UNIVERSITÀ DEGLI STUDI DI PADOVA

Sede Amministrativa: Università degli Studi di Padova
Dipartimento di Medicina Animale, Produzioni e Salute

SCUOLA DI DOTTORATO DI RICERCA IN SCIENZE ANIMALI E AGROALIMENTARI

INDIRIZZO DI SCIENZE ANIMALI

CICLO XXVI

PAIN ASSESSMENT METHODS:
THE CASE OF SURGICAL CASTRATION IN PIGLETS

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Summary

Surgical castration of piglets is a high topic due to the higher attention of both the consumers and the public opinion on the animal welfare. The management practice is usually performed for the reduction of aggressiveness in adult subjects and to preserve meat from boar taint, a sensory defect caused by the sexual hormone androstenone and the intestinal catabolite skatole. The “European declaration on alternatives to surgical castration”, signed in 2011 by all the principal actors of swine production system, require the abandon of surgical castration from the 1st of January 2018;moreover, the Declaration still recognizes the inevitability of mutilation in the case of pigs for the production of meat registered under "traditional specialties guaranteed" or with "geographical indications" (Protected Geographical Indication (PGI) or Protected Designation of Origin (PDO)), that represent the majority of Italian pig production. In any case, for such production, alternatives to surgical castration with analgesia and/or anaesthesia should be introduced. The choice of the most effective drugs is strictly connected to the degree of pain experienced by piglets. Although many studies demonstrated the presence of pain due to castration regardless pigs age, no standard method for the pain assessment exist yet, and consequently there are not method to evaluate its reduction using drugs.

In the first three chapter of the present work different methods for pain assessment were tested both physiological/emetic and behavioural, comparing piglets subjected to surgical castration to handled piglets. Among tested parameters the analysis of blood cortisol, considered the most important stress hormone, revealed a pick in castrated piglets within the first hour after mutilation, demonstrating to be a useful parameter to evaluate differences between the two group just immediately post mutilation. The analysis of blood lactate and glucose after the painful stimulation did not underline the expected increase as well as body temperature which revealed differences between castrated and handled just 3 hours after the treatment, probably due to a voluntary longer permanence of mutilated piglets under the heat lamp in the nest. The measurement of eye temperature through the use of a infrared thermacam, never used before in a similar study, pointed out a temperature higher in castrated piglets. Among behavioural parameters, mutilated piglets needed more time to start walking when placed back to the farrowing crate, and they demonstrated alterations in posture and walking up to one hour after the mutilation.

The evaluation of facial expression, method widely used in human unconscious patient and infants as well as recently in laboratory animals as mouse, rats and rabbit but never in pigs, revealed a major presence of check tension in castrated subjects in comparison to manipulated ones.

The last chapter focused on the effect of a non steroidal anti-inflammatory drug through the analysis of blood cortisol and of general behaviour recorded using the scan sampling method. Tolfenamic

acid demonstrated its utility in the reduction of pain locating treated piglets in intermediated levels between piglets castrated without drugs and handled piglets both as cortisol level and time used performing different behaviours.

Concluding, the evaluation of pain in pigs is of difficult interpretation, but the combination of different methods seems to give useful indications to study drug efficacy for the reduction of such a pain.

Riassunto

La castrazione chirurgica dei suinetti è un argomento di elevata attualità data l'attenzione sempre più elevata dei consumatori, e quindi dell'opinione pubblica, in tema di benessere animale. Tale pratica zootecnica viene routinariamente effettuata per la riduzione dell'aggressività nei soggetti adulti e per preservare le carni del cosiddetto "odore di verro", difetto sensoriale causato dall'ormone sessuale androstenone e dal catabolita intestinale skatolo. La "Dichiarazione volontaria di abbandono della castrazione", sottoscritta nel 2011 da tutti i principali attori della filiera suinicola dei diversi stati europei, impone l'abbandono della pratica chirurgica a partire dal 1 Gennaio 2018, ma riconosce la necessità della mutilazione per la produzione di prodotti di alta qualità quali DOP e IGP, destino della maggior parte della produzione suinicola italiana. Per le realtà produttive esonerate dall'obbligo di abbandono è comunque previsto l'utilizzo di anestesia e/o analgesia prolungata. La scelta dei farmaci più efficaci a tale scopo è strettamente vincolata alla valutazione del grado di dolore provato dai suinetti. Attualmente, però, nonostante numerosi studi dimostrino la presenza di dolore associato alla pratica a prescindere dall'età in cui venga effettuata, non esistono metodi standard per valutarne l'intensità e conseguentemente l'eventuale riduzione derivante dall'utilizzo di farmaci.

Nei primi tre capitoli del presente lavoro sono stati testati diversi metodi di valutazione del dolore sia di carattere fisiologico/ematico che di carattere comportamentale confrontando suini sottoposti a castrazione chirurgica e suini in cui la pratica veniva simulata. Tra i diversi parametri testati la valutazione del cortisolo ematico, considerato ormone dello stress per eccellenza, ha rilevato un picco nei suini castrati rispetto ai non castrati unicamente entro la prima ora dal trattamento, dimostrandosi un parametro utile ai fini delle differenze tra trattamenti solo nell'immediato post castrazione. L'analisi ematica di lattato e glucosio in seguito alla stimolazione dolorifica non ha evidenziato l'innalzamento previsto, così come la temperatura corporea ha rivelato delle differenze tra suini sottoposti a castrazione o manipolati solo a 3h dal trattamento, probabilmente a causa di una maggior permanenza volontaria dei suini doloranti sotto la lampada riscaldante del nido. Il rilevamento della temperatura oculare attraverso l'utilizzo di una termocamera a infrarossi, mai utilizzata prima d'ora con questo scopo, ha invece evidenziato una temperatura tendenzialmente più elevata nei suini mutilati. Tra le valutazioni di carattere comportamentale, i suini castrati impiegavano più tempo a riprendere a camminare appena appoggiati nella gabbia parto, e dimostravano alterazioni in posture e atteggiamenti fino a un'ora dopo la mutilazione. La valutazione delle espressioni facciali, metodica ampliamente utilizzata nei pazienti umani incoscienti e nei neonati nonché ultimamente negli animali da laboratorio quali topi ratti e conigli

ma mai nei suini, ha rivelato una maggior presenza di tensione a livello guanciale nei soggetti castrati rispetto ai manipolati.

L'ultimo capitolo, attraverso l'analisi del cortisolo ematico e del comportamento generale valutato mediante tecnica dello scan sampling, si è concentrato sull'effetto di un farmaco antiinfiammatorio non steroideo. L' acido tolfenamico si è dimostrato utile nella riduzione del dolore, localizzando i suini trattati col farmaco a livelli intermedi tra i castrati senza farmaco e i manipolati sia come valori di cortisolo sia come tempo impiegato nei diversi comportamenti.

Concludendo, la valutazione del dolore negli animali, e nei suini nello specifico, è tutt'altro che di facile interpretazione, ma l'accostamento di diversi metodi sembra poter fornire indicazioni utili allo studio sull'efficacia di farmaci destinati alla riduzione di tale sofferenza.

Chapter 1

GENERAL INTRODUCTION AND AIM

INTRODUCTION

1.1 The effects to rear entire male pigs

1.1.1 Meat quality

The castration of male piglets by surgical removal of gonads is a management practice widespread in pig farms with the aim of reducing aggressive behaviour and the so-called "boar taint" in the finished product (Lundström & Haugen, 2009) considered by many consumers an unacceptable sensory defect (Dijksterhuis *et al.*, 2000; Lunde et al., 2009. The smell is a sensory characteristic determinant in the acceptance of a food product by the consumer. The possibility that meat from a sexually mature male pig might be foul smelling is not acceptable, it is therefore necessary to eliminate or reduce boar taint risk, while maintaining the quality of the product (Zamaratskaia *et al.*, 2009).

The substances responsible of boar taint are androstenone (Patterson, 1968) and skatole (Vold, 1970; Walstra *et al.*, 1970; EFSA, 2004). The first is a steroid hormone derived from testosterone produced in the testes under stimulation of the hypothalamic-pituitary axis; it gives urine-like odour to the meat (Patterson, 1968; Lundström *et al.*, 2009). In the organism androstenone tends to concentrate in the salivary glands and adipose tissue, from which it is released during the cooking of meat allowing to form the typical smell. Differently, skatole is a metabolite of the tryptophan amino-acid due to bacterial fermentation in the large intestine (Jensen *et al.*, 1995; Yokoyama and Carlson, 1979). Part of produced skatole is metabolised in liver (Zamaratskaia *et al.*, 2009). A part can also accumulate in adipose tissue , giving , in this case, a faecal odour to the meat (Vold, 1970; Walstra and Maarse, 1970, Squires, 1999; Lundström *et al.*, 2009; Zamaratskaia and Lundström , 2006).

The development of the gonads and behavioral changes of the pigs become significant between 3 and 5 months, with an increase on the size of the testes and a rapid maturation of the seminiferous tubules (Ford and Wise, 2011). After reaching sexual maturity, there is a high production of androstenone and testosterone (EFSA, 2004). Over the past few years it has been studied how sex steroids may influence the level of skatole (Zamaratskaia *et al.*, 2004 a, b). According to recent studies (Rasmussen et al. , 2011), some authors report a correlation between androstenone or other steroids and the regulation of metabolism of skatole (Babol *et al.*, 1999; Doran *et al.*, 2002). Androstenone would seem, in fact, influence the activity of cytochrome P450E1, an essential enzyme in the metabolism of skatole (Doran *et al.*, 2002; Tambyrajah *et al.*, 2004; Brunius *et al.*, 2012). For this reason, castration affects the levels of skatole, lowering it to the point of eliminating the problem of the smell that comes from it.

The incidence of boar taint in pig carcasses varied from 10 to 75% and seems to be influenced by many factors , including feeding and slaughter weight (Squires *et al.*, 1995; Xue *et al.*, 1996; EFSA, 2004).
The perception of the defect varies greatly depending on the type of product and it is further complicated by the presence of porcine products very different in European countries (PIGCAS , 2008). Moreover, marked individual differences in the perception of boar taint confirm that the sensitivity varies widely by nationality , gender and age of consumers (Font I Furnols *et al.*, 2000; Matthews *et al.*, 2000; Weiler *et al.*, 2000; Font I Furnols *et al.*, 2003). The majority of people are sensitive to skatole, but not to androstenone since a percentage of subjects was found to be anosmatic against the latter compound. In accordance with the stated anosmia to androstenone is genetically determined (ORD7D4 genotype by Keller *et al.*, 2007), and the percentage of anosmatic women would seem to be lower (variability of 11-66 %) than men (18-74 %) (Bekaert *et al.*, 2010; Bremner *et al.*, 2003; Font I Furnols *et al.*, 2003; Lunde *et al.*, 2009; Weiler *et al.*, 2000) . Surveys conducted among consumers had revealed that there is a greater sensitivity of women than men (Weiler et al. , 2000; Font I Furnols *et al.*, 2009; Lunde *et al.*, 2010; Font I Furnols , 2012). For exemple, in Norway 46% of women declared to be sensitive compared with 36% of men (Lunde *et al.*, 2009), in France 53% vs . 45% (Chevillon *et al.*, 2010) , in Belgium 51 % vs . 38 % (Baeker *et al.*, 2011).
In the UK, the percentage of consumers sensitive to androstenone odour is less than in France and Spain (Bonneau *et al.*, 2000; Blanch *et al.*, 2012).

1.1.2 Behaviour

From the animal welfare point of view, castration leads to the reduction in aggression and sexual behaviours , which increases with the onset of puberty (Zamaratskaia *et al.*, 2008). This change in behaviour is positive for breeders because it simplifies the management, but it is especially positive for the animals in terms of reduction of aggressiveness and dominance problems limiting consequently the risk of trauma, both for animals and for the staff (Carroll *et al.*, 2006). The attention of researchers has focused much on the behaviour of intensively reared males concluding that entire pigs are much more aggressive than the castrated ones. Moreover, it seems that the time devoted to the game during young age is employed in fighting and competing in adulthood (Cronin *et al.*, 2003) , resulting in very serious skin lesions (Warriss, 2000; Andersson *et al.*, 2003; Fredriksen *et al.*, 2003). Several authors have confirmed the occurrence of increased aggressiveness of castrated and entire males compared to females, and consequently the higher incidence of skin and limb lesions (Tuyttens *et al.*, 2008; Boyle & Björklund, 2007; Llamas Moya *et al.*, 2008). In addition, the expression of aggressive behaviour, as demonstrated by Claus *et al.* (1994) and Giersing *et al.* (2000) , would lead to an increase in the production of androstenone.

1.2 Surgical castration: a tool to prevent the boar taint

The surgical castration involves the incision of the scrotum and, depending on the technique, of the tunica vaginalis to allow the removal of the testes, which are removed by pulling , cutting or twisting (Jäggin *et al.*, 2006). The duration of the procedure is short, as the veterinarian or trained operator takes less than 30 seconds. The surgery begins with the containment of the piglet; then, two vertical incisions (in the majority of cases, 78%) of around 2 cm of length are practiced on the scrotum, (Fredriksen *et al.*, 2009); rarely it is performed only a horizontal incision (22 %). Following castration the use of disinfectants to prevent infections, generally chlorhexidine, is common in all European countries, unlike the administration of antibiotics. In the Netherlands, Estonia and in Italy, however , more than 50% of breeders use antibiotics, especially amoxicillin (Fredriksen *et al.*, 2009).

Actually among 125 millions of male pigs reared in Europe (CE excluding Malta, Rumania and Bulgaria and including Switzerland e Norway), almost 20% are not castrated whereas less than 3% are castrated with anaesthesia and the remaining without anaesthesia or analgesia (PICGAS, 2008).

In the United Kingdom and in the most of Ireland castration is not performed, because the slaughter is carry out within 85 Kg of weight: entire pigs can boast a faster growth, a better feed conversion and leaner carcasses. In Spain and Portugal just a small percentage of pigs is castrated for the exportation of high quality meat (24% and 18% respectively).

Castration with anaesthesia include almost 3 millions pigs every year. In Norway from 2002 it is mandatory the administration of anaesthesia by a veterinarian before the procedure (Fredriksen *et al.,* 2009). It is use to carry out a intratesticular or subcutaneous lidocaine injection (Fredriksen & Nafstad, 2006). In Olanda since 2007 local anaesthesia is compulsory and since 2009 castration without general anaesthesia is forbidden (Spoolder & Baltusse, 2008). Also in Switzerland (PICGAS, 2008; Fredriksen *et al.*, 2009) the mutilation without anaesthesia or analgesia is forbidden since 2009, but from 2007 it is allowed the use of immunocastration (Improvac®; Pfizer) (Fredriksen *et al.,* 2009; Von Borrell *et al.,* 2009). The European Declaration on alternatives to surgical castration of pigs, signed by the majority of European actors (EU Commission, 2010) introduced the obligation from 2012 to use prolonged analgesia and/or anaesthesia in piglets subjected to the mutilation, exhorting to abandon the practice from the year 2018. However, the same document, signed by Italy as well, recognises unavoidability of castration in case of pig meat produced for traditional products: pigs reared under protocols for "traditional specialties guaranteed" or for "geographical indications" (Protected Geographical Indication (PGI) or Protected

Designation of Origin (PDO)) should be therefore submitted to surgical castration using analgesia and/or anaesthesia. For other production, the surgical procedure will be abandoned, and the main alternatives to the surgical procedure considered by European Union are:

- To rear entire males (EFSA, 2004).
- Control of nutrition (EFSA, 2004).
- Semen sexing (EFSA, 2004; ter Beek , 2007; von Borell *et al.,* 2009; Zamaratskaia and Squires, 2009).
- Slaughter <85 Kg (Dunshea *et al.,* 2001).
- Local anaethesia (White *et al.,* 1995; Prunier *et al.,* 2006; Leidig *et al.,* 2009; Sutherland *et al.,* 2010; EFSA, 2004, Haga *et al.,* 2005 ;Heinritzi et al. 2006b).
- Generale anaesthesia (McGlone *et al.,* 1993; Walker *et al.,* 2004; Hodgson, 2007; Gerritzen *et al.,* 2008,; Schulz et al. 2007a and 2007b ; Kupper and Spring, 2008; Axiak *et al.*, 2007).
- Immunocastration (EFSA, 2004; Zamaratskaia *et al.,* 2008; Velarde *et al.*, 2007).

Such a change will interest a big number of pigs. The Eurostat census of 2012 reports a total of 148.5 million pigs (EU27) reared in the European Community. Germany has the largest number of pigs with at least 27 million , Spain with 25 million is at the second place despite the decline in the number of animals compared to 2008. Following there are France (13,967), Poland (13,056), Denmark (12,348) and the Netherlands (12,013). The Italian pig population amounts to 9.3508 million heads. More than 80 % of the national pig production is concentrated in the regions of Northern Italy (Lombardia, Emilia- Romagna, Piemonte and Veneto). Italian pig farming , especially in the central and northern areas, differs considerably from other European countries. Italy directs its production towards a pig animal weight so high that is typically called "heavy pig" that is necessary in particular for the production of high quality hams, differently from the rest of Europe where pigs are slaughtered at weights below 100 kg and are selected to be sold as fresh meat.

1.3 Pain assessment in piglets

Surgical castration is practiced within seven days of age by veterinarians or trained personnel, without the use of anaesthesia or analgesia, according to the in force regulations (Dir. 2008/120/EU). In Europe the increasing change of opinion and interest of consumers, with regard to animal welfare, focused on the topic of surgical castration: consumer wants to be informed in regard to the real needs to perform such practice. Many researchers have therefore focused upon this issue concluding, based on the observation of behavioural and physiological parameters, that surgical castration is painful and stressful (White *et al.*, 1995, Horn *et al.*, 1999, Fitzgerald *et al.*, 2001;

Lessard et al., 2002; Prunier *et al.*, 2005; Prunier *et al.*, 2006; Von Borell *et al.*, 2009) during (Weary *et al.*, 1998; Horn *et al.*, 1999; Taylor *et al.*, 2000; Taylor *et al.*, 2001) and after the surgery (McGlone *et al.*, 1993; Hay *et al.*, 2003). It was also demonstrated that it is painful at any age (EFSA, 2004; Carroll *et al.*, 2006; Prunier *et al.*, 2006).

The identification and quantification of pain in animals is complex especially for the lack of an objective parameter and the inability to directly measure the subjective experience. Animals have different reactions to nociceptive stimuli, they exhibit a pain clinical semeiologic variability and they have different responses to analgesics treatment (ACVA, Position Statement, 2006) .

Pain may be associated with a condition of stress in animals. In fact, stress can be defined as a generalized and non-specific response to any factor which disturb or may disturb the compensatory ability of an animal to maintain homeostasis, or as a prolonged state of alert, characterized by excessive and persistent mobilization of physical and mental resources that the individual puts in place to answer to the environment real or perceived requests (Panzera and Albertini, 2008).

The acute response produced by the body against, for example, a painful stimulus, is represented by a mobilization of energy that is required to implement an adaptive behavioral response (Panzera and Albertini, 2008). The physiological response involves the activation of two main systems: one immediate and short-term , represented by the sympathetic nervous system and the activation of the adrenal gland to release catecholamines (adrenaline and noradrenaline), the other, slower and prolonged in time, represented by the activation of the hypothalamus-pituitary-adrenal axis with the release of glucocorticoids , as cortisol and corticosterone (Panzera and Albertini, 2008).

The first animal reaction may be an active behavior, such as escape or attack, or passive, ceasing to manifest normal behaviors and being able to get immobility (freezing). Animals can also show the increase of urination, defecation, vocalization or tremble.

Different methods have been investigated both experimentally and in clinical practice , with the aim to identify objective measures of pain assessment. These include the use of direct or animal based indicators such as observation and recording of physiological responses (heart and breathing rate, blood pressure, capillary perfusion , pulmonary ventilation, pupil dilation, etc.), neuroendocrine (cortisol, β -endorphin, catecholamines, glucagon, ADH, etc.), metabolic (metabolism of glucose, lipids and proteins) and behavioral (Short, 1998; Fernandez *et al.*, 2007). Therefore, it is possible to divide such indicators into physiological , neuroendocrine and behavioral.

1.3.1 Physiological indicators

Physiological indicators are represented by changes such as heart and breathing rate, blood pressure, capillary perfusion, and body temperature (Ferrante, 2008).

During surgeries the monitoring of physiological parameters is essential to assess the presence of pain and the depth of anesthesia and analgesia. Several studies have been conducted regarding the association between castration and the use of local anesthetics (White *et al.*, 1995; Ranheim & Haga 2006; Schiele *et al.*, 2010; Sutherland *et al.*, 2010; Hansson *et al.*, 2011), analgesics (Axiak *et al.*, 2007; Keita *et al.*, 2010; Sutherland *et al.*, 2012), general anaesthesia by injection (Mc Glone *et al.*, 1993;Axiak *et al.*, 2007; Heinonen *et al.*, 2009; Driessen *et al.*, 2010; Nussbaumer *et al.*, 2012) and by inhalation (Hodgson, 2007; Mühlbauer *et al.*, 2010; Waldmann *et al.*, 2010). Recent studies in veterinary field have introduced the use of infrared camera to detect body temperature (Eddy *et al.*, 2001; Morgante *et al.*, 2006; Morgante *et al.*, 2008 Gatto *et al.*, 2010). Thermography is a non-invasive diagnostic technique that does not have direct contact between the thermacam and the body surface of the animal, capable of detecting and measuring the heat emitted as infrared electromagnetic radiation (Eddy *et al.*, 2001). The result is the creation of a visual map of temperature gradients existing on the body surface (Morgante *et al.*, 2006). All objects emit infrared radiations; these radiations, invisible to human eyes, are detected by the camera that elaborates and represents it on a screen. The thermacam does not directly measure the temperature, but the intensity of the infrared radiation emitted by the body depending on the characteristics of the body itself, as well as on its temperature (Gatto *et al.*, 2010, Morgante *et al.*, 2006).

In animals body surface temperature is the expression of blood flow and metabolic rate of the underlying tissues, and the detection of patterns of abnormal temperature may indicate an underlying inflammatory process (Eddy *et al.*, 2001). In pig production it is used as a research tool to investigate the physiology of the energy use (Tivey and Benhazi, 2002) and to detect changes in average surface temperature of the body (Loughmiller *et al.*, 2001). Thermography has been applied to evaluate the relationship between piglets skin temperature and environmental temperature in a vehicle in transit (Costa *et al.*, 2011) and to assess the teeth temperature during resection by grinding (Radaelli *et al.*, 2012).

1.3.2 Neuroendocrine indicators

In the case of painful stimulus, as the surgical castration, the sympathetic nervous system and the hypothalamus-pituitary-adrenal axis are activated. The preganglionic neurons of the sympathetic nervous system stimulate postganglionic neurons that constitute secretory cells of the adrenal medulla. The latter release its transmitters, catecholamines, directly into the blood system, regulating the intermediary metabolism of the animal and the responses to acute stress. In particular, adrenaline acting on glucose metabolism promoting hepatic glycogenolysis and gluconeogenesis. Furthermore, stimulating glycogenolysis of skeletal muscle leads to the production of hepatic lactate used for the production of more glucose (Cunningham, 2006).

In the hypothalamus-pituitary-adrenal axis activation, through the release of CRH, the hypothalamus stimulates the pituitary to release ACTH, which leads to the release of glucocorticoids from the adrenal cortex. Among glucocorticoids the most representative for acute stress is cortisol. In physiological conditions, cortisol regulates itself by a negative feedback system by inhibiting the release of hypothalamic CRH and ACTH at the pituitary level. In conjunction with this regulatory mechanism there is the preset circadian rhythm (alternations sleep-wakefulness), resulting in a lower concentration of glucocorticoids in the evening and higher in the early hours of the morning (Cunningham, 2006). Glucocorticoids are degraded in the liver and partly conjugated with glucuronic acid and eliminated via the bile and then feces. The non-conjugated reach the kidney to be excreted through the urine (Panzera & Albertini, 2008). Following painful stimulation, cortisol concentrations increase quickly, in a few minutes. The actions of glucocorticoids are wide-ranging, involving glucose metabolism, lipid and protein. One of the most important is the stimulation of hepatic gluconeogenesis with the result of an increase of hepatic glycogen. Moreover, they have significant clinical effects, in particular inhibiting the inflammatory response.

The actions of both catecholamines and glucocorticoids are multiple and can be used to discriminate the responses resulting from a stressful event. The most commonly used is cortisol, but with the same purpose are also considered glucose, lactate, catecholamines, and ACTH (Ruis *et al.*, 1997; Hay *et al.*, 2003; Prunier *et al.*, 2005; Carroll *et al.*, 2006; Moya *et al.*, 2008; Marchant-Forde *et al.*, 2009; Mühlbauer *et al.*, 2010).

1.3.3 Behavioural indicators

Behavioural studies may be a useful method for the assessment of pain consequent to surgical castration (Rault *et al.*, 2011). Several studies have used both vocalizations emitted by piglets (Weary *et al.*, 1998; Taylor A.A. & Weary D.M. 2000; Puppe *et al.*, 2005; Schon *et al.*, 2006; Marchant - Forde *et al.*, 2009) and behavioural observation (McGlone *et al.*, 1993; Taylor *et al.*, 2001; Hay *et al.*, 2003; Llamas Moya *et al.*, 2008; Leidig *et al.*, 2009 ; Sutherland *et al.*, 2010; Sutherland *et al.*, 2012) .

The analysis and evaluation of animal behaviour can use several methods of observation, both directly and indirectly. The observations are often carried out indirectly through the use of video recording in order to reduce the effect of the observer. The indirect method provides the possibility to obtain a lot of data and for prolonged times; moreover it allows the observation of very rapid or complex actions. By contrast, in some cases, video recordings do not allow to resume some specific behaviors in detail (Mattiello & Panzera, 2008). In general, to start direct or indirect observations it is necessary, at first, to identify which subjects you want to watch and when. On the basis of the research's purpose, the choice of the sampling method is essential; secondly it is necessary to establish how to record the behaviour, if with continuous recording or time sampling (Martin & Bateson , 2007). In the scan sampling method, the observer records the activity of one or more individuals at regular time intervals. Through the scan, it is possible to record animal's behavioural state (behaviours that have an appreciable duration in time) and events particularly on a frequent manner. It is important to underline that this method does not provide neither the length, or the frequency of observed behaviours, or their sequence, but it can only give the idea of the percentage of time spent on different activities. This idea will be more accurate as the sample points are closer: they must be close enough to allow a realistic estimate of the percentage of time spent on various activities. The use of a timer can be useful to facilitate observations, reminding to the operator the exact moment of the scan. In the case it is required to observe several animals at once, or in the case of focal observations, it may be necessary to identify subjects individually. Such operations should be performed a few days prior to the sessions of observation to contain the effects of marking on animal behaviour (Martin & Bateson, 2007; Mattiello & Panzera, 2008). In the initial phase , the choice of behaviours to record will vary depending on the objective of the research. It 's always essential before starting the observations to define and to classify these behaviours within well distinct categories. This is important to make the observations repeatable by others who wish to perform similar experiments, in order to obtain comparable results (Mattiello & Panzera, 2008) .

Recently studies on laboratory animals have introduced facial expression as indirect tool for pain assessment. Actually at least 3 specific pain scales were validated which include the 'Mouse Grimace Scale' (Figure 1; Langford *et al.*, 2010), the 'Rat Grimace Scale' (Figure 1; Sotocinal *et al.*, 2011) and the 'Rabbit Grimace Scale' (Keating *et al.*, 2012). Considering the Mouse Grimace Scale (MGS) as an example, it consists in the observation of pictures of animals taken at different time, for example before anesthesia and after surgery, to which the scorer assigned a value from 0 to 2 for each of the action units (see below): a score of 0 indicates high confidence of the scorer that the action unit was absent; 1 indicates a moderate appearance of the action unit or an equivocation over its presence or absence, while a score of 2 indicates a marked appearance of the action unit. The final MGS score was the average score across the 4 or 5 action units, where mean difference scores were obtained by subtracting baseline (presurgery, predrug, or preanesthesia) MGS scores from those after surgery, treatment, or anesthesia. The Mouse Grimace Scale includes 5 Action Unit:

- "Orbital Tightening" mouse must display a narrowing of the orbital area, a tightly closed eyelid, or an eye squeeze. An eye squeeze is defined as the orbital muscles around the eyes being contracted. A wrinkle may be visible around the eye. As a guideline, any eye closure that reduces the eye size by more than half should be coded as a "2";

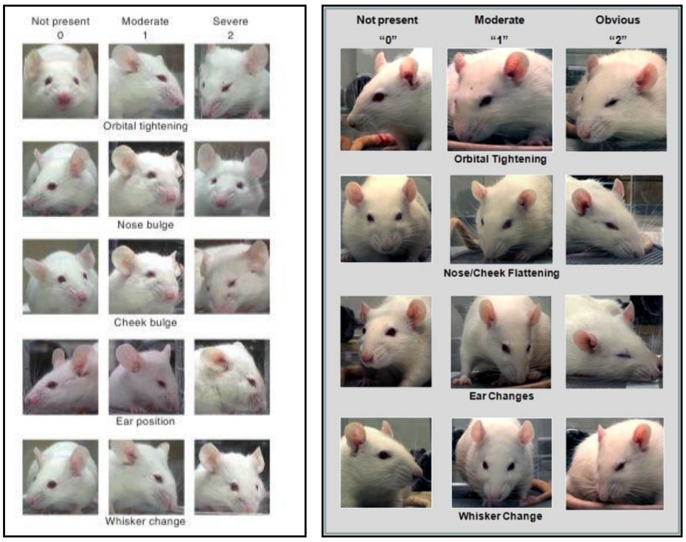
•"Nose Bulge" Mouse must display a bulge on top of the nose. The skin and muscles around the nose will be contracted creating a rounded extension of skin visible on the bridge of the nose;

•"Cheek Bulge" The cheek muscle is contracted and extended relative to the baseline condition; it will appear to be convex from its neutral position;

•"Ear Position" Ears may be pulled back from their baseline position, or may be seen as laid flat against the head;

•"Whisker Change" Whiskers must have moved from the baseline position. They could either be pulled back to lay flat against the cheek or pulled forward as if to be "standing on end". Whiskers may also clump together compared to baseline whiskers, which tend to be fairly evenly spaced.

Figure 1 Mouse and Rat Grimace Scale



Even if the technique is quite long and not of easy interpretation, it seems a good, non invasive method for pain assessment in animals as well as in non communicative humans (infants and unconscious patients). Actually there are no validated scale for pigs.

Among behavioural indicators of pain researcher have focused also in the study of posture considering that animals tend to assume protective position to avoid or reduce the stimulation of painful tissues (Figure 2; Molony and Kent, 1997; Mellor *et al.*, 2000). Moreover, alteration in latency between inactivity and movement after a procedure is considered an indication of distress in pigs (Chaloupkovà *et al.*, 2007).

Figure 2 Exemples of alterated postures

Aim

In the last thirty years there has been a significant increase in the public interest for animal welfare, and the European Declaration on alternatives to surgical castration in pigs is a practical consequence. In the case of Italy, where it will be maintained the procedure, it is necessary to introduce the use of analgesia and/or anaesthesia. Actually there are not specific drugs registered in pigs for such practice, and the choice of drugs should be influenced by the degree of pain that is present (Flecknell *et al*., 1999). Animals are unable to directly communicate their experiences of pain with us, therefore reliance must be made on behavioral and physiological indices of pain (Flecknell *et al.*, 1994).

The aim of this experimental study was to detect different methods of pain assessment in piglets subjected to surgical castration, including physiological and behavioral methods. Moreover, the reduction of pain through a non-steroidal anti-inflammatory drug has been tested.

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Chapter 2

PHYSIOLOGICAL AND BEHAVIOURAL RESPONSES IN PIGLETS SUBMITTED TO CASTRATION: PRELIMINARY STUDY

PHYSIOLOGICAL AND BEHAVIOURAL RESPONSES IN PIGLETS SUBMITTED TO CASTRATION: PRELIMINARY STUDY

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Published on Agriculturae Conspectus Scientificus Vol. 76 (2011) No. 4 (341-344)

Abstract

With the perspective to test drugs that may reduce pain due to surgical castration, this preliminary study tries to find out a robust and valid method for pain assessment in piglets. In the present study three treatments were applied: handling (H), tail docking (TD) and surgical castration + tail docking (CTD). To evaluate pain response to the treatments different variables were analyzed: movement latency (time from placing back the piglet inside the farrowing crate aft er treatments and its first movement towards the nest or the sow), rectal temperature and plasma cortisol and lactate levels. Movement latency was measured for all treatments. Rectal temperature was measured before treatments H and CTD, and 1, 3, 5, 24 hours later. Blood samples for cortisol and lactate determination were collected 1 hour before treatments H and CTD, right after and 3, 5, 24 hours later.

The significant increase of movement latency for CTD compared to H showed that pain can be assessed by this type of measure. Rectal temperature was significantly affected by time ($P < 0.01$) but not by treatment likely due to several factors that might have confounded the studied effect. Cortisol was significantly affected by interaction time*treatment ($P < 0.01$) particularly due to the high peak for CTD right after the surgical procedure. Lactate was modified only by time ($P < 0.01$).

This preliminary study suggests that a non invasive and easy measure such as movement latency is a promising method to assess pain in piglets after surgical castration and tail docking.

Keywords: piglets, castration, cortisol, lactate, pain assessment

Aim

Surgical castration is a painful practice in piglets considering that it is carried out without using anesthesia therefore it represents an important welfare issue (EFSA, 2004). With the perspective to test drugs that may reduce pain during and after castration, it is necessary to establish a protocol for the assessment of pain in piglets in a standardized way. The objective of this study was to identify a robust and valid method for the evaluation of pain in piglets after surgical castration and tail docking carried out without the use of anesthesia and performed before seven days of age according to D.lgs 53/04.

Material and methods

Animal, housing and variables

The study was carried out between January and April 2011 in a commercial sows' farm located in San Vito al Tagliamento in the Province of Pordenone (PN) in the north-east of Italy. The experimental measurements were taken in different farrowing rooms since farrowings occur every week in different rooms taking in account for the rotational management of the farm.

All piglets in the study belonged to a commercial hybrid (75% Large White 25% Landrace Belga) and were aged between four and seven days. Castration and tail docking procedures were carried out without administration of anesthesia or painkillers according to D.lgs 53/04.

Three different treatments were considered: handling (H), tail docking (TD) and tail docking + surgical castration (CTD).

The variables detected to evaluate signs of pain for each treatment were: movement latency, rectal temperature, and plasma levels of cortisol and lactate. Movement latency was measured for all treatments while the other variables were recorded only for H and CTD.

Movement latency

The time required by the piglets to make the first step after placing them back to the farrowing crate subsequent to handling (H), tail docking (TD) and castration + tail docking (CTD) procedures was recorded. This measure was taken on all males (257 piglets) of 35 litters chosen and assigned randomly to the different treatments: 31 piglets were only handled, 83 were tail docked, and 143 were surgically castrated and tail docked.

Rectal temperature

The measurement was carried out on all male piglets (32) of nine litters chosen and assigned randomly to the different treatments. Twelve piglets were only handled (H) and twenty were surgically castrated and tail docked (CTD). Male piglets of all litters were identified by numbers before mutilations and then individual rectal temperature was measured in order to obtain the basal temperature. The same measurement was repeated 1, 3, 5, and 24 hours after the treatment in all piglets.

Cortisol and lactate

Cortisol and lactate were measured on blood samples collected from all male piglets (32) of nine litters after their identification by numbers. Twelve piglets were only handled (H) and twenty were surgically castrated and tail docked (CTD). Blood samples were collected from the anterior vena cava using 2.5 ml syringe and then stored in vacuum tubes (Vacutest Kimasrl, Arzergrande, PD, Italy). The sampling was repeated 1 h before, immediately after castration (time 0) and 3, 5, 24 hours post treatment. At the laboratory, blood samples were centrifuged at 2500 × g for 10 min at 20°C. Serum cortisol concentration was determined with chemiluminescent assay (LKCO1, Medical System, Genova, Italy) performed with automated analyzer Immulite One (Medical System, Genova, Italy). Lactate was determined with a commercial kit for colorimetric assay (L-Lactate, Randox Laboratories Ltd., Co Antrim, UK) performed with automated analyzer Cobas 501 (Roche Diagnostics, Mannheim).

Statistical analysis

The normal distribution of all variables was tested using PROC UNIVARIATE (SAS, 2008). Variables with Shapiro-Wilk values (W) ≥ 0.95 were considered as normal, whereas all other variables were log transformed before analysis. Movement latency was analyzed using a mixed model procedure (SAS, 2008) considering the treatment (H, TD, and CTD) as fixed and the litter as random effect. Data on rectal temperature, cortisol and lactate were analyzed using the same statistical procedure considering the effects of treatment (H vs. CTD), time from the treatment, the interaction time*treatment and the random combined effect of animal - litter within treatment.

Results and discussion

Movement latency

Data reported in Figure 1 show that there is a different latency between piglets that were only handled compared to those surgically castrated and tail docked. Alterations of the normal behaviour of piglets are reported in the literature as indicators of discomfort subsequent to surgical castration (McGlone *et al.*, 1993; Keita *et al.*, 2010), therefore it is assumed that the increased movement latency observed in our study is an indicator of pain and distress. This is supported also by the fact that piglets after surgical castration show a change in locomotion, posture, contact with the sow and they display pain related activities such as tremors or spasms and vocalizations (Taylor and Weary, 2000; Llamas Moya *et al.*, 2008; Waldmann *et al.*, 2010). From results obtained in the present study it is clear that piglets subjected only to tail docking are always slower to resume movement after the mutilation but their reaction time has an intermediate value between handled and surgically castrated piglets. This underlines that the real difference is due to the pain experienced by males subjected to castration.

Rectal temperature

Rectal temperature was significantly affected by time ($P < 0.01$) (Figure 2) but not by treatment or by the interaction time*treatment. Although literature reports a temperature increase due to stress in animals (Takakazu *et al.*, 2001), in the present study it is likely that several other factors affected rectal temperature that did not allow us to find a relation between the temperature and the surgical procedure. These factors could be the presence of heat lamps, time from milk intake, activity performed by the piglet just before the measurement (sleeping or walking). It seems therefore that body temperature is not a useful indicator of stress/pain in piglets after castration.

Cortisol and lactate

As shown in Figure 3, cortisol levels of piglets that were only handled remained fairly constant but higher than those reported by several studies (Prunier *et al.*, 2005; Llamas Moya et al., 2008; Waldmann *et al.*, 2010), probably because handled piglets were sampled after the surgically castrated piglets but in the same room, so they heard all the vocalizations of the CTD piglets. As expected, piglets of CTD have had a significantly higher cortisol peak (P<0.01) right after mutilations and its concentration returned to baseline three hours post treatment. This result is in agreement with findings by Prunier et al. (2005) and Waldmann *et al.* (2010) while Llamas Moya *et al.* (2008) describes also a second peak three hours after the treatment.

Lactate was significantly affected only by time (Figure 4) whereas CTD piglets showed similar concentrations to those of H pigs. This result is in contrast with the literature that reports an increase of the lactate concentration after castration as a consequence of the release of the glycogen reserve (Prunier *et al.*, 2005).

Probably it is necessary to repeat the experiment with larger number of animals to see the real effect of CTD on blood parameters.

Conclusions

According to our results movement latency seems to be a reliable parameter. However the same measurement should be repeated at different time before and after castration in order to evaluate the duration of animal pain. Significant changes in cortisol levels have been observed only immediately after castrations therefore cortisol can be used as marker of acute stress.

Acknowledgements

The authors are grateful to Animal Ethics Pty Ltd for their financial support to the study and the pig farm “Azienda Agricola Milani, di Milani Maurizio Orazio Enzo” for the friendly hospitality.

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Figure 1 Latency (sec) from placing back the piglet to the farrowing crate and the first movement towards the nest or the sow after handling (H), tail docking (TD) and surgical castration + tail docking (CTD). Means with different letters differ significantly (*P* < 0.05) according to the Bonferroni test

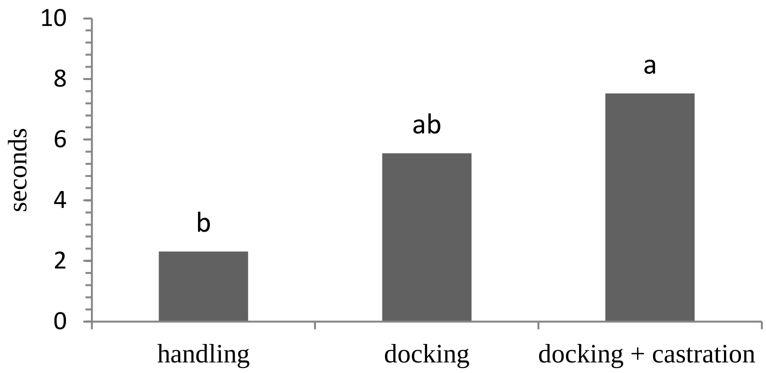


Figure 2 Rectal temperature (C°) at different hours from the treatment (H vs. CTD). Means with different letters differ significantly (*P* < 0.05) according to the Bonferroni test

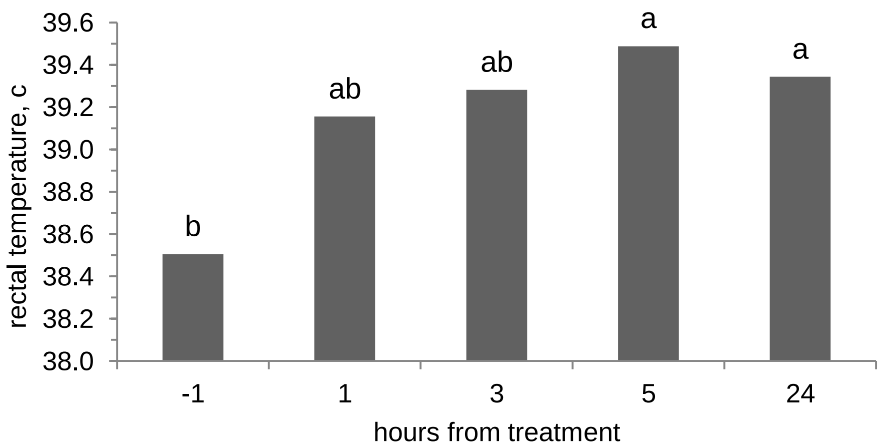


Figure 3 Blood cortisol levels (nmol/l) in piglets subjected to handling (H) and surgical castration + tail docking (CTD). Means with different letters differ significantly ($P < 0.01$) according to the Bonferroni test

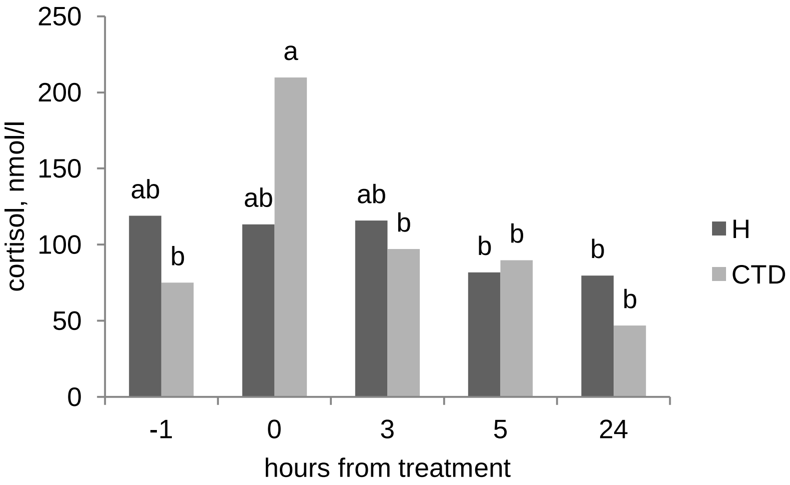
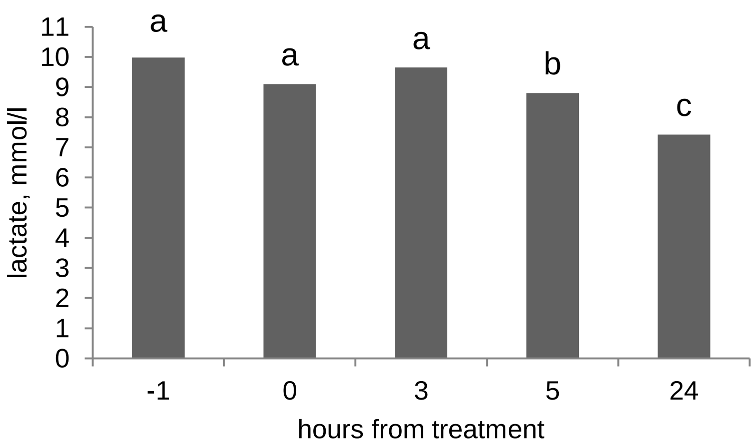


Figure 4 Lactate level (mmol/l) in piglets at different hours from treatment. Means with different letters differ significantly ($P < 0.05$) according to the Bonferroni test



Chapter 3

USE OF SEVERAL PARAMETERS FOR CASTRATION PAIN ASSESSMENT IN PIGLETS

USE OF SEVERAL PARAMETERS FOR CASTRATION PAIN ASSESSMENT IN PIGLETS

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Submitted to Animal Journal

Abstract

Given that surgical castration is a painful practice concerning millions of pigs every year, the need to validate objective pain assessment tools exists in order to formulate animal welfare policies and to test anaesthetic and analgesic protocols that may reduce related pain. Two treatments were applied: handling (H) and surgical castration (C), and physiological (cortisol, lactate, glycaemia, rectal and eye temperature) and behavioural variables (latency to move after treatment and alterations in posture and walking) were analysed. Cortisol showed a peak in C piglets right after the surgical procedure and a positive correlation with lactate and glycaemia concentrations. Eye temperature was higher in C piglets, and the same difference was detected for rectal temperature 3 hrs after castration. Among behavioural parameters, C piglets showed an increased latency to move and an higher risk of alteration in posture and walking. The study identified during the first three hours from castration the time interval in which both behavioural and physiological measurements concentrate detectable alterations. The latency to move, the alterations in posture and walking and eye temperature appear to give additional and useful information in piglets pain assessment. However, differently from the behavioural parameters considered, eye temperature needs several manipulation of the animals and a long process for temperature acquiring.

Keywords: *animal welfare, piglets, castration, pain assessment, thermography*

Implications

Surgical castration is a painful practice concerning millions of pigs every year and finding objective and scientifically robust pain assessment tools is necessary to develop further on effective pain reduction protocols. Results of this study showed that the time interval in which detectable behavioural and physiological alterations occur is within the first three hours from castration. Latency to move, alterations in posture and walking, and infrared thermography for the detection of eye temperature are promising tools for pain assessment in piglets. However, infrared thermography needs additional handling of the animals during the process for temperature acquiring.

Introduction

Surgical castration in piglets is the most commonly performed management practice in order to avoid boar taint in swine meat. It involves 96 million male pigs in Europe every year. This practice is widely executed without pain relief, even if it has been considered a distressing practice regardless of piglets' age (Carrol *et al.*, 2006; von Borell *et al.*, 2009). Moreover, it represents an animal welfare issue with an increasing negative public perception due to animal sufferings (Guatteo *et al.* 2012; Sutherland *et al.*, 2012). That's why the European Declaration on alternatives to surgical castration of pigs (EU Commission, 2010) exhorts to abandon the practice from the year 2018. However, the same document recognises unavoidability of castration in case of pig meat produced for traditional products, in order to meet their current quality standards. For this reason pigs reared under protocols for "traditional guaranteed specialties " or for "geographical indications" (Protected Geographical Indication (PGI) or Protected Designation of Origin (PDO)) should be therefore submitted to surgical castration using analgesia and/or anaesthesia. The waiver include the Italian heavy pig production for the typical cured ham in which castration is unavoidable considering that pigs are slaughtered at around 160 kg and 9 months of age, when the sexual maturity has been reached and might be responsible of pronounced boar taint, but an appropriate protocol for pain reduction during castration is required.

During the past years, methods to reduce pain with anaesthesia and/or analgesia were investigated (Sutherland *et al.*, 2010, Kluivers-Poodt *et al.*, 2012). Up to now the measurements used for pain assessment in piglets in such studies have mainly focalized on the evaluation of pain during the surgery itself. However, pain is a complex and individual sensation, which means that it is difficult to measure and compare it among animals (Sneddon and Gentle, 2000). Management could also interfere with the behavioural and physiological responses of young pigs (Llamas Moya *et al.*, 2008).

In order to monitor suffering after mutilation, main pain indicators commonly considered are physiological (including a clinical and a neuroendocrine approach) and behavioural parameters (Sutherland *et al.*, 2012; Weary *et al.*, 2006).

Within physiological parameters, clinical indicators such as rectal temperature, heart rate, respiratory rate, arterial pressure, capillary perfusion and pupillary dilatation, are usually assessed during general anaesthesia (Hodgson, 2007) or sedation (Axiak *et al.*, 2007), but they are difficult to assess in awake piglets. Regarding neuroendocrine indicators, surgical castration has shown to cause the activation of the hypothalamus-pituitary-adrenal and the sympathetic axes (Prunier *et al.*, 2005). This leads to several consequences such as the increase of ACTH and plasma cortisol up to 60 minutes after castration (Marchant-Forde *et al.*, 2009; Keita *et al.*, 2010; Sutherland *et al.*, 2012) with a possible second peak 3 hours later (Llamas Moya *et al.*, 2008), the increase of lactate due to glycogen mobilization (Prunier *et al.*, 2005), and the rapid and transient increase of epinephrine

followed by a longer lasting increase in plasma noradrenaline (Prunier *et al.*, 2002; Muhlbauer *et al.*, 2010). Moreover, variations of sympathetic and parasympathetic systems seem to alter eye temperature in animal subjected to painful practice, as recently described in cattle by Stewart *et al.* (2010). Surgical castration causes also behavioural changes (Taylor *et al.,* 2001; Keita *et al.,* 2010; Sutherland *et al.,* 2012). Changes in behaviour are considered relevant parameters to assess the overall pain and discomfort following this practice (Keita *et al.,* 2010) and are studied with methodologies as scan or focal sampling (Hay *et al.*, 2003; Llamas Moya *et al.*, 2008; Sutherland *et al.*, 2012). Alteration in latency between inactivity and movement is considered to indicate distress in pigs (Chaloupkovà *et al.*, 2007). Increased restlessness in animals suffering from pain could be interpreted as an adaptation attempt to stop other animals from inflicting more pain. Moreover, stiffness can be considered as protective, allowing the animals to avoid or reduce the stimulation of painful tissues after castration (Molony and Kent, 1997; Mellor *et al.*, 2000). Moreover, Keita *et al.* (2010) observed that an animal in pain assumes a different standing position and changes walking behaviour in order to relive pain after castration.
The present study aimed to test some innovative parameters such as latency to move and eye temperature, that, to author's knowledge, have never been used for the detection of surgical castration's pain in piglets. These parameters have been compared with the commonly used indicators of pain in order to evaluate a complete pain assessment. Considering the pressure of the European Declaration (EU Commission, 2010), which required to perform research on alternatives to surgical castration in piglets destined to traditional high quality products, the study was conducted in the specific rearing context of the heavy pig, with the perspective to subsequently test analgesic and anaesthetic protocols.

Material and methods

Animal, housing and surgical procedure

All procedures carried out on the animals were approved by the Ethical Committee for Animal Experiments of the University of Padova with the permission number 56 BIS-2012.
The study was conducted in a 2500 sows commercial pig farm located in the north-east of Italy. Data were collected over a 16 week period. All piglets in the study belonged to a commercial hybrid (75% Large White and 25% Belgian Landrace) selected for the production of the Protected Designation of Origin (PDO) cured ham.
Farrowing accommodations consisted of eight identical rooms. Each room held 12 farrowing crates, with two rows of six crates separated by an aisle. Crates had fully slatted floors made of wire mesh covered with a plastic carpet. Ventilation and temperature were automatically controlled by fans and air heating, and an electric radiant lamp was used to heat the creep area in each pen.

Immediately after farrowing, fostering was performed in litters for size and body weight of piglets. At day 4 after birth, piglets were subjected to intramuscular injection of iron dextran and males were castrated. For the present study 530 male piglets were randomly allocated to the two treatment groups: handling (H) and surgical castration (C). At the catching, piglets were visually marked with a different coloured number on their back depending on treatment group. Underweight piglets or piglets with visible clinical problems were excluded from the study. Surgical castration of the C group was performed following the common castration technique without using anaesthesia or painkillers, as allowed by the European Directive in force (EU Council Directive 2008/120/EC) if castration is carried out before 7 days of age of piglets. The piglets were restrained between the handler's legs in a head down position, then an incision on each side of the scrotum was carried out with a scalpel to extract testicles and removed them by cutting the spermatic cord. Finally a chlorhexidine-based antiseptic was applied on both the open wounds. In handled piglets (H) castration was simulated without incisions but handling and antiseptic application were performed in the same way of C group. After the procedure, piglets returned to their pen. To avoid confounding effects due to manipulations during experimental procedures (i.e. blood samples collection), behavioural and physiological measurements were carried out on different piglets. A summary of number of piglets used for different experimental measurements at different time from castration within experimental treatments is reported in Table 1.

Experimental measurements

Plasma cortisol, lactate and glucose levels were measured on samples collected from 32 male piglets belonging to eight randomly chosen litters. In every litter, four male piglets were chosen and randomly allotted to H (n=12) or C group (n=20). Blood samples were collected from the anterior vena cava using 2.5 ml syringe and then stored in vacuum tubes without anticoagulant (Vacutest Kima srl, Arzergrande, PD, Italy). Blood was sampled 1 hour before treatment (T-1) to obtain individual basal levels and then repeated within 30 minutes after castration (T0). To decrease the stress due to repeated blood sampling, half piglets were then sampled 3 hours after treatment (T3), whereas the other half 5 hours after treatment (T5). Twenty-four hours after treatment (T24) all the piglets were sampled again. Glycaemia was immediately measured at farm using a human self-monitoring system for assessing glycemic status (Accu-Chek®), never used in swine but already validated in cattle as a convenient field monitor (Rumsey *et al.*, 1999). The system allowed to measure glucose level instantaneously using a drop of blood remained in the syringe after the collection. For cortisol and lactate, blood samples were stored at 4°C and transferred to the laboratory where they were centrifuged at 2500 × *g* for 10 min at 20°C. Serum cortisol concentration was determined with chemiluminescent assay (LKCO1, Medical System, Genova, Italy) performed with automated analyzer Immulite One (Medical System Genova, Italy). Lactate was determined with a commercial kit for colorimetric assay (L-Lactate, Randox Laboratories Ltd., Co Antrim, UK) performed with automated analyzer Cobas 501 (Roche Diagnostics, Mannheim).

Rectal temperature was measured using a digital paediatric thermometer which was disinfected after each measurement and lubricated with gel for rectal examination in order to decrease the stress to the animals. In the first part of the experiment, rectal temperature was measured on 10 handled piglets and 14 surgically castrated piglets belonging to 6 litters. The measurement was performed 1 hour before treatment in order to obtain the basal temperature (T-1), then it was repeated immediately after treatment (T0), 4 hours (T4) and 24 hours after (T24). On the basis of the results of the first set, showing an increase in temperature around T4 (see the result section for details), a second part of the experiment was scheduled in order to investigate the temperature's trend around the fourth hour after treatment in more detail. Therefore, rectal temperature was measured 1 h before treatment (T-1), 1 hour after (T1), 3 (T3), 5 (T5) and 24 (T24) hours after treatment in other 19 handled and 43 surgically castrated piglets belonging to 16 litters.

Eye temperature was measured using a infrared camera on 28 piglets belonging to seven litters and randomly assigned to handling treatment (n=12) or surgical castration treatment (n=16). Piglets eyes' images were scanned using a hand-held portable infrared camera (Flir System, Model ThermaCam P25), which was calibrated to room temperature and absorptive conditions on each sampling day. While piglets were restrained by an operator, a second operator took two pictures of piglets' left half of the face. The distance between the piglet and the camera was 80 cm at a right angle and it was kept constant for all the piglets and all the time of measurement. Images were then processed and transferred on the computer. Maximum and mean eye temperatures (°C) of the medial posterior palpebral margin of the lower eyelid and the lacrimal caruncle (Stewart *et al.*, 2008) were then extracted from images. Image analysis software (ThermaCam Researcher Basic Software, Flir System, Inc. 27700 SW, Parkway Avenue Wilsonville, OR 97070) was then used . Only images depicting completely open eyes were considered. The measurement was carried out before treatment (T-1), immediately after (T0) and then repeated 2 (T2) and 4 (T4) hours after treatment.

Latency to move was measured as the time required by piglets to make the first step when replaced back within the farrowing crate after treatment (handling or castration). This measure was collected to express how fast after treatment (T0) the animal has recovered. The test was carried out on 287 piglets from 48 litters. Piglets were randomly allotted to H (n=130) or C group (n=157). Alterations in posture and gait after castration were observed. A total of 97 male piglets belonging to 25 litters were involved in this part of the study. Piglets were randomly allotted to H (n=20) or C group (n=77). Direct observation of standing position and walking behaviour of each piglet put back in the farrowing crate was carried out by a veterinarian before treatment (T-1), right after (T0), and 1 (T1), 2 (T2), 3 (T3), 5 (T5) and 24 (T24) hours after treatment. In particular, the occurrence of weaker and protracted forward hind limbs (hind limbs positioned more forward than normal under the belly), hind limb non weight bearing (walking with one hind limb off the ground), hind tip-toe walking pattern (contact with the floor only by the distal part of the hoof) and kyphosis (hunchback) were observed. When a piglet showed at least one alteration it was classified as "altered", otherwise as "normal".

Statistical analysis

The normal distribution of all variables were tested by Proc Univariate (SAS 9.2, SAS Institute Inc., Cary NC). Variables with Shapiro-Wilk values W≥0.90 were considered as normal, whereas all not normally distributed variables (glucose and latency to move) were log transformed before analysis. Data on cortisol, lactate, glucose, rectal temperature and eye temperature were covariated on the individual basal value and analyzed using an unequally spaced repeated measures model (PROC MIXED of SAS) in which treatment, time and interaction treatment×time were the fixed effects, the litter was the random effect and the animal the repeated factor. Pearson correlations within treatment were also calculated among blood parameters (cortisol, lactate and glucose) and between rectal and eye temperature. The variable latency to move was analyzed by using a mixed model procedure considering the treatment as fixed effect and the litter as random. For statistical differences ($P<0.05$) showed by time and time×treatment effects, contrast were also calculated. Risk rations of the presence of locomotory alterations were calculated between handled and surgically castrated piglets at different time of observation.

Results

Cortisol and lactate were not significantly affected by treatment, but both variables were affected by time of sampling and by interaction time×treatment. Considering C piglets, the cortisol concentration showed a peak ($P<0.001$) at the first sampling after surgery (T0), whereas it returned to the baseline level three hours later (Figure 1A). Regardless of treatment, cortisol had a higher concentration at T0 (171 nmol/l) compared to all other samplings ($P<0.001$). Lactate level of piglets was different ($P<0.05$) 24 hours after treatments and its concentration was significantly lower in handled piglets than in castrated (Figure 1B). Glucose was not affected by treatment or by interaction treatment×time, but it was affected by time ($P<0.001$; figure 1C). In particular, the concentration was higher at T0 in comparison to those measured in samples collected at 3, 5 and 24 hours. Moreover, in C piglets glucose was positively correlated to lactate concentration ($r_p = 0.30$; $P<0.05$) and cortisol concentration ($r_p = 0.38$; $P<0.01$).

In the first step of the study in which rectal temperature was recorded at time 4 hours after castration, the parameter was significantly affected by time ($P<0.001$), but not by treatment or interaction time×treatment (Figure 2A). In particular, rectal temperature at T4 was higher (39.5±0.1) compared to those at T0 (39.1±0.1) and T24 (39.2±0.01). In the second batch, rectal temperature was affected by interaction time×treatment (Figure 2B), in particular at T3 it was higher in castrated compared to handled piglets ($P<0.01$). As shown in Table 2, regardless of treatment, eye temperature was affected by time of measurement ($P<0.001$). Mean and maximum temperature were higher 4 hours after treatments compared to the previous measurements. Maximum temperature was higher in castrated piglets compared to handled ($P<0.05$). In the same piglets, rectal temperature was measured with the same time interval, and it resulted correlated to maximum eye temperature ($P<0.01$) and to mean eye temperature ($P<0.05$) in both H and C piglets (Table 3).

Latency to move was significantly affected by treatment ($P<0.01$), and H piglets spent 2.6 (CI 0.35-0.39) seconds before moving after being handled, whereas C piglets spent 3.9 (CI 0.45-0.5) seconds (Figure 3). The observation of locomotory behaviours (postures and gait) showed that surgically castrated piglets had a higher risk to show alterations than handled piglets, both at T0 and one hour after treatment (Table 4). From T3 onwards, the risk was not statistically different between handled and castrated piglets.

Discussion

Cortisol concentration in castrated piglets showed a peak right after castration, suggesting an increased adrenal output because of the surgical procedure. As stated by Prunier *et al.* (2005), this variation was due to castration itself because cortisol level was lower in pigs submitted to sham castration, suggesting a relation to pain or tissue damage. Results are in agreement with a recent study in which a cortisol peak was found between 30 and 60 minutes after castration (Sutherland *et al.*, 2012), and confirmed usefulness of cortisol measurement in identifying and quantifying acute pain in livestock animals (Wood *et al.*, 1991; Molony *et al.*, 1995). Moreover, the increment of plasma cortisol after castration was short-lasting: from three hours after castration onwards, the cortisol level decreased to that of handled piglets, in agreement with results by Prunier *et al.* (2005) and Waldmann *et al.* (2010). After a painful event the epinephrine stimulate the mobilization of muscular glycogen and its consequent hepatic metabolism, with the result of an increase in lactate and glucose concentrations (Mayes, 1995). In the present study the absence of a significant treatment effect at T0 for glucose and lactate could be due to the insufficient level of glycogen stores in so young pigs, as described by Prunier *et al.* (2005). However, the positive correlations among cortisol, glucose and lactate levels in C piglets suggest a congruent physiological response to distress of all blood parameters measured in the present study. The highest level of lactate in C piglets recorded only 24 hours after castration could be due to its accumulation when abdominal muscle tension is experienced. Cervero and Laird (1999) indeed reported a display of abdominal muscle tension in case of visceral pain states in humans. A further hypothesis that could explain this trend of lactate, could be found in the results of Llams Moya *et al.* (2008) who observed a peak in TNF-α (Tumor Necrosis Factor- α) level in piglets 3 hours after castration. The TNF-α seems to be effective in accelerating muscle proteolysis with a following increased serum lactate, as demonstrated by Goodman (1991) in rats.

Increase in body temperature is mediated by the autonomic nervous system and occurs during exposure to anxiogenic or stress-inducing stimuli, like noise, heat, handling, novelty or pain (Olivier *et al.*, 2003). In the present study the body temperature was investigated throughout rectal measurements and eye thermography. The rectal temperature in castrated piglets at T3 was higher compared to that of handled ones and this difference was short-lasting and disappeared in about one hour. The eye temperature was also higher in castrated compared to handled piglets, but the chosen timing of sampling was not useful to identify the exact time in which this difference arise. Stewart *et al.* (2010) and Dockweiler *et al.* (2013) described an increase of eye temperature in calves right after castration (up to 10 minutes) due to an increase in parasympathetic nervous system activity associated with deep visceral pain. Starting from these results, it seems necessary to better investigate the eye temperature in castrated piglets between T0 and T2 increasing the number of scan samplings. Literature reports a temperature increase due to stress in animals (Takakazu *et al.*, 2001), however in the present study the evidence of a transient and tardive hyperthermia in castrated piglets might be also related to the inflammatory response to the tissue damage. Roth *et al.* (2009) stated that hyperthermia is observed in response to injury, such as surgery and trauma, for the activation of the endogenous mechanisms related to inflammation. Among inflammatory mediators, TNF-α and IL-1β are considered the main endogenous pyrogens (Roth *et al.*, 2009), and according to the research carried out by Llams Moya *et al.* (2008), they increase in piglets 3 hours after castration, which could explain the tardive hyperthermia recorded in the present study. Even if the physiological explanation of results described above seem to support the output of the study, it is not possible to exclude that other external factors, such as exposure to heat lamps or time from milk intake, might have affected the body temperature. Considering eye thermography, the presence of different quantity of tears might have influenced eye temperature recorded, as found by Kamao *et al.* (2011) studying the dry eye syndrome in humans.

Among behavioural parameters, latency to move showed difference between treatments. Castrated piglets needed more seconds than handled ones to start walking after treatment. The fact that handled piglets were more prompt to move suggest that it was not the manipulation the cause of pain at the hind legs but the castration *per se.* Walking is considered a non-specific behaviour normally expressed by the piglets but easily involved in alterations, probably due to pain to the hind region, as indicators of discomfort subsequent to surgical castration (McGlone *et al.*, 1993; Keita *et al.*, 2010). Before treatments, none of the piglets showed alterations such as weaker and protracted forward hind limbs, hind limb non-weight-bearing, hind tiptoe walking and kyphosis. Immediately after treatment and 1 hour later, castrated piglets had a higher risk of locomotory alterations' occurrence compared to handled piglets. The fact that from T2 onward there was no walking or postural difference between treatments seems to support the hypothesis that detectable signs of pain due to castration persist less than 2 hours. Similar results were found Kluivers-Poodt *et al.* (2013) although they are in contrast with those of Hay *et al.* (2003) that reported abnormal behaviour for a few days.

In conclusion, given that surgical castration is a painful practice concerning millions of pigs every year, the scientific interest in finding objective pain assessment tools is increasing. The same tools could be applied to evaluate the effectiveness of anaesthesia and analgesia protocols to adopt during piglets castration as requested by European Declaration (EU Commission, 2010) in case of pig meat produced for traditional products. The study identifies in the first three hours from castration the time interval in which behavioural and physiological measurements concentrate detectable alterations. From a methodological point of view, besides to the most commonly used pain indicator such as cortisol, the latency to move and the alterations in posture and walking appear to give additional information. Moreover, these latter parameters are non-invasive and easy to apply, allowing their repetition several times for the monitoring of pain over time. Infrared thermography applied for the evaluation of alterations of eye temperature also seems a promising tool for pain assessment in piglets, even if it requires further investigations right after castration in order to assess transient and quick variations more precisely. However, differently from latency and alteration in posture and walking, eye temperature needs several manipulations of the animals and a long process for temperature acquiring.

Acknowledgements

The authors would like to thank Animal Ethics Pty Ltd (Australia) for funding this project. We are grateful to Dr. Meredith Sheil for advice and comments.

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Table 1 Number of piglets used within treatment (handled vs castrated) at different times for different experimental measurements

	Handled								Castrated							
Time of measurements	T-1	T0	T1	T2	T3	T4	T5	T24	T-1	T0	T1	T2	T3	T4	T5	T24
Blood parameters:																
Cortisol	12	12	-	-	6	-	6	12	20	20	-	-	10	-	10	20
Lactate	12	12	-	-	6	-	6	12	20	20	-	-	10	-	10	20
Glucose	12	12	-	-	6	-	6	12	20	20	-	-	10	-	10	20
Physiological parameters:																
Rectal temperature (1)	10	10	-	-	-	10	-	10	14	14	-	-	-	14	-	14
Rectal temperature (2)	19	-	19	-	19	-	19	19	43	-	43	-	43	-	43	43
Eye temperature	12	12	-	12	-	12	-	-	16	16	-	16	-	16	-	-
Behavioural parameters:																
Latency to move	-	130	-	-	-	-	-	-	-	157	-	-	-	-	-	-
Difference in posture and walking	20	20	20	-	20	-	20	20	77	77	77	-	77	-	77	77

Table 2 Effects of treatment and time of sampling on eye temperature in piglets subjected to handling (H) or surgical castration (C)

	Treatment		Time[1]			RMSE[2]	*P*	
	H	C	T0	T2	T4		treatment	time
Eye temperature:								
Maximum	35.5	35.8	35.5^{B}	35.5^{B}	35.9^{A}	0.11	0.037	<0.001
Mean	34.1	34.6	34.3^{B}	34.1^{B}	34.7^{A}	0.13	0.120	<0.001

[1]Time: right after treatments (T0), 2 (T2), and 4 (T4) hours after treatments

[2]RMSE: Root Mean Square Error

Least square means in the same row with different superscript letters differ significantly ($^{A,\ B}$: $P<0.001$)

Table 3 Correlations between rectal and eye temperature in handled (H) and surgically castrated (C) piglets

	Eye temperature			
	Maximum		Mean	
	H	C	H	C
Rectal temperature	0.45**	0.31**	0.38*	0.28*
Eye temperature:				
Maximum	1	1	0.72***	0.86***
Mean			1	1

*: *P*<0.05; **: *P*<0.01; ***: *P*<0.001.

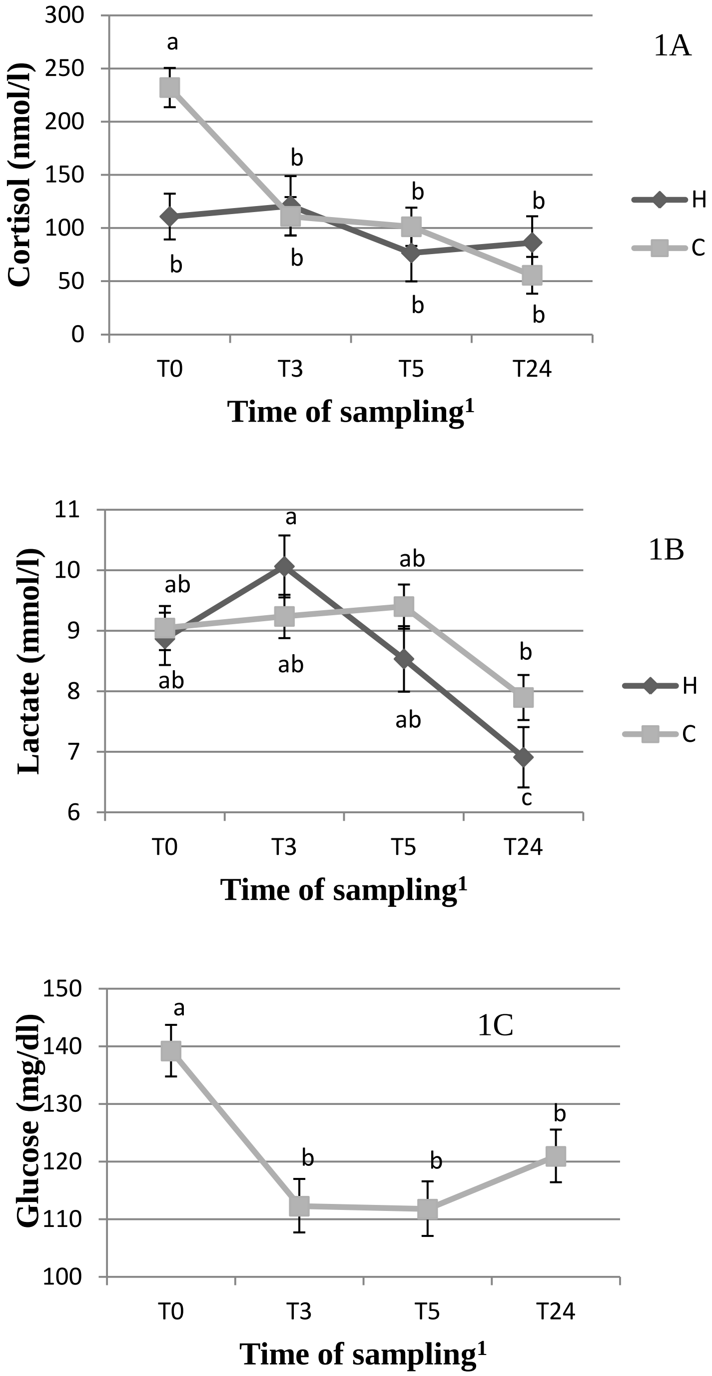
Table 4 Relative risk (RR) of locomotory behavior alterations in surgically castrated piglets (C) compared to handled piglets (H) at different time of observation

Time of observation[1]	% of castrated with alterations	RR	Confidence interval (95%)		*P*
			Lower limit	Upper limit	
T0	56	11.17	1.64	76.23	***
T1	39	7.79	1.13	53.72	**
T3	42	4.68	0.66	32.95	ns
T5	32	1.82	0.45	7.36	ns
T24	21	2.34	0.31	17.39	ns

[1]Time of observation: right after treatments (T0), 1 (T1), 3 (T3), 5 (T5) and 24 (T24) after treatment

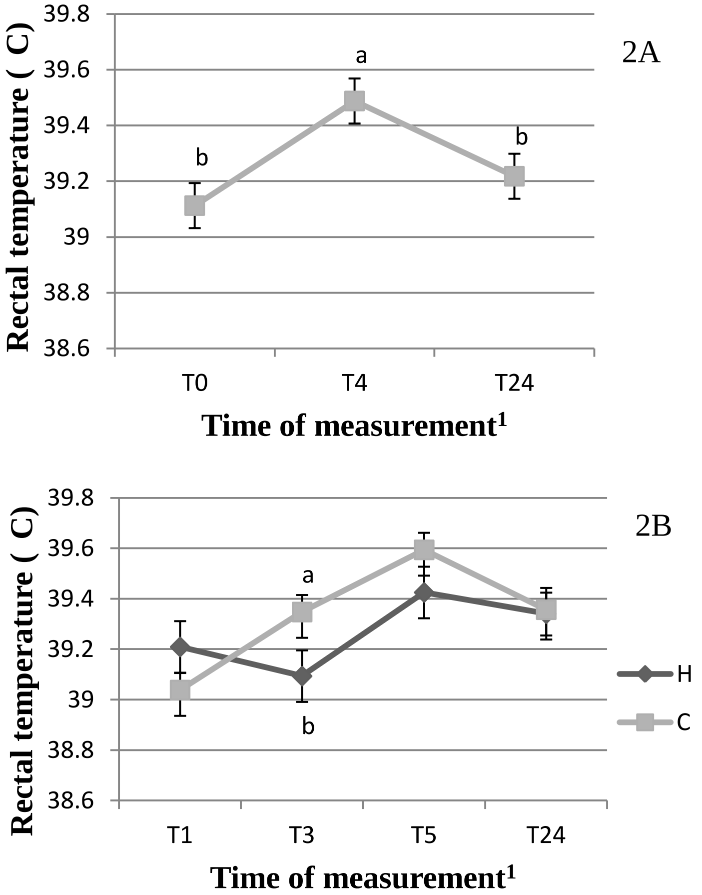
: *P*<0.01; *: *P*<0.001.

Figure 1 Effects of interaction treatment×time on cortisol (1A), lactate (1B) and glucose (1C) in handled (H) and castrated (C) piglets at different time of sampling. Least square means with different letters differ significantly per $P<0.05$



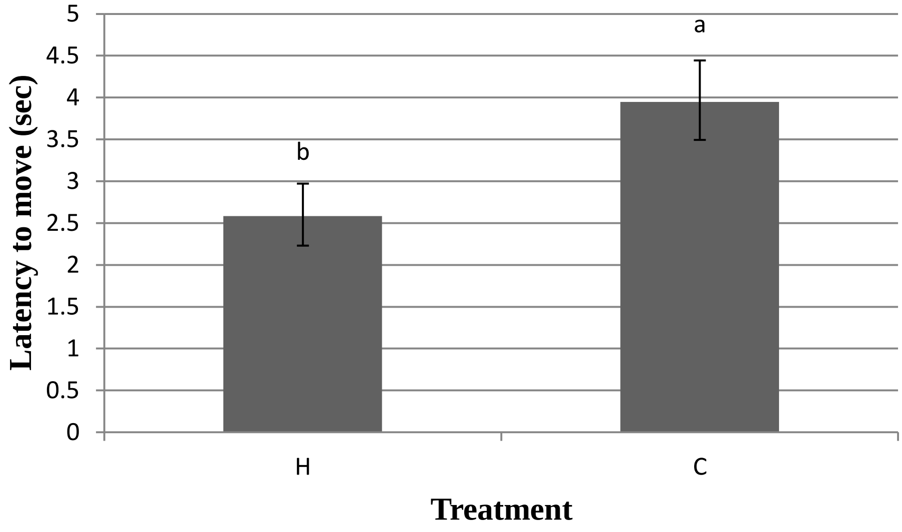
[1]Time of sampling: 20-50 minutes after treatments (T0), 3 (T3), 5 (T5) and 24 (T24) hours after treatments

Figure 2 Effect of time (2A) on rectal temperature in piglets of the first part of the experiment and interaction treatment×time in the second part (2B). Least square means with different letters differ significantly per *P*<0.01



[1]Time of measurement: right after treatments (T0), 1 (T1), 3 (T3), 4 (T4), 5 (T5) and 24 (T24) after treatment

Figure 3 Latency to move in piglets immediately after handling (H) or surgical castration (C). Least square means with different letters differ significantly ($P<0.01$)



Chapter 4

A PRELIMINARY STUDY ON THE 'GRIMACE SCALE': DO PIGLETS IN PAIN CHANGE THEIR FACIAL EXPRESSION?

A PRELIMINARY STUDY ON THE ‘GRIMACE SCALE’: DO PIGLETS IN PAIN CHANGE THEIR FACIAL EXPRESSION?

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Abstract

With the increase in attention towards animal welfare, researchers have focused their interest on the assessment of pain in farm animals. The possibility to assess pain through changes of facial expression has been studied in humans and animals. Although pigs have fewer muscles for facial expression, there are subtle changes in appearance there are currently no published pain scales based on facial expression in pigs. The aims of this research were to investigate if it is possible to observe changes in piglets' facial expressions immediately after painful procedures and subsequently the possibility to use facial expressions for pain assessment in piglets subjected to surgical castration with or without three different non steroidal anti-inflammatory drugs. In the first trial, 18 piglets were subjected to surgical castration. Images of faces were taken at different times in respect to the surgery: before, immediately after, 30 minutes, 1 hour, 2 hours and 4 hours after castration (126 pictures in total). These images were sorted and those in which piglets had closed eyes were excluded. Images were evaluated by three treatment-blind observers, scoring eye tightening (eye), cheek tension (cheek) and overall pain (pain) on a 3-point scale (0 = not evident, 1 = evident, and 2 = very evident). Data were submitted to statistical analysis with the non-parametric Wilcoxon Signed Rank Test, and results showed that the cheek tension ($P<0.01$) and the overall pain score ($P<0.001$) were significantly different at different time of detection. In the second trial, facial expressions were used by an observer to assess pain differences in 80 male piglets belonging to 16 litters and subjected to one of the 5 treatments: ordinary surgical castration (C), meloxicam plus surgical castration (M), ketoprofen plus surgical castration (K), tolfenamic acid plus surgical castration (T), handling (H).

Keywords: *facial expression, piglets, castration, pain*

Introduction

Facial expressions are a behavioural source of evidence about pain (Lewis *et al.*, 1990; Rosenstein *et al.*, 1988). As described by Prkachin (2009), changes in facial expressions are routinely used to assess pain in humans who cannot self-report, such as infants and unconscious patients. With the increase in attention towards animal welfare, the quality of pain management in laboratory animals has become highly important, however it is complicated greatly by the inability to recognize pain and dissociate it from other sources of distress (Matsumiya *et al.,* 2012). Differently from commercial pigs that can be subjected to tail docking or castration without using analgesia or anaesthesia, in laboratory animals the legislation requires avoiding or alleviation of unnecessary pain (Dir. 2010/63 EU). Even if analgesic protocols are used, it remains difficult to understand their real efficacy because of the lack of incontrovertible signs of pain. Therefore the research activity in this field has been addressed to assess pain according to objective and blinded scoring of facial expressions using the facial action coding system starting from those proposed for humans by Ekman and Friesen (1978). Initially, thanks to the similar anatomy, a scale based on changes in facial expression has been developed for non-human primate such as the chimpanzee (Vick et al., 2007) and the macaque (Parr *et al.,* 2010). Then, other 3 pain scales were developed which include the 'Mouse Grimace Scale' (MGS; Langford *et al.,* 2010), the 'Rat Grimace Scale' (RGS; Sotocinal *et al.,* 2011) and the 'Rabbit Grimace Scale' (RbtGS; Keating *et al.,* 2012). Although with some species differences, the three scales included four (RGS) or five (MGS and RbtGS) Facial Action Units (FAUs) focus on the eyes, nose, cheeks, ears and whiskers of an animal.

The scale, developed and validated using routine nociceptive tests, demonstrated a significant increase in MGS score from pre to post procedure, with high reliability (ICC: 0.9) and, depending on experience and video quality, accuracy between 72 and 97% (Langford *et al.,* 2010). In the rat grimace scale, cheek and nose are included in the same action unit "Cheek/nose flattening": opposite to mouse, rats in pain present a lack of bulge on top of the nose (i.e., a flattening of the nose) and the bridge of the nose flattens and elongates. Using the scale Sotocinal *et al.,* (2011) demonstrated a significant increase in RGS score from pre to post pain procedure and the effect of analgesia. Like the MGS, RGS is highly reliable (ICC: 0.9) and accurate (>81%) (Sotocinal *et al.,* 2011).

The objectives of the current study were to evaluate the possibility to use observations of the facial expression for pain assessment in piglets subjected to painful stimuli (castration) and to assess differences in facial expression of piglets using three non steroidal anti-inflammatory drugs to reduce pain.

Material and methods

The study has been carried out in two parts

2.1 First experiment

The first experiment was conducted in a 400 sows commercial pig farm located in the north-east of Italy. All piglets in the study belonged to a commercial hybrid (75% Large White and 25% Belgian Landrace) selected for the production of the Protected Designation of Origin (PDO) cured ham. Farrowing accommodations consisted of eight identical rooms. Each room held 12 farrowing crates, with two rows of 6 crates separated by an aisle. Crates had fully slatted floors made of wire mesh covered with a plastic carpet. Ventilation and temperature were automatically controlled by fans and air heating, and an electric radiant lamp was used to heat the creep area in each pen. Immediately after farrowing, fostering was performed in litters for size and body weight of piglets. At day 4 after birth, piglets were subjected to intramuscular injection of iron dextran and males

were castrated. For the present experiment 18 male piglets were subjected to routine surgical castration without using anaesthesia or painkillers, as allowed by the regulation in force if castration is carried out before 7 days of age of piglets (Legislative Decree 122/2011). The method for the evaluation of facial expression was the one developed for MGS, RGS and RbtGS (Langford *et al.,* 2010; Sotocinal *et al.,* 2011; Keating *et al.,* 2012). At least 4 pictures of of each piglet face were taken at different time respect the surgery: before, immediately after, 30 minutes later, 1 hour, 2 hours and 4 hours after castration (126 pictures in total). These images were sorted and those in which piglets had closed eyes were excluded. From included images, two pictures per piglet were evaluated by three treatment-blind observers, scoring eye tightening (eye), cheek tension (cheek) and overall pain (pain) from 0 to 2 (0 = not evident, 1 = evident & 2 = very evident; figure 1).
When assessor was unable to see a particular FAU clearly, he was asked not to score it and to state that he could not determine it.

2.3 Second experiment

The second experiment was conducted in the same field condition of experiment 1. In this case 80 male piglets belonging to 16 litters were considered. In every litter 5 treatments were included: ordinary surgical castration (C), meloxicam plus surgical castration (M), ketoprofen plus surgical castration (K), tolfenamic acid plus surgical castration (T), handling (H).
At least 4 pictures of face of each piglet were taken before drugs injection (T-1), immediately after castration (T0) and then repeated 10 minutes, 20 minutes, 1 hour and 3 hours after the surgery. These images were sorted and those in which piglets had closed eyes were excluded. From the extracted images 2 pictures per piglet for every time (960 images in total) were randomly scored by a trained treatment- and session- blind observer. As in the first experiment the observer scored from 0 to 2 the three different facial action units: eye tightening (eye), cheek tension (cheek) and overall pain (pain).

Statistical analysis

In the first experiment a Friedman's test was used to analyse the scores for each measure eye tightening (eye), cheek tension (cheek) and overall pain (pain) over different time points. Wilcoxon Signed Rank Tests (inc. Bonferroni correction for multiple comparisons) were used to compare differences in mean score for each measure between pre, immediately post, 30 mins, 1h, 2h and 4h post procedure.
In the second trial, treatments within time were analyzed by non parametric Wilcoxon Kruskal–Wallis test (PROC NPAR1WAY). Differences were considered significant at $P<0.05$ and trends presented if $P<0.10$.

Results

Results on piglets subjected to castration showed that across the three observers the scores for cheek tension were significantly different (table 1). In particular the mean score immediately post castration resulted higher compared to pre, 30 min, 2h and 4h post castration but not compared to 1h post castration (table 2). Mean scores for overall pain differed at different time of observation (table 1), and were significantly higher at post castration compared to all the other time points (table 2). The observation of eye tightening in the first experiment did not reveale differences between castrated and not castrated piglets. Considering methodological aspects evaluation of reliability between the three observers (table 3) showed that observers were not scoring the eyes reliably (23% agreement), they scores cheeks more reliably than eyes but is still very poor (47% agreement), but they were scoring pain consistently (74% agreement).

Differently from previous results, analysis on piglets treated with different NSAID (Table 4) did not show difference in cheek tension, but eye tightening score presented significant differences at 20 and 180 minutes after the procedure ($P<0.05$), and a tendency ($P<0.10$) of an effect 60 minutes after castration. Overall pain showed a significant effect only 60 minutes after the procedure. As represented in Figure 2 (A, B, C, D), 20 minutes after the procedure eye mean rank of piglets treated with meloxicam was the highest and statistically different from piglets treated with ketoprofen. Piglets of handling, castration and tolfenamic treatments showed intermediate values . Even if not statistically significant, at 60 minutes there was the same trend. At 180 minutes after the procedure the situation was exactly the same of 20 minutes, with meloxicam score higher than ketoprofen and the other treatments in a middle position.

Discussion

Observing facial expression with a painful procedure in relation to surgical castration carried out without anaesthesia or analgesia, cheek area before surgical castration looked relaxed and flat, differently after the procedure it looked hollowed and in tension. This difference was more intensive immediately post castration compared to 30 minutes after the surgery. Results are similar to what found in rats in which cheek region display bulging at baseline and flatten after a painful stimuli (Sotocinal *et al.,* 2011). Contrarily in the mouse cheeks at baseline have a smooth appearance and in the presence of pain bulge is noted (Langford *et al.,* 2010). Mean scores of overall pain and cheek tension were very similar but comparing different time of observation, overall pain resulted more significant and also different between immediately post castration and one hour post castration.

Leach *et al.,* (2012) underlined that pain assessment through the observation of facial expression has some advantages in comparison to classical behavioural analysis: the short time required for the analysis, the needless of perfectly trained observers and the natural tendency of humans to focus the attention on the face when interacting with animals. As humans used to give an interpretation of the emotional state of the persons, it seemed quite spontaneous for the observers to give a score to overall pain of piglets. This spontaneity in the expression assessment might be confirmed by the high reliability of the observation between the three observers in this study, and it probably originates, born from the fact that face-to-face interaction with a parent occurs from the earliest moment of existence. Parents monitor indeed their infants' facial expressions also because they provide cues to the changes in their infants' affective states (Rosenstein *et al.*, 1988; Lewis *et al.*, 1990).

Results of the second trial may however contradict results of the first one considering that the 3 NSAID can acted as pain relievers. In particular cheek tension, which allowed to discriminate castrated and handled piglets did not reveal differences among NSAID treatments. On the contrary eye tightening which was similar in the first experiment showed that piglets at 20 min treated with ketoprofen had a lower scores, while meloxicam the highest. Such difference could be due to the faster time of action of ketoprofen compared to the others and meloxicam in particular, in fact the time passed from the injection (10 minutes before castration) and the considered detection (20 minutes after castration) coincide with the time required by the drug to reach its maximum concentration and likely its max efficacy. Also 180 minutes after the procedure meloxicam piglets presented higher scores for eye tightening which is difficult to explain giving it a straight forward biological meaning particularly if we expected that the drug acted as described in its indicative drug facts. Overall pain expression seemed to be higher 60 minutes after castration in piglets treated with meloxicam. Even if results of the second trial, that consider just one observer, could be not credible, it is likely that any affect linked to meloxicam treatment was present.

The MGS is now considered a quick and easy method of assessing post-surgical pain and the efficacy of analgesic treatment in mice (Leach *et al.*, 2012). Even if this was a preliminary study, in pigs it is possible to confirm that, as described by Flecknell and Watermann-Pearson (2000), although they have fewer muscles for facial expression there are subtle changes, and this may offer a useful hint for future studies for acute pain assessment in pigs.

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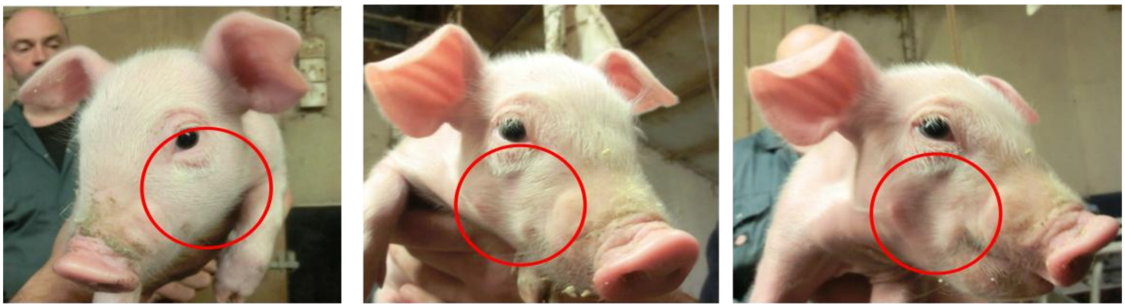
Figure 1 Cheek tension scores from 0 to 2 (0 was no evident tension and 2 very evident tension) in piglets



0 **1** **2**

Table 1 Mean rank score for each measure before (1), immediately post (2), 30 minutes (3), 1 hour (4), 2 hours (5) and 4 hours (6) post castration

Measure	Time of measurement						Chi-square	*P*
	1	2	3	4	5	6		
Eye tightening	3.49	3.81	3.22	3.83	3.11	3.54	8.31	ns
Cheek tension	3.38	4.44	3.38	3.38	2.94	3.49	15.714	<0.01
Overall pain	3.33	4.61	3.49	3.3	2.94	3.32	31.262	<0.001

Table 2 Comparison between the significant mean scores immediately post castration (2) compared with the other time points (before (1), 30 minutes (3), 1 hour (4), 2 hours (5) and 4 hours (6) post castration)

Measure		Comparison				
		1 vs 2	3 vs 2	4 vs 2	5 vs 2	6 vs 2
Cheek tension	Z	-2.818	-2.963	-2.353	-3.092	-3.035
	P	<0.01	<0.01	ns	<0.01	<0.01
Overall pain	Z	-3.821	-3.238	-3.418	-3.873	-3.755
	P	<0.001	<0.01	<0.01	<0.001	<0.001

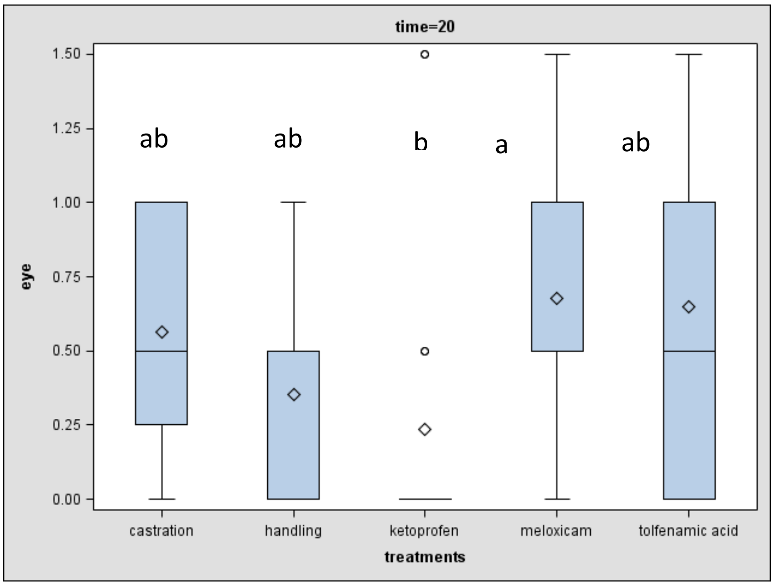
Table 3 Reliability between the three observers

Measure	interclass correlation	95% Confidence interval	
		lower bound	upper bound
Eye tightening	0.229	0.032	0.391
Cheek tension	0.472	0.337	0.583
Overall pain	0.736	0.669	0.792

Table 4 Statistical value of Wilcoxon Kruscal Wallis Test (*: $P<0.05$; °: $P<0.10$)

Time of detection	**Measure**		
	eye	**cheek**	**pain**
pre	6.2	0.64	1.13
0	3.83	1.14	2.13
10	1.64	6.51	3.69
20	11.71*	4.56	2.97
60	7.82°	1.49	11.11*
180	10.41*	4.71	0.91

Figure 2 Box plot of treatment effect on eye (A, B, C) and pain (D) at different time. Different letters mean significant difference at *P*<0.05



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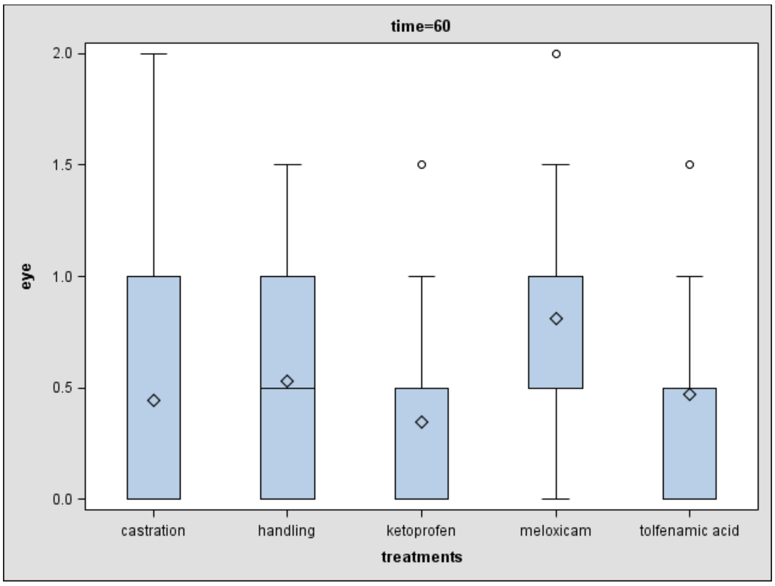

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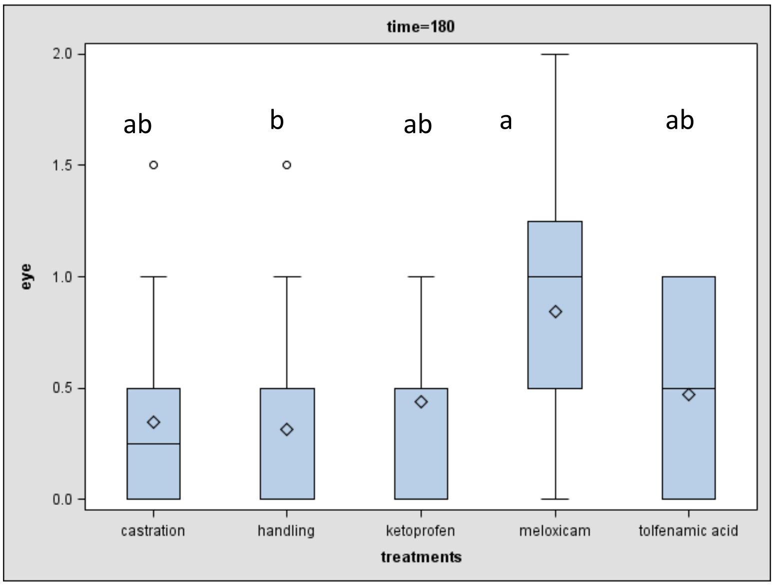
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1.5
1.0
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ab
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a
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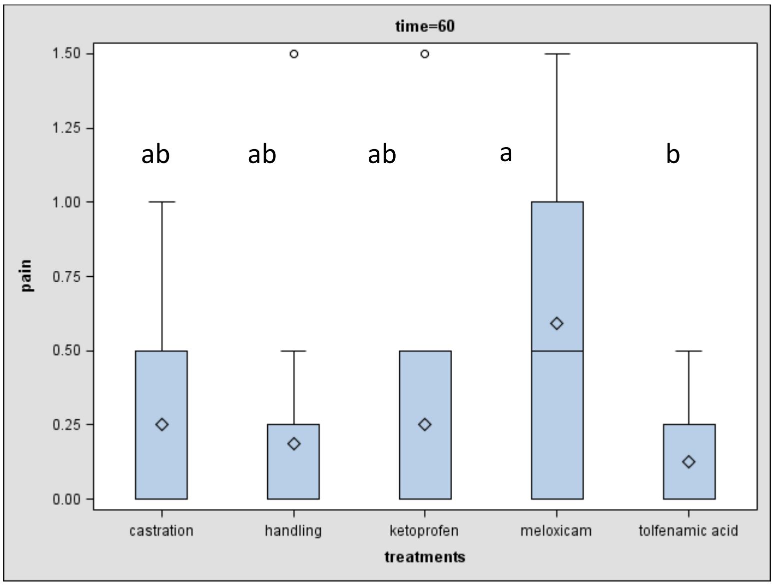
castration
handling
ketoprofen
meloxicam
tolfenamic acid
treatments

C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1.25
1.00
0.75
0.50
0.25
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ab
ab
ab
a
b
castration
handling
ketoprofen
meloxicam
tolfenamic acid
treatments

D

Chapter 5

ASSESSMENT OF PAIN AND STRESS DURING CASTRATION IN PIGLETS: PREEMPTIVE ANALGESIA USING TOLFENAMIC ACID

ASSESSMENT OF PAIN AND STRESS DURING CASTRATION IN PIGLETS: PRE EMPTIVE ANALGESIA USING TOLFENAMIC ACID

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Abstract

For the increasing animal welfare concerns and the public opinion pressure, alternative methods to surgical castration of piglets will have to be implemented. Aim of this study was to evaluate, by behavioural and physiological indicators, the effectiveness of tolfenamic acid administered before castration to alleviate the pain. The study involved 96 male piglets, divided into 4 groups: handled only (M), castrated without analgesia (C), treated with tolfenamic acid before castration (T), and treated with placebo before castration (P). Behaviour was observed for 15 minutes at 4 time point from castration (0h00, 0h16, 1h00, 1h15). Blood samples were collected 0h30 and 4h00 after castration for cortisol concentration. Results showed significant differences for all the parameters in H group compared to C and P groups (P < 0,001), excluding cortisol level at 4h00 which was similar in each group. Behaviours such as walking and standing, and cortisol at 0h30, showed no significant difference between T group and H group (P > 0,05). In conclusion, administration of tolfenamic acid before castration reduces alterations in behavioural and physiological indicators of pain in piglets. The findings suggest that tolfenamic acid could decrease the pain induced by castration in piglets, improving the welfare of piglets in practice.

Key words: *castration, analgesia, piglet, tolfenamic acid*

Introduction

Surgical castration of pigs is one of the most debated topics for its impact on animal welfare because about 100 million pigs are castrated in the European Union yearly, mostly without the use of anaesthesia or analgesia (Fredriksen *et al.*, 2009). The sustainability of this practice is being widely questioned (de Roest *et al.*, 2009) due to the impact of ethical considerations. Therefore the research is thus oriented towards the identification of methods that can reduce pain due to castration such as the use of local or general anaesthesia and/or analgesia. Unfortunately, up to now there is not an elective analgesic treatment for the painful practice, and no many drugs are licensed to be administered in pigs (Candotti and Rotanodari, 2010). One of them is the NSAID Tolfenamic acid, a long-acting non-specific COX inhibitor. Just a few data are available on the effect of tolfenamic acid in piglets subjected to castration, but a recent study described the positive effect of the drug on the reduction of time spent crying and standing isolated (Wavreille *et al.*, 2012).

Aim of the study was to increase the knowledge on the effects on pain response of the use of analgesia in piglets subjected to castration. In particular, behavioural and physiological indicators were used to assess the efficacy of tolfenamic acid a NSAID with a long lasting effect.

Materials and methods

Animal, housing and treatments

The trial involved 96 piglets 5 days old and weighted on average 2.1 ± 0.4 kg belonged to 24 litters of commercial hybrid (Goland ®). The piglets were reared in a farm located in the North-east of Italy, addressed to the production of the Protected Designation of Origin (PDO) cured ham.

Castration was performed as allowed by the regulation in force (Dir. 2008/120/EU and Legislative Decree 122/2011). For surgical procedure, piglets were restrained between the handler's legs in a head down position, then an incision on each side of the scrotum was carried out with a scalpel to extract testicles and removed them by cutting the spermatic cord. Finally a chlorhexidine-based antiseptic was applied on both the open wounds. After the procedure, piglets returned to their farrowing cage.

Other procedures usually performed at the same time of castration such as injection of iron dextrano and tail docking, have been carried out at the end of the experiment, in order to avoid any influence of these practices on the evaluated parameters.

In the study four experimental treatments were considered: Conventional castration (C) in which none drug was administered; Handling (H), castration was simulated with the same handling technique and with the same timing without tissue incisions and testicles removal; Physiological (P) Intramuscular administration with an insulin syringe of 0.05 ml/kg physiological solution to each subject 1 hour before castration; Tolfenamic acid (T) intramuscular administration with an insulin syringe of 2 mg/kg tolfenamic acid to each subject 1 hour before castration, corresponding to 0.05 ml/kg.

Each treatment groups was represented by 24 piglets one for each litter in order to remove the litter effect. One hour before the test, each piglet was marked a serial number on the back with a special marker for the individual identification. All piglets have been restrained, castrated and injected always by the same operator in order to avoid the possible effect due to manipulation.

Experimental measurements

All piglets were individually weighed before castration in order to calculate the correct drug dose. Forty-eight piglets (12 for each treatment) have been used for behavioural observations while the remaining were undergone to blood sampling for cortisol evaluation. The two groups were created to avoid possible influence between the types of collected data.

Four direct behavioural observations session, lasting 15 minutes each at 0h00, 0h16, 1h 00, 1h15 after castration, were carried out. The observations were performed by 2 trained observers using the scan sampling method adapted from Sutherland *et al.* (2012) with an interval sampling of one minute. At every scan the posture, activity and location performed by each piglets were recorded (Table 1).

Moreover postures and pain-relieving attitudes (kyphosis, arts underneath themselves, no or partial leaning on a limb and arched tail tucked/tail flexed between the back limbs) were recorded in the same subjects 0h30 and 1h30 after castration.

Blood samples were collected from vena cava using a 2.5 ml syringe in 48 piglets at 0h30 and 4h00 from castration. The samples were stored in vacuum tubes without anticoagulant (Vacutest Kima srl, Arzergrande, PD, Italy) and they were immediately refrigerated. Transferred to the laboratory, they were centrifuged at 2500 × *g* for 10 min at 20°C. Serum cortisol concentration was determined with chemiluminescent assay (LKCO1, Medical System, Genova, Italy) performed with automated analyzer Immulite One (Medical System Genova, Italy).

Statistical Analysis

The data were initially analysed to assess their Gaussian distribution using a PROC UNIVARIATE (SAS 9.2). Because of the non normal distribution, data on cortisol were corrected through a logarithmic transformation. The data were then statistically processed using PROC MIXED (SAS 9.2), evaluating the effects of the treatments, time of observation and their interactions. LS means were compared using the Bonferroni test.

Results

The behaviours that proved to be more variable according to treatments were walking and standing (Fig. 1A, B). The handled group (H) showed greater walking activity than both the conventionally castrated group (C) ($P < 0.001$) and the piglets which had been preventively administered the physiological solution (P) ($P < 0.01$). On the other hand, the Tolfedine group (T) showed intermediate levels of activity, proving to be the only treatment with no statistical difference with the handled group. Standing showed the same result: in this case, the group H spent less time standing when compared to the groups C ($P < 0.001$) and P ($P < 0.05$), but was not statistically different from the T group (Fig. 1A, B).

All the other behavioural variables were not statistically affected by treatment but they were modified by time from castration (Table 2). The time spent by the piglets in sitting and isolated behaviours was very high in the first 15 minutes of observation and then decreased gradually in the subsequent observations ($P < 0.01$). On the contrary, the time recorded lying and feeding increased ($P < 0.001$).

The serum cortisol values measured at 30 minutes after castration in groups C and P differ from those obtained by the H group. The value that did not show statistical differences from the H group is that of the T group, which was intermediate between the extremes (Fig. 2). No difference due to treatment was instead observed concerning the cortisol detected 4 hours after castration.

Discussion

The surgical castration of piglets is a procedure that causes physiological and behavioural changes that indicate acute pain (Fredriksen *et al.*, 2011). Even in the present work, castration without the use of analgesia (C) led to the alteration of some parameters in respect to the values observed in the only handled group (H). The piglets of the C group has shown a lower locomotory activity, as observed in a previous study (Moya *et al.*, 2008), with concomitant increase of standing motionless . Several authors ascribe the alteration of these non-specific activities, normally expressed by the piglets, to the response to pain caused by surgical castration (McGlone *et al.*, 1993; Taylor *et al.*, 2001; Hay *et al.*, 2003). It is possible that some activities, especially walking, cause further pain to the piglet and they are therefore avoided (Moya *et al.*, 2008). This behavioural adaptation is described by Mellor *et al.* (2000) as a protective process that allows the animals to reduce the pain of the tissues involved. In agreement with other authors, castration without pain relief administration did not alter other behavioural parameters and location of the piglets in the farrowing cage (McGlone and Hellman, 1988; Taylor *et al.*, 2001; Hay *et al.*, 2003). Considering the percentage average observed, most of the behaviours were altered in the first 15 minutes after castration, although Hay *et al.* (2003) report a continuation of this alteration for piglets castrated over 2 hours and 30 minutes for standing and isolation, and up to 2 days for other behaviour responses to pain such as back-scratching and tail-shaking. Results suggest a limited behavioural response to the stress of castration in terms of time.

In this work, the C group also raised the serum cortisol levels measured 30 minutes after castration in respect to the values registered in the group of manipulated piglets, a difference that is no longer observable in the blood drawn after 4 hours. These findings are consistent with those reported by Sutherland *et al.* (2012), who only registered the increase in cortisol in the first 2 post-castration hours, and they are confirmed by the data collected by Prunier *et al.* (2005), Carroll *et al.* (2006), and Marchant-Forde *et al.* (2009). Data collected for the C group were basically similar to the ones of the group treated with a physiological solution before castration (P), as far as both the behavioural and physiological parameters are concerned, suggesting that the additional manual injection with an insulin syringe does not constitute a source of additional stress or pain for the castrated piglets.

When previously treated with tolfenamic acid (T), the piglets showed less pronounced behaviour and serum cortisol alterations, with intermediate percentages between the C group and the M group. However, as to both walking, standing and the cortisol, this alteration was not enough to demonstrate significant differences between the T group and the manipulated group, suggesting a weak perception of pain in the treated piglets. A similar result is also reported by Wavreille *et al.* (2012) who observed minor differences in the behavioural responses to the castration pain stimulus between piglets treated with tolfenamic acid and manipulated only piglets, showing similar percentage of time spent isolated from the group and similar tail-shaking.

In conclusion, the obtained results confirm that castration causes behavioural and physiological alterations that suggest a condition of pain related to the procedure. The use of tolfenamic acid for analgesia during the castration of piglets reduces the pain and stress caused by the procedure, limiting the animal pain manifestations associated with the attempt to minimize suffering after the operation. The prior administration of tolfenamic acid is thus desirable to improve the welfare of piglets during and after castration.

Acknowledgements

The authors would like to thank English and Italian farmer for the availability and students for the help.

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Table 1 Observed behaviours and their description

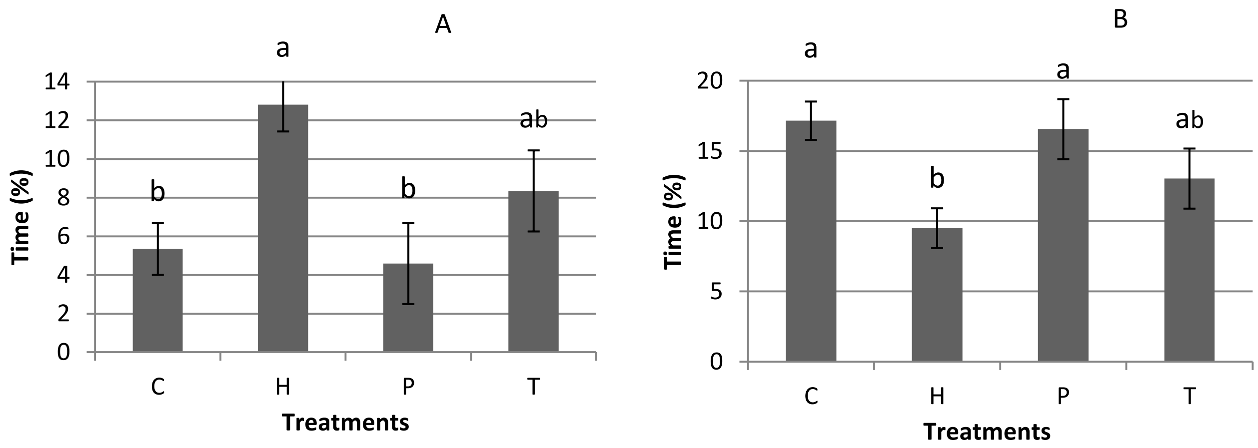
Observed behaviours		Description
Posture		
	Standing	The piglet stands
	Sitting	The piglet sits still
	Lying	The piglet is in lateral or sternum decubitus
Activities		
	Walking	The piglet walks
	Feeding	The piglet is milk suckling
	Painful	The piglet is showing pain: shaking, scratching, tail-shaking
Location		
	Nest	The piglet is in the nest
	Isolated	The piglet is isolated from pen-mates and sow

Table 2 Average percentage of the time spent by the piglets for each behaviour, according treatment and observation interval

	Treatment[1]					**Observation interval[2]**				
Behaviour	**C (%)**	**H (%)**	**T (%)**	**P (%)**	**SEM**	**1 (%)**	**2 (%)**	**3 (%)**	**4 (%)**	**SEM**
Lying	65	62	62	70	3,94	40^{b}	75^{a}	76^{a}	68^{a}	3,47
Feeding	9	14	14	7	2,37	4^{b}	13^{a}	10^{ab}	17^{a}	2,30
Sitting	3	2	2	2	0,76	4^{a}	2^{b}	1^{b}	2^{ab}	0,69
Nest	58	50	54	59	6,60	50	61	57	54	4,80
Isolated	6	2	0	4	1,63	6^{a}	4^{ab}	1^{b}	2^{ab}	1,34
Painful	2	0	0	0	1,06	1	1	0	0	0,81

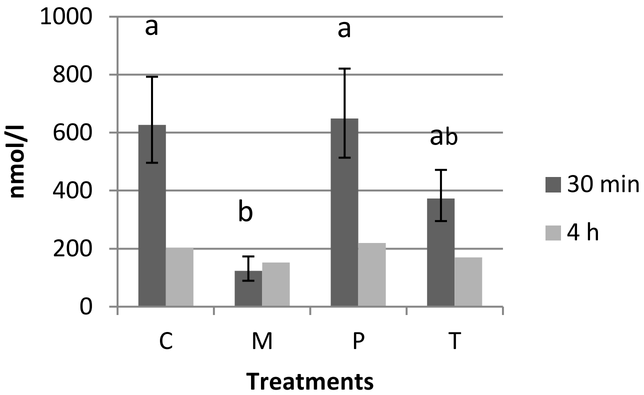
[1] Treatment: castration (C); handling (H); Tolfedine (T), physiological (P).
[2] Observation interval: 1 = from 0h00 to 0h15 minutes after castration, 2 = from 0h16 to 0h30 minutes after castration, 3 = from 1h00 to 1h15 minutes after castration; 4 = from 1h16 to 1h30 minutes after castration.
a,b Different letters show significant statistical differences (P <0.05)

Figure 1 Means time percentage for walking (A) and standing (B)



Treatments: castration (C); handling (H); tolfenamic acid (T); physiological (P).
[a,b] Different letters show significant statistical differences (P <0.05)

Figure 2 Serum cortisol concentration (nmol/l) for each treatment at 00h30 and 4h00 after castration.



Treatments: castration (C); handling (H); tolfenamic acid (T); physiological (P).
[a,b] Different letters show significant statistical differences (P <0.05).

General conclusion

The assessment of pain in farm animals and its possible reduction are concepts of utmost importance due to the growing interest towards their welfare. With regard to surgical castration of pigs, public opinion calls for the abandonment of the practice, but the alternative techniques today available make such a request unsuitable for Italy because of the prolonged fattening period for the production of high quality products as IGP and DOP.

From our studies, it is evident that the evaluation of such pain is difficult because of both the lack of applicable standard methods and the subjectivity of the manifestations of animals in response to that stimulus. Among tested methods, analysis of stress hormone cortisol seems useful indicator of acute pain considering that its level increase only immediately after castration. The observation of pain posture seem to be able to detect sign of pain until 1 hour from castration. Promising and not invasive methods of pain evaluation are the eye temperature and the observation of facial expression; the latter has never been used in pigs but it is widely use in humans and laboratory animals. Both methods required further investigations mainly address to standardize the observation technique.

The results obtained throughout the experiments carried out in this PhD thesis seems to indicate that the pain related to castration is detectable considering behaviour (pain posture and alteration of walking resting) and cortisol until 1 hour after castration It seems clear, therefore , that castration carried out without drugs is actually painful, but it seems equally clear the short duration of pain or of its manifestation. The use of the NSAID tolfenamic acid may decrease the suffering of animals, but did not kill it completely. It is therefore considered necessary to proceed with studies concerning both the standardization of methods of pain assessment applicable to pigs and the use of different analgesic and anaesthetic molecules, without forgetting economic, management and legal concern.

Acknowledgments

I first thank Dr. Meredith Sheil and Animal Ethics Pty Ltd (Australia) because my project on pain in pigs started thank to their support.
A huge thanks to the whole group, Marta, Annalisa, Barbara and Prof. Giulio Cozzi, and more than anyone else a special thanks to Prof. Flaviana Gottardo, for the opportunity, the patience, the help and the support.
On so many occasions you have been a friend and a "mother" as well as my supervisor...thanks ! Without all of you I would never have given this thesis. Thanks also to all of you from Londra for having accepted her “into the team”.
Thanks to Prof. Sandra Edwards, Prof. Paul Flecknell and Dottor Matthew Leach for the important experience in England.
A thought full and a big thanks to the fantastic members of the Carolo family, which in addition to open the doors of their livestock they have become friends...in particular thanks to Luigi for wine, to Mariarosa for the fantastic lunches, Marco for the huge portions of pasta, and Luca for accepting me in his farrowing rooms!
To my family, for their support, for the understanding and the help of these years and of recent months in particular.
To my friends, PhD students and not, for have always been, to students Chiara, Alessandro and Greta for the help, and to Flavio, fantastic translator!
To new colleagues Francesco, Mattia, Alfredo, Silvia and Elisa, for tolerating my nervousness of this period, and for giving me two days of vacancy to deliver!
A special thought to Londra, faithful companion of adventures across all my veterinarian course always and without conditions!
Finally, a special thanks to T.A.: in the last months, your support has been my salvation!

Ringraziamenti

Ringrazio innanzitutto la Dott.ssa Meredith Sheil e la Animal Ethics Pty Ltd (Australia), perché il mio progetto sul dolore nei maiali è partito grazie al loro sostegno.
Un gigantesco grazie a tutto il gruppo, Marta, Annalisa, Barbara e il Prof. Giulio Cozzi, e più di chiunque altro un grazie speciale alla Prof.ssa Flaviana Gottardo, per l’opportunità datami, per la pazienza portata, per l’aiuto e per il sostegno. In tante occasioni sei stata un’amica e una “mamma” oltre che il mio supervisore…grazie! Senza tutti voi non avrei mai consegnato questa tesi. Un grazie a tutti voi anche da Londra per averla “accettata nel team”.
Grazie alla Prof.ssa Sandra Edwards, al Prof. Paul Flecknell e al Dott. Matthew Leach per l’importante esperienza in Inghilterra.
Un pensiero e un grazie di cuore ai fantastici componenti della famiglia Carolo, che oltre ad aprirmi le porte del loro allevamento sono diventati degli amici…in particolare a Luigi per il vino, a Mariarosa per i fantastici pranzi, a Marco per le porzioni di pasta da due etti, e a Luca per avermi accettato nelle sue sale parto!
Alla mia famiglia, per il sostegno, la comprensione e l’aiuto di questi anni e di questi ultimi mesi in particolare.
Ai miei amici, dottorandi e non, per esserci sempre stati, ai tesisti Chiara, Alessandro e Greta per l’aiuto, e a Flavio, eccezionale interprete e traduttore!
Ai nuovi colleghi Francesco, Mattia, Alfredo, Silvia ed Elisa, per aver sopportato il mio nervosismo di questo periodo, e per avermi concesso due giorni di ferie per consegnare!
Un pensiero speciale a Londra, fedele compagna di avventure in tutto il mio percorso veterinario sempre e comunque!
Infine, un particolare grazie a T.A.: in questi ultimi mesi il tuo supporto è stata la mia salvezza!