T2 fro the placebo group, while the decrease is significantly minor for the DAP group at T2. Asterisks and circles represent extreme and outlier values.

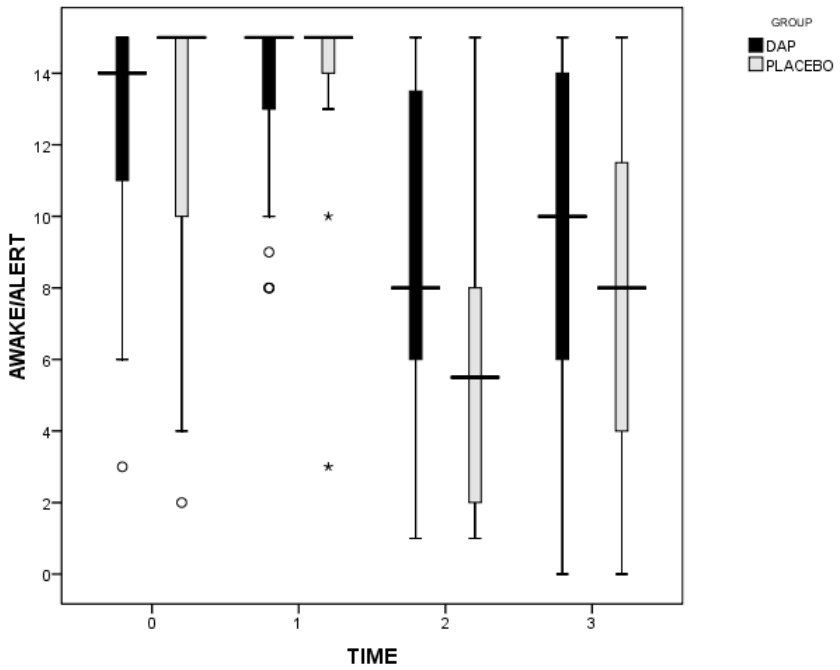



**Figure 6.3:** Variations of awake/alert over time for DAP and Placebo groups. The postoperative decrease experienced at T2 is significantly greater for the placebo group when compared to the DAP group. Asterisks and circles represent extreme and outlier values.

No significant effect of the DAP treatment was evidenced on the GPS score (1.58 ± 0.23 at T1 vs. 2.73 ± 0.35 at T2 for the DAP group; 1.02 ± 0.20 at T1 vs. 3.65 ± 1.09 at T2 for the placebo group; Wald chi-square = 0.435; P = 0.509). Similarly, the treatment with the synthetic pheromone had no significant influence on the perioperative variation of any of the GPS behavioural categories ($P > 0.05$ for all the categories). It is also relevant that there was a significant effect of time on the GPS score (1.31 ± 0.16 at T1 vs. 3.17 ± 0.55 at T2; Wald chi-square = 17.712, P = 0.000).

### *Hematological and biochemical data*

Geometrical means loaded on a semi-log graphic are used to present logarithmically transformed data (Figures 6.4-6.12, except 6.5), to visualize clearly the differences in variation among different times studied between the verum and placebo groups.

Regarding the effect of the synthetic pheromone on the biomarkers evaluated, no statistically significant effect of the DAP treatment on the cortisol response (F = 1.067; P = 0.363, see Figure 6.4), or on the glucose response (F = 0.939; P = 0.384, see Figure 6.5), was detected.

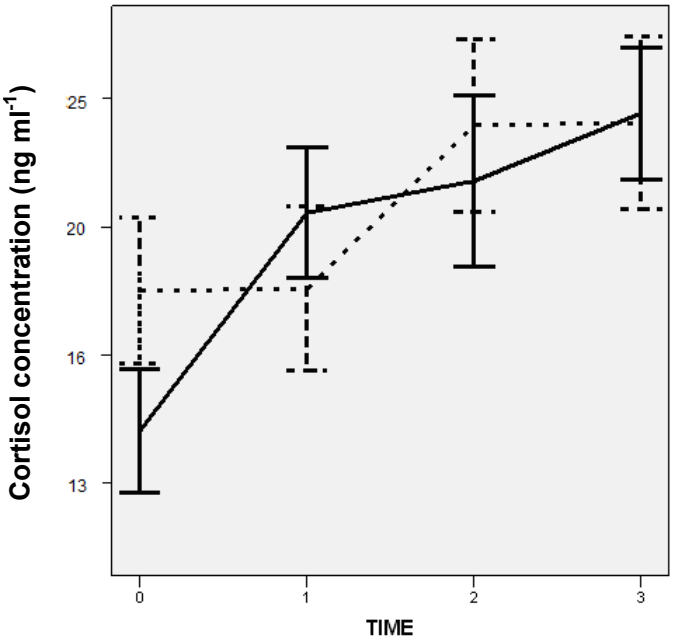



**Figure 6.4:** Salivary cortisol response in DAP (solid line) and placebo (dotted line) groups at times T0, T1, T2 and T3 (geometrical mean value ± SE).

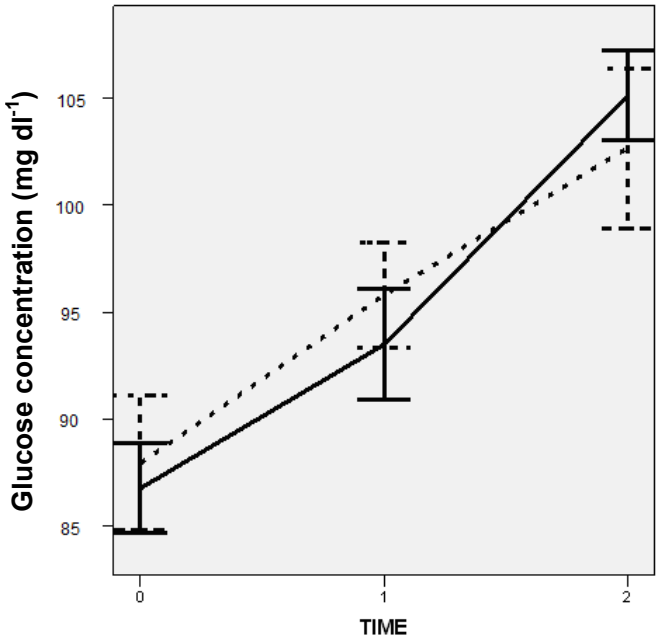



**Figure 6.5:** Serum glucose response in DAP (solid line) and placebo (dotted line) groups at times T0, T1 and T2 (mean value ± SE).

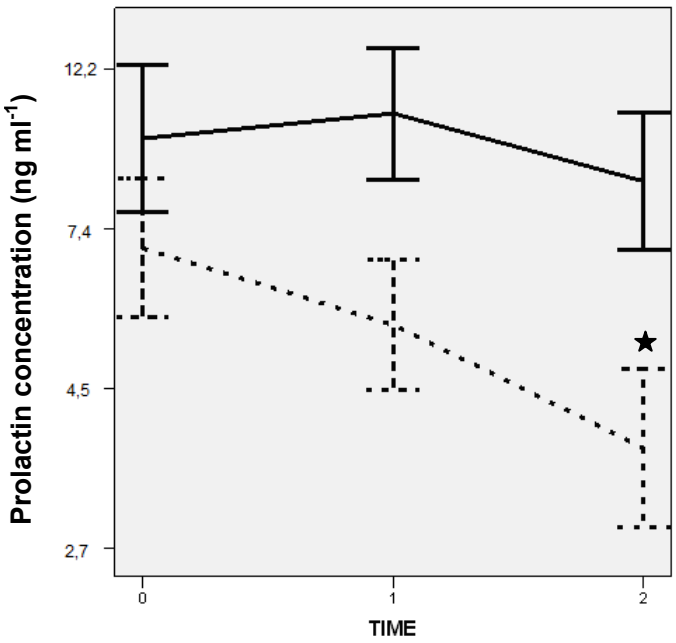



**Figure 6.6:** Serum prolactin response in DAP (solid line) and placebo (dotted line) groups at times T0, T1 and T2 (geometrical mean value ± SE). The star indicates the values showing statistical significant difference with the correspondent basal value at T0.

By contrast, prolactin variation over time was significantly affected by the pheromone treatment (F = 3.375; P = 0.046, see Figure 6.6). Prolactin values decreased significantly after surgery, when compared to the basal value at T0, for the placebo group (P = 0.007), while the postoperative decrease was not significant for the DAP group. No significant difference between prolactin response to the DAP treatment was found between male and female dogs (F = 0.626; P = 0.517).

TWBC, neutrophils, lymphocytes and neutrophil/lymphocyte ratio were not influenced by DAP treatment (F = 0.397 and P = 0.762 for TWBC, see Figure 6.7; F = 0.622 and P = 0.603 for neutrophils, see Figure 6.8; F = 0.276 and P = 0.888 for lymphocytes, see Figure 6.9; F = 0.750 and P = 0.532 for neutrophil/lymphocyte ratio, see Figure 6.10).

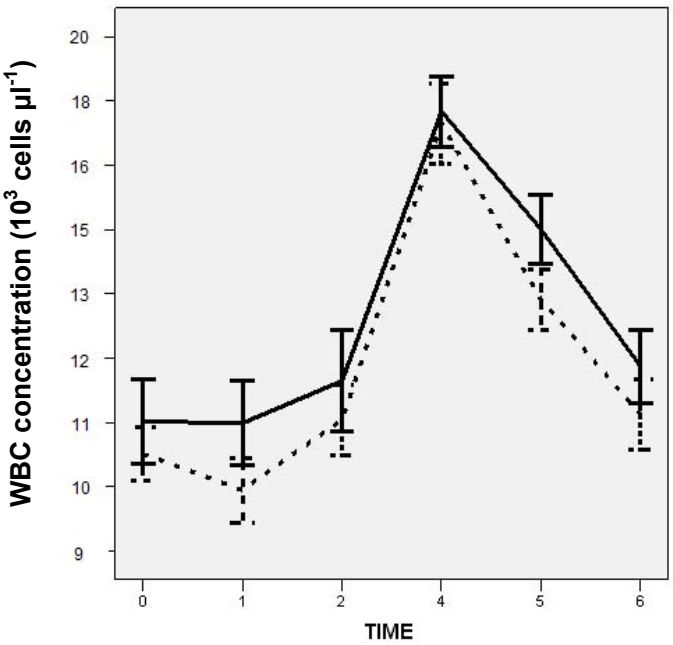



**Figure 6.7:** Total white blood cells response in DAP (solid line) and placebo (dotted line) groups at times T0, T1, T2, T4, T5 and T6 (geometrical mean value ± SE).

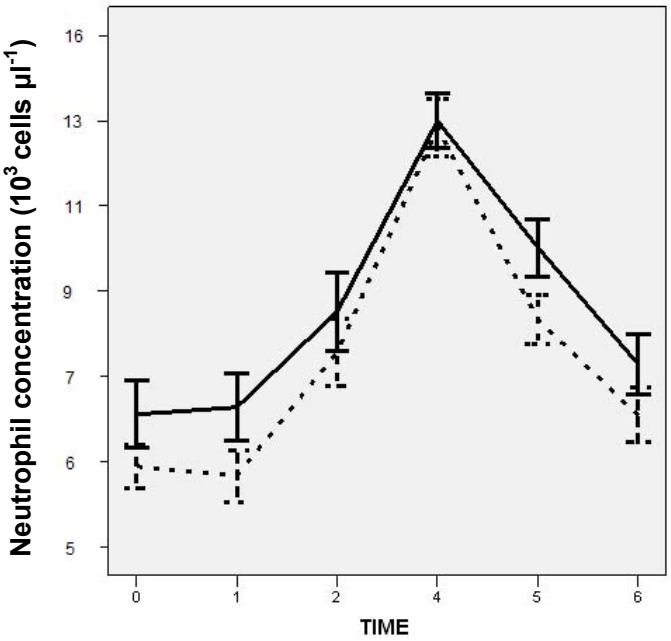



**Figure 6.8:** Neutrophil response in DAP (solid line) and placebo (dotted line) groups at times T0, T1, T2, T4, T5 and T6 (geometrical mean value ± SE).

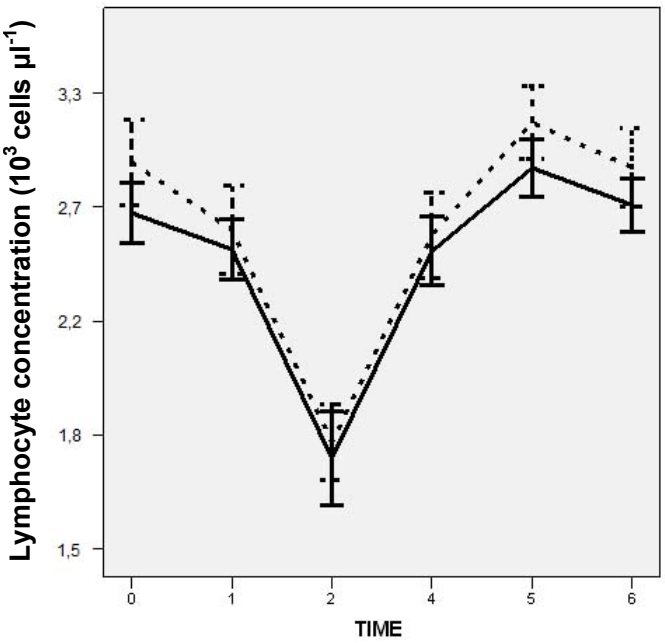



**Figure 6.9:** Lymphocyte response in DAP (solid line) and placebo (dotted line) groups at times T0, T1, T2, T4, T5 and T6 (geometrical mean value ± SE).

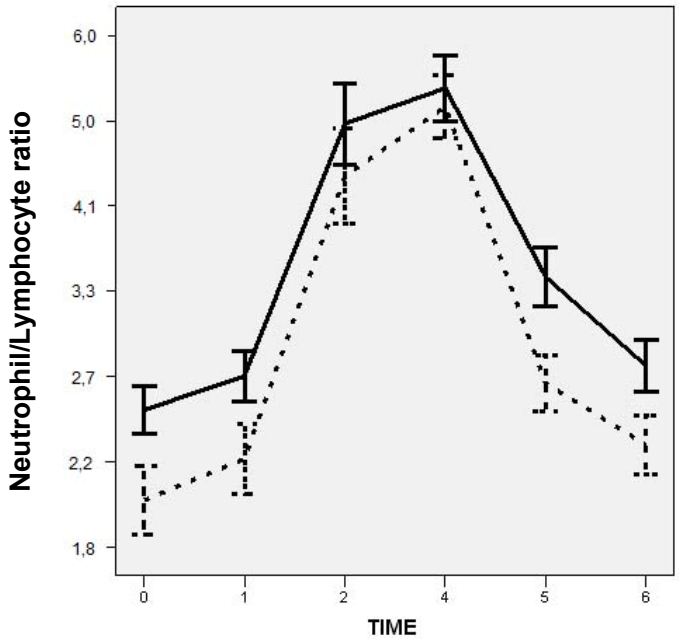



**Figure 6.10:** Variations in neutrophil/lymphocyte ratio in DAP (solid line) and placebo (dotted line) groups at times T0, T1, T2, T4, T5 and T6 (geometrical mean value ± SE).

Finally, neither the CRP response (F = 0.616; P = 0.560, see Figure 6.11) nor the Hp response (F = 0.343; P = 0.760, see Figure 6.12) showed any influence of the pheromone treatment.

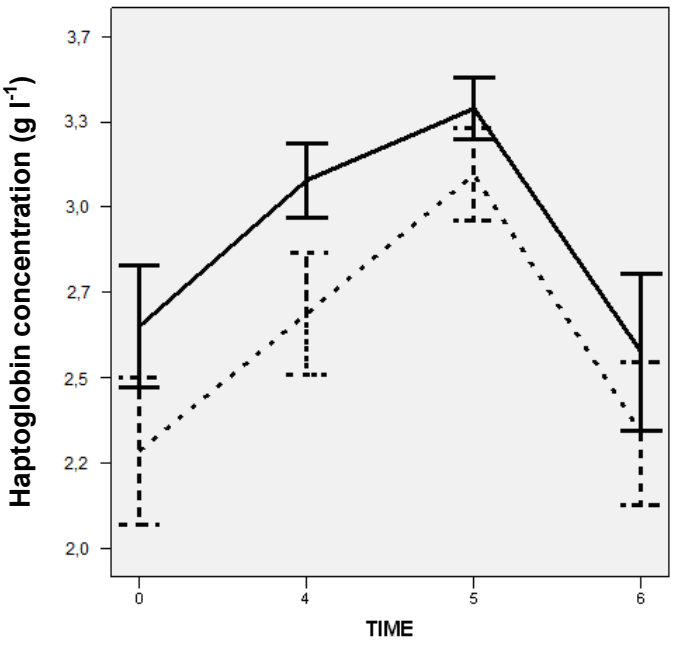



**Figure 6.11:** Serum haptoglobin response in DAP (solid line) and placebo (dotted line) groups at times T0, T4, T5 and T6 (mean value ± SE).

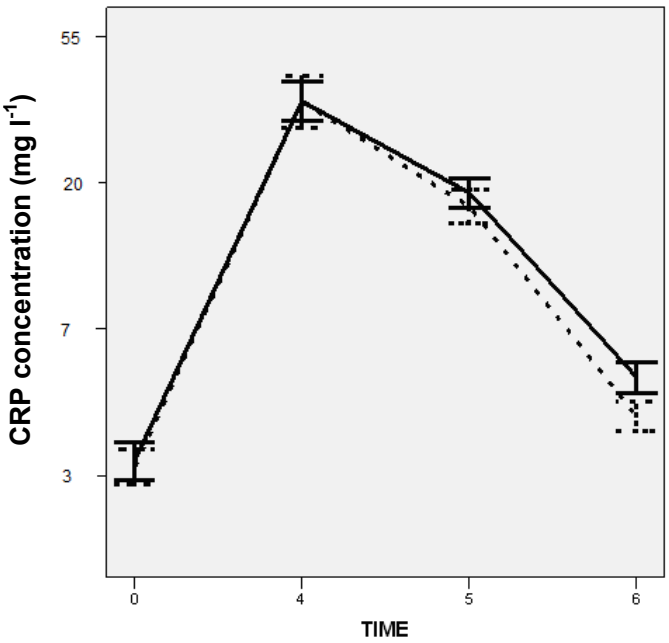



**Figure 6.12:** Serum CRP response in DAP (solid line) and placebo (dotted line) groups at times T0, T4, T5 and T6 (geometrical mean value ± SE).

## 6.4. DISCUSSION

The results presented in this study suggest that the Dog Appeasing Pheromone modifies the perioperative stress response in dogs undergoing elective surgery. Nevertheless, sensitivity to the synthetic pheromone varies greatly among the different components of the stress response. Alert and visual explorative behaviors were affected by the DAP treatment. As to the neuroendocrine, immune and acute phase stress responses, only the lactotropic axis, a component of the neuroendocrine response, proved to be sensitive. This was not the case with the other biomarkers studied. The relationship between pheromones and the neuroendocrine response has been demonstrated in the case of many mammals (Wyatt 2003), but never for the domestic dog. To our knowledge, this is the first evidence of an interaction between dog pheromones and the endocrine system.

Regarding the behavioural response, *visual scanning* and *awake/alert* behaviors were significantly affected by the DAP. The placebo group showed a major postoperative decrease for both categories when compared to the DAP group that was affected by a smaller decrease. These results suggest that the use of the DAP favored postoperative alert and visual explorative behaviors. According to the results presented in Siracusa *et al* 2008, alert and visual scanning behaviors are among the most sensitive behavioural parameters to detect postoperative stress in dogs when pain is a major component. However, the interaction test for pain evaluation performed in this study did not detect any influence of the DAP on pain perception, neither by analyzing the frequency of the GPS behavioural categories, nor the GPS score. It is possible that the GPS was not sensitive enough to detect a minor change in behaviour, when compared to the sensitivity of *visual scanning* and *awake/alert* behavioural categories. This difference could be due to the observational method on which the GPS is based. A one-zero recording method was in fact used to collect GPS behavioural data, according to the instructions provided with the scale (Holton *et al* 2001; Morton *et al* 2005), while all the other behavioural samples used in this trial were

collected by continuous observation, which is more accurate than the one-zero method (Martin and Bateson 1993).

Cortisol and glucose have often been used as markers to assess the neuroendocrine HPA axis activation. In Siracusa *et al* 2008, an increase in serum or salivary cortisol and serum glucose has been described in dogs exposed to perioperative stress, in agreement with previous studies (Hansen *et al* 1997; Väisänen *et al* 2002; Ambrisko *et al* 2005; Devitt *et al* 2005; Sibanda *et al* 2006). Cortisol is known to be sensitive to selective serotonin reuptake inhibitors (Weber *et al* 2006), a common pharmacological treatment for canine stress, fear- and anxiety-related problems (Overall 1997; Crowell-Davis and Murray 2006). Moreover, chemosignals can influence salivary cortisol concentration in humans (Moshkin *et al* 2006; Wyart *et al* 2007). Despite this existing evidence, the DAP treatment - a synthetic chemosignal with calming effects - did not show any significant effect on cortisol and glucose responses in our trial. A possible lack of influence of the DAP's clinical pharmacology on the HPA axis could account for this finding.

The sensitivity of prolactin to the DAP treatment suggests that the synthetic pheromone modifies the lactotropic response to perioperative stress. Stress activation of the lactotropic axis is a consistent observation, since prolactin has been used for stress assessment in many mammalian species (Matteri *et al* 2000), but few studies have been published for the dog. Prolactin is known to be involved in the emotional response of dogs, and increases during positive interaction with humans (Odendaal, and Meintjes 2003; Pageat 2005). Animals with generalised anxiety show hyperprolactinaemia, while dogs with phobias or mild anxiety do not (Pageat and Gaultier 2003b; Pageat *et al* 2007). Postoperative increases in prolactin have been reported in humans (Marrocco-Trischitta *et al* 2004), while a perioperative decrease in dogs undergoing elective surgery has been described in Siracusa *et al* 2008. In the latter study a slight preoperative downward tendency was caused by psychological stressors such as handling and confinement, but a greater postoperative decrease evidenced the major influence of postoperative

pain and anaesthesia-induced dysphoria. In the present study a similar decrease was observed in the prolactin response of the placebo group, but not in the DAP group, since the decrease observed in this group was significantly smaller. The downward tendency observed in the prolactin response of the placebo group was evident as early as 30 minutes after moving the animal from its usual environment to the ICU cage (T1) and persisted after surgery (T2, see Figure 6.5). By way of contrast, the DAP group showed a slight upward tendency after preoperative confinement in the ICU cage (T1), followed by a decrease after surgery (T2, see Figure 6.5). These variations show how the effect of DAP was focused on the psychological stress response caused by uncontrolled handling and confinement, being prevalent after the pre-surgery stay in the ICU cage (T1). After surgery, when pain and dysphoria prevail, we can observe a mild prolactin decrease also in dogs treated with the DAP (T3, see Figure 6.5). However, the synthetic pheromone is still able to modulate the lactotropic response, as this decrease (T3) is not significant when compared with basal values (T0).

Since DAP clinical pharmacology is still unknown, as is the action of the natural dog-appeasing pheromone, it is not easy to understand why the effects seen in our study are focused solely on the lactotropic axis. The secretion of pituitary prolactin, used as a marker for lactotropic axis activation, is regulated by the suppressive effect of hypothalamic dopamine and the stimulatory effect of TRH, neurophysin, substance P and other factors (Matteri *et al* 2000). Thus, the DAP treatment could have a direct effect on prolactin or regulate its secretion indirectly, via the dopaminergic system. The direct influence of prolactin on oxytocin secretion was also documented, and both hormones have been shown to modulate the neuroendocrine acute stress response influencing maternal behaviour. An increase in prolactin and oxytocin concentrations showed an anxiolytic effect in pregnant and lactating rats (Grattan 2002; Neumann *et al* 2000). In turn, the increase in oxytocin after parturition in sheep seems to be triggered by stimulation of olfactory cues from the lamb and the amniotic fluid (Wyatt 2003). Thus natural pheromones, prolactin and oxytocin could be involved in controlling the acute stress

response after parturition, although their interaction is not yet well understood. A similar mechanism could be responsible for the prolactin response seen in dogs given DAP treatment, but not limited to females as showed by the absence of significant interaction between variation of prolactin over time and sex of animals.

Perioperative changes in immune function and variation of C-reactive protein and haptoglobin in dogs have been described in previous studies (Ceròn *et al* 2005; Murata *et al* 2004; Siracusa *et al* 2008). The immune response, easily assessed by using total WBC, neutrophils and lymphocytes counts (Blecha 2000), did not appear to be influenced by the pheromone treatment, as was the case with the neutrophil/lymphocyte ratio. If the perioperative neutrophil response is unlikely to be sensitive to the DAP treatment, because of the major influence of post-surgery tissue inflammation on it, the lymphocyte response, a better indicator of perioperative psychological stress according to the result presented in the previous chapter, could have been potentially sensitive to the calming effect of the DAP. However, this hypothesis has been rejected in our study. On the other hand, the lack of sensitivity of lymphocytes concurs with the absence of DAP influence on cortisol response, as the HPA axis is the main regulatory system involved in the stress response of these blood cells (Blecha 2000; Matteri *et al* 2000).

Acute phase proteins may also be elevated in association with physical and psychological stress in humans, cattle, rats and mice (Marrocco-Trischitta *et al* 2004; Murata *et al* 2004; Ceròn *et al* 2005). An increase in the hepatic synthesis of acute phase proteins in response to cytokine-mediated HPA axis activation has been proposed as the mechanism involved in the acute phase response to stress (Murata *et al* 2004). In such cases, the lack of sensitivity to DAP treatment seen in this trial could concur yet again with the cortisol response observed here. Moreover, the major influence of the postoperative inflammation on C-reactive protein and haptoglobin responses, together with their long-term activation shown in the results presented in Siracusa *et al* 2008,

makes it more difficult to detect a possible minor change due to a short-acting treatment such as the DAP to control psychological stress (Pageat and Gaultier 2003a). Through analysis of immune and acute phase responses, we can affirm that the DAP treatment had no influence on the postoperative inflammatory response.

Although an extensive literature on the benefits of using the DAP to reduce stress has been published (Sheppard and Mills 2003; Gaultier *et al* 2005; Tod *et al* 2005; Levine et al 2006; Mills et al 2006; Taylor and Mills 2006; Gaultier *et al* 2008), it is difficult to compare our results with previous ones, because of the different methods used for stress assessment. Qualitative methods, mainly questionnaires submitted to owners, have been used to assess the efficacy of DAP (Gaultier *et al* 2005; Levine *et al* 2006; Mills *et al* 2006; Taylor and Mills 2006; Gaultier *et al* 2008). To our knowledge this is the first study using a multiple quantitative responses to analyze the canine response to DAP.

## 6.5 CONCLUSION

Evidence is provided in this study to support the hypothesis that the Dog-Appeasing Pheromone influences the perioperative stress response in dogs undergoing elective orchiectomy and ovariohysterectomy. In these dogs the DAP favours postoperative alert and visual explorative behaviors and decreases the magnitude of the lactotropic axis activation due to perioperative stress. However, neither the HPA axis, nor the immune and the acute phase responses were affected by treatment with the synthetic pheromone. These findings suggest that the DAP could be effective in controlling the perioperative stress response in dogs undergoing elective surgery. Thus, the use of this product in a clinical setting could improve the welfare of dogs undergoing elective surgery.

# 7. GENERAL DISCUSSION

# 7. GENERAL DISCUSSION

The results presented in this study provide evidence that perioperative stress in dogs represents a major challenge for their homeostasis, especially during the post-surgery period. It has also been shown that the use of a synthetic dog appeasing pheromone can be a valid contribution to alleviating the neuroendocrine and behavioural signs of perioperative stress.

Although an extensive discussion followed the results previously presented in this study, we would like to add in this section some thoughts about the general perioperative stress response observed in the two trials presented. At the same time we want to speculate whether this response is ascribable to a general type of stress response observed in other species, in relation to the nature of the coping strategy used: reactive or proactive.

Another interesting point that deserves to be discussed is the possibility of using the dog appeasing pheromone as a complement to the pharmacological control of the stress response. It is in fact controversial whether the perioperative stress response should be eliminated or mildly modulated by the use of medications. In this light, the DAP could represent a valid complementary and "natural" aid.

Finally, a comparison of our results with previously published ones, together with the strengths and limitations of this study will be discussed, to favour a prudent and appropriate interpretation of the results presented.

## 7.1. The perioperative stress response: agreement between the responses observed in the two trials presented.

When we consider the overall perioperative stress responses observed in this study, we can see that the variations evidenced in all biomarkers

studied followed the same tendencies in both trials, with the exception of the cortisol response. In the first trial the cortisol mean value peaked immediately after surgery but decreased to the basal value as early as 30 minutes after taking the animal back to its usual environment. This drop was not observed in the results presented in the DAP trial. Differences in postoperative pain perception between the two trials could explain this divergent cortisol response.

This hypothesis is also confirmed by the GPS score registered in the two trials. In the first clinical trial there was no significant difference between pre- and postoperative GPS scores, while in the DAP trial the GPS score was significantly increased after surgery. The analgesic and anaesthetic protocols used could have caused this discordant postoperative pain perception between the animals enrolled in the two trials. According to previous studies, different forms of pain management can have different effects on pain perception measured by neuroendocrine or behavioural parameters (Yardeni *et al* 2007; Sibanda *et al* 2006; Shin *et al* 2008). Buprenorphine, the analgesic treatment used in the preliminary study, was more efficient than morphine used in the DAP study in controlling the postoperative stress response, where pain is a major activating stimulus, due to a longer-lasting effect than morphine (Roughan and Flecknell 2002).

## 7.2. The Hawks and Doves game: does the stress response observed in dogs ascribe to this theory?

The overall findings of the behavioural, haematological and biochemical studies can be uniformly accounted for by recourse to the evolutionary theory concerning stress response as described in Korte *et al* 2005. Two major behavioral strategies for coping with stress have been described as being widespread in the animal kingdom - *Hawks* and *Doves*. *Hawks* are high-aggressive (fight-flight) and *proactive* coping individuals, while *Doves* are low-aggressive and cooperative animals, adopting *reactive* coping strategies with cautious and thorough explorative behaviour.

Studies conducted in birds and rodents (Korte *et al* 2005) have also evidenced that two divergent neuroendocrine and immune responses correspond to these behavioral traits. When compared to *Hawks*, *Doves* are characterised, among other elements, by a higher activation of the HPA axis and the dopaminergic system, together with a lower activation of the neurosympathetic system.

The high occurrence of explorative behaviors, high-pitched vocalizations (whining), oral behaviors, stiffening position (freezing) and the absence of an aggressive response, together with a high activation of the HPA axis and the dopaminergic-lactotropic system, evidenced in the individuals enrolled in our study, suggest that a *Dove*-type response could be typical of the canine species when coping with perioperative stress. The inclusion criterion of non-aggressive dogs used in this study could have influenced the stress response observed. However, it has also been demonstrated that the genetic manipulation operated by humans on Wistar rats selected for easy handling, resulted in a prevalent Dove-type in this species (De Boer *et al* 2003). Similarly, the genetic selection operated on the domestic dog along its evolution, promoting the reproduction of low-aggressive and docile individuals (Lindsay 2000; Trut 2001), could have been accompanied by the selection of a *Dove*-type stress response. Further studies are necessary to confirm the hypothesis of the existence of both *Hawk* and *Dove* types in the domestic dog.

## 7.3. The risk related to the pharmacological attenuation of the perioperative stress response: the possible contribution of the DAP.

In the second part of this study we propose the use of the DAP as a valid tool to increase the control over perioperative stress, complementary to an effective anaesthetic and analgesic protocol, cautious handling and to a comfortable confinement in the ICU.

Using pharmacological means to attenuate or abolish the stress response is not without risk. The stress response was in fact programmed in

higher organisms to provide homeostatic adjustments to factors such as cold exposure, volume loss, hypoglycemia, and inflammation. When using pharmacological stress-modifying tools (anaesthesia and analgesia), providers must be keenly aware of the potential problems associated with cold exposure, hemorrhage, hypovolemia and sepsis in the treated patients. This places responsibility on the care providers to minimize these and other potential external stressors and, if necessary, to respond therapeutically in an appropriate manner if an unexpected incident would occur. Protocols should be devised to treat these potential problems if they were to occur in patients undergoing stress reduction therapy.

This point was emphasized by Cannon 70 years ago (Cannon 1932). He devised a method to totally sympathectomize cats, and these animals could be maintained in a carefully controlled laboratory without difficulty. However, they were unable to defend against hypoxia, fluid restriction or the stress associated with changes in environmental temperature, hemorrhage and severe exercise.

In this light, the use of appropriate behavioural and environmental modification could contribute to modulation of the perioperative stress in a safer way, reducing the need for drastic pharmacological intervention. This could contribute to decreasing the postoperative risk and favouring the recovery of patients.

It is important to remember that the control of perioperative stress begins with a careful preoperative handling and confinement. We have seen in this study how cortisol can reach a significant preoperative increase, underlying an intense activation of the HPA axis. Non-threatening handling accomplished by avoiding sudden movements, using a calm tone of voice, preferring a lateral and horizontal approach toward the animal, and learning to interpret the dog's body language, is the first element that needs to be adequately considered. Moreover, environmental interventions in the ICU, such as easy-to-clean surfaces to eliminate pollution by alarm pheromones, or reducing the intensity

of environmental visual and acoustic stimulation, can also help to minimize stress starting before surgery.

It has already been emphasized how the knowledge of stress behaviours is a powerful tool to detect the effect of major postoperative stressors, such as pain. Obviously, considerations about handling and confinement highlighted as useful during the pre-surgery period are even more relevant during the post-surgery period. Promoting and implementing the use of behavioural tools for detection and control of stress and pain in veterinary medicine represents an important step toward optimizing animal welfare.

According to the results presented in this study, the DAP could provide a further contribution to minimize the risk related to the control of perioperative stress. As a natural chemical message, with no side effects reported, it elicits a biological response that can be self-modulated by the recipient (Pageat and Gaultier 2003a). Undoubtedly, the DAP cannot represent a substitute for analgesics and anaesthetics for controlling perioperative stress, but it can surely be a complementary aid. Behavioural categories significantly affected by anaesthesia and analgesia, *awake/alert* and *visual scanning*, were in fact positively influenced by the treatment with DAP, showing that the synthetic pheromone favours a more rapid recovery from anaesthesia, promoting awakening and visual environmental exploration. Similarly, the DAP was able to modulate the post-operative lactotropic axis activation, as shown by the prolactin response.

## 7.4. The efficacy of the DAP for stress control: a comparison of our results with previous studies.

When we attempted to compare the results presented in this study with previously published results, we faced some difficulties mainly related to the different methods used to assess the efficacy of the DAP. Many studies published are based on a qualitative evaluation that uses a questionnaire filled in by dog owners (Taylor and Mills 2006; Levine *et*

*al* 2006; Denenberg and Landsberg 2008; Gualtier *et al* 2008). In these studies the efficacy of the DAP in reducing behavioural signs related to stress, fear and anxiety in different contexts, e.g. social isolation, training and long-term socialization, adoption, fear of fireworks and loud noises, were proven. Although our conclusions agree with the same general statement that the DAP is an effective tool for stress and anxiety control, a direct quantitative comparison is impossible. Behaviours were studied in different contexts and with different assessment methods (qualitative vs. quantitative), and no haematological and/or biochemical markers were used in previous studies.

An exception is represented by a study published by Tod *et al* 2005, in which a quantitative assessment of the DAP effectiveness was realized. In this study the efficacy of the DAP in reducing stress and fear-related behaviour in shelter dogs has been evaluated. A quantitative analysis was used to determine the occurrence of behaviours and characteristics of vocalizations. A reduction in frequency and amplitude of barking was evidenced in dogs exposed to DAP. These dogs also showed less barking, more resting and more sniffing in response to a friendly stranger. From a quantitative point of view, our results are divergent from these results. The dogs enrolled in our trial showed no significant effect of DAP on vocalizations and olfactory exploration; while resting/sleeping was significantly reduced after surgery in dogs treated with DAP. However, due to the different nature of the stress experienced (acute in case of surgery vs. chronic in case of sheltering) and to the different contexts in which the studies were realized, these differences in results do not lead to different conclusions. As stated by the same Tod *et al* 2005, studies using behavioural and biochemical markers of stress for DAP evaluation can give more conclusive results about its efficacy and facilitate comparison among results obtained in different studies.

## 7.5. Strengths and limitations of the study.

To help with the interpretation of the results presented in our study, it is relevant to highlight some strengths and limitations of the two clinical

trials presented. We are strongly convinced that one of the main positive aspects of the study is having analysed a complex phenomenon like perioperative stress, using multiple biomarkers for stress assessment. The parameters used in this study are related to different systems activated by a stressor (Matteri 2000) and provide a wider range of tools in order to analyse the stress response in a more accurate fashion. It is well known in fact that the behavioural, the neuroendocrine and the immune stress responses are not always activated together by a unique stressor, and when activated the magnitude of their response can be extremely variable (Moberg 2000). Therefore, using multiple tools increased the sensitivity of our trial in detecting perturbations of homeostasis related to perioperative stress.

With regard to this aspect, it is interesting to note that cortisol, probably the most commonly used parameter for stress assessment (Matteri 2000), confirmed to be a highly sensitive biomarker, was the only one of the parameters studied to show significant preoperative variations. However, cortisol was not sensitive to the DAP treatment, and therefore not useful to detect the efficacy of the synthetic pheromone, that was instead confirmed by the sensitivity of prolactin and behaviour. These findings show the importance of a multifactorial approach to stress analysis to avoid an underestimation of a stressor or a treatment's efficacy.

The longitudinal design of the study is also a positive element, which helps to reduce the effect of the high individual variability in the stress response (Moberg 2000). This variability affects especially the behavioural response. For instance, a behaviour that is usually displayed by an individual in response to handling, e.g. a stiff posture or reluctance to move, could be misinterpreted after surgery as a pain-related behaviour. Therefore, studying the individual response both pre and post-surgery, applying a sequence of stressors of increasing intensity (handling, confinement, surgery, anaesthesia and analgesia, pain), can help to minimize the impact of the individual variability (Mich and Hellyer 2009). Moreover, this design reproduces the ideal perioperative

clinical approach, where signs of stress should be observed both before and after surgery.

Although the clinical design of the study has the advantage of providing the veterinarian with useful tools for stress evaluation in daily clinical practice, it also carries an important limitation. Our sample collection was in fact influenced by this choice. According to the recommendation of the Ethical Committee of our institution, the Autonomous University of Barcelona, we were required to limit all the procedures used in this study to the normal surgery protocol adopted by the hosting shelter. Any additional procedure that could compromise the welfare of the sheltered dogs enrolled in the study was not included in our protocol. For this reason, the postoperative blood sample collection was limited to one sample in the early post surgery period and no sample was collected after reintroduction of dogs to their usual environment, i.e. to minimise the risk of postoperative complications.

Using laboratory animals could have increased the uniformity of the population and allowed wider limits in sample collection. But it could also have influenced the magnitude of the stress response and the quality of the stress behaviour displayed. Dogs presented as clinical patients commonly display a repertoire of fear- and stress-related behaviours, which are instead limited in laboratory dogs selected for being easily confined and handled by humans. Therefore, some important behavioural indicators of stress in clinical practice could have been inadequately predicted using laboratory animals.

A population of sheltered dogs was used for this study. Using dogs that have experienced chronic stress due to long term confinement (Hubrecht *et al* 1992; Beerda *et al* 1999) could have influenced the response to an acute stress like the perioperative one (Matteri *et al* 2000). For example, prolactin response in dogs exposed to an acute stressor can be influenced by the release of dopamine due to underlying chronic stress (Cuadra *et al* 1999). To minimize the confounding effect of chronic confinement, dogs that were showing overt behavioural signs

of chronic stress (e.g. stereotypies) were not included in this trial. Moreover, only dogs that spent more than 20 days in the shelter facilities were enrolled in this study, based on previous results reporting a peak in cortisol response within the first 17 days after relinquishment, followed by a steady decline (Stephen and Ledger 2006; Hennessy *et al* 1997).

A technical problem is related to the use of synthetic pheromones, such as the DAP, and affects their clinical use, as well as the studies about their efficacy. In nature the secretion of pheromones is associated with other behavioural or chemical signals, emphasizing signals (Pageat and Gaultier 2003a; Wyatt 2003). These signals are usually represented by a body posture (e.g. the marking posture), showing a part of the body that is usually hidden (e.g. the anal area), modifying the marked substrate (e.g. scratching it) or expelling some individual odours associated with the chemical signal. The manufacturer of the synthetic pheromone tried to compensate for the lack of these associated emphasizing signals by increasing the concentration of the pheromone and its odour (Pageat and Gaultier 2003a). However, there is no evidence that the intensity and efficacy of the message sent by the synthetic pheromone is not compromised when compared to the natural context. During the lactation of puppies, appeasine's effect could be magnified by vocalizations or tactile stimulations from the bitch. For example, high-pitched vocalisations are used by puppies and bitches as signals to elicit the "searching response", with a consequent decreasing of distance and calming effect (Lindsay 2000). Further studies to clarify the nature and influence of the emphasizing signals on the dog appeasine could contribute to improving the efficacy of pheromonotherapy with the DAP.

In spite of the limitations discussed, the study presented in this work provides valuable new data about the perioperative stress response in dogs and on the use of a non-conventional therapy to control stress. In human medicine the study of perioperative stress had been receiving attention since it was first described by Cuthbertson in the late 1920s,

and efforts have been made to modify the stress response and improve patient outcome (Douglas 2002). Conversely, in veterinary medicine perioperative stress and pain management have been receiving attention only in the last decades. It is interesting to note that the International Pain Society supported the Cartesian belief that possession of language is a precondition for the ability to feel pain in its definition of pain, until 2001. The denial of the experience of pain and perioperative stress by animals in veterinary medicine was so powerful that when the first textbooks of veterinary anaesthesia were published in the United States by Lumb (1964) and Lumb and Jones (1972), they did not list the control of pain as a reason for using anaesthesia and had no discussion of analgesia (Rollin 2009). Similarly, many veterinarians still use sedation, but not analgesia, during surgery. Other concerns in human medicine, such as the use of drugs like ketamine that causes flashbacks and hallucinations reported as negative experiences by human patients, are not yet perceived as a problem in veterinary medicine (Rollin 2009). Providing objective data about perioperative stress in dogs and showing that, even with an appropriate surgery protocol, there is still a major activation of the stress response, can be useful elements to increase sensitivity in veterinary circles toward these issues.

# 8. CONCLUSIONS

# 8. CONCLUSIONS

1. Behavioural changes together with haematological and biochemical markers of the neuroendocrine, immune and acute phase stress responses are sensitive and useful tools for assessing perioperative stress in dogs undergoing elective orchiectomy and ovariohysterectomy.

2. Changes in explorative and communicative behaviours, as well as alterations in waking/sleeping pattern, activity and active interaction with a handler are the most relevant postoperative behavioural variations.

3. Cortisol was the only biomarker to show a significant preoperative change. This finding suggests an important preoperative activation of the HPA axis and proves that cortisol is the most useful tool for psychological stress assessment.

4. After surgery, lymphocytes and eosinophils showed a great sensitivity for early postoperative psychological stress detection, while neutrophils, monocytes, C-reactive protein and haptoglobin proved to be good markers for inflammation. It has also been evidenced that neutrophil/lymphocyte ratio represents a useful and inexpensive tool for postoperative stress assessment.

5. Postoperative changes observed in dogs undergoing elective surgery showed that pain, analgesia- and anaesthesia-induced dysphoria, tissue damage, along with persistent psychological stressors, represented a major challenge to the animals' homeostatic balance.

6. The Dog Appeasing Pheromone (DAP) was able to modulate the perioperative stress response in dogs undergoing elective

orchiectomy and ovariohysterectomy. It favoured postoperative alert and visual explorative behaviors and decreased the magnitude of lactotropic axis activation due to perioperative stress. However, neither the HPA axis, nor the immune and the acute phase responses were affected by treatment with the synthetic pheromone.

7. Behavioural and neuroendocrine changes observed in dogs treated with the DAP suggest that this synthetic pheromone could represent an effective aid complementary to the pharmacological control of perioperative stress, contributing to improving the welfare and safety of dogs undergoing elective surgery.

# 9. REFERENCES

# 9. REFERENCES

**Alleva R, Tomasetti M, Solenghi MD, et al** 2003 Lymphocyte DNA damage precedes DNA repair or cell death after orthopaedic surgery under general anaesthesia. *Mutagenesis 18*: 423-428.

**Ambrisko TD, Hikasa Y and Sato K** 2005 Influence of medetomidine on stress-related neurohormonal and metabolic effects caused by butorphanol, fentanyl, and ketamine administration in dogs. *The American Journal of Veterinary Research 66*: 406-12.

**Anand KJS, Phil D and Hickey** 1992 Halothane-morphine compared with high-dose sufentanil for anaesthesia and postoperative analgesia in neonatal cardiac surgery. *The New England Journal of medicine 326*: 1-9.

**Beerda B, Schilder MBH, Janssen NSCRM, et al** 1996 The use of saliva cortisol and catecholamine measurements for a noninvasive assessment of stress response in dogs. *Hormones and Behaviour 30*: 272-279.

**Beerda B, Schilder MB, van Hooff JARAM, et al** 1997 Manifestation of chronic and acute stress in dogs. *Applied Animal Behaviour Science 52*: 307-319.

**Beerda B, Schilder MB, van Hooff JARAM, et al** 1998 Behavioural, saliva cortisol and heart rate responses to different types of stimuli in dogs. *Applied Animal Behaviour Science 58*: 365-381.

**Beerda B, Schilder MB, van Hooff JARAM, et al** 1999 Chronic stress in dogs subjected to social and spatial restriction. I. Behavioural response.*Physiology and behavior 66*: 233 – 242.

**Blackburn-Munro G** 2004 Pain-like behaviours in animals – how human are they? Trends in Pharmacological Science 25: 299-305.

**Blecha F** 2000 Immune system response to stress. In Moberg GP and Mench JA (eds) *The Biology Of Animal Stress* pp 111-122. CABI Publishing, Wallingford, UK.

**Brodner G, van Haken H, Hertle L et al** 2001 Multimodality perioperative management - combined thoracic epidural anesthesia, forced mobilization, and oral nutrition – reduces hormonal and metabolic stress and improves convalescence after major urologic surgery. *Anesthesia and Analgesia 92*: 1594-1600.

**Broom DM and Johnson KG** 1993 *Stress And Animal Welfare.* Klumer Academic Publisher, Dordrecht, The Netherlands.

**Butler KL** 2003 Surgical stress: a basic critical concept or is it just wodoo? *Current Surgery 0149-7944*: 551-554.

**Cannon WB** 1932 *The wisdom of the body.* WW Norton, New York, USA.

**Cerón JJ, Eckersall DP and Martinez-Subiela S** 2005 Acute phase proteins in dogs and cats: current knowledge and future perspectives. *Veterinary Clinical Pathology 34*: 85-99.

**Conner JG and Eckersall PD** 1988 Acute phase response in the dog following surgical trauma. *Research in Veterinary Science 45*: 107-110.

**Coppola CL, Grandin T and Enns RM** 2006 Human interaction and cortisol: Can human contact reduce stress for shelter dogs? *Physiology and Behavior 87*: 537-541.

**Crowell-Davis S and Mrray T** 2006 *Veterinary psychopharmacology.* Blackwell Publishing: Ames, Iowa, USA.

**Cuadra G, Zurita A, Lacerra C, et al** 1999 Chronic stress sensitizes frontal cortex dopamine release in response to a subsequent novel stressor: reversal by naloxone. *Brain Research Bulletin 48*: 303-308.

**Dantzer R and Mormede P** 1995 Psychoneuroimmunology of stress. In Leonard B and Miller K (eds) *Stress, the Immune System and Psychiatry* pp 47-67. John Wiley and Sons Ltd., New York, USA.

**Dawkins MS** 1995 *Unravelling animal behavior, 2nd edition.* Longamn Scientific & Technical, Harlow.

**De Boer SF, vna der Vegt BJ and Koolhaas JM** 2003 Individual variation in aggression of feral rodent strains: a standard for the genetics of aggression and vilence? *Behavior and Genetics 33*: 485-501.

**Delogu G, Moretti S, Famularo G, et al** 2001 Mitochondrial perturbations and oxidant stress in lymphocytes from patients undergoing surgery and general anesthesia. *Archives of Surgery 136*: 1190-1196.

**Desborough JP** 2000 The stress response to trauma and surgery. *British Journal of Anaesthesia 85*: 109-17.

**Devitt CM, Cox RE and Hailey JJ** 2005 Duration, complication, stress and pain of open ovariohysterectomy versus a simple method of laparoscopic-assisted ovariohysterectomy in dogs. *Journal of Veterinary Medical Association 227*: 921-27.

**Douglas W and Wilmore MD** 2002 From Cuthbertson to fast-track surgery: 70 years of progress in reducing stress in surgical patients. *Annals of Surgery 236*: 643-648.

**Egdahl RH** 1959 Pituitary-adrenal response following trauma to the isolated leg. *Surgery 46*: 9-21.

**Elena GA, Acosta AP, Antoniazzi S et al** 2006 Hemodynamic, immunologic and systemic stress response during surgery under total intravenous anaesthesia with midazolam-ketamine-fentanyl or remifentanil-midazolam. *Revista Española de Anestesiología y Reanimación 53*: 275-282.

**Firth AM and Haldane SL** 1999 Development Of A Scale To Evaluate Postoperative Pain In Dogs. *Journal of American Veterinary Association* 214: 651-659.

**Fox MS, Mellor DG, Lawoko CRO, et al** 1998 Changes in plasma cortisol concentrations in bitches in response to different combinations of halothane and butorphanol, with or without ovariohysterectomy. *Research in Veterinary Science 65*: 125-133.

**Gaultier E, Bonnafous L, Bougrat L, et al** 2005 Comparison of the efficacy of a synthetic dog-appeasing pheromone with clomipramine for the treatment of separation-related disorders in dogs. *Veterinary Record 156*: 533-538.

**Gaultier E, Bannafous L, Vienet-Legué D, et al** 2008 Efficacy of dog-appeasing pheromone in reducing stress associated with social isolation in newly adopted puppies. *Veterinary Record 163*: 73-80.

**Gauter-Fleckenstein B, Kaviani R, Weiss C et al** 2007 Perioperative patient management. Evaluation of subjective stress and demands of patients undergoing elective gynaecological surgery. *Der Anaesthesist 56*: 562-570.

**Giescke K, Hamberger B, Jamberg PD, et al** 1988 High and low dose fentanyl anaesthesia: hormonal and metabolic response during cholecystectomy. *British Journal of Aneasthesia 61*: 575-582.

**Goodmann PA, Klinghhammer E and Willard J** 2002 *Wolf Ethogram, Ethology Series No. 3.* EH Hess Institute of Ethology, Battle Ground, USA.

**Grattan DR** 2002 Behavioural significance of prolactin signaling in the central nervous system during pregnancy and lactation. *Reproduction 123*: 497-506.

**Griffis CA, Compton P and Doering L** 2006: The effect of pain on leukocyte cellular adhesion molecules. *Biological Research for Nursing 7*: 297-312.

**Hansen BD, Hardie EM and Carroll GS** 1997 Physiological measurements after ovariohysterectomy in dogs: what's normal? *Applied Animal Behaviour Science 51*: 101-109.

**Hardie EM, Hansen BD, Carrol GS** 1997 Behaviour after ovariohysterectomy in the dog: what's normal? *Applied Animal Behavioural Science 51*: 11-128.

**Hart BL** 1985 Behavioural indications for phenotiazine and benzodiazepine tranquilizers in dogs. *Journal of American Veterinary Medical Association 186*: 192-94.

**Haverbeke A, Diederich C, Depiereux E, et al** 2008 Cortisol and behavioral responses of working dogs to environmental challenges. *Physiology and Behavior 93*: 59-67.

**Hellyer PW** 2005 Pain Idebtification. In Ettinger SJ and Feldman EC (edts.)*: Textbook Of Veterinary Internal Medicine, 6th Edition.* USA pp 16-21. WB Saunders Company, Philadelphia, Pennsylvania, USA.

**Hennessy MB, Davis HN, Williams MT, et al** 1997 Plasma cortisol levels of dogs at a county animal shelter. *Physiology and Behavior 62*: 485-490.

**Hetts S, Clark Derrel J, Calpin JP, et al** 1992 Influence of housing conditions on beagle behaviour. *Applied Animal Behavioural Science 34*: 137-155.

**Holton L, Reid J, Scott EM, et al** 2001 Development of a behaviour-based scale to measure acute pain in dogs. *Veterinary Record 148*: 525-531.

**Horvat Z, Igyarto BZ, Magyar A, et al** 2007 Three different coping styles in police dogs exposed to a short-term challenge. *Hormones and Behavior 52*: 621-630.

**Hubrecht RC, Serpell JA and Poole T** 1992 Correlates of pen size and housing conditions in the behaviour of kennelled dogs. *Applied Animal Behavioural Science 34*: 365-383.

**Hume DM** 1953 The neuro-endocrine response to injury: present status of the problem. *Annals of Surgery 138*: 548-557.

**Jain NC** 1989 Acute Phase Proteins. In Kirk RW (ed.): *Current Veterinary Therapy X, Small Animal Practice* pp 468-471.WB Saunders, Philadelphia, USA.

**Karlson P amd Luscher M** 1959 "Pheromones": a new term for a class of biologically active substances. *Nature 183*: 155-156.

**Kehlet H and Wilmore DW** 2002 Multimodal strategy to improve surgical outcome. *American Journal of Surgery 183*: 630-641.

**Kobelt AJ, Hemsworth PH, Barnett JL, et al** 2003 Sources of sampling variation in saliva cortisol in dogs. *Research in Veterinary Science 75*: 157 – 161.

**Korte SM, Koolhaas JM, Wingfield JC, et al** 2005 The Darwinian concept of stress: benefit of allostasis and cost of allostatic load and the trade-offs in health and disease. *Neuroscience and Biobehavioral Reviews 29*: 3-38.

**Lascelles BDX, Cripps PJ, Jones A, et al** 1998 Efficacy and kinetics of caprofen, administrered preoperatively or postoperatively, for the prevention of pain in dogs undergoing ovariohysterectomy. *Veterinary Surgery 27*: 568-582.

**Levine ED, Ramos D and Mills DS** 2006 A prospective study of two self-help CD based desensitization and counter-conditioning programmes with the use of Dog Appeasing Pheromone for the treatment of firework fears in dogs (Canis familiaris). *Applied Animal Behavior Science 105*: 311-329.

**Lindsay SR** 2000 *Applied Dog Behavior and Training, Vol. 1* pp 12-22, 136-145. Iowa State Press, Annes, Iowa, USA.

**Lumb WV** 1963 *Small animal anesthesia.* Lea & Febiger, Philadelphia, USA.

**Lumb WV and Jones EW** 1973 *Veterinary anesthesia.* Lea & Febiger, Philadelphia, USA.

**Mandel I** 1990 The diagnostic use of saliva. *Journal of Oral Phatology Medicine 19*: 119-125.

**Marrocco-Trischitta MM, Tiezzi A, Svampa MG, et al** 2004 Perioperative stress response to carotid endarterectomy: the impact of anestethic modality. *Journal of Vascular Surgery 39*: 1295 – 1304.

**Martin P and Bateson P** 1993 *Measuring Behaviour, An Introductory Guide. 2nd Edition.* Cambridge University Press, Cambridge, UK.

**Mason WA** 2000 Early developmental influences of experience on behavior, temperament and stress. In Moberg GP and Mench JA (eds) *The Biology Of Animal Stress* pp 269-290. CABI Publishing, Wallingford, UK.

**Mathews KA** 2000 Pain assessment and general approach to management. *Veterinary Clinics of North America: Small Animal Practice 30*: 729 – 755.

**Matteri RL, Carroll JA and Dyer CJ** 2000 Neuroendocrine response to stress. In Moberg GP and Mench JA (eds) *The Biology Of Animal Stress* pp 43-76. CABI Publishing, Wallingford, UK.

**Mellor DJ, Cook CJ and Stafford KJ** 2000 Quantifying some responses to pain as a stressor. In Moberg GP and Mench JA (eds) *The Biology Of Animal Stress* pp 171-198. CABI Publishing, Wallingford, UK.

**Mich PM and Hellyer PW** 2009 Objective, categoric methods for assessing pain and analgesia. In Gaynor GS and Muir III WW (eds) *Handbook of veterinary pain management* pp 78-112. Mosby Elsevier, St Louis, Missouri.

**Mills DS, Ramos D, Gandia Estelles M, et al** 2006 A triple blind placebo-controlled investigation into the assessment of the effect of Dog Appeasing Pheromone (DAP) on anxiety related behavior of problem dogs in the veterinary clinic. *Applied Animal Behavior Science 98*: 114-126.

**Moberg GP** 1985 Biological Response To Stress: Key To Assessment Of Animal Well-Being? In Moberg GP (ed) *Animal Stress* pp 27-49. American Physiological Society, Bethesda, Maryland, USA.

**Moberg GP** 2000 Biological response to stress: implications for animal welfare. In Moberg GP and Mench JA (eds) *The Biology Of Animal Stress* pp 1-22. CABI Publishing, Wallingford, UK.

**Mormede P, Andanson S, Auperin B, et al** 2008 Exploration of the hypothalamic-pituitary-adrenal function as tool to evaluate animal welfare. *Physiology and Behavior 92*: 317-339.

**Morton DB and Griffiths PHM** 1985 Guidelines on the recognition of pain, distress and discomfort in experimental animals and a hypothesis for assessment. *Veterinary Record 20*: 431-436.

**Morton CM, Reid J, Scott EM, et al** 2005 Application of a scaling model to establish and validate an interval level pain scale for assessment of acute pain in dogs. *American Journal of Veterinary Research 66*: 2154 – 2166.

**Moshkin MP, Gerlinskaia LA, Kolosova IE, et al** 2006 Scent attractiveness and endocrine status in male students before and during a stress situation (in Russian, with English abstract). *Rossiĭskii fiziologicheskiĭ zhurnal imeni I.M. Sechenova / Rossiĭskaia akademiia nauk 92*: 1250-1259.

**Murata H, Shimada H and Yoshioka** 2004 Current research on acute phase proteins in veterinary diagnosis: an overview. *The Veterinary Journal 168*: 28-40.

**Neumann ID, Torner L and Wigger A** 2000 Brain oxytocin: differential inhibition of neuroendocrine stress responses and anxiety-related behavior in virgin, pregnant and lactating rats. *Neuroscience 95*: 567-575.

**Odendaal JS and Meintjes RA** 2003 Neurophysiological correlates of affiliative behaviour between humans and dogs. *The Veterinary Journal 165*: 296-301.

**Overall K** 1997 *Clinical behavioural medicine for small animals* pp 301-316. Mosby, St. Louis, Missouri, USA.

**Pageat P** 1998 *Pathologie Du Comportement Du Chien, 2nd edition*. Le Point Vétérinaire, Maisons-Alfort, France.

**Pageat P** 2000 *Pig appeasing pheromone to decrease stress, anxiety and aggressiveness.* Unites States Patent 6,077,867.

**Pageat P** 2005 Assessing prolactinemia in anxious dogs (*Canis familiaris*): interest in diagnostic value and use in the selection of the most appropriate psychotropic drug. In Mills D, Levine E, Landsberg G, Horwitz D, Duxbury M, Mertens P, Meyer X, Huntley LR, Reich M and Willard J (eds) *Current issues and research in veterinary behavioural medicine. Papers presented at the 5th International Behaviour Meeting.* Purdue University Press, West Lafayette, Indiana, USA.

**Pageat P and Gaultier E** 2003a Current research in canine and feline pheromones. *Veterinary Clinics of North America - Small Animal Practice 33*: 187-211.

**Pageat and Gaultier** 2003b Using prolactin blood level in the diagnosis of anxiety related disorders in dogs pp 35-36. In Heath S amd DeKeuster T (eds) *Therapeutic Approaches in Veterinary Behavioural Medicine. Proceedings of 9th ESVCE congress, 19 September, Salzburg, Austria.* ESVCE, Lovendegem, Belgium.

**Pageat P, Lafont C, Falewée C, et al** 2007 An evaluation of serum prolactin in anxious dogs and response to treatment with selegiline or fluoxetine. *Applied Animal Behaviour Science 105*: 342-350.

**Rasmussen LS, O'Brien JT, Silverstein JH et al** 2005 Is perioperative cortisol secretion related to postoperative cognitive dysfunction? *Acta Anaesthesiologica Scandinavica 49:* 1225-31.

**Rogers A, Walker N, Schug S et al** 2000 Reduction of postoperative mortality and morbidity with epidural or spinal anaesthesia: results from overview of randomized trials. *British Medical Journal 321*: 1493-1504.

**Rollin BE** 2009 *The ethics of pain management.* In Gaynor GS and Muir III WW (eds) Handbook of veterinary pain management 2nd edition pp 2-12. Mosby Elsevier, St Louis, Missouri, USA.

**Roughan JV and Flecknell PA** 2002 Buprenorphine: a reappraisal of its antinociceptive effects and therapeuric use in alleviating post-operative pain in animals. *Laboratory Animals 36*: 322-343.

**Roughan JV and Flecknell PA** 2003 Evaluation of a short duration behaviour-based post-operative pain scoring system in rats. *European Journal of Pain 7:* 397-406.

**Rushen** J 2000 Some Issues In The Interpretation Of Behavioural Response To Stress. In Moberg GP and Mench JA (eds) *The Biology Of Animal Stress* pp 23-41. CABI Publishing, Wallingford, UK.

**Sanford J, Ewbank R, Molony V, et al** 1986 Guidelines for the recognition and assessment of pain in animals. *Veterinary Record 118:* 334-338.

**Schultze AE** 2000 Interpretation of canine leukocyte responses. In Feldman BJ, Zinkl JG and Jain NC (eds) *Shalm's Veterinary Ematology* pp 366-381. Lippincott Williams and Wilkins, Baltimora, USA.

**Servei Metereologic de Catalunya**. 2007 *Dades d'estacions metereològiques automàtiques (EMA) dels anys 2006 i 2007.* URL: http://www.meteocat.com/marcs/marc_dades.html.

**Sheppard G and Mills D** 2003 Evaluation of dog-appeasing pheromone as a potential treatment for dogs fearful of fireworks. *Veterinary Record 152*: 432-436.

**Shin AC, Robertson S, Isaza N, et al** 2008 Comparison between analgesic effects of buprenorphine, caprofen, and buprenorphine with caprofen

for canine ovariohysterectomy. *Veterinary Anaesthesia and Analgesia 35*: 69-79.

**Schultze AE** 2000 Interpretation Of Canine Leukocyte Responses. In Feldman BJ, Zinkl JG, Jain NC (eds) *Schalm's Veterinary Hematology* pp 366-381. Lippincott Williams and Wilkins, Baltimora, USA.

**Sibanda S, Hughes JM, Pawson PE, et al** 2006 The effects of preoperative extradural bupivocaine and morphine on the stress response in dogs undergoing femoro-tibial joint surgery. *Veterinary Anaesthesia and Analgesia 33*: 246-57.

**Siracusa C, Manteca X, Cerón J, et al** 2008 Perioperative stress response in dogs undergoing elective surgery: variations in behavioural, neuroendocrine, immune and acute phase responses. *Animal Welfare 17*: 259-273.

**Stephen JM and Ledger RA** 2006 A longitudinal evaluation of urinary cortisol in kennelled dogs, *Canis Familiaris*. *Physiology and Behavior 87*: 911-916.

**Stockham SL, Keeton KS and Szlatovits B** 2003 Clinical assessment of leukocytosis: distinguishing leukocytoses caused by inflammatory, glucocorticoid, physiologic and leukemic disorders or conditions. *Veterinary Clinics of North America: Small Animal Practice 33*: 1335-1357.

**Stover SM, Steffey EP, Dybdal NO, et al** 1998 Hematologic and serum biochemical alterations associated with multiple halothane anesthesia exposures and minor surgical trauma in horses. *American Journal of Veterinary Research 49*: 236-241.

**Tayama E, Hayashida N, Oda T, et al** 1999 Recovery from lymphocytopenia following extracorporeal circulation: simple indicator to assess surgical stress. *Artificial Organs 23*: 736-740.

**Taylor PM** 1998 Effects of surgery on endocrine and metabolic responses to anaesthesia in horses and ponies. *Research in Veterinary Science 64*: 133-140.

**Taylor K and Mills DS** 2006 A placebo-controlled study to investigate the effect of Dog Appeasing Pheromone and other environmental and management factors on the reports of disturbance and house soiling during the night in recently adopted puppies (Canis familiaris). *Applied Animal Behavior Science 105*: 358-368.

**Thomas JS** 2000 Overview Of Plasma Proteins. In Feldman BJ, Zinkl JG, Jain NC (eds) *Shalm's Veterinary Ematology* pp 891-898. Lippincott Williams and Wilkins, Baltimora, USA.

**Thompson SB** 1998 Pharmacological treatment of phobias. In Dodman NH and Shuster L (eds) *Psychopharmacology of Animal Behaviour Disorders* pp 141-184. Blackwell Science Inc., Malden, USA.

**Tod E, Brander D and Waran N** 2005 Efficacy of dog appeasing pheromone in reducing stress and fear related behavior in shelter dogs. *Applied Animal Behavior Science 93*: 295-308.

**Trut LN** 2001 Experimental studies on early canid domestication. In Rowinsky A and Sampson J (eds) *The genetics of the dog* pp 15-41. CABI International, Wallingford, UK.

**Väisänen MN, Raekallio M, Kuusela E, et al** 2002 Evaluation of the perioperative stress response in dogs administered medetomidine or acepromazine as part of the preanesthetic medication. *American Journal of Veterinary Research 63*: 969-975.

**Väisänen MA, Valros AE, Hakaoja E, et al** 2005 Pre-operative stress in dogs - a preliminary investigation of behaviour and heart rate variability

in healthy hospitalized dogs. *Veterinary Anaesthesia and Analgesia 32*: 158-167.

**Vetter TR and Heiner EJ** 1996 Discordance between patient self reported visual analogue scale pain scores and observed pain-related behavior in older children after surgery. *Journal of Clinical Anesthesia 8*: 371-375.

**Weber CC, Eckert GP and Muller WE** 2006 Effects of antidepressants on the brain/plasma distribution of corticosterone. *Neuropsychopharmacology 31*: 2443-2448.

**Wells DL** 2004 A review of environmental enrichment for kennel dogs, canis familiaris. *Applied Animal Behaviour Science 85*: 307-317.

**Wyart C, Webster WW, Chen JH, et al** 2007 Smelling a single component of male sweat alters levels of cortisol in women. *Journal of Neuroscience 27*: 1261-1265.

**Wyatt TD** 2003 *Pheromones and Animal Behavior* pp 164-205. Cambridge University Press, Cambridge, UK.

**Yamamoto S, Shida T, Miyaji S, et al** 1993 Changes in serum C-reactive protein levels in dogs with various disorders and surgical traumas. *Veterinary Research Communications 17*: 85-93.

**Yardeni IZ, Shavit Y, Bessler H, et al** 2007 Comparison of postoperative pain management techniques on endocrine response to surgery: a randomised controlled trial. *International Journal of Surgery 5*: 239-243.

**Zahorec R** 2001 Ratio of neutrophil to lymphocyte counts: rapid and simple parameter of systemic inflammation and stress in critically ill. *Bratislava Medical Journal 102*: 5-14.

**Zakowsi SG, McAllister CG, Deal M, et al** 1992 Stress, reactivity, and immune function in healthy men. *Health Psychology 11*: 223-232.