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Doctor of Philosophy thesis:

Whole-blood leukocyte coping capacity chemiluminescence: an innovative tool for assessing pain in animals?

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RIASSUNTO

La valutazione del dolore negli animali è una procedura estremamente difficile, complicata dalla incapacità di comunicare verbalmente da parte degli animali. Nonostante la sua spiccata difficoltà, il riconoscimento del dolore nelle specie animali è un dovere necessario per garantire loro una buona qualità di vita, la quale, per poter essere definita tale, deve essere priva di dolore. Ad oggi, non sono ancora presenti tecniche "gold standard" per la valutazione del dolore negli animali. Infatti, al fine di valutare l'entità e i diversi aspetti del dolore, in medicina veterinaria viene effettuata una valutazione congiunta di molteplici parametri comportamentali e fisiologici. Negli ultimi anni è stata messa a punto una tecnica in grado di valutare lo stress psicogeno in varie specie animali e nell'uomo: la "whole-blood leukocyte coping capacity (LCC)". Questa tecnica ha il vantaggio di poter essere eseguita direttamente in campo su minime quantità di sangue intero, permettendo una rapida valutazione dello stato psico-fisiologico dell'individuo tramite l'analisi degli effetti dello stress sul sistema immunitario. Infatti, la valutazione viene eseguita attraverso la misurazione della produzione delle specie reattive dell'ossigeno da parte dei granulociti. Il dolore è considerato da diversi autori come un agente stressogeno per le sue ripercussioni sia a livello fisico che psicologico. Quindi, considerando la capacità dell'LCC di misurare lo stress psicogeno, e considerando il dolore come agente stressogeno, il nostro team di ricerca si è proposto di valutare il potenziale della succitata tecnica nella valutazione del dolore in due diverse specie animali (il bovino e il cavallo) sottoposte a due tipi diversi di stimolo algico (castrazione non chirurgica e chirurgica). I risultati ottenuti dalla tecnica di LCC sono stati correlati e confrontati con quelli derivanti da altri parametri per la valutazione di stress e dolore. I risultati promettenti degli studi di questa tesi hanno sottolineato come la tecnica di LCC possa fornire informazioni preziose per la valutazione del dolore animale, in particolar modo quando questa viene utilizzata in

concomitanza ad altri parametri. L'approccio multi-parametrico ha infatti permesso di cogliere condizioni anomale dello stato psico-fisiologico dell'animale, e ha dimostrato come, in determinate condizioni, l'LCC potrebbe essere più sensibile di altri indicatori nella valutazione del dolore animale. Nonostante i risultati molto promettenti, per migliorare la sensibilità specie-specifica della tecnica di LCC, questa dovrebbe essere sottoposta a procedura di validazione e testata su altre tipologie di dolore. Si crede che la ricerca presentata in questa tesi di dottorato possa fungere da base sulla quale sviluppare nuovi filoni di ricerca atti al miglioramento del benessere animale attraverso il perfezionamento della valutazione del dolore.

SUMMARY

Assessing pain in animals is an extremely difficult task due to their inability to verbally communicate. Despite its challenging nature, the task of assessing pain in animals is compulsory seen the importance of granting them a good quality of life, which should be free of pain. At present, no gold standard technique for pain assessment in animals is available and researchers and clinicians have been relying on the conjunct use of behavioural and physiological assessments to grasp the extent and different aspects of the multifaceted nature of pain. In the last years, a new technique for assessing psychological stress has been evaluated and tested on different animal species and humans: the whole-blood leukocyte coping capacity (LCC). This technique can be performed directly in the field on a small amount of whole-blood, allowing for a rapid assessment of the individual's psycho-physiological status and its stress-related changes over time by measuring granulocytes' oxidative burst. Pain has been recognised worldwide as a stressor by several authors due to its short and long-term effects on the individual's mental status and physiology. Considering that the LCC technique allows for detection psychological stress, and considering that pain is a stressor, the research team decided to evaluate the potential of the LCC technique as a pain assessment tool in two animal species (the cattle and the horse) undergoing different castration procedures (non-surgical and surgical castration).The LCC results were correlated and compared with ones from other well-known pain and stress indicators. The studies of this thesis revealed that the whole-blood LCC seemed to provide very promising results, especially when used concurrently with other pain and stress assessment tools. In certain circumstances, the LCC seemed to allow for pain assessment even when some of the other parameters failed to detect it. Despite the promising results, the whole-blood technique should undergo a species-specific validation process and be tested on different pain types (*e.g.* non-inflammatory pain) for further confirmation of its sensitivity. It is believed

that the research presented in this PhD thesis could set the basis for further studies aimed at ameliorating animal welfare through further refinement of pain assessment.

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1 State of the art

1.1 Pain: definition and classification

Pain has been defined by the IASP (International Association for the Study of Pain) as "An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage." (IASP 1994). Furthermore, the IASP highlighted the fact that if an individual is unable to verbally communicate does not negate the possibility that it is experiencing pain. This note pertains to pain experienced by human infants, non-communicative human patients, and animals.

Pain is unquestionably a sensation in a part or parts of the body, but it is also always unpleasant and therefore also an emotional experience. This definition does not tie pain to a nociceptive stimulus, on the contrary, it embraces the existence of psychological pain. The latter comes from reports of many people experiencing pain in the absence of tissue damage or any likely pathophysiological cause. This points to the fact that nociception and pain are two intertwined but also distinct aspects. Nociception, which is defined by the Kyoto Protocol of IASP Basic Terminology as "the neural process of encoding and processing noxious stimuli" (Loeser and Treede 2008), can be the initiating process leading to pain perception. Nevertheless, as mentioned earlier, nociception is a necessary but not sufficient condition for generating pain.

There are multiple ways to classify the different pain types, but according to the World Health Organization (WHO), the etiologic-, anatomic-, duration-, and pathophysiological-based are the most used classification systems (World Health Organization 2012).

1.1.1 Acute and Chronic pain

Acute pain is generally associated with tissue damage, or the threat of it, and serves the function of altering the animal's behaviour in order to avoid or minimize damage and optimizing the

conditions in which healing can take place. Acute pain varies in its severity from mild to excruciating. It is frequently associated with surgery, trauma, and/or some medical conditions. It is evoked by a specific disease or injury and it is self-limiting (Grimm *et al.* 2015; Portenoy and Ahmed 2018).

Chronic pain is a type of pain which lasts for longer periods and is classically associated with a chronic inflammatory disease or a degenerative condition or following nerve injury or damage. The IASP pragmatically defined chronic pain as pain that persists or recurs for more than three months (IASP Task Force on Taxonomy 1994). It can also represent pain that persists beyond the expected course of an acute disease process. It has no biological purpose and no clear end-point, and evidence from human medicine indicates that it can have a significant impact upon the quality of life of the sufferer. For this reason, chronic pain is considered a disease state. During the lifetime of an individual, animal or human, acute exacerbations of the chronic pain state may occur (breakthrough pain), or new sources of acute pain may occur independently (acute on chronic pain), jeopardizing effective pain management strategies (Grimm *et al.* 2015). Due to the multifaceted aspects of chronic pain and the need for a standardization of different chronic pain states, the International Classification of Diseases (ICD) of the WHO found a rational principle of classification in the following categories: chronic primary pain, chronic cancer pain, chronic post-surgical and post-traumatic pain, chronic neuropathic pain, chronic headache and orofacial pain, chronic visceral pain, and chronic musculoskeletal pain (Treede *et al.* 2015).

1.1.2 Nociceptive, Neuropathic, and Mixed pain

Nociceptive pain originates from tissue injury which activates specific pain receptors called nociceptors. Nociceptors can respond to heat, cold, vibration, stretch stimuli and chemical substances released from tissues in response to oxygen deprivation, tissue disruption or inflammation (inflammatory pain; see Chapter 1.5.1 for further details). Nociceptive pain can

be subdivided into somatic and visceral pain depending on the location of activated nociceptors. Somatic pain is caused by the activation of nociceptors in either surface tissues (skin, mucosa of mouth, nose, urethra, anus, etc.) or deep tissues such as bone, joint, muscle or connective tissue. Visceral pain is caused by the activation of nociceptors located in the viscera, can occur following infection, distension from fluid or gas, and stretching or compression (Portenoy and Ahmed 2018).

The most recent and widely accepted definition of *neuropathic pain* is pain caused by a lesion or disease of the somatosensory system. This system is what allows for the perception of touch, pressure, pain, temperature, position, movement and vibration. In human medicine, common conditions associated with neuropathic pain include postherpetic neuralgia, trigeminal neuralgia, diabetic neuropathy, HIV infection, leprosy, amputation, peripheral nerve injury pain and central post-stroke pain (Colloca *et al.* 2017). The risk of persistent postsurgical pain in animals following surgery has not been quantified; however, seen the similarities between aetiology and pathophysiology of some human and animal diseases, it is likely to occur in some animals with the potential for this to adversely impact on their quality of life (Grimm *et al.* 2015).

In some patients, nociceptive and neuropathic pain can coexist, producing a *mixed* type of pain. For instance, in burns, damage is dealt to both skin (nociceptive somatic pain) and nerves (neuropathic pain). Another example of mixed pain is cancer pain. In cancer, nerves compression and degeneration may be concurrent, leading to a mixed type of pain. Cancer pain syndromes are defined by the relationship to the tumour, pain pathophysiology, temporal features, and quality. In domestic animals, pain secondary to cancer is a key concern and needs to be promptly addressed to alleviate suffering, stress, and anxiety as well as to improve quality of life (Portenoy and Ahmed 2018).

1.2 Pain and stress

Acute and chronic pain are capable of producing a significant stress response in domesticated species. Severe pain has been found to cause profound physiologic effects on the endocrine system, such as initial hyperarousal of the hypothalamic-pituitary-adrenal (HPA) system which is the most important stress control mechanism of the body (Tennant 2013). This biologic response is exhibited by animals when their homeostasis is threatened by stressors, representing an unfavourable and damaging condition for the physical and psychological homeostasis.

If pain persists for too long, the hormonal system becomes unable to cope with the stress of pain, and hormone production may be decreased causing serum hormone levels to drop below normal. The biologic purpose of this system is to produce additional hormones in different organs like the thyroid, adrenals, and gonads, and pour them into the serum as these compounds are needed by for many pain-control functions, such as protection and regeneration of injured tissue, immunologic activity, and metabolic controls (Tennant 2013).

On a situational basis, stress has been demonstrated to either enhance (stress-induced hyperalgesia) or suppress (stress-induced analgesia) pain depending on the severity of pain and the animal's prior experience with stressful, painful, or environmental stimuli. Both these phenomena have been demonstrated in animals (Ahmad and Zakaria 2015). For example, acute stress caused by inescapable holding and chronic stress caused by repeated swim stress induced hyperalgesia in rats (Vidal and Jacob 1982; Quintero *et al.* 2000). On the contrary, different stressors led to stress-induced analgesia in the same species (Abbott *et al.* 1986).

The response to pain is not only represented by changes in the autonomous nervous systems, but also in the mental state, and in the behaviour of the animal (Martini *et al.* 2000; Gaynor and Muir III 2015). For example, a mouse who is exposed to a thermal nociceptive stimulus will change its behaviour towards the environment he is interacting with, in the attempt to escape and foresee the upcoming painful stimulation (Vierck *et al.* 2010).

Stress and pain are two extremely intertwined dimensions sharing several afferent and efferent pathways and continuously communicating with each other. Another example for this is that nocireceptive spinal cells project to the limbic system (*e.g.* the amygdala) other than the abovementioned hypothalamus, both of which are important components of central stress circuits, acting via brain stem nuclei to modulate reflex responses (Chapman *et al.* 2008). Despite the extreme complexity and multifaceted nature of both pain and stress, several authors concur upon the fact that pain has to be considered a stressor in animals (Vierck et *al.* 2010).

1.3 Pain physiopathology

The most classic and thoroughly described pain pathway consists of a three-neuron chain that transmits pain signals from the periphery to the cerebral cortex (see Figure 1). The first-order neuron resides in the dorsal root ganglion and has two axons, one that projects distally to the tissue it innervates and the other that extends centrally to the dorsal horn (DH) of the spinal cord. At this level, this axon synapses with the second-order neuron, the axon of which crosses the spinal cord ascending through the spinothalamic tract to the thalamus. In the thalamus, it synapses with the third-order neuron, which projects to the cerebral cortex, where information is somatotopically organized (Cross 1994).

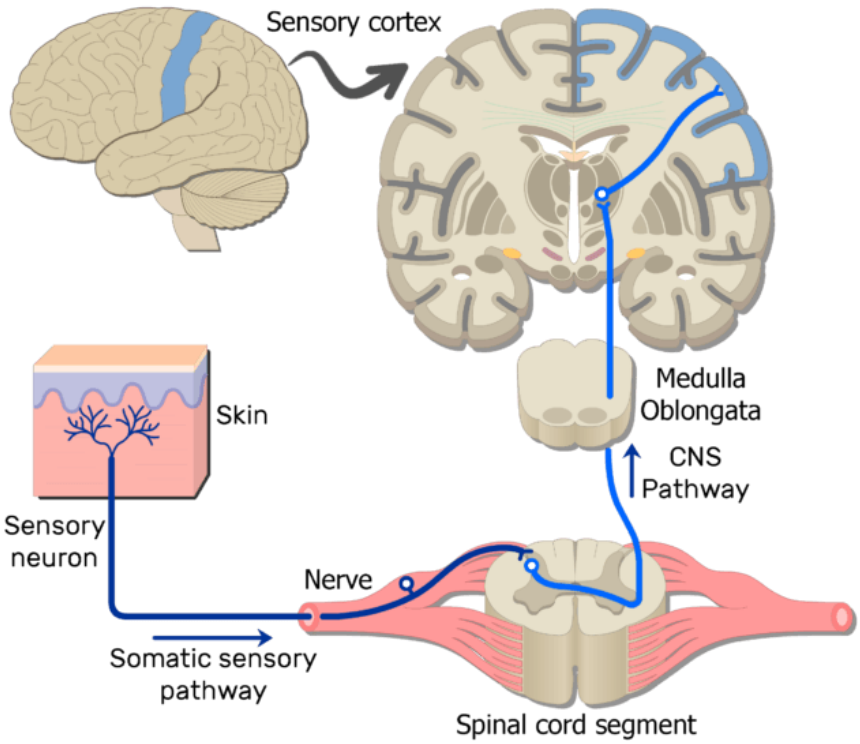

Figure 1. Ascending pain pathway

Visual display of the ascending pain pathway and the interconnections between neurons from the periphery to the cerebral cortex (source: www.getbodysmart.com).

The abovementioned process of transmitting and elaborating of painful stimuli is extremely complex and intricate, but can be summarized in the following steps: *transduction, transmission, modulation, perception.*

1.3.1 Transduction

Nociceptive transduction refers to the process by which external stimuli are converted to electrical signals that can be perceived as pain. It is a fundamental part of the process of detecting external stimuli. In fact, without transduction, the body would lose the ability to taste, touch, hear, see, and feel pain. Transduction takes place when, in response to a stimulus, somatosensory processes facilitate the opening of ion-gated channels, transforming the stimulus into an electrochemical signal that can be delivered to and perceived by higher-order nervous centres (McEntire *et al.* 2016). A visual representation of the process is provided in Figure 2.

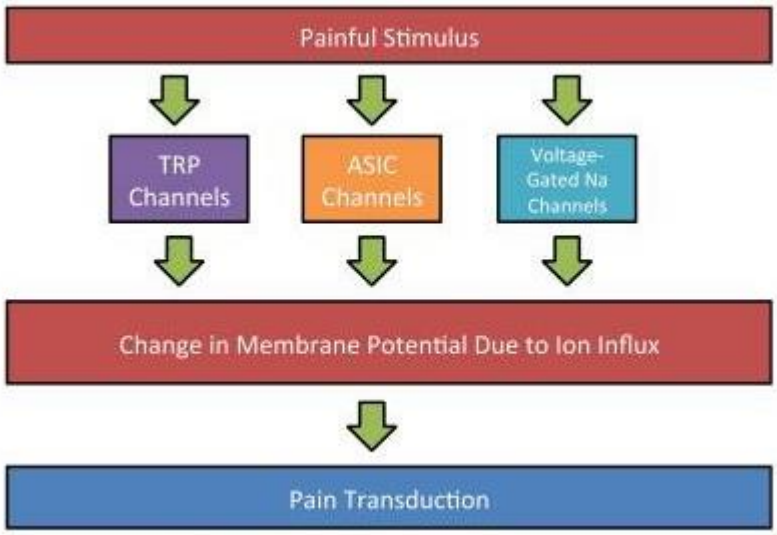

Figure 2. Nociceptive transduction

Visual display of the transduction process by which a painful stimulus is transformed (via specialized ion channels like the ones displayed in the figure) into a signal that can be carried (via transmission) to the central nervous system and perceived as pain [source: McEntire *et al.* (2016)].

There are several types of transduction pathways which involve different ions. In fact, the opening of these leads to changes to membrane potential, the opening of additional channels, and the eventual depolarization of the afferent nerve, which generates a nociceptive signal. Amongst these channels, the most clinically relevant ones are the followings: *acid-sensing ion channels (ASIC), transient receptor potential (TRP) cation channels*, and *Voltage-gated sodium (Na^+) channels*.

ASICs are non-voltage sensitive, proton-induced sodium channels found throughout the body (Wemmie *et al.* 2006). Their function is to detect changes in pH and they have been associated with various disease processes both in the central and peripheral nervous system such as epilepsy, depression, migraines, and neuropathic pain. The exact mechanism and role of this channel family are yet to be fully elucidated, but their importance in nociception is becoming more and more apparent (McEntire *et al.* 2016).

TRP channels are a broad family of channels that are involved not only in nociception but also in a plethora of physiological processes. In the nociceptive process, only some TRPs seem to

be involved, and these are the TRPV1, TRPA1, TRPV3, and TRPM8 channel. TRPV1 channels allow for the passage of calcium ions, playing a key role in nociceptive transduction. They are activated by heat, acidity, and molecules such as capsaicin (Caterina *et al.* 1997; Clapham 2003; Macpherson *et al.* 2007). Like TRPV1 channels, TRPA1 channels are sensitive to thermal, mechanical, and chemical stimuli (Macpherson *et al.* 2007). TRPV3s share different analogies with TRPV1, but do not react to capsaicin and acidic pH (Smith *et al.* 2002). TRPV3 channels respond to heat and are thought to participate in the transduction of warm sensation and heat pain. Also, they tend to be activated at a lower temperature (T< 33°C) than TRPV1 channels (T<44°C) (Peier *et al.* 2002; Smith *et al.* 2002). Ultimately, TRPM8 channels are the principal receptors accountable for environmental cold sensation and cold pain transduction (Clapham 2003).

Voltage-gated Na^+ channels are heavily involved in the transition from transduction to transmission and the generation of action potentials. In particular, nociceptive transduction mediated by TRP, ASIC, and other channels depolarize Na^+ channels leading to the formation of an action potential (McEntire *et al.* 2016).

1.3.2 Transmission

Transmission is the process that allows for the electrical signal to be transported towards higher centres and is made possible by afferent nerve fibres. These fibres have cell bodies in either the dorsal root ganglia or trigeminal ganglion and terminate in the DH of the spinal cord. Even though all the pain fibres terminate in the DH, their route to this end-point can vary. Most of them enter the DH at the level of the ventrolateral bundle of the dorsal root, however, 30% of the C fibres enter the spinal cord via the ventral root. After they have entered the spinal cord, the nerve roots can bifurcate into ascending and descending branches, which can enter the DH one or two segments higher or lower than the segment of origin (Steeds 2009).

The most important afferent nerve fibres are C, A-δ, and A-β fibres and are described here below:

C fibres have a small diameter; are unmyelinated and conduct impulses at the slow rate of 0.5-2 m s^{-1}. They have smaller receptive fields than the A-δ nociceptors and mostly terminate in lamina I, II, and V of the spinal DH (Zeilhofer 2005) (See Figure 3). Their activation results in a prolonged sensation of dull and burning pain. Most C fibres are activated by high-threshold mechanical, chemical stimuli, and by heat (39-41°C). Furthermore, they are heavily influenced by both the phenomena of sensitization (enhanced response to a lasting/repetitive stimulus of same intensity) and fatigue (reduced response to a lasting/repetitive stimulus of same intensity) (Vardeh and Naranjo 2017).

A-δ fibres are sensitive to thermal and high-threshold mechanical stimuli. They respond with higher discharge frequencies than C fibres and the discriminable information they provide to the central nervous system (CNS) is greater. Most of these fibres have polymodal properties (with high heat threshold at 40-50°C), are thinly myelinated (conduction velocity between 5 and 35 m s^{-1}) and terminate in lamina I and V of the DH (Zeilhofer 2005). Activation of these fibres results in a short sensation of sharp and pricking pain (Vardeh and Naranjo 2017).

A-β fibres are myelinated fibres (conduction velocity 35-75 m s^{-1}) and the fastest among the abovementioned. They terminate in lamina III, IV, and V, and play a major role in encoding muscle spindle information and vibration, therefore typically conducting innocuous mechanical stimuli. Nevertheless, some A-β fibres encode stimulus intensities in the noxious range and in some cases respond to noxious heating of the skin. A-β fibres are of major importance in mediating allodynia in states of central sensitization (refer to subchapter 1.3.3) (Vardeh and Naranjo 2017).

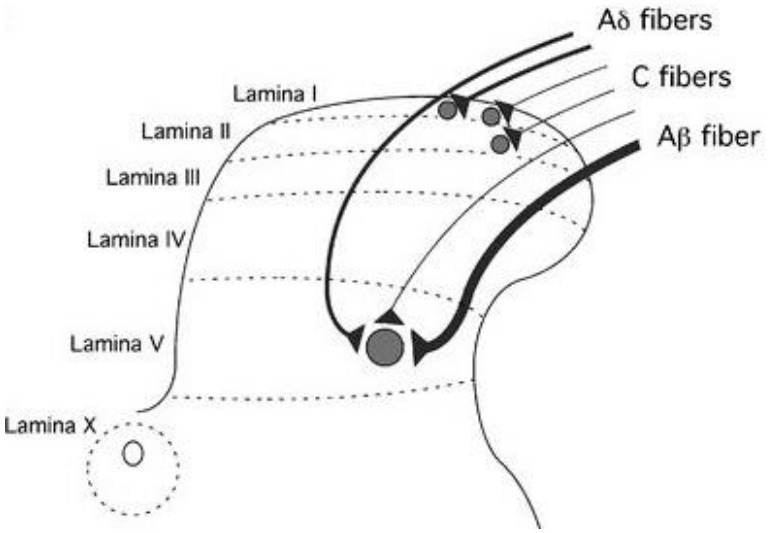

Figure 3. Afferent nerve fibres

Display of the innervation of the dorsal horn by primary afferent nerve fibres. C fibres terminate in laminae I, II, and V; A-δ fibres in laminae I and V; and A-β fibres in laminae III, IV, and V [Zeilhofer (2005)].

The nociceptive pathways that carry information to the brain are extremely complex and, at a certain degree, poorly understood. The *spinothalamic tract* carries sensory information and noxious stimuli from the periphery to the thalamus and is therefore of the utmost importance among the central pain pathways. Other important pathways are the *trigeminal pain and temperature systems* that carry information from the face to the thalamus (See Figure 4).

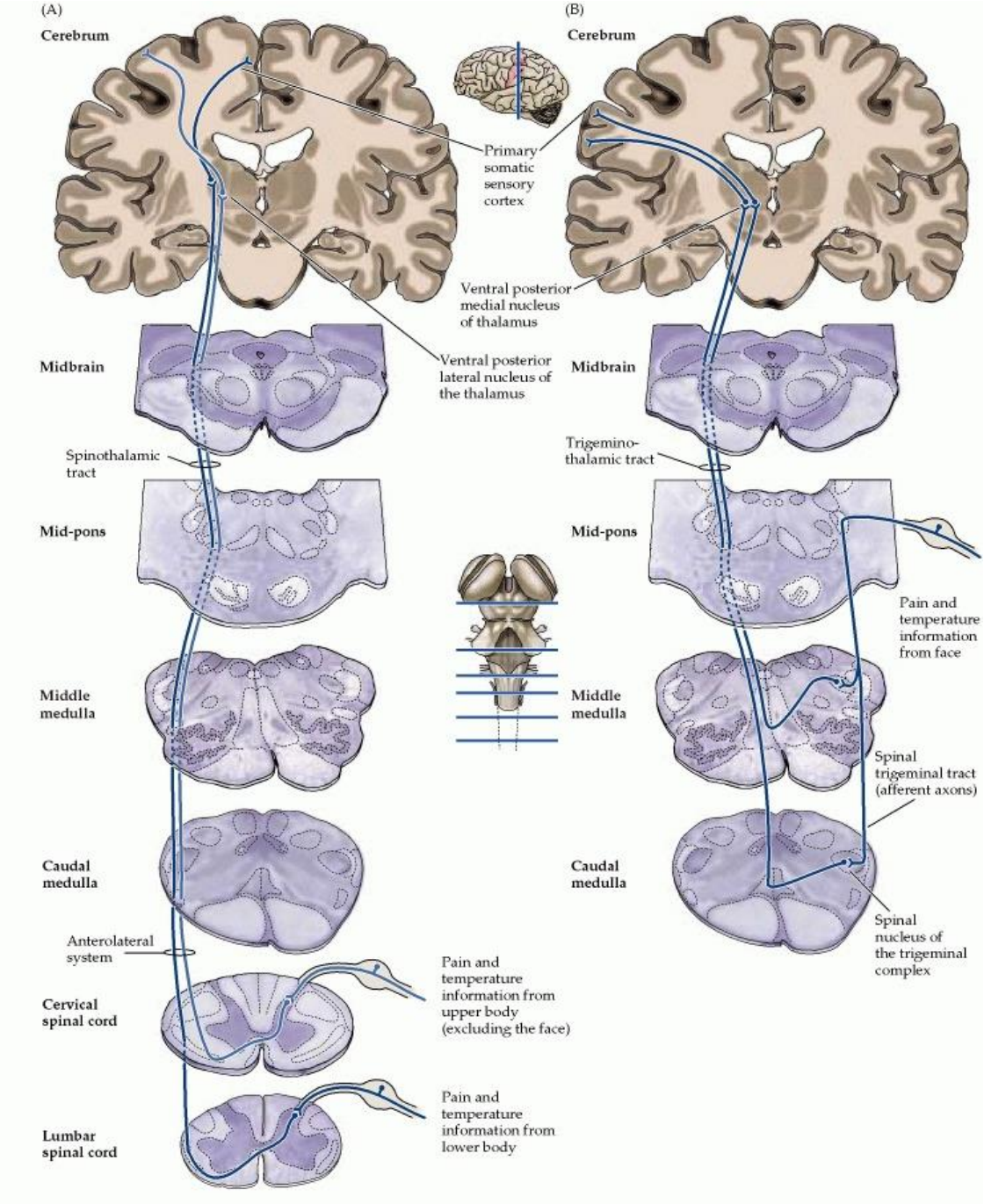

Figure 4. Spinothalamic and trigeminal pathways

Visual display of the ascending pathways for pain. (A) The spinothalamic system. (B) The trigeminal pain system and the temperature ascending pathway [Purves *et al.* (2011)].

There are several types of neurotransmitters that mediate pain. The most known ones up to date are described here below and in Table 1 as reported by Yam *et al.* (2018).

One of the most abundant species of neurotransmitters are *tachykinins* (*e.g.* Substance P, neurokinin A and B). These neuropeptides are produced from peripheral terminals of the

sensory nerve fibres and bind to their cognate receptors, which are G-protein coupled receptors. Tachykinins are important mediators of neurogenic-induced inflammation.

Calcitonin gene-related peptide (CGRP) is a neuropeptide widely produced in both the CNS and PNS; however, it is primarily located in the primary afferent nerves. In fact, it is mostly found at the level of the DH of the spinal cord and is associated with conduction of noxious stimulation.

Bradykinin is a well-known algogen and acts as one of the inflammatory mediators that are locally produced from the breakdown of kininogens in the site of the inflamed tissue. It binds to G-protein coupled receptors in the nociceptive afferent fibres and leads to sensitization.

Cytokines (*e.g.* serotonin, histamine, interleukin1β, tumour necrosis factor α) are produced during inflammation by degranulation of mast cells. This process leads to the release of platelet-activating factor which in turn causes serotonin (5-HT) to be produced by circulating platelets. 5-HT receptors are located on the nerve cells membrane and comprise mostly G-protein coupled receptors with the exception of the 5-HT_3 receptor which is a ligand-gated ion channel. Interleukin 1β and tumour necrosis factor α play a crucial role in exerting a pro-inflammatory effect leading to hyperalgesia.

Prostaglandins are produced by the cyclooxygenase pathway from the arachidonic acid. They can be found in several tissues and are considered as an archetypal sensitizing agent that reduces the nociceptive threshold. Prostaglandins enhance the effects of other mediators, such as 5-HT and bradykinins, as well as augments the release of neuropeptides. Also, the increase of the bradykinin causes prostaglandins to be released resulting in a "self-sensitizing" pathway.

Leukotriene B_4 is one of the eicosanoid inflammatory mediators produced by leukocytes from the arachidonic acid by means of lipoxygenase and subsequently leukotriene B_4 hydrolase. Leukotriene B_4 is responsible for recruiting neutrophils towards the site of the damaged tissue, whilst simultaneously promoting the production of cytokines. Leukotriene B_4 can cause

sensitization of the nociceptors by increasing the cyclic adenosine monophosphate (cAMP)/protein kinase A activities.

Protons are high in number in the injury site, this causes the pH of the lesion to be acidic. This stimulates ASICs channels which, as described in chapter 1.3.1, lead to increase of Na^+ influx at the level of the membrane of afferent fibres, thus generating an action potential and sensitization of the nerves.

Adenosine triphosphate (ATP) is released locally by the damaged tissues and directly stimulates its receptors. ATP is metabolized by ectonucleotidases into adenosine and binds to ionotropic purinergic receptors which are located at the peripheral site of the sensory neurons and centrally on the second-order neurons in the DH. When such receptors are activated, Na^+ crosses them inducing membrane depolarization and activating various Ca^{2+}-sensitive intracellular processes, leading both to pain and hyperalgesia. On the other hand, ATP produces a by-product from its metabolism, adenosine, which can have both an excitatory and inhibitory effect depending on which receptor it binds with.

The *nerve growth factor* is a well-known mediator for persistent pain. It is locally released at the site of injury by fibroblasts rapidly inducing mechanical and thermal hyperalgesia by activation of tropomyosin receptor kinase A. This receptor is widely expressed at the level of primary afferent neurons, suggesting its primary role in peripheral sensitization. Furthermore, because non-neuronal cells (*e.g.* keratinocytes, mast cells and circulating eosinophils) are also capable of expressing tropomyosin receptor kinase A, the nerve growth factor can lead to the enhancement of inflammatory signals, like production of 5-HT, histamine, and other nerve growth factor by positive feedback.

Glutamate is considered the most abundant excitatory neurotransmitters in vertebrates. It is present both at the periphery and the upper cores of the nervous system, being involved in more than 50% of the brain synapses. The receptor of glutamate is N-methyl-d-aspartate receptor, an

ion channel that requires a specific combination of events in order to be activated, and results in the increase of the intracellular Ca^{2+}, leading to central sensitization.

γ-aminobutyric acid (GABA) is, as opposed to glutamate, the most widely present inhibitory neurotransmitter in the mammalian CNS. It contributes to up to 40% of the brain synapses and can be found in the interneurons of the spinal cord, neocortex, and cerebellum. GABA is produced by GABAergic neurons and binds to GABA-receptor: $GABA_A$ and $GABA_B$. The inhibitory effect mediated by these receptors is achieved through different means. $GABA_A$ receptors cause an influx of Cl^- ions, leading to depolarization of the nervous fibre, whereas GABA receptors are inhibitory G-protein coupled receptors and cause inhibition towards the formation of cAMP.

Opioids are peptides that bind to specific receptors, such as μ-, δ-, and κ-opioid receptors. All of these receptors are inhibitory G-protein coupled receptors and therefore inhibit the formation of cAMP. These receptors are widely distributed in both the primary afferent neurons and the dendrites of postsynaptic neurons. These are two endogenous opioid peptides which are released into the interneurons of the DH: enkephalin and dynorphin. They inhibit the release of neurotransmitters from the afferent terminals, leading to a stop in the propagation of the signal.

Cannabinoids (*e.g.* tetrahydrocannabinol) are one of the major psychoactive components isolated from *Cannabis sativa*. They can bind to inhibitory G-protein coupled cannabinoid type 1 receptors, which are highly expressed in the pre- and post-synaptic level in the brain and spinal cord. They inhibit the formation of intracellular cAMP and consequently the propagation of noxious signals, and can prevent mast cells degranulation and the release of pro-inflammatory mediators, leading to a further reduction in pain sensation.

Norepinephrine is the principal neurotransmitter of the adrenergic pathways and is synthesized from phenylalanine in the nerve terminals. It binds to different stimulatory and inhibitory α- and β-receptors both at the level of pre- and post-synaptic neurons.

Table 1. Neurotransmitters

List of neurotransmitters that modulate nociception at the level of the central nervous system (CNS) and peripheral nervous system (PNS) [adapted from Yam *et al*. (2018)].

Neurotransmitter	**Location**	**Receptor**	**Effect**
Tachykinins	CNS and PNS	NK_1, NK_2, NK_3	Excitatory
CGRP	CNS and PNS	CALCRL	Excitatory
Bradykinin	CNS and PNS	B_1, B_2	Excitatory
Cytokines	CNS and PNS	H_1, 5-HT_{2A}, 5-HT_3	Excitatory
Prostaglandins	CNS and PNS	EP_1, EP_2, EP_3, EP_4	Excitatory/Inhibitory
Leukotriene B_4	PNS	LTB4-R_1, LTB4-R_2	Excitatory/Inhibitory
Protons	CNS and PNS	ASIC, VR1	Excitatory
ATP/Adenosine	CNS and PNS	$P2X_3$, A_1, A_2	Excitatory/Inhibitory
Nerve growth factor	CNS and PNS	TrkA	Excitatory
Glutamate	CNS and PNS	AMPA-R, NMDA-R,	Excitatory
GABA	CNS and PNS	$GABA_A$, $GABA_B$	Inhibitory
Opioids	CNS and PNS	μ, k, δ	Inhibitory
Cannabinoids	CNS and PNS	CB_1, CB_2	Inhibitory
Norepinephrine	CNS and PNS	α-1, α-2, β	Excitatory/Inhibitory

NK_1: neurokinin type 1 receptor; NK_2: neurokinin type 2 receptor; NK_3: neurokinin type 3 receptor; CGRP: Calcitonin gene-related peptide; CALCRL: calcitonin receptor-like receptor; B_1: bradykinin receptor type B_1; B_2: bradykinin receptor type B_2; H_1: histamine; 5-HT_{2A}: 5-hydroxytryptamine type 2A receptor; 5-HT_3: 5-hydroxytryptamine type 3 receptor; EP_1: prostaglandin E_2 receptor type 1; EP_2: prostaglandin E_2 receptor type 2; EP_3: prostaglandin E_2 receptor type 3; EP_4: prostaglandin E_2 receptor type 4; LTB_4-R_1: leukotriene B_4 type 1 receptor; LTB_4-R_2: leukotriene B_4 type 2 receptor; ASIC: acid-sensing ion channels; VR_1: vanilloid receptor for capsaicin; $P2X_3$: purino receptor; A_1: adenosine type 1 receptor; A_2: adenosine type 2 receptor; TrkA: tropomyosin receptor kinase A; AMPA-R: amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors; NMDA-R: N-methyl-D-aspartate receptors; $GABA_A$: γ-aminobutyric acid type A receptor; $GABA_B$: γ-aminobutyric acid type B receptor; μ: μ-opioid receptor, k: k-opioid receptor; δ: δ-opioid receptor; CB_1: cannabinoid type 1 receptors; CB_2: cannabinoid type 2 receptors; α-1: alpha-1 adrenergic receptor, α-2: alpha-1 adrenergic receptor, β: beta-adrenergic receptors.

1.3.3 Modulation

Pain modulation refers to the process by which the body alters a pain signal as it is transmitted along the pain pathway and explains the difference between individual responses to the same noxious stimulus. Modulation process can also explain why the activation of pain neurons do not always coincide with the sensory experience of pain. Furthermore, and most importantly, pain modulation elucidates the mechanisms of action underlying clinical analgesia (Kirkpatrick *et al.* 2015).

One of the first theories on pain modulation is the *gate control theory* and was proposed by Wall and Melzack in 1965. This theory suggests that nociceptive and non-nociceptive stimuli converge together into the *substantia gelatinosa,* in the spinal cord. Here, if nociceptive stimuli outweigh the non-nociceptive ones, the individual will experience pain. This theory also suggests that larger nerves carry non-nociceptive information whereas smaller ones carry nociceptive information (Melzack and Wall 1965). In fact, large afferent fibres excite *substantia gelatinosa* cells and elicit presynaptic inhibition, whereas small afferent fibres inhibit *substantia gelatinosa* cells removing presynaptic inhibition and eliciting presynaptic facilitation (Mendell 2014). This theory has been refined through the years and it is considered a powerful the scientific rationale behind the instinctive response to painful stimuli witnessed when individuals rub the affected area after having injured themselves (Kirkpatrick *et al.* 2015).

A fundamental means of inhibition or enhancement of painful stimuli is what is called *descending modulation*. This pathway involves several CNS regions either directly or indirectly, like prefrontal, anterior cingulate, and insular cortices, amygdala, periventricular and posterolateral hypothalamus, periaqueductal grey, dorsolateral pons, and rostroventral medulla. These areas exert influences on the perception of pain by inhibition or facilitation of the transmission of nociceptive inputs at the level of the DH. These effects are mediated by descending monoaminergic pathways that utilize serotonin, norepinephrine, or dopamine.

Endogenous opioids (*e.g.* enkephalins) are important modulators of pain processing in the spinal cord dorsal horn, but the cell types involved in their effects are yet unclear. One type of these cells has proven to be the DH interneuron. In fact, these cells mediate their effects on mechanical pain thresholds by inhibiting presynaptic transmitter release from primary sensory afferents. Nevertheless, these interneurons seem to be connected to the rostroventromedial medulla, which suggests a pathway through which neurons coming from the upper centres may inhibit the antinociceptive actions of spinal enkephalinergic interneurons. Both these inhibitory and facilitatory circuits were proven true by recording interneuronal activity in mice undergoing acute and chronic stress. Acute stress resulted in augmented activity from spinal enkephalinergic interneurons whereas chronic stress led to the opposite (Whalley 2017).

Other pain modulation effectors are *on* and *off-cells*. These cells have been identified by Fields *et al.* (1983) in the rostroventral medulla as descending pain modulatory neurons. Off-cells are in fact excited by opioids and inhibited by noxious stimuli. On-cells have the opposite response of off-cells. Off-cells inhibit ascending noxious stimuli from the periphery by triggering descending inhibition and on-cells are thought to trigger descending facilitation (Kwon *et al.* 2014). On-cells are thought to critically contribute to descending pain control by facilitating nociception, probably via glutamatergic neurotransmission and the excitation of primary afferent terminals and/or excitatory DH neurons (Heinricher *et al.* 2009).

Facilitation of noxious stimuli can take place through several mechanisms that can ultimately lead to different consequences, such as *allodynia* and *hyperalgesia*. *Allodynia* is referred to as "pain caused by a stimulus that does not usually provoke pain" and *hyperalgesia* is "increased pain from a stimulus that usually provokes pain" (Jensen and Finnerup 2014) (see Figure 5). Both these pain modulatory effects are often due to changes in neuronal expression of receptors, especially following injury or nerve damage.

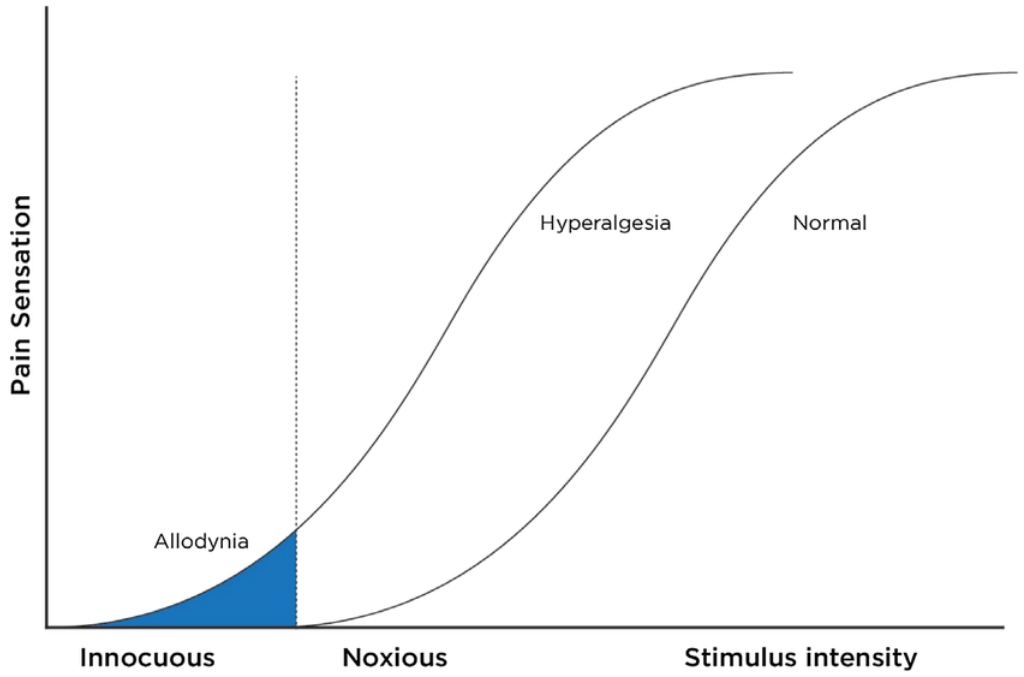

Figure 5. Allodynia and hyperalgesia

Graphical representation of allodynia and hyperalgesia phenomena. As shown, allodynia is elicited when non-noxious stimuli are applied, whereas hyperalgesia corresponds to noxious stimuli causing a greater pain sensation than the same stimulus normally would [source: Louw *et al.* (2017)].

Peripheral sensitization is that process caused by the repetitive exposure to noxious stimuli which causes the membrane depolarization along with Ca^{2+} influx, which in turn induces neurotransmitters release at the site of the injury and activates the surrounding nociceptors. This leads to a decrease in threshold to stimulation, as well as an increase of firing rate due to the enhanced sensitivity of primary afferent nociceptors. All these neuromodulators bind to their respective cognate receptors and subsequently augment the pain sensation.

Another type of sensitization is *central sensitization* which is the repetitive stimulation of the nociceptors that causes amplification in the nociceptive information, leading to the excitability of the projection neurons within the DH of the spinal cord (Yam *et al.* 2018).

1.3.4 Perception

Nociceptive pathways terminate in subdivisions of thalamic nuclei known as the ventral posterior lateral nucleus and the ventromedial nucleus (Willis and Westlund 1997). From these

nuclei, the information transferred to various cortical and subcortical regions, such as the amygdala, hypothalamus, periaqueductal grey, basal ganglia, and different regions of the cerebral cortex. One of the most important brain regions that are activated upon noxious stimulation and are associated with the subjective experience of pain is the insula and anterior cingulate cortex (Tracey and Mantyh 2007). All these integrated thalamo-cortical and cortico-limbic structures have been termed the pain *neuromatrix* (Melzack 1999).

Pain perception involves a number of psychological processes, including *attentional orienting* to the painful sensation and its source, *cognitive appraisal* of the meaning of the sensation, and the subsequent *emotional, psychophysiological, and behavioural reaction*.

Attentional modulation of pain experience correlates with changes in activation of the pain neuromatrix. In fact, a distraction from pain reduces activations in several brain regions. In contrast, attentional hypervigilance for pain leads to hyperalgesia and allodynia (Garland 2012).

Cognitive appraisal of pain is that process in which an individual consciously or unconsciously evaluates the meaning of sensory signals coming from the body to determine the extent to which they signify the presence of actual/potential harm. The manner in which the information is appraised influences whether it is experienced as unpleasant pain or not. For example, keeping constant the stimulus intensity, cognitive appraisal of that information can lead to altered activation of the anterior cingulate cortex, depending on whether the individual thinks he is able to cope with that stimulus or not. In fact, ventrolateral prefrontal cortex activation is positively associated with the extent to which pain is viewed as controllable and negatively correlated with subjective pain intensity (Garland 2012).

Pain is known to elicit a powerful emotional reaction that results in modulation of pain perception. Feelings of anger, sadness, and fear depend on how the pain is cognitively appraised. These emotions are associated with autonomic, endocrine, and immune responses which may amplify pain through a number of psychophysiological pathways. While stress and

negative emotions may temporarily dampen pain via norepinephrine release, a prolonged "fight or flight" response can increase blood flow to the muscle and increase muscle tension, aggravating the original injury. Negative emotions and stress elicit activation in the amygdala, anterior cingulate cortex, and anterior insula other than leading to an impairment of the prefrontal cortex function, all of which increases attention toward pain, intensifies pain unpleasantness, and may reduce the ability to regulate pain using higher order cognitive strategies like reappraisal or viewing the pain as controllable and surmountable (Arnsten 2009; Lawrence *et al.* 2011). Thus, anger, sadness, and fear may result from acute or chronic pain and in turn feedback into the bio-behavioural processes that influence pain perception to exacerbate anguish and suffering (Garland 2012).

1.4 Animal pain

In 1991, Bateson set out a basis upon which a wide range of researchers investigated the capacity for pain in animals. He stated several criteria for pain perception in different animals and it was hypothesized that animals that fulfilled all criteria should be capable of perceiving pain. The abovementioned criteria are the followings: possession of nociceptors, pathways from nociceptors to the brain, brain structures analogous to the human cerebral cortex that process pain, opioid receptors and endogenous opioid substances in a nociceptive neural system, a reduction in adverse behavioural and physiological effects after administration of analgesics or painkillers, learning to avoid potentially painful stimuli and that this learning is rapid and inelastic. Later on, in 2004, Sneddon added that animals undergoing pain should also suspend normal behaviour for a prolonged period and not only show a reflex response. It is with few doubts that a lot of researchers in the last decades affirmed that animals could perceive pain, and in 2011, Allen firmly concluded that it was "beyond a reasonable doubt that several animals belonging to different orders feel pain".

The aversive experience associated with pain is considered an important driver in ensuring that animals survive in a dangerous habitat, avoiding injury that may otherwise lead to ill health and mortality. Therefore, pain should not be considered a special property of humans, but it is likely that pain, and its associated motivational state, has an adaptive survival function for animals. It is believed that pain has an important protective and evolutionary function that also humans have (Rutherford 2002; Sneddon *et al.* 2014).

1.4.1 Animal pain assessment

Assessing how humans feel when they are experiencing pain can be challenging, but verbal assessment helps to address this issue quite well. The real challenge comes when non-verbalizing patients (*e.g.* neonates or verbally impaired individuals) have to be assessed for pain. A similar situation occurs when pain has to be assessed in animals, a task that can prove to be even more challenging since animals do not even share our language.

Over the last decades, societal concern has grown with regards to animal welfare. In fact, many laws have tried to address the important concerns of slaughter, housing, and transport of animals. As an example, in 1992 the Farm Animal Welfare Council defined the concept of welfare in animals as reported here below:

"*Welfare of animals includes both physical and mental aspects and implies that animals should be free from pain, injury or disease*"

To be alleviated, pain first must be identified and assessed, and this is very hard to do when a species tries its best to hide any pain sign. This kind of behaviour is seen in prey animals like farm animals (*e.g.* cattle, horse, sheep, etc.). A sick or injured animal is more prone to predation, and hiding signs of pain is none other than a survival strategy (Underwood 2002).

A pain assessment tool, in order to be efficient, needs to be characterized by important properties, which have been identified and refined over the 20th century (Dubner 1985). These properties include that the tool should be: sensitive to changes in stimulus intensity, sensitive

to changes throughout the range from threshold to tolerance, sensitive to manipulations that alter sensory-discriminative capacities of the subject, free from subject and experimenter bias, able to separate intensity from quality of pain, and characterized by absolute rather than relative scales to allow use across both time and individuals (Keefe *et al.* 1991).

The most used methods for pain assessment are divided into three main categories: *behavioural assessment*, *physiological assessment*, and *nociceptive threshold testing*.

1.4.2 Behavioural assessment

The behavioural expression of nociception varies depending upon the evolutionary advancement of the animal under study, the type of pain stimulus used, and any endogenous or exogenous factors that might modulate the pain response (Kavaliers 1988). Over the years numerous pain measuring systems have been developed, but very often, observational systems were developed for a single study and the psychometric properties of the system left unassessed. This led to consistent differences in results from very similar procedures.

One of the most reliable systems for pain assessment in animals is the psychometric approach. This system requires that instruments demonstrate the psychometric properties of *validity*, *reliability* and, *responsiveness to change*, before being adopted for clinical use.

Validity is the most fundamental attribute of an instrument because it provides evidence that the instrument is able to measure what it was designed to measure. It comprises three sub-properties (*e.g.* criterion, content validity, and construct). Criterion validity is the comparison of the new tool with some existing gold standard method; however, in the case of animal pain no such item exists, therefore, other forms of validity must be sought. Content validity focuses on the appropriateness and completeness of the items that characterize the system and is deemed to be present when those items cover all the relevant aspects being measured without including any extraneous features. Construct validity is demonstrated when hypotheses regarding the attribute in question are supported by the use of the instrument.

Reliability is a measure of whether an instrument can measure accurately and repeatedly what it is intended to measure, so that measurements of individuals on different occasions when their condition is unlikely to change or made by different observers at the same time, produce the same or similar results.

Responsiveness is the property that ensures that the instrument is sensitive enough to detect differences in health status that are not only statistically important but are also important to the clinician or to the patient.

In addition to possessing the abovementioned properties, a good clinical instrument must also be practical and easy to use and interpret (Reid *et al.* 2013).

Traditionally, pain assessment in veterinary medicine has relied on the use of *unidimensional scales* like the *simple descriptive scale (SDS),* the *numerical rating scale (NRS),* and the *visual analogue scale (VAS)* (see Figure 6).

The SDS assigns descriptors to the varying severities of pain such as none, mild, moderate, and severe. These descriptors are often assigned a number that is used for calculating the animal's pain score.

The NRS consists of multiple categories (*e.g.* 1 to 4 or 1 to 10) that may list various descriptors (*e.g.* no pain, mild pain, moderate pain, severe pain, very severe pain) about each category in an attempt to quantify a gradual increase in pain intensity. Unfortunately, NRS instruments are not weighted (*i.e.* each descriptor is treated equally) or robust (*i.e.* they do not apply to all circumstances).

The VAS is one of the most extensively used subjective pain scoring systems both in human and veterinary medicine. Both numeric and non-numeric versions of VAS exist. This scale consists of a horizontal line measuring 100 mm in length with a vertical line border at both ends. Identifiers are usually present at the left and right borders (*e.g.* no pain, worst pain possible). Occasionally VAS scales may have descriptors preplaced along the horizontal line,

but this practice is not recommended because it seemed to introduce bias. The VAS does not provide an objective quantitation of pain, making it difficult to compare the severity of pain and response to therapy among animals (Gaynor and Muir III 2015).

Figure 6. Unidimensional Pain Scales

Example of unidimensional pain scales; (top) simple descriptive scale; (middle) numerical rating scale; (bottom) visual analogue scale (source: http://www.animalpain.com.br).

Seen the multidimensional characteristics of pain, a scale which measures just an aspect of this complexity could be limiting. An attempt to address this problem was made by researchers over the years and resulted in the creation of *multidimensional pain scales*. These scales may incorporate both physiologic and behavioural parameters into several categories and the assessor has to assign a descriptor that approximates the animal's behaviour in each category. The value for the descriptor is added to the animal's total pain score (Gaynor and Muir III 2015). An example of multidimensional pain scale is the famous *Glasgow Composite Measure Pain Score (GCMPS)* for the assessment of post-operative pain in dogs designed by Holton *et al.* in 2001. This scale comprises seven behavioural categories with associated descriptors

which include 47 words, for each category, selected from a collection of 279 words to describe pain behaviours. This pain scale proved to be so useful in pain management in dogs that a few years later, Reid *et al.* (2007) decided to create a short and easy-to-use version of it (see Figure 7). Multidimensional pain scales were also designed for other animal species, such as horse, cattle, cat, and other animal species and are considered fundamental tools for pain assessment and management, nowadays (Sotocinal *et al.* 2011; de Oliveira *et al.* 2014; Dalla Costa *et al.* 2014; Reid *et al.* 2017).

The user of both unidimensional and multidimensional pain scales has to record a score for pain intensity that is purely subjective in nature and can lead to possible biases. In fact, factors such as age, gender, personal health and clinical experience can heavily influence the observer's judgement. The use of a proxy inevitably introduces some degree of inter-observer variability, limiting the reliability of the resulting scores. Importantly, studies on chronic pain have pointed out the importance of the owner, rather than a practitioner, being the proxy, since subtle behavioural alterations may only be apparent outside a clinical setting and changes in behaviour may appear only to someone familiar with the animal. Using the owner as a proxy also has the advantage of reducing the inter-observer variability (Reid *et al.* 2013).

SHORT FORM OF THE GLASGOW COMPOSITE PAIN SCALE

Dog's name _____

Hospital Number _____ **Date** / / **Time**

Surgery Yes/No (delete as appropriate)

Procedure or Condition_____

_____

In the sections below please circle the appropriate score in each list and sum these to give the total score.

A. Look at dog in Kennel

Is the dog?

(i)		(ii)	
Quiet	0	Ignoring any wound or painful area	0
Crying or whimpering	1	Looking at wound or painful area	1
Groaning	2	Licking wound or painful area	2
Screaming	3	Rubbing wound or painful area	3
		Chewing wound or painful area	4

In the case of spinal, pelvic or multiple limb fractures, or where assistance is required to aid locomotion do not carry out section **B** and proceed to **C**
Please tick if this is the case ☐ then proceed to C.

B. Put lead on dog and lead out of the kennel.

When the dog rises/walks is it?

(iii)	
Normal	0
Lame	1
Slow or reluctant	2
Stiff	3
It refuses to move	4

C. If it has a wound or painful area including abdomen, apply gentle pressure 2 inches round the site.

Does it?

(iv)	
Do nothing	0
Look round	1
Flinch	2
Growl or guard area	3
Snap	4
Cry	5

D. Overall

Is the dog? *(v)*		*Is the dog?* *(vi)*	
Happy and content or happy and bouncy	0	Comfortable	0
Quiet	1	Unsettled	1
Indifferent or non-responsive to surroundings	2	Restless	2
Nervous or anxious or fearful	3	Hunched or tense	3
Depressed or non-responsive to stimulation	4	Rigid	4

Total Score (i+ii+iii+iv+v+vi) = _____

Figure 7. Short Form of the Glasgow Composite Pain Scale

The easy- and rapid-to-use version of the Glasgow Composite Pain Scale for post-operative pain assessment in the dog designed by Reid *et al.* (2007).

An innovative approach for pain detection was developed in the last years and involves the concept that animals can show pain through facial expressions. The use of facial expressions as mean for pain assessment had already been used in human medicine, especially on non-verbalizing patients like new-borns and proved to be so sensitive and useful that some authors

consider it as a “gold-standard” in such type of patients and are now included in the majority of multiple composite observational pain assessment scales (Desrosiers *et al.* 2015). The first pain scale ever designed in veterinary medicine is the *Mouse Grimace Scale (MGS)* (Langford *et al.* 2010). The MGS has proved particularly useful in the laboratory setting and started the development of similar facial expression scales intended to detect pain in other animal species like the rat, rabbit, cat, horse (Sotocinal *et al.* 2011; Holden *et al.* 2014; Hampshire and Robertson 2015). As in human medicine, facial expressions of animals have subsequently been included in multidimensional pain scales, like in the Glasgow acute pain scale for cats designed by Reid *et al.* in 2017.

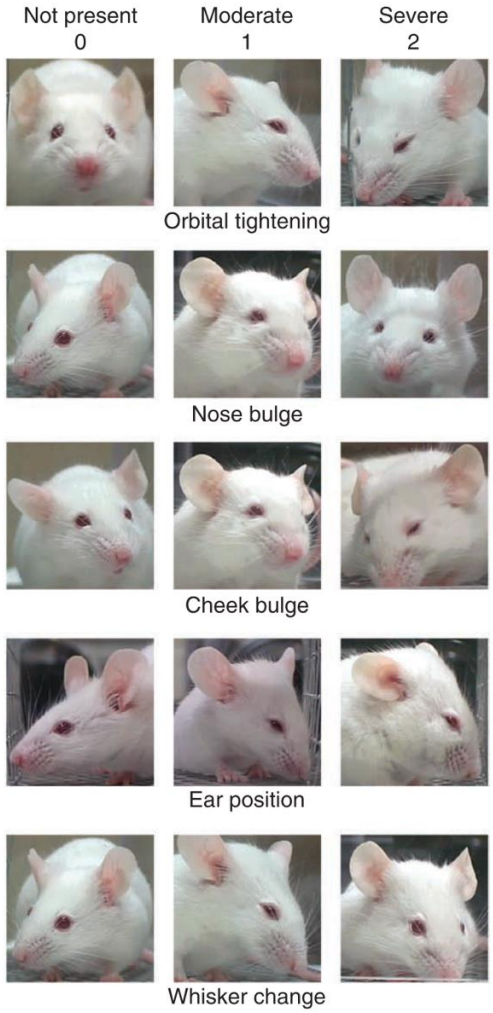

Figure 8. Mouse Grimace Scale

Standardized behavioural coding system using facial expressions designed for the assessment of pain in the mouse (Langford *et al.* 2010).

In recent years, the concept of *Quality of Life (QOL)* in both animals and humans has received increasing attention and its measurement has been focus of intensive research. The concept of QOL in humans was defined at the end of the 20th century by Birnbacher (1999). He described QOL as "objective living conditions and subjective satisfaction with them" and "the health-related subjective well-being of the individual". The QOL is affected by an individual's physical, psychological, and social state, and it is referred to in human medicine as an individual's perception. One of the definitions of animal QOL was provided by Wiseman-Orr *et al.* (2004) who stated that "Quality of life is the subjective and dynamic evaluation by the individual of its circumstances (internal and external) and the extent to which these meet its expectations (that may be innate or learned and that may or may not include anticipation of future events), which results in, or includes, an affective (emotional) response to those circumstances (the evaluation may be a conscious or an unconscious process, with a complexity appropriate to the cognitive capacity of the individual)". It is straightforward that pain is a cause of suffering and can lead to an impairment of the QOL. An example of disease which causes suffering and may severely affect an animal's QOL is cancer. In such a condition, QOL measurement can facilitate decision-making on the treatment, whether active or palliative, including decisions regarding the appropriateness of euthanasia (Scott *et al.* 2007). Psychometric methods have been successfully applied to develop tools that measure QOL among dogs and cats with different diseases (Giuffrida and Kerrigan 2014). Quantitation of an animal's QOL may be the most non-invasive and promising method for determining the severity of an animal's condition (Gaynor and Muir III 2015).

1.4.3 Physiological indicators of pain

The main neural substrates involved in the animal's response to aversive conditions including pain are the medial hypothalamus, amygdala, periaqueductal grey (PAG), and locus coeruleus. The activation of these centres is accountable to a cascade of events that ultimately lead to the

production of several biological mediators affecting the body and adapting it to address a situation, or not adapting to it at all and leading to an impairment of the individual via maladaptive systems. One of the most important systems activated by painful afferent signals is the HPA. The activation of HPA leads to the production of several mediators, such as *corticotropin-releasing factor (CRF), adrenocorticotropic hormone (ACTH), cortisol, catecholamines, glucagon, and growth hormone (GH)*. These and other mediators will be described and interaction among them elucidated here below:

The release of *CRF* from the locus coeruleus is one of the most important components of the stress response. It leads synergistically alongside to vasopressin to stimulate the production of ACTH and β-endorphin, enhancing survival and producing analgesic effects, respectively. CRF also stimulates the adrenal medulla to release ACTH and catecholamines. Also, CRF is an excitatory neurotransmitter which leads to cortical production of norepinephrine and excitation. *ACTH* main function is to stimulate the adrenal cortex to secrete cortisol, corticosterone, aldosterone, and androgenic substances. ACTH also stimulates the adrenal medulla to secrete catecholamines (Gaynor and Muir III 2015).

Cortisol is an indicator of the severity of the stress response in most species. It stimulates gluconeogenesis, increases proteolysis and lipolysis, facilitates catecholamine effects, and produces anti-inflammatory effects. It has been long used as the "gold standard" technique for stress assessment in animals, but because of high inter- and intra-individual variability its sensitivity as a tool might not be the highest. Cortisol levels can be examined via blood, saliva, and faecal sample collection (Schatz and Palme 2001; Bayazit 2009; Bonelli *et al.* 2019).

Catecholamines cause glycogenolysis, gluconeogenesis, inhibition of insulin release, peripheral insulin resistance, and lipolysis. Their increase into the systemic circulation is responsible for elevations in heart rate, respiratory rate, arterial blood pressure, and cardiac output. They increase blood flow into the muscles and thus prepare the animal for fight or flight

(Gaynor and Muir III 2015). Catecholamines have been used as a means for measuring post-operative pain and differentiating between varying pain degrees as shown by Ledowski *et al.* (2012).

Glucagon production by the pancreas can be induced by epinephrine, glucocorticoids, endogenous endorphins, and growth hormone. Acute pain, like surgical pain, cause an increase in glucagon secretion and a decrease in insulin secretion, which leads to hepatic glycogenolysis, gluconeogenesis, glucosaemia, and glucosuria (Gaynor and Muir III 2015; Greisen *et al.* 2001). Furthermore, acute severe pain decreases insulin sensitivity leading to insulin resistance, primarily by affecting nonoxidative glucose metabolism. This suggests that analgesia in pain states is important for the maintenance of normal glucose metabolism (Greisen *et al.* 2001).

Vasopressin is produced in conjunction with renin following painful stimuli and increases the circulating blood volume promoting water retention, vascular tone, and vascular responsiveness to catecholamines (Kendler *et al.* 1978; Waters *et al.* 1982; Gaynor and Muir III 2015).

Following injury, and due to neurohumoral changes, an increase in metabolism occurs. This leads to carbohydrates, lipids and protein breakdown. *Glucose* concentration in the blood increases following pain due to stress-induced production of glucagon and relative lack of insulin. *Glycerol* and *free fatty acids* are produced as a consequence of catecholamines, growth hormone, and cortisol secretion. The latter also causes an increase of protein breakdown, increasing the release of *amino acids*, which can be used to form new proteins or to produce glucose (Gaynor and Muir III 2015).

Changes in peripheral blood count may occur following exposure to stressors, like pain. *Stress leucocytosis* is associated with cortisol release which induces mature neutrophilia, lymphopenia and eosinopenia. Neutrophilia is the consequence of mobilization from the marginal pool, the reduced ability to migrate from the blood to the peripheral tissues and the increased mobilization of the population of the bone marrow reserve, which can be considered a mature

neutrophilia or neutrophilia with right shift. Lymphopenia is the result of lymphocyte sequestration from lymphoid tissues and the eosinopenia derives from the marginalization of eosinophils in the blood vessels and the decreased release from the bone marrow. This response appears between 2 and 4 hours after the elevation of the endogenous cortisol concentrations or after exogenous administration of corticoids (Welles 2000; Satué *et al.* 2014). As a consequence of this, several authors successfully evaluated the use of the neutrophils/lymphocytes (N:L) ratio as a stress measurement tool in different animal species. Its increase seems to be proportional to the level of glucocorticoid release (Davis *et al.* 2008; Lee *et al.* 2013; Hickman 2017).

Other physiological parameters indicative of homeostasis alteration and physiological stress are the *acute phase proteins (APPs)*. They are released from the liver following stimulation by inflammatory mediators, and serve different purposes, either positively or negatively impact the individual's physiological status. APPs are more sensitive than cytokines in indicating alteration of health status because last for a longer period in the bloodstream (up to 48 hours) than the latter which are cleared from the circulation within few hours (Gruys *et al.* 2005).

Body temperature, whether evaluated systemically or regionally, can be an important parameter to evaluate when assessing for pain. Regional body temperature can be evaluated by *infrared thermography* which is a non-invasive and safe diagnostic method which visualizes functional abnormalities and is used effectively in the diagnosis of numerous painful conditions. The mean temperature of the affected painful region of interest is usually compared with the mean value of the contralateral non-affected region (Nahm 2013; Soroko and Howell 2018).

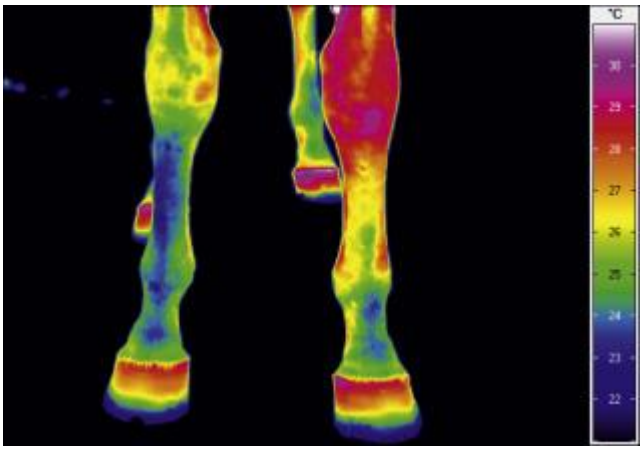

Figure 9. Infrared thermography

Infrared thermogram of the dorsal aspect of the distal part of the forelimbs. The image shows inflammation of the left carpus (Soroko and Howell 2018).

1.4.4 Nociceptive threshold testing

The nociceptive threshold test is an assay that evaluates the ability of an animal to perceive a noxious stimulus and allows for the evaluation of the animal's response to it. The animal response is considered the "end-point" of the procedure, and usually, comprises behavioural and/or physiological changes which can also be species-specific. The ideal nociceptive stimulus should be repeatable, reliable and easy to apply with a clear end-point (Beecher 1957). The test should be of an intensity that should be related to the perceived pain intensity, it should be able to detect the effects of weak analgesics, and should not result in lasting tissue damage or harm to the animal (Love *et al.* 2011). A common disadvantage that these tests come with is that none of them represents a clinical type of pain, therefore the tests may not detect the analgesic effects of all classes of analgesic drugs under clinical conditions. Due to the complexity of clinical pain, it is unrealistic to expect a single threshold testing to be able to reproduce the complete pain experience of the animal (Nielsen *et al.* 2009).

The most common nociceptive threshold tests deliver *mechanical*, *thermal*, or *chemical* noxious stimuli, all of which come with both advantages and disadvantages:

Mechanical stimulation is commonly used in the clinical practice to detect painful body areas or to elicit reactions when testing the peripheral nervous system function. However, that type of evaluation is a subjective one and to objectively assess an animal's response to mechanical stimuli the use of a validated method is compulsory. Devices have been developed for use under laboratory conditions to measure mechanical nociceptive thresholds on the surface of the skin by application of force over a given area (Love *et al.* 2011). Von Frey or Semmes Weinstein filaments are commonly used to measure mechanical sensory thresholds in humans and laboratory animals (Bove 2006). The tip of the filament is placed in contact with the skin and pressure applied until the filament bends. The mechanical threshold is determined by the size of filament at which a behavioural response is obtained (Love *et al.* 2011). Another method to measure somatic mechanical nociceptive threshold is using an algometer, such as the hand-held flat-ended probe algometer used in horses by Haussler and Erb (2006). Several methods to measure visceral mechanical nociceptive threshold were designed in horses and mostly consist of balloons, which following constant inflation procedure, causes distension of hollow viscera, which leads to pain and therefore a response from the animal (Sanchez and Merritt 2005). Unfortunately, mechanical stimulation causes non-specific activation of mechanoceptors, therefore also activating low-threshold non-nociceptive receptors, so care must be taken when elaborating results.

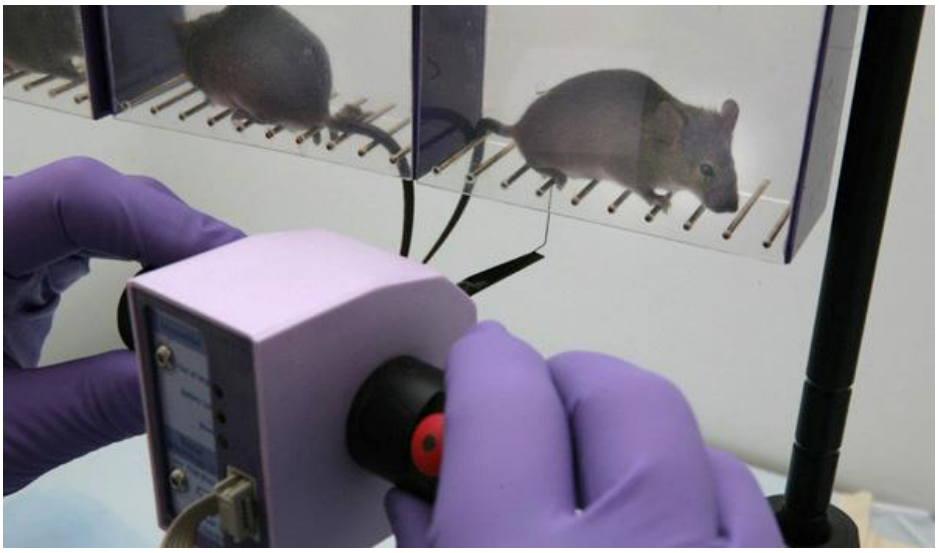

Figure 10. Electronic Von Frey system

Use of the electronic von Frey system on a mouse's paw to elicit a nociception-derived behavioural response (source: www.mousemet.co.uk).

Thermal stimulation is usually applied to the skin and evokes superficial cutaneous pain. There are two most common methods to perform this type of stimulation, being: latency to response following application of a constant temperature, and measurement of the temperature at which a response occurs when there is a ramped increase in temperature (Love *et al.* 2011). It has been reported that these two different methods affect different afferent nociceptive fibres in the rat. In fact, following exposure to sudden (6.5 °C second^{-1}) changes in superficial skin temperature, Aδ fibres are activated, whereas when slow (0.9 °C second^{-1}) changes in temperature are used, C fibres are involved (Yeomans and Proudfit 1996). There are several methods available to measure thermal stimulation, but for the purpose of this thesis, only a few will be reported. As an example, in 2013, Poller *et al.* described the use of a thermode based contact heat test system at different body regions (nostril, withers, coronary band) in the horse (Figure 11). Unfortunately, the concurrent activation of both non-nociceptive and nociceptive afferent fibres leads to bias and also the thermodes often have a flat surface that does not properly conform to the skin surface (Grimm *et al.* 2015). The radiant heat source can also be used, it is easy to build and only stimulates nociceptive fibres. Nevertheless, it produces infrared light that has low

penetrability in the skin and is subjected to several conditions that can bias the results, such as: initial temperature of the skin, its conduction properties, reflectance, transmittance and absorption properties of it and the electromagnetic spectrum emitted by the source of radiation (Grimm *et al.* 2015).

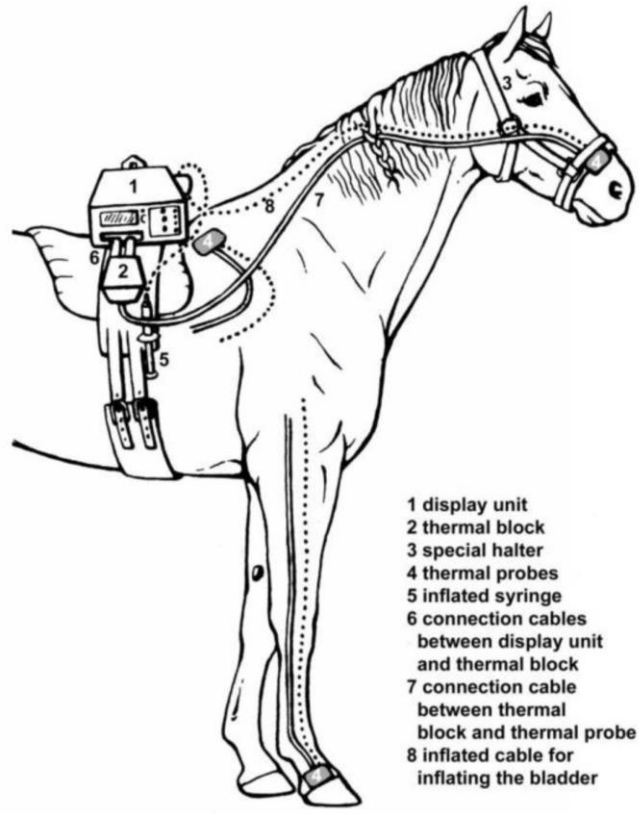

Figure 11. Thermal stimulation in the horse

Thermode-based contact heat test system used in the horse. Different probes are applied to different body parts (nostril, withers, coronary band) (Poller *et al.* 2013)

Electrical stimulation causes direct activation of all nerve fibres, bypassing transduction mechanisms at the peripheral nociceptor. It delivers a brief, controllable, and quantifiable stimulus, and very importantly it is easy to reproduce. Electrical stimulation leads to a synchronized pattern of neuronal activity that is sufficient to generate reflex responses. The response is proportional to the intensity of the stimulus delivered. Unfortunately, it also has drawbacks, because it activates indiscriminately all fibres types, it is of limited use to study peripheral pain mechanisms due to it bypassing the transduction part of the pathway, and it

elicits a neuronal activity that is not representative of that which happens in clinical states of pain. Also, electrical stimuli are also dependant on the impedance of the skin to which it is delivered (Grimm *et al.* 2015). See Figure 12 for an example of electrodes application in the rat and an increasing stimulus intensity administration.

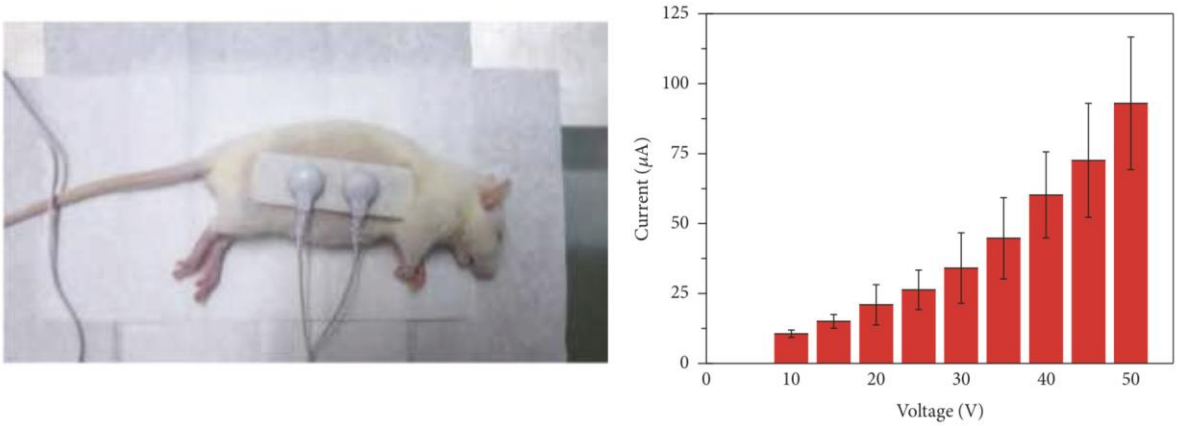

Figure 12. Electrical stimulation

Display of electrodes positioning on the flank of a rat (left) and increasing stimulus intensity delivered (right) (Lee *et al.* 2018).

Chemical stimulation is used to create pain models of inflammatory pain, visceral pain, and somatic pain (joints level; osteoarthritis pain). They are usually non-phasic, and lead to an inflammatory phase (*e.g.* formalin) and/or different extent of tissue damage. Carrageenan seems not to elicit a systemic immune response but causes thickening of the skin, which impedes repeated testing at the same site. Some chemical agents can cause desensitisation of the nociceptors or even neurotoxicity and neuronal cell death (Grimm *et al.* 2015).

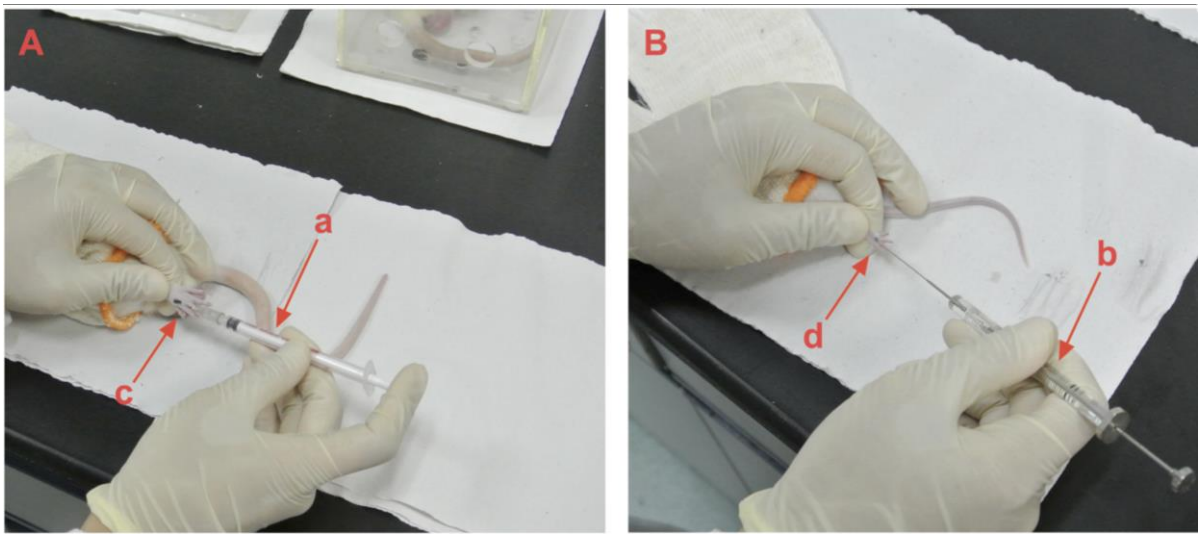

Figure 13. Formalin injection in the rat and mouse

Formalin injection in the rat (A) and mouse (B) at the level of the dorsal aspect of the hind foot (Gong *et al.* 2014).

1.5 The link between immune and nervous system

A relationship exists in which the immune system can modulate the nervous system and vice versa. The immune system not only is able to cause inflammation and therefore inflammatory pain but also functions as a diffusely distributed sense organ that communicates injury-related information to the brain. The immune system detects pain or injury in different ways, like: through blood-borne immune messengers originating at the site of pain or injury; through nociceptor-induced sympathetic activation and subsequent stimulation of immune tissues, and through endocrine signalling that triggers the acute-phase reaction (Gaynor and Muir III 2015).

1.5.1 Inflammatory pain

Tissue injury causes a release of endogenous danger signals, termed *alarmins* like heat shock proteins and high mobility group box 1 protein, which are recognised by toll-like receptors, expressed in the immune cells, triggering neurogenic inflammation and cytokines release. Cytokines, which are produced by activated leukocytes, fibroblasts, and endothelial cells represent the main effectors of the response to tissue injury with two opposing phenotypes: the pro-inflammatory, IL-1β, TNF, IL-6, IL-15, IL-17, IL-18 and IFN-γ; and anti-inflammatory,

IL-4, IL-10 and TGF-β (Xu *et al.* 2018). TNF-α, IL-1 and IL-6 also play a key role in the starting of the hepatic acute phase reaction, which involves the release from the liver of acute phase proteins. This inflammatory response is subsequently amplified by the migration of leukocytes into the inflamed tissue, by the production of cytokines, chemokines, growth factors (*e.g.* nerve growth factor), and tissue acidification. Leukocyte recruitment is not only mediated by chemokines, but also by other mediators such as complement or neuropeptides which can act as chemoattractants either by direct chemotactic effects on monocytes and granulocytes, increased expression of adhesion molecules, and augmentation of local chemokine production (Rittner *et al.* 2008).

Leukocytes, in turn, and especially macrophages and neutrophils contribute to peripheral nociceptive sensitization by releasing soluble factors and interacting directly with nociceptors. Evidence of this has been provided by Carreira *et al.* who reported that treatment of mice with an antagonist for the chemokine receptor CXCR1/2, expressed on the surface of neutrophils, led to a reduction in mechanical hyperalgesia and neutrophil infiltration (Carreira *et al.* 2013). Furthermore, it has been demonstrated through the use of NOX2 (NADPH-oxidase complex) deficient mice that macrophages contribute to neuropathic pain hypersensitivity after peripheral nerve injury. NOX2 generates reactive oxygen species (ROS) production in macrophages. Thus, mice lacking the ROS producing macrophages did not experience pain hypersensitivity suggesting their involvement in pain (Kallenborn-Gerhardt *et al.* 2014). ROS have been implicated in the development of persistent pain states that result from nerve injury or inflammatory insult. In fact, studies have shown that various antioxidants (*e.g.* phenyl N-tert-butylnitrone], 5,5-dimethyl-1-pyrroline-N-oxide, 4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl, and vitamin E) are very effective in alleviating mechanical hyperalgesia in spinal nerve–ligated neuropathic rats and capsaicin-induced secondary mechanical hyperalgesia in rats and mice. Also, several pieces of evidence indicate that the increased ROS in the spinal cord may

induce pain by reducing GABA inhibitory influence on *substantia gelatinosa* neurons that are involved in pain transmission (Yowtak *et al.* 2011). Therefore, much evidence suggests ROS are important for the development and maintenance of persistent pain (Yowtak *et al.* 2011).

Macrophages have proven to play an important role in non-inflammatory pain too when Gong *et al.* found that macrophages in the central nervous system that had been administered a toll-like receptor antagonist showed reduced hyperalgesia in mice (Gong *et al.* 2016). Resident macrophages are also referred to as microglia. Microglia cells are activated within 4 hours from peripheral nerve injury and seem to be important in chronic pain modulation due to brain microglia proliferation and activation at the cortex, thalamus, amygdala and hypothalamus, resulting in central pain sensitisation (Xu *et al.* 2018). These cells produce microglia-derived factors that can signal to astrocytes and neurons, enhancing neuronal firing through both direct and indirect mechanisms.

Pain can also occur as a result of direct IgG-induced injury of nociceptive fibres either during infection or following sterile antigen exposures. The exposure of the antigen by an antigen-presenting cell leads to the activation of naive T and B cells. Naive T cells then mature into different subtypes driving the immune response (*e.g.* antibodies production). IgG complexes seem to directly bind Fc-gamma receptors on nociceptive fibres in the dorsal root ganglion or their soma without axonal loss which leads to the painful condition (Xu *et al.* 2018).

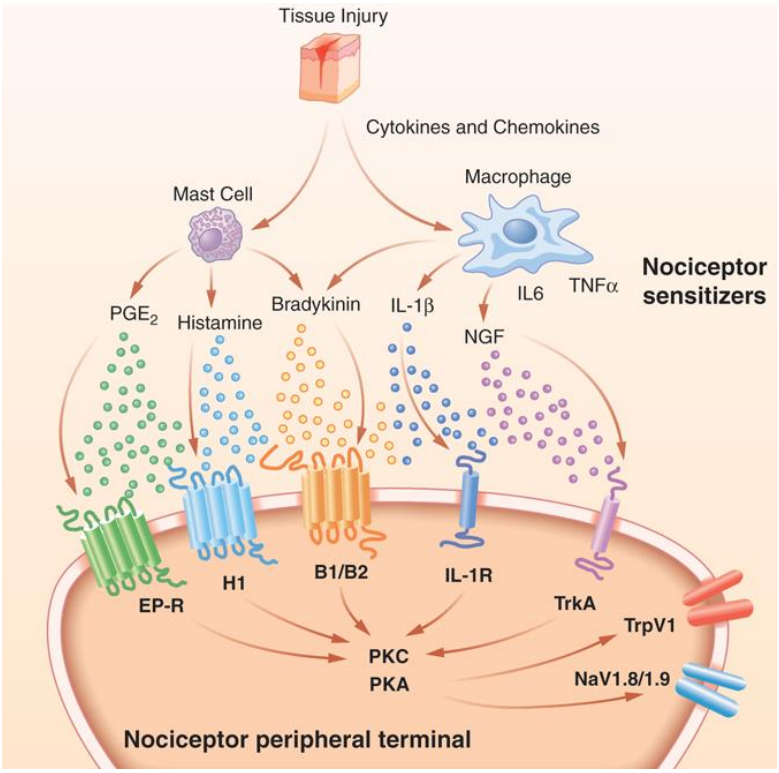

Figure 14. Immune activation and nociceptor sensitization after injury

Nociceptor sensitization after injury by liberation of cytokines and activation of mast cells and macrophages (Longnecker *et al.* 2012).

1.5.2 Immunomodulatory effect of the nervous system

It is clear now that the immune system heavily influences pain perception by acting on the nervous system, but can pain and stress exert modifications on immune cells too?

Inflammation is considered an adaptive biological response and key indicator not only to tissue injury but also to mental disease (Reader *et al.* 2015). The deriving immune response might differ depending on the nature, intensity, and duration of the stressor.

In the last decades, neurotransmitters resulted to have substantial roles in modulating immune cells and some of the different pathways of interactions between the endocrine, central nervous and immune systems have been elucidated: the HPA axis, hypothalamic–pituitary–gonadal axis, hypothalamic–pituitary–thyroid axis, and the hypothalamic–growth-hormone axis. Furthermore, the autonomic nervous system also communicates with the lymphoid

compartment through the release of norepinephrine and acetylcholine from sympathetic and parasympathetic nerves (Taub 2008).

Already in 1987 some authors tested the influence of cortisol, glucagon and catecholamines on leukocytes *in vitro*, and found that these caused different effects on leukocytes' migration and bactericidal activity (Deitch and Bridges 1987). Cortisol has long been referred to as an anti-inflammatory agent, but proof from recent research has shown that its interaction with white blood cells is much more complex than previously thought, evidencing its importance in the fluctuation of white blood cells trafficking and potentiation of the immune response in certain conditions. Whether it is true that glucocorticoids can act as anti-inflammatory agents in chronic conditions, it must be accounted that in certain cases such as during repeated social defeat stress in mice, leukocytes may become insensitive to them and therefore capable to produce high levels of proinflammatory cytokines and chemokines, creating an immunoenhancive environment within the periphery (Reader *et al.* 2015).

In 1989, substance P receptors were found on the surface of neutrophils. Substance P resulted to stimulate neutrophils-driven cytotoxicity and ROS production. Furthermore, the regulatory effects of Substance P on the neutrophil functions appeared to be similar to those of cytokines implicated in inflammation (Wozniak *et al.* 1989).

In 1991, Bayne and Levy discovered that trout neutrophils could be influenced by ACTH and catecholamine agonists administration which induced them to produce ROS, suggesting the presence and interaction of specific hormones/neurotransmitters with adrenergic receptors on the trout's neutrophils (Bayne and Levy 1991). The immunomodulatory effect of adrenaline seems to increase circulating neutrophil numbers and reduce lymphocyte numbers in blood, whereas noradrenaline was reported to increase both neutrophil and B cell numbers with distinct temporal profiles (Ince *et al.* 2019).

Dopamine and glutamate interact directly with T-cell-expressed receptors, activating/suppressing various T-cell functions like cytokine secretion, proliferation and integrin-mediated adhesion and migration (Franco *et al.* 2007). Also, serotonin and dopamine were found to serve controversial purposes in white blood cells modulation (Arreola *et al.* 2016; Herr *et al.* 2017).

Any disturbance in brain homeostasis evokes rapid changes to microglia morphology, gene expression, and production of inflammatory signalling molecules. Microglia are sensitive to the physiological response of stress as they express β-adrenergic and glucocorticoid receptors (Kettenmann *et al.* 2011). In response to stress, microglia cells were discovered to also produce ROS, showing how the immune system respiratory burst and neurotoxicity can be neurotransmitters-driven (Mead *et al.* 2012). Different types of stressful stimuli can induce the same microglial activated profile (Webe *et al.* 2017). Interestingly, chronic pain was found to attenuate dopamine transmission by microglial activation (Taylor *et al.* 2015).

Several studies reported that leukocytes are capable of producing a broad range of neurotransmitters themselves (*e.g.* acetylcholine, serotonin, dopamine, norepinephrine, and glutamate) (Franco *et al.* 2007; Fujii *et al.* 2017). This evidence supports the theory on the important exitance of cross-talk between the immune system and nervous system.

Unfortunately, very little is known on the relation between immune and nervous system yet, therefore exploring all the mechanisms by which these systems communicate seems to be a very promising field of study.

1.6 Whole-blood leukocyte coping capacity (LCC) chemiluminescence

The whole-blood leukocyte coping capacity (LCC) is a technique that allows for the assessment of ROS production by polymorphonuclear cells. These cells are the first line of innate immune protection in animals, and in mammals they come in the form of neutrophils (Mantovani *et al.* 2011). The process of ROS production is also called "*oxidative burst*" and serves the purpose

of generating agents that are microbicidal themselves or become microbicidal in combination with other components. The respiratory burst is the consumption of oxygen that leads to the production of superoxide (O_2^-) and is catalysed by the *NADPH-oxidase system*, a multi-protein system that exists in a disassembled state in quiescent cells and is rapidly assembled following phagocyte activation (Robinson 2008) (See Figure 15). The first reaction leading to ROS production is the following: $NADPH + 2O_2 \rightarrow NADP^+ + 2O_2^- + H^+$

This enzyme is lacking in humans affected by the genetic disorder called chronic granulomatous disease, leading to an incapacitated immune system unable to produce ROS and causing the organism to easily suffer from infections.

Superoxide is further processed by a dismutation reaction which transforms it into H_2O_2 as displayed in the formula: $O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2^-$

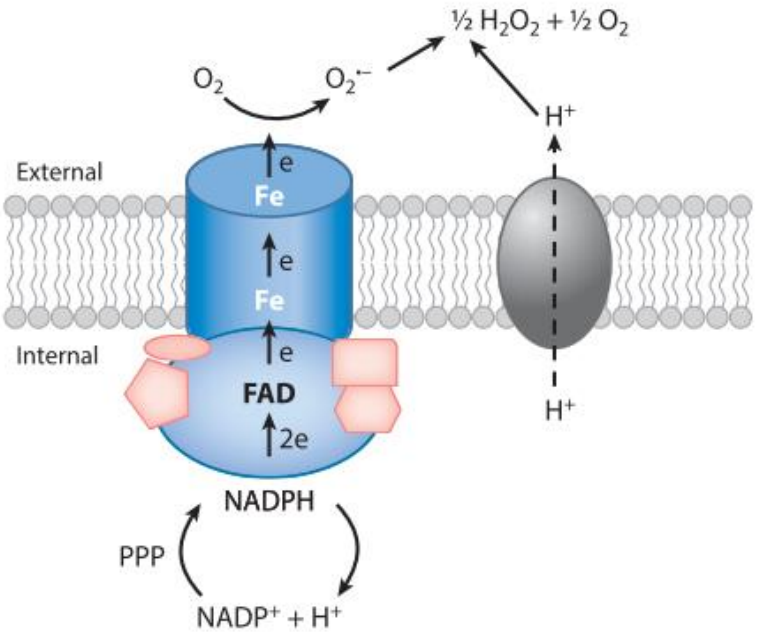

Figure 15. NAPDH-oxidase complex

Graphic representation of the NADPH-oxidase complex which catalyses the reduction of oxygen by a single electron, with the electrons passing through the complex from NADPH (Winterbourn *et al.* 2016).

H_2O_2 and O_2^- are both very strong and efficient anti-microbial agents, but leukocytes can even produce hypochlorous acid by the action of the enzyme *myeloperoxidases* (Figure 16):

$H_2O_2 + Cl^- + H^+ \rightarrow HOCl + H_2O$

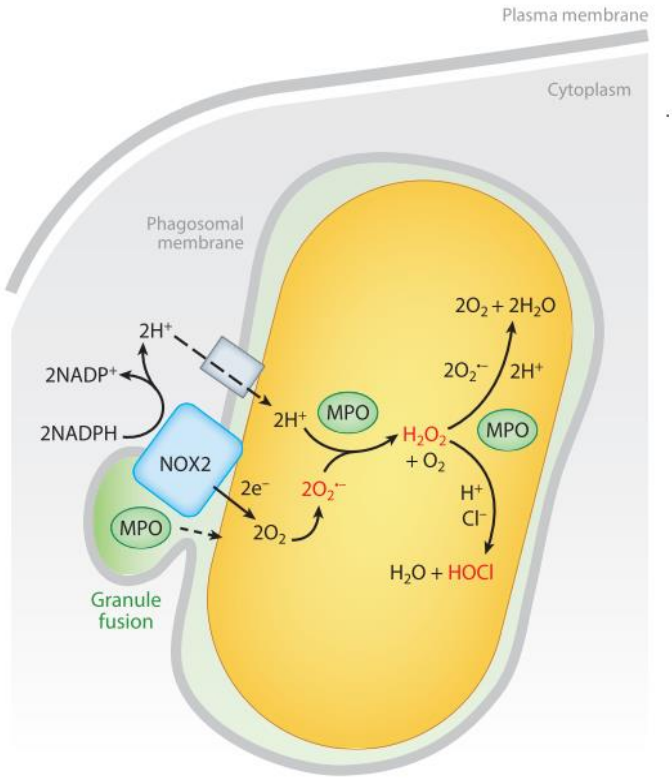

Figure 16. The oxidative process within the phagosome

Display of $O2^-$ and HClO production by NADPH-oxidase complex (NOX2) and myeloperoxidase (MPO), respectively (DeCoursey and Ligeti 2005).

Excessive production of free-radicals can be very harmful to the organism, therefore, following acute activation, ROS production is usually attenuated by, for example, de/hyper-phosphorylation of the complex (DeCoursey and Ligeti 2005).

Polymorphonuclear cells can be activated when binding to surface peptides of pathogens or by the stress-related activation of their α- and β-adreno- and glucocorticoid receptors. They have in fact been addressed as to biological indicators of stress. In fact, polymorphonuclear cells have over 150 different receptors which are sensitive to varied stress signals in the organism, including plasma endocrine factors, changes in blood biochemistry and red cell haemodynamics, changes of cytokine levels and mediators released by the HPA axis and the sympathetic nervous system (Mian *et al.* 2005).

The important role of neutrophils led McLaren *et al.* (2003) to develop the whole-blood LCC technique. He claimed that this technique could sensitively detect changes in ROS production by polymorphonuclear cells following an *in vitro* chemical stimulation. He also put a particular focus on decreasing trends in LCC results, which he addressed as indicative of lower ROS production. This "exhaustion" state follows the activation of leukocytes and leads to an increased susceptibility of the animal to pathogens infections. Unfortunately, it is still not clear whether the decrease in ROS detection is due to an actual decrease in their production, consequent to activation of a defence mechanisms aimed to inactivate the NADPH complex, harmful ROS, or both.

The whole-blood LCC comes with several advantages compared to other laboratory techniques that evaluate ROS production in isolated leukocytes. The most striking advantage of the whole-blood LCC is that neutrophils are maintained and analysed in their natural environment. This is crucial for maintaining *in vitro* the physiological interactions and cross-talk that happen *in vivo* among white blood cells and the molecules and other cells present in their natural environment. Therefore, this method does not necessitate centrifugation and can be easily performed in the field by means of a portable chemiluminometer (Figure 18) which detects photons emitted by the chemical interaction between luminol (a chemical that chemiluminesces when mixed with oxidizing agents) and ROS (See Figure 17). Luminol, due to its chemical structure, can cross biological membranes, allowing for detection of both extra- and intra-cellular reactive oxygen species (Pavelkova and Kubala 2004). This characteristic of luminol provides data reflective of the entire oxidative process taking place in the organism at the cellular and cytosolic level.

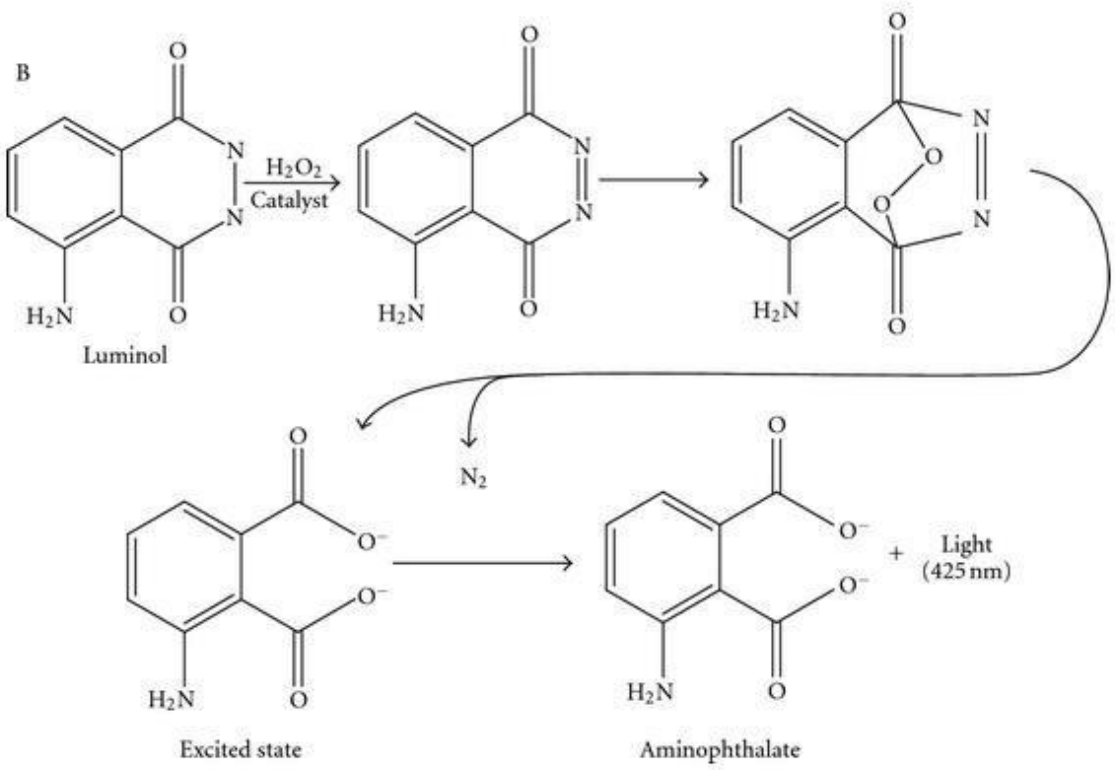

Figure 17. Luminol chemical reaction

Chemiluminescence reaction by luminol and a ROS (*e.g.* hydrogen peroxide, H_2O_2) interaction (Kazemi and Abedirad 2012).

The LCC essay does not demand large sample quantities and can be performed with amounts as small as 20 µL of blood in total, which makes the technique applicable to small animals like rodents or even small birds (See chapter 3.2.3 for a thorough description of the chemiluminescence method).

Figure 18. Portable Chemiluminometer

Picture of the portable chemiluminometer used to detect whole-blood LCC (Junior LB 9509, Berthold, Germany) (source: www.berthold-bio.com)

1.6.1 State of the art

In 1992, Lilius and Marnila reviewed the phagocyte chemiluminescence and stressed the concept that the respiratory burst of an individual can be reflective of its pathophysiological condition (Lilius and Marnila 1992). One year later, pursuing this line of thinking, Thompson *et al.* studied ROS production in the Atlantic salmon (*Salmo salar*) and revealed that the animals that had been subjected to a 2h period of confinement manifested a reduction in ROS produced by isolated polymorphonuclear cells. This further confirmed that stress somehow was capable to impair innate immune defence system and due to a lower oxidative burst capacity (Thompson *et al.* 1993).

The whole-blood LCC was used by Marnila *et al.* in 1995 to measured phagocyte activity in the frog (*Rana temporaria*) at different temperatures and enzymes concentrations. In 2003, McLaren *et al.* was the first one to use the whole-blood LCC in the field on wild badgers (*Meles meles*) to measure transport stress after capture. He set up a detailed protocol for use in mammals and found out that transported badgers had a lower ROS production, and therefore a lower LCC output than badgers sampled at the capture site. This result implied that transport

can be considered an additional stressor beyond the capture event. The whole-blood LCC method was also used in 2005 to compare stress between non-human primates, specifically rhesus macaques (*Macaca mulatta*), housed in open-room housing and traditional laboratory caging systems. This study found out that housing this animal species in open-room housing was less stressful and beneficial to the animal's physiological status (Honess *et al.* 2005). A study on bank voles (*Clethrionomys glareolus*) and wood mice (*Apodemus sylvaticus*) was performed resulting in lower LCC values in animals that had been handled than in those which had not been handled (Gelling *et al.* 2009). Other authors also used LCC in a different rodent species, the water vole (*Arvicola terrestris*), and found out that radio-collaring and larger social groups might impair the animal's immune status because of stress (Moorhouse *et al.* 2007; Gelling *et al.* 2010). The LCC technique proved a great success among wildlife researchers leading to the development of other more studies in other animal species. In 2016, Esteruelas *et al.* found that solitary free-ranging brown bears (*Ursus arctos*) displayed lower LCC values than those that lived in a group, pointing out that solitary brown bears could undergo a more stressful condition for their organism. In the following year, Huber *et al.* found that LCC levels were negatively impacted by the time of human presence at the capture site prior to the actual handling procedure in non-anesthetized European roe deer (*Capreolus capreolus*) (Huber *et al.* 2017b). This study brought evidence of the fact that human presence should be minimized when capturing this species. Recently this technique was tested in the house sparrow (*Passer domesticus*), showing a significant increase in LCC values after 30 minutes from the administration of the psychological stressor (handling following capture), implying that such a short psychogenic stimulus impaired the immune response only for a brief period of time (Huber *et al.* 2017a). The most recent study on whole blood LCC was carried out by Huber *et al.* (2019) in the kulan species (*Equus hemionus*). In this study, the technique was used to assess which individuals were highly stressed during captive housing and had to be excluded from the

transportation process. The author stated that the whole-blood LCC is an extremely useful method to deal manage wildlife and reduce the odds of casualties.

Just like in animals, the whole-blood LCC was also used in humans to test the effect of different psychological stressors. Shelton-Rayner *et al.*, on behalf of the automotive industry, quantified the mental load in humans undergoing different ergonomic evaluations by means of the whole-blood LCC (Shelton-Rayner *et al.* 2010, 2011, 2012).

This technique proved to deliver very promising and sensitive results in perceiving physio-psychogenic changes in white blood cells functionality (see Table 2 for a complete list of the studies available in the literature). Nevertheless, further studies are needed to confirm the hypothesized sensitivity and applicability of this technique to other animal species. Despite this, the whole-blood LCC seems to hold the potential to be considered amongst the more important and common parameters for stress and welfare assessment.

Table 2. Studies on the whole-blood LCC and stress assessment

Summary of the current literature on whole-blood LCC technique used to assess psychophysiological stress.

Reference	**Species**	**Stressor**	**Effect**
McLaren *et al.*, 2003	*Meles meles*	Transport	Decrease in ROS prodcution
Honess *et al.*, 2005	*Macaca mulatta*	Housing	Decrease in ROS prodcution
Gelling *et al.*, 2009	*Clethrionomys glareolus; Apodemus sylvaticus*	Handling	Decrease in ROS prodcution
Moorhouse *et al.* 2007	*Arvicola terrestris*	Captive housing, manipulation, radio-collaring	Decrease in ROS prodcution
Gelling *et al.* 2010	*Arvicola terrestris*	Captive housing	Decrease in ROS prodcution
Shelton-Rayner *et al.*, 2010, 2011, 2012	*Homo sapiens sapiens*	Mental loading	Decrease in ROS prodcution
Esteruelas *et al., 2016*	*Ursus arctos*	Solitary animals	Decrease in ROS prodcution
Huber *et al.*, 2017a	*Passer domesticus*	Handling	Decrease in ROS prodcution
Huber *et al.*, 2017b	*Capreolus capreolus*	Human presence at capture	Decrease in ROS prodcution
Huber *et al.*, 2019	*Equus hemonius*	Captive housing	Decrease in ROS prodcution
Huber *et al.*, 2019	*Capreolus capreolus*	Anaesthesia	Increase followed by decrease of ROS production

2 Aim of the thesis

The impelling need to improve pain assessment in veterinary medicine has driven the research team to focus their studies on testing an innovative technique able to measure psychological stress by measuring the immune system's capability to produce reactive oxygen species: the whole-blood leukocyte coping capacity (LCC). Seen the intertwined nature of stress and immune system, and since pain is a stressor, the aim of the present thesis was that of evaluating the LCC technique as a new tool for assessing pain in animals, specifically in the bovine and equine species.

The PhD thesis comprised of two major studies:

1. **Whole-blood LCC chemiluminescence in calves undergoing non-surgical castration;**
2. **Whole-blood LCC chemiluminescence in horse stallions undergoing surgical castration.**

The main hypothesis of the two studies was that the whole-blood LCC technique would be able to detect castration-induced pain by highlighting changes in the immune response of the animal. Furthermore, in these studies, LCC results were compared and discussed with results from traditional pain and stress assessment parameters.

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3 Whole-blood LCC chemiluminescence in calves undergoing non-surgical castration

Results of this study were published on *Journal of Animal Science* 2018, 96 (11), pp. 4579-4589.

3.1 Introduction

Pain and stress assessment in animals are considered an imperative issue and also a difficult challenge. Objective, quantitative, and practicable measures of sufferance are crucial to studies in many branches, including animal husbandry (Flecknell and Roughan 2004). The pain response in animals is currently assessed using a variety of techniques, including physiological, haematological, and behavioural parameters (Weary *et al.* 2006). Unfortunately, no gold standard technique for pain and stress assessment in animals has been validated nowadays. For this reason, researching effective and innovative methods is essential.

A new tool to assess stress in animals consists of measuring the leukocyte coping capacity (LCC). The LCC is defined as the ability of an animal's leukocytes to produce a respiratory burst in response to a bacterial-type challenge and has been used as a measure of stress (McLaren *et al.* 2003; Honess *et al.* 2005; Moorhouse *et al.* 2007; Shelton-Rayner *et al.* 2011; Esteruelas *et al.* 2016; Huber, Vetter, *et al.* 2017). This immune challenge is triggered, in vitro, by the use of a chemical stimulator and compared with the individuals' own baseline level of immune system activity (McLaren *et al.* 2003). It is reported how LCC results vary according to different stress levels. In fact, lower LCC values have been reported in stressful situations, thus proving how these conditions impair reactive oxygen species (ROS) production by the subject's immune system (mainly by granulocytes) (Shelton-Rayner *et al.* 2011). Studies in humans revealed that pain and stress are 2 distinguished yet overlapping processes sharing

multiple conceptual and physiological patterns (Abdallah and Geha 2017). Moreover, both can be considered double edged swords, as they can result in adaptive or maladaptive changes required to regain homeo- stasis and/or stability (Sinha and Jastreboff 2013). If maladaptive changes occur, changes in physiology and behaviour can be observed, resulting in suffering and compromised well-being of the subject (Knaster *et al.* 2012).

No reports on whole-blood chemiluminescence in the bovine species are available in the literature available to date; thus, how the magnitude of ROS production varies after stress and/or pain is still unknown.

The primary objective of this research was to investigate the validity and feasibility of this new methodology for pain assessment. In this experimental study, ring castration of calves, which has been considered by Marti *et al.* (2010) a source of pain, was used as nociceptive stimulus. Furthermore, comparisons between LCC results and some established methods to measure pain and pain-related stress were executed.

We hypothesized that: 1) calves after castration would show lower LCC values than before castration, 2) non-castrated animals would present higher LCC values compared to treated ones, and 3) there would be a meaningful correlation between LCC results and other parameters routinely used for stress (blood cortisol level, leukogram) and pain (pain scale) assessment.

3.2 Materials and Methods

3.2.1 Study design

The study was performed after the approval of the Animal-welfare Body of the University of Padua in a farm located in the Veneto region (Italy) between March and May 2017. Twenty (n = 20) 2-month-old male mix-breed Piemontese-Angus-Belgian Blue calves (*Bos taurus*) weighing 90 ± 4 kg (mean ± SD) were used. The animals were housed in 2 large group pens, fed mashed weaning feed and milk replacer; they also had free access to water and contact with herd mates and mothers. The animals had been living with the herd for at least 30 d when they

entered the study. Calves were considered healthy on the basis of clinical examination and blood exams results. The animals were randomly allocated in 2 groups composed of 10 subjects each as follows: ring castration group (CAS) and sham castration group (SHAM).

One hour before castration or sham procedure, all calves were assessed for pain using the UNESP-Botucatu unidimensional composite pain scale (de Oliveira *et al.* 2014) (Table 3).

Table 3. UNESP-Botucatu unidimensional composite pain scale for assessing postoperative pain in cattle

Pain scale used to assess post-castration pain in calves in the present study.

Item	Score/Criterion
Locomotion	▪ (0) Walking with no obviously abnormal gait.
	▪ (1) Walking with restriction, may be with hunched back and/or short steps.
	▪ (2) Reluctant to stand up, standing up with difficulty or not walking.
Interactive behaviour	▪ (0) Active; attention to tactile and/or visual and/or audible environmental stimuli; when near other animals, can interact with and/or accompany the group.
	▪ (1) Apathetic: may remain close to other animals, but interacts little when stimulated.
	▪ (2) Apathetic: may be isolated or may not accompany the other animals; does not react to tactile, visual and/or audible environmental stimuli.
Activity	▪ (0) Moves normally.
	▪ (1) Restless, moves more than normal or lies down and stands up with frequency.
	▪ (2) Moves less frequently in the pasture or only when stimulated.
Appetite	▪ (0) Normorexia and/or rumination.
	▪ (1) Hyporexia.
	▪ (2) Anorexia.
Miscellaneous behaviours	▪ Wagging the tail abruptly and repeatedly.
	▪ Licking the surgical wound.
	▪ Moves and arches the back when in standing posture.
	▪ Kicking/foot stamping.
	▪ Hind limbs extended caudally when in standing posture.
	▪ Head below the line of spinal column.
	▪ Lying down in ventral recumbency with full or partial extension of one or both hind limbs
	▪ Lying down with the head on/close to the ground.
	▪ Extends the neck and body forward when lying in ventral recumbency.
	(0) All of the above described behaviours are absent.
	(1) Presence of 1 of the behaviours described above.
	(2) Presence of 2 or more of the behaviours described above.

Before any manipulation, every animal was evaluated for pain assessment, using the above-mentioned scale that was performed for 25 min by 3 trained observers who did not interfere with the animals and used a scoring system ranging from 0 to 10. Each animal was then gently captured by means of a rope and restrained by 2 people for weighing, blood sampling, temperature measuring, and scrotal clinical evaluation. Scrotal and perineal (used as control

measure) temperatures were recorded by means of an infrared thermometer (DT8380, CAMMUO, SKU009011, China) at a distance of 20 cm from the skin. The scrotum was evaluated for clinical conditions using a scoring system ranging from 0 to 6 (Table 4).

Table 4. Scrotal lesion score

The subjective scoring system used to assess the scrotal condition in calves undergoing rubber ring castration and sham castration.

Score	Description
0	Clinically normal/wound healed
1	Small wound
2	Mummified scrotum
3	Mummified and shrunk scrotum with partial detachment from the abdominal wall
4	Dry scrotum, can be swollen
5	Swollen and dry scrotum, ulceration
6	Swollen, moist and hot scrotum

Castration was performed as described by Stafford *et al.* (2002). Briefly, while the animal was being restrained, the skin of the scrotum was disinfected with povidone iodine solution (Betadine 10%, Meda Manufacturing, Merignac, France) for 3 min and 2 rubber castration rings (Allflex New Zealand, Palmerston North, New Zealand) were placed simultaneously on the neck of the scrotum just proximal to the testes using an elastrator (Elastrator, Blenheim, New Zealand) (see Figure 19).

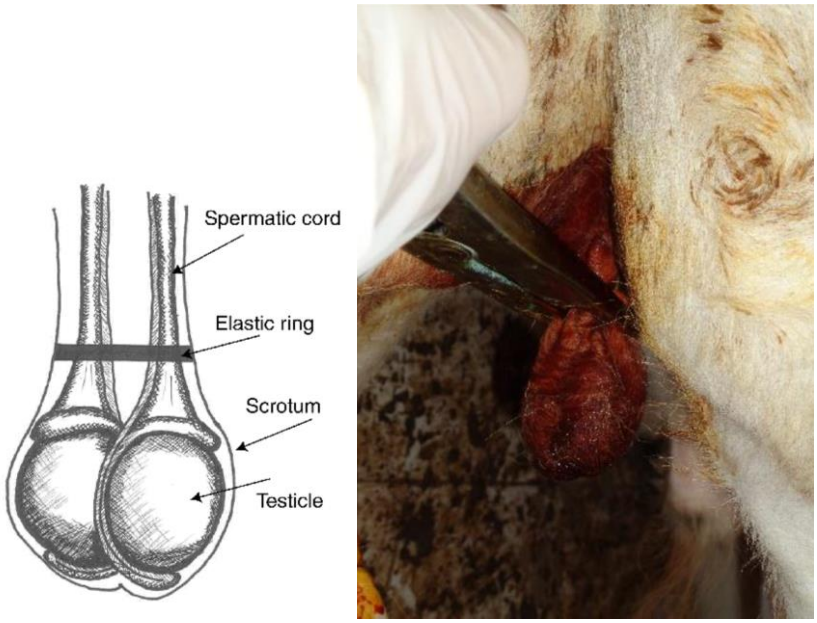

Figure 19. Rubber ring application

Pictures displaying the correct positioning of the rubber ring (left) and the actual procedure (right) following disinfection with povidone iodine solution, which causes a brownish-red coloration of the scrotum. The picture on the left has been taken from (Anderson, Rings, and Abrahamsen 2009).

After 10 d, the devitalized tissues of the scrotum and testes were surgically removed, before they naturally fell off, to spare the animal any kind of unnecessary discomfort accountable to tissues degeneration. Fourteen days after castration/sham, because of the absence of the scrotum, the wound temperature was evaluated instead.

Pain assessment, blood sampling (Figure 20), scrotal, perineal, and environmental temperature recording, and scrotal clinical evaluation were repeated as follows: 1 h before castration (−1 h), 30 min after castration (30 min) and at day 3 (3 d), 7 (7 d), and 14 (14 d) after rings application (see Figure 21).

An identical study design was applied to SHAM group (iodine solution application included), except for castration which was replaced with an equally long testicular manipulation.

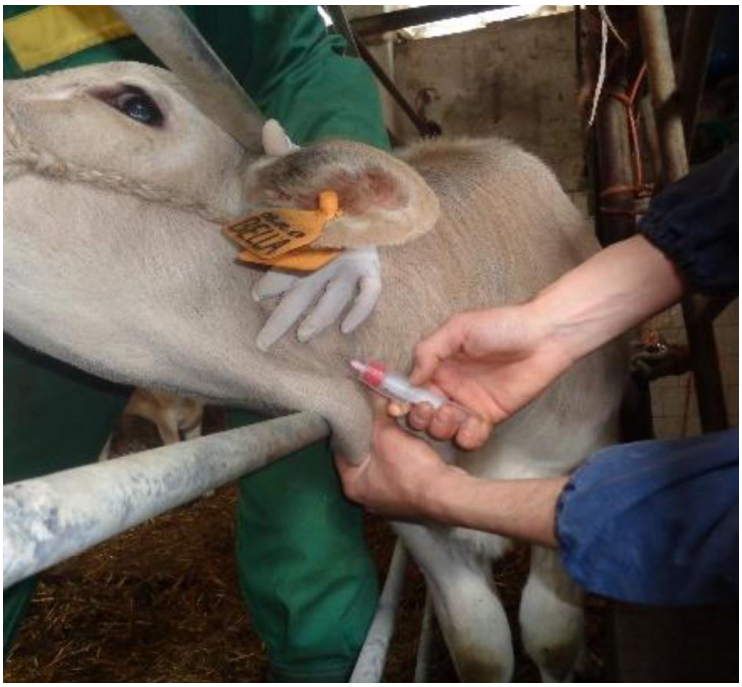

Figure 20. Venepuncture procedure

Blood sampling procedure during animal restraint.

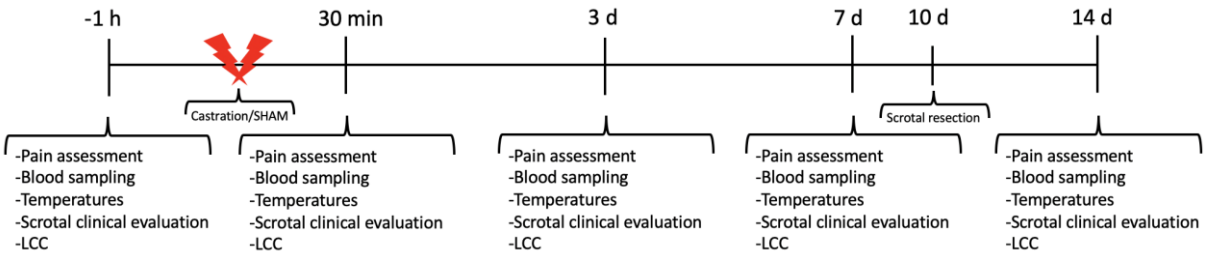

Figure 21. Workflow timeline diagram

Visual display of the study times (-1h: 1h prior to castration/SHAM; 30 min: 30 min post-castration/SHAM; 3d: 3 days post-castration/SHAM; 7d: 7 days post-castration; 10d: 10 days post-castration/SHAM; 14d: 14 days post-castration/SHAM) and the procedures performed at each one of them.

3.2.2 Blood samples processing

Three and 9-mL blood samples were aseptically collected from the external jugular vein in evacuated tubes containing 5.4 mg of K_3EDTA (Vacumed, FL MEDICAL s.r.l., Italy) and in evacuated tubes containing serum clot activator (Vacuette Z Serum Separator Clot Activator, Preanalitica s.r.l., Italy), respectively (see Figure 20). A small amount of blood stored in K_3EDTA tubes was used in the field for chemiluminescence assay, while the residual was

immediately refrigerated at 4 °C until blood count test was performed. A full haematological profile was provided by means of an automated cell counter (ADVIA 120 Hematology System, Siemens Healthcare GmbH, Germany). Clotted blood samples were centrifuged at 3,500 rpm for 15 min to obtain serum, which was stored at −20 °C and then thawed for cortisol concentrations to be measured by immunoassay (COBAS 6000-c601, Roche Diagnostics S.p.A., Italy).

3.2.3 Whole blood chemiluminescence assay

In order to understand an individual's LCC, the whole blood of the same individual was divided into 2 samples: the non-stimulated sample (ns) and the stimulated one (s).

The non-stimulated sample provided a baseline measure of the individual's LCC response. The sample was prepared as described by Shelton-Rayner *et al.* (2012): 10 µL of whole blood-K_3EDTA were transferred into a silicon antireflective tube (Lumivial, EG & G Berthold, Germany); 90 µL of 10^{-4} mol L^{-1} luminol (5-amino-2.3-dihydrophthalzine; Sigma A8511, Sigma-Aldrich, Oslo, Norway) diluted in phosphate-buffered saline (PBS) (Sigma, Sigma-Aldrich) were added and 10 µL of fresh PBS were used to bring the solution to a total volume of 110 µL. The tube was gently shaken to mix the reagents. Because it is known that luminol chemiluminesces if combined with an oxidizing agent to produce a low-intensity light reaction (Whitehead *et al.* 1992), phorbol 12-myristate 13-acetate (PMA, Sigma P8139, Sigma-Aldrich) was used as an activator to challenge granulocytes in the stimulated sample. The preparation of the latter was identical to that of the unstimulated sample, except for the replacement of fresh PBS with 10 µL of PMA at a concentration of 10^{-5} mol L^{-1}.

The PMA solution had been prepared in advance by diluting 1 mg of PMA in 8.106 mL of dimethyl sulfoxide (Sigma D 5879, Sigma-Aldrich) creating 0.1 mL aliquots (stock solutions) which were stored at −20 °C as long as necessary. A working solution of 10^{-5}M was produced

daily, by adding 9.9 mL of fresh PBS to 0.1 mL of 10^{-3}M PMA. PBS was prepared by adding 1 tablet to 200 mL distilled water and stored at −20 °C until needed. A stock solution of 10^{-2} M luminol was produced by dissolving 0.0177 g of luminol in 1 mL of dimethyl sulfoxide and 9 mL of fresh PBS, using a magnetic hotplate stirrer. Attention was paid to prevent luminol to be exposed to light and 0.2 mL aliquots were stored, wrapped in foil, at −20 °C. A luminol working solution of 10^{-4} M was produced from stock solution by dilution with 19.8 mL PBS and was refrigerated in the dark until required.

Chemiluminescence of both samples (ns and s), measured in relative light units (RLU), was recorded at intervals of 5 min using a high sensitivity portable chemiluminometer (Junior LB 9509, E G & G Berthold, Germany) for a total of 45 min in order to produce a luminescence profile (Figure 22). The measurements were done in the field (Figure 23) immediately after the blood samples were collected. When not in the chemiluminometer, tubes were incubated at 37 °C in a lightproof thermostatic bath.

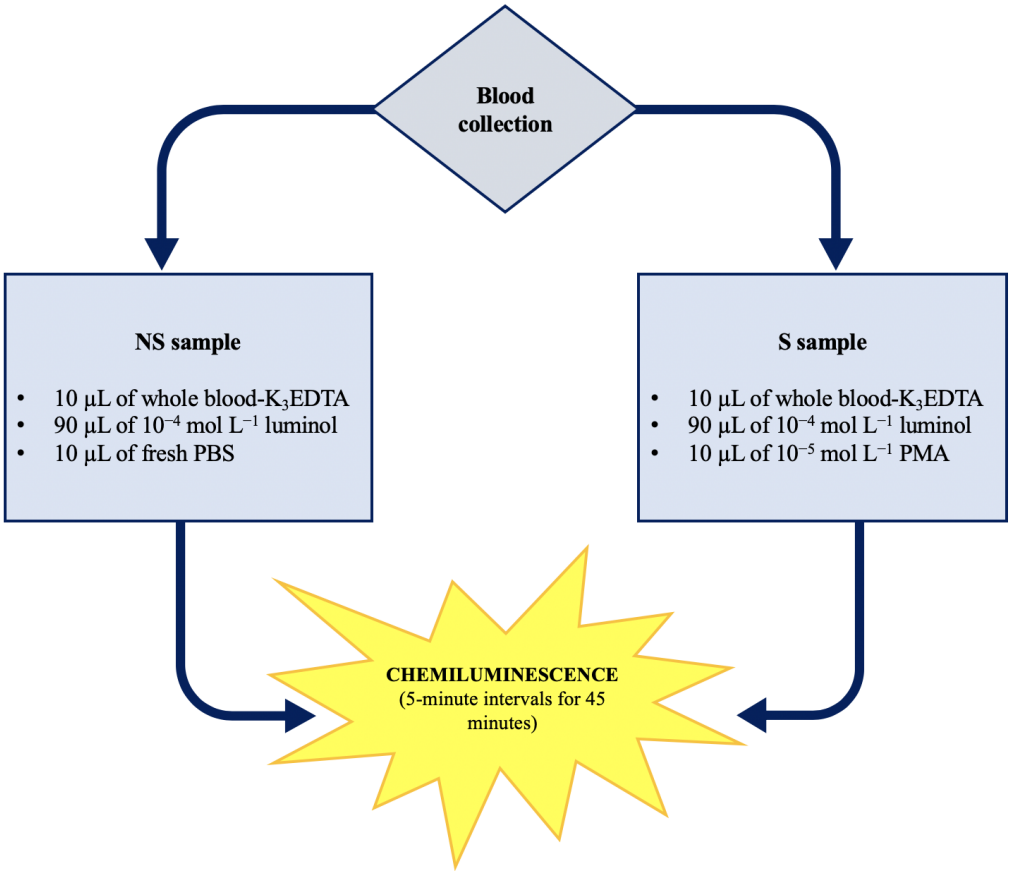

Figure 22. Chemiluminescence workflow diagram

Visual representation of samples preparation for the chemiluminescence essay.

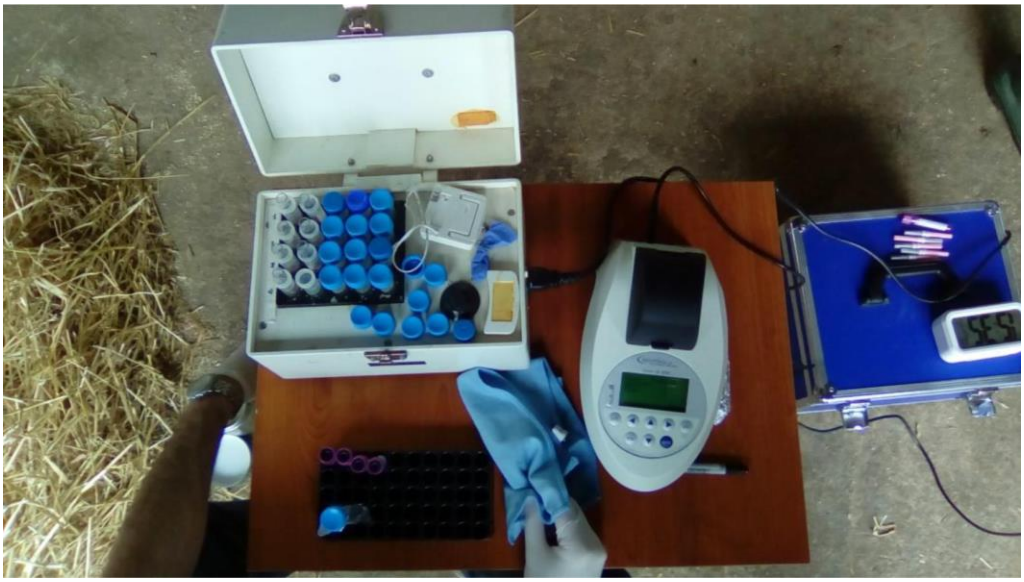

Figure 23. In situ whole-blood LCC

Picture of the field setting for the chemiluminescence essay. The portable thermostatic bath and the portable chemiluminometer are displayed from left to right, respectively.

3.2.4 Statistics

Data were analysed using the SAS statistical software (version 9.3, SAS Inst. Inc., Cary. NC). Normality of data distribution was assessed by adopting the Shapiro-Wilk test. For LCC data, some values were calculated: Delta LCC (the difference in response at each time interval between ns and s measures), Max Delta LCC (maximum Delta LCC value per time point), AUC (area under the Delta LCC curve), and AUC/GRAN (AUC/granulocytes number).

Analysis of repeated and normally distributed data (Delta LCC, white blood cells count and AUC/GRAN) was performed through a repeated type mixed model analysis of variance. Time points (−1 h, 30 min, 3 d, 7 d, 14 d), CAS/SHAM groups and their interaction were included in the model as fixed effects, intervals (0, 5, 10, up to 45 min) as time effect and animal as the random repeated effect. Non-repeated and normally distributed measures (Max Delta LCC, AUC, scrotal, perineal and environmental temperature) were analysed using a mixed model with time points (−1 h, 30 min, 3 d, 7 d, 14 d), CAS/SHAM groups and their interaction as fixed effects and animal as random effect. Non-normally distributed data were first log-transformed before being analysed through the same model.

Data were reported as least squares means ± standard error for normally distributed variables (transformed data were back log transformed).

Correlation among variables was calculated. Pearson coefficient was used for normally distributed data, whereas the Spearman rank correlation index was applied to non-normally distributed variables. Pain scores were analysed by computing the percentage of scores ≥ 1. These percentages were compared using the chi-square test and Marascuilo procedure.

3.3 Results

3.3.1 LCC

Analysis of data for CAS group revealed that Delta LCC values were associated with a significant ($P < 0.05$) increase at 3 d from −1 h and 30 min, and reduction at 7 d from −1 h, 30

min, and 3 d. Delta LCC values at −1 h, 30 min, and 14 d showed no statistically significant difference. Analysis of data for the SHAM group revealed that Delta LCC values did not significantly vary among the study times. Furthermore, significant differences in Delta LCC values between CAS and SHAM were noted only at 7 d ($P < 0.0001$); no differences between the experimental groups were documented at the other time points (Figure 24).

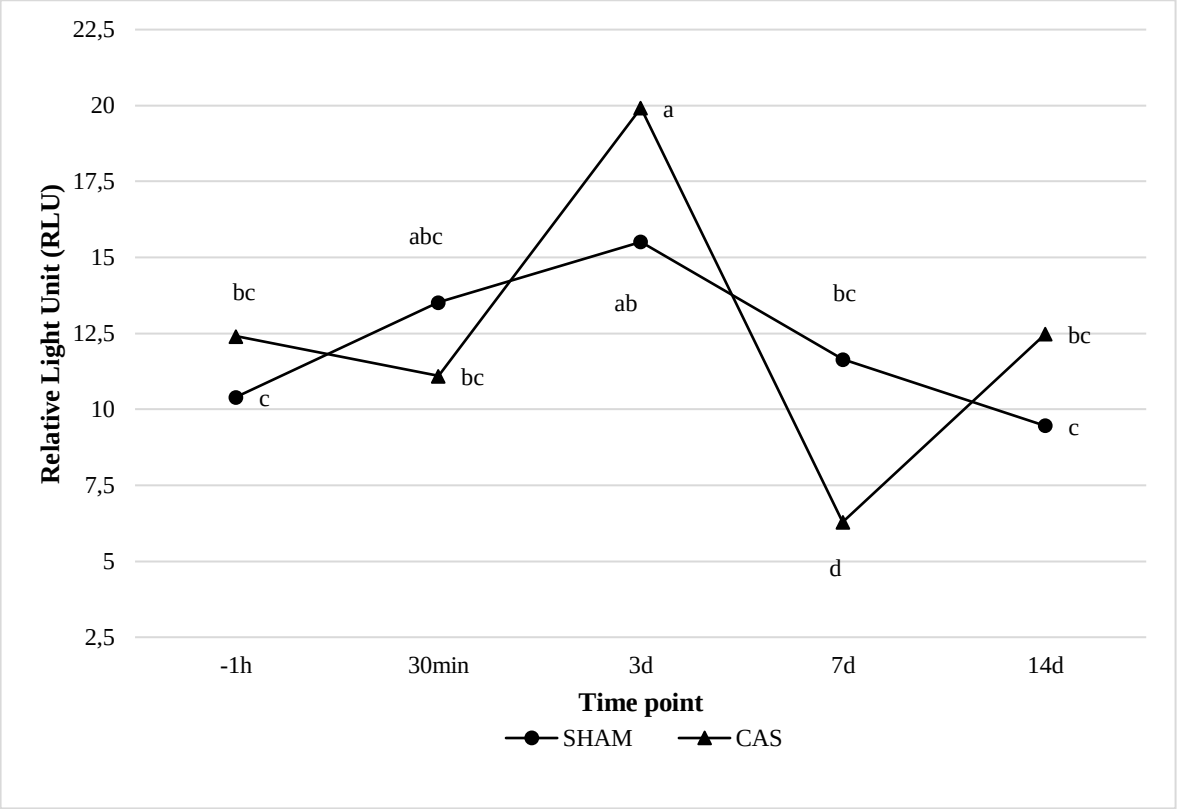

Figure 24. Delta LCC (calves)

Difference in Leukocyte Coping Capacity (LCC) luminescence values at each time point between non-stimulated and stimulated measures in both castrated (CAS) and control (SHAM) groups (Delta LCC). Results are reported in Relative Light Unit (RLU). Lowercase letters indicate differences between and within groups at different time points ($P<0.05$). Time points are reported as follows: -1h (1 hour before castration/manipulation), 30min, 3d, 7d, 14d (30 min, 3, 7, and 14 days post-castration/manipulation).

As far as Max Delta LCC is concerned, there were no differences neither inside each group nor be- tween CAS and SHAM. Recorded peak values were 37 and 29 RLU in CAS and SHAM, respectively, at 3 d.

AUC data analysis resulted similar to the one provided by Delta LCC for CAS and SHAM among time points: AUC values in CAS increased with statistical significance at 3 d and significantly diminished at 7 d ($P < 0.05$). Whereas, for SHAM, no statistically significant variation was noted among time points (Figure 25).

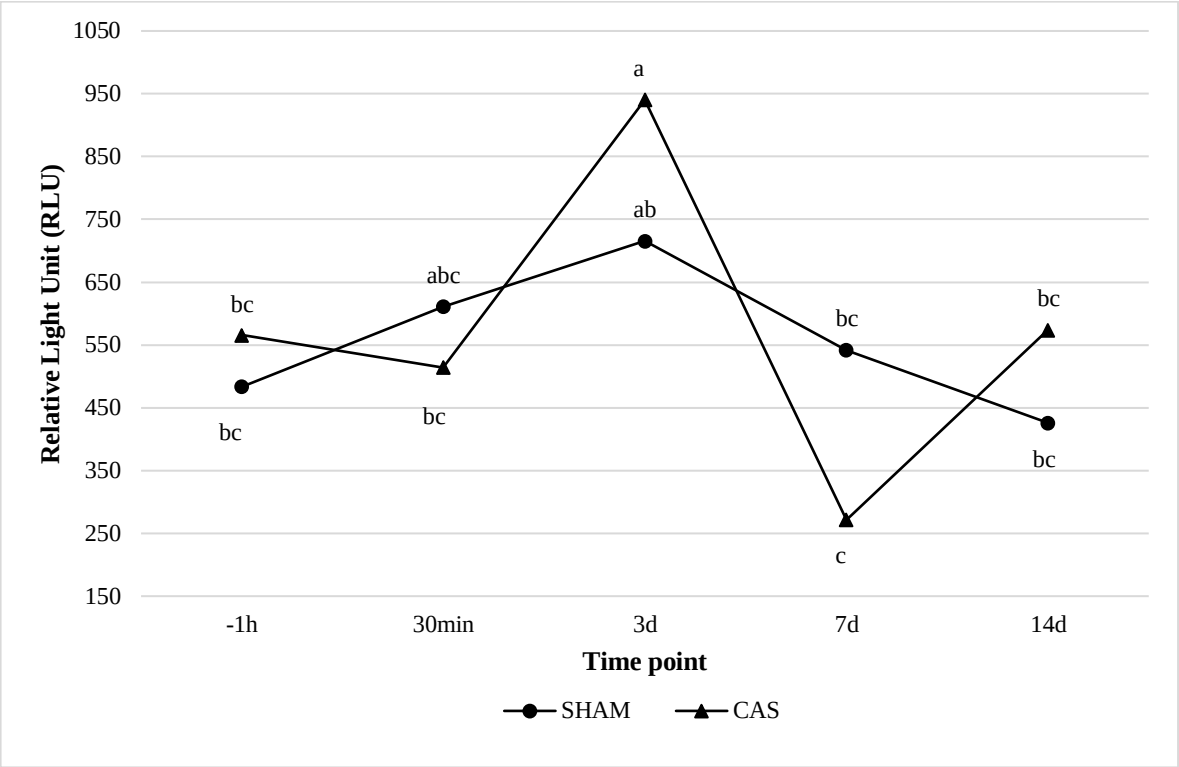

Figure 25. AUC (calves)

Area under the Delta LCC curve (AUC) values at each time point in both castrated (CAS) and control (SHAM) groups. Results are reported in Relative Light Unit (RLU). Lowercase letters indicate differences between and within groups at different time points ($P<0.05$). Time points are reported as follows: -1h (1 hour before castration/manipulation), 30min, 3d, 7d, 14d (30 min, 3, 7, and 14 days post-castration/manipulation).

3.3.2 Scrotal lesion score

No scrotal abnormalities were recorded at the first time point in CAS; all animals' scrotal sacs were considered to be in good condition after clinical examination (all scores were 0). At 30 min, no gross lesions were noted and the scrota presented a clinical condition similar to that recoded at −1 h (all scores were 0). Some scrotal swelling was documented at 3 d; the scrotal surface was dry and without any other apparent sign of inflammation (average score 3.8; range

3 to 4). Mummification and partial detachment of the scrotum were the most evident clinical signs at 7 d. Also, the scrotal sacs were cold and stiff at palpation (average score 2.7; range 2 to 3). After the surgical removal of the strangled portion at day 10, the abdominal wall began a healing process, resulting in a neat circular scar at 14 d (average score 0.2; range 0 to 1). SHAM exhibited normal scrotal condition throughout all the time points (all scores were 0) (Table 5).

3.3.3 Temperatures

No significant variation was noted in perineal temperature (35.5 ± 0.8 °C) at any time point within the SHAM group. In the same group, scrotal temperature significantly decreased at 30 min (28 ± 2.3 °C; $P < 0.05$) from −1 h (31.8 ± 1.8 °C), increased at 3 d (33.1 ± 1.9 °C; $P < 0.05$) from 30 min and at 14 d (average 33.8 ± 1.6 °C; $P < 0.05$) from −1 h and 30 min. No significant variation was noted in perineal temperature within CAS group (36.1 ± 1.3 °C), whereas the scrotal temperature significantly decreased at 30 min (28.2 ± 2.8 °C; $P < 0.05$) from −1 h (31.4 ± 3.2 °C), increased at 3 d (29.9 ± 1.9 °C; $P < 0.05$) from 30 min, but still remained significantly lower than at −1 h. Also, the temperature of the wound recorded at 14 d (35.8 ± 0.9 °C; $P < 0.05$) increased from the scrotal temperature registered at 7 d (30.1 ± 1.7 °C). Differences among the 2 groups were noted in scrotal temperatures only at 3, 7, and 14 d ($P < 0.05$) (Figure 26). The environmental temperature recorded throughout the study did not change significantly at any time point (22.1 ± 0.7 °C).

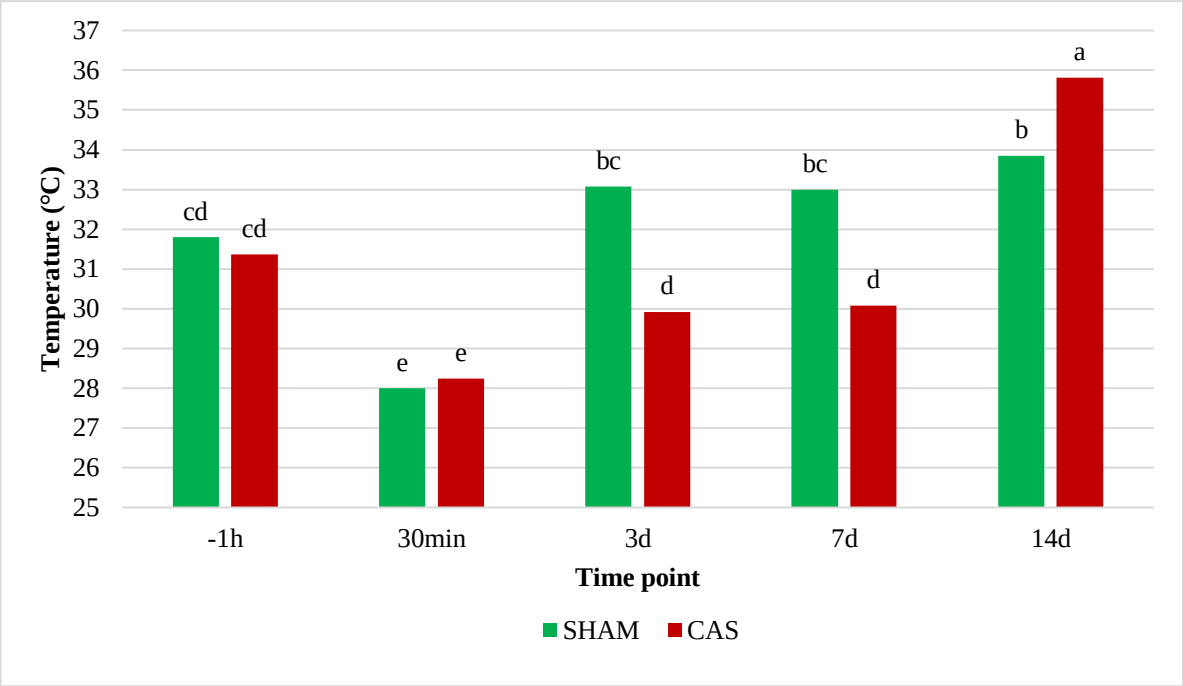

Figure 26. Scrotal and wound temperatures

Scrotal and wound temperature variation between time points in castrated (CAS) and control (SHAM) groups. Results are reported in Celsius degrees (°C). Lowercase letters indicate differences between and within groups at different time points ($P<0.05$). Time points are reported as follows: -1h (1 hour before castration/manipulation), 30min, 3d, 7d, 14d (30 min, 3, 7, and 14 days post-castration/manipulation).

3.3.4 Pain Scores

All 3 observers gave the animals the same score at each time point and, as expected, no pain signs were noted in the SHAM at any time point (all animals were scored 0 at each time point). The animals belonging to CAS showed very little pain signs at each experimental time. For this group, the mean value was 0.3 out of 10 at −1 h (range 0 to 2), 0.6 at 30 min (range 0 to 4), 0.3 at 3 d (range 0 to 2), 0.2 at 7 d (range 0 to 1), and 0.1 at 14 d (range 0 to 1) (Figure 27; Table 5). The percentage of animals which obtained ≥ 1 was: 10% at −1 h, 30% at 30 min, 20% at 3 and 7 d, and 10% at 14 d.

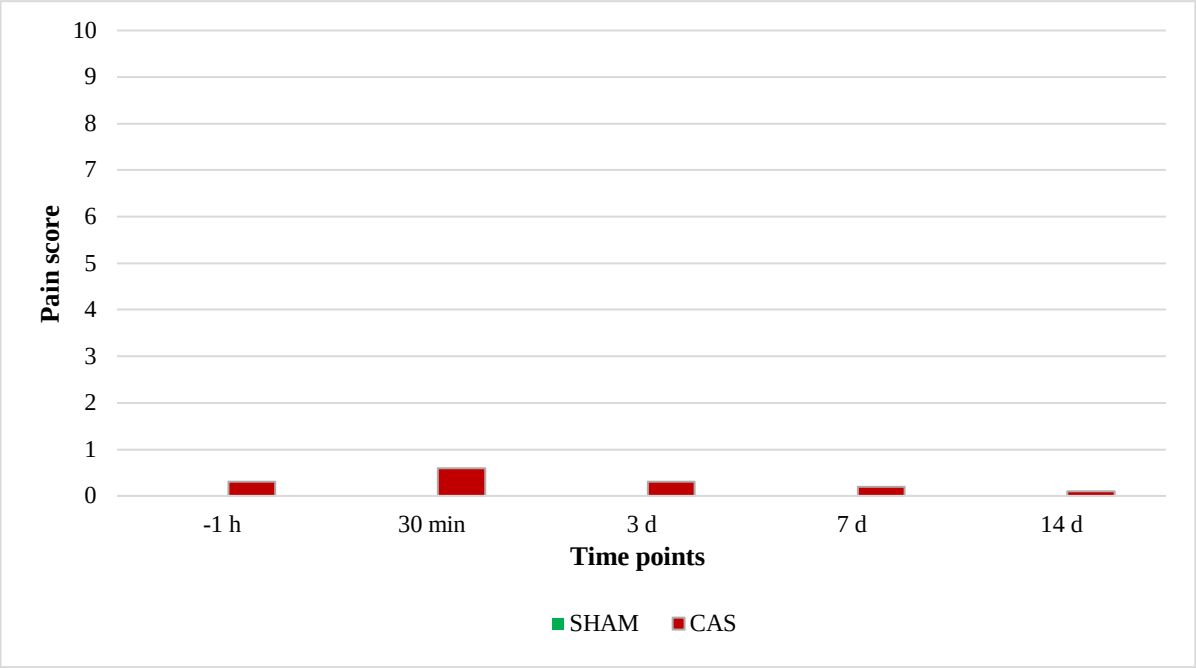

Figure 27. Pain scores

Mean pain scores recorded during the study times in cattle undergoing surgical castration (CAS) and in the control group (SHAM). Scores ranged from 0 to 10 according to the UNESP-Botucatu unidimensional composite pain scale for postoperative pain assessment in cattle.

3.3.5 Blood cortisol level

Cortisol data did not show any significant variation within CAS between time points. The average values were 7.54, 11.09, 8.60, 13.02, and 8.73 nmol/L at −1 h, 30 min, 3 d, 7 d, and 14 d, respectively. Whereas, in SHAM, differences were noted between 30 min and 3 d ($P < 0.05$) and also 30 min and 14 d ($P < 0.05$). The average values per time point were 8.48, 2.91, 10.98, 5.28, and 13.06 nmol L^{-1} at −1 h, 30 min, 3 d, 7 d, and 14 d, respectively (Figure 28; Table 5). No significant differences were recorded comparing CAS and SHAM at any time point. Spearman test did not reveal any correlation between cortisol and LCC AUC data, nor between cortisol and Max Delta LCC data.

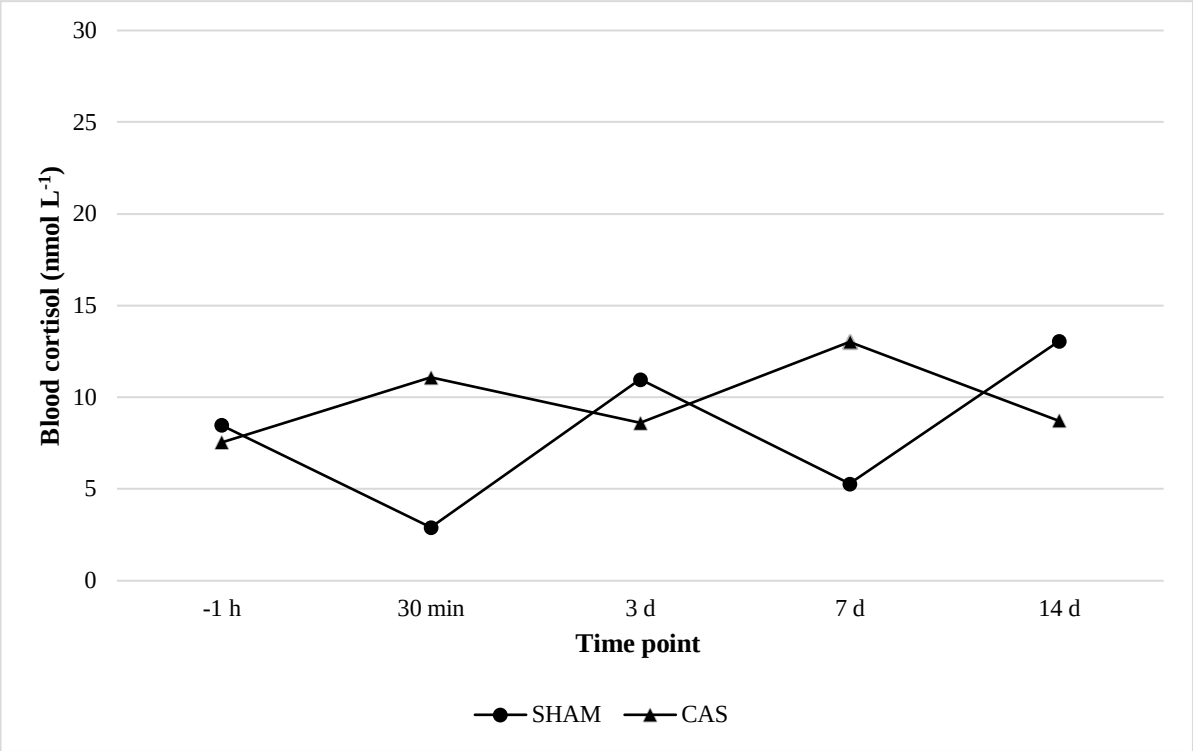

Figure 28. Blood cortisol (cattle)

Mean blood cortisol values recorded during the study times in cattle undergoing non-surgical castration.

3.3.6 White blood cells

Complete blood counts and biochemistry parameters were within the range of reference for the species (Kessell 2015). All animals were considered to be in good health status. No stress leukograms were noted at any of the time points in either CAS or SHAM. The prevailing white blood cell type was represented by lymphocytes (range 63.0 to 69.4%), followed by neutrophils (range 22.3 to 27.9%), monocytes (range 2.8 to 7.1%), eosinophils (range 1.2 to 3.1%), and basophils (0.8 to 1.1%).

In addition, no significant variation in white blood cells count was registered neither inside each group nor between CAS and SHAM (range 8.90 to 10.65 × 10^3cells μL^{-1} in CAS; range 8.45 to 9.39 × 10^3 cells μL^{-1} in SHAM) (Table 5).

No statistically significant differences were documented in the Neutrophil/Lymphocyte ratio (N:L) between time points in any of the groups (range 0.34 to 0.42 for CAS; range 0.39 to 0.49

for SHAM). AUC/GRAN did not change over time in CAS group, whereas it significantly increased (P = 0.0009) and decreased (P = 0.0095) at 3 and 7 d from values at -1 h, respectively. After correlating max Delta and AUC values with the number of both total white blood cells and every single white blood cell population, we observed that only a moderate correlation exists (Spearman correlation: WBC-maxDelta 45.99%; WBC-AUC 45.30%)

Table 5. Physiological parameters and scores (calves)

Total white blood cells count (WBC), different white blood cells populations (Neutrophils, Lymphocytes, Monocytes, Eosinophils and Basophils), blood cortisol, AUC/granulocytes number (AUC/GRAN), pain scale score, and scrotal lesion score at each time point in CAS and SHAM group.

	Time point									
	-1h		**30min**		**3d**		**7d**		**14d**	
	CAS	**SHAM**	**CAS**	**SHAM**	**CAS**	**SHAM**	**CAS**	**SHAM**	**CAS**	**SHAM**
WBC (10^3 cells μL^{-1})	8.90 ± 2.67	9.39 ± 2.49	8.89 ± 2.67	9.39 ± 2.49	9.01 ± 2.26	9.36 ± 1.48	9.37 ± 1.99	9.36 ± 1.48	10.65 ± 3.72	8.45 ± 1.69
Neutrophils (10^3 cells μL^{-1})	2.14 ± 1.43	2.66 ± 1.39	2.14 ± 1.43	2.66 ± 1.39	2.37 ± 1.37	2.65 ± 0.92	2.44 ± 1.13	2.65 ± 0.92	2.99 ± 2.78	2.10 ± 0.71
Lymphocytes (10^3 cells μL^{-1})	6.04 ± 1.23	5.84 ± 1.55	6.04 ± 1.23	5.84 ± 1.55	5.80 ± 1.66	5.90 ± 0.98	6.12 ± 1.52	5.89 ± 0.98	7.05 ± 1.55	5.57 ± 1.21
Monocytes (10^3 cells μL^{-1})	0.47 ± 0.19	0.52 ± 0.17	0.47 ± 0.19	0.52 ± 0.17	0.61 ± 0.20	0.39 ± 0.11	0.58 ± 0.20	0.39 ± 0.11	0.28 ± 0.15	0.42 ± 0.17
Eosinophils (10^3 cells μL^{-1})	0.15 ± 0.12	0.23 ± 0.28	0.15 ± 0.12	0.23 ± 0.28	0.1 ± 0.06	0.31 ± 0.24	0.12 ± 0.06	0.31 ± 0.24	0.20 ± 0.1	0.24 ± 0.26
Basophils (10^3 cells μL^{-1})	0.07 ± 0.02	0.11 ± 0.04	0.07 ± 0.02	0.11 ± 0.04	0.08 ± 0.04	0.10 ± 0.04	0.07 ± 0.03	0.1 ± 0.04	0.12 ± 0.04	0.08 ± 0.02
Blood cortisol level (nmol L^{-1})	7.54 ± 5.82	8.48 ± 8.24	11.08 ± 8.00	2.91 ± 3.82	8.60 ± 7.75	10.98 ± 9.61	13.02 ± 12.74	5.28 ± 5.36	8.73 ± 7.41	13.06 ± 9.46
AUC/GRAN (RLU/10^3 cells μL^{-1})	199.6 ± 63.2	136.7 ± 48.4	200.6 ± 73.6	173.5 ± 46.0	330.8 ± 128.9*	219.0 ± 82.7	87.3 ± 44.8*	168.3 ± 85.0	190.8 ± 80.7	160.4 ± 62.9
Pain Scores	0.3 (0-2)	0 (0-0)	0.6 (0-4)	0 (0-0)	0.3 (0-2)	0 (0-0)	0.2 (0-1)	0 (0-0)	0.1 (0-1)	0 (0-0)
Scrotal lesion score	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	3.8 (3-4)	0 (0-0)	2.7 (2-3)	0 (0-0)	0.2 (0-1)	0 (0-0)

3.4 Discussion

To our knowledge, this study is the first to present the whole-blood LCC assay in the bovine species in field conditions, although ROS production from isolated bovine leukocytes has been evaluated in the past years (Hoeben *et al.* 2000; Mehrzad *et al.* 2005; Rinaldi *et al.* 2007; Pang *et al.* 2009). The validity of this technique seems to reside in maintaining leukocytes in their natural environment, preserving cellular integrity, thus minimizing potential disruption to cell signalling pathways (Shelton-Rayner *et al.* 2011, 2012; Huber *et al.* 2017b). Studies on human leukocytes demonstrated that their surface is covered with over 150 receptors (Mian *et al.* 2005), capable of interacting with a large number of stress-sensitive factors, like: endocrine factors in the plasma, cytokines, and substances released from circulating and noncirculating cells (*e.g.* endothelial cells), and also changes in erythrocyte haemodynamics, blood biochemistry, hypothalamic-pituitary-adrenal axis, and the sympathetic nervous system. The constant exposure to each of these stimuli pertains to their effectiveness as stress indicators (Shelton-Rayner *et al.* 2012). In our study, LCC values at 3 d of the animals undergoing ring castration showed an increase when compared to their own LCC values at other time points and also to values of SHAM group animals. This result might seem in contrast with our first and second hypothesis. In fact, we would have expected a reduction in LCC values in CAS group after castration. Rubber ring gradual constriction of the scrotum might be responsible for the nonsignificant change in LCC values seen at 30 min. Whereas, a possible explanation to the augment in LCC values at 3 d might be attributable to the systemic diffusion of inflammatory factors coming from the scrotal sac (oedematous scrotal sac). We believe that the local inflammation/ischemia started a chain reaction that eventually led to a systemic activation of the immune system, inducing leukocytes to temporarily augment their ROS production (which is what we observed at 3 d). This fact might have led to a subsequent impairment in ROS production at 7 d, causing an "exhaustion state" in granulocytes, thus potentially resulting in

augmented susceptibility to infections (McLaren *et al.* 2003). Also, at 7 d, in addition to the persistent inflammation, mummification and partial detachment of the scrotum might have caused a stressful condition leading to a decrease in ROS production.

Our results at 7 d seem to confirm that LCC is affected by pain-related discomfort/stress, leading us to confirm our first and second hypothesis. In fact, we think that LCC technique might be able to detect physiological changes accountable to stress/pain caused by ring castration, especially during the degenerative phase of the scrotal tissues starting from the 7th day after castration. This is very similar to what was reported by Molony *et al.* (1995). They demonstrated that methods of castration involving the use of rubber rings in calves produced a long-lasting inflammation. Furthermore, he observed that if the scrotum is left untouched, wound healing could be incomplete even after 51 d, with a peak in lesion severity between 27 and 30 d. Similar results were obtained by Fisher *et al.* (2001) and Thüer *et al.* (2007). Considering this, we wanted to avoid unnecessary pain and stress to the animals opting for surgical removal of the degenerating tissues after 10 d from castration, leading to a complete wound sealing at 14 d.

Whole-blood LCC chemiluminescence seems to be an interesting tool for stress/pain assessment in calves after castration, even though it must be pointed out that results coming from this technique in the bovine species are not always easy to be interpreted. In fact, bovines are known to have an "inverted" white blood cell formula, in which lymphocytes are predominant whereas neutrophils are the second population in order of numerosity (Sjastaad *et al.* 2010; Roland *et al.* 2014). This fact might explain the lower whole blood chemiluminescence values, because of the lower number of neutrophils (which are known to be the main ROS producers) per blood volume, if compared to values coming from other mammalian species like bears, badgers, bank voles, or humans (Montes *et al.* 2004; Gelling *et al.* 2009; Shelton-Rayner *et al.* 2010, 2011; Esteruelas *et al.* 2016). Because this is the first

study that evaluates whole-blood LCC after castration, the exact time at which LCC values would have changed from baseline was not known to the authors. In fact, our study times were chosen on the basis of what reported in the literature about blood cortisol changes after castration (Molony *et al.* 1995; Fisher *et al.* 2001; Stafford *et al.* 2002; Marti *et al.* 2010; Becker *et al.* 2012). Hence, to better understand the effect of ring castration on ROS production observed in this study, it would be interesting to evaluate changes in LCC values at more frequent intervals.

As for temperature, its decrease recorded in both groups at 30 min may be accountable to the presence of the povidone-iodine detergent previously applied on the scrotal surface at castration and that led to heat loss. Furthermore, compression caused by rubber ring is known to be gradual and not sudden (Stafford *et al.* 2002), thus the temperature decrease seen at 30 min should not be due to blood flow impairment. Temperature returned to baseline values at 3 and 7 d in SHAM, but not in CAS. This result can be explained by the blood flow impairment at the level of the testicular artery caused by mechanical compression of the rubber ring, leading to hypoxia of the tissues (Marti *et al.* 2010; Fubini and Ducharme 2017). The temperature increase registered at 14 d in CAS from all other time points might be accountable to the healing process of the tissues resected at day 10 post-castration and the fact that the measurement was performed at the level of the wound, because of the absence of the scrotal sac.

Diagnosing pain in nonverbal patients has always been a challenge for veterinary practitioners. In fact, pain in animals can only be measured indirectly using pain scoring systems or pain scales based on behavioural assessment. These tools represent a valuable diagnostic aid, as they provide pain assessors with objective, ready-to-use tools (Della Rocca *et al.* 2017). Because methods of castration are typically associated with physical, chemical, or hormonal damage to the testicles (Stafford and Mellor 2005), it is legitimate to discuss whether these procedures can be a source of pain for the animal and, if necessary, it is important to efficiently assess its

presence. Animal behaviour has been shown to be a sensitive indicator of pain in response to castration in cattle (Robertson *et al.* 1994). In 2014, the UNESP-Botucatu Unidimensional Composite Pain Scale (UCPS) for assessing postoperative pain in cattle was developed and validated by de Oliveira *et al.* This pain scale is nowadays considered as the only tool specifically designed for pain assessment in bovine species (Della Rocca *et al.* 2017) although it is validated for postoperative pain only. It is not clear whether ring castration causes pain and what type of pain (*e.g.* chronic, acute or both) in calves (Mellor *et al.* 1991; Robertson *et al.* 1994; Molony *et al.* 1995; Stafford *et al.* 2002; Boesch *et al.* 2006; Thüer *et al.* 2007; Becker *et al.* 2012; Marti *et al.* 2017). A major hypothesis points out that occlusion of the blood vessels to the testes might not immediately disable the afferent nerves or nociceptors and increased afferent activity may be accountable to the sensitization of nociceptors after hypoxia of tissues (Gebhart and Ness 1991; Handwerker and Reeh 1991). Also, failure to seal the distal scrotal portion from the rest of the body might lead to exposure of living tissues to algogens, pathogens, and toxins carried by fluids coming from the degenerating scrotum (O'connor *et al.* 1993). Interestingly, in our study, the UNESP-Botucatu pain scale did not measure highly painful conditions at any time point. The highest score was 4 out of 10 measured at 30 min in 1 animal (CAS group) showing discomfort signs, probably due to the new condition caused by the rubber ring and povidone solution application, such as tail flicking, licking its scrotum and slight apathy. These results might be explained by the intrinsic nature of calves. Cattle are known to be grazing animals which are generally predated upon, and man might be easily considered a predator. For this reason, pain experiences can be expected to have different priorities in these species and to influence behaviour in different ways (Molony *et al.* 1995). In accordance with this, we believe that the incidence of pain manifestation in these animals might be low and subject to high variability. Thus, it is recommended to extend the observation period, provide different schedules of observation and always compare findings with physiological changes.

Also, it must be taken into account that the UNESP-Botucatu pain scale is specifically designed to measure postoperative pain. In fact, the low pain scale values can be attributable to bias due to the ring-castrated calves experiencing a different kind of pain to which the scale is not sensitive enough.

Serum cortisol measurement has been widely used as an indicator of stress in animals (Möstl and Palme 2002). Specifically, some reports using blood cortisol level as a tool for comparing different methods of castration in cattle are documented in the literature (Cohen *et al.* 1990; King *et al.* 1991; Faulkner *et al.* 1992; Robertson *et al.* 1994; Fisher *et al.* 1996, 1997, 2001; Stafford *et al.* 2002; Thüer *et al.* 2007; Becker *et al.* 2012). Also, it is reported that rubber banding castration may be less stressful than surgical castration if performed appropriately (Bretschneider 2005). Conflicting results can be found in the literature regarding blood cortisol variations after rubber ring castration. In fact, some authors report an increase in blood cortisol level which is greater in castrated animals than in control calves (Stafford *et al.* 2002; Thüer *et al.* 2007; Pang *et al.* 2009; González *et al.* 2010), whereas others did not find any significant change between the 2 groups (Mellor *et al.* 1991; Fisher *et al.* 2001; Becker *et al.* 2012). In our study, no significant difference in blood cortisol changes was found between the castrated and the control group. This result is in accordance with what already documented by Mellor *et al.* (1991), Fisher *et al.* (2001), and Becker *et al.* (2012). It is important to remember that cortisol is rapidly cleared from the bloodstream (Plumb 1994); therefore, the timing of blood sampling after ring castration might influence the interpretation of the results (Bretschneider 2005). For this reason, to better evaluate the cortisol response, the time intervals between blood samplings could be narrowed. In addition, no significant blood cortisol peaks were documented within animals from CAS group and their average values at 30 min (11.09 ± 8.00 nmol L^{-1}) post-castration were lower than those reported by other authors in rubber ring castrated cattle at the same study time (range 40 to 76 nmol L^{-1}), which seems to be the time at which blood cortisol

peaks (Stafford *et al.* 2002; Thüer *et al.* 2007). Evaluating blood cortisol level is indeed important when assessing stress in animals. Unfortunately, it is not a perfect tool. In fact, blood cortisol level might be influenced by many factors, including the mental status of the animal at sampling and a high individual variability. Furthermore, low blood cortisol values may be due to individuals having high pain thresholds (Stafford and Mellor 2005) and being less likely to get stressed by noxious stimuli like the one caused by rubber ring castration. Serum cortisol levels must be interpreted with caution, as they may not always accurately reflect the extent of the pain response in animals, because of this variability (Coetzee 2011). As a confirmation of this, blood cortisol levels of SHAM group significantly fluctuated between the study times even if no painful stimulus was inflicted to these animals.

Stress is known to cause neutrophilia, lymphopenia, and eosinopenia in the bovine species (Kessell 2015), conditions characterizing the so called "stress leukogram." As a consequence of this, an increased N:L can be observed (Tornquist and Rigas 2010). In the present study, neither stress leukogram nor altered N:L was documented at any time point in castrated animals or in SHAM group. This finding is in accordance with what reported by Wistuba *et al.* (2004) and (Pang *et al.* (2009) who did not find any white blood cell count variation in calves after band castration.

Even though HPA-mediated stress is known to be involved in several aspects of pain appraisal in animals and humans (Blackburn-Munro 2004) and downregulate immune system function (Sapolsky *et al.* 2000; Tsigos and Chrousos 2002), LCC values (AUC and max Delta LCC) did not correlate with cortisol values and weakly correlated with both the total number of white blood cells and each white blood cell population. Therefore, we rejected our third hypothesis that LCC values would correlate with the other physiological parameters. This is in accordance with what was found by Shelton-Rayner *et al.* (2012) and Esteruelas *et al.* (2016). They attributed the absence of correlation between LCC values and physiological variables to the fact

that they are influenced by a wide range of factors in addition to stress. Moreover, because of the weak correlation between LCC values and granulocytes, we hypothesize that, in our study, ROS production, and thus LCC values documented, might not have been predominantly related to the number of granulocytes present in the chemiluminescence tube, but rather to their current status and capability of producing ROS. As a further proof of this, AUC/GRAN values significantly decreased at 7 d in CAS group in the present study.

Gaining more insight into stress and pain caused by routine procedures in animal husbandry is fundamental, especially nowadays that societal concern regarding the moral and ethical treatment of animals has become more relevant (Rollin 2004). The model of pain we chose in this study is just one of the many procedures that veterinarians and zootechnicians must deal with during everyday practice. As suggested by our results, ring castration seems to cause long-lasting pain in calves, but its magnitude may not be easily detected by conventional pain assessment methods. For this reason, we argue that whole-blood LCC chemiluminescence might be a new useful tool for assessing pain and stress in farm animals undergoing ring castration and further studies should be carried out to test its efficacy on other routine husbandry procedures that are considered to be painful. Furthermore, as reported in the literature and suggested by our results, stress measures based on immune system alterations seem to be valid alternatives to measures based on the HPA axis, and may even be more suitable in certain circumstances (McLaren *et al.* 2003; Gelling *et al.* 2009). Nevertheless, given the complexity of the subject and to provide a clearer picture of the multifaceted effects of stress and pain, we suggest a combined approach using more than one parameter.

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4 Whole-blood LCC chemiluminescence in horse stallions undergoing surgical castration

4.1 Introduction

Pain assessment is imperative for good horse welfare and it is essential for treating horses in pain with appropriate analgesic therapy, which is adjusted to meet the needs of the individual patient (Daglish and Mama 2016; Guedes 2017; Gleerup 2019). Unfortunately, pain recognition in this species is particularly challenging as they have evolved not to openly express pain in order to avoid predation (Taylor *et al.* 2002). At present, there is no gold standard technique for assessing pain in horses, even though some authors would agree on the fact that behavioural traits seem to be the most informative clinical signs, therefore making pain scales a very important tool to be used (Gleerup 2019). Nevertheless, behavioural assessment is dependent on the observational skills of the practitioner, and thus it could be subjected to bias. Physiological parameters (*e.g.* cortisol, heart rate, etc.) used individually also showed not to be sensitive enough because of their inter-individual variability and interaction with confounding factors (Landa 2012; Gaynor and Muir III 2015). In order to address the lack of specific pain assessment tools, the conjunct evaluation of multiple physiological (*e.g.* blood cortisol level) and behavioural variables (quantified by pain scales) becomes essential.

The whole-blood leukocyte coping capacity (LCC) chemiluminescence is an innovative tool that allows for the assessment of stress through the evaluation of reactive oxygen species (ROS) production by granulocytes (mainly neutrophils). A decrease in ROS production is indicative of stress in humans and different animal species, like: water voles, bank voles, wood mice, roe deer, badgers, brown bears, and calves (McLaren *et al.* 2003; Montes *et al.* 2004; Moorhouse *et al.* 2007; Gelling *et al.* 2009; Shelton-Rayner *et al.* 2010, 2012; Esteruelas *et al.* 2016; Huber,

Vetter, *et al.* 2017; Gaudio *et al.* 2018). This tool has been addressed as an in vitro assessment of the individual's current physiological status by McLaren *et al.* (2003) and has the advantage of being quickly performable in the field by means of a portable chemiluminometer.

Pain and stress are closely related, and they affect each other as they share several pathways (*e.g.* the hypophyseal-pituitary axis) and effects (*e.g.* autonomic, emotional, sensory, motivational and motor effects). For the intertwined nature of these dimensions, pain is in fact considered a stressor (Johnson 2016; Vierck 2006; Vierck *et al.* 2010). How pain and stress affect the immune system is not completely understood yet, but it is believed that the constant exposure of white blood cells receptors to a large number of stress-sensitive factors (*e.g.* endocrine factors, cytokines, changes in erythrocyte haemodynamics, blood biochemistry, etc.) could be accountable to their effectiveness as stress indicators (Shelton-Rayner *et al.* 2012). Along this line of thinking, Gaudio *et al.* (2018) investigated the potential of the whole-blood LCC as an indicator of pain-related stress in calves following non-surgical castration and reported promising results.

The present study proposed to test the whole-blood LCC chemiluminescence as an innovative tool for pain assessment in horse stallions undergoing surgical castration. The LCC results have also been compared with conventional pain/stress assessment parameters (*e.g.* pain scale, blood cortisol, heart rate, leukogram analysis) for further verification.

4.2 Materials and methods

4.2.1 Study design

The study was performed after the approval of the Animal Welfare Body of the University of Padua at the "Ospedale Veterinario Universitario Didattico" animal hospital of the University of Padova between March and June 2017. Eight stallions (*Equus ferus caballus*) (average age: 4 years old) belonging to different breeds and weighing 418.7 ± 34.6 kg (mean ± SD) were used. The horses were all healthy on the basis of clinical examination and blood exam results

and were scheduled for elective surgical castration. The animals were transported from their owner's property to the animal hospital and individually housed in boxes (measuring 4x6 m) where they were fed hay, had free access to water and could acclimate for a period of 24 hours before surgery. The animals were starved starting at 8 hours prior to surgery.

One hour before castration, all animals were assessed for pain for by 3 trained observers, using a scoring system ranging from 0 to 17, by means of the UNESP-Botucatu unidimensional composite pain scale for assessing post-operative pain in horses (Taffarel *et al.* 2015) (Table 6). This evaluation comprised remote visual assessments as well as clinical examination, including HR (heart rate) and palpation of the painful region. For the latter, each animal was gently approached and restrained by means of a lead rope. During restraint, the animal was also weighed, and blood sampling performed.

Table 6. UNESP-Botucatu multidimensional composite pain scale for assessing postoperative pain in horses

Pain scale used in the present study to monitor post-operative pain in stallions.

Variable	Criteria	Score
Positioning in the stall	The horse's head is at the outside door	0
	The horse is inside the stall, but looking at the outside door, observing the environment	1
	The horse is eating	0
	The horse is not close to the outside stall door and does not look interested in the environment	2
Locomotion	The horse moves freely alone	0
	The horse does not move, or is reluctant to move	1
	The horse is agitated, restless	2
Locomotion when led by the evaluator	The horse moves freely when led	0
	The horse does not move, or is reluctant to move when led	1
	The horse is agitated, restless	2
Response to palpation of the painful area (approximately 3 cm besides the wound)	No response or change in relation to pre-procedure palpation response of the surgical wound	0
	Mild reaction to palpation of the surgical wound	1
	Violent reaction to palpation of the surgical wound	2
Looking at the flank	The horse does not look at the flank	0
	The horse looks at the flank	1
Kicking at the abdomen	The horse does not kick the abdomen	0
	The horse kicks at the abdomen	1
Lifting hind limbs	No lifting of hind limbs	0
	Lifting hind limbs	1
	Lifting hind limbs and extending the head	2
Head movement	Head straight ahead most of the time	0
	Lateral and/or vertical occasional head movements	1
	Lateral and/or vertical continuous head movements	2
Pawing on the floor (fore limbs)	Quietly standing, no pawing	0
	Pawing	1
Heart rate (compared to initial values)	25-50% increase	1
	>50% increase	2

The horse was intravenously tranquillized with acepromazine (0.04 mg kg^{-1}) (Prequillan, Fatro S.p.A, 40064 Ozzano dell'Emilia, Italy) and after 20 min a 15 gauge over-the-needle catheter was placed and affixed to the right jugular vein for drugs and intravenous (IV) fluids administration. The animal was then moved to the induction box, premedicated with detomidine (0.2 mg kg^{-1}) (Domosedan, ESTEVE, 0841 Barcelona, Spain), and then anaesthesia was induced with a mixture of ketamine (2.2 mg kg^{-1}) (Ketavet, Intervet productions s.r.l., 04011 Aprilia, Italy) and diazepam (Ziapam, Laboratoire TVM, 63370 Lempdes, France) IV. The animal was loaded onto the surgical table, an endotracheal tube inserted, and anaesthesia

maintained with isofluorane (Isofluorane vet, Merial Italia S.p.A, 20090 Assago, Italy). Before the start of the surgery, pre-emptive analgesia was provided with butorphanol intramuscular administration (0.2 mg kg^{-1}) (Dolorex, MSD Animal Health, 20090 Segrate, Italy).

Each surgery was performed at the same time of the day (9.00 a.m.) using the open technique as described by Searle *et al.* (1999) and lasted on average 30 minutes in total. At the end of the surgical procedure, the animal was moved again into the induction box, and while laterally recumbent, xylazine (0.2 mg kg^{-1}) (Rompun, Bayer S.p.A., 20100 Milano, Italy) was administered to smoothen recovery phase. Endotracheal tube was removed upon re-establishment of the swallowing reflex. Seven hours after the surgery, a nonsteroidal anti-inflammatory (flunixin meglumine; 1.5 mg kg^{-1}) (Alivios, Fatro S.p.A., 40064 Ozzano dell'Emilia, Italy) was administered.

Blood sampling was repeated at the following time points: 1 h before castration (−1 h), 0 min after surgery (0 min) and at 4 (4 h), 6 (6 h), and 24 h (24 h) after surgery. Pain and HR assessment were performed at the same time points, with the exception of 0 min, when these were not evaluated because the animal was recovering from anaesthesia (see Figure 29). In order to uniform the horses' cortisol circadian rhythms, each time point always corresponded to the same time of the day in all animals.

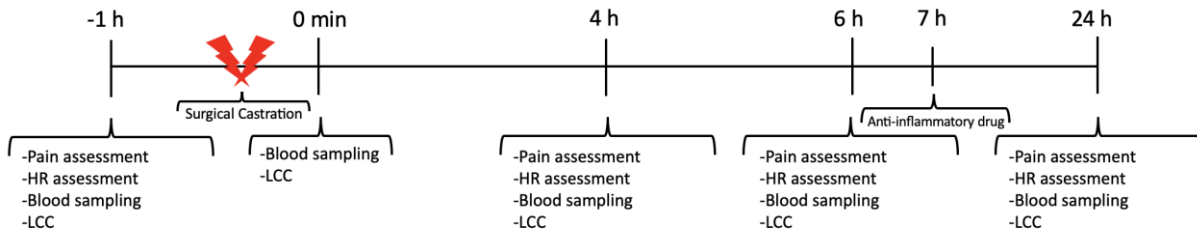

Figure 29. Workflow timeline diagram (horse)

Visual display of the study times (-1 h: 1h prior to castration; 0 min: 0 min post-castration/SHAM; 4 h: 4 hours post-castration; 6 h: 6 hours post-castration; 7 h: 7 hours post-castration; 24 h: 24 hours post-castration) and the procedures performed at each time point.

4.2.2 Blood samples

Three and 9-mL blood samples were aseptically collected from the external jugular vein in evacuated tubes containing 5.4 mg of K_3EDTA (Vacumed, FL MEDICAL s.r.l., 51100 Pistoia, Italy) and in evacuated tubes containing serum clot activator (Vacuette Z Serum Separator Clot Activator, Preanalitica s.r.l., 24043 Caravaggio, Italy), respectively, at each time point. A small amount of blood stored in K_3EDTA tubes was immediately used for chemiluminescence assay, while the residual was refrigerated at 4 °C until blood count test was performed. A full haematological profile was performed by an automated cell counter (ADVIA 120 Haematology System, Siemens Healthcare GmbH, 91052 Erlangen, Germany). Clotted blood samples were centrifuged at 3,000 rpm for 10 min to obtain serum, which was stored at −20 °C and then thawed for cortisol concentration measurement by immunoassay (IMMULITE 1000 Immunoassay System, Siemens Healthcare s.r.l., 20128 Milano, Italy).

4.2.3 Whole-blood chemiluminescence

In order to evaluate an individual's LCC at each time point, the whole blood of the same horse was divided into 2 samples: a non-stimulated sample (NS) and a stimulated one (S).

The NS sample provided a baseline measure of the individual's ROS production and this value subtracted to the S sample's one gave an outline of the individual's coping capacity (Delta LCC). Preparation of the reagents and samples was maintained identical to the procedure already described in Chapter 3.2.3 of this thesis (see Figure 22).

Chemiluminescence of both samples (NS and S), measured in relative light units (RLU), was recorded at 5-minute intervals by means of a high sensitivity portable chemiluminometer (Junior LB 9509, Berthold Technologies GmbH & Co. KG, Germany) for a total of 45 min in order to produce a luminescence profile. All measurements were performed immediately after blood collection at each time point at the veterinary hospital. When not being processed in the chemiluminometer, tubes were incubated at 37 °C in a lightproof water bath.

4.2.4 Statistics

Data were analysed using the SAS statistical software (version 9.3, SAS Inst. Inc., Cary. NC). Normality of data distribution was assessed by adopting the Shapiro-Wilk test. For LCC data, some parameters were calculated per time point: Delta LCC (mean value of the difference between S and NS), Max Delta LCC (maximum Delta LCC value), AUC (area under the Delta LCC curve), and AUC/GRAN (AUC/granulocytes number). In addition, to evaluate the presence of stress leukograms at each time point, the NLR (neutrophils/leukocytes ratio) was also calculated.

Analysis of repeated and normally distributed data (Delta LCC) was performed through a repeated type mixed model analysis of variance. Time points (−1 h, 0 min, 4 h, 6 h, 24 h) were included in the model as fixed effects, intervals (0, 5, 10, up to 45 min) as time effect and animal as the random repeated effect. Non repeated normally distributed measures (Max Delta LCC, AUC, cortisol, white blood cells count, AUC/GRAN, HR, and NLR) were analysed using a mixed model with time points (−1 h, 0 min, 4 h, 6 h, 24 h) as fixed effect and animal as random effect. Non-normally distributed data were first log-transformed before being analysed by the same model.

Data were reported as least squares means ± standard error for normally distributed variables. Non-normally distributed data (pain scores) were reported as median (range) and analysed using the Steel-Dwass-Critchlow-Fligner test.

Correlation among variables was calculated. Pearson coefficient was used for normally distributed data, whereas the Spearman rank correlation index was applied to non-normally distributed variables.

4.3 Results

4.3.1 LCC

Analysis of data revealed that Delta LCC increased significantly ($P < 0.0001$) at 4, 6, and 24 h from -1 h and 0 min. There was no difference between Delta LCC values at 4 and 6 h even

though a slight increase was noted at the latter time point. At 24 h, Delta LCC values started to significantly decrease ($P < 0.007$) from 6 h (Figure 30).

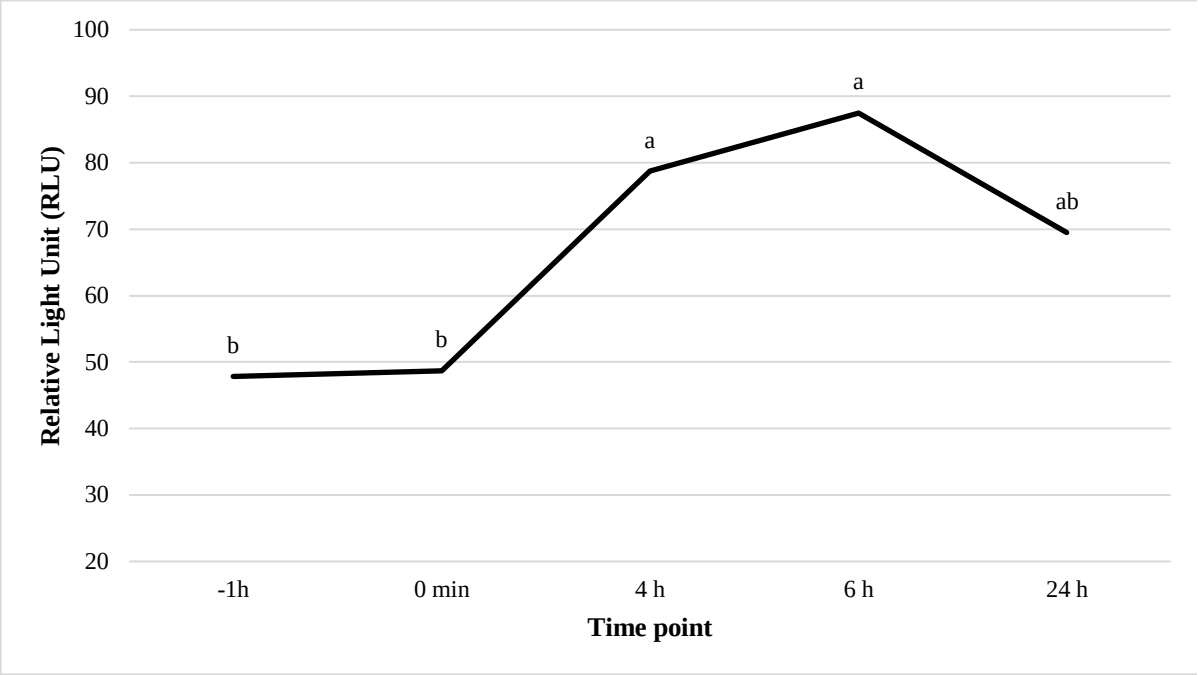

Figure 30. Delta LCC (horse)

Difference in leukocyte coping capacity (LCC) luminescence values at each time point between non-stimulated and stimulated measures (Delta LCC). Results are reported in Relative Light Unit (RLU). Lowercase letters indicate differences between and within groups at different time points ($P < 0.05$). Time points are reported as follows: −1 h (1 h before castration), 0 min, 4 h, 6 h, 24 h (0 min, 4, 6, and 24 h post-castration).

Max Delta LCC results did not significantly change between time points, except for values at 6 h which were different from -1 h ($P < 0.0008$) ones. The maximum light emission was recorded at 6 h with a Delta LCC peak of 146 RLU.

Delta AUC values had a similar trend to that of Delta LCC ones throughout the whole study, increasing significantly at ($P < 0.009$) at 4, 6, and 24 h from -1 h and 0 min, and decreasing, even though non significantly, at 24h from 6 h (Figure 31).

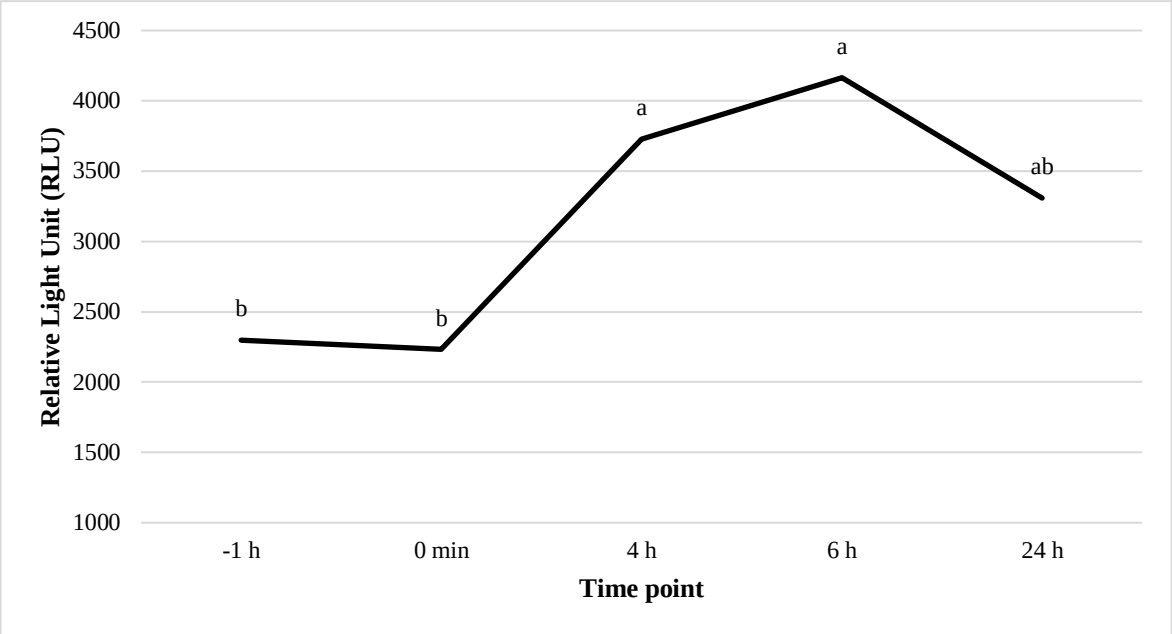

Figure 31. AUC (horse)

The area under the Delta LCC curve (AUC) values at each time point. Results are reported in Relative Light Unit (RLU). Lowercase letters indicate differences between and within groups ($P < 0.05$). Time points are reported as follows: −1 h (1 h before castration), 0 min, 4 h, 6 h, 24 h (0 min, 4, 6, and 24 h post-castration).

4.3.2 Blood cortisol level

Blood cortisol significantly increased at 4 ($P < 0.0001$) and 6 h ($P < 0.01$) from values at -1 h and 0 min, whereas a decrease was documented at 24 h from values at 6 h ($P < 0.01$). Mean blood cortisol values were as follows: 4.94, 4.60, 9.04, 7.41, and 4.79 μg dL^{-1} at -1 h, 0 min, 4 h, 6 h, and 24 h, respectively (Table 7).

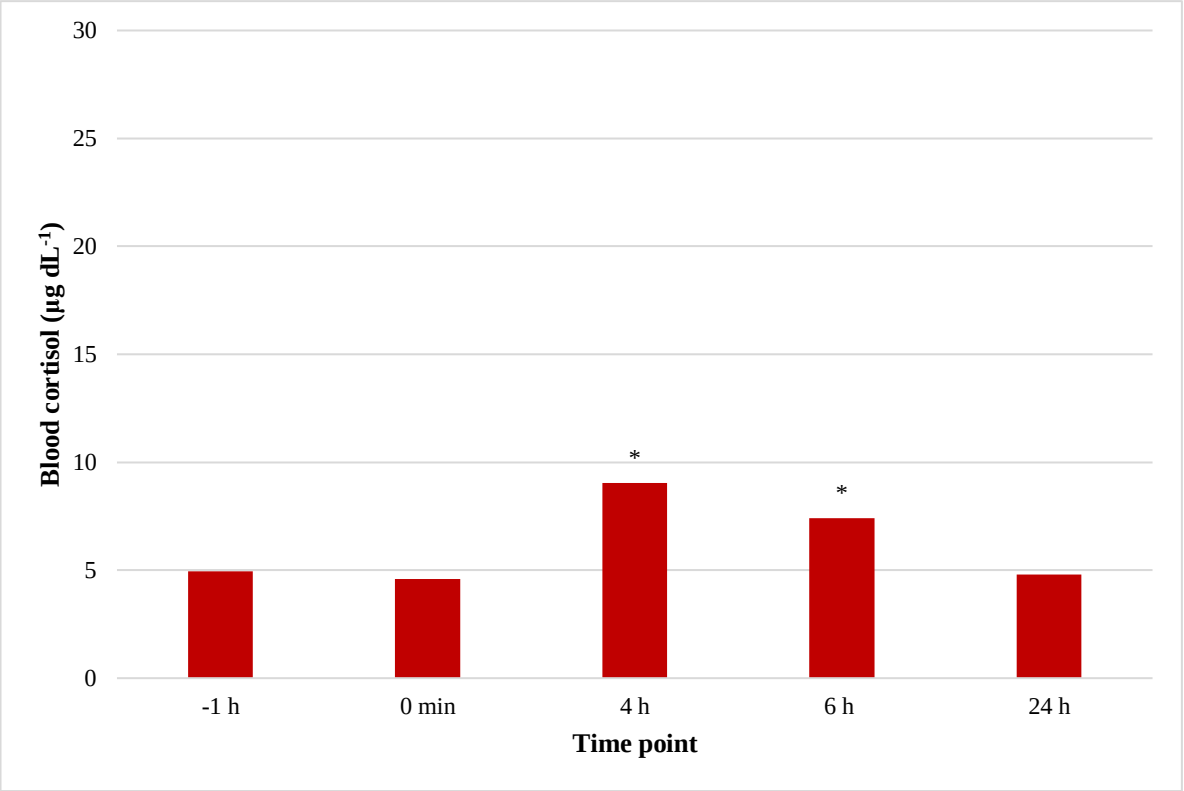

Figure 32. Blood cortisol (horse)

Mean blood cortisol values recorded during the study times in horses undergoing surgical castration. * indicates values significantly ($P < 0.05$) different from -1 h.

4.3.3 Heart rate

No significant changes were seen in HR between time points ($P = 0.38$). Nonetheless, an increasing trend was documented throughout the whole study. Mean HR values recorded were 46.0, 45.6, 48.6, 50.0 bpm min^{-1} at -1 h, 0 min, 4 h, 6 h, and 24 h, respectively (Figure 33; Table 7).

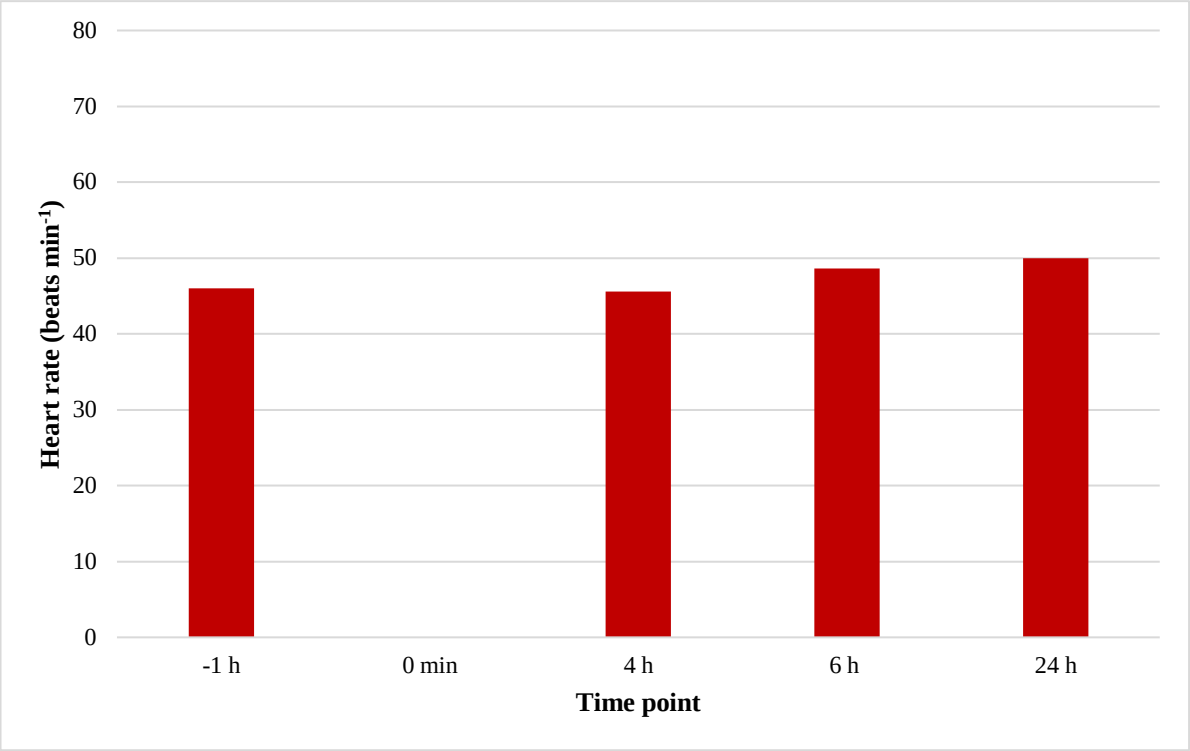

Figure 33. Heart rate (horse)

Mean heart rates recorded during the study times in horses undergoing surgical castration.

4.3.4 Pain scores

The animals were scored the same by the observers at each time point, and results showed a significant increase ($P < 0.0001$) at 4 h and 6 h from values at -1 h. At 24 h, an intermediate pain score between -1 h and 6 h values ($P > 0.05$) was recorded. Mean pain scores were as follows: 1.0, 4.88, 4.63, 3.38 at -1, 4, 6, and 24 h, respectively (Figure 34; Table 7).

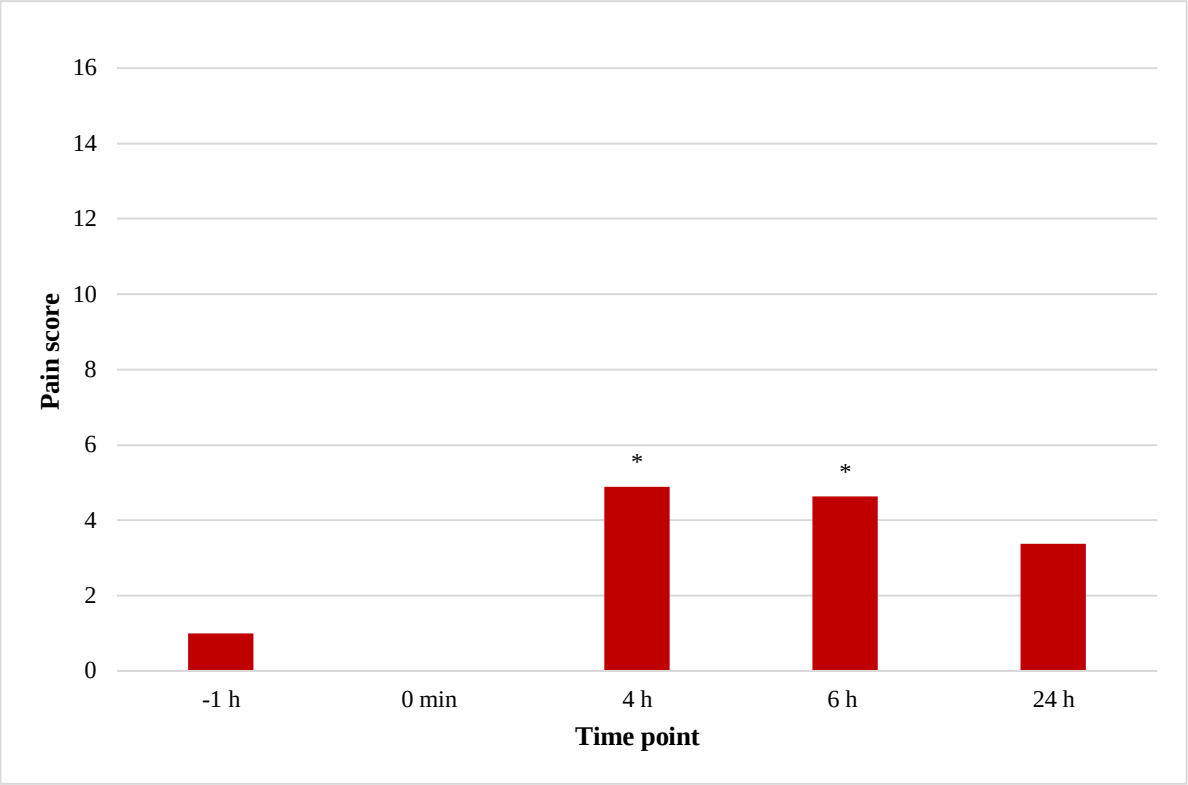

Figure 34. Blood cortisol (horse)

Mean blood cortisol values recorded during the study times in horses undergoing surgical castration. * indicates values significantly ($P < 0.05$) different from -1 h.

4.3.5 White blood cells

Presurgical whole blood counts and biochemistry values resulted in good health status of all horses. Over the study period, an increase, even though not statistically significant, of the total white blood cells populations was documented over the study times (range 10.40-7.45 x 10^3 cells μL^{-1}). The prevailing white blood cells population was represented by neutrophils (range 78.35-60.75%), followed by lymphocytes (range 32.2-16.58 %), monocytes (range 4.06-3.20 %), eosinophils (range 2.22-0.67 %), and basophils (range 0.88-0.35 %). Neutrophil percentage significantly increased ($P < 0.0001$) at 4, 6, 24 h from -1 h and 0 min, and neutrophil count values kept increasing reaching a significant difference from -1 h values at 24 h ($P < 0.05$). The opposite trend was seen with lymphocyte percentage and count which significantly decreased ($P < 0.0001$) over the study times from -1 h values (Table 7).

NLR significantly increased ($P < 0.0001$) at 4, 6, and 24 h from -1 h and 0 min, but its value at 24 h was significantly lower than that at 6 h ($P < 0.001$).

AUC/GRAN did not show any significant change over the evaluated study times.

4.3.6 Correlation between variables

Pearson and Spearman multivariate data analysis revealed that Delta LCC positively correlated with polymorphonuclear cells number ($P < 0.02$), NLR ($P < 0.001$), AUC/GRAN ($P < 0.001$), and pain score ($P < 0.01$). The latter being also positively correlated with cortisol, neutrophil absolute count and NLR ($P < 0.01$).

Table 7. Physiological parameters (horse)

Total white blood cells (WBC) count, different WBC populations (neutrophils, lymphocytes, monocytes, eosinophils, and basophils), NLR (neutrophils/lymphocytes ratio), AUC/GRAN (amount of relative light units produced by 10^3 polymorphonuclear cells), blood cortisol, and pain scale score at each time point. Time points are reported as follows: −1 h (1 h before castration), 0 min, 4 h, 6 h, 24 h (0 min, 4, 6, and 24 h post-castration). Asterisks indicate significant differences ($P < 0.05$) from values at -1 h.

	Time point				
	-1h	**0 min**	**4 h**	**6 h**	**24 h**
WBC					
(10^3 cells μL^{-1})	8.11 ± 1.34	6.35 ± 1.10	8.87 ± 2.78	8.65 ± 1.19	10.40 ± 2.41
Neutrophils					
(10^3 cells μL^{-1})	5.08 ± 1.36	3.98 ± 0.98	6.92 ± 2.48	6.87 ± 1.29	7.62 ± 1.96*
(%)	61.90 ± 8.61	62.20 ± 6.0	77.30 ± 4.8*	79.00 ± 4.34*	73.00 ± 3.86*
Lymphocytes					
(10^3 cells μL^{-1})	2.42 ± 0.61	1.94 ± 0.22	1.48 ± 0.26*	1.32 ± 0.07*	2.14 ± 0.38
(%)	30.40 ± 7.8	30.80 ± 2.54	17.20 ± 2.50*	15.50 ± 1.60*	20.80 ± 1.45*
Monocytes					
(10^3 cells μL^{-1})	0.34 ± 0.12	0.21 ± 0.08	0.27 ± 0.15	0.32 ± 0.15	0.4 ± 0.21
Eosinophils					
(10^3 cells μL^{-1})	0.17 ± 0.11	0.13 ± 0.10	0.06 ± 0.05*	0.05 ± 0.06*	0.13 ± 0.14
Basophils					
(10^3 cells μL^{-1})	0.03 ± 0.01	0.03 ± 0.01	0.08 ± 0.07	0.04 ± 0.02	0.04 ± 0.01
NLR	2.22 ± 0.85	2.04 ± 0.35	4.61 ± 0.91*	5.16 ± 0.75*	3.53 ± 0.40*
AUC/GRAN					
RLU (10^3 cells μL^{-1}) $^{-1}$	415.98 ± 100.57	471.81 ± 210.03	515.3 ± 219.82	608.41 ± 167.50	400.84 ± 68.23
Blood cortisol level					
($\mu g\ dL^{-1}$)	4.93 ± 1.48	4.60 ± 1.66	9.03 ± 1.47*	7.41 ± 1.94*	4.79 ± 0.87
HR					
(beats/minute)	46 ± 9	-	46 ± 7	49 ± 10	50 ± 8
Pain Score	0 (0-4)	-	5 (3-7)*	5 (2-7)*	3 (1-5)

4.4 Discussion

This research was the first to use the whole-blood LCC technique to evaluate its potential as a pain/stress assessment tool in the domestic horse. The respiratory burst has been evaluated in the past in horses which had been exposed to strenuous exercise, but focus has never been put on the assessment of changes in ROS production associated to nociception and pain-related stress in this species (Donovan *et al.* 2007; Raidal *et al.* 2000). Furthermore, in previous studies, the evaluation of ROS production was carried out following leukocytes isolation, a process that leads to deprivation of leukocytes' natural environment, which is fundamental for preserving the natural communication among white blood cells and their surroundings, allowing them to readily react to environmental changes. Human leukocytes are known to display over than 150 receptors (Mian *et al.* 2005) which interact with a large number of stress-sensitive parameters (*e.g.* endocrine factors, cytokines, changes in erythrocyte haemodynamics, blood biochemistry, etc.). The constant exposure to such factors is accountable to their effectiveness as stress indicators (Shelton-Rayner *et al.* 2012).

In the present study, LCC values increased at 4, 6, and 24 h from pre-operative values. This result seems to be in contrast with what would be expected from ROS production following exposure to a stressor. In fact, exposure to psychological stress is reported to impair ROS production and therefore, in this study, post-surgery LCC values were expected to be lower than pre-operative ones (McLaren *et al.* 2003; Shelton-Rayner 2009). The increase recorded at 4 and 6 h might be accountable to different causes. One explanation, although less plausible, could be that the animals were already psychologically stressed at -1 h due to the new environment they had been housed in, which led to a low ROS production. Nevertheless, the authors found consensus in another explanation for this result, which accounts the surgical-driven local/systemic inflammation and secretion of glucocorticoids as the main cause for the ROS production changes from pre-castration values. In fact, accumulating evidence seems to

indicate that glucocorticoids can have both permissive and stimulatory effects on the immune system under specific conditions (Bellavance and Rivest 2014). Furthermore, surgical procedures and trauma are known to acutely increase ROS production by activation of the complement system, polymorphonuclear cells, and macrophages (Rosenfeldt *et al.* 2013). Evidence of this was already found in 1997 by Azbill *et al.* who documented an augment in ROS production between 2 and 24 h after iatrogenic spinal cord injury in rats. This time frame is in line with the period of time in which ROS production was recorded at its peak in the present study. The findings of the present study are also in accordance with what reported by Gaudio *et al.* (2018), who measured the whole-blood LCC in calves undergoing ring castration. He found that ROS production increased when the scrotum was swollen and suggestive of an ongoing inflammatory process. It is believed that the immediate activation of post-surgical inflammatory processes could explain the increase in LCC results documented at 4 and 6h in the present study. Also, Gaudio *et al.* (2018) reported a decrease in LCC values subsequent to its initial increase. This phenomenon had already been associated with the cost of ROS release leading to a transient period of immunosuppression (McLaren *et al.* 2003). A similar decreasing trend in LCC values was documented in the present study at 24 h. Nevertheless, the magnitude of the "exhaustion" state recorded was not as pronounced (in the present study, LCC values were never lower than pre-operative ones). An explanation for this could be sought in the sampling period of the present study being too short. In fact, it could be hypothesized that a longer period of evaluation (beyond 24 h post-op) could have revealed LCC values lower than -1 h ones. Nevertheless, it must be taken into account that the administration of the anti-inflammatory drug at 7h post-surgery might be accountable to the ROS production decrease seen at 24h. This is a limit to this clinical study, but due to ethical reasons the horses could not be left untreated, especially since at 4 and 6 h blood cortisol and pain scores were reflective of post-surgical stress and pain. Another thing that must be considered when interpreting this study

results is that the number of animals used could be limiting, and that it was not possible to evaluate a control group of horses undergoing anaesthesia and sham castration for comparison purpose.

The UNESP-Botucatu multidimensional composite pain scale refined by Taffarel *et al.* (2015) is considered one of the most reliable and specific tools available for assessing acute, mild clinical pain in horses undergoing castration, nowadays. In the present study, pain scores significantly increased peaking at 4 and 6 h post-castration and the most encountered behaviours included reluctance to move, mild reaction to palpation of the wound, and lifting of the hind limbs. The fact that pain scores positively correlated with the increase in LCC values could suggest that acute inflammation might have played an important role in causing pain, therefore indicating that the LCC technique could be a useful indicator when assessing post-surgical castration pain in horses. Furthermore, the non-significant increase in pain scores seen at 24h might be due to administration of the anti-inflammatory drug at 7h post-surgery, which might have alleviated inflammatory pain.

Pain is reported to affect the activity of the sympathetic nervous system, which is known to promptly influence the cardiovascular system, inducing efferent cardiac sympathetic activation and ultimately, heart rate increase (Terkelsen *et al.* 2005). As a result, the heart rate can be an easy acute pain parameter to measure, but changes in its value have shown not to always be reliable. In fact, HR can be easily influenced by the conditions of the patient and psychological status, all of which could lead to false-positive results (Chen and Chen 2015). The authors believe that HR scarce sensitivity could be the reason for the lack of significant difference in HR values recorded among the study times in the present study.

Cortisol is a key factor in the stress response. It surges following exposure to physical or psychological threat and provides the energy and substrate necessary to cope with stressors (Jankord and Herman 2008). For this reason, cortisol has long been considered as one of the

most important stress indicators in animals (Möstl and Palme 2002). Pain itself is a stressor, and cortisol secretion in response to it can intensify its experience and cause a fear-based memory of pain (Hannibal and Bishop 2014). In this study, cortisol values increased over the study times from -1 h values, peaking at 6 h and then decreasing at 24 h. Blood cortisol values documented at -1 h (4.94 ± 1.48 μg dL^{-1}) are in line with normal physiological values reported by Padalino *et al.* (2017) in healthy horses (4.80 ± 0.60 μg dL^{-1}). This result could indicate that the animals were not stressed at -1 h, therefore LCC results at that time point are ought to be considered as normal resting values not biased by non-noxious psychological stress. The blood cortisol increase seen at 4 and 6 h is in accordance with what reported by Ayala *et al.* (2012) in horses 6 h post-castration and, in the present study, might also be explained by the fact that the analgesic effect of the anaesthetic drugs used had already worn off (Kaka *et al.* 1979; Valverde 2010; Sellon *et al.* 2009). Another interesting finding documented in the present study was the loss of normal cortisol diurnal variation. Blood cortisol concentration was expected to peak in the morning and then decrease, reaching its lowest value, in the afternoon. Contrariwise, in this study blood cortisol peaked in the afternoon (between 1.30-3.30 p.m., at 4 and 6 h) and decreased the next morning at 24 h (8.00 a.m.). Previous studies showed that blood cortisol increased in concentration in horses undergoing anaesthesia even without surgery. However, this effect seems to be of short duration with a return to control values within 2 h post-anaesthesia (Taylor 1989). For this reason, it is believed that the longer-lasting increase in blood cortisol concentration and the loss of its normal diurnal fluctuation recorded in the present study was more likely due to surgery-related pain and stress. The positive correlation found between cortisol and pain scores ($P < 0.001$) seems to further confirm this hypothesis. Furthermore, the decrease in cortisol values seen at 24 h is in accordance with what documented by Dias *et al.* (2014) at 19 hours post-orchiectomy in horses anaesthetized with a similar protocol (ketamine,

midazolam, butorphanol, isofluorane) to that used in this study, and administered with flunixin meglumine after surgery.

Leukogram analysis is an important and useful tool for assessing the psycho/physiological status of the horse. In fact, stress is known to cause neutrophilia, lymphopenia and eosinopenia, and these changes usually appear between 2 and 4 h after the increase of endogenous cortisol release (Satu *et al.* 2012). In accordance with this, an increase in NLR was documented starting at 4 h and peaking at 6 h, exactly 2 h after the recorded significant increase in blood cortisol. AUC/GRAN did not change over the study times, revealing that changes in LCC values might have mainly been dependent on the quantity of ROS-producing cells present in the chemiluminescence vial.

It is controversial which and how anaesthetic drugs influence blood cells distribution in different animal species, but there is evidence that some drug protocols can lead to a decrease in both red and white blood cell number, and ROS production (Dhumeaux *et al.* 2012; Sutil *et al.* 2017; Parry and Anderson 1983; Costa *et al.* 2013; Kurosawa and Kato 2008). The lack of significant decrease in blood cell values after surgery from pre-surgical values and the significant increase in neutrophil percentage recorded at 4, 6, and 24 h might indicate that in this study anaesthesia may not have biased results. Also, as suggested by the positive correlation found between blood cortisol, neutrophils percentage, NLR and pain scores, the variation in blood cells number seems to be mainly due to post-surgical stress and pain.

Whether anaesthetic drugs influenced ROS production in the present study is not clear, but the increase in ROS production recorded in the post-operative period seems to be in contrast to what was expected following exposure to anaesthetics, which had been reported to have an immunosuppressive effect (Kurosawa and Kato 2008). The present study results are similar to what reported by Esteruelas *et al.* (2016) who stated that even though anaesthetic drugs might have affected LCC values in brown bears after capture, their results were deemed to be

significant and the LCC technique considered sensitive enough to detect post-capture stress. It is hypothesized that even if anaesthetic drugs decreased ROS production in this study, surgery-related circulation of pro-inflammatory factors might have overshadowed the anaesthetics' effects, momentarily increasing the respiratory burst. Interestingly, in this study the administration of the anti-inflammatory drug at 7h did not cause a decrease in circulating white-blood cells at 24h. On the contrary, at 24 h neutrophils number was significantly higher than pre-surgical one. This seems to be in contrast with what suggested by AUC/GRAN results. In fact, as mentioned earlier, the lack of variation in AUC/GRAN values among the study times should indicate that the respiratory burst recorded was mainly dependent to the of granulocytes present in the vials rather than their individual capability of producing ROS. Nevertheless, the decrease in ROS production documented at 24 h was not concurrent to a decrease in granulocytes number. This discrepancy could either reflect a true impairment of the immune system at 24 h, or be either due to bias caused by the small animal number used in this study or the effects of the anti-inflammatory administered at 7 h.

These study results further highlight the difficulty of the task of assessing animal pain by means of physiological parameters. Future studies are needed to elucidate the immunomodulator effects of anaesthetic and anti-inflammatory drugs in different animal species.

Concluding, the lack of verbal self-report and a poor sensitivity or specificity of behavioural and physiological pain parameters are the biggest challenges that practitioners encounter when assessing animal pain (Flecknell and Waterman-Pearson 2000). For this reason, new and more specific tools for pain assessment are needed. The whole-blood LCC chemiluminescence is a fast and easy to perform innovative technique designed to detect psychological stress. Despite the need for further research on this topic, it is believed that the whole-blood LCC technique, if used concurrently with other parameters, could prove to be an important tool for assessing animal pain.

5 Comprehensive discussion

Pain assessment in animals is an extremely difficult challenge since animals do not verbally communicate. Also, no gold standard techniques for animal pain assessment are available, nowadays. For this reason, in order to allow for a better understanding of what the animal is undergoing, pain assessment often requires a multimodal approach, comprising the conjunct use of parameters from different medical disciplines such as physiology and ethology (Anil *et al.* 2005; Gigliuto *et al.* 2014; Fox 2014). This approach is meant to provide insight into both the psychological and physiological dimension from which pain perception and elaboration is originated (Weary *et al.* 2006). Despite the useful information provided by the multimodal approach, sometimes a complete understanding of the current status of the animal cannot be obtained. This is due to large individual variation in the stress response, differences in physiological strategies to cope with stressors, and the different period of time during which the same mediators operate in different species (Sapolsky 1994; Cockrem 2013; Houslay *et al.* 2019).

From this, it follows that the scientific community is still far from having gained a proper understanding on how to thoroughly assess animal pain, and therefore the need for new tools is mandatory.

The studies of this thesis focused on testing the whole-blood LCC technique; a tool that had previously been used with promising results in humans and animals for acute psychological stress assessment: the whole-blood LCC (McLaren *et al.* 2003; Montes *et al.* 2004; Moorhouse *et al.* 2007; Gelling *et al.* 2009; Shelton-Rayner *et al.* 2010, 2012; Esteruelas *et al.* 2016; Huber *et al.* 2017b). Seen the strong evidence of the overlapping nature of pain and stress (Vierck *et*

al. 2010), this thesis aimed at evaluating the whole-blood LCC in animals undergoing painful stimuli, like those derived from elective non-surgical and surgical castration.

In both studies the whole-blood LCC provided satisfying results indicative of alteration of the psycho-physiological status of the animal (McLaren *et al.* 2003). In order to prove this, the authors evaluated, in conjunction with the new technique, other parameters commonly used to detect stress (physiological parameters) and pain (species-and pain type-specific pain scales). As expected, results from physiological parameters and pain scales were not uniform among different species and even individuals. Specifically, physiological parameters were subjected to great variation and bias. An example of this was the diurnal fluctuation of cortisol level, its great inter-individual variation, and the influence of acute non-noxious psychological stressors on cortisol and other parameters (*e.g.* heart rate and white blood cells number) (Gaynor and Muir III 2015; Lee *et al.* 2015). Fortunately, whenever a parameter failed to detect changes in the animals' psycho-physiological status, another provided useful information in both studies, thus allowing the research team to evaluate the proper functioning of the LCC technique.

The complexity of the aims of this thesis was particularly evident in the study on calves, where, despite both significant changes in scrotal clinical condition and LCC values were suggestive of an abnormal psycho-physiological condition, no cortisol, white blood cells, or pain scale results were indicative of pain/stress. It is believed that in this study the pain scale failed to detect pain probably due to the prey nature of cattle, which led the animals to hide signs of pain from the assessors (Molony *et al.* 1995). Another hypothesis is that the scale might not have been sensitive enough to assess pain in animals undergoing a non-surgical type of castration. As for the high inter-individual variability seen in cortisol and the lack of stress leukograms recorded at the different time points, it is thought that the amount of pain experienced by the animals might have been too subtle to trigger a change in these parameters. If this was the case, considering the valuable information derived from the clinical evaluation of the scrotal tissues,

it could be assumed that the, in this specific setting, LCC technique might be a more sensitive parameter than the other ones used in this study.

Both physiological parameters and the pain scale seemed to be more sensitive in detecting pain in horses than in calves. In fact, the pain scale and cortisol results were significantly indicative of stress and pain in this species. This allowed for a better evaluation of the LCC results, which also seemed to be indicative of psycho-physiological alteration. The greater reliability of the pain scale used in the horse seems to be accountable to the fact that it was specifically designed to detect post-operative pain in this species. Also, it could be speculated that the horses were much more accustomed to human interaction, which might have made them more at ease with showing pain signs than the calves that had never been manipulated before the study.

It must be said that, even though the LCC technique seems to be sensitive with regards to the detection of alteration in the psycho-physiological status, and thus pain, a thorough species-specific validation of the method should be provided. In fact, at present, the potential sensitivity and diagnostic ability of the technique is still not known. As mentioned in Chapter 3.4, some difficulties were encountered while working with calves blood: a low chemiluminescence signal was recorded in this species throughout the study times, and was probably caused by the lower number of neutrophils per mL compared to other species (Sjastaad *et al.* 2010; Roland *et al.* 2014). For this reason, and also as suggested by Shelton-Rayner (2009), in order to optimize the technique for different species, it should be made sure that every reagent (*e.g.* K_3EDTA, DMSO, luminol, PMA, and PBS) does not interfere with the reaction and is used at the correct concentration so that it would not be rate-limiting or cytotoxic. Thus, it is important that the only rate-limiting factor of the reaction is the leukocyte's capacity for activation. That said, to thoroughly validate the technique for a species, the chemiluminescence reaction should be tested at different concentrations of each reagent.

With regards to the whole-blood LCC procedure itself, it should be considered that monitoring the respiratory burst at 5-minute intervals for 45 minutes could be a limiting factor, and a more frequent evaluation (*e.g.* 1-minute intervals) could be preferred to better evaluate the curve of ROS production over time. Nevertheless, when dealing with large animal numbers, the narrower the time interval between sampling times the more difficult it becomes to use the portable luminometer, because it can process only one sample at a time and the processing time for an individual sample is too long to analyse a high number of samples per minute. To address this issue, a microplate reader luminometer could be used instead, but in that case, a laboratory setting would be necessary to carry out the whole procedure. In the present studies, the choice of using a portable chemiluminometer was made because one of our aims was to test a tool that could quickly provide results in the field.

It is possible that different animal species present different ROS production patterns and peak times, which may require a longer *in vitro* evaluation time than 45 minutes. Thus, it would be interesting to evaluate and compare whole-blood LCC results from different animal species which blood has been processed for a longer period of time. This could cast new light on *in vitro* species-specific ROS production patterns under normal and abnormal psycho-physiological conditions.

Another factor that could increase the understanding on LCC results when tested on animal pain is the refining of the study times according to the procedure that needs to be evaluated. As seen in the study on calves, a long sampling period allowed for detection of a consistent increase and subsequent decrease in LCC values, but perhaps a narrower time interval between time points would have revealed a more precise variation in ROS production and other parameters, which could have allowed for a better pain assessment. Similarly, in the study performed on horses, a longer evaluation period might have revealed a marked neutrophil “exhaustion” state, such as that documented in calves, which was not recorded at the evaluated study times. Had it

been feasible, a longer evaluation period would have been chosen in this study. Nevertheless, as already mentioned, care must be taken when speculating on these results, since the administration of the anti-inflammatory drug at 7h might have biased the results recorded at 24h. Also, the influence of anaesthetic drugs on ROS production is not fully known yet.

The mechanism behind neutrophil "exhaustion" is not yet understood (McLaren *et al.* 2003; Honess *et al.* 2005; Moorhouse *et al.* 2007; Gelling *et al.* 2009; Gelling *et al.* 2010; Shelton-Rayner *et al.* 2011; Esteruelas *et al.* 2016; Huber *et al.* 2017a,b; Huber *et al.* 2019). In fact, the exact mechanism behind neutrophils' decrease in ROS production following exposure to a stressor and for how long this state of impairment lasts still requires clarification. Shelton-Rayner *et al.* suggested that human neutrophils would be able to re-establish their normal respiratory burst after 45 minutes from administration of a transient psychological stressor (Shelton-Rayner *et al.* 2010, 2011). Whether ROS production would return to normal activity after 45 minutes following transient psychological stressor in animals is not known, and it is possible that consistent differences may be present among different animal species. Furthermore, painful stimuli are different from non-noxious ones and might have different (*e.g.* more long-lasting) effects on the animal's immune system. That said, seen the lack of information, no further speculation on the results of the current studies can be done on this regard.

Despite the whole-blood LCC seems to provide promising results in the field of pain assessment, it must be pointed out that its efficacy in detecting pain following non-inflammatory noxious stimuli has not been tested yet. In fact, both the procedures evaluated during the present studies were associated with tissue inflammation, which systemic spread of inflammatory mediators may have influenced leukocytes' activity, and thus our results. In fact, neutrophils activation could be due to the interaction between leukocytes' receptors and inflammatory mediators (direct activation) rather than pain-related activation of leukocytes by

their binding with neuromodulators produced by the nervous system (indirect activation). Unfortunately, no specific markers for inflammation were measured in the studies of this thesis. Had it been feasible, acute phase proteins would have been measured to further confirm the ongoing inflammatory state and its magnitude at the different time points (Jain *et al.* 2011). Nevertheless, even though both direct and indirect neutrophil activation might have happened in the present studies, seen the concurrent variation of some of the other parameters evaluated (*e.g.* blood cortisol, pain scale, scrotal clinical evaluation, etc.), the research group believes that results obtained from the LCC technique were indicative of post-castration pain in both studies. In order to avoid future bias by inflammation it is suggested that the LCC technique be tested on non-inflammatory pain types such as that caused by noxious thermal stimulation (to an extent which is not harmful to tissues) and results should be correlated with results from scoring systems (behavioural assessment) and physiological parameters. The latter could include blood cortisol and catecholamines evaluation, which have already been used both in human and veterinary medicine to assess the pain response (Nakagawa and Hosokawa 1994; Mellor *et al.* 2002; Tennant 2013). Other interesting parameters that could assist in the interpretation of the whole-blood LCC results following painful stimuli could be the level of serum oxidised proteins (*e.g.* measurement of serum carbonyl concentration) and serum lactoferrin (Rebelo *et al.* 1995; Weber *et al.* 2015). Serum carbonyl is indicative of amount of damage dealt by ROS to the animal's proteins in the blood, tissues and cells, whereas, lactoferrin reflects the extent of neutrophils' activation.

It would be very interesting to evaluate the LCC technique's efficacy in detecting different pain types whether they be of iatrogenic nature or due to the animal's clinical condition. It is hypothesized that the LCC technique could show a different degree of reliability depending on pain type, aetiopathogenesis and location.

6 Conclusion

The whole-blood LCC technique seemed to be able to provide promising information with regards to the animal's psycho-physiological status and could be a valuable instrument for detection of animal pain.

Whether it is true that the technique still requires species-specific refinement and validation, when used in conjunction with other pain and stress assessment parameters it allowed for rapid *in situ* evaluation of pain and stress in calves and horses undergoing different castration procedures.

It is believed that, following amelioration, the whole-blood LCC technique could improve our understanding of animal pain in different species, and that the findings of these studies could set the basis for further investigation in many directions.

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7 References

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