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A study of an anti-GnRF vaccine as a more welfare friendly method of castration for ram lambs

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To my grandma, parents and husband

Declaration

I declare that I have undertaken and written the present thesis. This thesis is presenting my own work. Any assistance has been duly acknowledged. The work described has not been submitted for any other degree or professional qualification with exception of the Qualitative Behavioural Assessment study which has been carried out by Fabiana Mizzoni as part of her MSc thesis under supervision of myself and Prof Francoise Wemesfelder.

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Conference abstracts

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Chapter 1 General Introduction

Abstract

Castration of male lambs is performed in all major sheep producing counties as a standard management practice. The reasons to castrate may be different and will depend on the size and type of the farm. Castration gives more control over genetics of the flock, stops inbreeding, unwanted pregnancies and behaviours. It also gives improved carcass characteristics. However, it has been shown that castration is painful and distressing to the animals. Different techniques are used to castrate sheep at the present time such as rubber ring, Burdizzo, combined, short scrotum, and surgical castration. Due to changing attitudes towards animal pain and unnecessary suffering there is a need for further development and implementation of new castration methods, efficient pain assessment techniques, animal welfare codes of practice and legislative requirements to improve lamb well-being. Recent increase of public concern regarding animal welfare is putting pressure on many government bodies to strengthen research in this area and increase attempts to regulate by law unnecessary suffering during standard livestock management practices. Immunocastration with an anti-GnRF vaccine has the potential to be an alternative to common physical castration methods. Nonetheless there is little or no information about the impact of vaccination against GnRF on the physiology of lambs (rams' reproductive tract, endocrine regulation), emotionality (possible changes to normal behavioural patterns like increased aggression, anxiety) and health (is the vaccine safe to be used and if there are any adverse effects of vaccination like tissue damage, swelling, lesions etc.). There is also little or no information on how the vaccine affects sheep at the time of injection. This study investigates three main questions: Is Immunocastration a pain free alternative to traditional physical methods of castration? Is Immunocastration safe and practical to use? Does Immunocastration influence the male reproductive system in a way to achieve sterility without any negative impact on the ram natural behaviours, wellbeing and health?

Key Words: Animal Welfare, Castration, Pain, Immunization, Analgesia, GnRH, Rams

1.1 Introduction

Sheep production in Britain has a very long history. From the 14th century until around the time of the Second World War the major target of sheep production was wool; afterwards farmers moved their interests on to production of meat (Conington, 2010) due to changes in the market, declining need for British wool and the drive for more intense food production during the war. In 2008 one third of sheep meat produced in Europe was delivered by the UK making it one of the leading countries in production of sheep meat (Conington, 2010). At the present in the UK there are three main management systems for breeding of sheep: Hill system, Upland system and Lowland system (see details in table 1). The majority of husbandry systems in the UK are extensive, where sheep spend most of their time on the pasture without humans being present. There are however times when sheep need to be gathered for handling and management purposes (Dwyer, 2008).

Table 1 Husbandry systems of sheep production in UK (Dwyer, 2008)

System	Group	Characteristic	Justification for castration
Hill systems	Hill ewes producing pure-bred lambs	Bound (hefted) acclimatised stock on a high proportion of semi-natural pasture	- Prevention of unwanted and early pregnancies - Genetic control over animals - Male and female lambs can be reared together, allowing for later more natural weaning - Reduction of aggressive male behaviour during feeding and pasturing - Reduction of the risk of injury to animals and people - Reduction of taint, stronger consumer acceptance, improved meat quality * the barriers to farmers for keeping their male lambs entire are not the same for each system therefore justification for castrating rams might not be the same in different systems (as motioned in the introduction). The reasoning to use castration has been generalized in here across all systems
	Hill ewes producing some crossbred lambs	Bound stock, possibly partially de-hefted from hill. Usually a higher proportion of in bye (improved) land	
	Crofting	Smaller scale producer but often with larger sheep 'farmers' using most land in the area	
Upland	Draft hill ewes producing crossbred lambs	Unbound flock brought onto unit – grazing mainly sown pastures and fenced hill areas	
	Crossbred ewe mated to terminal sire	Unbound flock brought onto unit – grazing only sown pastures and fenced hill areas	
Lowland	Crossbred ewe mated to terminal sire	Grassland farm Mixed farm Arable unit	

Intensification of production and a general tendency to produce higher quantities of leaner, efficient, sustainable and disease resistant flocks (Conington, 2010) causes several problems related to farm animals' well-being, handling, management and economic value. It has been shown that gathering livestock for management practices influences their welfare in a negative way. Animals may show signs of stress such as raised heart rates (Goddard et al., 2000); the presence of dogs elevates cortisol levels as well as heart rate (Harlow et al., 1987; Komesaroff et al., 1998). Fear or anxiety can lead to injuries as animals may panic and attempt to escape (Grandin, 1987) which can affect

their health, productivity and commercial value (Bouissou, 1992). There is also a trend for "easy care" sheep which would be able to cope in extensive and adverse conditions without much human intervention (Dwyer, 2008). There is a tendency to reduce labour and human supervision in the extensive systems as well. Easier manageable stock would allow for lowering the costs of stockmanship. The time spent on individual care would also decrease in the flocks where animals are easier to manage. The reason for the trend towards easy care sheep is purely economic allowing extensive systems to achieve reasonable profit with reduced inputs. Castration of males appears to be a good alternative for the farmers and sheep breeders with an aspiration to have easier manageable flocks. However, there are differences in management between particular sheep breeding systems. Therefore, justification to use castration will also vary between systems. For example, in Hill systems (which may be dominated by ewe stock) it might not be possible for farmers to bring ram lambs to slaughter before they reach puberty as the rate of lamb growth is slower in comparison to the lowland system. Rams may not reach desired weight before puberty. Another issue that farmers are faced with is a matter of unfenced pastures where sheep have unlimited access to certain areas and are able to move freely within boundaries of that area. This may cause handling problems for the farmer who needs to manage separate groups of ewes and rams. The Hill farmer may favour castration to prevent inbreeding and unwanted pregnancies. It will also increase ease of stock management. Lowland farmers have more options to choose a management policy that would be appropriate for their particular situation. Castration may not be used at all as lowland lamb breeds may grow faster and it is possible to bring lambs to market before puberty. The practice of not castrating rams if it is not necessary has been advised and recommended to farmers by the Farm Animal Welfare Council (FAWC) and it is increasingly popular (FAWC, 2008). Nevertheless, altering male lambs is still very common practice in many countries such as the UK, Australia and New Zealand. There are many benefits of castration such as reduced levels of fighting (Godfrey et al., 1996) as castrated males are less aggressive. When there is a lower rate of aggression and fighting between individuals the risk of injury is lower as well and the productivity and the economic value may be greater (Bouissou, 1992). Several studies

have also shown that castration may have a beneficial impact on carcass conformation and meat quality (Kiyma et al., 2000; Thornton et al. 1999; Amatayakul-Chantler et al., 2012). In flocks where rams have been sterilised, indiscriminate breeding does not cause problems either. Transport of ewes to markets or slaughterhouses during the last stages of pregnancy is stressful and may result in abortion or premature birth. This very important issue affecting ewes' health, wellbeing, productivity and commercial value has been pointed out by FAWC to the sheep breeders' community (FAWC, 2008). Farmers have also genetic control over their sheep population in the flocks where castration is used. Lambs can be reared together, allowing for later more natural weaning as well and the issue of unfenced pastures can be avoided. Several physical methods of physical castration are being used to castrate male rams (such as rubber ring, Burdizzo, or short scrotum castration). Rubber ring castration (elastration) is the most popular method used in the UK. Accordingly, to a Defra Farm survey in 2005 over 95% of male lambs were castrated by elastration regardless of the farm size (small, medium or large). Nonetheless, it has been shown that physical castration induces acute and chronic pain as shown by altered lamb behavioural patterns and increased cortisol production (Kent et al., 2000; Kent et al., 2004; Thornton & Waterman-Pearson, 2002; Thornton & Waterman-Pearson, 1999; Molony et al., 1993; Molony et al., 1995; Molony et al., 1997; Molony et al., 1993; Molony et al., 2002; Molony et al., 2011). Physical castration is not only a cause of pain that may last for several days (Thornton & Waterman-Pearson, 1999), it also may have negative economic influence on the flock productivity as weathers (castrated rams) grow more slowly than entire males and have to be fattened for longer periods of time. This is because they lack the beneficial influences of testosterone on growth (Reece, 1997). Attempts to develop a welfare-friendly, painless method of castration, which would eliminate unwanted behaviours, and remove the sensation of pain at the same time, have been studied by many scientists. Use of analgesia or anaesthesia has been proposed as an alternative solution to traditional methods of sterilisation. The implementation of non-steroidal anti-inflammatory drugs like carprofen, meloxicam, ketoprofen etc. which are easy to administer could reduce the expression of pain behaviour and cortisol responses at least in the first few hours

following the procedure (Fisher et al., 2007; Molony et al., 1997; Stafford et al., 2002). However, use of analgesia or anaesthesia has proved to be difficult to be applied in practice. It is time consuming, costly or requires additional staffing which does not lie in agreement with a tendency to reduce stockmanship labour. Thus other solutions to traditional methods of castration have been proposed by experts. The emphasis is put on finding a method which would improve livestock welfare and allow simultaneously benefit from the influence of testosterone at the early stages of life. In this way the wellbeing of male lambs, as well as flock productivity would be improved. It is believed that immunocastration may be a welfare-friendly alternative for the physical methods of castration. Implementation of immunocastration has been investigated since 1970 (Fraser 1980 and 1986; Schanbacher, 1984; Chaffaux et al., 1985; D' Occhio, 1993; Thompson, 2000). The results of studies performed on species like cattle and goats have shown that active immunization against GnRH hormones reduces fertility, aggressive and unwanted behaviours and additionally improves carcass conformation (Thompson, 2000; Amatayakul-Chantler et al., 2013). It also significantly decreases testicular development, serum testosterone levels and physical activity (Janetta et al., 2012), however there is little or no information on the effect of immunocastration in sheep. Therefore, the objective of this thesis is to determine the efficacy of an experimental anti-GnRF vaccine developed by Zoetis as a more welfare friendly method of castration for ram lambs.

In this chapter I will present the most common castration techniques performed in the countries leading in sheep production. The perception of physiological (nociception) and emotional pain as well as the effects of different castration techniques on rams' behaviour will be discussed. The anatomy of the ram reproductive tract, reproductive development, function of testosterone in male reproduction, courtship and sexual behaviours along with alternatives to painful castration techniques, such as the use of analgesia/anaesthesia and its effect on animal behaviour will be mentioned. I will also consider legal requirements which need to be met while castration is performed. At the end of this review I will outline the key research questions and present the thesis

objectives which were formed to investigate the use of immunocastration for castration of ram lambs.

1.2 What is Animal Welfare?

The concern about livestock welfare has been rising since the 1960s. The intensification of production has changed the livestock management practices dramatically. The public opinion and attitude towards the concept of animal well-being have also changed throughout the years and the interest about animal welfare has been growing from the time when Ruth Harrison (1964) published her work "Animal Machines". Harrison's book in which she described the conditions of farm animals' housing, breeding and management was a turning point. After this publication the UK Government formed the Brambell Commission ''to enquire into the welfare of animals''. But what is Animal Welfare? It can be defined in many ways, for some it is "the state of an animal as it attempts to cope with its environment" (Broom, 1988, 1991). Others defined it as part of three main mechanisms of life related to physiological functioning, emotional states and natural living (Fraser, 2003). Marion Stamp Dawkins defines animal welfare by asking two questions. Is the animal healthy? Does the animal have the things it wants (Stamp-Dawkins, 2008)? The Farm Animal Welfare Council (FAWC) – which is a government advisory body – has identified animal welfare by setting codes of practice called "Five Freedoms". These codes should be followed in everyday livestock husbandry to safeguard animal welfare on the farm. The five freedoms are:

- Freedom from hunger and thirst – it means that animals must have access to fresh water at all times and a balanced or nutritious diet which will maintain the health and vigour of an animal
- Freedom from discomfort - shelter and a comfortable resting area should be provided
- Freedom from pain, injury or disease – it means that the prevention of illnesses, or rapid diagnosis and treatment will be provided if needed

- Freedom to express normal behaviour - sufficient space, proper facilities and company of the animal's own kind will be provided to allow animals to express a range of their natural behaviours
- Freedom from fear and distress – it means that in no circumstances should the animal be caused mental suffering

When all of those definitions are summarised it may be concluded that Animal Welfare is mostly referring to the quality of any individual's life. Furthermore, a good quality of life means that all of the physiological (i.e. access to food with appropriate nutrition, absence of disease, pain and distress, emotional and behavioural) needs of particular individuals are met (Mellor and Stafford, 2001). In other words, the animal is able to achieve a "life worth living" (concept presented by FAWC, 2009), which is focused on the positive aspects of animal welfare.

1.3 Assessing Animal Welfare

Similarly, to defining animal welfare, assessment of it is not an easy task. A lot of modern welfare assessments rely on the physiological measures as it is extremely difficult to measure another's feelings in an objective way. Scientists have been working on the development of the most efficient scientific approaches to assess animal welfare such as considering the "animal perspective" since the 1970s (Lawrence, 2008). The use of animal welfare indicators like stress physiology, behaviour, mortality, health and productivity is also very popular (Hemsworth et al., 1995) as by identification of particular problems associated with management systems there is the possibility to judge the level of welfare (Fregonesi and Leaver, 2001). Although there are some limitations to such an approach due to the nature of specific indicators i.e. milk production, (many other factors like genetics, disease etc. have influence on milk production) some indicators are believed to predict welfare in a better way or more efficiently than others. In this study the quality and quantity of pain inflicted by traditional castration techniques was investigated. It was believed that pain sensation is decreasing the welfare state of any individual subjected to castration. Therefore, assessment of pain was one of the main objectives of this thesis which will be explored in detail later on in this chapter.

1.4 Overview of legal requirements concerning animal welfare and castration procedure of lambs

1.4.1 Introduction

In the UK welfare of sheep is regulated by appropriate legislation which makes it an offence to cause unnecessary suffering to an animal. The welfare regulations, standards and codes of practise are also reviewed by the bodies such as The Farm Animal Welfare Council (FAWC). FAWC is an organization established in 1979 with primary focus on the review of current welfare standards and codes of practise to advise Great Britain's Rural Affairs Ministers of any legislative or other changes that may be necessary to improve welfare of farmed animals. In this section I will be focused on two particular reports published by FAWC that have led to change in the management recommendations for the sheep farmers in the topic of lambs' castration.

In 1994 FAWC report On the Welfare of sheep was published which revealed concern regarding pain induced by castration and tail docking of lambs. The report was based on the experimental evidence provided by various organizations and research sector. For example the age of animals at the time of castration, breed and influence of different castration techniques on plasma cortisol concentration as well as changes in behavioural patterns following castration were taken into consideration (Mellor & Murray 1989a and 1989b, Mellor et al., 1991; Molony et al., 1993).

The most important findings of the report were as follows:

1. Tail docking and castration without anaesthesia or analgesia inflict pain, "it is difficult to give general approval to any system of husbandry that relies on painful mutilations to sustain the system but we see no alternative until the results of research provide further guidance".

2. Careful consideration should be taken before any painful husbandry procedure is performed, "…at the outset, we wish to state that all farmers should consider carefully the necessity for performing any mutilation on sheep and we hope that

as many as possible will choose to avoid tail docking and castration". This message has led to changes in the Code of Recommendations for the Welfare of Livestock:

- Consideration should be taken by the owner's whether castration is necessary in flocks slaughtered before reaching puberty, "Farmers and shepherds should consider carefully whether castration is necessary within any particular flock. Castration is unlikely to be necessary where lambs will be finished and sent to slaughter before reaching sexual maturity. The procedure should only be carried out when lambs are likely to be retained after puberty and where it is necessary to avoid welfare problems associated with the management of entire males".
- Other factors affecting welfare of animals before, during and after castration procedure should also be considered, "Account should be taken not only of the pain and distress caused by castration but also the stress imposed by gathering and handling, and the potential risk of infection. For very young lambs gathered in large groups, there is a real risk of miss-mothering, which may lead ultimately to starvation and death."

3. Different methods of castration will induce different amounts and durations of pain in the individuals, "there is no doubt that lambs feel pain and distress as a result of castration and tail docking but the type of distress and duration vary according to the method used". The report also stated that, "the current legislation which limits the use of the rubber ring to the first week of life appears to be based on the erroneous impression that lambs of this age feel less pain than older lambs".

The findings of the report have led to increase of research focused on physiological and behavioural responses of lambs to tail docking and castration (i.e. Kent et al., 1995, 1998, 2000, 2004; Molony et al., 1995, 1997, 2002; Graham et al., 1997, 2002; Price and Nolan 2001, Thornton and Waterman-Pearson 1999, 2002).

The main objective of these investigations was to provide evidence of distressing and painful nature of both procedures and to evaluate possible mitigating factors. In 2004 a Working Group to investigate castration and tail docking in lambs was established by FAWC to carry out a public consultation. The experts in the field (industry, research and academic institutions, animal organizations) were asked to give oral and written evidence in the topic. In 2008 a new report on the Limitations of Castration and Tail Docking for the Welfare of Lambs was published by FAWC which has initiated Government response on behalf of the Governments of Scotland, England and Wales.

Most important recommendations of the report related to the castration of lambs were as follows:

1. Decision to castrate lambs should be agreed with the Private Veterinary Surgeon (PVS) as a part of routine Health plan after careful consideration of all the risk factors related to welfare and health of the flock.
2. When castration is considered necessary, it should be performed as early as practically possible. However time should be given to form maternal bond therefore castration should be carried out not earlier than 24 hours after birth
3. When lambs are 3 months and older, castration should be only performed by the PVS with use of pain relief.
4. Surgical castration should only be performed by PVS.
5. Use of pain relief should be considered wherever possible. Pain management should be discussed with PVS before any decision is made.
6. Until appropriate methods of pain relief administration under the farm conditions will be developed, existing methods of castration are permitted to continue. The Government, industry, and research sector should direct all of the efforts towards support and the development of appropriate methods of delivering local anaesthetic under field conditions.
7. Use of Rubber rings for castration, and the appropriate application equipment, should be designed and manufactured, to specifications, which result in an effective application and prevent use above defined age limits. It is also

recommended, to investigate alternative methods to rubber ring castration such as very tight rings, equivalent for pain relief through nerve destruction or immunocastration (together with potential consumer concerns regarding this method). Industry should promote welfare-focused practises avoiding mutilations if possible.

8. Retailers, farmers and farm assured schemes should apply welfare codes of practise, to avoid unnecessary suffering wherever possible.
9. The Government should introduce a policy that would allow for easy monitoring of castration practices in farming industry by the various methods, to determine if the improvements are being implemented.

The Government has published response to the report recommendations. The Government did agree with the need to create written health and welfare plan for each flock accordingly to veterinary advice included in the current Codes of Recommendations for the Welfare of Livestock for sheep species. It was the Government view that farmers may seek business advice on the possibility of finishing lambs at an earlier age depending on market conditions. The Government decided to introduce an appropriate amendment to the Welfare Code, when the Code will be reviewed. With regard to castration of lambs older than 3 months of age, which should be done by PVS only with use of local anaesthesia is already a legal requirement. Total ban of surgical castration will be considered by the Government during consultations on the legislative amendments, other methods of castration commonly used at the present time will continue to be permitted.

The Government approves use of pain relief during castration. However, it was also recognised that further research is needed to develop practical methods of pain relief administration under farm conditions. Promising results have been presented in the past ascertaining needleless local anaesthetic injectors as a possible solution. It was the Government opinion that further research is needed in conjunction with the industry to investigate the most appropriate methods of pain relief administration. Once appropriate methods are developed, current castration techniques should be re-assessed.

Pharmaceutical industry is encouraged by the government to develop suitable local anaesthetic that could be specifically authorised for use in sheep. Government supports as well investigating the potential use of very tight rings. Further research to promote additional potential more welfare friendly methods of castration such as immunocastration by the pharmaceutical industry would be supported by the government. Once appropriate product would become available, the Government would take part in the provision of information to reassure consumers about the safety of the product.

The Government agreed that monitoring system should be put in place to gather information on the types and frequencies of castration methods. Information gathered this way would allow for implementation of the necessary improvements. The government supports the work carried out in this area and encourages other sectors (retailers, food supply) to investigate impact of castration above certain age on the meat quality characteristics. Further studies supported by the industry on meat quality in uncastrated lambs reared in different conditions should also be carried out.

In summary, both reports of FAWC (1994 and 2008) as well as the government response to the presented in the 2008 report recommendations have led to increase in the funding of research aimed to investigate the impact of different castration methods on the health and welfare of sheep. The main conclusion from the 1994 report was that the castration is painful and distressful procedure. For example, following findings have been reported to support that conclusion:

Castrated lambs have shown significant increase in the plasma cortisol concentration, activity as well as hypertension in hind limbs. Castration induces substantial pain in lambs irrespectively of their age (Mellor & Murray 1989a,b; Mellor et al., 1991; Kent et al., 1993; Molony et al., 1993). Different methods of castration will induce different quality and quantity of pain i.e. combined methods of castration was proved to be less painful (Kent et al., 1993, Molony et al., 1993).

In the years following publication of 1994 report scientific research programs were focused on how pain inflicted during castration may be mitigated, how to assess pain, what alternatives to castration may be used that would be more welfare friendly etc. These findings gave evidence which was used to evaluate husbandry practices described in the report from 2008 and allowed to grasp new conclusions related to lambs health and well-being. For example, following findings have been described to support recommendations of 2008 report:

The study on the implications of rubber ring and combined castration in lambs groups of one, and four to six weeks of age was carried out. Both methods have been shown to be distressful, changing behavioural patterns of sheep in all age groups. Results of this study had significant impact on proposed by FAWC extension of the maximum legal age for rubber ring castration from one week to six weeks of age (Thornton and Waterman-Pearson 2002).

Further evaluation of combined method of castration (application of rubber rings followed by immediate crush of spermatic cord by Burdizzo clams) revealed that this method is less painful due to reduced duration of pain related behaviours (Kent et al., 1995, 1998) as well as quicker healing time of the lesions (Kent et al., 2000).

Investigation of different methods for reduction of acute pain following rubber ring castration i.e. administration of local anaesthetic (lidocaine) by needle and syringe or high pressure needless injector or innervation to the scrotum by powered bloodless castrator provided valuable insight on ease of use, practicality and effectiveness of such methods after rubber ring castration (Kent et al., 1998).

Field trial of two new techniques for the castration and tail docking of lambs less than two days of age with a 'Big Nipper' (bloodless castrator), and high-pressure jet injector (for the injection of 2% lignocaine with adrenaline) have revealed decrease in limb movement and expression of abnormal postures in comparison to traditional methods. It was also shown that shepherds testing new equipment preferred to use jet injector (Kent et al., 2004).

Administration of Local anaesthetic or NSAID i.e. diclofenac prior to castration procedure, may reduce plasma cortisol concentrations and duration of abnormal postures expressed by lambs (Molony et al., 1997).

This study is also one of initiatives founded by Zoetis and the Scottish Government aimed to promote more welfare friendly alternatives to painful physical castration methods i.e. rubber ring castration.

1.4.2 Legal requirements for castration of lambs in the UK

In the UK general welfare requirements for all farmed animals (including sheep) are outlined in the Welfare of Farmed Animals Regulations 2007.

The Animal Welfare Act 2006 makes it an offence to cause or allow unnecessary suffering to any animal. The Act introduces also a duty of care for all animals and livestock therefore farmers must take appropriate action to ensure that all animals under their control are treated with respect and that all welfare requirements are adhered to. Section 5 (1) and (2) of the Animal Welfare Act 2006 makes it an offence to carry out, or to cause or, in specified circumstances, permit another person to carry out, a prohibited procedure on a protected animal (a protected animal is any animal to which the Act refers to i.e. cattle, goats, poultry, sheep, horses, dogs etc.). A prohibited procedure is one which involves interference with the sensitive tissues or bone structure of the animal, otherwise than for the purpose of its medical treatment. The Animal Welfare Act 2006 also specifies the procedures to which the offences in section 5(1) and 5(2) do not apply. In sheep such procedures involve:

- Identification Procedures: Ear clipping, Ear notching, Ear tagging, Micro-chipping, Tattooing. Other methods of identification involving a mutilation required by law.
- Procedures for the Control of Reproduction: Castration, Implantation of a subcutaneous contraceptive, Vasectomy.
- Other Management Procedures: Dehorning, Disbudding, Removal of the insensitive tip of the horn, Tail docking.

Several farm assurance schemes and government bodies have published animal welfare codes of practice to guide farmers, breeders, hauliers and stakeholders on the appropriate course of action with regard to handling, transport, management, housing etc. of livestock. These sets of rules are designed to maintain and to help ensure that the welfare of any livestock is met at all times.

Defra has also published codes of practice for sheep breeders. Sheep owners are legally obliged to ensure that all staff attending their sheep are familiar with and have access to the relevant welfare codes.

Although The Animal Welfare Act 2006 as well as other government codes of practice permits castration, it has been demonstrated that castration causes distress and suffering to animals (Kent et al., 2004; Thornton & Waterman-Pearson, 2002) and changes in their natural behaviours. In recent years public opinion as well as the science sector in many countries have shown a great interest in the welfare of farm animals. The UK and other countries such as Australia and New Zealand leading in sheep production (see table 2) are working to develop and promote more welfare friendly methods of castration to minimize an animal's suffering and improve their wellbeing. Many countries have put in place regulations to protect animals' rights like DEFRA codes of practice, FAWC five freedoms etc. General recommendations are to follow legislation and codes of practice distributed by government organizations and assured schemes.

Stockmen and women should be trained in Welfare Codes of Practice and follow those rules in their everyday activities. Under the Protection of Animals (Anaesthetics) Act 1954, as amended, it is an offence to castrate lambs which have reached three months of age without use of an anaesthetic. The use of a rubber ring, or other device, which restricts blood flow to the scrotum or tail, is only permitted without an anaesthetic if the device is applied during the first week of life and it can be performed only by a competent person (Thornton & Waterman-Pearson, 2002). No person under the age of 17 should castrate. Under the Veterinary Surgeons Act 1966 only a veterinary surgeon can castrate animals older than three months. It is an offence to castrate animals without

anaesthesia when they have reached three months of age. Surgery or open castration can be performed by vets only (Thornton & Waterman-Pearson, 2002). FAWC in 2008 strongly recommended caution and good consideration to the farmers before any castration was performed. Also FAWC in 2008 recommended to exclude surgical castration as it causes more pain and distress in comparison to other castration techniques.

1.4.3 Legal requirements for castration of lambs in other countries with high sheep production

Similar to the UK other countries like Australia and New Zealand are concerned about the welfare of rams during and after the castration procedure. Appropriate laws and codes of practice are developed, implemented and enforced to protect animal rights. Equally UK public opinion is also increasingly interested in the standards of animal welfare and the management practices therefore there is an incentive to develop, implement, enforce and review animal welfare guidelines and codes by the appropriate institutions.

General welfare requirements for farmed animals in Australia are primarily regulated and enforced by individual states and territories. There is no national welfare legislation however there are national welfare standards and guidelines which are being implemented to improve the consistency of animal welfare laws across the country. Government bodies like Animal Health Australia (AHA) which is an organization formed by the collective Australian governments and the industry councils deals with the development of animal health services and management of national animal health programs. The key role of AHA is recognition and implementation of animal welfare standards and codes of practice across the country (Neumann, 2002). In 2013 there was a 60-day public consultation period run by AHA on the draft of the Australian Animal Welfare Standards and Guidelines for Cattle and for Sheep concerning castration, tail docking and dehorning.

In New Zealand the general welfare requirements for farm animals are outlined in the Animal Welfare Act 1999 and the Animal Welfare Amendment Act 2002. The National

Animal Welfare Advisory Committee has released a new draft code of welfare for sheep and beef cattle in 2010.

In Australia as well as in New Zealand according to recommended codes of practice castration is allowed to be performed in sheep less than six months of age providing that the practice is performed by a trained and skilled person or under the supervision of a skilled person with the use of approved techniques and equipment. Castration of sheep older than six months of age should be considered as a major surgical procedure which requires use of anaesthetics as well as pre and post-operative care (Animal Welfare (Painful Husbandry Procedures) Code of Welfare 2005, Geoff Neumann, 2002).

The animal welfare laws and codes of good management practices have also been debated in other countries with a high level of sheep production like China, India, Nigeria or Iran (See table 2). The animal welfare rights and laws in those countries are relatively new. However, implementation of welfare laws concerning specifically lamb castration in the developing countries may not be easy as there are many other issues and priorities which have to be resolved in the first instance.

In China the draft of the animal welfare law was proposed in 2009. The Chinese animal protection law recognizes castration as a potential welfare issue which is causing unnecessary pain. India and Nigeria have also considered animal protection laws and there are some regulations in place i.e. section 495 of the Criminal Code of Nigeria makes it an offence to cruelly beat an animal, overload, torture, bait and coerce animals to engage in fighting. In 2011 a new draft of the Animal Welfare Act was published for comments in India giving guidance to organizations and officials such as the police to help them interpret and apply the law, however there are no specific guidelines with regard to sheep castration.

Table 2 below represents the number of sheep stock which was recorded in 2008 by the Food and Agriculture Organization of the United Nations (FAO) for the countries leading in sheep production in the world.

Table 2 Production of global sheep stock in 2008

Global sheep stock in 2008 (in million)	
China	**1,364.0**
Australia	**79.0**
India	**65.0**
Iran	**53.8**
New Zealand	**34.1**
Nigeria	**33.9**
United Kingdom	**33.1**

1.5 Reproduction in male animals

In this section the reproductive system of male animals will be described to give an overview of the anatomy, physiology and general function and activity of the reproductive tract. The intention is to have a better understanding of the normal function of reproductive organs to fully consider the impact of castration on the ram's anatomy (changes in the structure and function of testicles), physiology (changes in endocrine system – lack of testosterone) and natural behaviours (i.e. sexual and courtship behaviours).

1.5.1 The ram's reproductive tract

The anatomy of the ram reproductive tract is shown in figure 1. The reproductive tract has in its structure two testicles, epididymis, vas deferens and accessory glands (seminal vesicle, bulbo-urethral gland and prostate). The testicles are based in the scrotal sac below the body and produce the male sex hormone, testosterone, and sperm (Cupps, 1991). Sperm matures in the epididymis and it is conducted to the urethra and penis by the vas deferens, also called the testicular cord. Accessory glands provide sperm with nutrition and medium which allows movement of the sperm. The testicles are held below the body for temperature control and production of fertile sperm as the spermatogenesis process requires an appropriate temperature to produce spermatozoa (Cupps, 1991). Primary spermatocytes and spermatids are very sensitive to the heat in comparison to spermatogonia which are fairly resistant (Bergh and Söder, 2007; Cupps, 1991). Tuinica dartos muscles are able to lower or raise testicles within the scrotal sac according to the temperature requirement. The testes can be held close to the abdominal cavity when it is

cold or lowered down to hang further away from the abdominal wall in hot conditions (Cupps, 1991). The scrotal skin is also very well equipped with temperature receptors which are involved in temperature control and maintenance (Cupps, 1991).

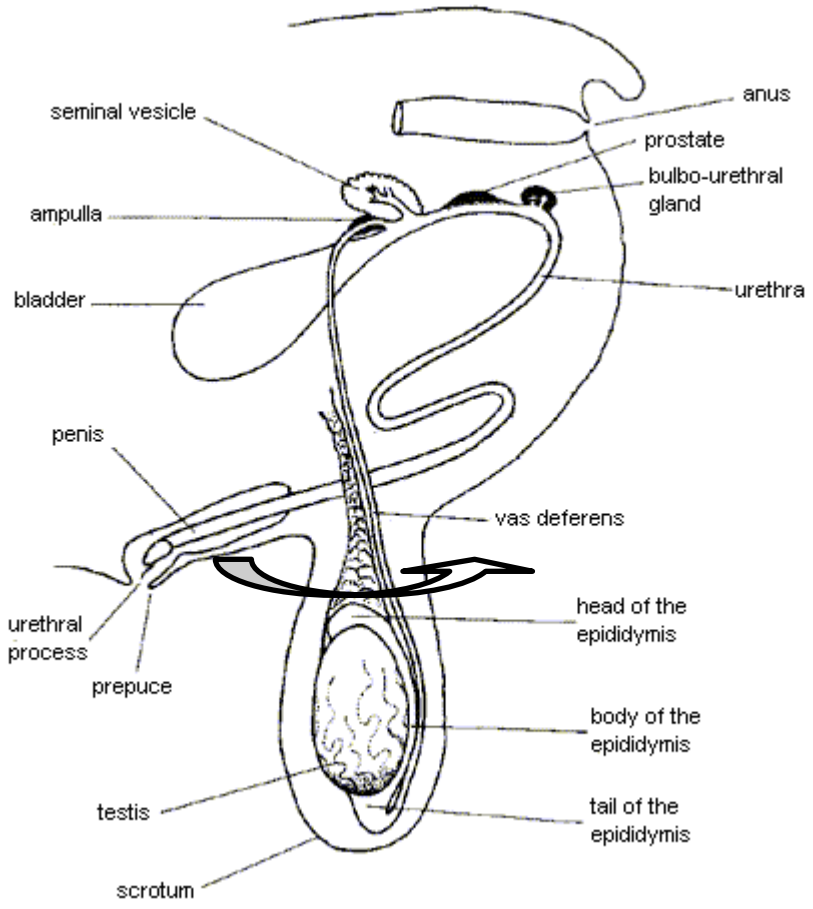

Figure 1 Reproductive tract of the ram. Black arrow represents the area where the rubber ring is applied.

The substance of each testicle is built by two main tissues: seminiferous tubules surrounded by peritubular myoid cells and interstitial tissue. Within the seminiferous tubules there are Germ cells and Sertoli cells (see figure 2 below). Germ cells will develop into spermatogonia, spermatocytes, spermatids and spermatozoon (see figure 2) through the process of spermatogenesis. The ultimate goal of Germ cells is formation of gametes (spermatozoon) which contain DNA material for fertilization of an ovum

(Hafez & Hafez, 2000). Sertoli cells, whose primary function is to support the Germ cell in their development into spermatozoa, divide seminiferous tubules into two compartments: the basal and the adluminal compartment. They also produce a fluid which is secreted into the adluminal compartment of the tubule and will take part in the development of spermatozoa (Reece, 1997).

Peritubular myoid cells surround the seminiferous tubules limiting each tubule and forming basement membrane (see figure 2) around the tubules. (Noakes, et al., 2009). Between seminiferous tubules there are interstitial cell layers containing Leydig (see figure 2) cells immature Leydig cells, interstitial macrophages, epithelial cells and blood vessels. Leydig cells are steroid cells that produce and secrete testosterone and other androgens important for sexual development and puberty. Leydig cells are also responsible for expression of secondary sexual characteristics, sexual behaviour and libido. They support spermatogenesis, erectile function and testicular volume (through testosterone) as well. In the ram Leidig cells can be seen as small groups of cells in the interstinal tissue surrounding blood vessels (Noakes et al., 2009).

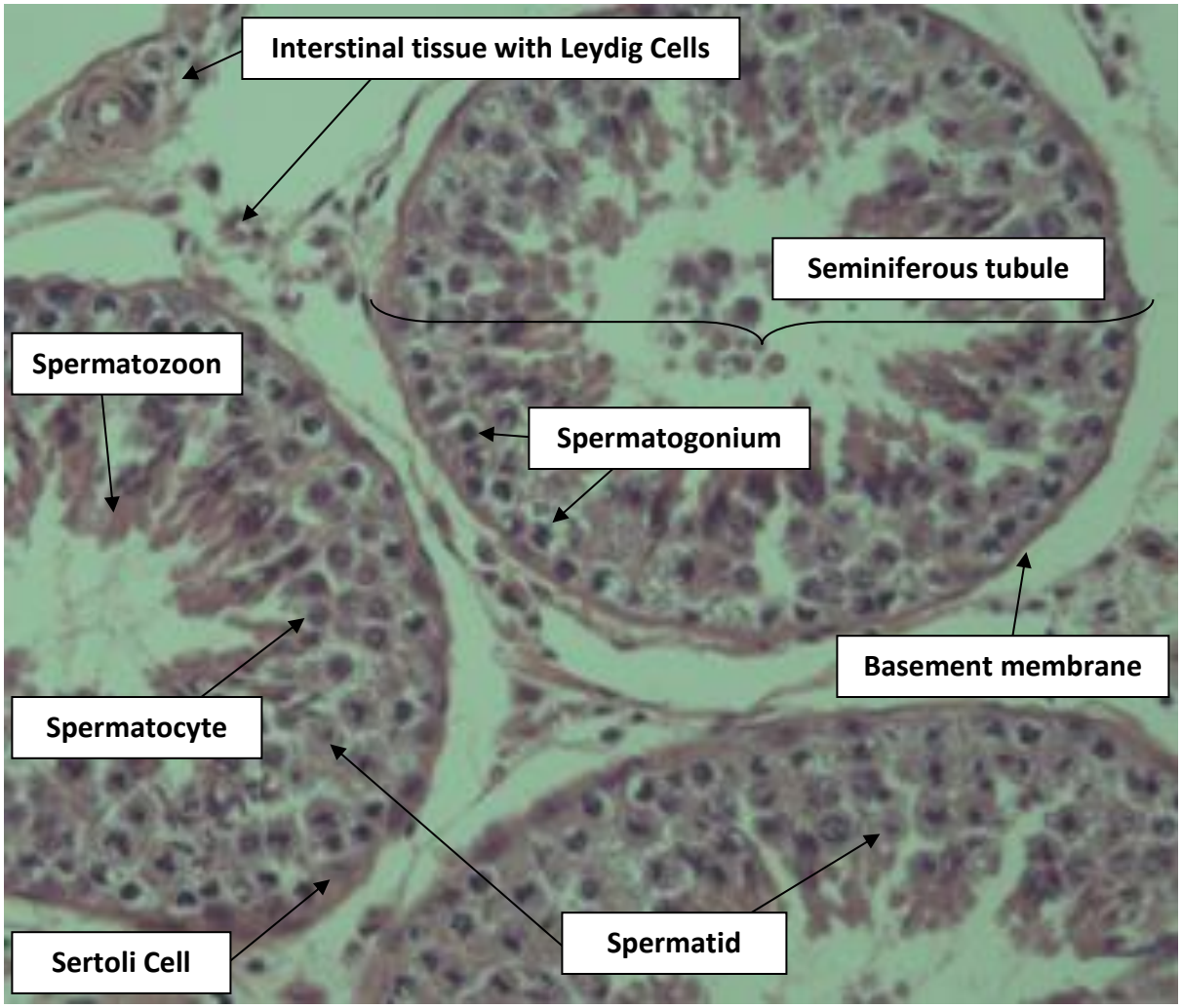

Figure 2 Representative transection of a ram testicle after puberty. (Image was taken on the second year of the thesis study in year 2012 and represents testicle tissue of control ram (entire male) at the age of 8 months)

1.5.2 Testosterone and its role in reproduction

Testosterone is a 19 carbon steroid hormone produced by Leydig cells in the testis. Most of the testosterone circulating in the blood is inactive. This is because testosterone secreted by Leydig cells is bound to a plasma protein for transport within the blood stream (Reece, 1997). Only 2% of testosterone is free to act on its target organs after it has been transformed to its active form, dihydro-testosterone. The major functions of testosterone in males are: stimulation of the late stages of spermatogenesis and prolonging the life span of the eipdidymal sperm, development of accessory glands and

stimulation of their activity. Testosterone also promotes growth, manifestation and maintenance of the secondary male body characteristics, libido and performance of courtship and sexual behaviours (Hafez & Hafez, 2000). Moreover, in embryonic development, the presence or absence of testosterone determines the development of the penis and scrotum in males or clitoris and vagina in females (Reece, 1997).

There is also evidence that testosterone influences regions of the brain which are responsible for special memory, higher motor action, cognitive behaviours, emotional behaviour, generalized emotional reaction, wakefulness and memory (Greenlee 2000; Azad et al., 2003). Therefore, its presence or absence may influence not only reproductive behaviours but also emotions, for example anxiety.

Libido is the sexual drive expressed by males at the time of mating. It can be easily eliminated by castration (absence of testosterone). However residual testosterone secreted by the adrenal gland may be sufficient enough for the manifestation of libido in some cases even in the absence of testis (Reece, 1997).

Male body characteristics influenced by testosterone activity are: increased weight and growth of bones, thicker skin, deeper voice and increased growth of muscles (Reece, 1997). Thicker skin and greater development of muscles can be explained by the anabolic functions of testosterone. This particular feature is very important for farmers, breeders and stakeholders as it means that entire male animals will grow faster and their muscularity may be greater in comparison to castrated males (Reece, 1997).

1.5.3 Hormonal Regulation of reproduction

Reproduction is regulated by the hormones secreted by the hypothalamus, particularly gonadotropin-releasing hormone (GnRH). GnRH is a decapeptide synthesized in the medial basal hypothalamus. It is secreted in a pulsatile manner and provides a humoral link between the neural and endocrine system (Hafez, & Hafez, 2000). GnRH acts on the pituitary gland and as a result of its pulsatile activity Gonadotropin hormones - Luteinising hormones (LH) and Follicle-stimulating hormone (FSH) are released from the anterior pituitary (see figure 3 below).

LH is a glycoprotein gonadotropin LH crucial for the synthesis and secretion of androgens, especially testosterone in males by acting on interstitial Leydig cells. Secreted testosterone acts in the way of negative feedback on the hypothalamus and pituitary gland to reduce the secretion of testosterone (see figure 3 below).

FSH is also a glycoprotein which acts on the germinal cells in the testis and it is responsible for the process of spermatogenesis until it reaches the stage of secondary spermatocytes. The final stages of spermatogenesis are supported by the androgens secreted by the testis. The process of spermatogenesis is also regulated by way of negative feedback (see figure 3 below). Inhibin, secreted by Sertoli cells, acts on the pituitary gland and the secretion of FSH is stopped (Hafez & Hafez, 2000).

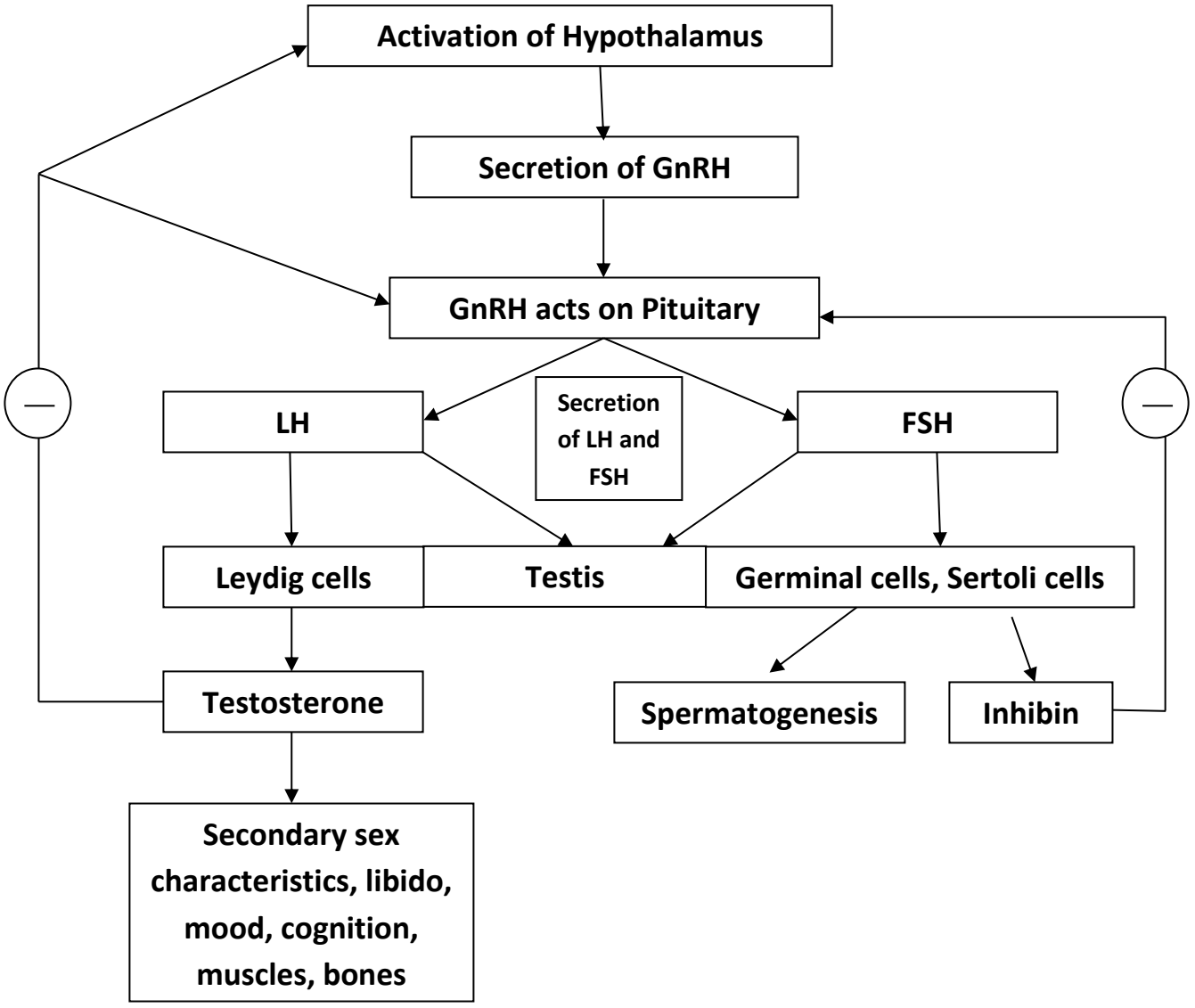

Figure 3 Hormonal regulation of ram reproductive system

1.5.4 Factors affecting testicular function

Sheep are seasonally polyoestrous animals and they mate in autumn. The length of daylight plays a significant role in the initiation of the mating season as well as other factors like nutrition, stress and social cues (Dwyer, 2008). Seasonality of breeding is also influenced by geographical location (Dwyer, 2008), and there may be a genetic component too as some breeds of temperate sheep are more seasonal than others i.e. Dorset Horn sheep show a long breeding season, whereas Scottish Blackface, Texel, Suffolk, Leicester, and Hampshire have a very short breeding season. There is also

genetic variation in when the breeding season begins and ends. Tropical breeds of sheep can mate throughout the whole year (Lynch et al., 1992). The sexual drive of rams (libido) is initiated by oestrus in females. During the breeding season males' fertility is fairly constant and production of gametes shows only slight variations (Lynch et al., 1992).

On the other hand, ewes' fertility varies a lot during the mating season and the ewe will be fertile only for a short period of time around the ovulation therefore seasonality of breeding is driven by the female's oestrus cycle (Dwyer, 2008).

In autumn the photoperiod is decreasing which releases the onset of sheep mating behaviours. Decreasing day length increases the secretion of melatonin. Melatonin influences GnRH (see diagram 3 for the details of GnRH action on LH and FSH) In females, an acute rise of LH triggers ovulation and development of the corpus luteum.

1.5.6 Courtship and Mating behaviours

During the mating season males and females perform a range of courtship and sexual behaviours allowing them to correctly identify individuals which are ready to mate and initiate mating, using visual and olfactory cues (Lynch et al., 1992). Reproductive signals can be categorized into four categories:

1. **Signals attracting opposite sex**

It has been argued by Katz and Tillbrook that there is some kind of preference in choice of mating partner and some individuals will appear more attractive to the opposite sex than others (Katz, 2008; Tillbrook, 1987 a, b). It has been shown that the length of the wool attracts males more than the level of oestrogen, although attractiveness was closely related to the presence or absence of oestrus in females (Tillbrook, 1987 a, b; Tilbrook & Cameron, 1989).

2. **Signals demonstrating dominance**

Showing dominance over conspecifics appears to be an obvious way of securing continuous access to females. Therefore, the ancestors of modern domestic livestock were seen to have frequent aggressive encounters and fights and by that securing his

reproductive success (Zuk, 1991; Iwasa and Pomiankowki, 1994; Jacobs,1996). Dominant rams will also affect the sexual behaviour of subordinate rams (Lindsay et al., 1976; Synnott and Fulkerson, 1984; Tilbrook et al., 1987; Ungerfeldnd and González-Pensado, 2008a) and disrupt mating activity of subordinate rams (Hogg, 1984). It has been shown that rams with greater social ranking had greater body weight, scrotal circumference, semen production and sexual behaviour at earlier ages of life (Ungerfeld and González-Pensado, 2008b).

3. Signals stimulating oestrus in females

Oestrus in ewes can be stimulated naturally by the presence of a ram. It is the ram's smell (ram pheromones) that influences the ewes most (Knight, 1983). This fact has been used widely among farmers and breeders in the practice of flocks' synchronization into oestrus.

4. Signals allowing correct identification of females in oestrus

Rams are able to distinguish between ewes in oestrus and ewes which are not in oestrus by visual cues and a series of olfactory behaviours. Vison plays a great role in recognition of the appropriate partner. It has been shown that lack of vison may reduce the mating capacity of a male (Chenoweth, 1981). Olfaction is another way of identification of an appropriate partner ready for mating. For example, flehmen behaviour when a ram raises its upper lip way to sense female pheromones. Flehmen is one of the most distinct courtship behaviours by which rams discriminate mating partners who are able and willing to mate. Another very distinct behaviour is sniffing and/or nosing which allows for sensing oestrus females by sniffing or rubbing their urine or genital areas with the muzzle (Lynch et al., 1992). The most common courtship and sexual behaviours of rams and ewes are described in Table 3 below. The frequency of occurrence of particular behaviours is specific for each animal and not all of the behaviours will be performed during the mating act (Lynch et al., 1992).

Table 3 Ewe and Ram Reproductive Behaviours (Lynch et al., 1992)

Ewe Reproductive Behaviours	
Squat/Crouch	Ewe takes a crouching posture, it may involve urination and usually occurs after nudging by the ram
Circling	Ewe turns back toward the ram, often nuzzling his flank, ram follows to retain his position behind the ewe
Tail fanning	The ewe tail is elevated and fanning in the presence of the ram
Head Turning	Ewe turns her head back toward the ram as he approaches to mount
Stand	Ewe stands firmly when ram attempts to mount
Following/Migration	Ewe follows the ram after initial contact by the ram, often in association with another ewe
Ram Reproductive Behaviours	
Sniff/Nose	Smells urine or perineal region of the ewe
Flehmen	After sniffing the ram arches his head and curls upper lip showing his teeth
Low Stretch	The neck is being held horizontal to the ground, muzzle is forward and raised, The head is often turned through 90 degrees
Nudging	Consist of one alone or combination of kicks with the forefeet used in pawing motion while standing parallel to the ewe, rubbing the flank and shoulders along or under the ewe's flank and low stretch
Lick	Licking ewe's flank, running tongue in or out
Mount	The ram brisket is in firm contact with the ewe's rump
Ejaculation	Visible pelvic thrust during mount accompanied by a rapid backward movement of the head

The mating begins as a sequence of behaviours which are initiated by the male who approaches the female. If the response of female is positive (female does not move away) he continues until reaching the stage of mounting and ejaculation. The factor that will determine and control the mating act is the ewe response to the ram's behaviour. The frequency and duration of male courtship behaviour depends on many factors like breed, libido, testosterone levels, and learned behaviours (Hulet, 1966; Lynch, Hinch & Adams, 1992).

It is also worth mentioning that young sheep at around four to six weeks of age will perform similar behaviours imitating courtship and sexual behaviour of adult sheep. This form of behaviour is sometimes called "sexual play". It appears very frequently in the first few weeks of life. It is associated with play activity and it is performed by male and female lambs. This kind of sexual play is not correlated with the hormonal status of animals (Orgeur et al., 1984; Hass et al., 1993; Orgeur, 1994).

1.6 Castration techniques

Castration of rams is a very popular practice in the UK and most farms perform the procedure regardless of the farm size (see table 4 for details). Altering rams' reproductive status gives more control over the flock management (unwanted behaviours, aggression, indiscriminate breeding) and genetics (inbreeding, meat quality and characteristics). In this section the most common castration techniques used to castrate lambs will be described.

Table 4 Summary of on farm castration practices survey 2005

% of sheep holdings which castrate				
	Farm Size			
	Small	**Medium**	**Large**	**All sizes**
lambs castrated	**91**	**91**	**96**	**92**
Age of castration				
Up to one week	**89**	**89**	**95**	**89**
1-2 weeks of age	**5**	**6**	**2**	**5**
2-4 weeks of age	**4**	**2**	**2**	**3**
% of performed Rubber Ring castration				
Rubber Ring	**95**	**96**	**99**	**96**

1.6.1 Rubber Ring Castration

Rubber ring castration is the most common method used in the UK (see table 3 for details). Elastration has been used by farmers for more than 40 years (FAWC, 2008). It is quick, easy to perform and economical (Archer et al., 2004). By law, rubber ring castration can only be performed until one week of age without anaesthesia. In reality hill flocks are normally gathered for husbandry practices when animals are older than one week so it is highly possible that the rings are applied without use of anaesthesia until lambs are 8 weeks old (FAWC, 2008). The ring is applied around the neck of the scrotum (see figure 1) by the stockperson using an elastrator. Castration can be done by a single person applying the ring or by two people where one is applying the ring and the other is holding the lamb. The ring stops arterial blood supply and venous drainage (Kent et al., 2004) resulting in ischaemic pain and tissue necrosis. Because of this, testicles will die and are shed within a few weeks after the procedure.

1.6.2 Short Scrotum Castration

The short scrotum method of castration artificially produces the naturally-occurring condition called cryptochoridism. In cryptochoridism one or both of the testicles do not descend to the scrotum sac and remain in the abdominal cavity. Animals with such a condition do produce sex hormones and sperm but the spermatocytes are not fertile as the testicular temperature is not appropriate (Lester et al., 1991). The short scrotum method is very similar to a standard rubber ring castration. The ring is applied around the scrotum neck but before that happens the testicles are pushed back to the abdominal cavity. The scrotum sac will then die and be shed within a few weeks as it would in standard rubber ring castration and the testicles will remain in the abdominal cavity producing testosterone and sperm but the animal will remain infertile. This method is widely used in New Zealand. About 41% of castrated lambs in New Zealand are castrated by this method (FAWC, 2008; Fisher et al., 2010).

1.6.3 Surgical castration

This is one of the oldest methods of castration which was used widely before rubber ring castration replaced it. By UK law surgical castration can be performed by vets with the

use of pain relief in the cases when it cannot be avoided or replaced by other suitable methods (FAWC, 2008). Usually two people are required. One of them holds and restrains the lamb and the other opens the scrotal sac by a single incision with the scalpel across the bottom of the scrotum or by two incisions on each side of the bottom of the scrotum. The testicles and epididymis with spermatic cord are pulled back and removed. The bleeding that follows from the procedure from the spermatic and epididymis arteries is stopped by use of an emasculator or cautery. The hygiene prior to and during surgical castration is essential for quick and easy healing of the wound without complications.

1.6.4 Bloodless method of castration

The bloodless method of castration is performed by the usage of a Burdizzo clamp which was invented for the castration of cattle (FAWC, 2008). Metal jaw clamps are applied through the skin of the scrotum neck on each side of the scrotum so that the spermatic cord of each side is crushed. This procedure is best performed by two people where one is holding the lamb and the other is applying the instrument. Good knowledge of anatomy is essential for the correct application and destruction of the spermatic cord. It is also advised to crush the spermatic cord twice, the second time a bit lower than the first. This method is believed to give less pain as the duration of the pain behaviours caused by castration is shorter (see chapter 3). It is because the nerves supplying the testicle tissue are crushed immediately after the rubber ring application so the information about the tissue injury cannot be transmitted further.

1.6.5 Combined rubber ring and Burdizzo castration method

The combined rubber ring and burdizzo clamps method of castration was previously described by J. Kent and V. Molony. It has 2 components. Firstly, the rubber ring is applied as in standard rubber ring castration (see Diagram 1). This is immediately followed by the crushing of the spermatic cord by the application of the burdizzo clamp proximal to the ring (Molony et al., 1993). Similarly, to the bloodless method of castration this technique was also observed to give less pain due to the shorter duration of the pain behaviours (Kent et al., 2004). This is caused by the lack of transmission of the information about the tissue damage.

1.6.6 Immunocastration

Immunocastration has been studied as a potential alternative to traditional methods of castration since 1970 (Fraser, 1980 and 1986; Schanbacher, 1984; Chaffaux et al., 1985; D' Occhio, 1993; Thompson, 2000). The basis of active or passive immunization is to prevent the binding of gonadotrophin hormones (like GnRH, LH and FSH) circulating in the blood to their primary target cells, the gonadotropes. The binding of the gonadotropin hormone like GnRH may be done in two ways with the use of (1) antibodies or (2) antagonists targeting specific reproductive hormones (GnRH, LH or FSH).

1. - The immune system of an animal vaccinated against GnRH is stimulated to produce antibodies which will recognize the GnRH molecule as a threat and bind it within the blood stream. This is achieved by the slight alteration of GnRH or attaching it to another molecule that is not part of the animal and will be recognized as foreign by the immune system.

2. - GnRH antagonists (receptor blockers) are similar to GnRH in structure, class of peptide compounds which will compete with natural GnRH for binding to GnRH receptors and by that blocking completely or decreasing function of GnRH in the body (blocking the release of LH and FSH).

The aim of Immunocastration in farm animals is to reduce or minimise pain induced by the castration procedure and the achievement of the desired castration effects. In this thesis primary interest was focused on the immunization against GnRH in the way of antibodies binding. This hormone (for details of hormone secretion and function see section 1.5.3 above) plays a key role in the Immunocastration procedure. When the GnRH neurones are excited, the peptide hormone is released into the extracellular space and capillary blood. The hormone travels to the secondary capillary plexus where some proportion of GnRH leaves the capillaries and becomes available for binding to its target cells. The period when GnRH leaves the capillaries is when it is most vulnerable to be attacked by the vaccine antibodies (Thompson, 2000). Binding with antibodies

neutralizes GnRH by preventing it from diffusing through the capillary walls or by masking the receptor binding site on the GnRH molecule (Thompson, 2000).

Schanbacher found that immunized rams had similar carcass quality to rams physically castrated; only the thickness of back fat in immunized rams were similar to intact males (Schanbacher, 1982). There was improvement in feed efficiency and carcass conformation in animals immunized against GnRH (Thompson, 2000). Godfrey immunized goat bucks with anti-GnRH vaccine (Vaxtrate®) and compared their behaviour and physiological characteristics with non-immunized males. Male odour as well as concentrations of LH, FSH and testosterone were decreased in immunized bucks (Godfrey et al., 1996). The weight of the testicles was also lower after booster vaccinations given after 2 or 4 week intervals (Godfrey et al., 1996). Jeffcote et al. (1982) immunized rams with anti-GnRH vaccine and found reductions in testicle size in immunized animals. Brown et al. (1994) has also shown delays in testicle growth in the males that were immunized before puberty.

In the commercially available vaccine products there are two components which are the main focus. These are: A) the adjuvant used and B) the number of booster injections which would need to be done for the vaccine to be effective. Adjuvant is a pharmaceutical or immunological agent that modifies the effects of other agents like the vaccine. Adjuvant can stimulate the immune system response to the target antigen. Many different adjuvants were used in the production of an anti-GnRH vaccine since this method of castration has first been introduced i.e. polyvinylpyrrolidone (Arimura et al., 1973), bovine serum albumin BSA (Fraser and Gunn, 1973), glutaraldehyde (Kerdelhue et al., 1976; Hotzel et al., 1997). Many of the investigated adjuvants have been shown to be successful in neutralizing GnRH antibodies, however there were other factors that need to be considered before vaccination against GnRH was applied. For example, FCA (Freund's Complete Adjuvant) contains Mycobacterium components therefore it may be not practical for use in the farm environment as it may affect tuberculosis tests results in cattle and it has damaging tissue effects (Thompson, 2000). McLachlan et al. (1995) in rats, Brown et al. (1994) in rams and Godfrey et al. (1996) in

goats used mycobacterium free adjuvant successfully and achieved reduction in testes size.

Achievement of desired outcomes may be also difficult due to the fact that the vaccination needs to be repeated to succeed; the strongest reaction comes from repeated exposures (vaccinations). However, if the immunization is done in the way of antibodies binding to GnRH or altered GnRH and the immune system already recognizes GnRH as part of its own, the vaccination may not be effective. Also vaccination may not be practical to be used in the farm environment as it may interfere with normal husbandry practices and additional gatherings will be needed to perform booster vaccinations. Therefore, finding an optimal regime of vaccination which would fit with normal husbandry practices should be considered.

At the present time there is a successfully implemented anti-GnRF vaccine for cattle (Bopriva). Bopriva is a vaccine made from an analogue of GnRF linked to a carrier protein. Like most immunocastration products Bopriva has two components, the adjuvant and the two dose regime of vaccination (primary and one booster) which need to be applied for the vaccine to be effective. The adjuvant which was used in the production of the vaccine is a synthetic aqueous adjuvant that increases the level and duration of immunity. The vaccination has to be applied in a two dose regime to be effective. Vaccine has been designed to eliminate or reduce such behaviours as fighting; sexual mounting; pawing; digging and pasture damage; bellowing; damage to infrastructure; dominance and territorial behaviour. It has been shown by Janetta et al. (2012) that Bopriva vaccination against gonadotropin-releasing factor (GnRF) significantly decreases testicular development, serum testosterone levels and physical activity in pubertal bulls. It also improved performance and carcass quality, leading to better carcass grading and loin fat cover (Amatayakul-Chantler et al., 2012). In summary, Bopriva provided a safe and sufficient alternative which had a beneficial impact on animal welfare in comparison to surgically castrated bulls without any negative effects on carcass or meat quality traits. Therefore, it was concluded that

Immunocastration is a safe, practical and economical method providing production gains to the farmers (Amatayakul-Chantler et al., 2013).

A similar product has been developed by Zoetis for the immunocastration of rams. The exact formulation of the vaccine has not been revealed due to commercial sensitivity. The product is very similar to cattle vaccine Bopriva which stimulates antibodies against GnRF. In the product developed for ram lambs GnRF is conjugated to a carrier protein and articulated with an adjuvant. Adjuvant used in the vaccine is immuno-stimulating complex required to enhance immune response. Primary dose of the vaccine primes the immune system. Secondary vaccination stimulated production of specific antibodies which will neutralise GnRF and by doing so activation of LH and FSH will be stopped and secretion of testosterone will not be possible. Experimental trials of Bopriva vaccine have shown that duration of the immunity effect will depend on the interval between primary and booster vaccination. Therefore, cattle farmers are encouraged to carefully consider their vaccination regime. To fit the vaccination in the management practices farmers will need to decide when they want the primary dose to be administrated and for how long the immunity effect is desired to last. Bopriva is recommended to be given at 3-8 weeks' intervals.

This study will be investigating most efficient vaccination regime as well as interval between primary and booster vaccination maximizing suspension of testosterone in ram lambs. There were attempts in the past to immunize sheep against GnRF with other available on the market products. For example, Janett et al. (2003) used another anti-GnRF vaccine developed by Zoetis, Improvac, (designed for pig males) in the study investigating possibility of immunocastration against GnRF in lamb rams. Improvac is an analogue of GnRF allied with a carrier protein and a synthetic aqueous adjuvant to increase the level and duration of immunity after booster vaccination. In the study by Janett et al., (2003) using this vaccine in sheep was successful for a period of at least 12 weeks. After 12 weeks the vaccine effect was reversed in 8 out of 10 lambs at the age of 3-7 months. It was concluded that for the immunity period to last for longer, third booster vaccination will be needed.

The background of the immune vaccine use in ram lambs is similar to comparable products used for immunocastration of pigs and cattle. The aim of immunization use in ram lambs is reduction of aggressive and sexual behaviours, elimination of unwanted mating (indiscriminate breeding) and enhancing lambs' productivity through improved growth, carcase and meat characteristics. It has to be noted that, primary objective is to reduce or minimize pain induced by physical castration methods. The influence of different castration techniques including immunization was investigated in sheep and cattle by Steiner and Janett (2013). It was concluded that cattle and sheep should be castrated at the age of 14 days or less with use of local anaesthesia i.e. lidocaine or NSAID i.e ketokarprofen. Immunization was found to be valuable welfare friendly and economical alternative to rubber ring castration allowing for suppression of testosterone for at least 12 weeks in 2 dose regime with 2-4 weeks' interval between primary and booster injection. However, there were no registered products on the market for the use in the ruminants in Europe.

For the purpose of this study Zoetis was the supplier of the novel anti-GnRF vaccine for ram lambs.

1.7 Castration procedure complications

Many scientists have already shown that physical castration is painful and causes a lot of distress to the animals (Thornton & Waterman-Pearson, 1999; Kent et al., 1995; Mellor and Murray, 1989; Molony et al., 2002) (for more details please see chapter 3). The duration and amount of pain-related responses which is caused by castration will depend on the method used. All traditional methods of castration may be the cause of phantom pains as there is a risk of continuing neuropathic nerve damage (Wood and Molony, 1991). Any castration technique will eventually damage the integrity of the animal tissues therefore careful consideration should be given first before any procedure is performed to establish general conditions of the housing sheds and the health state of the ewes and lambs. Any husbandry procedure should be delayed or withdrawn when the animal is unfit, when there are signs of weakness, disease (FAWC, 2008) and presence

of scrotal hernias (Henderson, 1990). The environment in which animals are kept plays a great role too as bad weather and unhygienic conditions can compromise the lambs' health. The pain induced by castration may stop the lambs from suckling for a time. This may be a cause of more serious diseases, like 'watery mouth' (infection by E. Coli in lambs), hypothermia or starvation therefore it is highly recommended to castrate animals when they are at least 12 hours old (Eales and Small, 1986). FAWC in 2008 recommended allowing ewe and lamb to form the bond first, before any procedure is carried out. Therefore, lambs should be at least 24 hours old before castration is performed.

Surgical castration creates an easy-to-enter route for bacterial flora. The rubber ring and Burdizzo castration do not break the skin immediately but the skin does breakdown over time and the testicles will be shed. Bacterial flora could enter through the wound that will be formed i.e. infection by clostridia (like *Clostridium tetani*) or by erysipelas which may lead to polyarthritis. Many farmers in the US apply tetanus toxoid injections and antimicrobial injections prior to elastration and surgical castration to avoid the possibility of lambs becoming infected with clostridium tetani or other infectious agents (Coetzee et al., 2010).

Rubber ring castration when applied too high may trap the rudimentary teats which will increase the pain and discomfort of the animal, also the wound and route of entry for bacterial flora will be bigger. It is also possible to trap the urethra which will lead to retention of urine in the bladder and finally to renal failure (Henderson, 1990).

In surgical castration haemorrhage is a large issue and can be a cause of lamb death. Clotted exudative and blood are also a perfect culture for bacterial growth. Bacterial flora can move to the abdominal cavity by the spermatic cord and cause peritonitis or chronic inflammation of the spermatic cord.

The Burdizzo method requires a lot of technical skill. Poor technique may be the cause of failure to castrate animals properly therefore it may not be suitable for lambs (Henderson, 1990). It can inflict unnecessarily pain due to poor application of the

instrument. It is recommended to crush spermatic cords twice to reduce the pain but there is a risk of too high application of the clamps which may damage the urethra or too low application of the clamps which could lead to damage of the testicle. Appropriate size of clamps and a good knowledge of anatomy as well as the appropriate time of application of the clamps to damage the nerves in spermatic cord is essential.

The effects of immunocastration on the ram's anatomy and physiology need to be further investigated. There is possible tissue damage due to injection. Lesions may be formed. There is also a possibility of inflammation around the injection. Behavioural patterns of immunized rams may also be changed due to the procedure.

1.8 Perception of pain

Pain is a sensation which serves a unique function. The attempt to define pain perception by the animals has been undertaken by many researchers (Molony and Kent, 1997; Rutherford, 2002; Molony et al., 2002). For the purpose of this study "Pain sensation" expressed by any individual being was defined as: "Aversive sensory and emotional experience representing an awareness by the animal of damage or threat to the integrity of its tissues… it changes the animal's physiology and behaviour to reduce or avoid the damage, to reduce the likelihood of recurrence and to promote recovery" (Molony, 1997). This particular definition was chosen to account for individual animal needs. Situations where animals do not feel pain when injured are dangerous and harmful to their wellbeing as it may provoke irreversible damage. Some scientists also suggest that avoidance of dangerous and harmful situations may be learned to some extent (Gregory, 2004). Rats that were raised in a pain-free environment showed lower avoidance of painful stimuli in comparison to rats raised in environments with painful stimuli, so pain may play an educational role to help protect the integrity of the body in many life threatening situations (Raisen and Zilbert, 1975). In consequence pain has to be an aversive feeling to stop the animal from inducing further damages to the integrity of its tissues. It causes vegetative, motor, emotional and behavioural reaction and may involve fear and anticipation of pain (Seksel, 2007).

Nociception is the physiological response to a painful stimulus. This term can be explained by situations where the animal has been anaesthetised so the pain reaction should not be expressed however the peripheral pain pathways are still active (Gregory, 2004) therefore nociception can be measured by physiological indicators, such as the level of cortisol in the blood.

Animals can express pain in many ways for example: escape, avoidance, withdrawal, seeking cover, sleeplessness, abnormal postures, behaviours (licking, biting, chewing, scratching, rolling, stamping, tail wagging, easing quarters, increased rate of breathing, muscle tension, tremor, twitching, spasm etc.) and vocalisations. Pain sensation will change movement and escape capability of prey animals therefore it is potentially deadly for them as they might not be able to flee in life threatening situations. It may cause problems during handling as a result of learnt aversion associated with a certain situation (Seksel, 2007). Pain may also lead to negative long term effects due to its influence on the immunological system increasing susceptibility to infections, diseases, parasites infestation etc.

Pain can be categorised as acute and chronic. For example, pain induced by castration or tail docking may be classed as acute for the first several hours following procedure however it may lead to complications like secondary infections which will prolong the time of healing causing pain sensation that may last for several days after the procedure.

1.8.1 Assessment of pain

The main objective of this thesis is to investigate whether Immunocastration will be a new, potentially pain free and more welfare friendly method of castration for ram lambs. To investigate the efficiency of this new alternative to physical castration the assessment of pain related behaviours and postures following the castration procedure was carried out. Assessment of pain in animals is not an easy task as the judgement may be clouded by several external factors. Therefore, it is worth exploring in more detail how the quality and quantity of pain can be evaluated.

There are few methods allowing for the assessment of amount and quality of pain. First of all, pain can be assessed by: overall body function such as weight gain, food and water intake. Secondly pain can be assessed by physiological responses like cortisol concentrations and lastly by the appraisal of specific behaviour i.e. vocalizations (Weary et al., 2006). However, there are some limitations with regard to the use of particular pain indicators. For instance, overall body function indicators are unable to represent the actual state of an animal's health and may only indicate what was happening with the animal in the past (Weary et al, 2006).

Physiological measures may be more useful in the overall assessment of the animal's actual state and well-being. Most commonly used physiological responses to pain sensation are endocrine responses. Spontaneous autonomic responses may be indicators of painful incidents as well. Physiological pain indicators may include measures of heart and respiratory rate (which are responses of the sympathetic-adrenomedullary system), changes in the cortisol level concentrations (which are responses of HPA axis (Gregory, 2004; Weary et al., 2006). Nevertheless, such measures may be more useful in the lab setting (with the environment controlled by humans) and during procedures which require restraining of an individual i.e. in wild species like deer (Woodbury et al., 2002). Physiological measures might be impractical in the farm environment as many standard husbandry practices are stressful to the animals i.e. sheep shearing and will increase heart rate and levels of cortisol due to handling but it does not mean that they inflict pain.

Behavioural measures have potential to give a good overview of an animal's emotional as well as physical state. Occurrence or absence of certain behaviours may be the first indication of pain existence and could be used by carers as an assessment tool to investigate pain related behaviours or animal reactions in worrying situations (Bateson, 1991; Rutherford, 2002; Anil et al., 2002; Weary et al., 2006; Vinuela-Fernández et al., 2007). It is also very important to remember that if the pain is to be measured by the behavioural means the observer should take into account the context in which particular sets of pain behaviours will be observed, all external factors and, if possible, rule out

confounding factors. To make sure that the observations are not biased it is crucial to be familiar with the species' specific behaviours before any pain assessment is carried out and to have individual knowledge about particular animals at the same time (Seksel, 2007). The most difficult part in recording pain behaviour is to distinguish it from the other range of animal behaviour. The observer should also have knowledge about physical injuries or diseases as well, as they can account for changes in normal behavioural patterns. For example, old injuries of the joints may lead to stiffness and difficulties in movement which are not necessary related to pain reactions (Weary et al., 2006). Indicators of pain will therefore depend on many factors such as pain duration, specific location, origin (what was the cause of the pain), species, age and breed (Gregory, 2004).

One way to check whether a particular stimulus is inducing pain is to provide local anaesthesia or analgesia and compare reactions in the situation before and after the animal has been anaesthetised. For instance, dehorning with the use of hot-iron is believed to be a very painful procedure in dairy herds which is leading to changes in the behavioural and physiological responses of animals (Morisse et al., 1995; McMeekan et al., 1998). When calves are given anaesthetics before dehorning they do not show higher frequencies of pain related behaviours like: head shaking, head rubbing and flicking ears (Morrise et al., 1995; Sylvester et al., 1998; Graf and Senn, 1999; McMeekan et al., 1999). Therefore, it can be argued that increased frequencies of these behaviours are indicators of pain. Another good example validating high frequencies of certain behaviours as pain related behaviours was shown by Roughan and Flecnell (2003) in the studies concerning behaviours of rats after abdominal surgery with or without use of analgesics. Rats which were given analgesics before surgery were showing lower frequencies of back arching, staggering and writhing in comparison to the group which was given a saline injection prior to surgery.

Pain behaviours can be measured in a few ways. First of all, there is objective and subjective assessment of pain related behaviour. Objective measures are more difficult to apply as they need to be quantitative i.e. stride length and duration in the pattern of

movement for specific species. The stride length is always the same when an individual is walking straight. The stride length is the distance between 2 successive placements of the same foot, consisting of 2 step lengths; measured between successive positions of the left foot is always the same as that measured by the right foot. Flower et al. (2005) found that stride length and duration of cows with painful ulcers differed from heathy animals. Subjective assessments are based on indirect measures of rating scales like the visual analogue scale (VAS), 5-point lameness scoring scale developed by Sprecher et al. (1997), and behavioural and physiological indicators. There are doubts with regard to the consistency and accuracy of subjective measures therefore it is always worth performing inter and intra-observer reliability to check how consistent viewers are in their behavioural assessments. Many researchers choose to be blinded with regard to the treatment while assessing behaviour to avoid any bias. Subjective measures are believed to be easy to apply and are very popular in the veterinary field (Weary et al., 2006; Vinuela-Fernandez et al., 2007).

In summary, pain behaviours may be measured easily by assessment of frequency and duration of relevant pain related behaviours and postures. For example, Roughan and Flecnell (2003) showed an increase of withdrawal behaviour in rats following abdominal surgery. A high frequency of calls following castration in piglets was shown by Taylor and Weary (2000) and defensive behaviours were shown by Thornton and Watherman-Pearson (1999) during assessment of the post castration site. Another very distinct indicator of pain may be the lack of activity or decrease in frequency and duration of specific behaviours (i.e. decreased motivation to perform favourable behaviour) (Weary et al., 2006). There are also studies using preference choice in the assessment of welfare and pain in animals such as the evaluation of self-medication used by animals to measure the amount of experienced pain. For example, Danbury et al. (2000) discovered that lame birds will prefer to eat feed which contains medication over normal not medicated feed in comparison to healthy birds.

There are a lot of external factors which need to be considered before pain assessment is carried out such as breed, environment, age and sex. It has been shown that new-born

animals showed different reactions to painful situations compared to older animals i.e. an adult dog would withdraw its limb if it is stimulated by mild electric shock. On the other hand, a five-day old puppy would try to crawl away, raise its head and there would be flexion of the limbs (Fuller et al., 1950). There are also differences in the expression of pain between sexes. Males may show different pain reactions to females i.e. aggression (Gregory, 2004).

For the purpose of this thesis the ethogram of pain related behaviours (associated with castration procedure and validated before by Molony et al. (2002) have been developed and implemented (see chapter 3 for more details). Careful consideration of species specific behaviours, breed and age was taken into account before scoring any behaviour. The pain related behaviours correlated to this thesis were assessed by a single observer who was blinded with regard to specific castration treatment during evaluation. Inter-observer reliability was also carried out.

1.9 Pain responses to different castration methods

As mentioned before castration is a painful and distressing procedure and this fact has been shown by many scientists. However, there are differences in duration and quality of pain induced by different techniques of castration. Rubber ring castration is thought to be more painful than the combined method of castration as the mean frequency of abnormal behaviours like foot stamping, tail wagging and head turning to the scrotum side was increased in rubber ring castration (Kent et al., 2000). It was also shown that lambs castrated with rubber rings and an injection of local anaesthetic performed less abnormal behaviours than lambs castrated with rubber rings only.

However, some argue that it is difficult to compare this kind of pain with other methods. The removed organs are not causing the pain. Nevertheless, lesions created during castration can be a source of inflammation and bacterial infections which may result in chronic pain (Wood and Molony, 1991). Thornton and Waterman-Pearson (1999) studied changes in behaviours, plasma cortisol concentrations and mechanical nociceptive thresholds after different castration techniques with or without the use of

anaesthesia. They have shown that surgical castration gave the highest negative response and this was followed by the rubber ring and combined method of castration. Use of local anaesthetic had no influence on reduction of pain after surgical castration. Short scrotum castration was found to induce a lower rise of cortisol in comparison with rubber ring together with tail docking (CTD), rubber ring alone (RR) and unilateral castration (C1) type of castration where one testicle is pushed back into the inguinal canal or region. The rubber ring is applied below the testis with one testicle remaining in the scrotum sac (Molony et al., 2002). It was also shown that cortisol concentration after short scrotum castration was higher than in lambs which were tail docked (TD), handled only (H) or castrated with use of local anaesthesia (CLA) (Molony et al., 2002). Behavioural observations also confirmed that CTD, RR and C1 castration were more painful than TD, H, and CLA treatment groups. The lip curling and easing quarters behaviours were seen more frequently in CTD, RR and C1 than in other groups, as well as trembling of the torso. Abnormal lying behaviours with full extension of the limbs were performed for longer periods of time after CTD and RR castration. It is also worth mentioning the time spent on abnormal standing was reduced in the CLA group. The persistence of pain-related responses after castration depends on the technique which was used. Molony et al. (1995) assessed acute and chronic pain responses in the four different castration methods (four groups of Ayrshire calves were castrated at 1 week of age by either surgery, crushing by a Burdizzo, rubber ring or a combination of the Burdizzo and rubber ring methods). It was shown that acute pain responses, such as changes in behaviours and postures as well as changes in plasma cortisol concentrations lasted up to 3 hours after the procedure. Chronic pain induced by rubber ring castration was shown to persist at least 42 days post-castration. Assessed animals were standing abnormally and licking the lesion site at a higher rate. The tail was moved more carefully, movement of the head and lifting of the hind limbs were also greater especially when the lesion was formed (Molony et al., 1995).

The breed of lamb may affect their responses to painful stimuli. It has been shown that lambs of different breeds, Suffolk or Charolais, had differences in recorded behaviour

patterns after rubber ring castration (Archer et al., 2004). Overall recorded behaviours were very similar and suggest that both breeds were experiencing acute pain however some of the reactions were different e.g. standing behaviour. Charolais lambs were more active after castration, their recovery time to normal postures was longer and their respiration rate was higher than in Suffolk lambs (Archer et al., 2004). These findings suggest that different breeds may experience pain in a different way although different type of temperament which characterise different breeds should also be taken into account during such assessments (Archer et al., 2004).

The age of animals is a very important factor too. Thornton & Waterman-Pearson (2002) studied the influence of rubber ring and combined method of castration on behaviours of lambs in different age groups. Two groups of animals were studied: one-week-old lambs and four-to six-week-old lambs. They recorded significant differences between different age groups in recoded behaviours and postures. One-week-old lambs performed less play behaviours and four-to six-week-old lambs performed less lying behaviour. The older group of animals also showed significant increase in abnormal postures. However, there were no significant differences between two castration methods. It has also been found that the scrotal lesions of younger lambs were less severe in comparison to older lambs (Kent et al., 2000), which probably relates more to lamb weight than age per se.

2.0 Usage of Analgesia/ anaesthesia and its effect on animals' behaviour

According to medical dictionaries analgesia and anaesthesia can be explained as follows:

Analgesia is absence of sensibility to pain or the relief of pain without loss of consciousness.

Anaesthesia is a loss of sensation in a part, or in the whole body, induced by the administration of a drug (an **anaesthetic agent**).

Analgesia is thought to be one of the solutions for the pain behaviours induced during castration (Wood et al., 1991). Mellema et al. (2006) showed that behavioural responses after Burdizzo and rubber ring castration were observed at a lower frequency when 5ml of diluted Lidocaine was injected prior to the procedure. Cortisol levels were also

reduced. It has been shown that local anaesthesia helps to modify behavioural and physiological reactions to painful stimuli. To reduce castration pain analgesia, general anaesthesia or non-steroidal anti-inflammatory drugs can be used. Anaesthesia influences the animals' response to pain by preventing afferent impulses from the injured tissues reaching the brain (Molony et al., 1997). There are attempts to adapt different techniques and different types of drugs to minimize pain reactions, however there are some obstacles in practical application of anaesthesia before castration on a large scale (Price and Nolan, 2001). It may not be economical as well as too time consuming a procedure in the farm environment, or may require greater labour than is available. Sedation is a good method of calming animals during husbandry procedures but it may not be sufficient to mitigate pain during castration (Mellor and Stafford, 1999). General anaesthesia is risky for ewe-lamb bonding and time consuming as well as not practical on the farm and does not prevent the occurrence of post castration pain (Mellor and Stafford, 1999). Non-steroidal anti-inflammatory drugs (NSAIDs) have been shown to reduce the cortisol response and abnormal behaviours after burdizzo castration but they might not be sufficient in rubber ring castration (Molony et al., 1997). In cattle a combination of NSAIDs and local anaesthesia applied before castration has been shown to be a good method of pain reduction (Stafford et al., 2002). Some non-steroidal anti-inflammatory drugs like carprofen, meloxicam, ketoprofen etc. could solve the problem as they are practical and easy to administer, but they are not licensed in the UK for sheep species therefore cannot be used (Price and Nolan, 2001) or they can be only used under the cascade system which may not be practical to use. There is not much data available on use of NSAIDs in sheep. Combinations of local anaesthesia and NSAIDs (i.e. carprofen) have been shown to be effective in pain moderation after mulesing (removal of strips of wool-bearing skin from around the breech to prevent fly strike) which is believed to cause similar acute stress response as shearing, mild fly strike and castration (Fisher et al., 2007). In the light of the issues related to traditional methods of castration, efforts to find alternatives to this painful procedure are important and relevant.

2.1 Summary and Conclusion

Castration is a painful procedure negatively affecting the animals' behaviours and welfare. Different types of castration induce pain in different ways and for different amounts of time. Post castration complications can prolong animals' suffering and are the cause for chronic pain. Introduction of analgesia or anaesthesia which would alleviate pain sensation and largely reduce lamb suffering have proved to be impractical to be applied on the farm. Immunocastration is believed to be potentially an almost pain-free new method of castration, however the effects of the vaccine on the ram behaviours, anatomy and physiology needs to be further investigated. In the light of these findings the following thesis objectives have been formed.

2.2 Thesis Objectives

The overall objective was to determine efficacy of an anti GnRF vaccine developed by Zoetis as a more welfare friendly method of castration for ram lambs. The impact of the vaccine on a range of lamb natural behaviours (development of pain behaviours, ewe-lamb bonding, anxiety, aggression, courtship and sexual behaviours), blood testosterone level, growth rate, carcass conformation and meat quality was investigated.

2.3 Overview of the key research questions

Chapter 1, 2 and 7 are informative chapters describing general information, general materials and methods, discussing results of this study and summarising findings to conclude the best solution for the issues related to ram castration in the future.

Chapter 3 Hypotheses

1. Pain behaviours and postures expressed by animals castrated with different castration techniques will vary in duration of time and frequency of occurrence.
2. QBA is able to distinguish between different castrations techniques, especially in the case when lambs do not show high frequencies of pain related behaviours after the castration procedure due to periods of immobility which is caused by severe pain sensation.

Chapter 4 Hypothesis

1. Seven month old male lambs immunized against GnRH (accordingly to agreed protocols at 6 and 12; 6, 12 and 22; 10 and 16; 10 and 20; 12 and 18; 12 and 22 weeks of age) show sexual behaviour comparable to a physically castrated male lamb (using rubber rings), and less sexual behaviour than entire male lambs of a similar age.
2. Male lambs that have been made cryptorchids will show intermediate sexual behaviour.
3. Histology of the testes may be different in immunocastrated rams in comparison to entire males and short scrotum castrated rams due to differences in concentration of circulating testosterone.
4. An optimal regime for Immunocastration of male lambs will extend the period of immunity and reduce the expression of sexual behaviours during this period.

Chapter 5 Hypothesis

1. Physically castrated males and ewe lambs will show greater fearfulness in standard tests compared to entire males and immunocastrated males will be intermediate.
2. Immunocastration of male lambs reduces the expression of aggression during the period of immunity.
3. Early post-natal pain experience and manipulation of circulating testosterone alters lamb behavioural development, particularly ewe-lamb bonding and anxiety behaviour.
4. Seven month old male lambs immunized against GnRH (accordingly to agreed protocols at 6 and 12; 6, 12 and 22; 10 and 16; 10 and 20; 12 and 18; 12 and 22 weeks of age) show agonistic behaviour comparable to a physically castrated male lamb (using rubber rings), and less aggressive behaviour than entire male lambs of a similar age.
5. Male lambs that have been made cryptorchids will show intermediate aggressive behaviour.

Chapter 6 Hypothesis

1. Carcass conformation and quality may be different in rams castrated with different castration techniques.
2. Immunocastration improves carcass conformation and meat quality in immunized animals in comparison to physically castrated and control males.
3. Growth rates may be different in rams castrated with different castration techniques in comparison to entire male lambs.

Chapter 2 General Materials and Methods

2.1 Introduction

In this chapter general materials and methods implemented to collect data for this thesis will be presented. Sheep husbandry and management practices, selection of animals as well as ethical review, risk assessments and biosecurity protocols and "the end point" procedure will be mentioned. Blinding of the videos, inter-observer reliability, overview of how experimental work have spanned over the course of the study and selection of statistical methods will be also discussed. Specific protocols used to gather physiological measures like: growth rates, assessment of lesion or injection sites of lambs subjected to both traditional and alternative methods of lamb castration , blood sampling techniques (for levels of testosterone to determine efficacy of the vaccine in reduction of fertility and for cortisol concentration to determine levels of anxiety in a challenging situation), immunization procedures, testosterone concentration analysis as well as scrotal circumference, testes consistency, assessment of testicular histology and ewes' induction into oestrus state will not be discussed here as they will be presented separately in subsequent chapters.

2.2 Ethical review, risk assessments and biosecurity protocol

The study was conducted at SRUC Woodhouselee farm in Edinburgh, Scotland. Data was collected from April 2011 until April 2014. All of the techniques used to collect data described in this thesis were performed under the Home Office Licence (Ref no 60/4081) and approved by the Scotland's Rural College Animal Experiments and Ethics Committee. The flocks were managed in accordance with the UK regulations on animal care and ethics of experimental animals' use. Risk assessments were considered for each experimental protocol and implemented during the course of the study. In case of any suffering, distress, injury or lasting harm there were procedures in place allowing for immediate action in such events. Veterinary care and attention was available at all times. Lambs and ewes' health were checked on a daily basis by the shepherd. The flocks were also monitored by myself and NACWO (Named Animal Care and Welfare Officer) once a week. NVS (Named Veterinary Surgeon) inspections were conducted regularly as well. In the event of lamb or ewe loss the post-mortem examination was carried out at SRUC Veterinary Investigation Centre and the post mortem report was presented to NVS, NACWO, the project manager and the project licence holder.

Risk assessment was carried out and implemented by Zoetis prior to vaccine administration. The operator was obligated to follow all recommendations with regard to spillage of the product on the hands or self-injection safeguarding the health and safety of the operator. In the event of accidental self-injection, needle stick-injury or spillage of the product on the hands it was recommended to wash out hands immediately and seek medical advice. Vaccination was carried out only with use of the safety auto vaccinator (® Simcro Limited) provided by Zoetis to minimize the risk of self-injection.

The manufacturer's instructions were applied with regard to health and safety during ewes' induction into oestrus with Chronogest CR 20 mg vaginal sponge for sheep (MSD Animal Health®).

Protective clothing, waterproof clothing and rigger boots were worn each time during lab work and animal handling. Farm-work protective clothing was not allowed to be worn at any other SRUC farm unit or other type of farm-work minimizing risk of cross

contamination of the Woodhouselee farm staff and animals with infectious agents. Boots were cleaned and disinfected every time before and after animal handling and other activities (i.e. behavioural observations on the fields) by dipping in a Blitz solution (Dawnland Partners in Farming Health®). The Blitz solution was prepared and used accordingly to manufacturer instructions. Hands were cleaned and disinfected with antimicrobial skin cleanser HIBISRUB® (Regent Medical Overseas Limited®) with 4% Chlorhexidine gluconate as an active agent before and after animal handling accordingly to manufacturer's instructions. Disposable gloves were used during all lab procedures, blood sampling, immunization procedure and ewe induction into oestrus. All equipment was cleaned and disinfected where necessary before and after use. Eating and drinking was not permitted in the animal handling areas and was restricted to a designated area only. All of the approved protocols were adhered to, safeguarding the health and safety of handlers and animals.

2.3 General husbandry procedures

2.3.1 Animals

In total 256 (Mule x terminal sire Texel or Suffolk) lambs were used in the study. Mule is a cross breed between a lowland ram like Bluefaced Leicester and a pedigree upland or hill ewe like Scottish Blackface. Breeding of mule ewes is widely favoured by the farmers, because of the characteristics of such ewe. Cross breeding brings together the best features from both breeds giving farmers a lot of advantages in their stock productivity. The Scottish Blackface is a breed with a high maternal instinct. However, it produces usually only one lamb due to extreme hill conditions. The Blueface Leicester on the other hand is a very fertile breed giving 1-3 lambs at lambing. Dams are also capable of sufficient milk production to rear this number of lambs. So it was only natural for the farmers to crossbreed both breeds to create ewes with a good number and size of offspring, high maternal instinct and substantial milk production. Mule ewes are usually cross bred afterwards with Suffolk or Texel sires which are meat type breeds. This practice is performed to give high quality market lambs desired by stakeholders.

2.3.2 Housing and Management

The physiological data collection and all of the behavioural observations and recordings for this thesis were conducted at SRUC Woodhouselee farm in Edinburgh, Scotland. Woodhouselee farm is a lowland type farm where ewes are housed inside for lambing and stay at pasture from April until late autumn. This kind of management is called a "shed lambing system". Lambs are born in a shed which gives the shepherd more control over a flock and a safe, weather independent, predator free environment for ewes and lambs. Each year prior to parturition ewes were gathered around January and housed in one of the sheds at Woodhouselee farm. Overall construction, design and maintenance of sheds follows appropriate protocols based on The Welfare of Farmed Animals (Scotland) Regulations 2000. Structures and activity safeguard animal welfare (consideration of adverse weather protection, adequate ventilation, suitable pen conditions, bedding, water, food provision, group segregation and stocking densities). There is an effective identification system in place to spot visible signs of illness, suffering or distress. Prompt action is taken to relieve pain and suffering where this is required. The following regulations were applied with regard to indoor housing facilities based on the Schedule 1 of the Welfare of Farmed Animals (Scotland) Regulations 2000.

Sheep had access to a lying area which was well maintained with dry bedding and well-drained (Schedule 1, paragraph 4). Sick or injured animals were immediately separated and placed in the isolation pen with dry comfortable bedding (Schedule 1, paragraph 6). The animals had a freedom of movement appropriate to their species and they were not restricted in any way as to cause them unnecessary suffering or injury (Schedule 1, paragraph 9). The space given to the animals was appropriate to their physiological needs in accordance with established experience and scientific knowledge (Schedule 1, paragraph 10). Air circulation, dust levels, temperature, relative air humidity and gas concentrations were kept within limits which were not harmful to the animals (Schedule 1, paragraph 13). Materials used for the construction of accommodation, and, in particular, for the construction of pens and equipment with which the animals may have come into contact, were not harmful to them and were capable of being thoroughly

cleaned and disinfected. There were no sharp edges or protrusions likely to cause injury (Schedule 1, paragraphs 11 and 12). Adequate lighting was available to enable inspection of sheep at any time (Schedule 1, paragraph 3). Animals were not kept in darkness and the natural light was available in buildings during the day. Artificial lightning was also available. Sheep were also given an appropriate period of rest from artificial lighting (Schedule 1, paragraphs 14-16).

Each shed at Woodhouselee farm could have been used as an experimental or commercial shed. As there were few of them and they did differ slightly between each other the number from 1 to 5 was allocated to each of the sheds so the location of particular animals was easy to trace. The following sheds were used in this study: shed no 1 with dimensions of approximately 18 x 50 metres, shed no 2 (18 x 35 metres), shed no 3 (18 x 45 metres), and shed no 5 (18 x 45 metres).

Shed no 2 was equipped with concrete floors and passageways. Sheds no 1, 3 and 5 also had concrete passageways but the floors were made of soil. Deep straw bedding was used to house sheep in all facilities regardless of flooring type.

All sheds were equipped with automatic drinkers and portable gates which could be adjusted allowing for construction of appropriately sized pens with the required space allowance for a single animal. The general rule was to give at least 1.3 square metres (for lambs <35kg) and 1.9 square metres (animals >35 kg) of floor space for a single sheep housed in a group of animals.

Ewes were ultrasound scanned between the 70th – 80th day of gestation to determine the number of offspring they will produce and to allocate them to appropriate groups (single, twin, triplet etc.) for pre-partum management. This allowed for facilitation of housing according to specific needs (more space was given to ewes expecting multiple offspring). During the lambing period ewes that were expected to lamb within 10 days were moved to shed no 5. The shed had 5 large straw bedded pens and concrete passageways. A general stocking density rule of 1.9 square metres was also applied here. The pens could accommodate 20-30 ewes. Every day new dry straw was added to each

pen to make sure ewes had a nice dry lying area. Lambs were born in large pens and moved to small 1.5x1.5m pen units together with dam and all siblings thereafter for individual housing. Small pens were constructed alongside holding pens for pregnant ewes in passageways. They were equipped with deep straw bedding, a bucket with water and a feeder. Water and hay were offered ad libitum. Ewes were also fed 0.5 kg per ewe 2 x a day of concentrated feed: XL Ewe 18% + Amino Green® Rolls (®East Coast Viners Animal Nutrition), with a high level of molassed sugar beet pulp for palatability, Soya for quality protein, 150 iu/kg vitamin E, and 0.5mg/kg selenium. Feed contains: 4.1 oil, 18 protein, 6.4 fibre and 7.3 ash, traces of Manganese, Cobalt, Iodine and Zinc crucial for good health, increased milk yield, better lactation and productivity of ewes. Dams and their lambs were closely monitored and when there were no concerns with regard to lambs' health (around 12-48 h after birth) lambs were allocated to specific treatment groups of this study.

2.3.3 Treatments

Lambs were treated as described below under agreed earlier specific protocols approved by the SRUC Animal Experiments and Ethics Committee.

Throughout the whole study animals were allocated to treatment groups balanced for litter size, maternal parity and sire breed when lambs were aged between 12 and 48 hours (see table 1 for more details). After allocation to a specific group, treatments were applied to the lambs as per agreed procedures (description of particular treatments and technique of application will be explained in relevant chapters). Control lambs were handled (this involved picking up a lamb from the ground and handling it in a manner mimicking physical castration without application of the rubber ring). Lambs from treatments requiring physical castration were castrated with or without anaesthesia depending on the protocol. Lambs from the immunocastration treatment and female siblings were untreated at this point.

Table 1 Allocation to treatment groups

Legend:

Birth litter: 1 – Single lamb, 2 – Twins, 3 – Triplets etc.

Rearing litter: 1 – Single lamb, 2 - Twins

Dam Parity: * - unknown, 0 - First lambing. 1 - Second lambing, 2 – Third lambing etc.

Sire: * - unknown, T – Texel, S – Suffolk

Group	**n**	**Lamb sex**	**Birth litter**	**Rearing litter**	**dam parity**	**sire breed**
Study of 2011						
Control	20	M	1 = 3 2 = 13 3 = 4	1 = 4 2 = 16	0 = 6, 1 = 2, 2 = 4 3 = 2 , 4 = 2, * = 4	T = 11 S = 9
Rubber Ring	20	M	1 = 3 2 = 14 3 = 4	1 = 3 2 = 17	0 = 7, 1 = 5, 2 = 4 * = 4	T = 13 S = 7
Short Scrotum	20	M	1 = 3 2 = 13 3 = 4	1 = 3 2 = 17	0 = 9, 1 = 1, 2 = 8 3 = 1, * = 1	T = 12 S = 8
Combined	20	M	1 = 3 2 = 13 3 = 4	1 = 3 2 = 17	0 = 8, 1 = 3, 2 = 5, 4 = 4	T = 11 S = 9
Immunocastration	20	M	1 = 3 2 = 13 3 = 4	1= 3 2= 17	0 = 7, 1 =, 5 2 = 2 4= 1, *= 5	T = 13 S = 7
Study of 2012						
Control	12	M	2 = 12	2 = 12	0 = 4, 1 = 3, 2 = 1 4 = 2, * = 2	T = 3 S = 9

Rubber Ring	12	M	2 = 12 3 = 2	2 = 12	0 = 3, 1 = 2, 2 = 2 4 = 1, 5 = 1, * = 3	T = 5 S = 6, *=1
Local Anaesthesia	12	M	2 = 12 3 = 2	2 = 12	0 = 3, 1 = 5, 2 = 3 4 = 1	T = 4 S = 8
Female	24	F	2 = 24 3 = 2	2 = 24	0 = 7, 1 = 4, 2 = 6 4 = 4, 5 = 1, * = 2	T = 11 S= 12,*=1
Immunocastration	12	M	2 = 12 3 = 2	2 = 12	0 = 3, 1 = 4, 2 = 4, * = 1	T = 3 S = 9
Study of 2013						
Control	14	M	2 = 12 3 = 2	2 = 14	0 = 6, 1 = 2, 2 = 4 3 = 2	T = 5 S = 9
Rubber Ring	14	M	2 = 12 3 = 2	2 = 14	0 = 5, 1 = 1, 2 = 4 3 = 4	T = 4 S = 10
Immunocastration 1	14	M	2 = 12 3 = 2	2 = 14	0 = 4, 1 = 3, 2 = 3 3 = 4	T = 5 S = 9
Immunocastration 2	14	M	2 = 12 3 = 2	2 = 14	0 = 4, 1 = 1, 2 = 7 3 = 2	T = 5 S = 9
Immunocastration 3	14	M	2 = 12 3 = 2	2 = 14	0 = 4, 1 = 2, 2 = 5	T = 7 S = 7

					3 = 3	
Immunocastration 4	14	M	2 = 12 3 = 2	2 = 14	0 = 3, 1 = 3, 2 = 5 3 = 3	T = 6 S = 8

The Immunocastration group was vaccinated thereafter to the appropriate procedures (see Chapter 4 for details). At a minimum of 2 hours after physical castration or handling lambs and their siblings were weighed, ear tagged with electronic identification tags Shearwell Data® (Animal Identification & Management Systems), and tail docked with rubber rings following administration of local anaesthesia. Lambs were also individually marked with use of sheep spray allowing for their traceability on the pasture for the general health management purposes as well as behavioural observations if needed.

In the first year of the study 2% lidocaine with adrenaline (Norbrook® 2.0% w/v Solution for Injection) was used as an anaesthetic agent. 1 ml was applied by needleless injector. In the subsequent years diluted "anaesthetic solution" of 2% Lidocaine (Lidocaine 100mg/5ml (2%) solution for injection Alliance Healthcare (Distribution) Ltd®) and 0.5% bupivacaine (Marcaine 0.5% AMPS 10x10ml AstraZeneca UK Limited®) was prepared and administered subcutaneously (detailed preparation and dose of the anaesthetic solution will be described later in this chapter). The day following treatments, lambs and their mothers were moved out to graze in a paddock. Each year due to the anthelminthic control system and rotation of fields' management strategy lambs were moved to graze on a different field. The general rule was to place 15 ewes with their offspring on a pasture of the size of at least 1 hectare (which is 10.000 square metres). In this way the field was sufficient enough in grass to sustain all of the sheep's nutrition needs. On the first year of study the field which was used for grazing was the size of 5 ha, on the second year of study 6 ha and on the third year 5 ha. Each pasture was equipped with through drinkers and portable floor standing feeding troughs as ewes were given supplementation feeds (details described below).

In a situation when lamb health did not allow for movement onto the paddock, the dam and its offspring were kept in a shed for additional time until transfer to the appropriate field took place. This happened on a few occasions when lambs had problems with suckling and there was a concern that their weight might drop dramatically when they were transferred to the pasture. Some of the ewes and lambs were removed from the study before any testing or behavioural observations took place and were replaced by a different set of animals. This happened on one occasion on the third year of the thesis during the time of allocation to specific treatments. Two of the selected ewes could not provide enough milk to feed both siblings on the pasture. As I was interested in twin pairs only and the selection of lambs had not finished yet those two ewes were removed from the study and replaced by additional animals.

One of the ewes was also replaced because one of its rams was discovered to be cryptochorid (its testicles did not descend therefore it could not be used in the tests crucial for this thesis: scrotal measures, testosterone concentration measures, and expression of sexual behaviours).

Ewes stayed on the pasture with their offspring until weaning time (3 - 4.5 months of age depending on the year of the study). Within that time ewes were given additional feed and supplements XL Ewe 18% + Amino Green® Rolls (®East Coast Viners Animal Nutrition 1/3 kg per ewe once a day until weaning) so all of the nutritional needs could have been sustained while suckling lambs. After weaning ewes were moved to different pasture so there could be no further contact between ewes and their lambs.

In addition to the specific requirements of the project protocols, lambs and ewes were also gathered for standard sheep husbandry practices e.g.: vaccinations, anthelminthic treatment, health checks, shearing etc. following farm health plan separately from the study protocols.

2.3.4 Weaning

The date of the weaning depended on the experimental design and specific study protocols. On the day of the weaning lambs were gathered in the weighting pens area

together with their mothers and sibling and manually separated from the ewes. Dams were placed on a different pasture to prevent further contact between them and their offspring. The time of weaning depended on the study design in a particular year of this thesis.

In 2011 lambs were weaned at approximately 3.5 months of age, Female siblings and the combined castration group were removed at that time from the flock.

In 2012 lambs were weaned at approximately 3.5 months of age

In 2013 lambs were weaned at 3 months of age.

2.3.5 The end point

Every year in autumn (September) lambs were moved from pasture into straw-bedded pens in a shed and stayed in the shed until the end of experimental procedures as per agreed protocols. Pens were made up of portable gates so appropriate sticking density could have been achieved. Again the agreed earlier rule of at least 1.3 and 1.9 square metres of floor space for a single sheep was applied. Pens could accommodate 20-30 sheep. Lambs were spread across 4 pens in more or less equal in numbers groups. Lambs were fed concentrate feed - Lamb Finisher (®East Coast Viners Animal Nutrition) ad libitum. The feed consists of: citrus pulp for desired sugar content and palatability, 4.4 oil, 15 protein, 7.6 fibre, 8.3 ash, 30 iu/kg vitamin E, 0.3 mg/kg selenium traces of Manganese, Cobalt, Iodine and Zinc essential for good health, productivity and carcass conformation of lambs. Water and hay were present ad libitum as well.

Each year at the end of a specific study lambs were slaughtered or euthanized. Lambs that were fit for human consumption (control rams and rams from physically castrated groups) went through the slaughter process (at ScotBeef abattoir Stirlingshire, Scotland). Immunocastrated lambs were euthanized on the Woodhouselee farm with an overdose of Euthatal (Merial Animal Health Ltd®, solution for injection 200mg in 1ml, active ingredient - Pentobarbital Sodium) in years 1 and 2 of this thesis. The Euthatal was administered by the NACWO in the presence of NVS intravenously (into the jugular vein, approximately 150 mg/kg bodyweight) as rapidly as possible. In the event of

individual resistance to the drug the same amount given by the intravenous route was administered directly to the lamb's heart (this happened on only one occasion on the second year of the study). On year 3 of the study (December 2013) all lambs went through a commercial slaughter process to enable a meat quality trial. Lambs were transported to the Bio Support Unit at the University of Nottingham, Sutton Bonington Campus where appropriate facilities were available. Transport of lambs to slaughter was widely discussed and approved by the SRUC Animal Experiments and Ethics Committee, SRUC as well as the University of Nottingham NVS and a Home Office Officer. Appropriate protocols were put in place with regard to Public Health and safety as well as waste disposal. Immunocastrated lambs did not enter the food chain but were killed and dressed in the same manner so the comparative analysis could have been performed. Rams were monitored throughout transport and housing at the Sutton Bonington Campus to ensure that the best code of practice was adhered to. The details of rams' housing at the University of Nottingham and the slaughter process will be discussed in Chapter 6.

2.4 Overview of how the experimental work covered the period of whole study

Tables 2-4 below represent how experimental data collection spanned over the period of the study. Only experimental data collection is shown in tables 2-4. Overview of how the analysis of collected data was carried out is not included. The Project has started in April 2011. I have joined the project in September 2011. All of the measures (video recording for the assessment of pain related behaviours and postures following different castration methods; severity of the lesions and reaction site to the immunization scoring; immunization procedure; testosterone concentration, scrotal circumference and testes consistency, growth rate measurements) taken before September 2011 (First year of thesis marked in yellow in table 2 below) were carried out by Prof Cathy Dwyer and the technical team at SRUC.

Preparation of video clips for QBA study was carried out by me. I selected appropriate time thresholds for the study and prepared video clips from videos collected for pain measurements analysis (mentioned above) in video movie maker software. The order of the Free choice profiling video-clips and the video-clips shown to observers for the purpose of terms generation were also prepared by me. Free choice profiling and data analysis was carried out by MSc student Fabiana Mizzoni and Prof Francoise Wemelsfelder.

Legend:

- **Study 1 - Assessment of different castration techniques and their impact on the welfare of male lambs**

a) Collection of video recordings of expressed behaviours and postures after handling, physical castration and immunization procedure (April 2011, videos software transformation and analysis was carried out January 2012-April2012)
b) Assessment of castration lesions and site reaction to vaccination (April 2011-September 2011; April 2012-September 2012)
c) Evaluation of QBA as a new technique to assess pain in castrated lambs (February 2013-April 2013, Free choice profiling and analysis April 2013- August 2013)

- **Study 2 - The effect of immunization against gonadotropin-releasing factor (GnRF) on circulating testosterone, histology of the testes and the development of sexual behaviour in ram lambs**

d) Immunization procedure (study conducted accordingly to agreed protocols; May and June 2011, May, June, September 2012, June, July, August 2013.
e) Scrotal circumference and testes consistency measures (May 2011-December 2011, May 2012-December 2012, May 2013-December 2013)
f) Testosterone concentration analysis (May 2011-December 2011, May 2012-December 2012, May 2013-December 2013)
g) Analysis of the testes histology and testicular measures after slaughter (December 2011 and 2012)
h) Assessment of expression of male sexual and courtship behaviours (November 2011,2012 and 2013)
i) Assessment of ewes' perception of rams form different treatment groups (November 2011, 2012)

- **Study 3 - Influence of castration method and sex on lamb behaviour, the development of ewe-lamb bond and stress responses**

j) Assessment of mother-lamb bonding behaviour (April-July 2012)
k) Anxiety fear tests (August 2012)
l) Plasma cortisol concentration measurements (August 2012)
m) Assessments of aggressive behaviours (October 2012)

- **Study 4 - The effects of immunization with novel anti GnRF vaccine developed by Zoetis on aggression, growth rate, carcass and meat quality characteristics in ram lambs**

n) Assessment of growth rate (April 2011-December 2011, April 2012-December 2012, April 2013-December 2013)
o) Assessment of aggressive behaviours expression (October 2013)
p) Assessment of carcase conformation and meat quality characteristics (December 2013)
q) CT scanning analysis (October 2011)

Table 2 Overview of how the experimental work spanned during the first year of the thesis

First year of Thesis	Lambs age in weeks																																	
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
Month	April					May				June				July				August				September				October				November				December		
Study 1																																				
a																																				
b																																				
Study 2																																				
d																																				
e																																				
f																																				
g																														Pilot						
h																														Study						
i																																				
Study 3																																				
m																									Pilot study											
Study 4																																				
n																																				
q																																				

Table 3 Overview of how the experimental work spanned during the second year of the thesis

Second year of Thesis	Lambs age in weeks																																
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
Month	April					May				June				July				August				September				October				November				December		
Study 1																																				
b																																				
Study 2																																				
d																																				
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g																																				
h																																				
i																																				
Study 3																																				
j																																				
k																																				
l																																				
m																																				
Study 4																																				
n																																				

Table 4 Overview of how the experimental work spanned during the third year of the thesis

Third year of Thesis	Lambs age in weeks																																
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
Month	April					May				June				July				August				September				October				November				December		
Study 1																																				
b																																				
Study 2																																				
d																																				
e																																				
f																																				
h																																				
Study 4																																				
n																																				
o																																				
p																																				

2.6 Blinding of the videos for the behavioural analysis

All of the behavioural data videos were recorded prior to scoring in the Observer program.

Pain related behavioural study was carried out before the thesis has started. The observer was given video-clips to score. Each lamb was identified by an individual number. The video-clips were scored with use of those individual numbers. The treatment groups which each lamb belonged to, were not revealed until behavioural scoring in the Observer program has finished. Thus the observer was blind to treatment for this study.

The study of aggressive, courtship and sexual behaviours as well as ewes' perception of entire and/or castrated/immunized males was carried out by the observer throughout the period of the whole thesis (please see table 2 for more details). Video-clips were scored in the Observer program. Each lamb/ewe was identified by the individual number. Although observer did carry out recordings of the particular behaviours because they were scored later on during the study it was impossible for the observer to remember individual numbers of each lamb/ewe and correlate them with the treatment group. There was however exceptions related to very unique coat markings of three individuals. During the scoring observer was aware to which treatment group those three individuals were allocated. The analysis of the final results was carried out with and without data collected for these specific individuals. There was no influence on the p-value therefore all of the data was included in the final analysis.

Similarly, to the study described above assessment of anxiety behaviours was carried out by the observer and analysed thereafter. Same three individuals were apparent and the observer was aware of their allocation to the treatment groups. However due to unaffected p-value all data has been included in the final statistical analysis.

Ewe-lamb bonding assessment was carried out by the single observer in the 3 periods by scan sampling. Lambs and their dams were identified by individual numbers. The observer was unaware of the treatment allocation of particular lambs during the scoring.

2.7 Inter-observer reliability

All of the behavioural analysis was carried out by a single observer. Inter observer reliability was carried out for each behavioural study. Randomly selected videos were re-scored at the end of each particular data analysis. Received data was then compared together to see how good was the observer in scoring particular behaviours. Percentage of agreement was calculated. The outcome was perceived as satisfactory if 90% of agreement was achieved by the observer. No statistical analysis has been carried out.

During the course of the study observer has achieved 90% of agreement in all behavioural studies. Before each behavioural scoring have commenced the observer spent 5 days to practise scoring of particular behaviours. This scoring was carried out on the randomly selected video clips across all treatments to allow the observer to familiarise herself with the range of behaviours expressed by the representative lambs from each treatment group.

2.8 Choice of statistical methods

Before statistical analyses have been carried out all data were checked to determine its characteristics. Anderson-Darling test was carried out in Minitab statistical package 16th edition (Minitab, Inc., State Collage, PA) to determine normality of data distribution. Anderson Darling test determines whether or not tested sample of data is drawn from a particular likelihood distribution.

In the event when the analysed data was not normally distributed there was an attempt to normalize data by square root and/or log transformation. If the data was found impossible to be transformed non-parametric statistical tests were used during analysis.

Careful consideration was given prior to choosing an appropriate statistical test to analyse the data. There were two types of data in this thesis parametric and non-parametric. For the parametric data ANOVA and REML analysis of variance were considered. When the data is balanced REML uses the analysis of variance facilities.

When there is no blocking in the design (i.e. there is only one random term) REML uses the GenStat regression facilities. Additionally, random effects are accounted for.

In this thesis collected data contained fixed factors for example, sire breed, ewe parturition, birth weight etc. and random factors such as repeated measurements of particular parameter over a period of time which was always collected under different circumstances (i.e. weather conditions; different staff helping in the collection of blood, weight measures, testes consistency and scrotal circumference scoring) that needed to be accounted for at the time of analysis. Therefore, REML analysis of variance was selected as appropriate test to find statistical significance between treatment groups during the course of the study.

For the non-parametric data, Kruskal-Wallis test (alternative to a one-way ANOVA and an extension of the Mann-Whitney test to allow the comparison of more than two independent groups.) was considered to determine whether the medians of two or more groups differ from each other. The test is used when the data is not symmetric, for instance skewed data. This test does not require the data to be normally distributed. It uses the rank of the data values instead of the actual data values for the analysis. In this thesis most of the data collected during the course of the study was non-parametric and skewed. Therefore, it was decided that data concerning only 2 experimental groups (i.e. analysis of the impact of immunocastration on the expression of pain related behaviours and restlessness) was analysed with use of Mann-Whitney test. Non-parametric data concerning three or more treatments was analysed with use of Kruskal-Wallis test (i.e. the impact of different castration method on the expresion of pain related behaviours and postures, development and expression of antagonistic behaviours, occurrence of courtship and mating behaviours etc.). Appropriate post-hoc analysis was carried out to determine significant differences between treatments (see below).

2.8.1 Selection of statistical tests used in the thesis to analyse data

Normally distributed data (i.e. time to shed testicles, live weight before slaughter, testes consistency, scrotal circumference etc.) was analysed with use of REML (Restricted or residual Maximum Likelihood Mixed Model, GenStat® 14th edition, Lawes

Agricultural Trust, VSN International Ltd, Oxford, UK) which is a form of maximum likelihood estimation. REML doesn't base estimates on a maximum likelihood fit of all the information, but on a likelihood function calculated from a transformed set of data. The birth weight, dam parity, sire breed and the litter size were taken into account during analysis as random effect factors. Where REML suggested that between treatments differences were significant two sample T-tests were carried out to determine significant differences between treatment groups. Each experimental chapter explains in more detail how data was analysed.

Weight gain in growth rate measurement was analysed with use of Repeated Measures tests in Genstat 14th edition Genstat (GenStat® 14th edition, Lawes Agricultural Trust, VSN International Ltd, Oxford, UK. Repeated measures test can be used when a random sample (monthly weight gain in this study measured through the period of the whole thesis ~ 3 years) measured under a number of different conditions. Because the sample was exposed to each condition in turn, the measurement of the dependent variable is repeated. Two sample T-tests were carried out to determine significant differences between treatment groups.

Correlation between plasma testosterone levels and expression of courtship and sexual behaviours were analysed by use of Spearman Rank Correlation test in Minitab statistical package 16th edition (Minitab, Inc., State Collage, PA). The test evaluates how well the association between two variables can be defined using a monotonic function. The sign "+" or "-" of the Spearman correlation indicates the direction of association. This is most commonly descried as positive or negative Spearman Rank Correlation. The Spearman correlation increases in magnitude as X and Y variables become closer to being perfect monotone functions of each other. When X and Y are perfectly monotonically related, the Spearman correlation coefficient becomes 1. In this study the cut off point for the correlation result to be regarded as meaningful was 0.4. Correlations between 0.4-0.65 were regarded as moderate, while correlations higher than 0.65 were regarded as strong.

Non-parametric data was analysed with use of Kruskal-Wallis and Mann-Whitney non-parametric tests (GenStat® 14th edition, Lawes Agricultural Trust, VSN International Ltd, Oxford, UK) to determine significant differences between treatment groups.

A Bonferroni correction was applied to account for the multiple testing or multiple comparisons. The new critical p-value for each specific experiment was indicated in the result section for each experiment. The problem of multiple testing arises from the fact that with increased number of hypotheses that are being tested, there is also an increase in the likelihood of a rare event, and therefore, the likelihood of incorrectly rejecting a null hypothesis (i.e. Type I error). The basis of Bonferroni correction is correcting for the error rate by testing each individual hypothesis at a statistical significance level of 1 / number of tested hypotheses x times the desired maximum overall level. For example, if a trial is testing 8 hypotheses (i.e. there are 8 treatment groups) with a desired p-value = 0.05, then the Bonferroni correction would test each individual hypothesis at $p = 0.05 / 8 = 0.00625$. Note that statistically significant simply means that a given result is unlikely to occur if the null hypothesis is true (i.e., no difference among groups, no effect of treatment, no relation among variables). In this thesis new amended p-value was given as appropriate in the statistical section of each experimental chapter and in the each figure or table that was presented.

Statistical Analysis of QBA technique was performed by Prof Francoise Wemelsfelder and MSc student Fabiana Mizzoni. To analyse these data a Generalized Procrustes Analysis (GPA) was used in Minitab statistical package 16th edition (Minitab, Inc., State Collage, PA). This method is explained in more detail in chapter 3. Detailed description was based on paper by Wemelsfelder et al., (2001).

One-way analysis of variance was carried out to determine whether dimension scores were significantly affected by treatment. Tukey post-hoc tests were carried out thereafter on both dimensions to investigate where any significant differences were.

Correlation of Quantitative and Qualitative pain assessment was analysed with use of Spearman Rank Correlation test. The test was used to investigate the correlation between

GPA scores (dim1 and dim2) and the quantitative scores (expressed duration and frequency of restlessness, pain related behaviours and postures) for each lamb.

Principal component analysis (PCA, correlation matrix, no rotation) was carried out to assess patterns of correlation between quantitative and qualitative (QBA) assessments (please see chapter 3 for more details). Dimension results were not normally distributed; therefore Kruskal-Wallis and Mann-Whitney tests were used to determine significant differences between treatment groups on the main components of the combined PCA.

Chapter 3 Assessment of different castration techniques and their impact on the welfare of male lambs

Abstract

Castration of male lambs is raising a lot of public concern due to its distressing nature. In this study investigation of how different methods of castration affected lamb pain behaviours was carried out to assess which of the techniques would be less painful. One hundred 2-day-old male Mule (Scottish Blackface x Bluefaced Leicester) x Suffolk or Texel lambs were allocated to one of 5 groups (n=20 per treatment): handled only (control, C), castrated using conventional rubber rings (RR), short-scrotum castration (SSC) and rubber rings castration combined with Burdizzo (COM) and immunocastration group (VAC – vaccinated at 6 weeks of age by s.c. injection in the neck). The duration and frequency of lamb behaviours were recorded continuously for 30 minutes after the procedure for C, RR, SSC, and COM group as well as for 15 minutes for VAC and C after the primary vaccination was administered. Recorded behaviours were focussed on measures believed to be related to the expression of acute pain like: foot stamping/kicking, easing quarters, wagging tail, head turning, trembling, dog sitting, abnormal standing, abnormal lying, lateral lying etc. Kruskal-Wallis and Mann-Whitney tests were used to determine significant differences between treatment groups. RR and SSC lambs showed greater active pain behaviours (summation of foot stamping/kicking, wagging tail, head turning, easing quarters, trembling, dog sitting, median frequency: C=24.0; RR=110.5, COM=25.0, SSC=97.0, P< 0.001), and restlessness (median frequency of postural changes: C=9.5, RR=33.0, COM=10.0, SSC=22.0, P<0.001), than C or COM lambs. There were no significant differences between C and VAC group in expressed pain related behaviours at the time of primary vaccination with the exception of restlessness (median frequency C=1.5; VAC=2, P=0.038). The implementation of Qualitative Behavioural Assessment (QBA) as a new technique for pain recognition was also investigated. It was hypothesized that QBA will be able to find differences between treatment groups. Two-minute-long video clips were extracted from the footage recorded for 30 min for 79 lambs following castration. Nine blinded (to the applied treatments) observers provided QBA analysis. A Free Choice Profiling (FCP) approach was used to assess lamb expressive demeanour and Generalized Procrustes Analysis (GPA) to calculate a consensus profile providing two

main dimensions of behavioural expression. The GPA consensus profile explained 65.12% of the variation in observer scoring patterns, which differed significantly from the mean randomised profile (27.81+0.13%, t99=103.16, P<0.001). Two main dimensions of behavioural expression accounted for the 80.6% of the variation between lambs (44.8 % of the variation is accounted for dimension 1 and 35.8 % for dimension 2). Dimension 1 ranged from 'Restless'/'Painful' to 'Comfortable'/'Calm' and Dimension 2 from 'Lethargic'/'Tired' to 'Interested'/'Curious'. RR treatment was correlated with more negative behavioural expression (high negative scores on Dimension 1 showing more restless, painful, tense and agitated demeanour) in comparison to C treatment which was associated with more positive behavioural expression (higher scores for interested and curious demeanour on Dimension 2. QBA also evaluated the behavioural pattern of the COM group in more detail which was not possible with use of the quantitative scoring method. COM treatment was associated by observers with lethargic/tired expressions indicating that the observers identified that lambs from this treatment did not show restlessness but they have not associated this behaviour with positive welfare in contrast to C group lambs. Overall results have also indicated that there was a moderate to strong correlation between quantitative and qualitative (QBA) measures of pain related behavioural expression of rams following castration (several meaningful and significant moderate correlations between QBA dimensions and quantified physical behaviours were observed). In conclusion different castration techniques were associated with different expressive behavioural responses in lambs, with the RR method being identified as the most painful. Furthermore, this study supports the use of QBA as a pain recognition and assessment method in castrated lambs.

Key Words: Animal Welfare, Castration, Pain, Immunization, QBA, Pain assessment, GnRF

3.1 Introduction

Castration of male lambs is commonly practised in lamb production systems to avoid indiscriminate breeding, undesirable behaviours such as aggression, fighting or reproductive behaviours. It is also believed that castration may improve carcass characteristics and quality which is very important due to increased demand for leaner meat. However, it has been proven that castration may be extremely distressing therefore alternatives to this procedure are still one of the main interests of much research. Several methods are used by farmers to castrate male lambs in the pre-pubertal period to avoid difficulties in management and minimize unwanted behaviours (Godfrey et al., 1996). The most common method (accordingly to DEFRA farm survey 2005) used in the UK is rubber ring castration (elastration). By law it can be used up to one week of age (DEFRA Codes of Recommendations for Welfare of Sheep 2008). An elastic band is applied to the neck of scrotum, causing blockage of the venous drainage and arterial blood supply (Kent et al., 2004). When blood supply is blocked ischemia and necrosis of the tissues occurs in the affected area distal to the ring and the scrotum will detach 4-6 weeks after the procedure. Elastration is quick, easy to perform and economical (Archer et al., 2004). Nonetheless it inflicts pain and distress in castrated animals (Kent et al., 2004; Thornton & Waterman-Pearson, 2002) which may last for a few days after application (Thornton & Waterman-Pearson, 1999). There have been several trials to develop more welfare-friendly methods of castration. Attempts to apply other castration techniques like short scrotum castration (testicles are pushed back into abdominal cavity and the rubber ring is applied only on the scrotum sac), unilateral castration (one testicle is pushed back into the inguinal canal or region and the rubber ring is applied below the testis with one testicle remaining in the scrotum sac), Burdizzo castration (crush of spermatic cords with Burdizzo clamps) or combined method of castration (rubber ring technique followed by immediate crush of spermatic cords with Burdizzo clamps) were investigated. Use of medication in the mitigation of pain induced by castration was also studied, for example analgesia is able to minimize pain behaviours and postures (Wood et al., 1991). Administration of anaesthesia before castration can minimize pain responses (Molony et al., 1997) but it is not a practical technique to be performed on a

large scale in the farm environment (Price and Nolan, 2001). It is because it takes much more time to implement castration with the use of anaesthesia than i.e. single banding or Burdizzo as the operator is forced to wait at least few minutes after administration of anaesthetic before castration can be done to allow for the pain mitigation to be effective. It may also be too expensive as anaesthesia can be used under veterinary supervision only. Non-steroidal anti-inflammatory drugs like carprofen, meloxicam, ketocarprofen etc. are a very good technique of pain alleviation (Price and Nolan 2001; Fisher et al., 2007). They are also thought to be practical and easy to administer in the field setting but they are not licensed in the UK for sheep species therefore cannot be used (Price and Nolan, 2001). In the light of the issues related to traditional methods of castration, efforts to find alternatives to this painful procedure are important and relevant. Immunization against gonadotropin releasing factor (GnRF) is a potential alternative to traditional physical methods of castration in farm species which may be theoretically painless to animals. Several studies in other species (i.e. pigs, goats, cattle) have shown that active immunization against GnRF hormones reduces fertility, aggressive and sexual behaviours as well as male taint in meat. It is also believed that characteristics of the carcass in castrated or immunized animals in comparison to intact males is better, which is leading to stronger consumer acceptance (Kiyma et al., 2000; Thornton et al. 1999; Amatayakul-Chantler et al., 2012). However, there is little information on immunocastration in lambs, and also very little or no information on how the anti-GnRF vaccine influences the physical development of animals e.g. bone growth or muscularity. Behavioural development of lambs may be also interrupted due to vaccination and therefore expression of natural behaviours in immunized animals may vary from intact males so there is a need for further study.

The first part of this study was evaluation of different castration methods to assess which of the investigated techniques would be less painful or even pain free. Rubber ring, Short scrotum, Combined method of castration and immunization with novel anti-GnRF vaccine were assessed. The intention was to compare techniques which were believed to induce different amounts of acute and chronic pain and evaluate for the first time the

immunization method with the use of a new product developed by Zoetis. The focus was placed on the most possible pain reduction of acute and chronic pain and practicality of administration. For the purpose of this study acute pain was determined by measuring frequencies and durations expressed by lambs' postures and behaviours which were validated in the past. It has been shown that animals that had undergone castration show higher rates of behaviours and postures which may indicate that they are expressing pain such as: stamping, kicking, lateral lying with hind limbs extended, tail wagging, head turning (Molony et al., 1993; Kent et al., 1995; Kent et al., 2000; Lester et al., 1996). Painful stimuli can also invoke changes in skin resistance, peripheral blood flow and pupillary diameter. The heart rate and cortisol levels can be elevated as well (Molony and Kent, 1997). These changes have been observed in many castration studies together with behavioural changes. Molony et al. (2002) reported that behavioural parameters were the best indicators of pain related expression able to discriminate the different treatments associated with altered behavioural patterns. In this study only behavioural expression was assessed due to its practicality and perhaps lowest negative impact on health and emotionality of lambs as young as 2 days old. Chronic pain was assessed by the evaluation of length of time taken to completely heal the castration wound. A completely healed wound was observed when the skin on the post castration site was intact and there was no presence of swelling, reddening or residual scab. The size and severity of wounds induced by different castration techniques and site reaction to immunization was also measured.

The second part of this study was the evaluation of the Qualitative Behavioural Assessment (QBA) as a novel method for recognition of pain and distress in farm animals. QBA is a method first proposed by Wemelsfelder et al., (2001) then taken up by many others (Rutherford et al., 2012). This method was never used to assess pain in farm animals before. This study is the first attempt to use QBA in pain severity evaluation. In summary, QBA may give an overview of "whole animal" level of organization (Wemelsfelder et al., 2001). The foundation of QBA technique lies in defining animals' behaviour as an expressive process and describing it with the use of

behavioural descriptors such as “playful, confident, calm, anxious, nervous” etc. In this way different features of an animal dynamic can be captured. Throughout analysis of the animal dynamic there is possibility to understand how particular behaviours and postures are performed by specific individuals in their interaction with a specific environment or situation (Wemelsfelder et al., 2001). In this particular study lambs’ expressive behaviour was used to investigate the usefulness of QBA as an assessment tool for recognition of pain and distress in castrated lambs. It was hypothesized that QBA may be able to detect subtle expressive clues in the lambs’ behaviour, and therefore potentially serve as a powerful method for detecting emotional state of castrated lambs. In this way QBA may also help to interpret the emotional meaning of measured physical behaviours i.e. immobility performed by the lambs after the castration procedure was applied. The basis of this technique is the ability of human observers to judge and evaluate behavioural expression of castrated lambs (through scoring of their body language) and translate this judgement into vital information about their welfare. The observers used descriptors like tense, agitated, calm, relaxed, confident curious etc. to describe behavioural expression of castrated rams. Several studies in pigs and other species have shown that QBA was successfully implemented as a welfare assessment tool providing reliable information which was correlating to other measures (Rutherford et al., 2012; Napolitano et al., 2012; Fleming et al., 2013, Wemelsfelder and Mullan 2014). Rutherford et al. (2012) have reported that QBA was effective in the evaluation of emotional expression of pigs exposed to stressful situations. Blinded to the existence of experimental treatments, observers were able to detect changes in the behavioural expression between different treatment groups. It was shown that pigs which had undergone administration of a neuroleptic drug were more confident during the open field and elevated plus maze test in comparison to pigs that were given sham-control - saline. However, QBA has never been used before as a pain recognition and assessment technique for farm animals. Therefore, application of the QBA technique to rams which have undergone the traditional castration procedure may give a new insight into the emotional state of lambs and provide an alternative and practical tool to assess pain.

Chapter 3 Hypotheses

1. Pain behaviours and postures expressed by animals castrated with different castration techniques will vary in duration of time and frequency of occurrence.

2. QBA is able to distinguish between different castrations techniques, especially in the case when lambs do not show high frequencies of pain related behaviours after the castration procedure due to periods of immobility which is caused by severe pain sensation.

Chapter 3 Objectives

1. Evaluation of different castration methods to assess which of the investigated techniques would be less painful.
2. Assessment of the Qualitative Behavioural Assessment (QBA) as a novel method for recognition of pain and distress in farm animals.

3.2 Materials and methods

3.2.1 Animals, housing and management

Husbandry and management practices are described in detail in Chapter 2. After lambing lambs were moved into small pens (approx. 1.5 x 1.5 m) with their mother and siblings (if present – triplet lambs were reduced to 2 lambs prior to treatment). For male-male twins both lambs were assigned to the same treatment group, but treatments to litter mates were applied at least 2 hours apart. Pens were equipped with deep straw bedding. Water and hay were offered ad libitum. Ewes were also fed 0.5 kg per ewe (twice per day) concentrated feed: XL Ewe 18% + Amino Green® Rolls (East Coast Viners Animal Nutrition, UK).

20 (mule x terminal sire (Texel or Suffolk) lambs per treatment were used in the study. Twin, triplet and single born lambs were used (for details see section 2.3.3 and table 1 in Chapter 2). Five experimental groups were formed:

1. Positive controls (C) – lambs were handled only.

2. Negative controls (RR) – lambs were castrated using standard rubber rings at 24-48 h of age.

3. Short scrotum castration (SSC) – the testes were pushed back into the abdominal cavity and a standard rubber ring was applied around the scrotum only at 24-48 h of age.

4. Combined method (COM) – lambs were castrated by application of novel tight rubber rings (Molony et al., 2012) followed immediately by nerve crush using a Burdizzo proximal to the ring at 24-48 h of age.

5. Immunocastration (VAC) – lambs were vaccinated with an anti-GnRH vaccine developed by Zoetis at the age of 6 weeks.

3.2.2 Selection of the treatments

First of all, this thesis is investigating effectives of an anti-GnRF vaccine as a more welfare friendly method for castration of ram lambs. The main focus of the study was to evaluate how practical, economic and welfare friendly is the immunization in comparison to other methods commonly used by the farmers i.e. short scrotum, burdizzo or combined treatment which were already reported in the past to be cheap and less painful. The intention was to select particular treatment groups to provide the overview of most practical and economic castration options for the farmers. Factors like cost of application, practicality, time of application, impact on the welfare and economical return at the time of slaughter were taken into account before final selection of the treatments was decided. In summary easier management requires castration to be performed. Moreover, the method which is easy to apply, quick and economic would be preferred by the farmers. However some of the quick and economic methods of castration are often very painful and the methods that would be less distressing were found to be less economic and time consuming.

During the process of treatments selections following factors were considered:

1. Rubber ring castration

Advantages - quick, easy, cheap (box of 500 rings costs approximately 9.00 £), Disadvantages - slower weight gain, distressful nature

2. Short scrotum castration

Advantages - quick, easy, cheap

Disadvantages – distressful

3. Combined method of castration

Advantages - economic (cost of Burdizzo ~ 15.00£ - 155.00£ depending on manufacturer), less painful

Disadvantages - requires a skilled stockman, possible post application complications slower weight gain

4. Immunization against GnRF

Advantages - believed to be less painful, good weight gain, good feed conversion, improved meat quality

Disadvantages - possibly more expensive and more time consuming

5. Entire males

Treatment acting as a positive control of the study.

Secondly the intention was to investigate weather QBA used for the first time as a new method for recognition of pain in livestock will be able to recognize distress caused by castration. The particular interest was focused on the ability of QBA to distinguish between different levels of pain inflicted by different castration methods. Quantitative methods of behavioural assessments with use of ethogram of behaviours and postures have been shown in the past do distinguish between different levels of pain. To achieve the objective of assessing QBA as a new method of pain recognition it was considered necessary to use methods which have been shown earlier to induce different quality and quantity of pain (i.e. rubber ring and combined method). With this approach the assessment of pain and distress in several different scenarios could have been accomplished.

In this study the following methods of traditional castration were used: Rubber ring castration, Short scrotum castration, Combined Rubber ring and Burdizzo castration as well as Immunocastration. For specific details of particular castration techniques please see Chapter 1. All of these methods were applied without local anaesthesia. Application of pre castration anaesthesia or analgesia was considered. However due to impracticality of use (lambs would have to be handled twice as time is required for the anaesthesia to start working before castration is applied) and higher cost of this method (cost of anaesthetic, time and extra labour) it was deliberated that it is not the best choice for the farmers. Therefore, technique was not included as one of the treatments.

3.2.3 Immunization protocol

The Immunization procedure consisted of primary and booster vaccination given at 6 and 12 weeks of age. At the time of primary vaccination (6 weeks of age) the control group and lambs in the immunocastration group (together with their dams and siblings) were brought inside overnight and penned and managed in the same small pens (approx. 1.5 x 1.5 m) used for the castration assessments conducted in neonatal lambs. Lambs were vaccinated by a single s.c. (sub-cutaneous) injection on the left side of the neck with an anti-GnRF vaccine.

3.2.4 Assessment of physical castration lesions and site reaction to the vaccination

For the first 10 weeks following castration treatments animals were gathered and the size and severity of castration lesions were scored on a scale from 0 to 5 as described by Kent et al., 2000 (see Table 1 below). The presence or absence of the testes was noted each week until 10 weeks of age. In the event of the scrotum not being shed and healed at week 10, testes and the wound were checked until shed. Length and width of each lesion was recorded as follows: the lateral width of the scrotal lesion was measured (to the nearest mm) with callipers (Vernier Callipers®) from one edge of the open lesion to the other (or width of the rubber ring and enclosed tissue; Kent et al., 2000).

Table 1 Lesion scoring system (Kent et al., 2000)

Score	Description
0	Intact skin with no swelling or reddening. Complete healing with no residual scab.
1	Swelling but intact skin or healing lesion with a scab.
2	Severe swelling but skin intact or a narrow, red, ulcerated wound round the perimeter of the ring with little or no exudate and only slight swelling of the surrounding tissues. A healing lesion showed a large scab with underlying granulation tissue and exudate.
3	A wider band of red, ulcerated skin surrounding the ring, but no purulent exudate present. If the scrotum or tail was lost, a large granulated ulcer with exudate and swelling of the surrounding tissue.
3.5	A narrow band of red ulcerated skin with evidence of a small amount of pus round the perimeter of the ring. Limited swelling of the surrounding tissue.
4	A red, ulcerated lesion covered by a purulent exudate. Swelling of the surrounding tissues.
5	A large, red, ulcerated lesion with much pus and exudate and a strong smell of necrotic.

During the assessment of the lesions induced by a particular castration technique no scores of 4 and 5 were ever recorded.

Site reactions of the tissues in immunocastrated lambs were assessed on 7, 14 and 28 days after vaccination in the study of 2011 and on 0, 7, 14, 28, 35, 42 and 56 days after vaccination in the studies of 2012 and 2013. The presence or absence of the local tissue reaction to the vaccination was noted each week. These lambs were also vaccinated a second time accordingly to agreed protocols, and site reactions checked at the same times after the second vaccination. The site reaction was judged visually and by manual palpation of the injection location by the same handler. During the course of the study all of the assessed lambs were given score of '0'; Table 2 below describes the lesion

scoring scale used to assess the site reaction to the vaccination procedure. The scale is based on Kent et al., 2000 and practical knowledge.

Table 2 Scoring scale of local reaction of the tissues to immunization

Score	Description
0	Intact skin with no swelling or reddening.
1	Swelling but intact skin with or without reddening.
2	Swelling, intact skin with clear or pussy exudative.
3	Severe swelling but skin intact with clear or pussy exudative.
3.5	Swelling ulcerated skin with evidence of a small amount of pus.
4	Swelling, a red ulcerated lesion covered by a purulent exudate.
5	Swelling, a large, red, ulcerated lesion with much pus and exudate and a strong smell of necrotic.

3.2.5 Assessment of expressed behaviours and postures after handling, physical castration and immunization procedure

For the purpose of this study an ethogram of pain related behaviours and postures was formed after the literature review (Table 3) to allow for the assessment of the influence of the particular castration technique on the lambs' expression of pain related behaviours. The frequency and duration of performed postures and behaviours were the main focus of this study.

Table 3 Ethogram of behaviours and postures (after Molony et al., 2002)

Behaviour	Description
Easing Quarters	One action was recorded each time a front or hind limb, including the shoulder and hindquarters, was moved in a less forceful manner than stamping or kicking or the whole body was shifted or eased without moving from the place of rest, tensing of leg muscles was also included. Stretching with the forelegs forward and with the hind limbs back, back arching was included in this category.
Stamping/Kicking	One action was recorded when one of the feet was lifted and forcefully placed on the ground or kicked if animals were lying.
Teat seeking	Lamb places its head in the udder area with or without suckling.
Head Turning	Movement of the head beyond the shoulder both looking and touching; the source of pain was included.
Jumping	Lamb moves forward using bouncy hops with its hind limbs.
Shaking	The presence of trembling/shaking (torso, head or limbs) produced by major muscles was recorded.

Wagging Tail	A single side-to-side tail movement was recorded as one action; a continuous series of tail movements, without obvious pause with the tail hanging down, was counted as one action. Tail wags while suckling were not included.

Posture	**Description**
Normal st**anding**	Behaviours performed whilst maintaining a standing posture (standing, walking and playing, eating or investigating) with no apparent abnormalities.
Abnormal Standing	Standing or walking unsteadily sometimes with tail wagging, walking backwards; walking on knees; moving forwards with bunny hops, circling; leaning on a support or falling. Abnormal standing was often associated with frequent foot stamping, kicking and tail wagging.
Dog sitting	Ventral recumbency with the lamb keeping the scrotal region off the ground.
Normal Lying	Ventral (sternal) recumbency with the legs tucked in and the head down, either round to one side or directly in front or head up.
Abnormal Lying	Ventral recumbency with the hind limbs partially or fully extended.
Lateral Lying	Lateral recumbency with one shoulder on the ground and with the head up or down. The forelegs were usually extended.

3.2.6 Video recordings

Video recordings were taken for the first 30 minutes after treatments were applied for treatments 1-4 beginning when the handler had left the pen. For the Immunocastration and C group at the time of primary vaccination video recordings were taken for 15 minutes after immunization or handling. The main area of interest was the time interval representing the peak of expressed pain related behaviours and restlessness (Molony et al., 2002). Recordings were made with a Canon XM2 3CCD Digital Video Camcorder (Canon Inc., Japan). The camcorder was placed on a tripod and directed towards the pen in such way to allow for full vision of the pen. It has to be noted that video-recordings were taken by Prof Cathy Dwyer and the technical team at SRUC before this thesis has begun as a part of a different project. Recordings were then scored using the Observer XT 9 (Noldus Information Technology®) program by a single observer for the presence, duration and the frequency of occurrence of pain behaviours and postures (Table 1).

3.2.7 Evaluation of QBA as a new technique to assess pain in castrated lambs

Data for this section were collected and analysed (QBA analysis) by a MSc student of the Applied Animal Behaviour and Animal Welfare course at the Royal (Dick) School of Veterinary Studies (Fabiana Mizzoni) under Dr Francoise Wemelsfelder and my supervision. The student carried out the free Choice Profiling (FCP) with blinded observers from video footage prepared and arranged in a particular order (by myself) as a part of her dissertation thesis. For the full details of FCP methodology please see the sections below (see also "Assessing the 'whole animal': a free choice profiling approach", Wemelsfelder et al., 2001).

For the control group video-clips were extracted starting from the 2nd minute after handling. This time was believed to be most uncomfortable for lambs as no other treatment was applied to these animals. For the physically castrated groups – RR, SSC, COM the video clips were extracted starting from the 16th minute after the castration procedure. This was believed to be the peak of pain sensation induced by castration according to previous experience and the literature review. Three sets of video-clips were formulated, one for the term's generation session and two sets for the two sessions when the scoring of the behavioural expressions took place. Selected video clips were arranged into a specific randomized order. At the start of each session a neutral clip was shown first (showing activities of the control treatment with a set of natural positive behaviours like normal lying, normal standing, normal steady walking, sleeping etc.), followed by the clip with one of the castration treatments. The order of the clips with recording of specific treatments was randomized across each session so no more than two clips of the same treatment were shown consecutively. The desirable outcome was finding significant agreement between observers in their valuation of lambs' behavioural expression.

3.2.7.1 Observers

Nine observers were used in this study. They were postgraduate students of the Applied Animal Behaviour and Animal Welfare course at the Royal (Dick) school of Veterinary Studies so they have some (not specified) earlier experience in animal behaviour

observations. No student had previous specific experience in recognition of pain induced by physical castration procedures. At the beginning of the study observers were gathered to receive instructions which would allow them to carry out behavioural expression assessment. The instruction specified that the focus should be placed on "how" the animal is behaving and not "what" it is doing. Observers were told that lambs which would be scored had undergone a variety of on-farm husbandry procedures which may potentially inflict varying levels of pain and distress, however neither castration or castration methods, nor the existence of an untreated control group, were explicitly mentioned at any point during the instructions. After receiving instructions observers were required to generate their own descriptors of behavioural expression (see table 4 for details). To assure independence of particular assessments viewers were told not to discuss with each other the generated terms throughout whole study. During the FCP sessions observers were blind to the specific treatment groups of lambs of which behavioural expressions they were scoring.

3.2.7.2 Free choice profiling

Free Choice Profiling (FCP) was the method used in this study to assess behaviour expression and consists of 2 phases.

Phase 1

In this phase observers were gathered for the terms' generation session and introduction to the behavioural expression scoring technique. For the purpose of this particular meeting 15 video-clips were prepared and arranged in a specific order for the observers. The clips were selected from the 79 video-clips which were prepared to perform this study. This selection of 15 clips was a representative sample of footage of wide range of behavioural expression of rams (or their untreated siblings) across all of the treatment groups showing comprehensive range of expressions.

After viewing each clip each observer was asked to write down their own terms which fully described the quality of the behaviour of a lamb shown in the clip. This process continued until all video-clips were presented to the observers. During the introduction

to the technique observers were made aware that they are free to decide on new terms for each lamb or use the ones already selected before. In consequence each observer created a list of descriptors defining the expressive range of behaviours for the lambs they observed (see table 7 below for the list created by the viewers' descriptors).

Phase 2

The second phase of FCP consisted of two parts which took place on two separate days. Prepared footage (79 2-min long video clips) was split in two single sets of video-clips (as it was impossible and not practical to assess all the clips in one session) which were assessed on two separate days. Each set of clips was a footage of behavioural expression of rams across all of the treatment groups (the existence of which observers were unaware of). In this phase viewers used the lists of their own descriptors (generated in Phase I) to score expressive behaviours of lambs castrated with different physical castration methods. Scoring sheets prepared earlier were given to the observers at the beginning of each session. Each sheet contained an observer's terms placed next to visual scales; a line of 12.5 cm ranging from "minimum" (left end of the scale) to "maximum" (right end of the scale). The video-clips were shown and the viewers were asked to score each clip using the list of their terms and assess each individual ram on every term they generated. Table 4 contains all of the descriptors which were formed by the 9 observer during the terms' generation session. (Figures in brackets indicate how many observers were using the term. The terms with no number allocated to them were used only by one observer).

Table 4 Total list of descriptors generated by all 9 observers

Descriptors
Calm (7), Comfortable (7), Relaxed (5), Sleepy (2), Quiet, Drowsy, Content, Curious (8), Interested (6), Inquisitive (4), Active (4), Responsive (3), Alert (3), Eager, (2), Engaged (2), Playful (2), Happy (2), Aware (2), Investigative, Exploratory, Interactive, Energised, Attentive, Excited, Restless (9), Painful (7), Irritable (7), Agitated (6), Tense (3), Twitchy (3), Aroused (2), Stressed (2), Sore (2), Annoyed (2), Fidgety, Distressed, Uneasy, Disturbed, Desperate, Suffering, Miserable, Alert, Jumpy, Aware, Content, Tired (3), Quiet (2), Lethargic (2), Listless,

Sleepy, Still, Subdued, Sad

Note: numbers in the brackets indicate how many observers were using the term to evaluate behaviour of lambs

3.2.8 Comparison of Qualitative and Quantitative assessments of pain in lambs after castration procedure

To perform comparison and correlation of QBA analysis results with quantitative behavioural assessment the same 2 min periods of the video clips (which were used to generate the QBA data) were re-analysed from the larger data set (described in sections 3.2.4) in Observer XT 9 (Noldus Information Technology®) by restricting the time interval to this period. The data were then analysed (data distribution, Kruskal-Wallis and Mann-Whitney test) in Minitab statistical package 16th edition and Genstat 14th edition (GenStat® 14th edition, Lawes Agricultural Trust, VSN International Ltd, Oxford, UK and Minitab, Inc., State Collage, PA) to check whether there were significant differences between treatments in expressed behaviours and postures. Correlation of qualitative behavioural assessment (QBA results) with quantitative behavioural scoring was carried out thereafter to allow for the comparison of both types of assessment. Correlation was made for two separate sets of quantitative data; 2 min video-clips used in QBA as well as 30 min video-clips used in the primary quantitative assessment of different castration techniques (these sets of data will be called further 2 min data and 30 min data respectively).

3.3 Statistical Analysis

3.3.1 Analysis of the frequency and duration of pain related behaviours and postures and the severity of the lesions (time to heal, time to shed testicles)

Normally distributed data (time to shed testicles) was analysed with use of REML (Restricted or residual Maximum Likelihood Mixed Model, GenStat® 14th edition, Lawes Agricultural Trust, VSN International Ltd, Oxford, UK). The birth weight, dam parity, sire breed and the litter size were taken into account during analysis as random

effect factors. Where REML suggested that between treatments differences were significant two sample T-tests were carried out to determine significant differences between treatment groups. Bonferoni corrections were applied to account for multiple testing. The Anderson darling test was used to check the normality of data distribution in Minitab statistical package 16th edition (Minitab, Inc., State Collage, PA). Frequency of performed pain related behaviours/postures and duration of performed postures as well as severity and size of post castration lesions data were not normally distributed. It was impossible to normalize the data therefore Kruskal-Wallis and Mann-Whitney non-parametric tests were used to determine significant differences between treatment groups. A Bonfrerroni correction was also applied to account for the multiple testing. In this chapter due to different number of treatment groups in particular experiments the new critical p-value will be as follows; a) for experiments with 4 treatment groups new p – value would be $P<0.0125$; b) for experiments with 3 treatment groups new p – value would be $P<0.016$. For the experiment with 2 treatment groups the p – value would be $P<0.05$. The Bonferroni correction does not need to be applied in this situation.

3.3.2 Statistical Analysis of QBA technique

Statistical analysis of FCP of lambs' expressive behaviours were performed by Dr Francoise Wemelsfelder and MSc student Fabiana Mizzoni. The score of each term for each lamb was determined by measuring the distance in mm between the "minimum" end of the visual scale and the point where the viewer placed a cross or line marking his judgement of the behavioural expression of lamb behaviour with regard to a particular term (please see table 1a and 1b in Annex 1 as an example of original data obtained form one of the observers). Nine data matrices were prepared (one for each observer). To analyse these data a Generalized Procrustes Analysis (GPA) was used in Minitab statistical package 16th edition (Minitab, Inc., State Collage, PA). GPA is a type of multivariate statistical technique which does not rely on fixed variables and it is used as a pattern matching technique (Wemelsfelder et al., 2001). The basis of this method lies in the assumption that the distances between samples (lambs) will be comparable even though observers may have used different terms in scoring behavioural expression because the sample (in this case lambs) are always the same. In summary the GPA

identifies a consensus profile, which can be understood as a 'best fit' between all multi-dimensional scoring profiles generated by individual observers (please see figure 1-9 in Annex 1 as an examples of consensus profiles obtained form each observer. Figure 10 represents "the best fit" between all multidimensional scores provided by individual observers). Subsequently a randomization test is carried out to specify the significance of the consensus profile (Dijksterhuis and Heiser, 1995). The details of GPA methodology were previously described in the literature (Wemelsfelder et al., 2000, 2001, 2009; Rutherford et al., 2012; Napolitano et al., 2012; Fleming et al., 2013). The randomization test produces new data matrices by randomizing each observer scores. The next step is the calculation of a 'randomised' consensus profile by application of GPA to the randomized matrices. This procedure requires to be repeated 100 times to provide a distribution of the Procrustes Statistic specifying how likely it is to find an observer consensus based on chance alone. Then a one-way t-test is performed to define if the actual observer consensus profile falls significantly outside the distribution of randomised profiles. The total number of dimensions of the consensus profile can be reduced to a few main dimensions using Principal Component Analysis (PCA). In this particular study the number of dimensions of the consensus profile is condensed to two dimensions (dim1 and dim2). Then the consensus dimensions can be interpreted by selecting terms for each observer that correlate strongly with the consensus dimensions. Finally, PCA assigns a quantitative score to each animal (lamb) on each of the two (in this particular situation) consensus dimensions.

One-way analysis of variance was carried out to determine whether dimension scores were significantly affected by treatment. Tukey post-hoc tests were carried out thereafter on both dimensions to investigate where any significant differences were.

3.3.3 Correlation of Quantitative and Qualitative pain assessment

Spearman Rank Correlation was carried out to investigate the correlation between GPA scores (dim1 and dim2) and the quantitative scores (expressed duration and frequency of restlessness, pain related behaviours and postures) for each lamb. Please see table 2 and 3 in Annex 1. Tables 2 and 3 are examples of how data was organized to carry out the

analysis. In the first instance the 2 min data of pain related postures and behaviours (recorded for each lamb following different method of castration or handling) was extracted form the Observer Nodulus XT programme which was used to score the frequency and duration of pain related behaviours and postures expressed by each lamb. Spearman rank Correlation was used to analyse data in Mini Tab statistical package 16th edition (Minitab, Inc., State Collage, PA). Similar procedure was carried out to investigate correlation between GPA scores (Dim 1 and Dim 2) and quantitative scores for the full 30 min of pain related behaviours and postures data obtained form Observer Nodulus XT programme for each lamb following different castration or handling.

Principal component analysis (PCA, correlation matrix, no rotation) was carried out to assess patterns of correlation between quantitative and qualitative (QBA) assessment and demonstrate graphical representation of the correlation pattern (see figure 11 and 12). PCA is a statistical test, which uses an orthogonal transformation to convert a set of variables (in this case results of frequencies and durations of recorded behaviours and postures as well as QBA dimensions) into a set of linearly uncorrelated variables called principal components. The first principal component accounts for as much of the variability in the data as possible. Each following variable will have in return the highest probable variance under the restraint that it is orthogonal to the following components.

Dimension results were not normally distributed; therefore Kruskal-Wallis and Mann-Whitney tests were used to determine significant differences between treatment groups on the main components of the combined PCA.

3.4 Results

3.4.1 Assessment of Pain behaviours and Postures (Acute pain assessment)

Analysis has revealed significant differences between treatment groups in expressed frequency and duration of behaviours and postures (Tables 5a-c) with the exception of the frequency and duration of Normal Standing. Therefore, the Mann-Whitney test was carried out to determine significant differences between particular treatments. Foot

stamping/kicking, wagging tail, head turning, easing quarters, trembling, dog sitting behaviours were combined together and formed a new category, which was called "active pain" behaviour. The number of postural changes was summarised as restlessness. Figures 1 and 2 below are showing the comparison of expressed active pain behaviour and restlessness between treatments; RR treatment was observed to express significantly greater frequencies of active pain H=46.5, Df=3, **P< 0.001** than C and COM group. Frequency of restlessness reported for RR rams was also increased H=47.55 Df=3, **P< 0.001** in comparison to C, COM and SSC treatments respectively.

Table 5a Frequency of pain related behaviours recorded in the first 30 minutes following handling (C), rubber ring castration (RR), short scrotum castration (SSC) or combined castration (COM) of lambs at 2days of age. Data are medians with Q1 and Q3.

Behaviour	C[1]	COM[1]	RR[1]	SSC[1]	P-Value[2]
Easing quarters	7.5(6.2-12.2)a	14.0(11-32)b	53.0(40.2-60.7)c	40.0(29.2-50.5)c	**P< 0.001**
Foot stamping/kicking	0.0(0.0-1.0)a	0.0(0.0-0.8)ac	28.5(14.2-42.5)b	7.5(1.2-14.7)c	**P< 0.001**
Head turning	1.0(0.0-2.0)a	2.0(0.0-4.40)ab	3.5(1.0-9.0)b	6.0(3.4-8.0)bc	**P< 0.001**
Jumping	0.0(0.0-0.0)a	1.0(0.0-1.0)b	0.0(.0.0-0.0)ab	0.0(0.0-1.5)a	**P= 0.005**
Teat Seeking	2.0(2.0-3.0)a	0.0(0.0-1.0)b	0.5(0.0-2.7)b	3.0(1.0-4.7)a	**P< 0.001**
Shaking/trembling	5.0(2.0-8.5)a	1.0(0.0-2.7)b	1.0(1.0-2.0)b	5.5(2.5-10.5)a	**P< 0.001**
Wagging tail	4.5(2.0-13.0)a	4.0(1.2-10.7)a	21.1(7.0-27.5)b	15.5(7.0-33.5)b	**P< 0.001**

[1]Median frequency (Q1-Q3). [2](amended p-value due to Bonferroni correction P<0.0125). Different letters within a row indicate significant differences between treatments.

Table 5b Frequency of pain related postures recorded in the first 30 minutes following handling (C), rubber ring castration (RR), short scrotum castration (SSC) or combined castration (COM) of lambs at 2days of age. Data are medians with Q1 and Q3.

Posture	C[1]	COM[1]	RR[1]	SSC[1]	P-Value[2]
Abnormal standing	2.0(1.0-6.0)a	3.0(2.0-6.5)a	14.0(11.0-15.0)b	10.0(6.7-13.2)c	**p< 0.001**
Normal Standing	2.5 (1.0-4.0)	1.0(0.0-2.7)	1.0(1.0-3.0)	2.0(1.0-3.0)	NS
Dog sitting	0.0(0.0-1.0)a	1.0(0.2-2.7)b	5.0(3.0-8.0)c	5.0(2.5-9.2)c	**P< 0.001**
Normal lying	0.0(0.0-1.0)	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.2)	**NS**
Abnormal lying	3.0(1.0-4.5a	3.0(2.0-6.0)a	10.5(8.0-15.5)b	10.0(7.0-16.0)b	**P< 0.001**
Lateral lying	0.0(0.0-0.0.0)a	0.0(0.0-1.7)a	4.0(2.5-7.0)b	0.0(0.0-1.0)a	**P< 0.001**

[1]Median frequency (Q1-Q3). [2](amended p-value due to Bonferroni correction P<0.0125), NS not significant. Different letters within a row indicate significant differences between treatments.

Table 5c Duration of pain related postures (measured in seconds) recorded in the first 30 minutes following handling (C), rubber ring castration (RR), short scrotum castration (SSC) or combined castration (COM) of lambs at 2days of age. Data are medians with Q1 and Q3.

Posture	C[1]	COM[1]	RR[1]	SSC[1]	P-Value[2]
Abnormal standing	160.2(56.4-331.3)a	174.8(93.3-324.7)a	325.6(211.0-404.6)b	414.0(235.1-737.3)b	**P= 0.004**

Normal standing	35.1(0.0-286.6)	129.4(15.1-294.1)	15.4(6.5-79.8)	49.0(5.7-138.6)	**NS**
Dog sitting	0.0(0.0-4.1)a	6.5(0.6-13.9)b	23.5(10.2-24.9)c	22.4(11.0-52.8)c	**P< 0.001**
Normal lying	0.0(0.0-0.0)	0.0(0.0-26.6)	0.0(0.0-0.0)	0.0(0.0-2.8)	**NS**
Abnormal lying	1195.0(469.3-1331.0)	894.4(666.6-1215.0)	612.6(436.5-753.0)	762.2(432.6-1035.0)	**NS**
Lateral lying	0.0(0.0-0.0)a	0.0(0.0-279.8)a	308.7(146.2-502.5)b	0.0(0.0-19.6)c	**P< 0.001**

[1]Median frequency (Q1-Q3). [2](amended p-value due to Bonferroni correction P<0.0125), NS not significant. Different letters indicate significant differences between treatments.

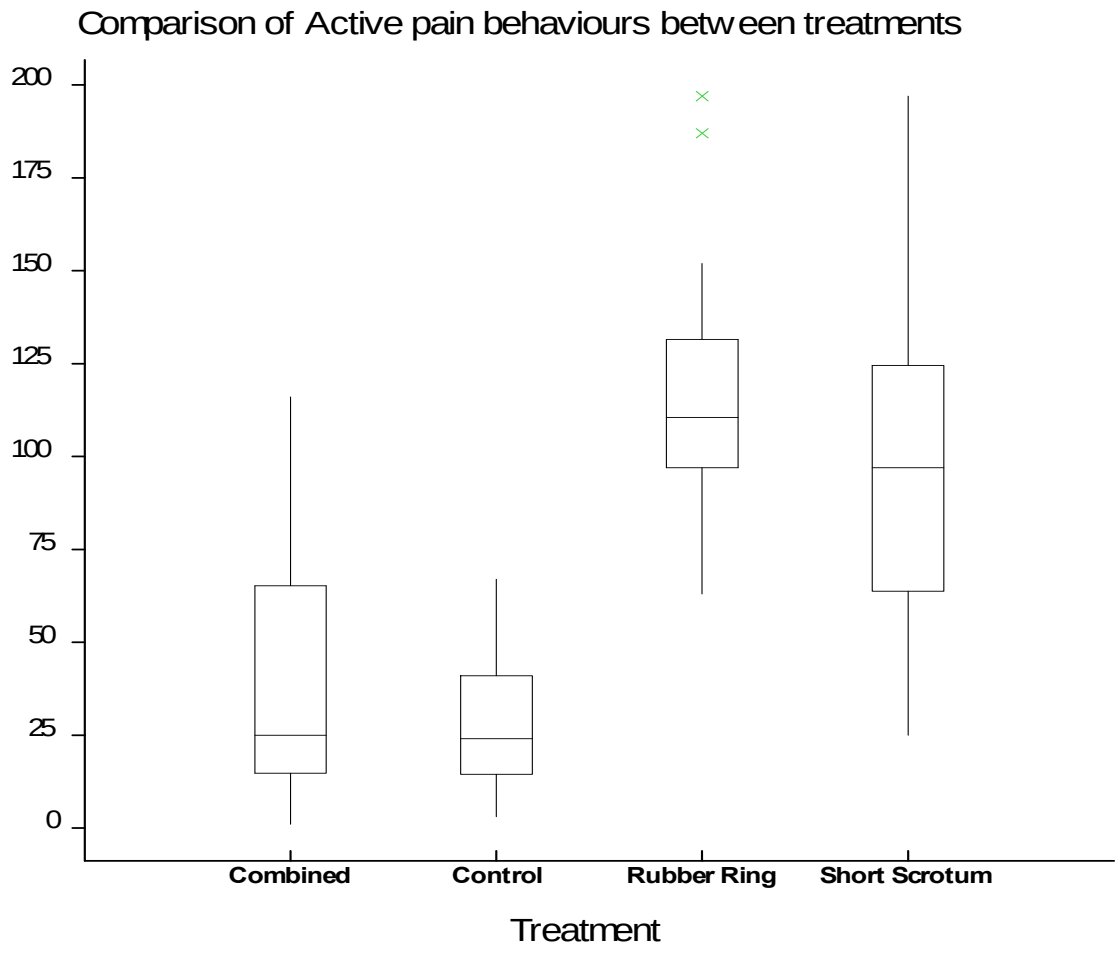

Figure 1 Box plot of active pain behaviours recorded in the first 30 minutes following handling (C), rubber ring castration (RR), short scrotum castration (SSC) or combined castration (COM) of lambs at 2 days of age. Data are medians with Q1 and Q3. Whiskers represent upper and lower extreme. ^{x} outlier/single data point.

C and COM group are showing less active pain behaviours than RR and SSC group median frequency: Combined=25, Control=24.0; Rubber Ring=110.5, Short Scrotum=97, **P<0.001**.

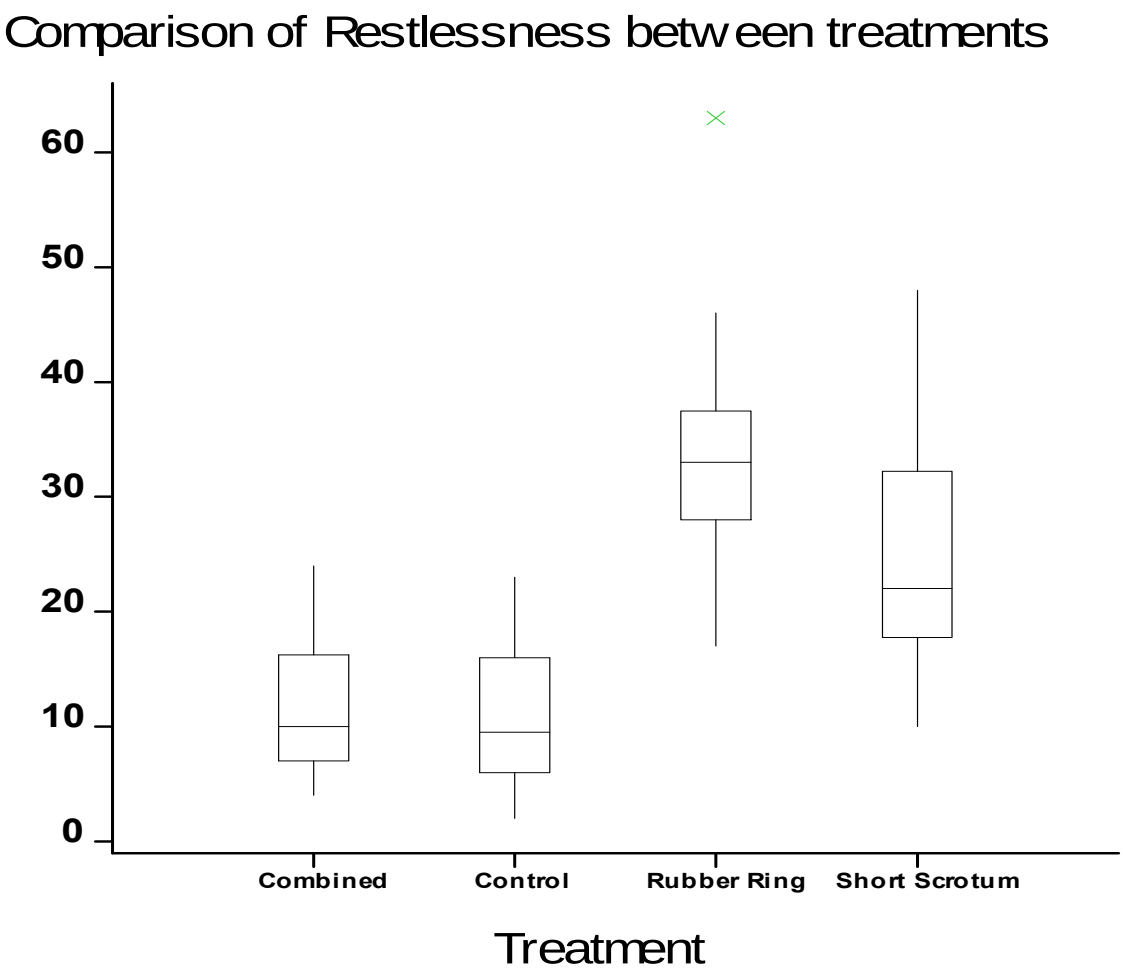

Figure 2 Box plot of expressed restlessness recorded in the first 30 minutes following handling (C), rubber ring castration (RR), short scrotum castration (SSC) or combined castration (COM) of lambs at 2 days of age. Data are medians with Q1 and Q3. Whiskers represent upper and lower extreme. x outlier/single data point.

C and COM group were less restless than RR and SSC treatment median frequency of postural changes: Combined=10, Control=9.5, Rubber Ring=33, Short Scrotum=22, **P<0.001**, RR rams were also more restless than SSC rams **P<0.001**.

3.4.2 Assessment of Pain Behaviours and Postures Between C and Vac Group at the Time of Primary Vaccination

The assessment of frequency and duration of expressed behaviours and postures following handling or immunization with ant-GnRH vaccine showed that there were no significant differences between treatment groups in expressed behaviours and postures

with the exception of restlessness behaviour **P=0.038**, U=100.0**.** Tables 6 a-c below show results for the expression of frequency and duration of recorded behaviours and postures, recorded for 15 min following handling or immunization. The Mann-Whitney test was carried out to determine significant differences between treatments. Behaviours like foot stamping/kicking, wagging tail, head turning, easing quarters, trembling, and dog sitting were combined together and formed a new category which was called “active pain” behaviour. Restlessness (median frequency of postural changes) was also calculated for each lamb. Figure 3 below is showing the comparison of expressed restlessness between treatments.

Table 6a Frequency of pain related behaviours recorded in the first 15 minutes following handling (C) or immunocastration (Vac) of lambs at 6 weeks of age. Data are medians with Q1 and Q3.

Behaviour	C[1]	Vac[1]	P-Value[2]
Easing quarters	05(0.0-1.0)	0.0(0.0-1.0)	**NS**
Foot stamping/kicking	0.0(.0.-0.0)	0.0(0.0-0.0)	**NS**
Head turning	0.0(.0.-0.0)	0.0(0.0-0.0)	**NS**
Jumping	0.0(.0.-0.0)	0.0(0.0-0.0)	**NS**
Teat seeking	1.0(0.0-1.0)	1.0(0.0-2.0)	**NS**
Shaking/trembling	0.0(.0.-0.0)	0.0(0.0-0.0)	**NS**
Wagging tail	0.0(.0.-0.0)	0.0(0.0-0.0)	**NS**

[1]Median frequency (Q1-Q3). [2](P<0.05) NS not significant.

Table 6b Frequency of pain related postures recorded in the first 15 minutes following handling (C) or immunocastration (Vac) of lambs at 6 weeks of age. Data are medians with Q1 and Q3.

Posture	C[1]	Vac[1]	P-Value[2]
Abnormal standing	0.0(.0.-0.0)	0.0(0.0-0.0)	**NS**
Normal Standing	10.0(1.0-3.0)	1.0(0.0-1.0)	**NS**
Dog sitting	0.0(.0.-0.0)	0.0(0.0-0.0)	**NS**

Normal lying	0.0(.0.-1.0)	1.0(1.0-1.0)	**NS**
Abnormal lying	0.0(.0.-1.0)	0.5(0.0-1.0)	**NS**
Lateral lying	0.0(.0.-0.0)	0.0(0.0-0.0)	**NS**

[1]Median frequency (Q1-Q3). [2] (P<0.05), NS not significant.

Table 6c Duration of pain related postures (measured in seconds), recorded in the first 15 minutes following handling (C) or immunocastration (Vac) of lambs at 6 weeks of age. Data are medians with Q1 and Q3.

Posture	**C[1]**	**Vac[1]**	**P-Value[2]**
Abnormal standing	0.0(.0.-0.0)	0.0(0.0-0.0)	**NS**
Normal standing	125/7(71.1-194.5)	149.7(103.8.274.3)	**NS**
Dog sitting	0.0(.0.-0.0)	0.0(0.0-0.0)	**NS**
Normal lying	0.0(.0.-0.0)	0.0(0.0-26.8)	**NS**
Abnormal lying	0.0(0.0-119.0)	1.8(0.0-66.2)	**NS**
Lateral lying	0.0(.0.-0.0)	0.0(0.0-0.0)	**NS**

[1]Median frequency (Q1-Q3). [2] (P<0.05), NS not significant.

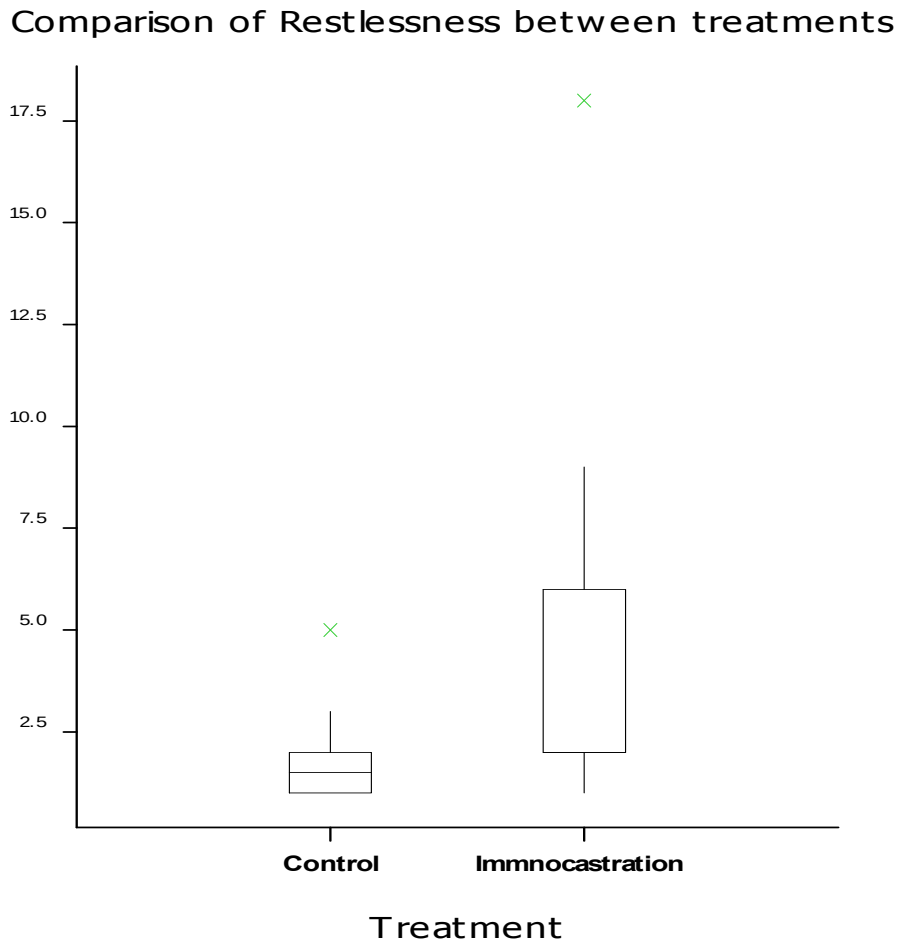

Figure 3 Box plot of restlessness recorded in the first 15 minutes following handling (C), or immunocastration (Vac) of lambs at 6 weeks of age. Data are medians with Q1 and Q3. Whiskers represent upper and lower extreme. ˣ outlier/single data point.

3.4 3 Assessment of the severity of lesions caused by particular castration technique (Chronic pain assessment)

Analyses have shown significant differences in severity and time course of lesion scores between treatments (Figure 4). COM lambs developed lesions more quickly than other groups (comparisons from table 7 below), although this resolved earlier in this group (comparisons at a suitable time point). Lesions of RR rams were still not fully healed at the time of the last evaluation in comparison to other treatments (see figure 4 and table 7 below).

Table 7 Effect of combined castration (COM), rubber ring castration (RR) or short scrotum castration (SSC) on the severity of scrotal lesions recorded for 7 weeks following treatments. Data are medians with Q1 and Q3, significance was determined by Kruskal Wallis tests..

Weeks post-castration	COM[1]	RR[1]	SSC[1]	P-Value[2]
1 week	1.25(0.5-1.5)a	0.5(0.0-0.5)b	0.5(0.0-1.0)b	**P<0.001**
2 weeks	2.0(1.0-2.0)a	1.0(0.0-2.0)ab	0.5(0.0-1.2)b	**P<0.02**
3 weeks	1.75(1.0-2.5)	2.0(1.7-2.5)	1.5(1.0-2.0)	**NS**
4 weeks	1.0(1.0-1.7)a	2.0(2.0-2.5)b	1.2(1.0-2.0)a	**P<0.001**
5 weeks	0.25(0.0-0.5)a	0.5(0.0-1.0)b	0.0(0.0-0.5)a	**P<0.001**
6 weeks	0.0(0.0-0.5)a	0.5(0.0-0.5)ab	0.0(0.0-0.0)ac	**P<0.004**
7 weeks	0.0(0.0-0.0)a	0.5(0.0-1.0)b	0.0(0.0-0.0)a	**P<0.005**

[1]Median frequency (Q1-Q3). [2](amended p-value due to Bonferroni correction P<0.016), NS not significant. Different letters indicate significant differences between treatments.

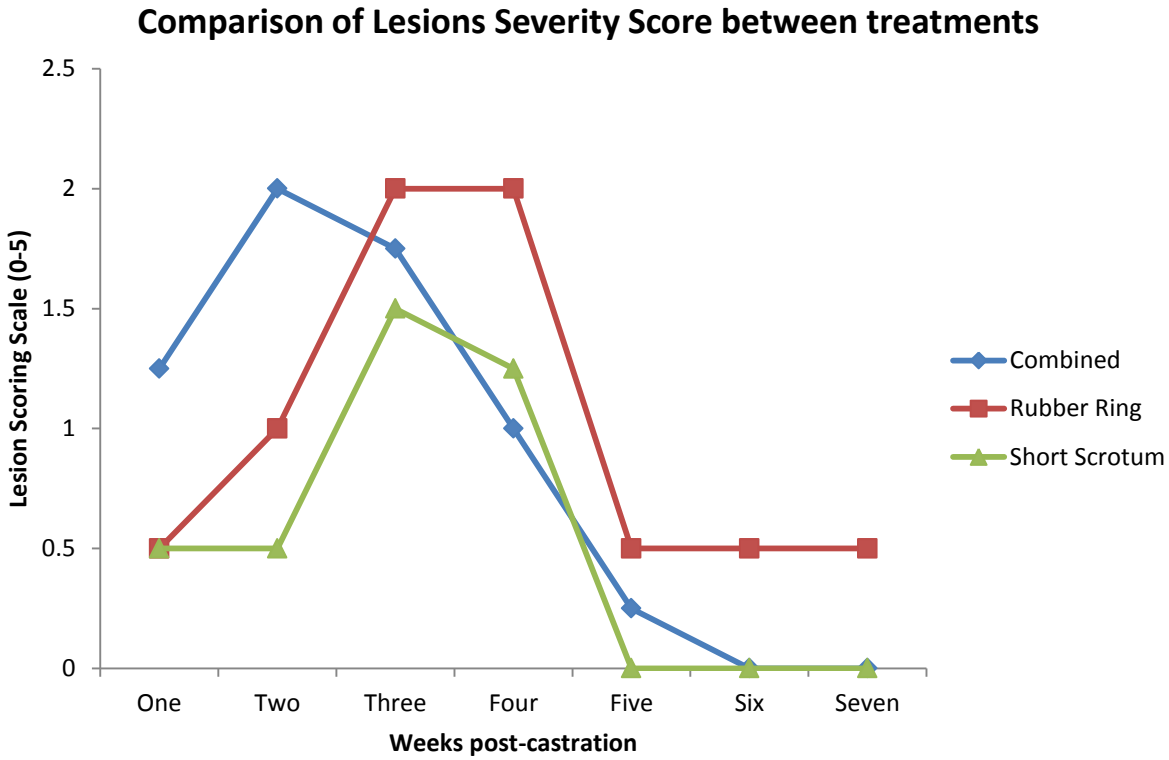

Figure 4 Comparison of lesion severity caused by specific castration techniques. Data are median scores for severity assessed at each time point after castration (application of

castration = d0). For clarity the error bars have not been included here, but the interquartile ranges around the medians for each treatment are shown in Figure 5a-c.

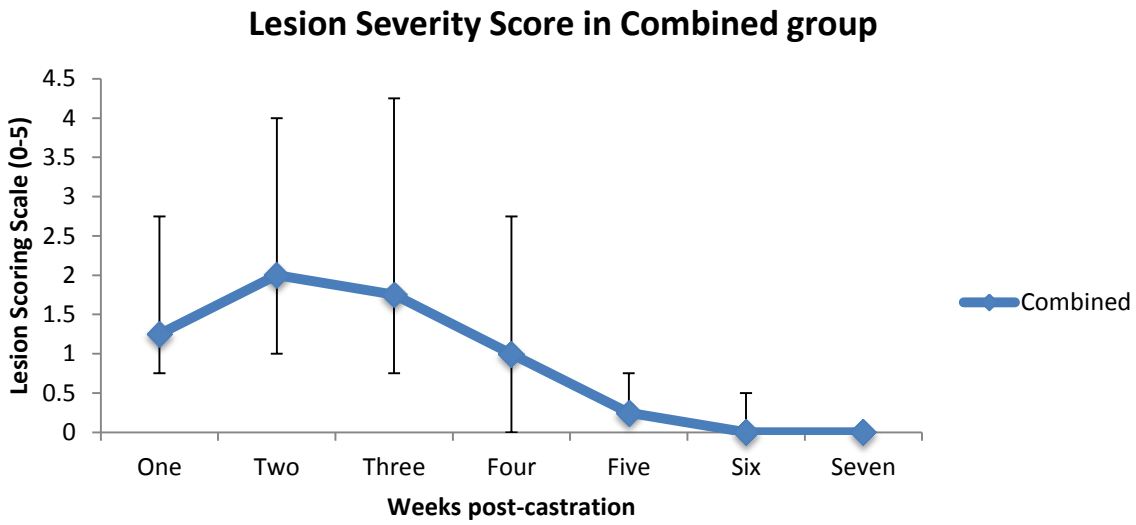

Figure 5a Lesion severity score for the combined castration method measured weekly from the time of treatment application d0. Data are medians with Q1 and Q3.

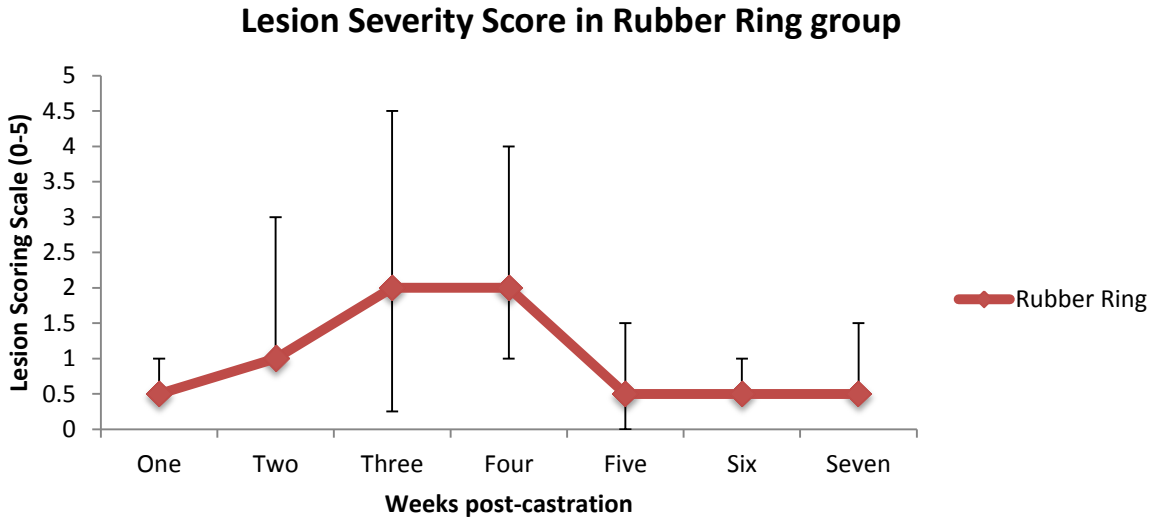

Figure 5b Lesion severity score for Rubber Ring castration method measured weekly from the time of treatment application d0. Data are medians with Q1 and Q3.

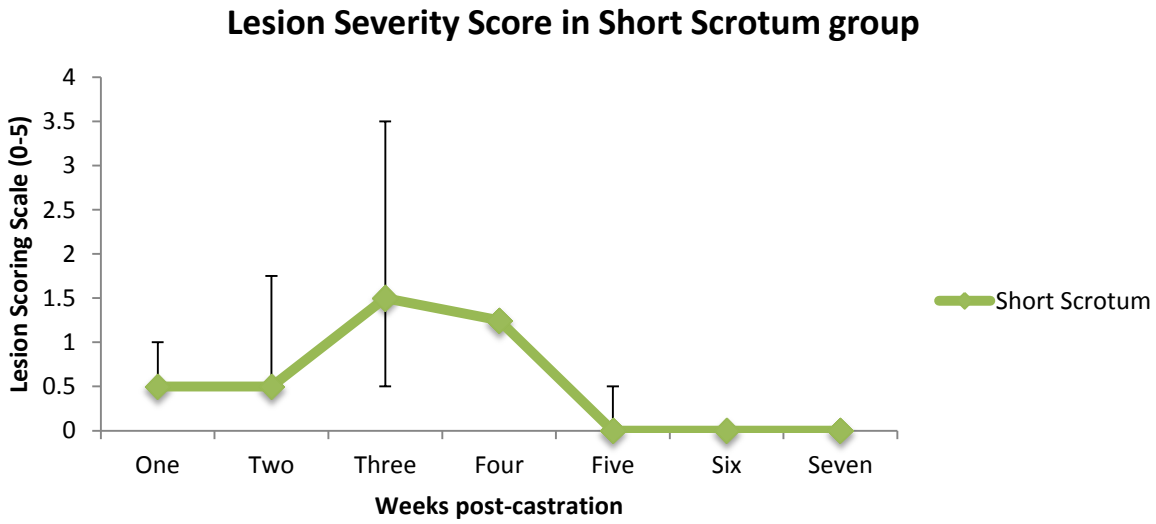

Figure 5c Lesion severity score for the Short Scrotum castration method measured weekly from the time of treatment application d0. Data are medians with Q1 and Q3.

3.4.4 Assessment of the size of lesions caused by particular castration technique (Chronic pain assessment)

Assessment of the lesion size caused by a particular castration technique showed that there were significant differences between treatment groups in the size of the lesions in all of the time points when the wound dimensions were measured (see table 8 and figure 6, 7a-c). Overall assessment showed that rubber ring castration caused development of a bigger wound which lasted for longer and was still not completely healed at the time of the last inspection at around 7 weeks' post-castration.

Table 8 Effects of combined castration (COM), rubber ring castration (RR) or short scrotum castration (SSC) on the size of scrotal lesions recorded for 7 weeks followingtreatment at 24-48 h of age. Data are medians with Q1 and Q3, significance was determined by Kruskal Wallis tests.

Weeks post-castration	COM[1]	RR[1]	SSC[1]	P-Value[2]
1 week	12.3(11.0-13.6)a	14.4(14.1-14.6)b	14.2(13.5-14.8)b	**P<0.001**
2 weeks	12.0(10.7-14.8)a	14.7(14.2-15.7)b	14.5(14.0-14.8)ab	**P=0.011**
3 weeks	12.0(10.4-14.0)a	15.0(14.4-16.2)b	14.3(13.7-14.6)c	**P<0.001**
4 weeks	7.4(5.5-11.2)a	14.0(14.2-15.7)b	11.4(3.6-14.7)a	**P<0.001**
5 weeks	1.2(0.0-5.3)a	8.0(5.6-13.6)b	0.0(0.0-4.2)a	**P<0.001**
6 weeks	0.0(0.0-4.2)a	6.5(0.0-8.6)ab	0.0(0.0-0.0)ac	**P<0.017**
7 weeks	0.0(0.0-0.0)a	3.5(0.0-7.5)b	0.0(0.0-0.0)a	**P=0.005**

[1]Median frequency (Q1-Q3). [2](amended p-value due to Bonferroni correction P<0.016), NS not significant. Different letters indicate significant differences between treatments.

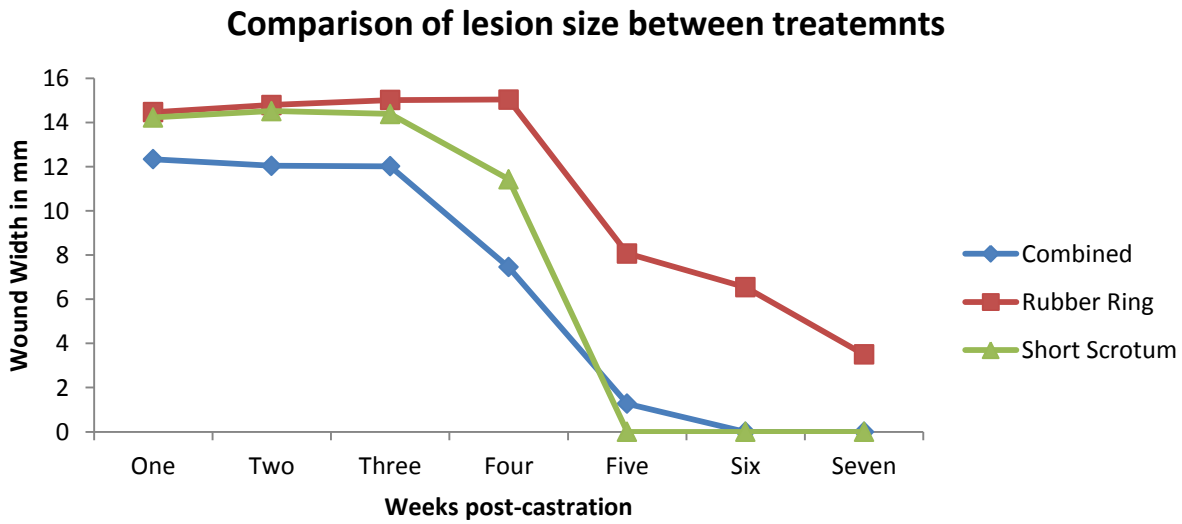

Figure 6 Comparison of lesion size caused by specific castration techniques. Data are median scores for severity assessed at each time point after castration (application of castration = d0). For clarity the error bars have not been included here, but the interquartile ranges around the medians for each treatment are shown in Figure 7a-c.

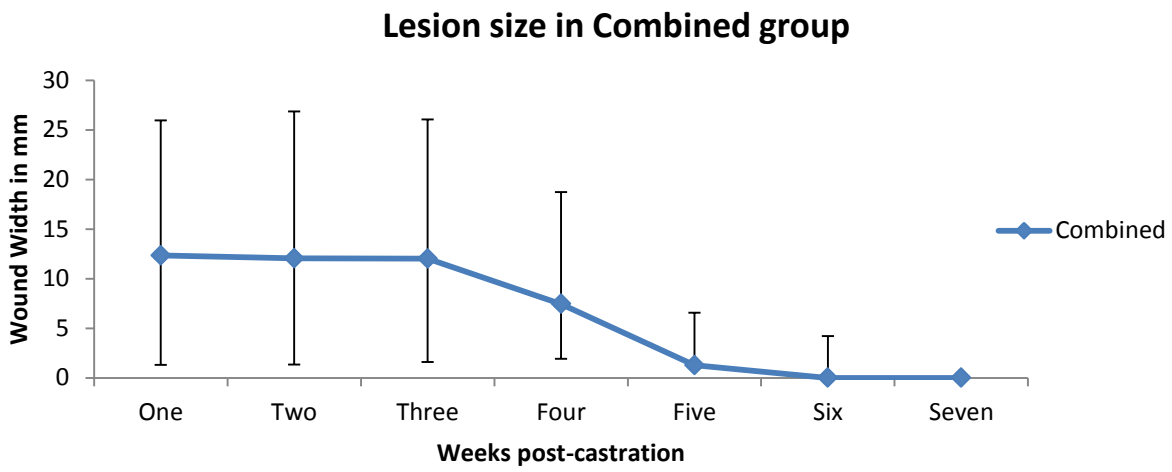

Figure 7a Recorded lesions size for the COM castration technique measured weekly from the time of treatment application d0. Data are medians with Q1 and Q3.

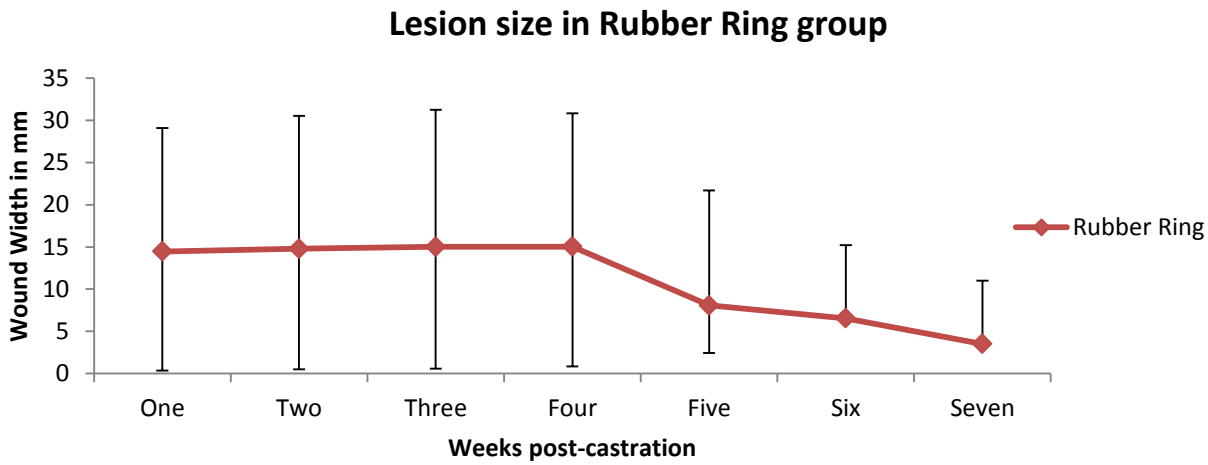

Figure 7b Recorded lesions size for the RR castration technique measured weekly from the time of treatment application d0. Data are medians with Q1 and Q3.

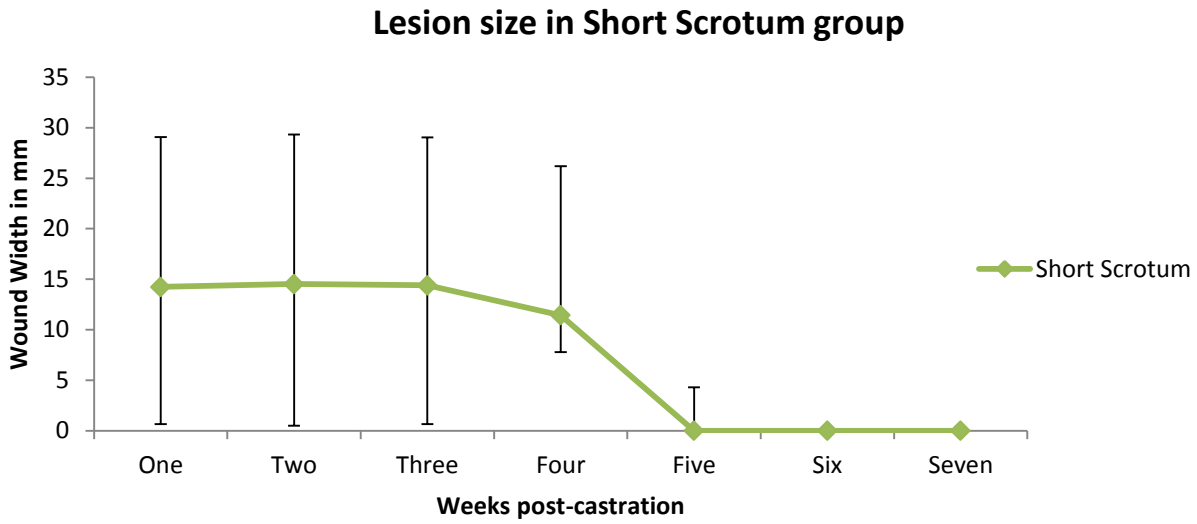

Figure 7c Recorded lesions size for the Short Scrotum castration technique measured weekly from the time of treatment application d0. Data are medians with Q1 and Q3.

3.4.5 Assessment of the immunization site

Assessments of the immunization site have shown that there was no site reaction of the tissues to the immunization agent in any of the time points when the evaluation was carried out (0, 7, 14 and 28 days after vaccination).

3.4.6 Assessment of time to heal the castration wound (chronic pain assessment)

Reported results showed significant differences between treatments in the time taken for lesions to heal (H=16.33; df=2; **P<0.001).** Post hoc analysis revealed that lesions caused by the RR method took significantly longer to heal than either of the other two castration treatments (U=83.5; **P=0.012** and U=36.0; **P<0.001** for COM and SSC respectively). Figure 8 below is representing those differences.

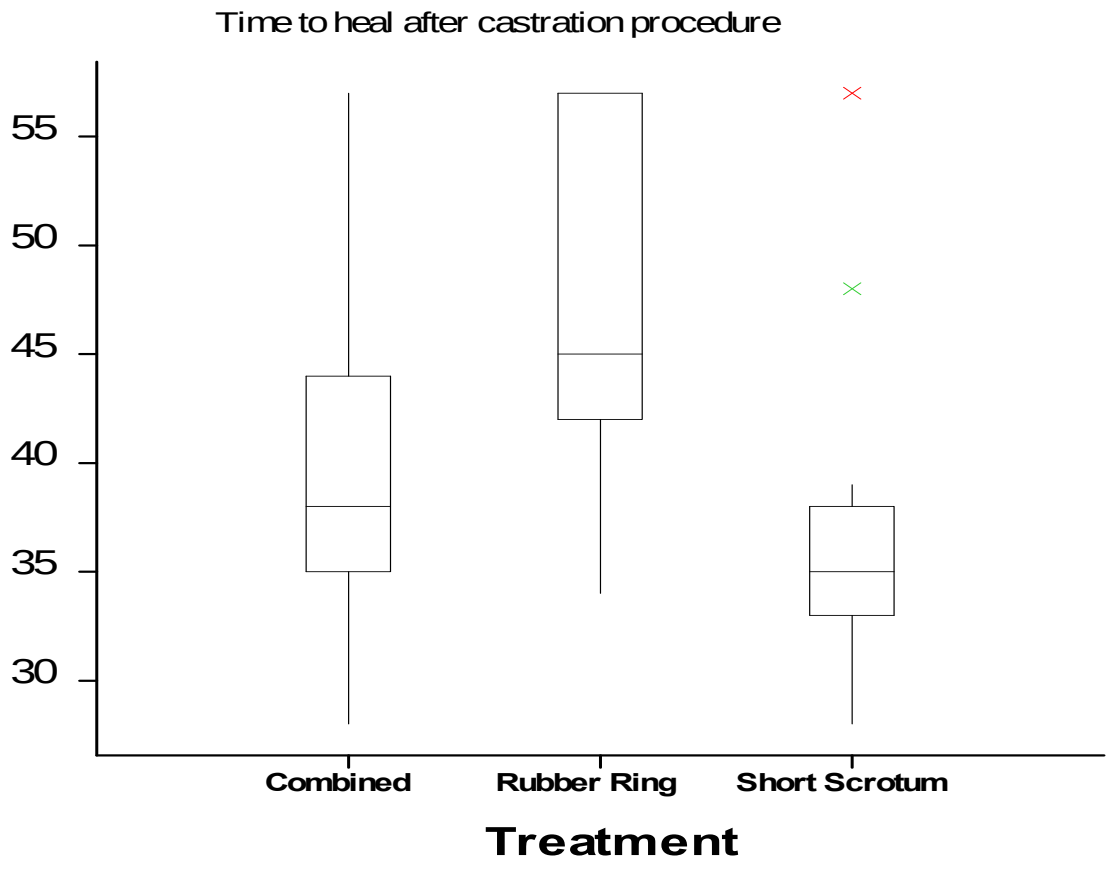

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Figure 8 Box plot of time to heal the lesion after castration procedure measured for 7 weeks following rubber ring castration (RR), short scrotum castration (SSC) or combined castration (COM). Data are medians with Q1 and Q3. Whiskers represent upper and lower extreme. ^{x} outlier/single data point.

3.4.7 Assessment of time to shed testicles after particular castration technique (chronic pain assessment)

There were significant differences between treatment groups in the time that was taken to shed the testicle and/or scrotum sac (for SSC group) following castration **(**Figure 9: Wald statistic= 9.45, d.f. 2, **P<0.013)**. Lambs from the RR group took longer to shed the scrotum in comparison to other treatments (t = -2.60, **P=0.013** and t=-2.80, **P=0.008** for COM and SSC respectively).

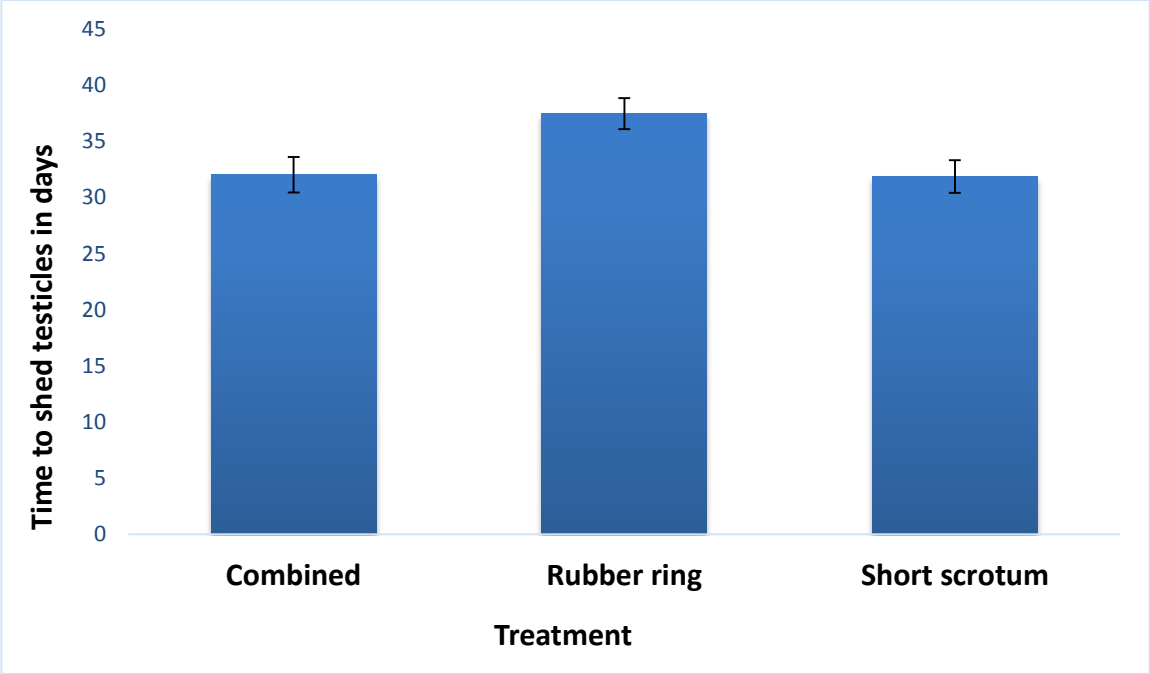

Figure 9 Differences between combined (COM), rubber ring (RR) and short scrotum castration group (SSC) in time to shed testicles following castration measured weekly form the day of the treatment d0 until 7 weeks of age. Data are Means with standard errors.

3.4.8 Qualitative Behavioural Assessment (QBA)

The GPA consensus profile explained 65.12% of the variation in observer scoring patterns, which differed significantly from the mean randomised profile (**27.81 +0.13%, t99 = 103.16, P < 0.001**). The figure 22 below represents the QBA analysis of the effect of the castration method on lambs' demeanour. All observers (except 2 outliers) have been found within a 95% confidence region showing a high level of agreement. Two main dimensions of behavioural expression accounted for the 80.6% of the variation between lambs (44.8 % of the variation is accounted for dimension 1 and 35.8 % for dimension 2, see table 9 for more details). This confirms that the observers were able to evaluate lambs' behaviour using their own terms which had similar meanings when particular behavioural expression was shown. The terms most commonly associated with dimension 1 was: calm, comfortable (positive) and restless, painful (negative). The terms most commonly associated with dimension 2 were interested, curious (positive)

and lethargic, tired (negative). These terms were then used as a label (see figure 10) to give an overall understanding of the significant points with regard to each dimension.

Table 9 Correlation with dimensions 1 and 2 of the consensus profile. (Figures in brackets indicate how many of observers were using the term)

Dimension	Positive correlation	Negative correlation
Dimension 1	Calm (7), Comfortable (7), Relaxed (5), Sleepy (2), Quiet, Drowsy, Content	Restless (9), Painful (7), Irritable (7), Agitated (6), Tense(3), Twitchy (3), Aroused (2), Stressed (2), Sore (2), Annoyed (2), Fidgety, Distressed, Uneasy, Disturbed, Desperate, Suffering, Miserable, Alert, Jumpy, Aware, Content
Dimension 2	Curious (8), Interested (6), Inquisitive (4), Active (4), Responsive (3), Alert (3), Eager, (2), Engaged (2), Playful (2), Happy (2), Aware (2), Investigative, Exploratory, Interactive, Energised, Attentive, Excited	Tired (3), Quiet (2), Lethargic (2), Listless, Sleepy, Still, Subdued, Sad

Please note: Dim1 44.4%, r>0.6 correlation with the consensus axis (this means that dimension 1 explained 44.4% of the variation), Dim2 showed 35.8%, r>0.6 correlation with consensus axis (this means that dimension 2 explained 35.8% of the variation).

The analysis of the pattern of the impact of different castration procedures or handling on dimension 1 and 2 revealed that there is a significant effect of the treatment on dimension 1 ($F_{2,78}=13.12$, **p=0.001**) and dimension 2 ($F_{2,78}=13.12$, **p=0.001**). Post hoc analysis of the specific differences between treatments have revealed that with regard to dimension 1 RR treatment had the lowest score in comparison to COM, SSC and C rams (T=-6.213, **P=0.001**, T=3.458, **P=0.005**, T=-3.805, **P=0.001**). Furthermore, there was a tendency for SSC treatment to be scored as second lower as well when compared with COM rams (T = -2.8, P=0.03). With regard to dimension 2 post hoc analysis found significantly lower scores for RR and SSC rams in contrast with C treatment (T = -7.535, **P=0.001**, T = -5.904, **P=0.001**), It was also shown that emotional expression of C

group scored higher on dimension 2 than COM treatment (T=7.01, P=**0.001**). Figure 10 below is representing that pattern.

It was found that the RR castration treatment was negatively associated (lower scores on both dimensions) with restless, painful expressions (Dim1) and tired, lethargic expressions (Dim2). The COM castration treatment was scored in the majority positively on dimension 1 (calm, comfortable) and negatively on dimension 2 (tired, lethargic).A 1/3 of SSC clips was correlated with the top left quadrant of the expressive pattern (calm/comfortable and lethargic/tired), another 1/3 of SSC clips was associated with the bottom left quadrant (lethargic/tired and restless/painful) , while a final 1/3 of lambs in this treatment was associated with the positive region on dimension 2 (interested, curious). C treatment was scored positively on both dimensions, falling into calm, comfortable region on dimension 1 in 2/3 and.in 1/3 into interested, curious region of dimension 2.

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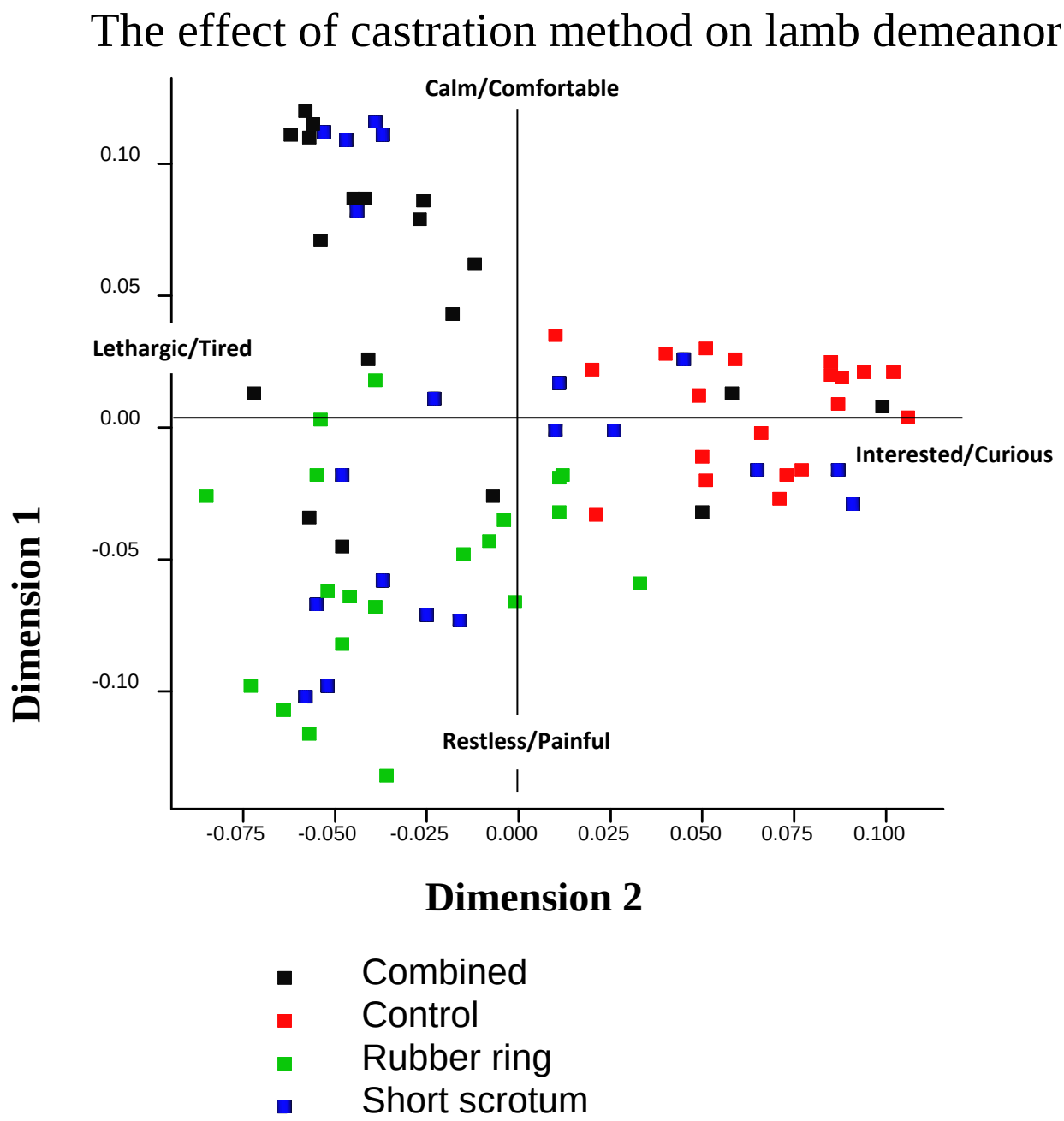


Figure 10 QBA analysis of lambs' behaviour after castration procedure or handling only. Black squares represent COM treatment, Red=C, Green=RR and Blue=SSC group. The figure is a sample plot of merged assessments conducted by all observers with the indication of how particular treatments were scored by the viewers. Axes reflect the level at which QBA terms load on to Principal Component's analysis

3.4.9 Comparison and correlation of Qualitative and Quantitative behavioural assessment

The table 10a-c below represents the results of the Kruskal-Wallis test showing significant differences between treatment groups in expressed duration and frequency of pain related behaviours and postures in the 2 min video clips. The Mann-Whitney test was then carried out to determine specific differences between treatment groups. Overall findings are similar to those presented for 30 min video recordings showing that the RR group was more painful than other treatments followed by SSC and COM treatment.

Table 10a Differences in the frequency of pain related behaviours expressed by entire males (C), rubber ring (RR), combined (COM) and short scrotum (SSC) treatment. Records are 2 min video-clips extracted from the 30-minute video footage documented at 2 days of age following castration or handling. Data are medians with Q1 and Q3.

Behaviour	C[1]	COM[1]	RR[1]	SSC[1]	P-Value[2]
Easing quarters	0.0(0.00-1.0)a	1.0(0.0-2.5)a	5.0(2.5-7.0)b	5.0(3.0-5.0)b	**P< 0.001**
Foot stamping/kicking	0.0(0.0-0.0)a	0.0(0.0-0.7)ac	3.0(2.0-6.0)b	0.5(0.0-2.5)c	**P< 0.001**
Head turning	0.0(0.0-0.0)a	0.0(0.0-0.0)a	0.0(0.0-1.0)ab	1.0(0.0-1.5)b	**P= 0.002**
Jumping	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)	**NS**
Teat seeking	0.5(0.0-1.0)a	0.0(0.0-0.0)b	0.0(0.0-0.0)b	0.0(0.0-0.0)b	**P< 0.001**
Shaking/trembling	0.0(0.0-2.0)a	0.0(0.0-0.0)b	0.0(0.0-0.0)b	0.0(0.0-1.0)b	**P= 0.002**
Wagging tail	2.0(0.0-4.0)a	0.0(0.0-0.0)b	0.5(0.0-1.0)b	0.0(0.0-3.0)ab	**P= 0.017**

[1]Median frequency (Q1-Q3). [2](amended p-value due to Bonferroni correction P<0.0125), NS not significant. Different letters indicate significant differences between treatments.

Table 10b Differences in the frequency of pain related postures expressed by entire males (C), rubber ring (RR), combined (COM) and short scrotum (SSC) treatment. Records are 2 min video-clips extracted from the 30-minute video footage documented at 2 days of age following castration or handling. Data are medians with Q1 and Q3.

Posture	C[1]	COM[1]	RR[1]	SSC[1]	P-Value[2]
Abnormal standing	1.0(0.0-1.0)	0.0(0.0-1.0)	1.0(0.0-2.0)	1.0(0.0-2.0)	**NS**
Normal standing	1.0(0.0-2.0)a	0.0(0.0-0.0)b	0.0(0.0-0.0)b	0.0(0.0-0.0)b	**P< 0.001**
Dog sitting	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-1.0)	0.0(0.0-0.5)	NS
Normal lying	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)	NS

Abnormal lying	0.0(0.0-0.0)a	1.0(0.0-1.0)b	1.0(0.5-2.5)b	1.0(1.0-1.5)b	**P< 0.001**
Lateral lying	0.0(0.0-0.0)a	0.0(0.0-0.0)a	0.5(0.0-1.0)b	0.0(0.0-0.0)a	**P< 0.001**

[1]Median frequency (Q1-Q3). [2](amended p-value due to Bonferroni correction P<0.0125), NS not significant. Different letters indicate significant differences between treatments.

Table 10c Differences in the duration of pain related postures (measured in seconds) expressed by entire males (C), rubber ring (RR), combined (COM) and short scrotum (SSC) treatment. Records are 2 min video-clips extracted from the 30-minute video footage documented at 2 days of age following castration or handling. Data are medians with Q1 and Q3.

Posture	**C[1]**	**COM[1]**	**RR[1]**	**SSC[1]**	**P-Value[2]**
Abnormal standing	35.0(0.0-91.7)	0.0(0.0-21.7)	22.6(0.0-34.0)	15.3(0.0-48.8)	**NS**
Normal standing	18.4(0.0-85.7)a	0.0(0.0-0.0)b	0.0(0.0-0.0)b	0.0(0.0-0.0)b	**P< 0.001**
Dog sitting	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-2.1)	0.0(0.0-1.1)	NS
Normal lying	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)	NS
Abnormal lying	0.0(0.0-0.0)a	80.1(0.0-120.0)b	55.0(5.1-93.1)b	84.9(15.8-120.0)b	**P< 0.001**
Lateral lying	0.0(0.0-0.0)a	0.0(0.0-0.0)b	1.1(0.0-41.3)c	0.0(0.0-0.0)ab	**p< 0.001**

[1]Median duration (Q1-Q3). [2](amended p-value due to Bonferroni correction P<0.0125), NS not significant. Different letters indicate significant differences between treatments.

Analysis of the correlation between 2 min data and the QBA data (Table 11) revealed that frequency of the quantitative measures of abnormal standing, foot stamping/kicking, lateral lying; easing quarters, active pain (combined behaviours of foot stamping/kicking, wagging tail, head turning, easing quarters, trembling, dog sitting), restlessness (frequency of postural changes) and the duration of lateral lying were negatively correlated with dimension 1 (Table 9). The frequency of the behaviours normal standing, teat seeking and trembling/shaking, and the duration of Abnormal standing and Normal standing were positively correlated with dimension 2 and the frequency of foot stamping/kicking behaviour, and duration of Abnormal ventral lying were negatively correlated with dimension 2 (Table 11).

Table 11 Spearman Ranks Correlation of QBA dimensions with quantitative data (2 min video-clips extracted from the 30-minute video footage documented at 2 days of age following castration or handling)

Frequency of Postures and Behaviours	Correlation with Dimension 1	Correlation with Dimension 2	P – Value Dimension 1	P – Value Dimension 2
Abnormal standing	**-0.468**	0.300	**0.000**	0.007
Normal standing	0.171	**0.518**	0.133	**0.000**
Dog sitting	-0.316	-0.226	0.005	0.046
Normal lying	0.164	-0.154	0.149	0.176
Abnormal lying	-0.195	-0.290	0.085	0.010
Lateral lying	**-0.505**	-0.293	**0.000**	0.009
Easing quarters	**-0.566**	-0.242	**0.000**	0.032
Foot stamping/kicking	**-0.753**	**-0.429**	**0.000**	**0.000**
Head turning	-0.264	-0.139	0.019	0.222
Jumping	0.080	0.173	0.481	0.126
Teat seeking	0.018	**0.533**	0.878	**0.000**
Shaking/trembling	-0.035	**0.435**	0.758	**0.000**
Wagging tail	-0.211	0.345	0.062	0.002
Active pain	**-0.739**	-0.073	**0.000**	0.523
Restlessness	**-0.506**	0.139	**0.000**	0.223
Duration of Postures	**Dimension 1**	**Dimension 2**	**P – Value Dimension 1**	**P – Value Dimension 2**
Abnormal standing	-0.318	**0.520**	0.004	**0.000**
Normal standing	0.170	**0.519**	0.135	**0.000**
Dog sitting	-0.333	-0.221	0.003	0.050
Normal lying	0.164	-0.154	0.149	0.176

Abnormal lying	0.144	**-0.466**	0.206	**0.000**
Lateral lying	**-0.491**	-0.312	**0.000**	0.005

Note: the cut off point for the correlation result to be regarded as meaningful in this study was 0.4. Correlations between 0.4-0.65 were regarded as moderate, while correlations higher than 0.65 were regarded as strong.

Analysis of the correlation between 30 min data and the QBA data (Table 12) revealed that the overall outcome of the analysis is very similar to the correlation of QBA with 2 min video clips. The differences lie in the following findings: There was no significant correlation between frequency and duration of the dog sitting posture as well as frequency of abnormal lying behaviour with dimension 1 in the 2 min footage in comparison to 30 min videos. Furthermore, behaviours like normal standing, trembling/shaking, and the duration of abnormal standing, normal standing and abnormal lying scored in 2 min clips were correlated with dimension 2 which was not observed in the correlation of QBA and 30 min recordings.

Table 12 Spearman Ranks Correlation of QBA dimensions with quantitative data (30-minute video footage documented at 2 days of age following castration or handling.

Frequency of Postures and Behaviours	**Correlation with Dimension 1**	**Correlation with Dimension 2**	**P – Value Dimension 1**	**P – Value Dimension 2**
Abnormal standing	**-0.513**	-0.227	**0.000**	0.044
Normal standing	0.107	0.154	0.350	0.175
Dog sitting	**-0.479**	-0.355	**0.000**	0.001
Normal lying	0.237	0.119	0.035	0.296
Abnormal lying	**-0.465**	-0.314	**0.000**	0.005
Lateral lying	**-0.566**	-0.314	**0.000**	0.005
Easing quarters	**-0.585**	-0.392	**0.000**	0.000
Foot stamping/kicking	**-0.578**	**-0.413**	**0.000**	**0.000**
Head turning	-0.186	-0.211	0.101	0.062
Jumping	0.094	0.360	0.409	0.001
Teat seeking	0.126	**0.410**	0.268	**0.000**
Shaking/trembling	0.057	0.339	0.617	0.002
Wagging tail	-0.357	-0.148	0.001	0.194
Active pain	**-0.605**	-0.298	**0.000**	0.008
Restlessness	**-0.551**	-0.281	**0.000**	0.012
Duration of Postures	**Dimension 1**	**Dimension 2**	**P – Value Dimension 1**	**P – Value Dimension 2**
Abnormal standing	-0.240	0.014	0.033	0.903

Normal standing	0.126	0.213	0.269	0.060
Dog sitting	**-0.417**	-0.291	**0.000**	0.009
Normal lying	0.273	0.121	0.015	0.286
Abnormal lying	0.257	0.081	0.022	0.478
Lateral lying	**-0.524**	-0.310	**0.000**	0.005

Note: the cut off point for the correlation result to be regarded as meaningful in this study was 0.4. Correlations between 0.4-0.65 were regarded as moderate, while correlations higher than 0.65 were regarded as strong.

Figure 11 below is representing the pattern of correlation between quantitative and qualitative assessments. The positive end of QBA dimension 1 was related to positive emotional state. The positive end of QBA dimension 2 was associated with curious, interested emotional state.

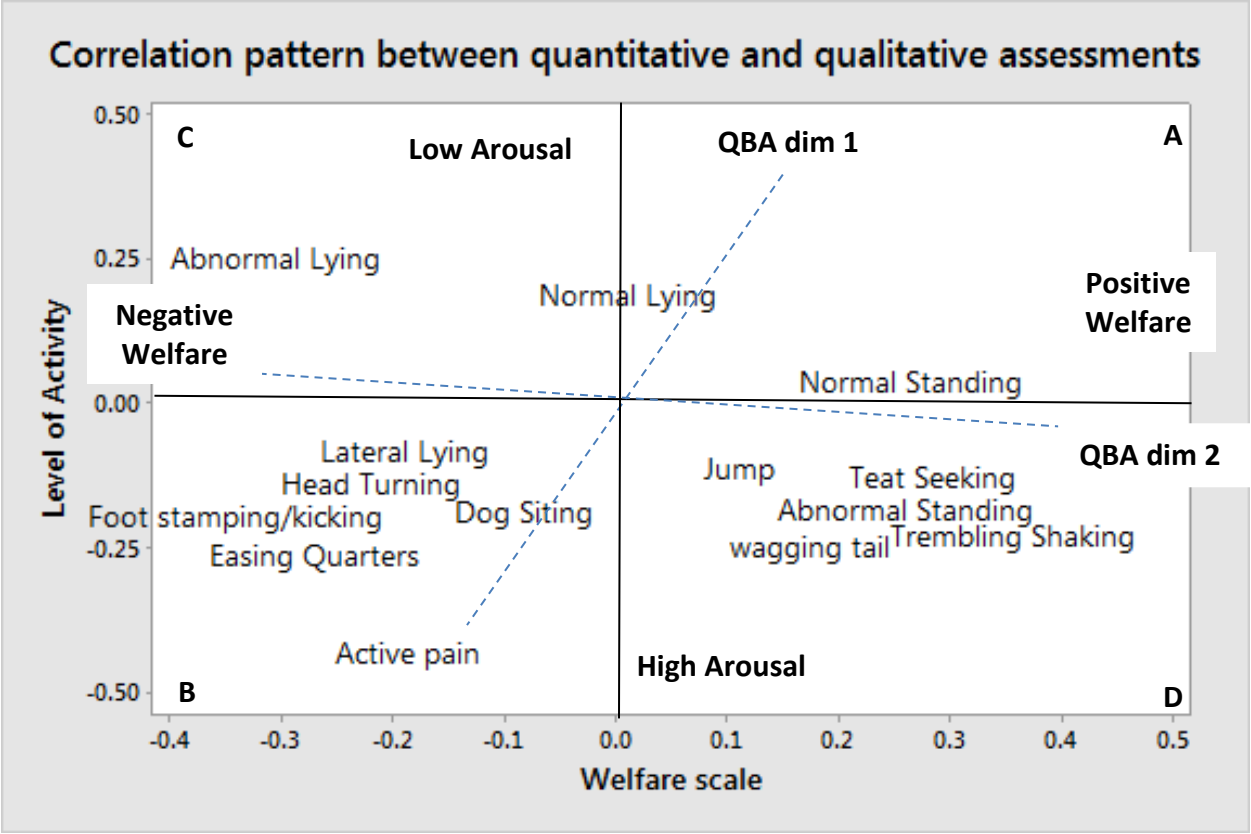


Figure 11 Pattern of correlation between quantitative and qualitative behaviour assessments. Letters A, B, C and D are used to indicate the 4 different quadrants of this correlational pattern. (A – QBA Calm/Comfortable, Normal standing; B – QBA

Restless/Painful, Dog sitting, Easing quarters, Foot stamping/kicking, Head turning, Lateral lying; C – QBA Lethargic/Tired, Abnormal lying; D – QBA Interested/Curious, Abnormal standing, Jumping, Wagging tail, Trembling/Shaking, Teat seeking). The endings of X and Y axis were labelled as Negative/Positive welfare and High/Low Arousal accordingly.

The analysis of the distribution pattern of the lambs from different treatments on the high/low arousal and positive/negative welfare axes are shown in figure 12. First of all, we can see that there is clear distinction between the control group and other treatments on both dimensions' dim 1 and dim 2. Kruskal-Wallis results for dim 1 were as follows: $P<0.001$, $H = 27.89$, $DF = 3$, median frequency C=0.1, COM=0.06, RR= - 0.05, SSC= - 0.008. The following results of Kruskal-Wallis test for dim 2 were noted: $P<0.001$ $H = 33.94$, $DF = 3$, median frequency C=0.06, COM= - 0.04, RR= - 0.03, SSC= - 0.03 (the minus mark in front of the results indicates a negative association with a particular dimension). The Mann-Whitney test results revealed significant differences between C treatment and all other treatments with regard to the median frequency of specific behaviours. Only the C group have shown significantly higher association with the positive side of the welfare scale in comparison to COM, SSC and RR treatment respectively ($P<0.001$, $U= 31.5$; $P<0.001$, $U=6.0$; $P<0.001$, $U=50.0$). the RR treatment has shown a significant association with the negative site of the welfare scale in comparison to C, COM and SSC treatment respectively ($P<0.001$, $U=30.0$; $P<0.001$, $U=31.0$; $P=0.01$, $U=106.5$). There was also a tendency for the COM treatment to show significantly higher scores on the negative site of the welfare scale in comparison to C lambs $P=0.02$, $U=112.5$.

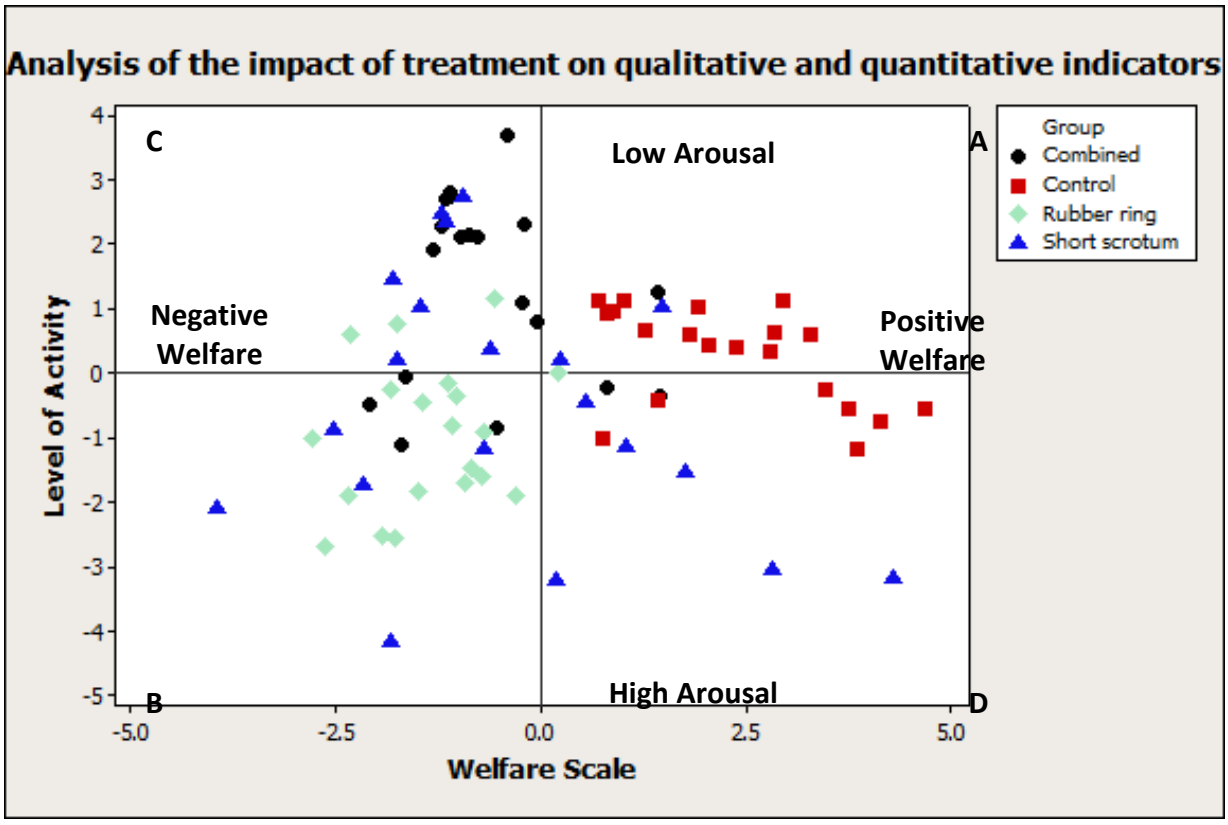


Figure 12. Distribution of lambs along the two main dimensions (welfare and arousal) of the combined PCA of qualitative and quantitative behaviour assessments.

3.5 Discussion

3.5.1 Assessment of different castration techniques

Overall findings reported in this study agree with previously described changes in the behavioural pattern of animals castrated with the use of different techniques showing that different castration methods will cause different quality and quantity of pain, having a negative impact on animals' emotionality and health at the same time (Molony et al., 1993; Kent et al., 1995; Lester et al., 1996; Molony & Kent, 1997; Thornton & Waterman-Pearson 1999; Kent et al., 2000, Thornton & Waterman-Pearson, 2002, Molony et al., 2002, Kent et al., 2004; Molony et al., 2011). According to the literature (DEFRA farm survey 2005) the rubber ring method is the most popular technique for lamb castration used in the UK. More than 95% of lambs in Great Britain are castrated

with use of rubber rings (DEFRA farm survey 2005). In this study the rubber ring castration method was found to be the most painful technique inflicting higher frequencies of pain related behaviours measured as a single behaviour or combined into one group of "active pain behaviours" (summary of the behaviours foot stamping/kicking, wagging tail, head turning, easing quarters, trembling/shaking and dog sitting) or restlessness (number of postural changes). This finding is consistent with the findings of increased scores of restlessness, foot stamping/ kicking and easing quarters reported by Molony et al., (2002, 2011). Similarly, Kent et al., (2000) have shown an increase in the frequencies of behaviours like foot stamping, tail wagging and head turning in lambs following rubber ring castration. Such increases of frequencies in these specific behaviours are indicators of severe acute pain. The duration of lateral lying posture observed in this study was also increased in the rubber ring treatment which may be highly linked with severe pain. This was also previously shown by Molony et al., (2002) who concluded that lateral lying with fully extended hind limbs is a good indicator of acute pain and when combined with other indicators is able to allocate lambs to groups of severe, mild and moderate pain. Elastration was also correlated with the most severe chronic pain sensation in this study assessed by measuring severity and size of the lesions as well as time to shed testicles and /or scrotum and heal the post castration wound. RR treatment was found to shed the scrotum later than COM and SSC rams. Similarly, time to heal the post-elastration wound was also longer in the RR treatment in contrast with the COM and SSC method. Furthermore, the size of the lesions inflicted by the RR technique was also greater. The short scrotum technique gave similar results to RR group. These results are consistent with previous reports describing the duration of chronic pain (measured by a single observer scoring animals' response to the palpation of scrotum and scrotum neck with use of visual analogue scale up to 72 h following castration which have lasted for a few days after the procedure (Thornton & Waterman-Pearson, 1999) and even up to 40 days after application of the rings (Kent et al., 2000).

SSC rams expressed greater amount of teat seeking behaviour in comparison to C, COM and RR treatments. Suckling has an analgesic effect and is thought to help lambs to cope with stressful situations (Gregory, 2004). Although the control group also expressed high levels of teat seeking behaviour it was mostly observed immediately after handling which is consistent with findings of Molony et al., (2002) who recorded increased suckling behaviour in handled only group of lambs in comparison to castrated lambs. It is possible that SSC method may be classified as the second most painful procedure which was assessed in this study. This is consistent with the findings shown by Molony et al., (2002) where the short scrotum technique was associated with a lower increase of cortisol concentration in comparison to elastration and a higher increase of cortisol when compared to tail docked, control (handled only) and lambs castrated with use of rubber rings and local anaesthesia.

The frequencies and durations of expressed behaviours and postures shown by the combined castration group were particularly difficult to assess due to long periods of immobility which may lead to the conclusion that this method was in fact painless. However, it was observed in this study that COM rams developed post-castration lesions which may be associated with some levels of pain therefore it is impossible to conclude that the combined method of castration influenced lambs in the same way as the group which was handled only even though they appear to give similar results especially for restlessness and active pain behaviour (see figure 1 and 2). It is highly probable that this technique of castration was also painful but it could not be fully assessed due to the type of chosen behavioural assessment protocol.

In the literature there is almost no information about the influence of the immunocastration technique on pain related behaviours and postures in vaccinated animals. In the present study the age when the lambs were physically castrated was different from the time when the rams were vaccinated therefore direct comparison of the methods was not possible. However, the comparison between handled animals and vaccinated lambs suggested almost no differences in behaviour thus it is likely that immunocastration was associated with very little pain or discomfort. One way to

investigate whether immunocastration is able to induce significant amounts of pain during the procedure is an evaluation of the reactions which will be initiated by the vaccination process. The immunocastration method with anti-GnRH vaccine (i.e. Improvac) is designed to promote specific reactions in the immune system which will lead to the formation and secretion of GnRH-specific antibodies that bind the hormones within the blood stream. immunocastrated animals have been previously found to have elevated levels of acute phase protein (in this case a α2-globulin also called Pig-MAP – a major acute phase protein in pigs) after vaccination (Faberga et al., 2010). Pig-MAP has been found to be a reliable marker of different pig pathologies (Alava et al., 2007). Secretion of acute phase proteins may be stimulated by adrenocorticotropic hormone, glucocorticoids (Gruys et al., 1994), inflammatory and neoplastic processes, stress, tissue damage and immune response (Le Floc'h, 2003). Acute phase proteins increase (positive acute phase proteins) or decrease (negative acute phase proteins) in response to inflammation. Inflammatory processes may be regarded as one of the main causes which are able to induce a significant pain sensation (Gregory, 2004). It has been shown that the use of surgical castration resulted in the increase of acute phase proteins (Faberga et al., 2010) therefore it is possible that such elevated levels of acute phase proteins may be a good indicator of pain. However, an increase in acute phase protein in both surgical and immunocastration may be explained by different causes. In the case of surgical castration, a rise in the levels of acute phase proteins may be associated with stress, infection and inflammatory process (Geers et al., 2003) which are likely to happen after the procedure. In the case of Improvac® an increase of acute phase proteins may be explained by the secretion of GnRH-specific antibodies. It is possible that increased levels of acute phase proteins was caused by this process and not by inflammation associated with tissue damage and pain (Faberga et al., 2010). This is also strengthened by the fact that Improvac® as an aqueous suspension induces only a very small response at the vaccination site (Dunshea et al., 2001).

Assessment of a novel immunocastration method for ram lambs with use of anti-GnRH vaccine developed by Zoetis showed that this method may be a more welfare friendly

alternative to traditional physical castration techniques. There were no significant differences between the control and vaccinated group in the expressed frequencies and durations of behaviours and postures assessed as a single event. There were no significant differences in expressed active pain behaviours as well. Immunization did not induce any negative impact on the lambs' tissues. There were no visible lesions found at the vaccination site in any of immunized rams throughout the whole study.

3.5.2 Assessment of QBA as a new pain recognition technique

In this study the Qualitative Behavioural Assessment was used for the first time to assess pain in farm animals. The results clearly showed that the blinded to the treatment observers were able to differentiate between control lambs and lambs which had undergone physical castration. Furthermore, the technique was able to differentiate the different castration treatments and allocate lambs to appropriate groups associated with mild or severe pain. The pattern of the impact of different castration procedures or handling on dimension 1 and 2 revealed a significant effect of the treatment on both dimensions. Clear distinction between lambs showing greater pain related behaviours and lambs with no pain related emotional state indicates that the observers achieved high level of agreement in their evaluation of post-castration or handling behavioural expression. QBA has been reported in the past to reach high level of agreement between observers in their assessment of sheep behavioural expression (Pythian et al., 2013) although these studies were not related to pain expression therefore could not have been directly compared. Moreover, the current study found a stronger dimension 2 (35.8% variation between lambs) than has been found previously in any other published QBA study. In contrast, in the most recent studies using QBA to evaluate emotionality in race horses, dimension 2 captured only 14.5% of the variation between animals (Fleming et al., 2013). The clear distinction between the control group, as well as the different castration treatments, which was established by the viewers, is the underlying cause for such strong dimension 2 in this study.

Overall results indicate that there is a moderate to strong correlation between quantitative and qualitative (QBA) measures of pain related behavioural expression of

rams following castration. Moderate to strong correlation of quantitative and qualitative measures has been also reported in the past adding to the value of QBA. Napolitano et al. (2012) described good correlation between QBA and frequencies of flight attempts as well as vocalizations in buffalos; similarly, Stockman et al. (2012) found correlation of QBA and plasma lactate concentration in dairy cattle before slaughter. Furthermore, the results shown in this study revealed good correlation between QBA and 30 minutes' footage which suggests that QBA may be used as an indicator of welfare in a wider timeframe and it may be successful tool in the assessment and recognition of different levels of pain and distress.

Combined PCA of quantitative and qualitative scores allowed for better understanding of the behavioural expression of lambs assessed in this study than any of those measures separately. Combined PCA also provided clearer picture particular in relation to behavioural pattern of lambs form the COM group. Whereas in the quantitative assessment the combined treatment showed similar results to the control treatment and evaluation of pain related postures and behaviours expressed by this group was very challenging. The judgement if the method is truly less painful was difficult due to low activity and low frequencies/durations of expressed postures and behaviours in the quantitative assessment. Correlation of both quantitative and qualitative methods of assessment added to the validity of QBA as a new pain recognition technique by showing clear distinction between the control and combined treatments. In the QBA COM group was scored by observers to express a low frequency of pain related behaviours Calm/Comfortable, similarly to controls, but fitted entirely in to the negative welfare site associated with Tired/Lethargic behaviours. This may indicate poor welfare that may be linked with low activity after the castration procedure. In contrast C lambs were associated with Interested/Curious behaviours indicating good welfare state.

Development of good pain assessment protocol is not an easy task. Scientists are in constant search of the best practical and economic method for the assessment of behavioural expression of pain and distress. In the past, researchers have applied several new approaches to assess animal welfare like: 'animal perspective' (Dawkins, 2008),

'cognitive bias' (Mendl et al., 2009) or 'appraisal theory' (Boissy et al., 2007). This study has presented QBA as a new method for assessing pain in castrated rams. QBA in this study used affective states of rams in the evaluation of animals' behavioural expression. QBA offered the 'whole animal' approach, which is able to integrate the whole range of animal behavioural expressions (like body language) and provide a valuable measure of emotional state (Wemelsfelder et al., 2001).

It has been shown in this study that investigating methods to mitigate pain in animals is a very important and relevant matter in animal welfare. Although some scientists do not acknowledge the capability of animals to feel pain (Bermond, 1997, 2001) arguing that animals are only capable of feeling an unpleasant painful stimulus but are not able to consciously transfer this information into the emotion of suffering. In recent years attitudes towards animal suffering and pain have been changing. This recent increase in prevention of unnecessary pain was caused by changing attitudes of public opinion towards preventable distress of animals. There are however difficulties with recognition and assessment of pain as well as use of pain mitigation techniques to minimize it. Whay et al., (2008) reported in the cattle clinicians' survey that half of the respondents admit to having inadequate knowledge of pain and its management. It was also concluded that the situation could have been improved by providing more information about pain, its assessment techniques, appropriate pain management and mitigation. Similarly, Hugonnard et al., (2004) in the survey of small animal clinicians showed that 84% of respondents would use analgesics following orthopaedic surgery of dogs and cats but only 17% would use analgesics following castration even though 96% of them were concerned about recognition and mitigation of pain in their patents. Results of this study have presented QBA as a new reliable method of pain recognition which may be used successfully in evaluation of different levels of pain following different castration procedures. QBA was able to detect subtle clues which were difficult to analyse or describe with use of other measures i.e. in relation to immobility behaviour. QBA was able to distinguish between calm comfortable periods of immobility and tired lethargic periods of immobility. Quantitative assessment used in this study was unable to

distinguish between those subtle clues which may have led to difficulties with regard to the evaluation of the level of pain inflicted by particular treatments. Moreover, result presented in here have shown that QBA could be potentially used to assess levels of pain related behaviours/postures during other husbandry practices in the farm setting (i.e. disbudding, dehorning, tail docking, mulesing) or by the small animal clinicians after surgical procedures which would allow for use of appropriate pain mitigation. QBA would also be a quick and economical technique that could be effectively implemented with only brief training at the beginning.

3.6 Conclusion

In conclusion it seems that the anti-GnRH vaccine tested in this trial causes less pain than any of the physical castration methods. It also suggests that the vaccine is safe to use in animals as young as 6 weeks of age as there was no site reaction to the active agent of the vaccine throughout the study period. The rubber ring castration was found to be the most severe method causing highest levels of acute (behavioural and postural changes) and chronic (size and severity of the post castration wound) pain. It was also found in this study that healing time and time to shed the scrotum and or scrotum sac following the RR castration procedure was extended in comparison to the other investigated methods. Assessment of alternative or modified physical castration techniques: short scrotum and the combined technique used in this study also showed that they were not pain free. Therefore, there is still a need to develop less painful methods to castrate lambs and improve lamb welfare. QBA was found to be a valuable new assessment method for recognizing pain in lambs which had undergone painful husbandry procedures by assessing the negative and positive emotional states of lambs. Evaluation of quantitative and qualitative methods of pain recognition and assessment has shown that both methods were successful in recognizing lambs' suffering, pain and distress. Qualitative Behavioural Assessment as well as use of quantitative behavioural scoring demonstrated a very clear distinction between control lambs and lambs experiencing the most painful castration treatment. Moreover, when both analyses were combined through PCA they gave stronger and better understanding of animal emotional

state which allowed for more robust assessment of the combined method of castration by evaluation of subtle clues which were difficult to score with use of quantitative measures only. Therefore, combining both types of assessment would be recommended as it provided stronger analysis of emotional state of lambs in this study allowing for more accurate conclusions in relation to inflicted levels of pain by particular castration treatment. The next step would be implementation of the new assessment method in the farm setting to evaluate the practicality and possible economic limitations of their use.

Chapter 4 The effect of immunization against gonadotropin-releasing factor (GnRF) on circulating testosterone, histology of the testes and the development of sexual behaviour in ram lambs

Abstract

Neonate lambs are often subjected to painful practices. Public opinion has become increasingly interested in recent years in mitigation of pain and distress in everyday husbandry practices. The effect of a novel anti-GnRF vaccine for castration of male lambs on circulating testosterone, testicular measures, development of sexual behaviour and the perception of the immunized rams by ewes during mating tests were investigated. The study involved 3 experiments conducted over three years. In total 188 mule x terminal sire (Texel or Suffolk) lambs took part in the study aged between 6 and 32 weeks. On the first year of the study the following treatments were applied: entire males - control group (C), rubber ring castrated rams (RR), short scrotum castrated rams (SSC), immunized rams with 0.5ml of anti-GnRF vaccine at 6 and 12 weeks of age (Vac1), n=20 per treatment. On the second year of the study the following treatments were formed entire males - control group (C) and group of immunized rams with 0.5ml of anti-GnRF vaccine at 6, 12 and 22 weeks of age (Vac2), n=12 per treatment. On the third year of the study the following treatments were formed: entire males - control group (C), rubber ring castrated rams (RR) and 6 groups of immunized rams with 0.5ml of anti-GnRF vaccine in a 2 dose regime accordingly to agreed protocol between 10 and 22 weeks of age (Vac3-6), n=14 per treatment. Testosterone concentration, scrotal circumference, and testes consistency were determined each year for C, SSC, Vac1-6 treatments. In addition, post-mortem testicular measures (weight, volume, width and length) and histology (average number and size of seminiferous tubules) were collected on the first (for C, SSC and Vac1 group) and second year (for C and Vac2 treatment) of the study. Frequency and duration of occurrence of reproductive behaviours were also measured each year (for C, RR, SSC, Vac1 on the first year of the study, C and Vac2 on the second year of the study and C, RR and Vac3-6 on the third year of the study) when rams were approximately 7 months of age. Vaccination against GnRF significantly influenced all measured parameters. Testosterone concentration, testicular circumference and testes consistency were suppressed from the time of the booster vaccination until 20 weeks of age on the 1st year of the study and until the end of trial in the following years (28-32 weeks). There was a significant difference in the number and the size of

seminiferous tubules between C and immunized rams with a higher number and smaller size of tubules (per slide/section) in the immunized group and reduced number and bigger size of tubules (per slide/section) in C rams. Reproductive behaviours were impaired in immunized rams and the control group showed consistently higher courtship and mounting behaviours than other groups. The level of reproductive behaviour suppression in immunized rams depended on the vaccination regime and the immunity period. Ewes' perceptions of rams during mating were also altered. Ewes directed a significantly greater frequency of aggression towards immunized and RR rams in comparison to entire males. Immunized rams were also observed to vocalize and attempting to escape at the frequency comparable to RR castrated rams.

Keywords: Ram, Reproductive behaviour, Testosterone, Testes, Immunization, GnRF

4.1. Introduction

Castration of pre-pubertal males is a routine on-farm practice allowing for the ease of management by reduction of aggression and fighting (Godfrey et al., 1996). Furthermore, it allows a better control of the flock genetics through elimination of inbreeding and unwanted pregnancies. Castration has been also found to improve carcass quality (Kiyma et al., 2000; Thornton et al. 1999; Amatayakul-Chantler et al., 2012). On the other hand, the most commonly used physical castration techniques (i.e. surgical castration, rubber ring castration, burdizzo castration) have been shown to be distressing and painful (Thornton & Waterman-Pearson 2002; Thornton & Waterman-Pearson 1999; Kent et al., 2000; Kent et al., 2004; Molony et al., 1993; Molony et al., 1995; Molony et al., 1997; Molony et al., 2002; Molony et al., 2011. See chapter 3 for more details). Increased interest of consumers in the suffering of castrated males doubled the number of studies attempting to find replacements of traditional castration techniques (Prunier et al., 2006). In 2008 in the UK the Farm Animal Welfare Council released a report (FAWC 2008) regarding sheep welfare where physical castration was considered to be very painful. Farmers were advised not to castrate their animals in situations when it is not crucial for the stock management. In other countries castration of certain species is forbidden i.e. in Norway surgical castration of pigs was banned in 2009 and in Switzerland castration of piglets without use of pain relief has been illegal since 2010 (Fabrega et al., 2010). Elimination of castration from standard husbandry management may seem to be a very good solution to the problem of reduced wellbeing and unnecessary suffering of livestock. However, banning castration even though it causes pain is not always possible due to economic circumstances, type of species and farming, livestock management practices, consumers demand for leaner meat and lack of viable alternative methods that do not cause distress. Therefore, techniques of pain mitigation following castration have been investigated by many scientists to improve the welfare of castrated animals. Non-steroidal anti-inflammatory drugs like carprofen, meloxicam, and ketocarprofen have been shown to minimize or reduce pain in the few hours after the procedure (Fisher et al., 2007; Molony et al., 1997; Stafford et al., 2002). However, there are some constrains on administration of such drugs in practice as this

requires legal approval, and additional costs, staff and time. For economic reasons use of non-steroidal anti-inflammatory drugs is not the best alternative to traditional castration methods. Equally non-steroidal anti-inflammatory administration of local anaesthesia products i.e. intratesticular and intrafunicular lidocaine injection is not practical due to further costs (supplementary staff and time). Similarly, attempts to apply other possibly less painful physical castration techniques, such as short scrotum castration (where the testicles are pushed back into abdominal cavity and the rubber ring is applied only to the neck of the scrotum sac) and the rubber ring is applied below the testis with one testicle remaining in the scrotum sac), the combined method of castration (rubber ring technique followed by immediate crush of the spermatic cords with burdizzo clamps) have been shown to be distressing as well. Although some of these techniques may induce lower frequencies of pain related behaviours and postures in comparison to rubber ring castration (the method commonly used by farmers due to its practicality and low costs of application) they are not pain free. In the light of these findings the search for the best possible pain free alternative is still valid and important.

Immunization against Gonadotropin realising hormone/factor (GnRH/GnRF) was believed to be a very promising new technique. Although immunization has been studied since 1970 (Fraser 1980 and 1986; Schanbacher, 1984; Chaffaux et al., 1985; D' Occhio, 1993; Thompson, 2000) implementation of this procedure is still limited. Immunocastration has been successfully implemented in species like cattle, pigs and goats. Immunized animals have shown improved feed efficiency and carcass conformation (Thompson 2000; Amatayakul-Chantler et al., 2013) and quality were found to be equal to entire males (Schanbacher, 1982). Immunization also decreased male taint and the concentrations of the hormones Luteinizing hormone (LH), Follicle-stimulating hormone (FSH) and testosterone in the blood (Godfrey et al., 1996; Janetta, et al., 2012). Testicle weight and size is reduced (Godfrey et al., 1996; Jeffcote et al., 1982) and the growth of testicles is delayed (Brown et al., 1994) leading to the conclusion that immunized males may be temporarily or permanently infertile which would be the desired outcome of the immunization. At the present time there are few

products available on the market that has been successfully used to immunocastrate males: Vaxtrate® for bucks, Improvac® for pigs and Bopriva® for cattle. However, there is little or no information on the effect of immunization as a castration method in sheep. The objective of this study was to evaluate efficacy of a new anti-GnRF vaccine (developed by Zoetis) for castration of ram lambs. To assess the ability of the vaccine to induce sterility, the impact of the vaccine on circulating testosterone, the growth and histology of the testes, the development of reproductive behaviour in rams and male attractiveness during mating behaviour tests were investigated.

Chapter 4 Hypothesis

1. Seven month old male lambs immunized against GnRH (accordingly to agreed protocols at 6 and 12; 6, 12 and 22; 10 and 16; 10 and 20; 12 and 18; 12 and 22 weeks of age) show sexual behaviour comparable to a physically castrated male lamb (using rubber rings), and less sexual behaviour than entire male lambs of a similar age.

2. Male lambs that have been made cryptorchids will show intermediate sexual behaviour.

3. Histology of the testes may be different in immunocastrated rams in comparison to entire males and short scrotum castrated rams due to differences in concentration of circulating testosterone.

4. An optimal regime for Immunocastration of male lambs will extend the period of immunity and reduce the expression of sexual behaviours during this period.

Chapter 4 Objectives

1. Evaluation of a new anti-GnRF vaccine (developed by Zoetis) for castration of ram lambs.
2. Assessment of the vaccine ability to induce sterility, the impact of the vaccine on circulating testosterone, the growth and histology of the testes.

3. Assessment of the impact of the vaccine on the development and expression of reproductive and aggressive behaviour in rams.
4. Assessment of the attractiveness of males from different treatments during mating behaviour observations.

4.2. Materials and methods

4.2.1 Housing and management

General husbandry procedures are described in section 2.3 of Chapter 2 where specific details of housing and management (section 2.3.2), allocation and implementation of treatments (section 2.3.3 see also table 1), time of weaning (section 2.3.4) and "the end point" (section 2.3.5) are defined. The study was conducted on the Woodhouselee farm (property of SRUC) Edinburgh, Scotland between April 2011 and December 2013. Separate groups of animals were selected each year (2011, 2012 and 2013) and allocated to specific treatment groups, to address specific hypotheses. The number of animals and the treatment groups were different each year and therefore they will be described separately. Although similar outcome measures were made in each year, as separate groups of animals were used every year to address specific questions, measures were analysed separately in each year.

4.2.2 Animals

Experiment 1 - 20 lambs per treatment were selected, balanced for maternal parity and sire breed (see table 1 in Chapter 2). Twin and single litters were used (if present – triplet lambs were reduced to 2 lambs prior to treatment). Four experimental groups were formed:

1. Positive controls (C) – lambs were handled only at 24-48 h of age.

2. Negative controls (RR) – lambs were castrated using standard rubber rings at 24-48 h of age (this group took part in the behavioural observations for the expression of sexual behaviour only. No other measures were taken).

3. Short scrotum castration (SSC) – the testes are pushed back into the abdominal cavity and a standard rubber ring was applied around the scrotum only (done at 24-48 h of age).

4. Immunocastration (Vac 1) – lambs were vaccinated at 6 and 12 weeks of age with an anti-GnRH vaccine.

Experiment 2 - 24 ram lambs were selected at birth, lambs were balanced for maternal parity and sire breed (see table 1 in Chapter 2). Only twin lambs were selected. Within litter, same sex twins received different treatments to allow comparison of possible maternal effects. Treatments were applied when lamb were between 24 and 48 hours old. Two groups of lambs were formed (n = 12 per treatment).

1. Positive controls (C) – rams were handled only at 24-48 h of age.

2. Immunocastration (Vac 2) – rams were vaccinated with the anti-GnRH vaccine at 6, 12 and 22 weeks of age.

Experiment 3 - 14 rams per treatment group were selected. Twin litters were used (if present – triplet lambs were reduced to 2 lambs prior to treatment). Lambs were identified as suitable at birth (i.e. healthy male twin-born lambs) and allocated to treatment group balanced for maternal parity and sire breed (see table 1 in Chapter 2). Six experimental groups were formed:

1. Positive controls (C) – lambs were handled only at 24-48 h of age.

2. Negative controls (RR) – lambs were castrated using standard rubber rings at 24-48 h of age (this group took part in the behavioural observations for the expression of sexual behaviour only).

3. Immunocastration (Vac 3) - lambs immunized at 10 and 16 weeks of age.

4. Immunocastration (Vac 4) - lambs immunized at 10 and 20 weeks of age.

5. Immunocastration (Vac 5) - lambs immunized at 12 and 18 weeks of age.

6. Immunocastration (Vac 6) - lambs immunized at 12 and 22 weeks of age.

4.3 Datasets description

4.3.1 Vaccination protocol selection

During the course of the study, 3 separate vaccination protocols were tested. On the first year of the study two-dose regime protocol was assessed. Lambs were vaccinated at 6 and 12 weeks of age with 0.5 ml of anti-GnRF vaccine. It was believed that this type of vaccine administration would best fit standard husbandry practices and mimics regimes used with similar products in other livestock species.

On the second year of the study, lambs were vaccinated at 6, 12 and 22 weeks of age 0.5 ml of anti-GnRF vaccine. The testosterone concentration, scrotal measures and histology data from the previous year of the study was preliminary analysed before the protocol was agreed. The vaccine was effective for 12 weeks. After 12 weeks, the immunity effect was no longer present. At this point, some of the lambs did not reach desired weight for slaughter. In a commercial setting, those lambs would have to be kept longer on the farm to gain appropriate weight. From the farm management point of view this meant that rams would have to be separated from the female lambs to avoid indiscriminate breeding which would add additional activities (i.e. separation of the stock) to normal husbandry practices. Before the vaccination protocol was agreed, the influence of the vaccine on the tissues as well as practicality of 2^{nd} booster injections was considered. There was concern regarding toxicity of the vaccine therefore larger doses were not used. It was decided that additional booster injection would be used at the time when, according to preliminary analysis of the first year study data, testosterone concentration was starting to rise again. This was thus a 'proof of principle' vaccination regime to test if testosterone could be suppressed throughout the rearing period in entire male lambs by vaccination.

In the third year of the study, the aim was to develop a two-dose regime which would fit the vaccination procedure into standard husbandry practices which might occur on a farm, to allow for more efficient administration of the vaccine without additional staffing, time and multiple gatherings of the flock. The main objective of this study was to find the most effective vaccination regime with use of 2-doses of the vaccine. There

was a concern that three dose regime of vaccination may have not be practical and economic therefore not useful in a farm setting. The intention was as well to vaccinate rams in the most practical way that will not lead to additional gatherings of the stock which would be time consuming and costly. Therefore, four different immunization regimes for primary and secondary vaccination were chosen using a 2 x 2 Latin square design to investigate the impact of weaning and delay in the time of booster vaccination administration on the immunity period. Rams were vaccinated at 10 and 12 weeks of age with 6 and 10 weeks' interval between primary and booster vaccination.

4.3.2 Immunization procedure

Each year lambs were vaccinated, using a Simcro® Safety Autovaccinator with 0.5 ml of a novel anti-GnRH vaccine developed by Zoetis administered subcutaneously on one side of the neck caudal to the ear (see figure 2b below). The Sekurus injector is designed to take 20-gauge x ½" hypodermic needles (Simcro®). The booster vaccination was administered at the same side of the neck as the primary vaccination after an interval of 6 – 10 weeks depending on experiment. During the vaccine administration process, lambs went through a race in small batches so no lamb would experience separation anxiety. There was an exception of the first year of the study when lambs were placed with their mother and sibling in a small pen approximately the size of 1.5x1.5m so the behavioural observations could be recorded. Immunization was performed by a single operator in each year.

Immunization was performed as per agreed protocols in each year of the study. Immunization was administered on the left side of the neck for the first 2 years and on the right side of the neck in the last year. Different intervals between primary and booster vacination were investigated in different experiments.

4.3.3 Scrotal Circumference and Testes consistency measures

Scrotal circumference was measured every 4 weeks commencing from 8 weeks of age by a single observer taking measure of the scrotal sack circumference with a metre tape. The measure was taken in the middle of a scrotal sack. Testes consistency was measured every four weeks as well starting from 8 weeks of age by manual palpation and scored

on a scale from 1 (very soft) to 4 (firm and hard). Table 1 below describes the testes consistency scoring scale which was used in the study. The scale was developed using practical knowledge and experience.

Table 1 Testicle consistency scoring scale

Score	Description
1	Very soft and easy to deform the original shape by gentle pressure.
2	Soft but elastic with some small tenderness, still easy to deform the original shape by gentle pressure but testicle goes back to its original shape after palpation quicker than in score 1.
3	Tender with some plasticity, not so easy to deform original shape (bouncy feeling, testicle goes back to its original shape very quickly after palpation).
4	Hard and firm with no plasticity at all, impossible to change original shape of testicle by palpation.

4.3.4 Collection of blood for testosterone concentration

Blood for testosterone concentration was collected every 4 weeks beginning when lambs were 4 weeks old and continuing until lambs left the study. The (BD Vacutainer®) 21G 1inch were used. For each lamb two serum separator (BD Vacutainer®) vacutainer tubes of 8.5ml volume were collected. Blood was taken only from male lambs, which did not undergo physical castration (with the exception of the short scrotum treatment group in 2011). Each tube was marked with a lamb ear tag ID, treatment code and the date, so the samples could be easily traced back. During the collection, harvested samples were placed into a tray in a cool box, which contained ice blocks to keep samples cool. When the collection was finished samples were loaded into a centrifuge. The settings were checked and set up for 2500 rpm, 4 º C and 20 minutes. Disposable pipettes (per lamb) were used to take out the serum from the vacutainers to the Thermo Scientific® 4.5 ml external thread cryo-tubes taking care not to get any blood cells in the sample. The serum separator tubes put a sticky clear plug over the top of the blood cells to facilitate serum collection. In the event of the sample being cloudy, or having obvious blood cells still free floating, a second spin was performed (this happened only on one occasion).

Serum samples were stored at -20ºC in a freezer until the time of shipment to Zoetis, Melbourne, Australia where further analysis of serum occurred.

4.3.5 Testosterone concentration analysis

Total serum testosterone concentration in the samples was determined using a DIAsource TESTO-EASIA (DIAsource ImmunoAssays S.A®) kit in Zoetis, Melbourne, Australia. In men testosterone comes in 90% from the testis. The rest of the circulating testosterone is secreted by peripheral tissues and the adrenal cortex. Therefore, in theory any test designed to detect concentration of circulating testosterone in the serum sample will sense testosterone levels secreted by testis in 90% and by other tissues in 10%. The principles of a method used to identify levels of testosterone were as follows. A fixed amount of testosterone was labelled with horseradish peroxidase (HRP) and compete with unlabelled testosterone present in the calibrators (used to make sure that the measured testosterone concentration is equally reliable and accurate across the plate wells), controls (reagent with known amount concentration of testosterone) and serum samples (samples of serum taken in the course of the study) for a limited number of binding sites on a specific antibody. The amount of substrate turnover was then determined by measuring the absorbance, which was inversely proportional to the testosterone concentration. A calibration curve was also plotted and Testosterone concentration in samples was determined by interpolation from the calibration curve. The test was carried out accordingly to the manufacturer's procedure. All reagents (calibrators, serum samples, see table 2 for more details) required to perform the test were kept at room temperature prior to use. The wells were identified and marked on a prepared earlier template representing particular wells. The 50µl of each calibrator, control and immunized animals' serum sample was pipetted into appropriate wells. Then 100µl of HRP conjugate solution was added (see table 2) to all wells. The Microtiter plate was incubated for 1 hour at room temperature. After that time the plate was washed 3 times by dispensing 0.4 ml of washing solution provided by manufacturers into each well (see table 2). Afterwards 100µl of the chromogenic solution – TMB was added (see table 2) to all wells and incubated for 30 minutes at room temperature and in the dark. After incubation time was finished 100µl of stop solution (see table 2) was added to all wells. The last step was to read the absorbance at the 450nm wavelength and the calibration curve was plotted to allow results identification.

Table 2 Reagents provided by manufacturers for the TESTO-EASIA test

Reagent	96 wells test kit	Recommendation	Colour code
Microtitemplate with 96 anti-TESTO coated breakable wells	**96 wells and a frame for it**	**Ready for use**	**Blue**
Conjugate - HRP labelled TESTO(HLPC grade) in TRIS-maleate buffer with bovine gelatine and thymol	**1 vial 1.2ml**	**Dilute 0.1ml in 1.0ml of conjugate buffer**	**Red**
Conjugate buffer - Phosphate buffer with bovine gelatine and thymol	**1 vial 21ml**	**Ready for use**	**Red**
Zero calibrator in human serum and thymol	**1 vial lyophilized**	**Add 1.0ml distilled water**	**Yellow**
Calibrator N = 1 to 5 (See exact value on vial label) in human serum and thymol	**5 vials lyophilized**	**Add 0.5ml distilled water**	**Yellow**
Wash Solution TRIS-HCL	**1 vial 10ml**	**Dilute 200 x with distilled water (use a magnetic flee0**	**Brown**
Controls – N = 1 or 2 in human serum and thymol	**2 vials lyophilized**	**Add 0.5ml distilled water**	**Silver**
Chromogenic TMB solution	**1 vial 23ml**	**Ready for use**	**White**
Stop Solution HCL 2N	**1 vial 25ml**	**Ready for use**	**White**

Preparation of reagents

Calibrators – Reconstitute the Zero calibrator with 1.0ml of distilled water. Reconstitute the other calibrators with 0.5ml of distilled water.

Controls - Reconstitute the controls with 0.5ml of distilled water.

Working TESTO-HRP conjugate – Prepare adequate volume of conjugate solution by adding 100µl of a concentrated TESTO-HRP to 1ml of conjugate buffer Use vortex to homogenize Extemporaneous preparation is recommended.

Working Wash Solution – Prepare adequate volume of Wash solution by adding 199 volumes of distilled water to 1 volume of Wash solution (200g) Use a magnetic flee to homogenize. Discard unused Working Wash solution at the end of the day.

4.3.6 Testes tissue collection

In December 2011 and 2012 lambs (physically castrated and the control group) were sent for slaughter at the age of 8 months to a commercial abattoir. The immunocastration group was euthanized on the Woodhouselee farm the following day (as these lambs could not enter the human food chain because the vaccine is not commercially available and so is not licensed for use in sheep destined for human consumption). Lambs were euthanized by the NACWO in the presence of NVS by overdose of Euthatal (Merial Animal Health Ltd®, solution for injection 200mg in 1ml, active ingredient - Pentobarbital Sodium. For details of the procedure please see the relevant section of this chapter).

Both testicles of each ram were collected in the years of 2011 and 2012. In the 2011 the collection involved testicle tissue of short scrotum (n=8), control (n=8) and Immunocastration treatment (n=8). In 2012 the collection involved tissue of control (n=10) and Immunocastration group (n=12).

A slaughter man manually collected the testicles on the line during the normal abattoir operation (in the case of treatments that were fit for human consumption) and passed on to me. I manually collected the testicles of immunized treatment groups in the post-

mortem facilities of the SRUC VI (Veterinary Investigation) centre. During collection, each testicle was placed in an individual bag with an L (left) or R (right) mark, kill number and/or the number of an ear tag so they could be related to each particular ram. The 1x1x1cm square, tissue samples of proximal, medial and distal transverse section of each left testicle were harvested. A separate room was assembled in the abattoir to allow for quick and efficient tissue collection and preservation. The epididymis was dissected free and the weight of each testis was recorded on an electronic scale (Sartorius Laboratory®). The length and width of each testicle was also measured by the electronic digital callipers (Vernier Callipers®). The volume of each testis was estimated by immersion in normal saline solution (solution of 0.9% NaCL/1l Norbrook®) in a graduated vessel.

Proximal, medial and distal transverse sections were taken (1x1x1 cm piece of tissue from slightly off-centre towards the periphery of each section) and placed in a labelled 50 ml universal tube (Cellarstar® Tubes) containing 15ml of freshly prepared Methacarn solution (60% Methanol, 30% Chloroform, 10% Acetic acid solution). Then the samples were placed in a chiller (Design Environmental®) for 24 hours. The temperature was monitored and ranged between 2-5º Celsius. The following day samples were taken out from the chiller and Methacarn was discarded. Samples were then placed in a plastic tissue embedding cassette (Tissue-Tek® Paraform® Sectionable Cassette System), labelled with the ram identification number, date of slaughter or euthanasia and submerged in a graduated vessel containing 70% ethanol for 4 hours. Afterwards samples were placed in a Tissue Processor (Leica® Jung TP 1050) which automatically processes tissue through a sequence of fixation, dehydration, clearing and paraffin impregnation steps. The processor was programed to process samples using various concentrations of ethanol to remove residual Methacarn. The program was set to last for 48 hours and submerged the samples in liquid paraffin (Lamb Wax Fisher Scientific®) resulting in samples being embedded in liquid paraffin. The cassettes containing tissues were taken out from the Tissue processor and placed in a Reicherd-Jung Tissues embedding Centre (IMEB INC.®). Each tissue sample was then taken out of the cassette

and placed in a metal container of the same size as the tissue cassette. A small amount of liquid paraffin was poured into the container and the tissue sample was gently pressed against the bottom of the container with dissection forceps for around 30s. This allowed for the fixation of the sample to the bottom of the container. Then the same tissue cassette was placed on top of the container and the remaining liquid paraffin was poured on top of the cassette. The structure was then placed on the ice surface of the Reicherd-Jung Tissues embedding Centre (IMEB INC.®) and left to cool down for half an hour. After that time the formed paraffin block could be easily taken out from the metal container. The tissue cassette formed a basis part of the paraffin block and contained information about ram identity (as it was labelled earlier; please see figure 1a, b). The paraffin blocks with embedded tissue inside were left in the fridge for few days at temperature of 2-5º C until further processing took place. Afterwards transverse histological sections (5µm) were cut from each sample using a Microm® Microtome. Cut section were carefully placed in the water bath (Thermo Scientific® Tissue Floatation Bath) set at 45º C and transferred to the histological glass slides (Thermo scientific® Menzel-Glaser Polysine® slides with dimension of 25x25x10 mm). Each slide was labelled with the same information as on the tissue cassettes. For each ram at least 10 transverse sections were harvested. Slides were then placed in the laboratory oven set at 50-60º C and left in the oven overnight so the paraffin could melt down leaving only tissue sample on the slide. Sections were stained with eosin and haematoxylin staining program in Leica® automatic Steiner XL and left out under the fume hood for one day. After 24 hours, stained sections were covered with Clearvue® Mountant XYL (Thermo Shandon Limited) and a cover glass (Thermo Scientific® Manzel-Glaser Cover glasses with dimensions of 22 x 50 mm) and left under the fume hood for another 24 hours.

Each section was then analysed under the microscope. For any individual animal 3 sections were selected to take part in further analysis. Digital images of each slide were made (please see figure 2-5 for representative samples) In total twelve images for each ram were taken (3 repetitions of 4 directions North, South, East and West of each slide)

and analysed using a Nikon® E600 microscope. The microscope was calibrated before images were taken (20x magnification lens was used).

The total number of tubules and the tubules area was manually counted in the ImageJ public domain program which is designed to display, edit, analyse, process, and save images. ImageJ can calculate area and pixel value statistics of user-defined selections and intensity threshold objects as well as measure distances and angles. The 20 µm scale was applied to each image. The average number of tubules as well as spaces taken by the tubules in the area of 20µm was calculated for each ram by adding results of the counts.

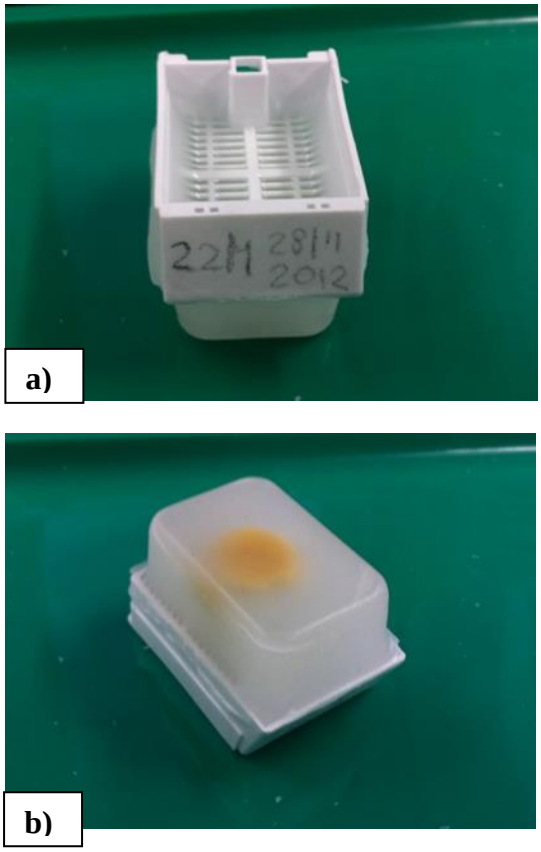
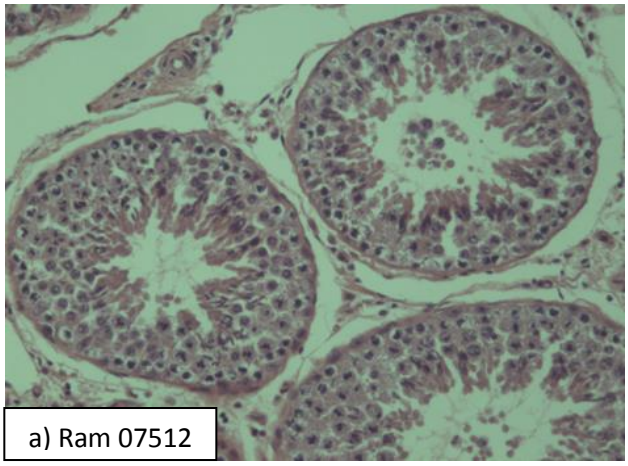
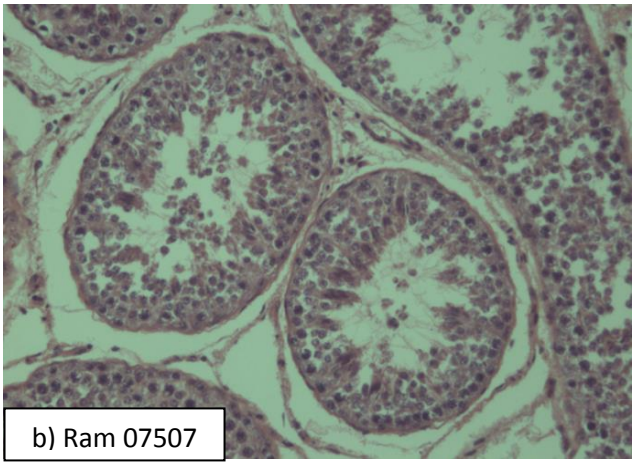
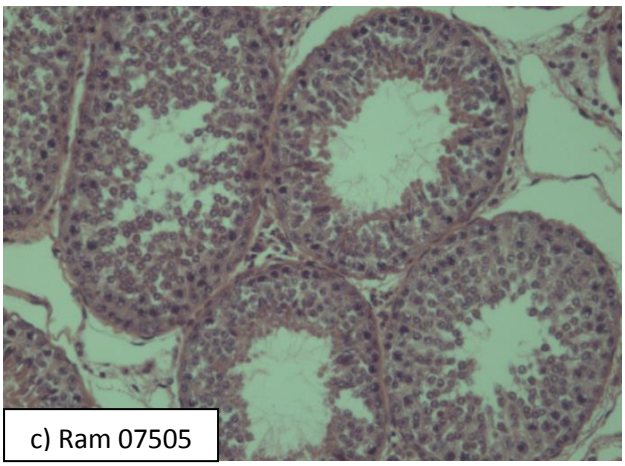


Figure 1 a, b Paraffin block with embedded testicle tissue sample







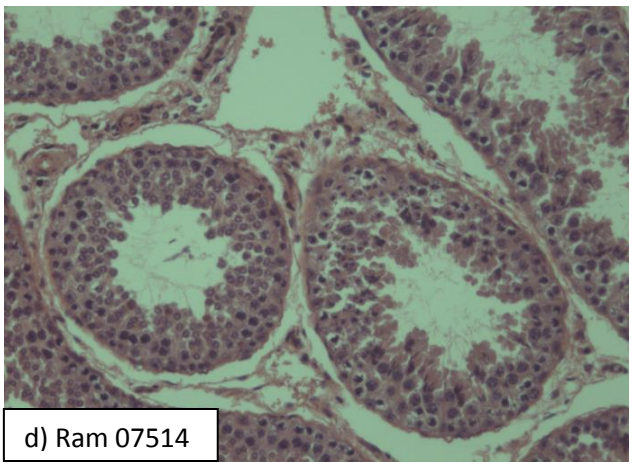
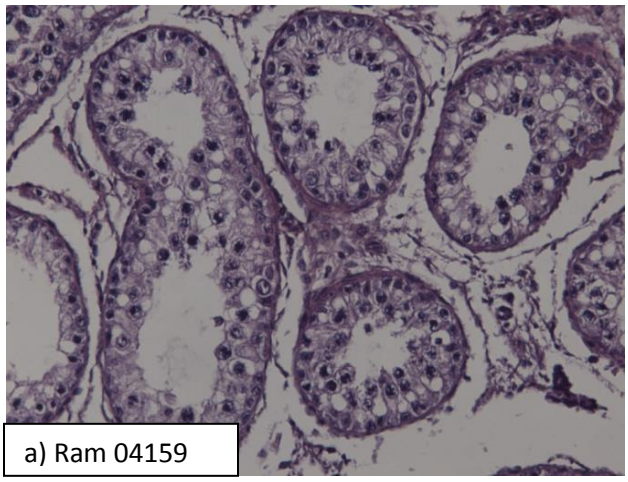
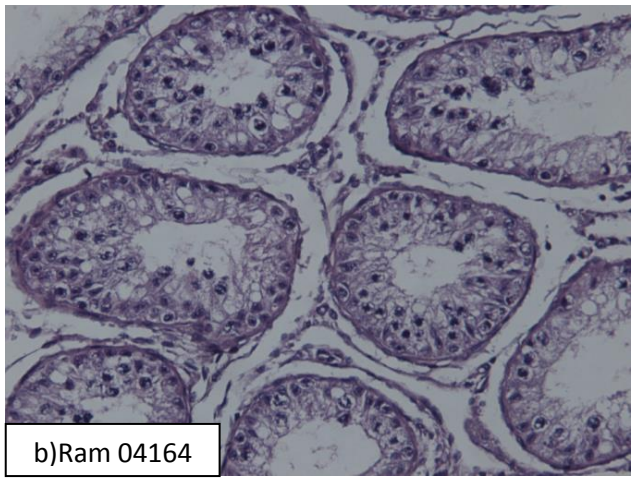
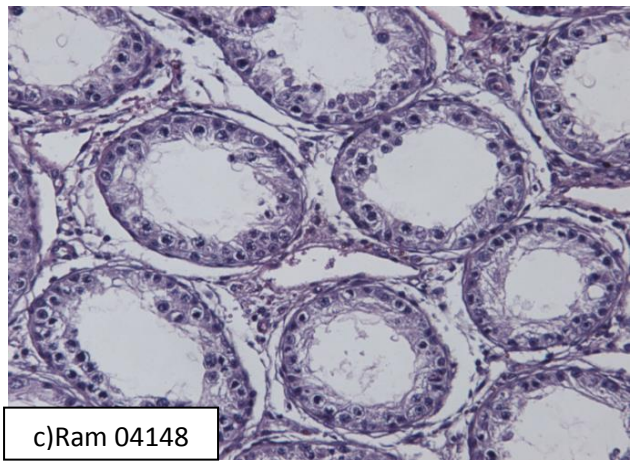


Figure 2 a-d presents representative trans-sections of Testis Tissue of control group (entire male).







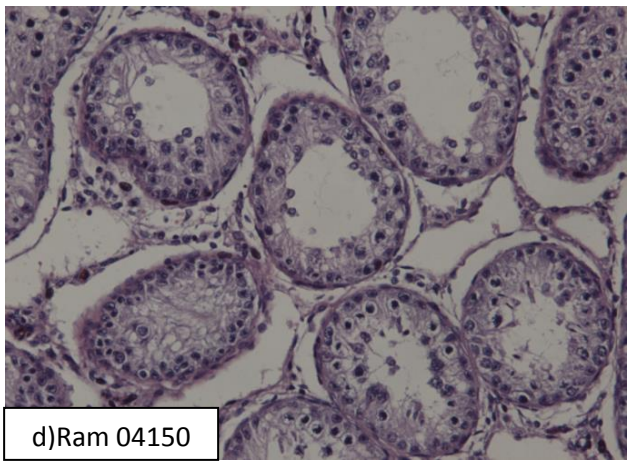


Figure 3 a-d Representative trans-sections of Testis Tissue of short scrotum castration group

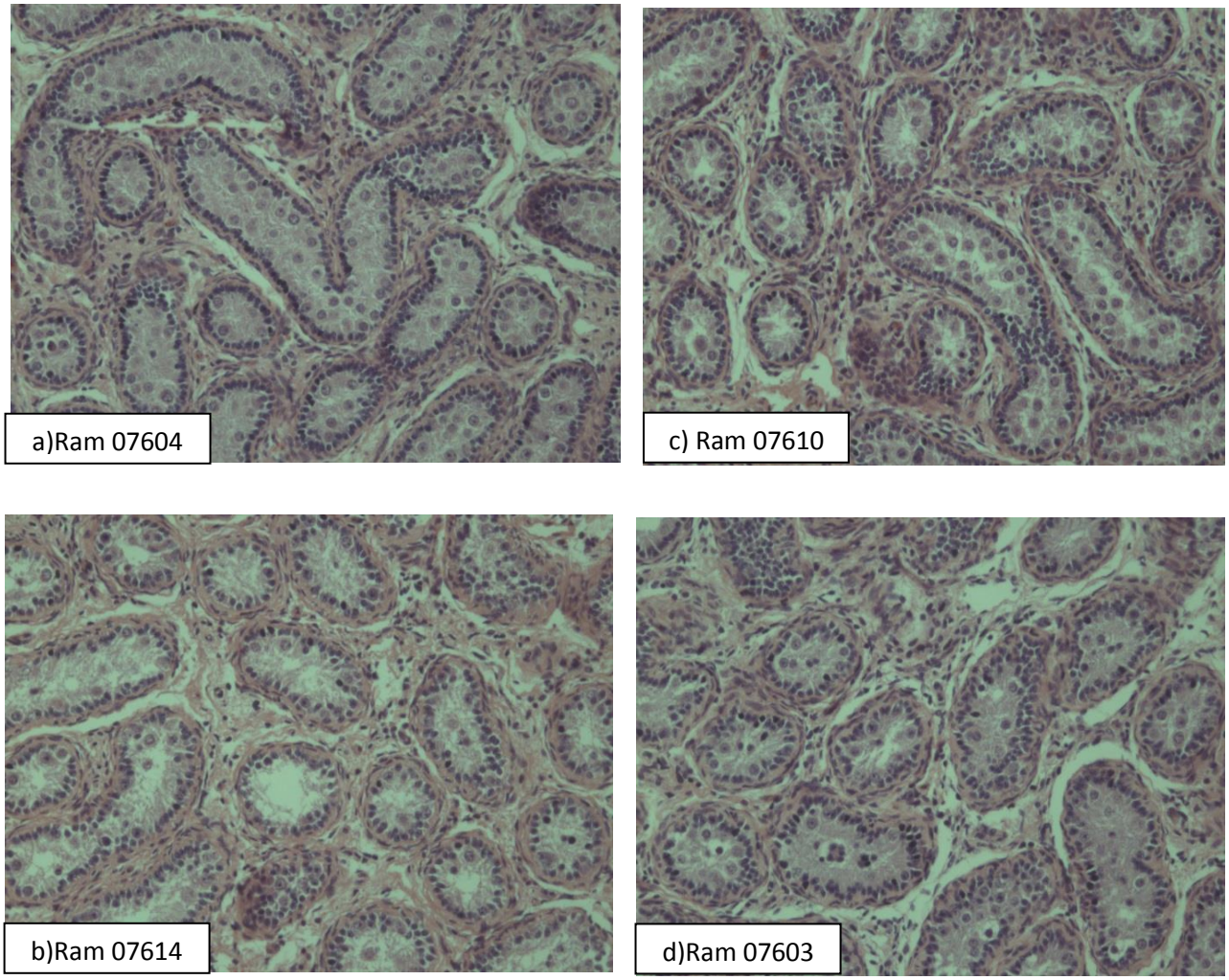
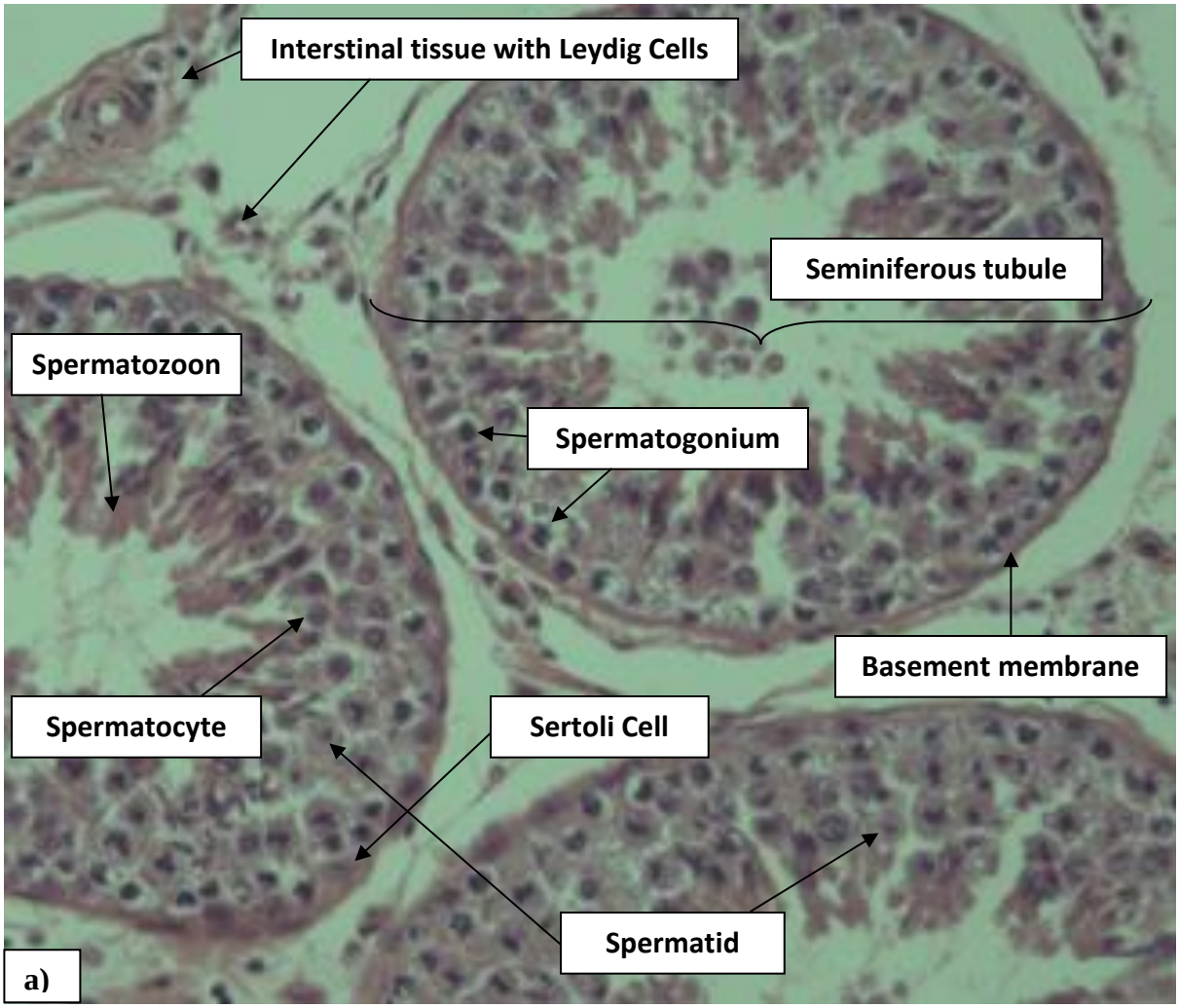


Figure 4 a-d. Representative trans-sections of Testis Tissue of Immunocastration group


Interstinal tissue with Leydig Cells
Seminiferous tubule
Spermatozoon
Spermatogonium
Basement membrane
Spermatocyte
Sertoli Cell
Spermatid
a)

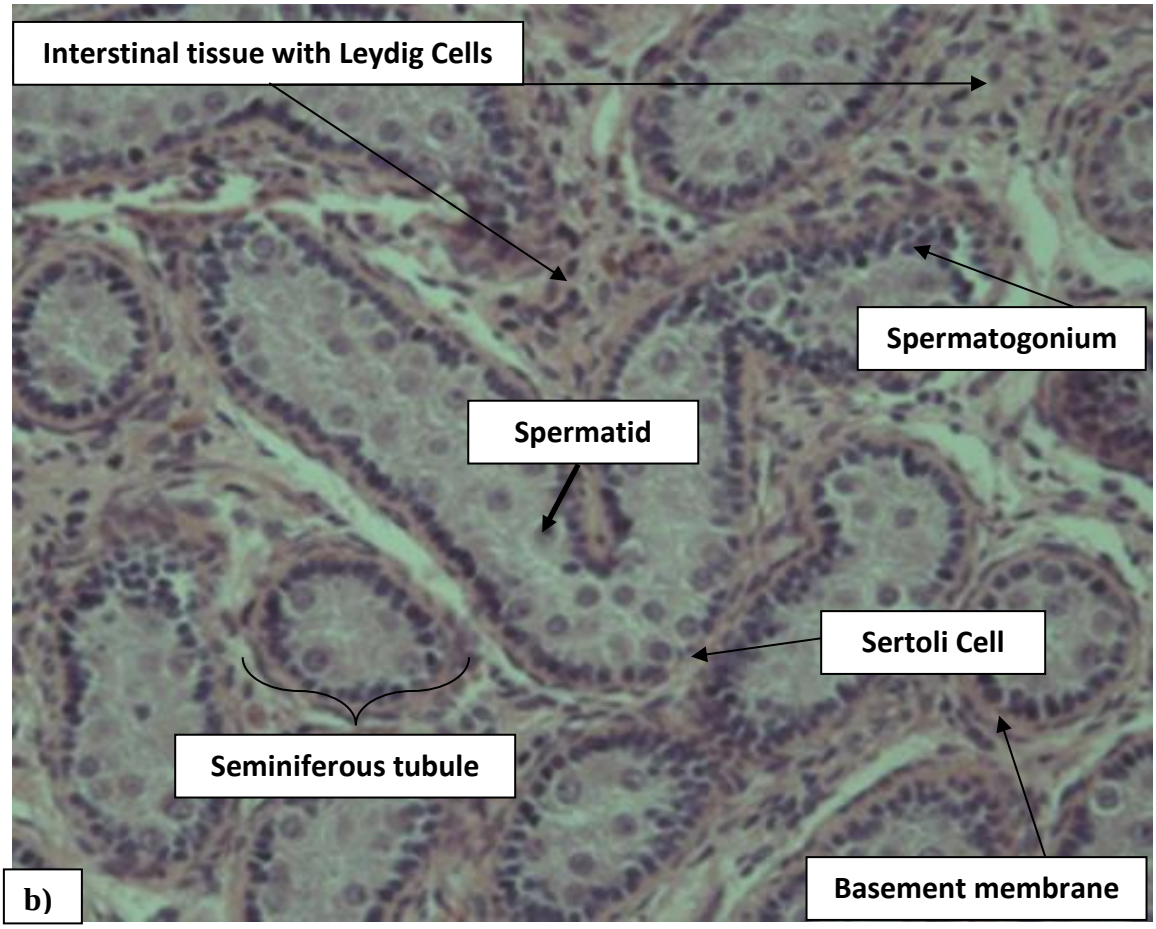


Figure 5 a, b Comparison of representative Testicle tissue trans-sections of control (a) and immunocastrated ram (b). In the figure b we cannot find spermatocytes and spermatozoon cells. The tubules size and number are also different (the scale of 20µm was applied to each image to allow the comparison).

4.3.7 Expression of Sexual Behaviours

4.3.7.1 Selection of the females

Each year Mule ewes were selected to take part in the mating behaviour tests. Each year different numbers of ewes were selected to take part in the sexual behaviour observations (Experiment 1: 16 ewes; Experiment 2: 15 ewes; Experiment 3: 30 ewes).

4.3.7.2 Oestrus induction

One of the objectives of this thesis was to determine if manipulation of circulating testosterone will have an effect on rams' sexual behaviours at the time of puberty. The aim was to define quality and quantity of sexual behaviours expressed by the rams immunized against GnRF. To test this specific area of interest rams had to be exposed to the females which would be in the state of oestrus mimicking naturally occurring reproductive behaviours of sheep. Therefore, each year selected ewes were induced into oestrus (in October/November) with Chronogest CR 20 mg vaginal sponge for sheep (MSD Animal Health®). The Chronogest vaginal sponges contain the Pregnant Mare Serum Gonadotropin (PMSG) which is a gonadotropic hormone produced in the chorion of pregnant mares used to induce ovulation in livestock prior to artificial insemination. This method of inducing ovulation by administration of PMSG is widely used in species like sheep, goats, swine and cattle. PMSG in mentioned species activates both follicle-stimulating hormone (FSH) and luteinizing hormone (LH). To induce oestrus in selected animals the following procedure was performed. Ewes were brought into the shed no 2 (described earlier) on the first and second year of the study and into the shed no 1 (also described earlier) on the third year of the study. The stocking densities and housing followed the same rules described in the section 2.3.2 of this chapter. Water and hay were offered ad libitum, additionally ewes were fed 0.5 kg per ewe 2 x a day of concentrated feed: XL Ewe 18% + Amino Green® Rolls (®East Coast Viners Animal Nutrition). Chosen ewes stayed in the shed throughout the induction process as well as during behavioural observation until all rams had been tested for the presence or absence

of sexual behaviours. The bedding was changed every third day (fresh straw was added) to make sure that the dry and comfortable lying space was present at all times.

Sponges were applied by same operator every year for ten consecutive days to allow secretion of PMSG accordingly to agreed protocol (day 1 was the day of sponge application and day 11 was the day of sponge removal). Afterwards sponges were withdrawn and the presence of oestrus was expected to occur after 48 hours from removal. In the event of delayed oestrus occurrence (this was determined by the willingness of ewes to mate with the presented rams) ewes were not used in the tests with the rams but were replaced with additional ewes also induced into oestrus at the same time showing willingness to mate with rams. This situation happened on two occasions during the whole study. The number of ewes which went through the oestrus induction process each year will be given in the relevant chapter.

4.3.7.3 Male Sexual behaviour observations

In the first and third experiments eight animals from each sub-group took part in the sexual behaviour tests, for practical reasons and to limit the number of ewes required to be brought into oestrus. Previous work has shown that this is a sufficient number to show statistical differences between treatments in the expression of courtship and sexual behaviours. Specific ram lambs were randomly selected to take part in the behavioural observations with use of the R statistical programme. In the second experiment all rams from the control (n=10) and immunized treatments (Vac2, n=12) were scored for the expression of courtship and sexual behaviours. Three groups of n=8 (4 control and 4 Vac2 rams), n=7 (3 control and 4 Vac 2 rams) and n=7 (3 control and 4 Vac 2 rams) were formed and tested one after another. All sexual behaviour tests were performed in November when rams were approximately 7 months old. In total 32 animals were observed in the first experiment, 22 rams in the second and 48 in the third.

One day before testing rams were brought to the housing shed and kept there overnight in a large straw-bedded pen, with oestrus ewes (housed in separate pens but in the same shed) before the testing period. All animals had access to hay and water ad libitum. Rams had physical access to ewes only during testing. Two testing pens (2m x 2m) were

prepared. The pens were blinded from each other so rams could only smell the presence of one other but there was no visual contact.

Tests in experiments 1 and 2 involved 6 repeated sessions of observations each 20 min long over 2 days (3 tests per day). During the testing period individual ewes were changed after a single test (each ewe took part in 3 tests a day). Each ram was tested individually 3 times a day with a different ewe.

Tests in the 3rd experiment involved 24 rams (4 rams from each sub group) tested once a day for 20 min over 6 consecutive days. In total all rams were tested in two - 6 days long sessions. Similarly, to previous years each ewe was changed after a single test and took part in 12 tests a day. The frequency of courtship responses and mounting attempts (table 20) were video recorded and scored thereafter.

All animals were marked with spray marks on their backs so they could be individually identified from the video. Behaviours were recorded continuously for 20 min by a Canon XM2 3CCD Digital Video Camcorder (Canon Inc., Japan). The order of the testing was randomized for each ram so the influence of time of the day on the performance of rams was minimized. This also allowed individual rams to be tested with different ewes in different pens. For each test the number and type of courtship behaviours and actual mounting were recorded (see table 3 for the reproductive behaviour ethogram). Moreover, due to fact that ewe responses to ram courtship may affect male attractiveness to the ewes, ewe behaviours during the tests in the 1st and 2nd experiments were also scored and analysed. Table 4 shows the ethogram of female reproductive behaviours that were considered during scoring. Additionally, to reproductive behaviours, received aggression, expressed aggression, vocalizations and escape attempts were recorded as well (see Table 22 for ethogram of those behaviours).

All of the recordings were then scored in the Observer XT 9 (Noldus Information Technology®) program by a single observer for the presence, duration and the frequency of occurrence of courtship, sexual and other behaviours (table 3, 4 and 5 below for details).

Table 3 Ethogram of Male Sexual behaviours (based on Lynch et al., 1992)

Behaviour	Description
Sniff/nose	Touch the muzzle to urine or perineal region of ewe with or without licking. Rub the perineal region of a ewe with its muzzle.
Flehmen	After sniffing the ram arches his head up and curls the upper lip showing his teeth.
Mount attempt	Ram is attempting to mount (set of back and forward movements of the pelvis while standing in front of or to the side of ewe's rump), jumps on the back of the ewe with or without firm contact of ram brisket with ewe's rump.
Mounting movements	Ram stands on the ground with all four legs and performs series of back and forward movements with its pelvis and limbs. Usually seen just before or after mounting attempt.
Lick	Licking ewe's flank, running tongue in and out.
Nudging	Combination of kick (forefeet used in a pawing motion while standing parallel to ewe), rubbing (the ram rubs head and shoulders along or under ewe's flank).
Low Stretch	Neck being held horizontal to the ground with the muzzle forward and raised. Head can be turned through 90° as well, with or without running tongue in or out.
Male follow	Ram is following female usually in a circling movement.

Table 4 Ethogram of Female Courtship and Sexual Behaviours (based on Lynch et al., 1992)

Ewe Reproductive Behaviours	
Crouch	Ewe takes a crouching posture, it may involve urination and usually occurs after nudging by the ram.
Circling	Ewe turns back toward the ram, often nuzzling his flank, ram follows to retain his position behind the ewe.
Tail wagging	The ewe tail is elevated and fanning in the presence of the ram. One action was counted for one series of fanning until it stops.
Head Turning	Ewe turns her head back toward the ram usually when ram is standing behind or at the side of the ewe.
Standing firm	Ewe stands firmly with four feet on the ground and does not move when ram attempts to mount and during mounting behaviour.

Following male	Ewe follows the ram attempting to make initial contact with the ram. Usually seen when ram is moving away and does not make any physical contact with ewe or contact is very limited.
Moving away	Ewe moves forward while male is attempting to mount.

Table 5 Ethogram of other behaviours

Flight or Fight Behaviours	
Received aggression	Individual is being butted, pushed, or made to leave its position by another individual.
Escape attempt	Individual jumps on the side of the pen, each jump was counted as one action, attempts to leave the pen under the barriers (put head under the barriers and performs up and down movements with its head which was counted as one action), actual escape from the pen is recorded.
Vocalizations	High pitch vocalizations.
Avoiding	Individual is leaving its position and does not attempt to make physical contact with another individual during courtship, sexual or received aggression encounters.
Expressed aggression	Every single head butt, push with head or whole body, displacement of another individual was counted as one action.

4.3.8 Correlation of plasma testosterone concentration and expression of sexual behaviours

The four most distinct sexual behaviours were selected from the male sexual behaviours ethogram to take part in the analysis (mounting attempts, nudge, fehmen and low stretch). It was believed that reduction or lack of circulating testosterone due to physical castration or immunization against GnRF will result in the reduction or absence of these behaviours during exposure of rams to oestrus ewes. For each individual ram frequency of expressed sexual/courtship behaviour was correlated with plasma testosterone level

measured for that individual ram approximately at the time of the sexual behaviours observations (~28 weeks of age).

4.4 Statistical analyses

The distribution of data was analysed in Minitab statistical package 16th edition (Minitab, Inc, State Collage, PA). The Anderson Darling test was used to test the distribution of the data. It was impossible to normalize the testosterone and behavioural data therefore it was decided that Kruskal-Wallis and Mann-Whitney non parametric tests were done to determine significant differences between treatment groups using Genstat 14th edition Genstat (GenStat® 14th edition, Lawes Agricultural Trust, VSN International Ltd, Oxford, UK). All non-parametric data was analysed this way. The Bonferroni correction was also applied to account for the multiple testing. In practice to correct for multiple comparisons the critical p-value of 0.05 has to be divided by the number of comparisons (treatment groups of the particular study). In this chapter due to different number of treatment groups in particular experiments the new critical p-value will be as follows; a) for experiments with 6 treatment groups new p – value would be $P<0.008$; b) for experiments with 5 treatment groups new p – value would be $P<0.01$; c) for experiments with 4 treatment groups new p – value would be $P<0.0125$; d) for experiments with 3 treatment groups new p – value would be $P<0.016$. For the experiment with 2 treatment groups the p – value would be $P<0.05$. The Bonferroni correction does not need to be applied in this situation. Normally distributed data (i.e. scrotal circumference, testes consistency, size of seminiferous tubules) was analysed with the use of REML (Restricted or residual Maximum Likelihood Mixed Model, GenStat® 14th edition, Lawes Agricultural Trust, VSN International Ltd, Oxford, UK) statistics. Ewe parturition (number of previous pregnancies) sire breed and litter size were fitted as fixed effects. Birth weight was fitted as a covariate. Two sample T-tests were carried out to determine significant differences between treatment groups. Bonferoni correction was also taken into account. Sperman rank correlation was carried out in Minitab statistical package 17th edition (Minitab, Inc, State Collage, PA) to detect

whether there was a relationship between expression of courtship and sexual behaviours and plasma testosterone concentration.

4.5 Results

4.5.1 Testosterone concentration analysis

Experiment 1

Circulating testosterone concentrations were low in all animals for the first 16 weeks of life. Significant differences between treatments were seen at 12, 16 and 18 weeks of age respectively (Kruskal-Wallis test results H=27.72 P<0.001; H=19.15 P<0.001; H=7.24 P=0.02). There were no differences between treatment groups in the median level of testosterone when lambs were 4, 8, 24 and 28 weeks of age (see figure 6 below). Post Hoc analysis (pairwise comparisons with Mann-Whitney tests) demonstrated that the Vac1 group had a lower testosterone concentration in comparison to C and SSC respectively at 12 and 16 weeks of age (12 weeks: U=26.0, **P<0.001** and U=34.0**, P<0.001,** 16 weeks: U=55.0**, P<0.001 and** U=66.0, **P<0.001).** There were no differences between C and SSC treatment (figure 6). At 20 weeks Vac1 lambs had a lower testosterone concentration than the SSC group (figure 6, U=100.0 **P=0.011**), but did not differ significantly from C lambs (figure 6, U=119.0, P=0.047). There were no significant differences between C and SSC lambs.

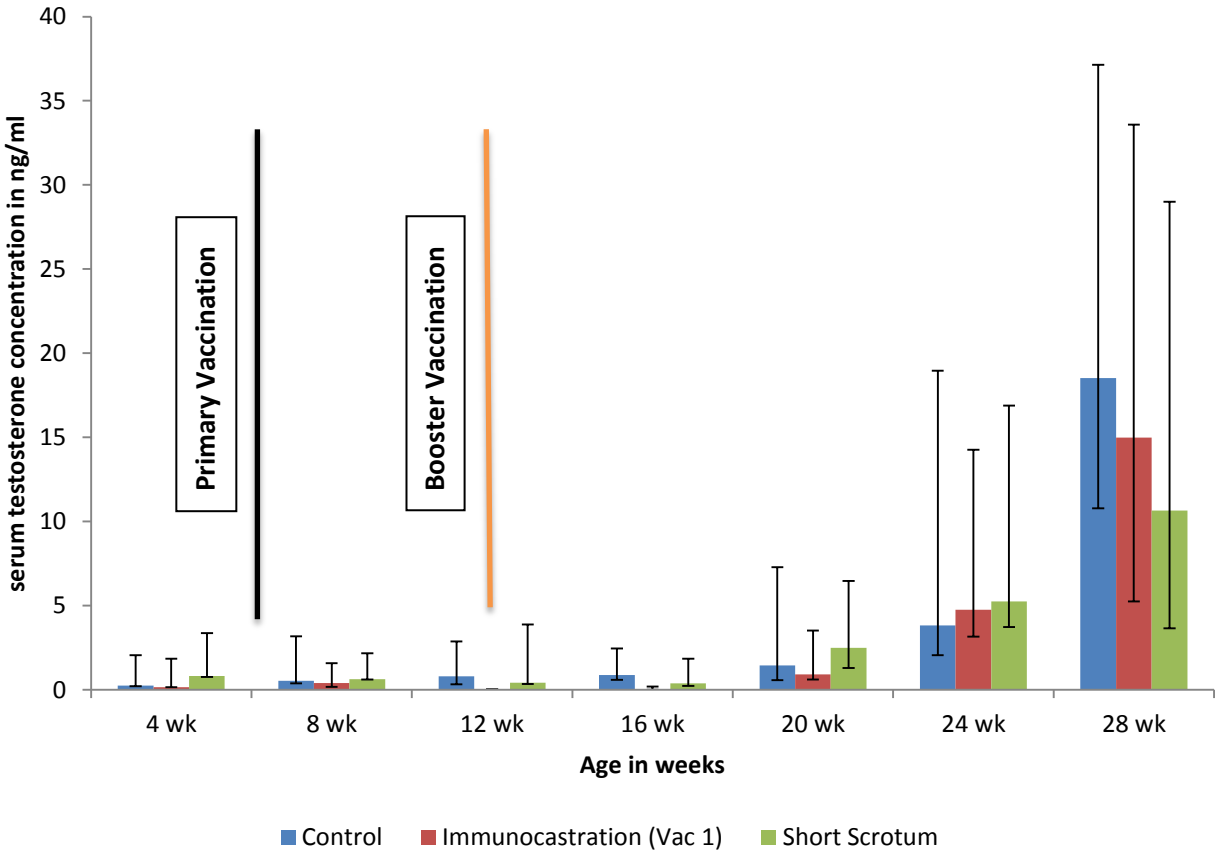


Figure 6 Differences in testosterone concentrations (ng/ml) between Control, Short Scrotum and immunocastration treatment recorded monthly until 28 weeks of age. Data are medians with upper and lower inter-quartile ranges Q1 and Q3. Timing of primary booster is indicated by the black vertical line and of the booster vaccination by an orange vertical line.

Experiment 2

In experiment 2, when a second booster vaccination was given at 22 weeks of age the Immunized group had lower serum testosterone concentrations than the control group from 12 weeks onwards, until the experiment ended at 28 weeks of age respectively U=12.0 P<0.001; U=13.0 P<0.001; U=4.0 P<0.001; U=0.0 P<0.001; U=0.0 P<0.001 (see figure 7).

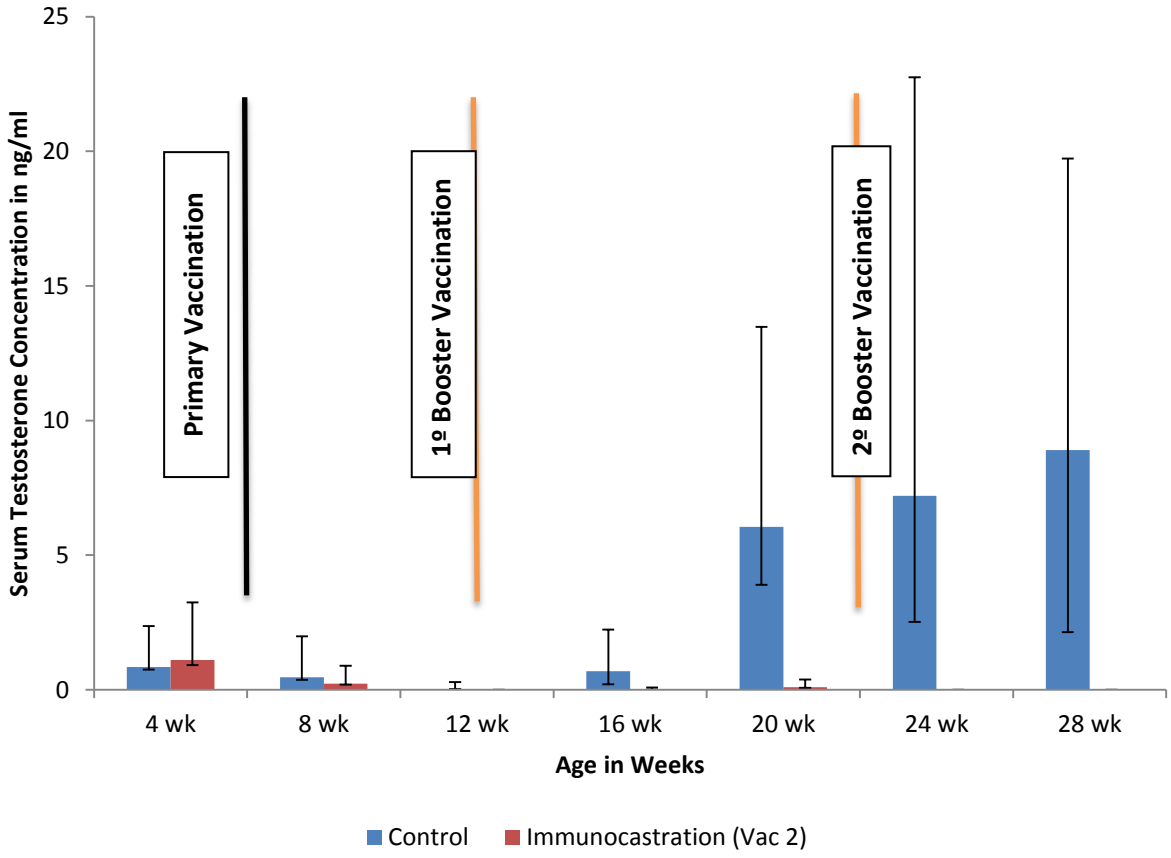


Figure 7 Differences in testosterone concentrations between Control and immunocastration treatment recorded monthly until 28 weeks of age. Data are medians with upper and lower inter-quartile ranges Q1 and Q3. Timing of primary booster is indicated by the black vertical line and of the booster vaccination by an orange vertical line.

Experiment 3

The effect of the different vaccination regimes used in Experiment 3 on circulating testosterone concentration is shown in fig 8. Significant treatment differences in testosterone concentrations were seen from 20 weeks of age until the experiment ended at 32 weeks (H=25.45 P<0.001; H=16.87 P=0.002; H=19.82 P<0.001; H=25.59 P<0.001).

At 20 weeks of age testosterone concentration was higher in Control lambs in comparison to Vac 3 and Vac 5 respectively (U=21.0, **P<0.001** and U=30.0**, P=0.002).**

In October when rams were 24 weeks old the C group had a significantly greater level of testosterone concentration than all other immunized rams (Vac 3-6 respectively: U=25.5, **P<0.001**, U=30.0, **P=0.007**, U=15, **P<0.001**, U=28.5, **P=0.009**). This difference persisted until at least 32 weeks of age (Vac 3-6 respectively: 28 weeks of age: U=38.5, **P=0.009**, U=21.0, **P<0.001**, U=23.5, **P=0.001**, U=6.0, **P<0.001**;32 weeks of age: U=39.5, **P=0.01**, U=17.0, **P<0.001**, U=23.5, **P=0.001**, U=4.0, **P<0.001**).

There were also significant differences between immunized groups in particular time points. At 20 weeks Vac 3 and Vac 5 had lower testosterone concentrations than Vac 4 and Vac 6 (Vac 3 vs Vac 4 and Vac6 respectively: U=38.0, **P = 0.005;** U=10.0, **P<0.001**; Vac 5 vs Vac 3 and Vac 6: U=42.0, **P=0.017** and U=21.0, **P<0.001**). All vaccinated groups had similar concentrations of testosterone during weeks 24 and 28, but testosterone was greater in Vac 5 compared to Vac 4 and Vac 6 in week 32 (Vac 5 vs Vac 4 and Vac 6 respectively: U=41.5, **P=0.015**; U=37.0, **P=0.014**).

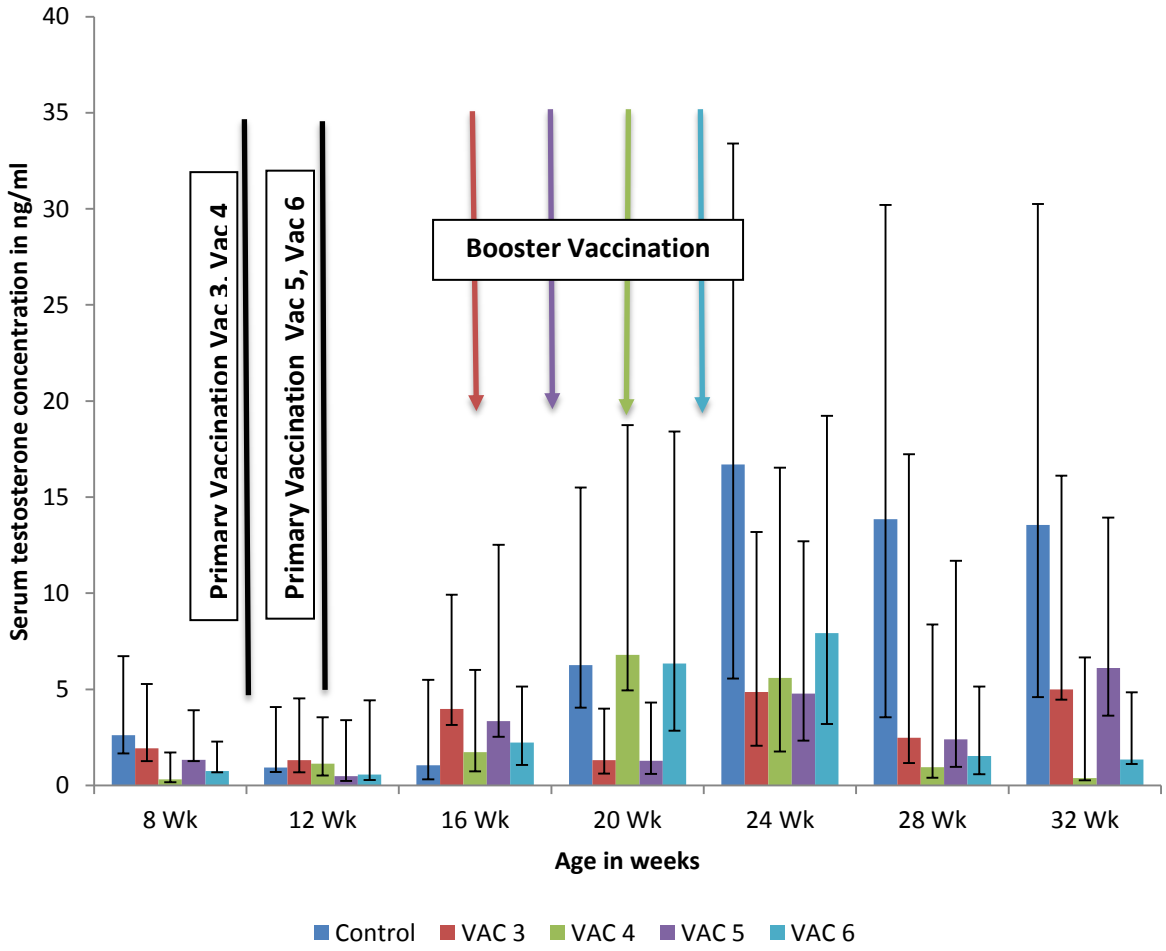


Figure 8 Differences in testosterone concentrations between Control and immunocastration treatments Vac3-6 recorded monthly until 32 weeks of age. Data are medians with upper and lower inter-quartile ranges Q1 and Q3. Black lines are indicating the time of primary vaccination (at 10 weeks of age Vac 3, Vac 4 and at 12 weeks of age Vac 5, Vac 6). The time of booster vaccination is marked in a different coloured arrow for a particular immunization group correlating to the colour of the result line of testosterone concentration.

4.5.2 Scrotal Circumference

Experiment 1

There was a significant effect of time (Wald statistic =1355.87, df=6 **P<0.001**) and treatment group (Wald statistic=21.84, df=1 **P<0.001**) on scrotal circumference (Fig 9). Scrotal circumference increased in an approximately linear fashion in C lambs. However, the growth of the testes was halted in immunized rams for an 8 week period,

and Vac1 lambs had a significantly smaller scrotal circumference when lambs were 16 (t=6.70, d.f=38, **P<0.001**), 20 (t=7.59, d.f=37, **P<0.001**) and 24 (t=3.17, df=37, **P=0.003**) weeks old in comparison to the control group. No differences were found between treatments when lambs were 4, 8, 12 and 28 weeks old.

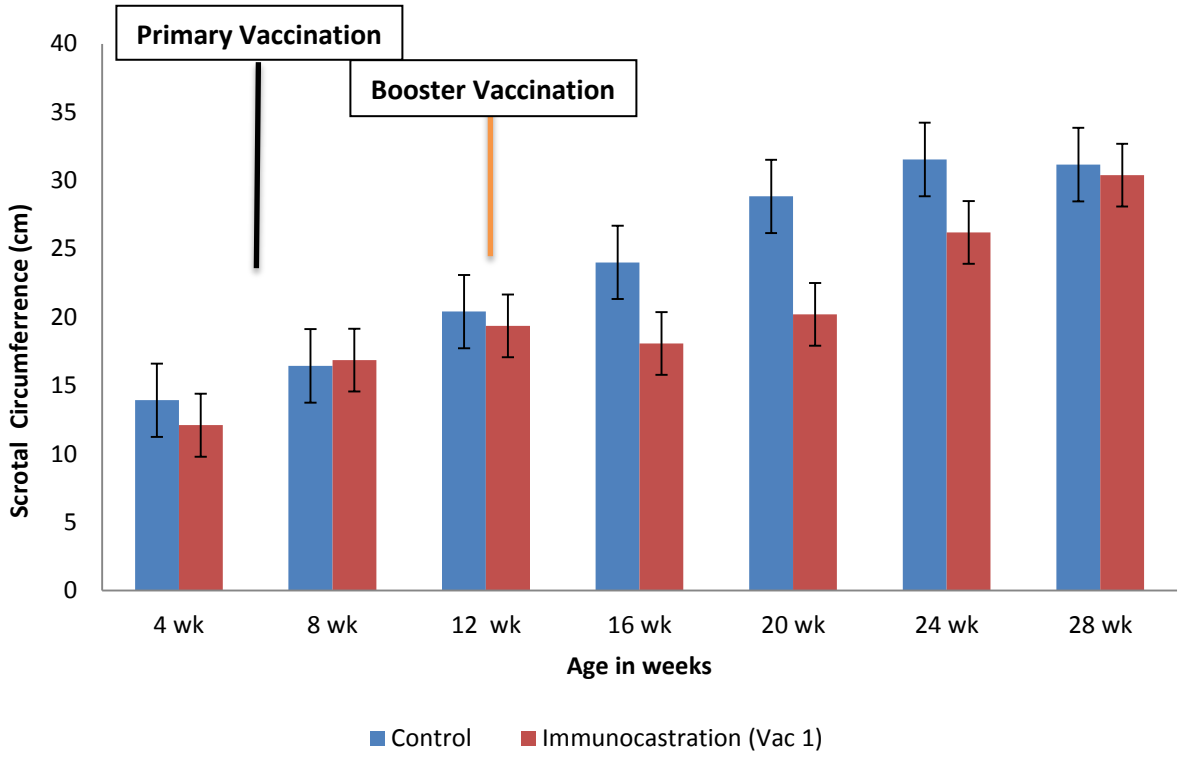


Figure 9 Differences in scrotal circumference between Control and Vac 1 treatment groups recorded monthly until 28 weeks of age. Data are Means with standard errors. Timing of primary is indicated by the black vertical line and of the booster vaccination by an orange vertical line.

Experiment 2

Scrotal circumference was significantly affected by the time (Wald statistic=1205.64, df=6, **P<0.001**) and treatment (Wald statistic=174.77, df=1, **P<0.001**) (see Fig 10). The additional booster vaccination extended the period when the increase in scrotal circumference was suppressed such that Vac 2 lambs had a significantly lower scrotal circumference than C rams at 16 (t=10.90, d.f=20, **P<0.001**), 20 (t=10.03, d.f=20,

P<0.001), 24 (t=15.57, d.f=20, **P<0.001**) and 28 weeks of age (t=17.88, d.f=20, **P<0.001**).

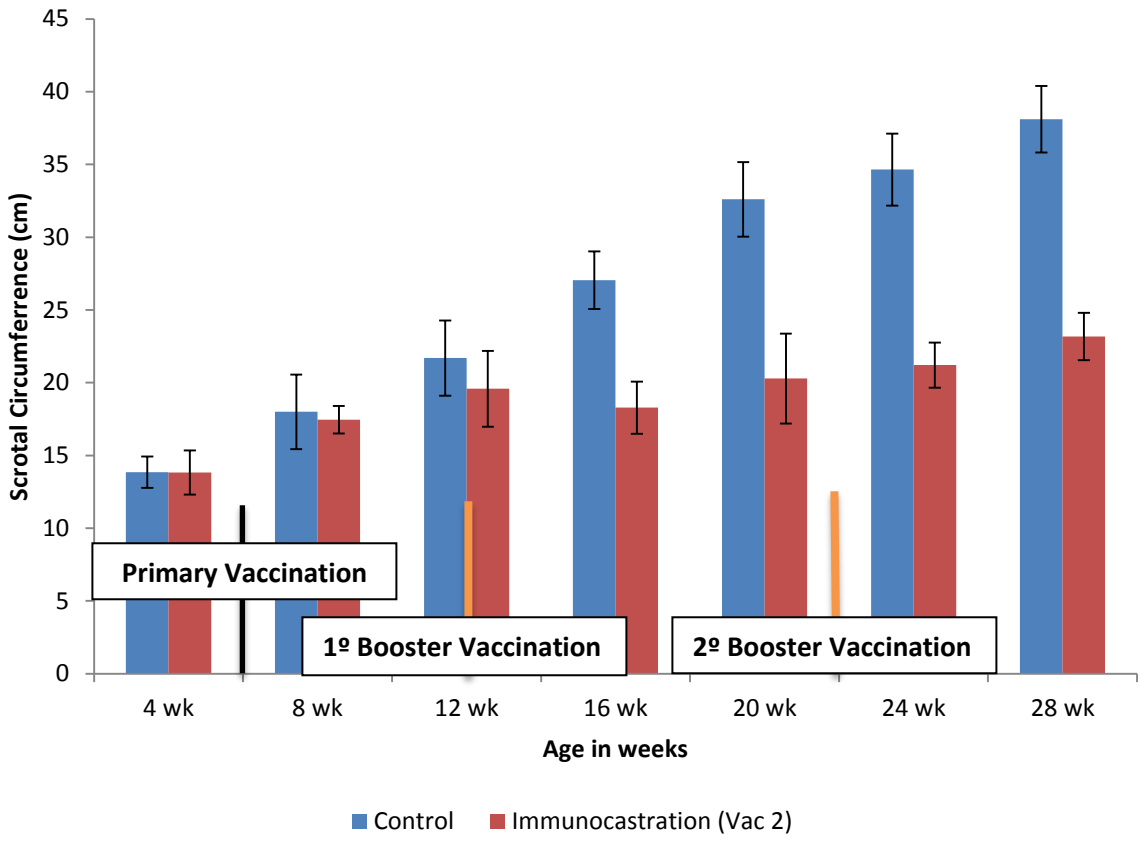


Figure 10 Differences in scrotal circumference between Control and Vac 2 treatment groups recorded monthly until 28 weeks of age. Data are Means with standard errors. Timing of primary vaccination is indicated by the black vertical line and of the booster vaccination by an orange vertical line.

Experiment 3

Similarly, to the previous experiments, there was a significant effect of time (Wald statistic=796.2, df=6, **P<0.001**) and treatment (Wald statistic=62.69, df=4, **P<0.001**) on scrotal circumference (figure 11). Scrotal circumference in Control rams was increasing until 24 weeks of age, whereas immunocastration treatment disrupted scrotal growth. It was observed that scrotal size in immunocastrated males was not growing in a similar manner in comparison to control rams. The first detectable difference in the size of

scrotal circumference can be seen at approximately 12 weeks of age. It was also observed that the differences between scrotal circumference and development depended as well on the time of booster vaccination. A further drop in scrotal circumference was perceived approximately at 16 weeks of age in Vac 3 and Vac 4 group which were vaccinated earlier in live than Vac 5 and Vac 6 treatment. At 12 and 16 weeks of age scrotal circumference was greater in C lambs than the vaccinated groups which were given the primary vaccination at 10 weeks: Vac 3 (12 weeks: t=2.23, d.f=26, **P=0.035**; 16 weeks: t=2.6, d.f=18.38, P=0.015) and Vac 4 (12 weeks: t = 2.50, d.f=26, **P=0.019**; t=3.88, d.f=19.77, **P<0.001**).

At the age of 20 weeks C rams had a greater scrotal circumference than Vac 3 (booster given at 16 weeks, t=8.87, d.f=26, **P<0.001**) and Vac 5 (booster given at 18 weeks, t=4.50, d.f=25, **P<0.001**). Vac 3 lambs also had a smaller scrotal circumference than all other immunized groups (Vac 4-6 rams respectively: t = -5.38, d.f=26, **P<0.001,** t= -4.14, d.f=25, **P<0.001,** t= -7.85, d.f=26, **P<0.001**). The scrotal circumference of Vac 5 were also smaller than Vac 6 (t= -3.32, d.f=25, **P=0.003**).

At 24 weeks (October) measures taken for C rams were larger than all other groups (Vac 3-6 respectively: t=10.9, d.f=24, **P<0.001,** t=6.54, d.f=24, **P<0.001**, t=7.12, d.f=24, **P<0.001**, t=2.71, d.f=24, P=0.012). Additionally scrotal measures of Vac 3 rams were smaller than Vac 4 and Vac 6 (t= -5.70, d.f=24, **P<0.001**, t= -4.89, d.f=15.69, **P<0.001**) and the scrotal circumference of Vac 5 rams was smaller than Vac 6 (t = -2.97, d.f=24, **P=0.007**). The increased scrotal circumference of C rams in comparison to all vaccinated groups was also apparent at 28 and 32 weeks of age (Vac 3-6 respectively: 28 weeks: t=9.69, d.f=25, **P<0.001**, t=8.78, 25 d.f=25, **P<0.001**, t=6.89, d.f=24, **P<0.001**, t=5.55, d.f=24, **P<0.001;** 32 weeks: t=7.33, d.f=25, **P<0.001**, t=8.74, d.f=25, **P<0.001**, t=5.25, d.f=24, **P<0.001**, t=4.36, d.f=18.38, **P<0.001).**

At 28 weeks Vac 3 rams had a larger scrotal circumference than Vac 5 lambs (t= -2.42, d.f=25, P=0.023).

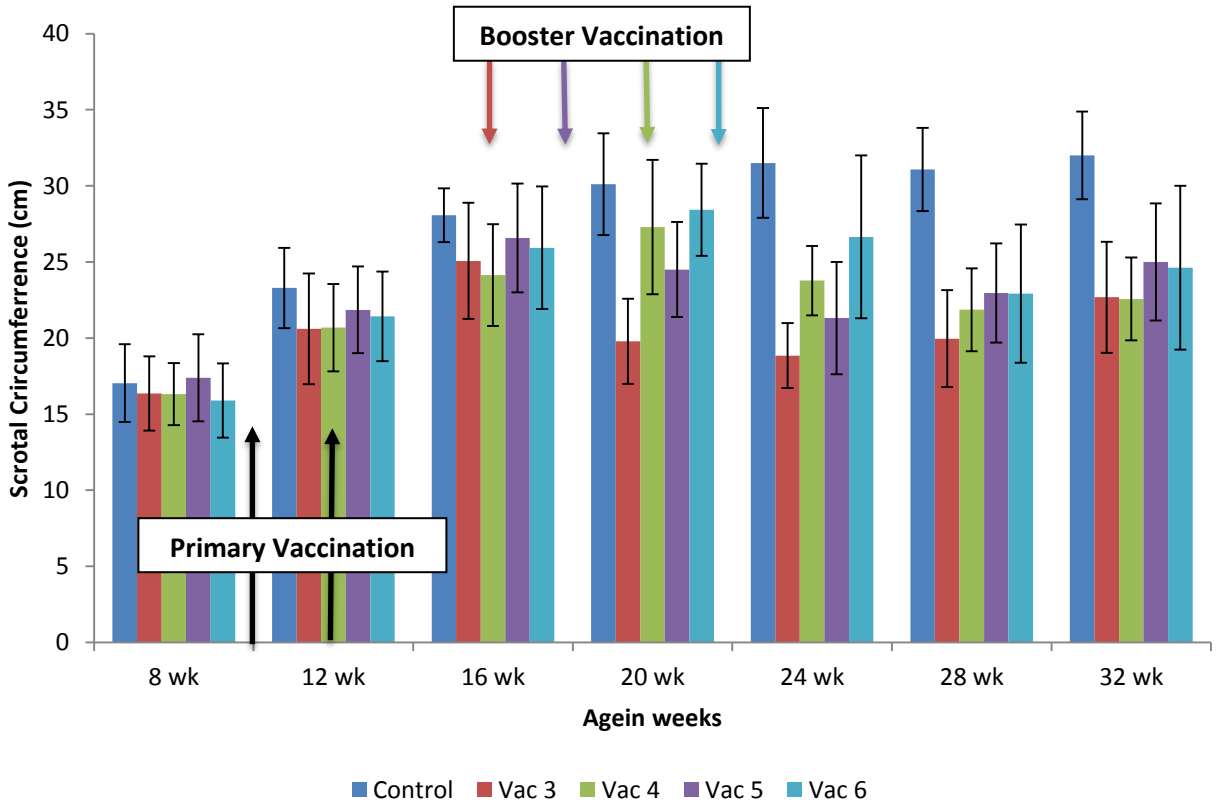


Figure 11 Differences in scrotal circumference between Control and Vac3-6 treatment groups recorded monthly until 32 weeks of age. Data are Means with standard errors. Black lines are indicating the time of primary vaccination (at 10 weeks of age Vac 3, Vac 4 and at 12 weeks of age Vac 5, Vac 6). The time of booster vaccination is marked in a different colour for each particular immunization group correlating to the colour of result line of scrotal circumference.

4.5.3 Testes Consistency

Experiment 1

Overall there were significant effects of time (Wald statistic=42.80, df=5, P<0.001) and the treatment (Wald statistic=49.26, df=1, P<0.001) on testes consistency (fig 12). The consistency of the testes increased for the C group between 12 weeks and 28 weeks of age and were significantly firmer than in Vac 1 (12 weeks: t=5.27, d.f=25.11, **P<0.001**, 16 weeks: t=6.67, d.f=38, **P<0.001**, 20 weeks: t=7.22, d.f=29.59, **P<0.001**, 24 weeks: t= 3.90, d.f=27.13, **P<0.001**, 28 weeks: t=3.03, d.f=28.87, **P=0.005**).

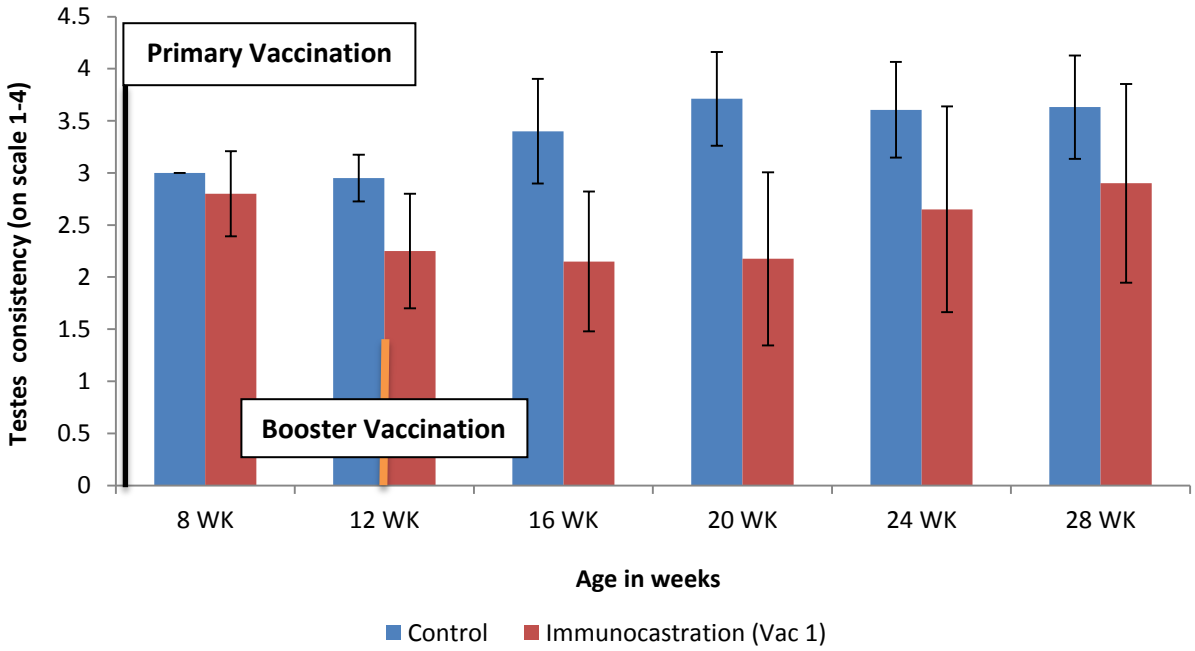


Figure 12 Differences in testes consistency between Control and Vac 1 treatment group recorded monthly until 28 weeks of age on a scale form 1 (minimum) to 4 (maximum). Data are Means with standard errors. Timing of primary is indicated by the black vertical line and of the booster vaccination by an orange vertical line.

Experiment 2

There were significant effects of time (Wald statistic=119.58, df=1 P<0.001) and treatment (Wald statistic=235.10, df=6, P<0.001) on testes consistency (fig 13). From 8 weeks of age testes consistency decreased in the Vac 2 group and increased in the C group, following the 2nd booster vaccination there was a further decrease in testes consistency in Vac 2 lambs. From July when lambs were approximately 12 weeks until 28 weeks old testes consistency was significantly greater in C than Vac 2 lambs (12 weeks: t=3.92, df=16.30, **P=0.001**, 16 weeks: t=7.71, d.f=20, **P<0.001**, 20 weeks: t=9.94, d.f=14.93, **P<0.001**, 24 weeks: t=10.34, d.f=20, **P<0.001**, 28 weeks: t=10.85, d.f=20, **P<0.001**).

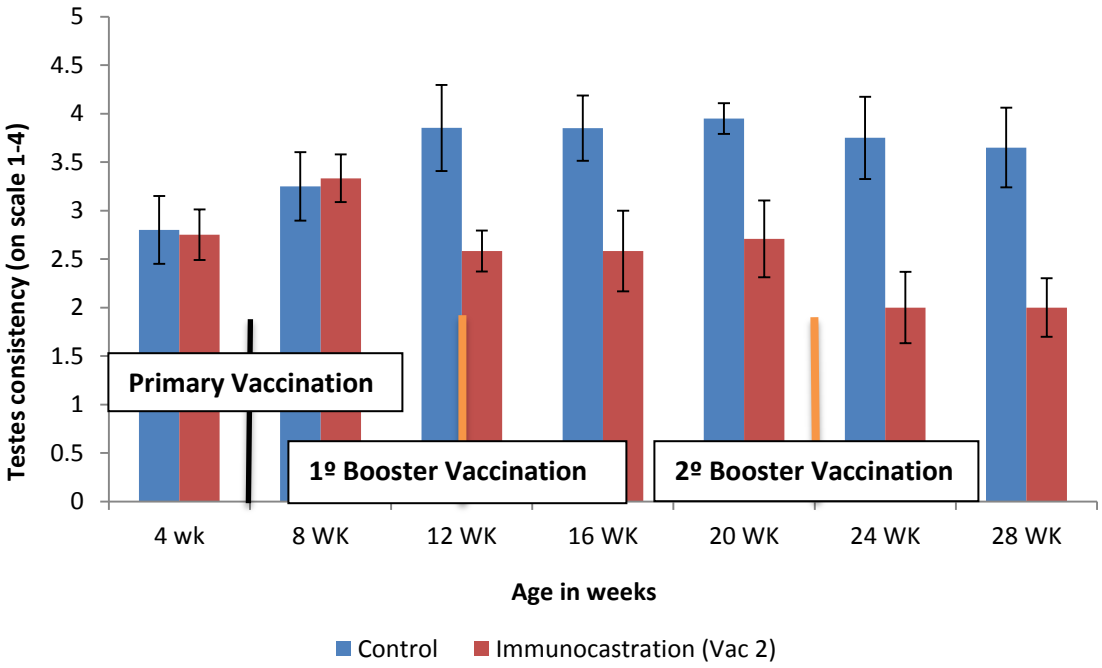


Figure 13 Differences in testes consistency between Control and Vac 2 treatment group recorded monthly until 28 weeks of age on a scale form 1 (minimum) to 4 (maximum). Data are Means with standard errors. Timing of primary is indicated by the black vertical line and of the booster vaccination by an orange vertical line.

Experiment 3

There was significant time (Wald statistic=347.23, df=6, P<0/001) and treatment (Wald statistic=62.14, df=4, **P<0.001**) effect on testes consistency in this experiment (fig. 14). The control lambs had greater testes consistency scores than all immunized rams from 20 weeks of age until 32 weeks for Vac 3-6 respectively (20 weeks: t = 9.85, d.f=26, **P<0.001**; t=3.86, 26 d.f=26, **P<0.001**; t=6.63, d.f=25, **P<0.001**; t= 2.60, d.f=26, **P=0.015; 24 weeks** t=14.57, d.f=24, **P<0.001**, t=5.83, df=19.26, **P<0.001**, t=7.70, d.f=24, **P<0.001**, t=2.58, d.f=18.13, **P=0.019; 28 weeks**t=5.59, d.f=25, **P<0.001**, t=6.61, d.f=17.64, **P<0.001**, t=5.83, d.f=17.33, **P<0.001**, t=5.31, d.f=24, **P<0.001**;, 32 weeks:t=5.85, d.f=25, **P<0.001**, t=5.91, df=25, **P<0.001**, t=3.39, d.f=18.42, **P=0.003**, t=5.87, df=23, **P<0.001**).

At 20 weeks scores were lower in Vac3 and Vac5 compared to Vac4 and Vac6, (t= -6.63, d.f=26, P<0.001, t= -4.24, d.f=25, P<0.001, t= -7.30, d.f=26, P<0.001) and lower in Vac3 than Vac5 (t=2.88, d.f=25, **P=0.008**, t= -3.83, d.f=25, **P<0.001**).

At the age of 28 weeks the Vac 3 group had significantly greater testes consistency than Vac 4 (t= -2.52, d.f=16.55, P=0.022) and Vac 6 lambs (t= -6.11, d.f=15.45, **P<0.001**). Vac4 and Vac6 rams and also had lower consistency scores than Vac5 lambs (t= -2.64, d.f=24, P=0.014).

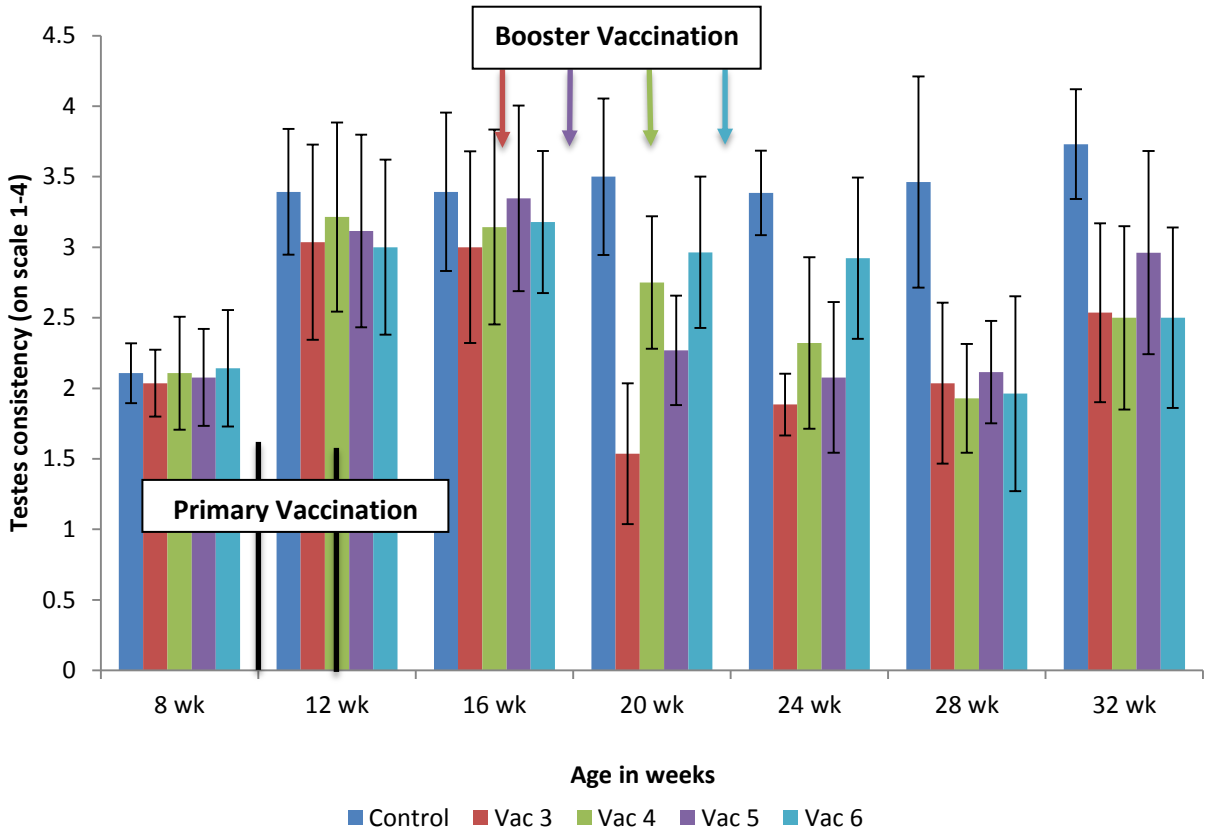


Figure 14 Differences in testes consistency between Control and Vac 3-6 treatment groups recorded monthly until 28 weeks of age on a scale form 1 (minimum) to 4 (maximum). Data are Means with standard errors. Timing of primary is indicated by the black vertical line and of the booster vaccination by a coloured vertical line.

4.5.4 Analysis of the testes Histology

Analysis revealed differences in the measured tubules size between treatments in both experiments. Moreover, the average number of counted tubules also differed which would be further evidence of the significant differences in the tubules dimension.

Experiment 1

The average count of seminiferous tubules (per slide) was significantly higher in the short scrotum treatment than in the control rams (figure 15, U= 3.0, **P<0.001**). There was also tendency for the short scrotum group to have a greater number of tubules than the immunized Vac 1 rams (figure 10, U= 15.5, P=0.08) (see also representative trans-sections of Testis Tissue of short scrotum castration and control group figure 1-5). There were no significant differences between the Control and Vac 1 group (U=27.0, P=0.645) in the average number of tubules.

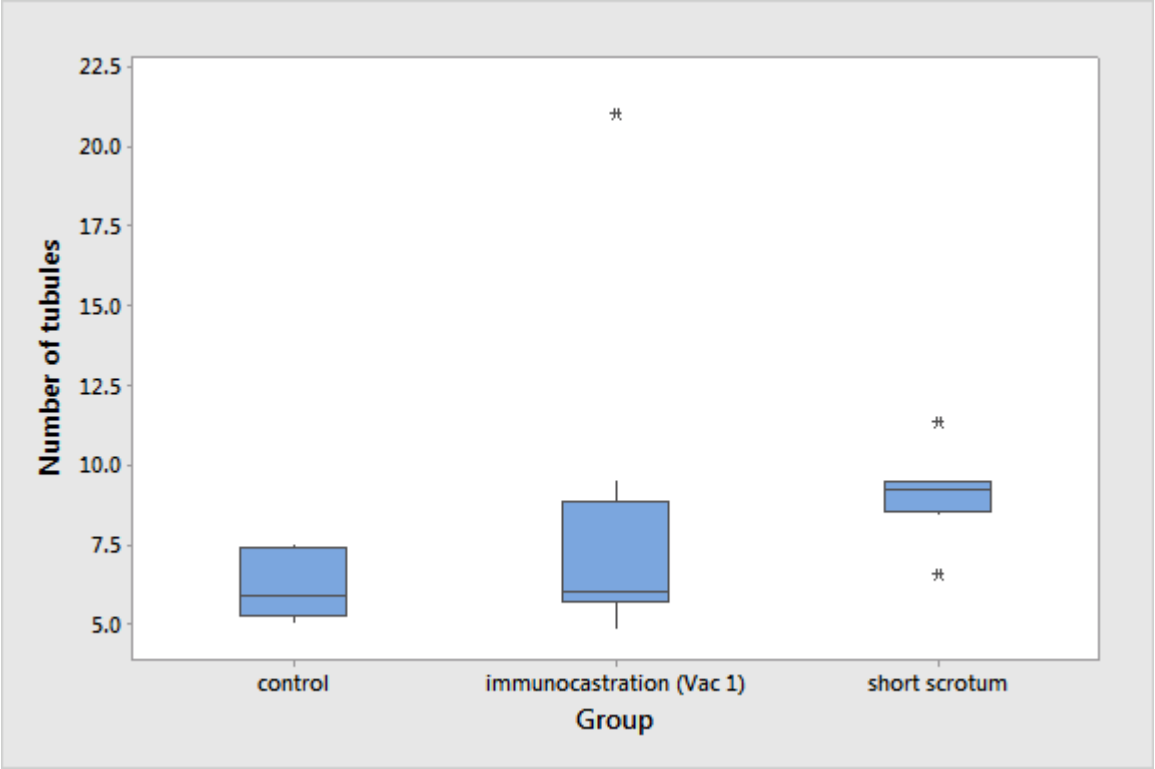


Figure 15 Box plot of average count of seminiferous tubules (per slide) measured in µm for control (C), short scrotum castration (SSC) and immunocastration (Vac 1) group. Data are medians with Q1 and Q3. Whiskers represent upper and lower extreme. x-outlier/single data point.

The size of the tubules were also significantly smaller in SSC than in the Control treatment (Figure 16, t = 2.77, d.f=14, **P= 0.015**) but there were no differences between SSC and Vac1 treatment. It has to be noted as well that the variability in the average size and number of the tubules per (slide) in the Vac 1 group was higher in comparison to other treatments. The basis of this outcome lies in the still visible changes in the testicular histology of some rams in that group at the time of slaughter.

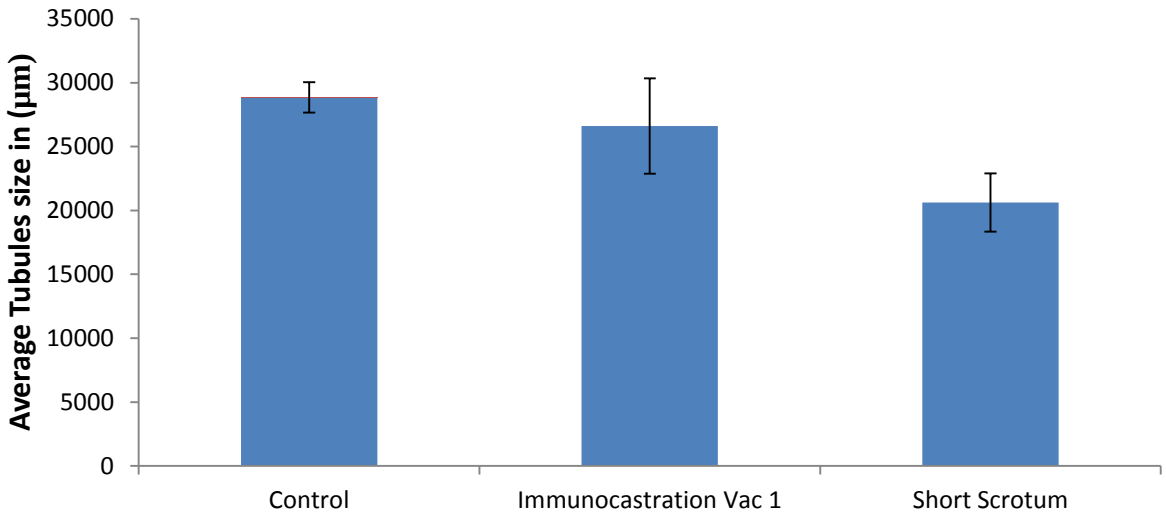


Figure 16 Average mean size (per slide, ±standard error) of seminiferous tubules measured in µm for each treatment.

Experiment 2

The average number of tubules was significantly greater for Vac 2 rams compared to C lambs (U=0.0 **P<0.001,** figure 17. See also representative trans-section of testis tissue of control and immunized Vac 2 rams figure 1-5) and the average size of the tubules was significantly greater in C rams (U=0.0 **P<0.001)** than in the Vac 2 group (figure 18).

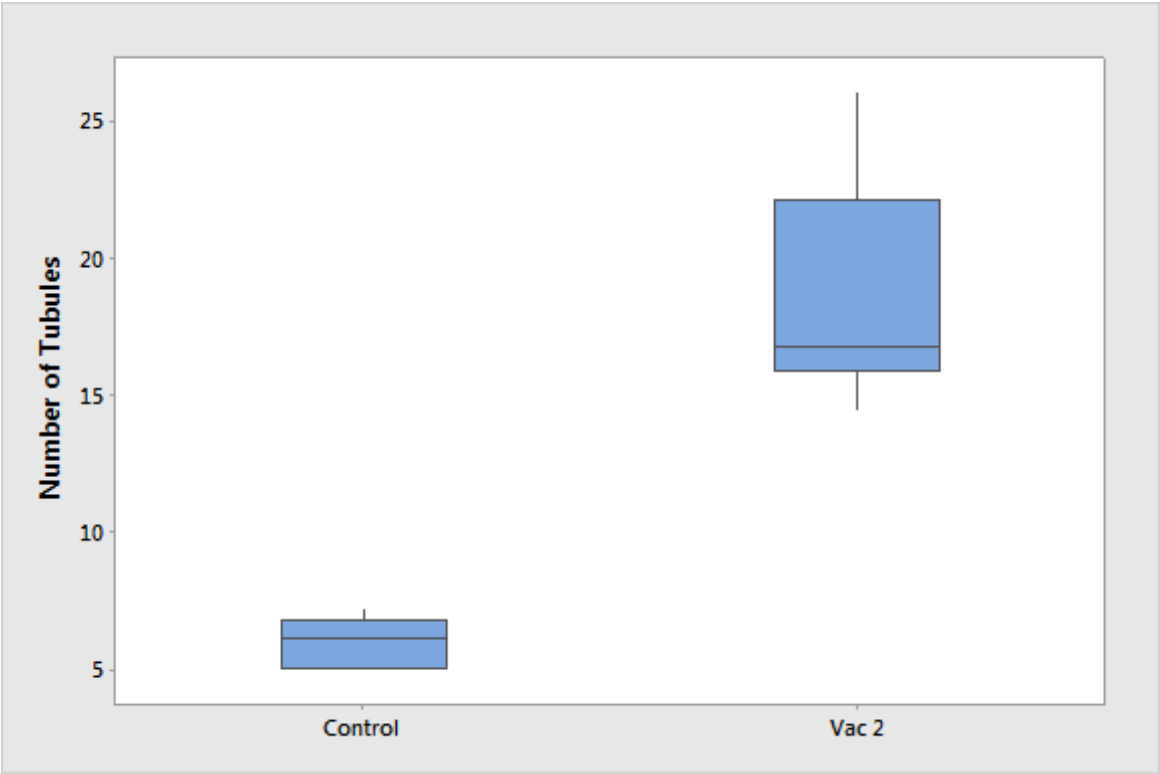


Figure 17 Box plot of average count of seminiferous tubules (per slide) measured in μm for control (C) and immunocastration (Vac 2) group. Data are medians with Q1 and Q3. Whiskers represent upper and lower extreme. x - outlier/single data point.

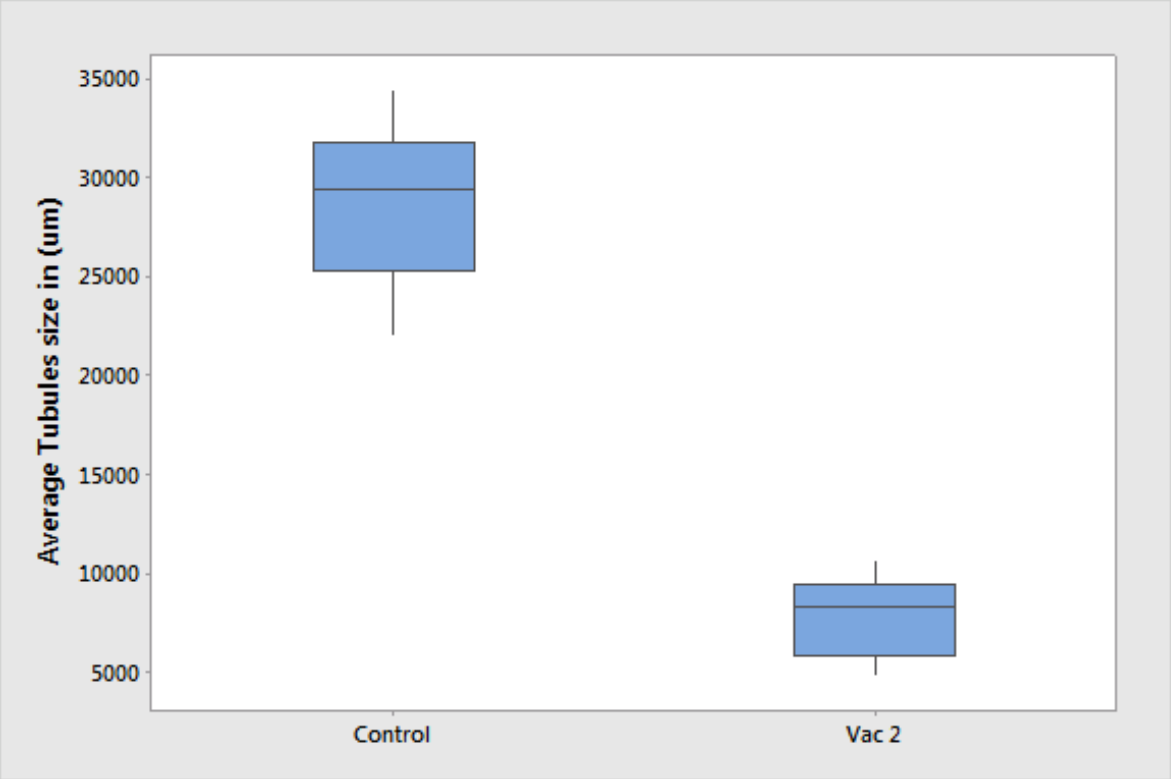


Figure 18 Box plot of average size of seminiferous tubules (per slide) measured in µm for control (C) and immunocastration (Vac 2) group. Data are medians with Q1 and Q3. Whiskers represent upper and lower extreme. x - outlier/single data point.

4.5.5 Testicular weight, length and volume

Experiment 1

The length of the right and left testicles was smaller in the SSC group in comparison to C rams t = 6.31, d.f=12, P < **0.001** and t = 8.66, d.f =14, **P < 0.001**. However, there were no significant differences between SSC and Vac 1.

Similarly the volume of the right and left testicles was smaller in the SSC group than controls t = 3.61, d.f = 12, **P = 0.004** and t = 4.96, d.f = 14, **P< 0.001)** and the Vac 1 group t = 3.22, 8.04 d.f = 8.04, **P= 0.012** and t = -2.56 on 13 d.f., **P = 0.024**.

The weight of the right and left testicles was reduced in SSC compared to Control t = 4.00, d.f= 12, **P= 0.002** and t = 5.26, d.f= 14, **P< 0.001** for right and left respectively. There was also a tendency for the SSC rams to have decreased weight of right and left

testicles in comparison to Vac 1 group t = -2.04, d.f = 9.53, P= 0.07 and t = -2.47, d.f = 8.16, P= 0.038.

The width of the left testicle in the SSC group was smaller than C rams t = 2.99, d.f=14, **P=0.01** and there was a tendency for the width of the right testicle in the SSC group to be reduced as well t = 1.94, d.f = 12, P= 0.07. There were no significant differences between SSC and Vac 1 treatment.

Experiment 2

The weight, volume, length and width of the left and right testicles were significantly reduced in the Vac2 groups compared to Controls respectively: U=0.0, **P<0.001.**

4.5.6 Expression of Sexual Behaviours

4.5.6.1 Male Sexual behaviour observations

Experiment 1

Overall results have shown that lambs in the RR treatment performed less courtship and sexual behaviours than other treatment groups (see table 6 below). RR also vocalized and received aggression at a significantly higher level during the test in comparison to other rams.

Table 6 Differences in the frequency of courtship and mating behaviours expressed by entire males (C), rubber ring castration (RR), short scrotum (SSC) and immunized (Vac 1) treatment, recorded for 30 minutes at ~28 weeks of age. Data are medians with Q1 and Q3.

Behaviour/ Posture	**C[1]**	**RR[1]**	**SSC[1]**	**Vac 1[1]**	**P–Value[2]**
Sniff/Nose	9.7(7.0-16.2)a	0.4(0.2-0.6)b	5.9(4.7-8.0)a	11.2(3.8-22.6)a	**P= 0.001**
Flehmen	0.8(0.5-1.3)a	0.0(0.0-0.0)b	0.2(0.0-1.0)ab	0.6(0.1-1.0)a	**P< 0.005**
Nudge	37.0(16.5-47.9)a	0.0(0.0-0.0)b	10.0(5.6-13.2)a	18.5(1.4-29.1)a	**P< 0.001**
Low stretch	45.4(21.0-55.7)a	0.0(0.0-0.0)b	24.0(17.5-28.5)a	24.5(2.8-33.7)a	**P< 0.001**
Lick	3.4(1.7-3.6)a	0.0(0.0-0.1)b	0.0(0.0-0.3)b	1.9(0.0-4.9)a	**P< 0.002**
Mounting	2.3(0.0-6.2)a	0.0(0.0-0.0)b	1.0(0.0-4.5)a	0.5(0.0-0.8)a	**P= 0.009**
Movements Mount	16.1(8.5-19.5)a	0.0(0.0-0.0)b	1.9(0.4-6.2)c	5.6(1.0-8.5)c	**P= 0.001**

Attempts					
Vocalizations	0.0(0.0-0.0)a	2.0(0.0-13.9)b	0.0(0.0-0.4)ab	0.0(0.0-0.0)a	**P= 0.009**
Escape Attempts	0.0(0.0-0.0)	0.0(0.0-0.5)	0.0(0.0-0.2)	0.0(0.0-0.0)	**NS**
Received Aggression	0.0(0.0-0.0)a	0.3(0.3-1.6)b	0.0(0.0-0.0)a	0.0(0.0-0.2)a	**P< 0.001**
Aggression	1.2(0.0-3.6)	0.0(0.0-0.1)	0.0(0.0-0.0)	0.0(0.0-0.1)	**NS**

[1]Median frequency (Q1-Q3). [2](amended p-value due to Bonferroni correction P<0.0125), NS not significant. Different letters indicate significant differences between treatments.

Experiment 2

Table 7 below represents significant results for the expression of courtship and sexual behaviours (recorded for control and immunized Vac 2 rams). Overall findings show that Vac 2 group expressed significantly lower frequencies of courtship and mounting behaviour showing greater amount of vocalizations, escape attempts and received aggression at the same time.

Table 7 Differences in the frequency of courtship and mating behaviours expressed by entire males (C) and immunized (Vac 1) treatment, recorded for 30 minutes at ~28 weeks of age. Data are medians with Q1 and Q3.

Behaviour/ Posture	**C[1]**	**Vac 2[1]**	**P-Value[2]**
Sniff/Nose	5.2(4.1-11.3)	0.7(0.2-3.8)	**P= 0.001**
Flehmen	0.5(0.0-1.0)	0.0(0.0-0.0)	**P= 0.006**
Nudge	25.2(8.1-44.3)	0.0(0.0-1.5)	**P= 0.012**
Low stretch	40.6(26.1-72.8)	0.0(0.0-2.5)	**P< 0.001**
Lick	2.2(1.8-3.6)	1.6(0.7-3.0)	**P< 0.001**
Mounting Movements	2.7(2.3-8.1)	0.0(0.0-0.0)	**P< 0.002**
Mount Attempts	7.7(4.5-21.0)	0.0(0.0-1.0)	**P= 0.004**
Vocalizations	0.0(0.0-0.0)	18.9(8.1-39.6)	**P= 0.001**
Escape Attempts	0.0(0.0-0.1)	1.5(0.3-4.6)	**P< 0.001**
Received Aggression	0.0(0.0-0.0)	4.0.(0.5-15.5)	**NS**

Aggression	1.4(0.1-5.5)	0.1(0.0-0.7)	**P< 0.001**

[1]Median frequency (Q1-Q3). [2](P<0.05), NS not significant.

Experiment 3

Similarly, to previous experiments, overall results have revealed that Control rams had the highest frequencies of courtship and mating behaviours whereas RR rams expressed the lowest frequencies of courtship and mating. RR rams were also observed to receive a significantly larger amount of aggression from females in comparison to other treatments. They vocalize and attempt to escape more than other groups as well.

Table 8 Differences in the frequency of courtship and mating behaviours expressed by entire males (C), rubber ring castration (RR), and immunocastration (Vac 3-6) treatment, recorded for 30 minutes at ~28 weeks of age. Data are medians with Q1 and Q3.

Behaviour/ Posture	**C[1]**	**RR[1]**	**Vac 3[1]**	**Vac 4[1]**	**Vac 5[1]**	**Vac 6[1]**	**P – Value[2]**
Sniff/Nose	19.0(16.9-26.0)a	2.5(2.0-4.4)b	5.3(2.4-10.9)b	4.2(2.0-8.5)b	4.6(2.0-9.4)b	3.6(2.5-8.2)b	**P= 0.002**
Flehmen	1.6(0.6-2.2)a	0.0(0.0-0.0)b	0.1(0.0-0.5)ab	0.0(0.0-0.2)b	0.1(0.0-0.3)ab	0.0(0.0-0.5)b	**P= 0.007**
Nudge	31.2(18.7-49.0)a	0.0(0.0-0.0)b	3.9(0.0-13.2)b	0.0(0.0-1.2)b	2.0(0.1-9.0)b	0.2(0.0-9.2)b	**P< 0.001**
Low stretch	64.2(56.8-80.5)a	0.0(0.0-0.0)b	15.6(0.0-39.5)bc	0.1(0.0-3.5)bc	5.6(0.6-20.5)c	1.0(0.0-15.9)bc	**P< 0.001**
Lick	0.7(0.2-2.0)	0.0(0.0-0.5)	0.0(0.0-0.1)	0.1(0.0-0.5)	0.1(0.0-0.2)	0.0(0.0-0.5)	**NS**
Mounting	3.9(2.0-5.3)a	0.0(0.0-0.0)b	0.7(0.0-1.6)b	0.0(0.0-0.2)b	0.0(0.0-2.7)b	0.0(0.0-1.0)b	**P< 0.001**
Movements	10.0(7.7-19.4)a	0.0(0.0-0.0)b	0.5(0.0-3.0)b	0.0(0.0-0.0)b	0.1(0.0-2.3)b	0.0(0.0-4.5)b	**P< 0.001**
Mount Attempts							
Male Follow	2.4(0.6-27.2)a	0.0(0.0-0.0)b	0.0(0.0-0.9)ab	0.0(0.0-0.0)b	0.0(0.0-0.0)b	0.0(0.0-0.6)ab	**P= 0.003**
Vocalizations	0.0(0.0-0.0)a	4.5(0.3-	1.8(0.0-8.0)b	6.7(0.5-15.3)b	3.6(1.9-7.3)b	3.2(0.5-4.1)b	**P= 0.008**

		325)b					
Escape Attempts	0.0(0.0-0.3)	0.2(0.0-1.3)	0.0(0.0-0.5)	0.0(0.0-1.0)	0.7(0.0-0.9)	0.1(0.0-0.3)	**NS**
Received Aggression	0.0(0.0-0.2)	4.4(1.8-8.6)	0.9(0.0-8.0)	0.2(0.1-6.6)	1.9(0.6-4.5)	0.8(0.0-7.5)	**NS**
Aggression	4.7(1.6-7.4)	0.0(0.0-0.9)	1.4(0.0-2.6)	0.2(0.0-2.3)	0.5(0.1-2.8)	0.1(0.0-0.4)	**NS**

[1]Median frequency (Q1-Q3). [2](amended p-value due to Bonferroni correction $P<0.008$), NS not significant. Different letters indicate significant differences between treatments.

Post hoc analysis has revealed that C rams expressed a significantly greater frequency of flehmen in comparison to RR, Vac 4 and Vac 6 rams (U=5.0, **P=0.003**, U=7.5, **P=0.007**, U=9.5, **P=0.014**). There was also a tendency for Vac 3 and 5 group to show less flehmen behaviour than C group (U=11.0, P=0.026, U=10.5, P=0.021).

Control rams showed a significantly greater amount of nudging and sniff/nose behaviour in contrast to all other ram groups (Nudging RR, Vac 3-6 respectively: U=0.0, **P<0.001**, U=11.0, P=0.028, U=3.0, **P=0.001**, U=6.0, **P=0.005**, U=4.0, **P=0.002**; Sniff/nose: U=0.0, **P<0.001**, U=3.0, **P=0.001,** U=2.0, **P<0.001**, U=3.0, **P=0.001**, U=2.5, **P<0.001**). Low stretch behaviour was performed at a higher frequency for the C treatment in comparison to all other groups (RR, Vac 3-6: figure 56, U=0.0, **P<0.001**, U=6.0, **P=0.005**, U=2.0, **P<0.001**, U=6.0, **P=0.005**, U=1.0, **P<0.001**). The frequency of low stretch behaviour was also higher for Vac 5 treatment in comparison to RR rams (U=5.0, **P=0.002).**

Duration of male follow behaviour was significantly higher for C rams than RR, Vac 4 and Vac 5 rams (U=4.0, **P=0.001**, U=7.5, **P=0.005**, U=10.5, **P=0.013**). There was also a tendency for the Vac 3 and Vac 6 rams to express a lower level of male follow behaviour in comparison to the C group (U=11.0, P=0.022, U=12.0, P=0.030). Control rams attempted to mount more frequently than rams from all other groups (RR, Vac 3-6 U=0.0, **P<0.001**, U=5.0, **P=0.003**, U=0.5, **P<0.001**, U=3.0, **P=0.001**, U=4.0, **P=0.002**). Control rams also expressed a higher rate of mounting movements in comparison to other treatments (RR, Vac3, Vac 4 and Vac 6: U=0.0, **P<0.001**, U=5.5, **P=0.003**, U=4.0, **P=0.002**, U=2.4, **P<0.001**) and there was a tendency for Vac 5 rams to have a lower rate of mounting movements than C (=10.5, P=0.021).

C lambs were observed to have the lowest level of vocalizations compared to all other treatments (RR Vac 3-6 respectively: U=0.0, **P<0.001,** U=13.5, P=0.026, U=4.5, **P=0.001**, U=4.5, **P=0.001**, U=9.0, **P=0.007**).

4.5.6.2 Correlation of plasma testosterone concentration and expression of sexual behaviours

Analyses have shown that there was a very good positive correlation between plasma testosterone concentration and expression of distinct sexual/courtship behaviours which were expressed at the time of the sexual behaviours observation conducted in the study of 2012 and 2013. There was no correlation between plasma testosterone concentration and sexual/courtship behaviours in the study of 2011. The results for each particular experiment are shown in tables 9-11 below.

Table 9 Correlation of plasma testosterone concentration and expression of sexual behaviours (2011 study)

Behaviour Frequency	Correlation[1] (age 28 weeks)	P – Value [2]
Mounting attempts	**0.384**	P=0.06
Nudge	**0.1**	P=0.6
Flehmen	**0.340**	P=0.1
Low stretch	**0.01**	P=0.9
Overall*	**0.09**	P=0.6

*sum of all presented sexual behaviours. [1]The cut off point for the correlation result to be regarded as meaningful in this study was 0.4. Correlations between 0.4-0.65 were regarded as moderate, while correlations higher than 0.65 were regarded as strong. [2] (P<0.05)

Table 10 Correlation of plasma testosterone concentration and expression of sexual behaviours (2012 study)

Behaviour Frequency	Correlation [1] (age 28 weeks)	P – Value [2]
Mounting attempts	**0.666**	**P=0.001**
Nudge	**0.801**	**P=0.000**
Flehmen	**0.502**	**P=0.017**
Low stretch	**0.794**	**P=0.000**
Overall*	**0.796**	**P=0.000**

**sum of all presented sexual behaviours.*[1]The cut off point for the correlation result to be regarded as meaningful in this study was 0.4. Correlations between 0.4-0.65 were regarded as moderate, while correlations higher than 0.65 were regarded as strong. [2] (P<0.05)

Table 11 Correlation of plasma testosterone concentration and expression of sexual behaviours (2013 study)

Behaviour Frequency	Correlation [1] (age 28 weeks)	P – Value [2]
Mounting attempts	**0.656**	**P=0.000**

Nudge	**0.744**	**P=0.000**
Flehmen	**0.699**	**P=0.000**
Low stretch	**0.738**	**P=0.000**
Overall*	**0.754**	**P=0.000**

**sum of all presented sexual behaviours.*[1]The cut off point for the correlation result to be regarded as meaningful in this study was 0.4. Correlations between 0.4-0.65 were regarded as moderate, while correlations higher than 0.65 were regarded as strong.[2] (P<0.05)

4.5.6.3. Female reproductive behaviours

Experiment 1

Ewes were observed to show a significantly lower frequency of courtship and sexual behaviours in the presence of RR rams compared to rams from all other treatments (table 12).

Table 12 Differences in the frequency of courtship and mating behaviours expressed by ewes in the presence of rams from different treatments (control –C, rubber ring –RR, short scrotum- SSC and immunocastration – Vac 1), recorded for 30 min when rams were ~ 28 weeks of age. Data are medians with Q1 and Q3.

Behaviour/ Posture	**C**[1]	**RR**[1]	**SSC**[1]	**Vac 1**[1]	**P–Value**[2]
Head Turning	16.0(11.1-25.8)a	5.0(3.5-6.8)b	10.0(7.8-14.3)a	12.1(8.0-17.0)a	**P<0.001**
Wagging Tail	18.6(14.0-32.0)a	0.3(0.1-2.1)b	5.5(0.3-18.6)bc	4.8(1.0-19.1)c	**P<0.001**
Crouch	1.0(0.5-1.3)a	0.3(0.0-0.3)b	1.6(0.5-3.3)a	1.0(0.5-2.6)a	**P<0.001**
Standing Firm	8.0(3.6-17.6)a	0.0(0.0-0.0)b	2.0(0.0-5.5a)c	2.1(0.1-5.1)c	**P<0.001**
Follow Male	0.0(0.0-0.0)a	1.1(0.3-2.8)b	0.0(0.0-0.3)a	0.0(0.0-0.3)a	**P<0.001**
Circulating	0.0(0.0-0.3)a	0.0(0.0-0.0)b	0.0(0.0-1.0)a	0.0(0.0-1.6)a	**P<0.001**
Moving Away	1.6(0.6-2.6)a	0.0(0.0-0.0)b	0.0(0.0-0.0)b	0.0(0.0-1.0)b	**P<0.001**
Avoiding	2.3(0.0-10.3)a	0.0(0.0-0.0)b	0.5(0.0-4.5)a	0.8(0.0-11.6)a	**P=0.002**
Vocalizations	0.0(0.0-0.0)a	0.0(0.0-0.0)ab	0.0(0.0-0.0)b	0.0(0.0-1.0)ab	**P=0.006**
Received Aggression	0.0(0.0-2.6)a	0.0(0.0-0.0)a	0.0(0.0-0.0)b	0.0(0.0-0.0)a	**P=0.01**

Performed Aggression	0.0(0.0-0.0)a	0.8(0.3-1.6)b	0.0(0.0-0.0)a	0.0(0.0-0.1)a	**P<0.001**

[1]Median frequency (Q1-Q3). [2] amended p-value due to Bonferroni correction (P<0.0125), NS not significant. Different letters indicate significant differences between treatments.

Experiment 2

Overall findings show that ewes expressed more courtship behaviours during mating behaviour tests performed with C rams, with the exception of crouch and following male behaviour, than with Vac2 rams (Table 13). C rams directed a significantly higher level of aggressive behaviours towards ewes compared to Vac 2 rams, whereas ewes directed an increased number of aggressions towards Vac 2 rams.

Table 13 Differences in the frequency of courtship and mating behaviours expressed by ewes in the presence of rams from different treatments (control – C and immunocastration – Vac 2), recorded for 30 min when rams were ~ 28 weeks of age. Data are medians with Q1 and Q3.

Behaviour/ Posture	**C[1]**	**Vac 2[1]**	**P – Value[2]**
Head Turning	25.6(15.8-36.3)	10.3(6.5-15.5)	**P< 0.001**
Wagging Tail	12.3(4.8-21.1)	0.3(0.0-1.8)	**P< 0.001**
Crouch	0.3(0.0-0.3)	0.6(0.0-1.0)	**P= 0.03**
Standing Firm	5.8(3.3-16.6)	0.0(0.0-0.0)	**P< 0.001**
Follow Male	0.0(0.0-0.0)	0.3(0.0-0.5)	**P= 0.003**
Moving Away	0.1(0.0-0.6)	0.0(0.0-0.0)	**P= 0.004**
Received Aggression	0.3(0.0-3.8)	0.0(0.0-0.0)	**P= 0.001**
Performed Aggression	0.0(0.0-0.0)	0.8(0.1-8.3)	**P< 0.001**

[1]Median frequency (Q1-Q3). [2](P<0.05), NS not significant

4.7. Discussion

Overall analysis of the results has shown that vaccination against GnRF effectively suppresses testicular development, testosterone concentration and the occurrence of reproductive behaviours compared to entire male rams. Furthermore, the testosterone concentration in the blood recorded for immunized group was also lower in comparison to SSC treatment. Levels of testosterone were successfully reduced to concentrations of less than 5ng/ml approximately 2 weeks after administration of a booster vaccination. This is also consistent with previous studies investigating vaccination against GnRF in cattle (Amatayakul-Chantler et al., 2012; Amatayakul-Chantler et al., 2013; Janett et al., 2012). Moreover, testosterone concentration remained suppressed for 12 weeks (until approximately 20 weeks of age) in the first experiment and until the end of the experimental period (28-32 weeks of age) in the following studies. Similar findings were reported by Amatayakul-Chantler et al. (2012) who showed that testosterone concentrations recorded for Bos taurus immunized against GnRF remained supressed for 15 weeks (the end of the trial). More importantly, such a long duration of testosterone suppression as described in this study could be achieved with only two doses of the vaccine when administered at approximately 10-12 weeks of age and with a longer interval between the primary and booster vaccination (approximately 10 weeks). This suggests that the timing of the primary vaccination and the interval between the primary and booster vaccination is a very important factor having a significant impact on the length of the immunity period as well as the practicality of on farm use. Results of the 3rd experiment showed that primary vaccination at weaning was an effective method of vaccination. It allowed for maintaining testosterone and testicular suppression through the fattening period. Other studies evaluating the use of anti-GnRF vaccine in cattle reported a need for 3 booster repetitions (Ribeiro et al., 2004) or 4 booster repetitions (Hernandez et al., 2005) to achieve the effect of extended immunity of up to 15 weeks. These results are important from an economic and practical point of view as it may be impractical and too expensive to gather animals for vaccination more than twice. Multiple repetitions of booster vaccinations may also be a cause of concern regarding the levels of toxicity and the impact of vaccination on the injection site. However, even a

3 dose regime evaluated in this study did not lead to any unwanted effects like post-injection lesions. This finding is in contrast with post-castration wounds observed in RR and SSC treatments (as reported in Chapter 3) which may have strong adverse effects on lambs' welfare, health and productivity despite the fact that the application of the rubber rings used in both methods involves only one gathering. The results of this study have also shown that the stress of weaning did not have a negative impact on the production of antibodies (for more details see Chapter 5).

Similarly, to the reduced levels of testosterone, testicular consistency and scrotal circumference were shown to be suppressed by anti-GnRH treatment, which is consistent with previous findings reported for cattle, sheep and pigs Kiyma et al., 2000; Ülker, et al., 2009; Ülker et al., 2005).

Moreover, results of this study have shown that testicular measures remained reduced in the immunized groups for the same time periods reported for testosterone concentrations. This was also found by Amatayky-Chantler et al. (2012) and Janett et al. (2012) investigating effects of anti-GnRF vaccination in cattle. Reduction in testicular measures may have been a consequence of changes in the development of testes. It has already been described by Brown et al. (1994) that immunization delays testicular development. The testicles of the immunized rams in the current study appeared to be immature with marked seminiferous tubules atrophy and lack of spermatozoa. The post-mortem testicular appearance seemed to be altered. This manifested in a lower volume, length, width and weight of testicles in immunized lambs when compared with testicles of entire males. This fact may be used by the farmers, or livestock workers, as an indicator of the vaccine efficiency which would be quick and easy to evaluate during normal husbandry practices by visual assessment and/or palpation. Reduced testicular size, depletion of epithelium layers, reduced seminiferous tubules diameter and absence of sperm after immunization were also previously reported in pigs (Einarsson et al., 2009; Fang et al., 2010), bucks (Ülker, et al., 2009) and rams (Kiyma et al., 2000; Ülker, et al, 2001; Ülker et al., 2009; Ülker et al., 2005).

Evaluation of reproductive behaviour patterns suggested that suppression of testosterone led to significant depression of courtship and mounting behaviour expressed by rams in the presence of oestrus ewes (as shown in table 4-6). Similar outcomes have been also described in the past in camels (Ghoneim et al., 2012), rams (Kiyma et al., 2000; Parthasarathy et al., 2002) and horses (Turkstra et al., 2005; Janett et al., 2009). It must be noted that there was some variability in expression of reproductive behaviours in each year of the study, which was related to the immunity period and the levels of circulating testosterone at the time of the test. However, it can be argued that courtship and mounting behaviour in immunized rams were consistently reduced throughout the whole study period in comparison to control rams who showed overall higher frequencies of reproductive behaviours than all other treatments throughout the whole trial. Visible reduction or lack of interest in the oestrus females represented by immunized and rubber ring castrated rams may be explained by a reduced testosterone level which may have led to a reduction or total lack of libido. Furthermore, the immunized rams have not only showed a reduction in sexual drive but an increase in flight behaviours like vocalizations and escape attempts. Moreover, similarly to RR rams immunized lambs were observed to receive aggression at higher frequencies as well.

This study reports also for the first time the impact of the immunization on the ewes' perception of rams' attractiveness. Results showed that ewes directed more aggression towards immunized rams. Similar behaviour was observed during the mating behaviour tests performed with physically castrated males. Furthermore, ewes expressed more follow male behaviour towards castrated rams which may indicate that females were attempting to initiate contact with RR males to elicit escalation of courtship and sexual behaviours. This contrasts with the entire males when presented to the ewes where a significantly greater amount of aggressive encounters was directed by C rams towards females. Moreover, ewes expressed more reproductive behaviours like standing firm while rams were attempting to mount, head turning and wagging tail behaviour in the presence of entire males. This may be a response to ram behaviour which would further reflect whether the rams showed sexual behaviour in the presence of a female. It is also

possible that C rams performed better during mating tests because ewes were more attentive towards them and did not direct any significant levels of aggression. It is also probable that ewes were more attentive to C rams because they had somehow a different appearance related to the presence or absence of testosterone. Similar findings related to ewes' appearance were reported previously. It was found that attractiveness of ewes may be altered by the presence or absence of oestrus (Tillbrook, 1987 a, b; Tilbrook and Cameron, 1989) which may be related to secretion of specific pheromones. It has also been shown that ram smell (ram pheromones) is the most crucial factor for initiation of oestrus (Knight, 1983) stimulating ewes to initiate reproductive behaviours like courtship. In addition, C rams had greater scrotal circumference, possibly greater semen production, body weight and social rank at earlier ages of life. All of these could potentially lead to a more attractive appearance as shown previously by Ungerfeld and González-Pensado (2008b). Therefore, it is very likely that ewes were aware of the impaired capacity of immunized and RR castrated rams to perform courtship from the time of the first contact. It is also possible that the smell of RR rams may have been completely different. Equally, scrotal circumference, semen production in immunized rams and possibly body weight in the RR group was also largely reduced. This may have been the cause of increased aggression towards immunized and RR treatment as well as greater frequencies of vocalizations and escape attempts expressed by those groups.

4.8. Conclusion

The objective of this study was to evaluate efficacy of a new anti-GnRF vaccine for castration of ram lambs. The ability of the vaccine to induce sterility was assessed. The impact of the vaccine on circulating testosterone, the growth and histology of the testes, the development of reproductive behaviour in rams was also evaluated. Furthermore, the male attractiveness during mating behaviour tests was investigated for the first time. It was hypothesized that immunocastrated males will show reduced frequencies of courtship and sexual behaviours in comparison to entire males due to reduced levels of circulating testosterone. It was also assumed that immunized rams will have impaired development of testes which will be manifested in smaller testes size, testes consistency and histology (impaired testes growth, presence of immature cells not capable to produce testosterone, absence of spermatocytes etc.).

Findings reported here confirm that an anti-GnRH vaccine for ram lambs evaluated in this study was effective in achieving sterility by reduction of circulating testosterone leading to impaired testes consistency, scrotal circumference and testicular histology. Alteration of testosterone levels resulted in suppression of reproductive behaviours (mounting attempts, flehmen, low stretch, nudging and male follow) as well. It was also shown that an extended interval between primary and booster vaccination prolonged the immunity period and the primary vaccination given at weaning was the most effective and practical method of vaccine administration. This finding is very important from the practical point of view. It allows for the vaccine to be administrated at the time when the flock needs to be gathered for general management. This permits for economical use of time and resources. It does not cause unnecessary stress to the animals by additional gathering as well. Evaluation of testosterone concentrations after administration of particular vaccination regime have shown that testosterone levels remained suppressed for at least 12 weeks (after vaccination) on the first year of the study, 20 weeks (after vaccination) on the second year of the study and until 28-32 weeks of age in the following year. This outcome was also achieved with only 2 vaccinations which again would be very important from the farmers' point of view as it may be impractical and

too expensive to gather animals for vaccination more than twice. Testicular measures remained reduced in the immunized groups for the same time periods reported for testosterone concentrations. Reduced testicular measures are very good indicator of the vaccine efficiency which could be use by the farmers and farm staff to assess if the vaccination was successful. This study reported also for the first time the impact of the immunization on the ewes' perception of rams' attractiveness. Ewes' perception of male attractiveness was influenced by immunization affecting females' behavioural pattern during the courtship and sexual behaviours observations. Ewes were more aggressive towards immunized or physically castrated males in comparison to entire rams. Moreover, ewes expressed more reproductive behaviours like standing firm while rams were attempting to mount, head turning and wagging tail behaviour in the presence of entire males in comparison to physically castrated and immunized rams.

On the basis of the results evaluated in this study it has been demonstrated that immunization against GnRF is a good alternative to traditional physical castration methods for example rubber ring, combined Burdizzo and, rubber ring or short scrotum castration in achieving male lamb sterility. An evaluation of the optimal vaccination regime will be given in Chapter 7. The intention was to assess all factors measured during the course of the study which may be crucial for the final judgement of most efficient vaccination regime, for example, impact of different castration method on carcase conformation and meat quality measures described in chapter 6. Therefore, the estimation of the most efficient way of vaccine administration will be done in chapter 7 where all gathered data from particular chapters will be summarised and included in the final evaluation.

Chapter 5 Influence of castration method and sex on lamb behaviour, the development of ewe-lamb behaviour and stress responses

Abstract

Castration alters the hormonal status of ram lambs, and most methods cause pain, which may have a longer-term impact on lamb behaviour. Seventy-two Mule (Scottish Blackface x Bluefaced Leicester) x Suffolk or Texel lambs were allocated to one of 5 groups at 2 days old (n=12 per treatment or n=24 in female group): entire male controls (C), male lambs castrated using rubber rings (RR) without anaesthesia, male lambs castrated using rubber rings and local anaesthesia (LA), immunological castration using an experimental anti-GnRF vaccine (VAC 2) or female lambs (F). Ewe-lamb bonding was recorded by one observer for 10 days in each of 3 time periods (1-immediately after birth; 2-at 6 weeks of age; 3-at 12 weeks of age) by scan sampling 3x a day. Duration, frequency and latency of lamb anxiety behaviours were recorded after weaning in 3 testing situations (isolation, novel object and unfamiliar human). Kruskal–Wallis tests were used to determine significant differences between treatment groups. C lambs tended to be further from the ewe than VAC2, LA or F lambs in Period 1 (H=15.54, P=0.05 median distance, m[Q1-Q3]: C=3.0 [0.2-10], VAC 2=0.5 [0-7], LA=1 [0.1-7], RR=1.5 [0.1-8], F=1.5 [0-8]). There were no significant differences between treatments in periods 2 and 3. Furthermore, treatment significantly affected stress responses after weaning. C lambs were consistently scored (on a scale from 1 not fearful to 4 most fearful) as less fearful than F lambs (measure taken just after the fear eliciting situations test had finished and lamb had to pass by the handler to return to home pen): isolation **P=0.01** (median frequency, m[Q1-Q3]: C=1.5[1.0-2.0], F=3.0[2.2-3.7]), surprise test **P=0.002** (median frequency, m[Q1-Q3]: C=2.0[1.0-2.0], F=3.0[2.0-4.0]) and unfamiliar human **P=0.01** (median frequency, m[Q1-Q3]: C=1.0[1.0-2.5], F=3.0[2.0-4.0]). However, there were no significant differences between treatments in other anxiety measures. C lambs were shown to have a significantly greater frequency of mounting behaviour than all other treatments during the feed competition test which may be related to aggression at this stage of life (**P=0.01** median frequency, m[Q1-Q3]: C = 1.0[0.0-8.2], Vac 2 = 0.0[0.0-0.5], RR=0.0[0.0-0.0], LA=0.0[0.0-0.0], F=0.0[0.0-0.0]). In conclusion, castration method, and lamb sex had no longer term impact on ewe-lamb

behaviours. Long term testosterone exposure had some influence on expression of social behaviours (aggression) and stress responses.

Key Words: Animal Welfare, Castration, Ewe-lamb bonding, Anxiety

5.1 Introduction

Most EU countries permit castration of the male stock in the first week of their lives (EC, 2001) due to public demand for leaner meat and improved carcass characteristics (Kiyma et al., 2000; Thornton et al. 1999; Amatayakul-Chantler et al., 2012) as well as ease of management (Godfrey et al., 1996). However, castration will alter the hormonal status of male lambs by the cessation of testosterone production. Testosterone is a steroid hormone which has beneficial effects on the development of bones, muscles, libido, cognitive functions, and mood and secondary male characteristics (Reed et al., 2006). Its secretion and presence appear to be strongest at puberty, when the hypothalamus increases secretion of gonadotropin releasing hormone (GnRH) which influences its target cells to produce and secrete testosterone by acting on Luteinizing hormone (LH). Due to the beneficial effect of testosterone on growth it is reasonable to suggest that lack of it may also contribute to economic consequences following castration. It has been shown that entire males have been reported to have better feed conversions (Bonneau, 1998), therefore they may grow faster and be slaughtered earlier adding to the farm profitability. Gonadal steroids may also impact on behavioural responses related to fear even if they appear later in life (Bouissou & Vandenheede, 1995). During the oestrus cycle, changes in hormonal status affects females' avoidance behaviours towards fear eliciting stimuli (Diaz-Veliz et al., 1991). If the normal hormonal changes during the female cycle are able to impact on their interaction with the environment what effect might castration of males have on their behavioural expression? Altered hormonal status may lead to exaggerated expression of such individual emotionality when faced with a fearful situation. It was shown by Vandenheede & Bouissou (1995) that wethers were more fearful than entire males. This was believed to be a consequence of differences in androgens which was further confirmed by the fact that female lambs were more fearful than male lambs. Moreover, androgen treated ewes were found to be less fearful than not treated entire males (Vandenheede & Bouissou, 1995). It seems that the easiest way to investigate the impact of gonadal steroids on behavioural reactions related to fear is to test entire males,

castrated males and female lambs in fear eliciting conditions. It can be argued that castrated males, due to lack of testosterone, should resemble reactions of female lambs when tested under the same circumstances. Sex differences in the response to fear eliciting situations have been already shown in the past (Vandenheede & Bouissou, 1995). Similarities in behavioural expression of wethers to females have been reported in the past as well. Castrated males are found to express higher frequency of activity during open field tests similar to female responses. Similarly, rearing reactions in castrated males are equal to female responses (Bengelloun et al., 1976). On the other hand, testosterone treatment has been found to reduce fear reactions in ewes (Bouissou & Vandenheede, 1993). Moreover, in cows it has been reported that administration of testosterone altered the social status of such animals by reduction of fear towards their conspecifics (Bouissou & Vandenheede, 1993). Fear has also been reported to influence other behaviours like maternal (Putu, 1990) or social behaviour (Bouissou, 1990). Fear may influence management procedures and everyday handling in a negative way leading to economic losses due to bruising and time wasting (Bouissou & Vandenheede, 1995).

The first objective of this study was to evaluate long term effects of the castration method including immunization against GnRF and sex on the expression of stress responses and social behaviours like aggression. Farm animals such as sheep are sentient beings although it is almost impossible to estimate what they really feel when faced with sudden unexpected situations. The response of an animal to handling which may be perceived as negative stimuli may give some indication about such animals' emotionality or so called temperament (Wolff et al., 1997; Boissy, 1998). Emotional response to handling in wethers has been studied before by Hargreaves and Hutson (1990). Fear may be used as an indication of when an animal is feeling unpleasant about certain situations. Fear may be seen as a tool preventing an animal from interacting with a potentially dangerous or threatening event (De´sire et al., 2002). The main emphasis of this study was to investigate the influence of early pain and testosterone exposure on lambs' behaviour later on in life. It was hypothesised that immunocastrated lambs will show similar levels of activity as entire males before the primary vaccination is

administered. It was also assumed that immunocastrated males will resemble castrated lambs and/or female lambs in the levels of aggressive behaviour and stress responses when faced with three fear eliciting situations, if fear is related to current circulating levels of testosterone rather than prior exposure. The Immunization technique allows lambs to be exposed to beneficial stimulatory effects of testosterone before the anti-GnRF vaccine is applied. The impact of immunization on the welfare of immunized males and their behavioural expression has not been investigated in depth (Prunier et al., 2006). Equally there are not many reports on the long term effects of immunocastration on the development of behavioural expression including behaviours like aggression, stress responses and development of ewe-lamb bonding. It has been shown that castration is a painful procedure (see chapter 3 for more details). Because of its distressing nature castration may have negative impact on the development of ewe-lamb bonding. It was suggested by Clark et al. (2014) that early pain exposure may have a long term effect on individual phenotype, health and welfare, especially in farm animals exposed to painful husbandry procedures on a daily basis. It was found by Clark et al. (2014) that variations in early life management may have an effect on well-being and health of production species. For example, ewes that were tail docked expressed increased pain related behaviour during parturition in comparison to control (not docked ewes). It was also suggested that fear responses of sheep later on in life may have been influenced by early life experiences.

In the UK, one of most popular castration technique is the rubber ring method (DEFRA farm survey 2005). Due to legal requirements rams need to be castrated within the first week of age which means that they must be gathered and the rings must be applied at the time when the bond with the ewe may still be in the process of development. Sheep are a precocial species, fairly well developed at the time of birth with the ability to stand up very quickly. This allows species like sheep to follow their mother and be at a close distance to her (Dwyer et al., 1999). It is crucial for sheep that their offspring are able to promptly establish the bond and recognize their mother as they will not remain at the birth site to grow up together with other young in the flock therefore their chances of

survival and welfare depend on their capability of mother recognition (Dwyer, 2008a). Their own ewe will be the only means of providing appropriate nutrition, protection and guidance during development. It is possible that the castration procedure may be a cause of disturbance in the normal bond formation as lambs will behave differently due to pain and distress caused by castration. Castrated lambs show higher frequencies of certain behaviours like easing quarters, foot stamping kicking or long periods of immobility after castration has been applied (as described in chapter 3). Although most mis-mothering behaviour of sheep is associated with inexperienced ewes it has been show that such instances have been also associated with nutritional status, breed and behaviour of the offspring (Dwyer, 2008b). As a consequence of altered behavioural patterns, lambs may not be able to express appropriate following or suckling behaviour (Dwyer, 2008b; FAWC, 2008). Due to pain, lambs may not suckle for several hours which may result in weakness and if this occurs in the first hours after birth it may affect immunity due to insufficient colostrum uptake which may end in higher mortality rates (Dwyer, 2008b; FAWC, 2008). The incidence of miss-mothering may be greater particularly in systems where ewe and lambs are not confined (FAWC, 2008). It has been shown in the past that castration is very painful and disrupts normal behavioural patterns in castrated animals (see chapter 3 for more details). In the light of these findings it was hypothesized that immunized lambs should form a bond with the ewe without disturbance or with minimal impact (related to environmental factors and individual characteristic of specific animals) equally to C lambs as the primary vaccination will be administered at the time when the bond is already formed. Hence, it would be a good alternative to traditional castration by preventing disturbances in the ewe-lamb bonding process. The following treatments groups have been formed to test whether presence of early pain and testosterone exposure have an effect on formation of ewe-lamb bond and expression of fear and anxiety behaviour after weaning: 1) Rubber ring treatment (RR - lambs experienced pain and reduction or absence in circulating testosterone); 2) Rubber ring treatments with use of local anaesthesia (LA - lambs did not experienced acute pain but there might have been chronic pain exposure and there was reduction or absence in circulating testosterone); 3) Entire males (C- lambs did not experience pain or absence

of circulating testosterone); 4) Immunocastration (Vac 2 - no pain and reduced testosterone later on in life), Female group (F- no pain experience and no presence of circulation testosterone in the plasma).

Chapter 5 Hypothesis

1. Physically castrated males and ewe lambs will show greater fearfulness in standard tests compared to entire males and immunocastrated males will be intermediate.

2. Immunocastration of male lambs reduces the expression of aggression during the period of immunity.

3. Early post-natal pain experience and manipulation of circulating testosterone alters lamb behavioural development, particularly ewe-lamb bonding and anxiety behaviour.

4. Seven month old male lambs immunized against GnRH (accordingly to agreed protocols at 6 and 12; 6, 12 and 22; 10 and 16; 10 and 20; 12 and 18; 12 and 22 weeks of age) show agonistic behaviour comparable to a physically castrated male lamb (using rubber rings), and less aggressive behaviour than entire male lambs of a similar age.

Chapter 5 Objectives

1. Assessment of the impact of different castration methods or handling and sex on formation of ewe-lamb bond.
2. Assessment of the impact of different castration methods or handling and sex on expression of anxiety/fear behaviours during three fearful situations.
3. Assessment of the impact of the different castration methods or handling and sex on the development and expression of aggressive behaviour during feed competition test.

5.2 Materials and Methods

5.2.1 Housing and management

Husbandry and management practices are described in detail in Chapter 2 where specific details of housing and management, allocation and implementation of treatments (see table 1), time of weaning and "the end point" are defined. The study was conducted on the Woodhouselee farm (property of SRUC), Edinburgh, Scotland between April and December 2012. After lambing, lambs were moved into small pens (approx. 1.5 x 1.5 m) with their mother and siblings (only twin pairs were selected). For male-male twins both lambs were assigned to different treatment groups. Pens were equipped with deep straw bedding. Water and hay were offered ad libitum. Ewes were also fed 0.5 kg per ewe (twice per day) concentrated feed: XL Ewe 18% + Amino Green® Rolls (East Coast Viners Animal Nutrition) UK.

5.2.2 Animals

72 male lambs (mule x terminal sire Texel or Suffolk) were selected at birth, lambs were balanced for maternal parity and sire breed across treatment (see table 1 in Chapter 2). Only twin lambs were selected. Treatments were applied when lambs were between 24 and 48 hours old. Five groups of lambs were formed (n = 12 for C, RR, LA, Vac2 groups and n=24 for F group).

1. Positive controls (C) – lambs were handled only at 24-48 h of age.
2. Negative controls (RR) - rams castrated with use of standard rubber rings without local anaesthesia at 24-48 h of age.
3. Lambs using rubber rings and local anaesthesia (LA)- rams castrated with use of standard rubber rings with use of local anaesthesia (see chapter 2 for more details) at 24-48 h of age. The mixture containing half the dose of bupivacaine and lidocaine associated with toxic signs after IV injection was prepared. The volume of 1.3 ml of the mixture was then injected into each spermatic cord and additional 2.4 ml to the neck of the scrotum (in total 5ml of mixture was administered to each lamb).

4. Immunocastration (Vac 2) – rams were vaccinated with an anti-GnRH vaccine as per agreed protocol at 6, 12 and 22 weeks of age.
5. Female lambs (F) - handled only at 24-48 h of age.

5.3 Dataset descriptions

5.3.1 Traditional castration

When lambs were aged between 12 and 48 hours control lambs were handled (this involved picking up a lamb from the ground and handling it in a manner mimicking physical castration without application of the rubber ring). Lambs from treatments requiring physical castration were castrated (rubber ring were applied) with or without anaesthesia depending on the treatment group. Lambs from the immunocastration treatment and female siblings were untreated at this point.

5.3.2 Immunization procedure

The Immunization procedure consisted of primary and 2-booster vaccination. Vaccine was administrated at 6, 12 and 22 weeks of age. Lambs were vaccinated by a single s.c. (sub-cutaneous) injection on the right side of the neck with an anti-GnRF vaccine. For more details related to the immunization procedure see chapter 4.

5.3.3 Local anaesthesia solution preparation

On the second year of the study I was particularly interested in the impact of early post-natal pain experience on lambs' behaviours later on in life. Moreover, I was interested whether manipulation of circulating testosterone alters lamb behavioural development, predominantly ewe-lamb bonding. To explore the influence of early pain exposure and manipulation of circulating testosterone on lambs' behaviour I had to form a treatment group that would be free from early pain experience and compare behaviours of such group with a control treatment and group which had undergone a painful procedure early in life such as rubber ring castration.

To undergo such a study, the use of local anaesthesia was discussed and agreed. Application of the following solution was approved by SRUC Animal Experiments and Ethics Committee. The intention was to prepare a safe and efficient anaesthetic solution

which would last for a few hours giving lambs pain free sensation and avoid any negative influence of used anaesthetics (related to the drug's toxicity) at the same time. Practical knowledge from previous studies and the literature review was used for assessment of available products and selection of appropriate active ingredients. A 2% lidocaine (Lidocaine 100mg/5ml solution for injection Alliance Healthcare Ltd®) and 0.5% bupivacaine (Marcaine 0.5% AMPS 10x10ml AstraZeneca UK Limited®) solutions for injections were chosen. The next step was to decide the appropriate dose which would be safe for lambs as young as 12-48 hours old. The mixture containing half the dose of bupivacaine and lidocaine associated with toxic signs after IV injection was prepared. The dose of 10 and 4 mg/kg lidocaine and bupivacaine respectively was mixed together. This gave a mixture of 0.25 (5/20) and 0.4 (2/5) ml of 2% lidocaine and 0.5% bupivacaine respectively (the volume of 0.75 mL for 1 kg of lamb weight). To control the toxicity levels even more, this volume was diluted 50% with normal saline solution (solution of 0.9% NaCL/1l Norbrook®), which gave in total 1.3 mL per kg of lamb.

After consideration of practical issues related to administration, the volume of 1.3 ml of the mixture was then injected into each spermatic cord and an additional 2.4 ml to the neck of the scrotum (in total 5ml of mixture was administered to each lamb). The 18G x 1-inch Veterinary Disposable Hypodermic Needles (Millpledge®) and 5 ml BD Discardit® syringes were used.

The anaesthetic solution was also used during tail docking of all treatments in the years of 2012/2013. 1mL (0.5 ml on each side of the tail) of the prepared mixture was given subcutaneously to all animals 5 minutes before tail docking.

5.3.4 Mother Lamb Bonding behaviour

The distance between the ewe and each lamb, and the posture and activity of ewes and lambs (see figure 1a, b, c, d and table 1 below for details) were recorded by scan sampling. Observations were carried out during 3 observation periods, each 10 days long. The start day was not the same for all animals as it depended on the date of lamb birth and allocation to the particular treatment.

All groups of lambs were taken with their siblings and dam to the grazing paddock (approximately 200x200m). General stocking density rule of 1.9 square metres were applied. The animals had a freedom of movement appropriate to their species and they were not restricted in any way. The space given to the animals was appropriate to their physiological needs. Water was offered ad libitum. Ewes could graze on the field and there was also concentrated feed provided 0.5 kg per ewe 1 x a day (XL Ewe 18% + Amino Green® Rolls ®East Coast Viners Animal Nutrition). In total there were three observation periods. The first period of observations took place shortly after the birth once handling (C and F group) or physical castration with or without anaesthesia (RR and LA group) have occurred when lambs were 24-48 h old. Vac 2 lambs were untreated at this stage. A second period of observations took place at the time of primary vaccination of Vac 2 treatment group when lambs were approximately 6 weeks old. The third period of observations took place 2 weeks before weaning (after administration of first booster vaccination in the Vac 2 treatment) when lambs were approximately 13 weeks old.

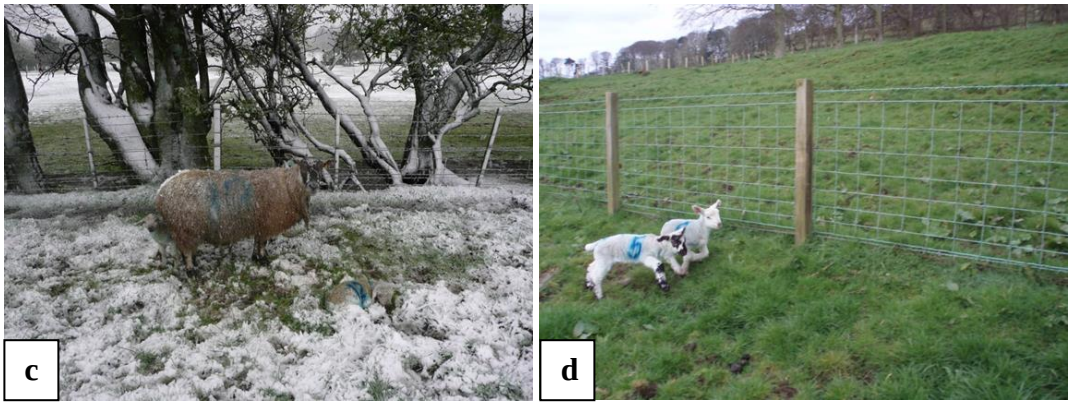


Figure 1a,b,c,d Examples of lambs' postures and behaviours during field observations of ewe-lamb behaviour: a) Suckling behaviour, b) walking behaviour, c) standing inactive (lamb behind ewe), lying sleeping (lamb in front of the ewe), d) play/jumping behaviour.

Table 1 Ethogram of Ewe and lamb behaviours

Ewe lamb bonding measurements	Description
Walking	Lamb moves forward in a, slow motion.
Standing	Lamb stands on the ground with all four legs.
Lying	Ventral (sternal) recumbence with the legs tucked in and the head down, either round to one side or directly in front or head up.
Running	Lamb moves forward in a faster motion than walking without jumps and hops.
Inactive/sleeping	Lamb lying with closed eyes and head down or up (sleeping) and standing or lying with open eyes with no other visible activity (inactive).
Play/Jumping	Lamb moves forward using hops and/or jumps.
Forage/Grazing	Lamb stands or walks with its head down sniffing or pulling at grass.
Suckling	Lamb head is in the region of ewe's udder, lamb is touching ewe udder with its muzzle and appears to be suckling.
Sniffing	Lamb head is directed towards a particular object i.e. ewe,

	sibling, fence, the nose is within 5 cm of the object.
Vocalizing	High pitched bleats.

Each day was divided into 3 2-h sections: Morning (9:00-11:00), Afternoon (12:00-14:00) and Evening (15:00-17:00). Three scans were performed every day, with one scan being performed in each time period. Data was collected for ten consecutive days (30 scans per lamb in total per observation block).

5.3.5 Anxiety - fear tests

The anxiety observations were carried out 2 weeks after weaning when lambs were approximately 4 months old. Labs were faced with 3 fear eliciting situations. Before the study was conducted, literature review was performed to define what anxiety and fear reactions are expressed by sheep and how they are manifested in this particular species. So what is anxiety/fear? According to Jones et al. (1981) fear is "an adaptive reaction to a perceived danger", Bouissou & Vandenheede 1995 defined fear as a “general characteristic of an individual which could influence its behaviour in the same manner in different situations”. Validated methodology was developed by Romeyer and Bouissou 1992 allowing for measurements of fear reactions by using three different fear stimulating behavioural tests. Several behaviours have been then correlated with these fear eliciting situations allowing for behavioural assessment of sheep emotional state to be conducted. In their research Bouissou and Vandenheede tested different factors for example: sex differences, castration of rams, testosterone treatment of ewes or environmental enrichment to investigate anxiety/fear reactions of sheep in three different tests: separation from conspecifics, presence of unfamiliar human and presence of unfamiliar object. In this study the ethogram of anxiety/fear behaviours was based on the previous studies of Bouissou & Vandenheede 1995 and Vandenheede 1998 (see table 2 for more details) investigating anxiety/fear reactions of sheep in a particular situation (i.e. separation).

Before testing animals were moved to the testing shed and placed in the home pen. The testing apparatus was set on one side of the shed (see figure 2). The other side of the shed was used to accommodate lambs for the whole period of testing. A three-day habituation period was used before testing so animals could adapt to the experimental environment in that time. During the habituation period all lambs were allowed to explore the testing area together with their conspecifics three times a day for approximately 20 min at a time (in the first day of habituation). On the second day of habituation all lambs were moved together with their conspecifics to the per-test pen, then the test pen and to the post-test pen three times a day again for approximately 20 min at a time. On the third day of habituation, animals were handled in pairs the same way as during the actual testing. Concentrate feed was presented to the animals in the feeding area at the back of the testing pen and post-test pen each time when animals were exploring the testing apparatus.

On the day of the test, 5 min was given to the group of animals to leave the pre-test area (area between home pen and pre-test pen). If no animal voluntarily left the pre-test pen they were gently pushed towards the entrance by the same handler so one of them eventually entered the pre-test pen (see figure 2) area in front of the starting cage. Each animal was then given a further 5 min to enter the starting cage voluntarily before it was gently encouraged to enter the cage by the experimenter. Once the lamb was in the starting cage the gate to the test pen was opened. Each lamb was given time to enter the testing pen and if there was no voluntary movement after 30s, the animal was encouraged to enter the testing pen by closing the space behind it. The time to enter the cage was noted by the observer. Each lamb was also given a fear score when the test was finished (see table 2). This was also noted by the same observer during the whole testing period. Single tests lasted for 4 minutes. The test pen (4 m x 4 m) was divided into 9 equally sized squares (cm each) marked on the floor of the pen. The squares were then added to form 3 testing areas 1, 2, 3 (see figure 2). The lambs were recorded for the duration of each test on a camcorder. The following behaviours were then derived from the recordings: anxiety behaviours (see table 2), latency of time to perform specific

behaviour, frequency and duration of time spent in each area of the testing apparatus by particular lambs recorded by video camera (Canon XM2 3CCD Digital Video Camcorder, Canon Inc, Japan, mounted on a tripod). Recordings were then scored using the Observer XT 9 (Noldus Information Technology®) program by a single observer.

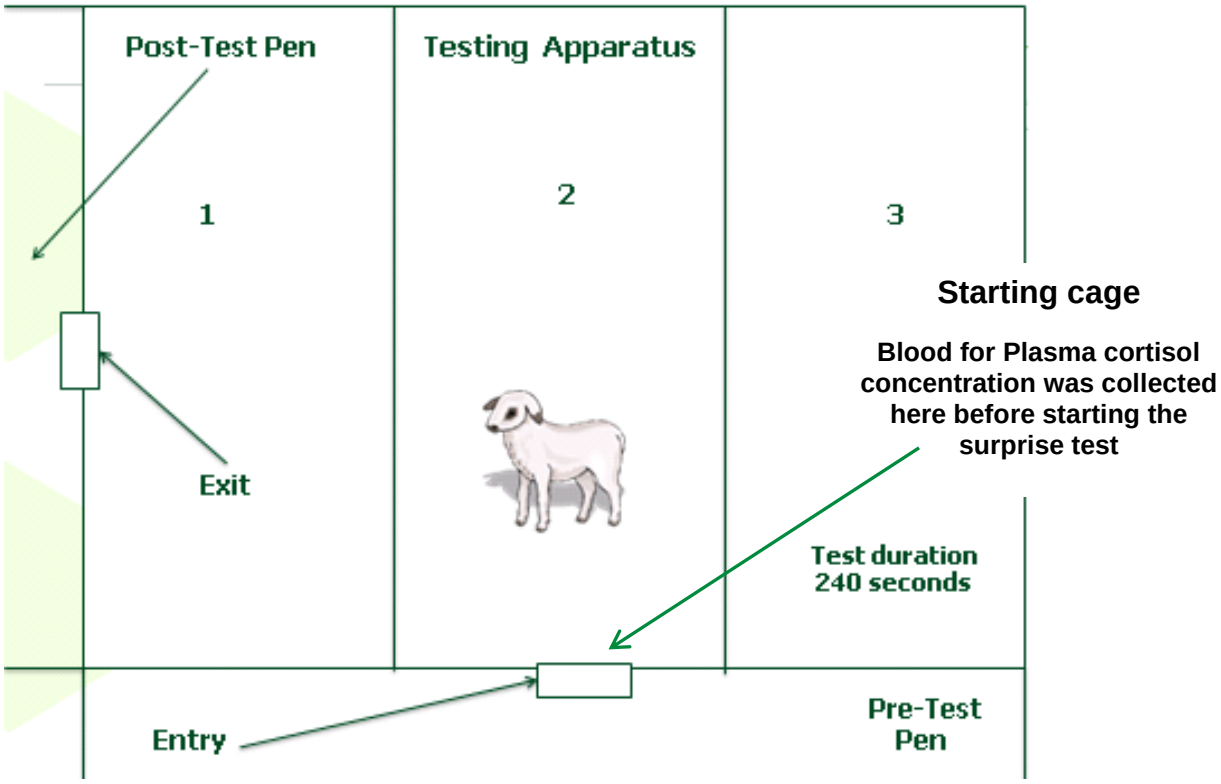


Figure 2 Testing apparatus (4x4m) used in anxiety fear tests.

Table 2 Ethogram of Anxiety Measurements (based on Bouissou & Vandenheede 1995, Vandenheede 1998)

Anxiety Measurements	Description
Latency to enter test pen	Time measured in seconds from the moment when the gates to the testing pen were opened to the moment when the lamb entered the testing apparatus with its front and back legs.
Walking	Lamb moves forward in a slow motion.
Standing	Lamb stands on the ground with all four legs.
Vocalizing	High pitched bleats.
Jumping/climbing on gates	Lamb climbs on gates with its fore legs or jump on

	it.
Sniffing Exploring Test Pen	Lamb stands or walks around the testing pen. Lamb head is directed towards a particular object i.e. floor or fence, the nose is within 5 cm of the object.
Number of times entering area 1,2 or 3	Frequency of crossing into a particular area of the test pen.
Duration of time spent in area 1,2,3	Time spent in seconds in particular area of the testing apparatus.
Fear Score	Fear Score of 1 to 4 was given to each lamb on the basis of lamb reaction to the handler in the post-test pen standing behind the exit gate in such a way that the lamb was able to see the handler once the gate was closed. 1 - passing observer with confidence. 2 - passing observer with caution. 3 - passing observer after stepping back and running away from observer. 4 - jumping on gates trying to escape.

Anxiety Measurements specific for the surprise test	**Description**
Sniffing/touching ball	Lamb head directed towards the ball, the nose is within 5 cm of the object with or without physical contact.
Looking in the direction of ball	Lamb head and ears are turned towards the ball.
Butting ball	A sharp downward movement of the head in the direction of the ball with or without physical contact.

Anxiety Measurements specific for the unfamiliar human test	**Description**
Sniffing/touching human	Lamb head directed towards the unfamiliar human with the nose within 5 cm of the unfamiliar human with or without physical contact.
Sniffing/licking feeder	Lamb head is in the feeder or within 5 cm of it with or without licking it.
Looking at the direction of human	Lamb head and ears are oriented towards the person.
Butting Human	A sharp, downward movement of the head in the direction of the human with or without physical contact.
Feeding	Lamb head is down in the feeder or up. The chewing movement is visible for at least 3 s.

Lambs were tested after weaning (2 weeks after weaning) when they were approximately between 3.5-4.5 months old in 3 fear eliciting situations. All tests were done on consecutive days. Lambs were divided into 4 groups of animals. Lambs from different treatments were randomized across all 4 groups with use of R statistical programme. Tests were always done in the same order. On the first day the isolation test was performed. On the second and third day the surprise test was done. On the fourth day the unfamiliar human test was carried out.

Separation from conspecifics.

Each animal was placed in the testing pen on its own. The test was performed without any additional stressor (see figure 3 below).

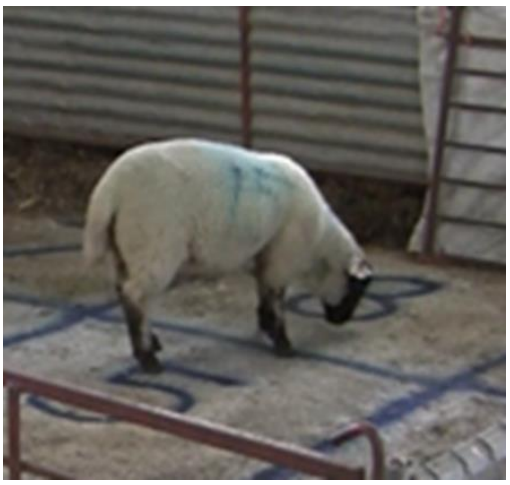


Figure 3 Lamb during Isolation test sniffing exploring testing pen

Surprise tests (unfamiliar object test)

The red ball was thrown in the middle of the testing pen one minute after the lamb has entered the pen (see figure 4 below).

Figure 4 Lamb during surprise (unfamiliar object) test looking at the ball

Presence of an unknown human.

The unfamiliar person made an entrance to the testing pen one minute after the lamb has entered the testing apparatus and sat down at the back of the testing pen by the feeder placing a small amount of feed (0.5 kg) in it (see figure 5 below). The intention was to create a conflict situation between motivation to eat and fear. A period of food withdrawal (12h) occurred before the start of the test. The unfamiliar human wore a grey hat and red overalls (fig 5), which was novel for the lamb as all the farm staff had blue or green overalls. The unfamiliar person had no previous contact with the lambs before the test. Throughout the whole study the same person took part in the unfamiliar human test.

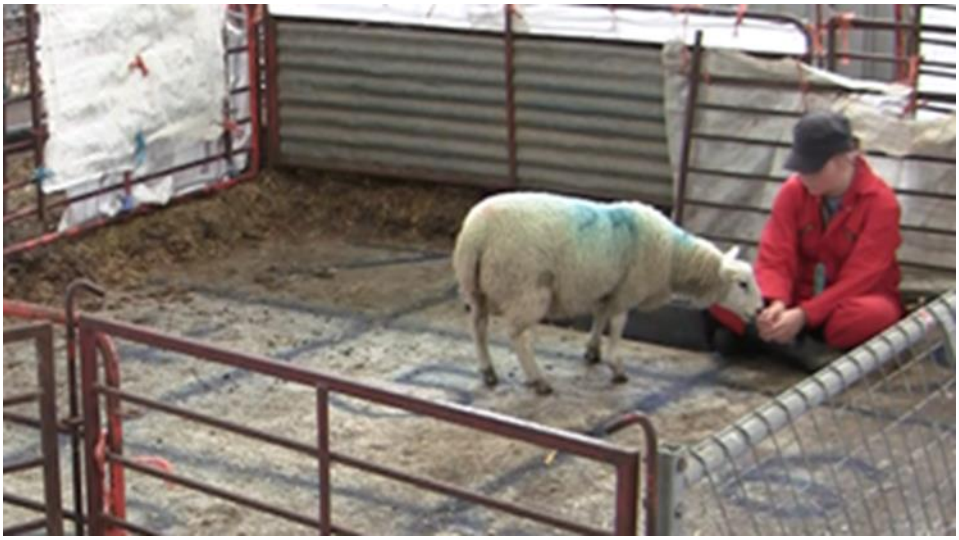


Figure 5 Lamb during unfamiliar human test sniffing unfamiliar human, (note the feeder behind the lamb where the unfamiliar human placed a small amount of feed before sitting down)

5.3.6 Plasma Cortisol concentration measurements

The secretion of glucocorticoid hormones i.e. cortisol or corticosterone during unpleasant situation or after encountering unpleasant stimuli is a classic physiological stress response commonly used to assess negative emotional states (i.e. stress response) (Georgia Mason, 2016). Glucocorticoid hormones can be used as a tool for validating behavioural assessments. In this study the intention was to investigate weather sex, different castration method or handling have an impact on lambs' emotional state. Plasma cortisol concentration just before and after the (surprise) novel object test was analysed alongside behavioural measures. Changes in the plasma cortisol concentration before stressful situation were reported in the past. For example, Bouissou & Vandenheede (1996) showed that castrated rams had higher cortisol concentration than entire rams before the surprise test. Therefore it was concluded that measuring plasma cortisol concentration will add validity to behavioural assessment and better understanding of lambs' behavioural pattern. It has to be noted that during the course of the study technical difficulties with the indirect ELISA method used to analyse cortisol concentration did not allow to show differences between treatments. Possible causes of technical difficulties related to indirect ELISA method are discussed in chapter 7.

Each of 4 groups was further divided into 2 sub-groups to allow for blood collection to analyse the concentration of cortisol in the plasma to be carried out. Due to the number of animals and practical issues it was impossible to collect blood and perform testing for all animals in one day therefore sub-groups were formed (lambs were randomly selected, in each subgroup there was at least one representative from different treatment. It was impossible to have an equal number of representatives because the number of lambs in each treatment was not equal. Blood samples were collected before and after the surprise object test only for the determination of plasma cortisol concentrations. The blood sample for the evaluation of pre-test cortisol levels was collected just before the surprise test when lambs entered the test pen. The lamb was held in the starting cage during the blood sample collection. The sample of blood for the evaluation of post-test cortisol levels was gathered 15 min after the test finished and the lamb was allowed to enter the post-test pen. For each lamb two tubes of 4 ml volume were collected. During collection samples were placed into a tray in a cool box which contained ice blocks to keep samples cool. When collection was finished samples were loaded into a centrifuge Thermo Scientific®. The settings were checked and set up for 2500 rpm, 4º C and 20 minutes. Disposable pipettes (per lamb) were used to take out the plasma from the tubes taking care not to get any blood platelets in the sample and placed in 2ml Eppendorf tubes. In the event of the plasma being cloudy, or having obvious blood platelets still free floating a second spin was performed (this happened only on one occasion). Harvested plasma samples were stored in a -20º C freezer until analysis. Samples were analysed using the indirect ELISA test.

5.3.7 Preparation of Plasma samples to carry out the Indirect ELISA test

On the day of sample preparation, they were taken out of the freezer and placed on ice to gently thaw. When samples were thawed 0.5 ml was measured and allocated into a 15 ml tube. To 0.5 ml of the plasma sample 5ml of diethyl ether was added. The tubes were vortexed (VMR VX 2500 Multitude Vortexer® vortex mixer) for 10 seconds and frozen at -80 º C overnight. The next day tubes were taken out of the freezer and placed onto dry ice (figure 6a) and the unfrozen portion was transferred into the glass tube under the fume hood (figure 6b). The unfrozen portion was then dried out with nitrogen

gas in a heating block (Figure 6c). Tubes with prepared dried samples were frozen at -20º C until the cortisol ELISA was performed. The day before carrying out the cortisol ELISA samples were taken out of the freezer and reconstituted with 250 µl of assay buffer. Reconstituted samples were then placed in a fridge at +3 º C overnight.

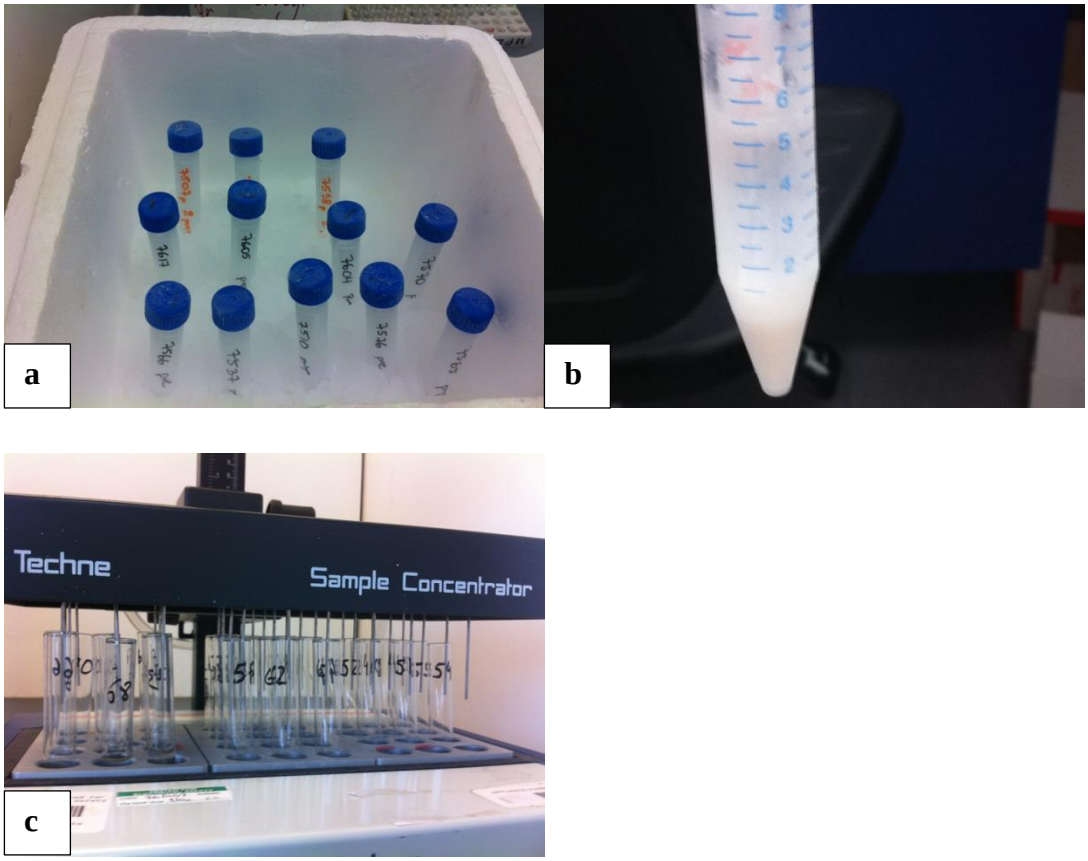


Figure 6 a, b, c Preparation of sheep plasma to conduct the ELISA test a) frozen plasma samples with added diethyl ether; b) unfrozen portion of the plasma sample (frozen portion is the excess of water); c) process of drying out the samples with nitrogen gas in a heating block Techne© Sample Concentrator.

5.3.8 Indirect method of Cortisol ELISA

Recipes of buffers and Dilutions

Specific and sensitive ELISA method for the estimation of cortisone levels has been developed and validated by Al-Dujaili et al. (2009); Al-Dujaili et al. (2012) has been used in this study.

Solvents and solutions

Coating buffer – 0.025 M phosphate buffer saline (PBS) pH 7.4.

Blocking buffer – 0.025 M PBS + 0.5% BSA (Bovine Serum Albumin).

Assay buffer - PBS pH 7.4 containing 0.1% bovine serum albumin (BSA).

Wash buffer - 0.015 M PBS pH 7.4 containing 0.05% Tween 20.

Antibody 1 anti-sheep cortisol as described by Emad AS Al-Dujaili et al., 2012, Al-Dujaili et al. (2009).

Enzyme donkey anti-sheep antibody + Horseradish peroxidase – HRP as described by Emad AS Al-Dujaili et al. (2012), Al-Dujaili et al. (2009).

Substrate buffer – 0.2 M acetate/citrate buffer pH 5.0.

TMB – 30mL of substrate buffer + 500 µl 3,3',5,5'-tetramethylbenzedine (0.4%) + 100 µl of H_2O_2. Mix tube gently on the vortex (Hook & Tucker Instruments® vortex mixer).

Stop solution – 900 ml of distilled water + 100 ml H2 SO4.

Steps of ELISA test

Step 1 – Coating: Plates (Micro Amp® 96-Well Reaction plate) were coated with 200uL of BSA (Bovine Serum Albumin) conjugate (0.5mg/ml) at a 1:200 dilution and incubated over night at 4 º C. Plate was cover with sticky film or Para film. All samples were assayed in duplicate.

Step 2 – Washing: Following overnight incubation contents of the plate was discarded and washed 3 times with 250uL of wash buffer.

Step 3 – Blocking: The plate was blocked with 200 µL of blocking buffer.

Step 4 – Incubation: The plate was incubated for 1 hour at 37 ºC. After 1-hour incubation the content of the plate was discarded and the plate was blotted to remove the extra droplets. No washing was required after this step.

Step 5 – Standards/Calibrators: All standards were added (50uL) as well as samples (100uL) to the wells assigned. The content of plate was mixed by gentle circulating hand movements.

Step – 6 Incubation: The plate was incubated for 2 hours at room temperature (in a dark place).

Step 7 – Washing: The plate was washed 3 times with 250uLwashing buffer.

Step 8 – Enzyme: 100µL HRP-anti-sheep IgG was added to each well and gently mixed by hand.

Step 9 – Incubation: Plate was incubated at room temperature for 1 hour.

Step 10 – Washing: Plate was washed 3 times with wash buffer and 100uL of substrate (TMB) was added to each well. The plate was then gently mixed by hand in a circulating movement.

Step 11- Incubation: The plate was incubated at room temperature in a dark place for 10 – 15 minutes.

Step 12 – Stopping process: 50uL of stop solution was added to each well (see figure 7) and the plates were read at 450nm using a microplate reader (Thermoscientific® Multiscan FC Microplate Photometer).

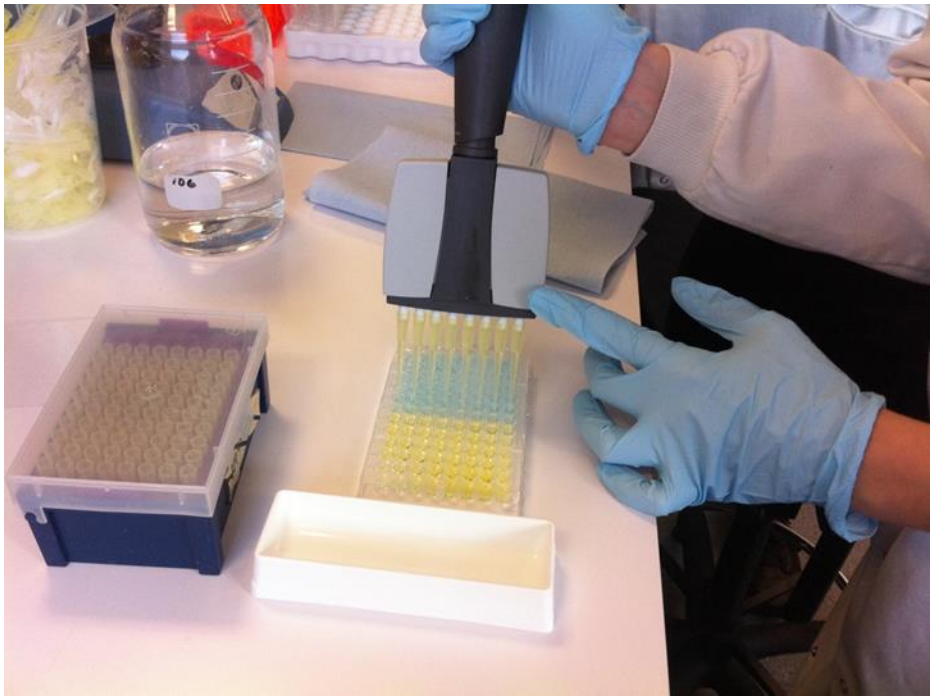


Figure 7 Stop solutions being added at the last step of indirect ELISA test to evaluate cortisol concentration in the blood of lambs before and after stressful situation

It must be noted that due to technical difficulties the number of obtained results may have not been sufficient enough to show the differences between treatments. Table 3 a, b is showing a number of samples in each treatment that were successfully analysed. It must be also noted that some of the samples pre and post-test did not belong to the same individual, which may have had a negative impact on analysis and the presented results as well.

Table 3 a Number of lambs in each treatment successfully analysed during Cortisol ELISA test for the cortisol concentration pre-unfamiliar object test

Treatment	n
C	7
F	19
Vac 2	9
LA	7
RR	8

Table 3 b Number of lambs in each treatment successfully analysed during Cortisol ELISA test for the cortisol concentration post-unfamiliar object test

Treatment	n
C	7
F	18
Vac 2	10
LA	6
RR	8

Concentration of the cortisol levels was determined as follows: The average cortisol concentration level (sum of each individual result measured for a particular lamb divided by the number of results that were obtained for this specific individual) was calculated and presented as a final result for further analysis. The final result for each individual was divided by 2 accordingly to the methods protocol (see section 5.2.5) to achieve a 1:1 relationship. The measure was presented in ng/ml.

5.3.9 Aggressive behaviours

Lambs were tested for the presence of agonistic behaviours on three consecutive days, when they were approximately 5.5 months old. One day before testing, animals were brought into the shed and segregated into home pens according to their treatment. Lambs from different treatments were not kept together. All animals had access to hay and water ad libitum during the night but the concentrate food was not present. Three testing pens (2m x 2m) were prepared on the opposite side to the pens in which animals were kept overnight. The testing pens were marked as 1, 2 and 3. During the testing sessions three lambs from each treatment were placed in the testing pen. The order of test pen and time allocation was randomized across all treatments with the use of R statistical programme to avoid any bias. Individuals were marked with the spray on their backs so they could be individually identified. A small amount of concentrate food (an equivalent quantity for each group – approx. weight of 0.5 kg) was placed in a feed trough for each trio, once all lambs were in their pens. Animals were allowed to compete for access to food for 30 min (although presented feed was eaten within the first couple of minutes of the test).

Recordings were made with a Canon XM2 3CCD Digital Video Camcorder (Canon Inc, Japan). The camcorder was placed on a tripod and directed towards the pen in such way to allow for full vision of the pen. Recordings were then scored using the Observer XT 9 (Noldus Information Technology®) program by a single observer. Table 4 below contains the ethogram which was divided into 5 categories: maintenance behaviours, performed aggression, returned aggression, male sexual-type behaviours and escape avoidance behaviours. The total number of aggressive encounters was also analysed by adding together behaviours from the performed aggression, returned aggression and sexual behaviours categories.

Table 4 Aggressive behaviours Ethogram

Behaviour/Posture	Description
Maintenance behaviours	
Walking/standing	Lamb standing on the ground with all four legs or moving forward in a slow motion.
Lying	Ventral (sternal) recumbence with the legs tucked in and the head up or down, either round to one side or directly in front.
Feeding	Lamb head is down in the feeder or up. The chewing movement is visible for at least 3 s.
Exploring feeder	Lamb head directed towards the feeder with the nose within 5 cm of the feeder with or without physical contact, licking the feeder was also included.
Ruminating	Lamb standing, walking or lying with its head up with visible chewing movement.
Inactive	Lamb standing, walking or lying and does not perform any other activity.
Performed Aggression	
Head Butt	A sharp downward movement with the forehead which makes contact with another lamb.
Displacement	Forcing another animal to leave its place of resting or feeding with or without physical contact.
Threat	A sharp, downward movement with the forehead directed at another lamb without physical contact.
Push	Pressing and/or pushing body against another lamb, head to head pushing, sometimes followed by head to neck pushing.
Returned aggression	
Butting back	A sharp, downward movement with the forehead making contact with a lamb which is also butting the focal lamb.
Pushing back	Pressing and/or pushing body against a lamb which is also pushing, or head to head pushing, sometimes followed by

head to neck pushing.

Sexual Behaviours	
Nudging	Combination of kick (forefeet used in a pawing motion while standing parallel to lamb), and rubbing (the lamb rubs head and shoulders along or under the flank of another lamb).
Low stretch	Neck held horizontal to the ground with the muzzle forward and raised. Head can be turned through 90° as well, with or without running tongue in or out.
Attempt to mount	Lamb attempts to mount (set of back and forward movements while standing in front of or to the side of the rump of another lamb), jumps on the back of the lamb with or without firm contact of the brisket with the rump.
Avoidance/escape behaviours	
Climbing/jumping on gates	Lamb places forelegs on gates or jumps on it.
Withdrawal	Lamb moves out of its current location (resting or feeding) following displacement activities by another lamb.
Avoidance	Lamb avoids physical contact with another lamb by changing its place of resting or feeding usually seen after threat.
Vocalizing	High pitched bleats.

5.4 Statistical analysis

The distribution of data was analysed in Minitab statistical package 16th edition (Minitab, Inc, State Collage, PA). The Anderson Darling test was used to test the normality of the data. All presented data were not parametric. It was impossible to normalize the behavioural data therefore it was decided that Kruskal-Wallis and Mann-Whitney non-parametric tests were carried out to determine significant differences between treatment groups using Genstat 14th edition Genstat (GenStat® 14th edition, Lawes Agricultural Trust, VSN International Ltd, Oxford, UK). The Bonferroni

correction was also applied to account for the multiple testing. In practice to correct for multiple comparisons the critical p-value of 0.05 has to be divided by the number of comparisons (treatment groups of the particular study). For example, if there were 5 treatment groups' critical p-value will be divided by 5. This will set a new critical p-value of 0.01 (0.05/5 = 0.01). In this chapter, 5 treatment groups were formed to carry out the analysis. Therefore the new p – value was set to be P<0.01.

5.5 Results

5.5.1 Development of Ewe-lamb behaviours

Overall treatment had no effect on lamb behaviours in any of the observation periods (Tables 6-8). There was a tendency for C lambs to be further from their mothers than lambs of the F and Vac 2 groups in period 1 (Table 5; due to Bonferroni correction this result cannot be treated as significant). However, there was an effect of time on the spatial proximity of lambs Wald statistic 47.89, ddf129.7, P<0.001.

Within treatment groups spatial proximity was changing over the period of the study (figure 8), LA lambs were observed in significantly greater spatial distance from the ewe in periods 2 and 3 in comparison to period 1 (U=15.5 **P=0.002).** Moreover, LA were seen to be further away from the ewe in period 2 compared to period 3 (U=25.0 **P=0.01).** Female lambs were observed to keep significantly closer spatial distance with their mothers during period 1 in comparison to periods 2 and 3 (U=141.5 **P=0.002**, and U=97.0 **P<0.001**, respectively) but there were no differences between periods 2 and 3. There were no significant differences found in spatial proximity between any observed periods in C, Vac 2 and RR lambs.

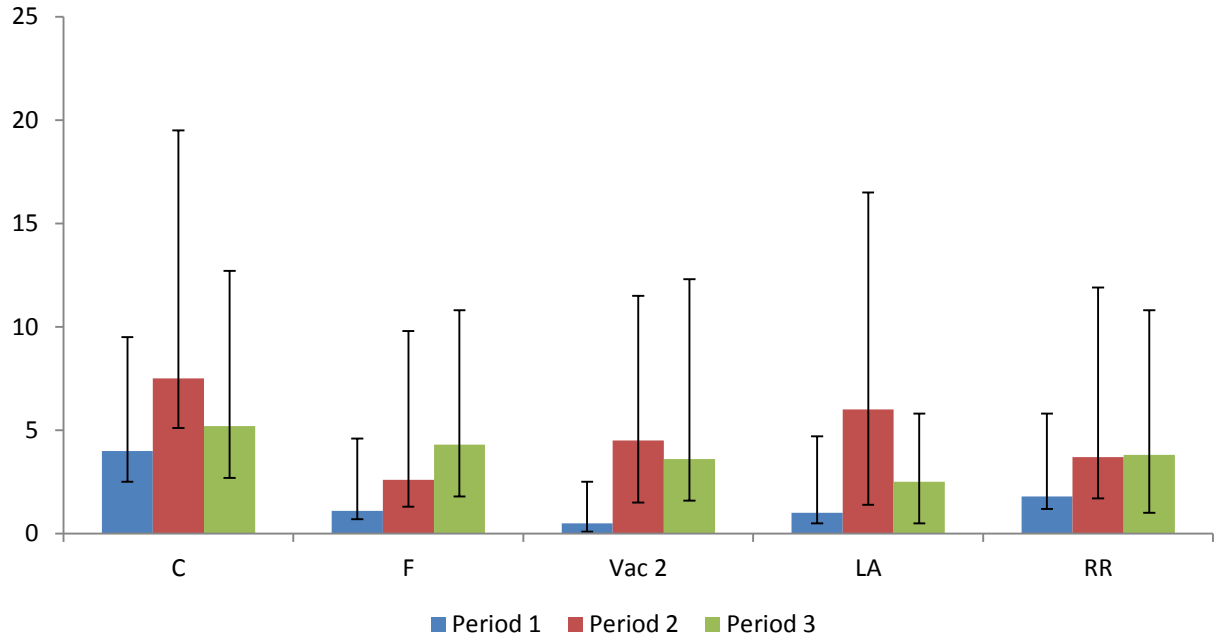


Figure 8 Changes in median spatial proximity between ewes and lambs form control (C), rubber ring (RR), Female (F), Local anaesthesia (LA) and immunocastration (Vac 2) treatments. The proximity was recorded over all 3 experimental periods each 10 days long. First period took place immediately after birth, second- after primary vaccination was administered to Vac 2 group ~ 6weeks of age, third – 2 weeks before weaning ~ 13 weeks of age. Data are medians with Q1 and Q3

Table 5 Differences between control (C), rubber ring castration (RR), local anaesthesia (LA), female (F) and immunocastration treatment (Vac 2) in the distance from the ewe measured in meters during all 3 experimental periods (1,2,3) respectively. Observations were recorded for 10 days at each period; immediately after birth (period1) at 6 (period 2) and 13 (period 3) weeks of age. Data are medians with Q1 and Q3.

Period	C[1]	RR[1]	LA[1]	F[1]	Vac 2[1]	P-Value[2]
1	4(1.5-5.5)a	1.8(0.6-4)ab	1(0.5-3.7)ab	1.1(0.4-3.5)b	0.5(0.4-2)b	P<0.05
2	7.5(2.4-12)	3.7(2-8.2)	6(4.6-10.5)	2.6(1.3-7.2)	4.5(3-7)	NS
3	5.2(2.4-7.5)	3.8(2.8-7)	2.5(2-3.3)	4.3(2.5-6.5)	3.6(2-8.7)	NS

[1]Median frequency (Q1-Q3). [2](amended p-value due to Bonferroni correction P<0.01), NS not significant. Different letters indicate significant differences between treatments.

Table 6 Differences in the frequency of behaviours expressed by entire males (C), rubber ring castration (RR), local anaesthesia (LA), female (F) and immunocastration treatment (Vac 2) measured for 10 days immediately after birth during 1st period of ewe-lamb bonding observations. Data are medians with Q1 and Q3.

behaviour	C[1]	RR[1]	LA[1]	F[1]	Vac 2[1]	P-Value[2]
Walking	3.0(2.5-4.0)	3.0(2.5-4.0)	3.0(1.0-4.0)	3.0(2.0-5.0)	3.0(1.5-4.0)	*NS*
Standing	10.0(8.0-12.0)	8.5(6.5-11.5)	10.5(8.5-13.0)	10.0(8.0-12.0)	8.5(8.0-11.5)	*NS*
Lying	15.0(13.0-16.5)	14.5(11.5-16.5)	15.5(13.5-17.0)	14.5(12.0-19.0)	16.0(15.0-17.5)	*NS*

Running	1.0(0.0-2.0)	2.0(1.0-3.0)	1.0(0.5-1.5)	1.0(0.0-2.0)	0.0(0.0-1.5)	*NS*
Inactive/sleeping	20.0(18.0-22.0)	19.5(18.0-20.0)	18.5(17.5-21.0)	19.5(16.5-21.0)	20.0(19.0-22.5)	*NS*
Play/Jumping	2.0(1.5-3.5)	3.0(1.5-5.0)	1.5(1.0-4.0)	2.0(2.0-3.0)	2.0(1.0-4.5)	*NS*
Forage/Grazing	0.0(0.0-0.0)	0.0(0.0-0.5)	0.0(0.0-0.0)	0.0(0.0-1.0)	0.0(0.0-0.0)	*NS*
Suckling	2.0(1.0-3.0)	1.5(1.0-3.0)	3.0(1.0-3.0)	2.0(0.0-2.0)	2.0(0.5-5.0)	*NS*
Sniffing	2.5(1.5-4.0)	3.0(1.5-4.0)	3.0(2.0-6.0)	3.0(2.0-4.0)	2.0(1.0-2.5)	*NS*
Vocalizing	1.0(0.0-2.0)	1.0(0.0-1.0)	0.0(0.0-1.0)	0.0(0.0-1.0)	0.0(0.0-1.0)	*NS*

[1]Median frequency (Q1-Q3). [2](amended p-value due to Bonferroni correction P<0.01), NS not significant. Different letters indicate significant differences between treatments.

Table 7 Differences in the frequency of behaviours expressed by entire males (C), rubber ring castration (RR), local anaesthesia (LA), female (F) and immunocastration treatment (Vac 2) measured for 10 days at 6 weeks of age during 2nd period of ewe-lamb bonding observations. Data are medians with Q1 and Q3.

Behaviour	**C[1]**	**RR[1]**	**LA[1]**	**F[1]**	**Vac 2[1]**	**P-Value[2]**
Walking	2.0(1.0-3.0)	2.0(1.0-3.0)	2,5(1.0-4.0)	2.0(1.0-3.0)	3.0(2.0-4.0)	*NS*
Standing	12.5(10.0-16.0)	13.5(11.5-15.5)	14.0(12.0-15.0)	13.0(11.5-15.5)	14.0(13.5-16.0)	*NS*
Lying	13.0(12.0-16.0)	14.0(13.0-15.0)	13.0(11.5-15.0)	14.0(12.5-15.0)	12.5(11.0-14.0)	*NS*

Running	0.5(0.0.-1.0)	0.0(0.0-1.0)	0.0(0.0-1.0)	0.0(0.0-1.0)	0.0(0.0-1.0)	*NS*
Inactive/sleeping	13.5(11.0-15.0)	14.0(12.5-16.0)	13.0(9.5-14.0)	13.0(9.5-15.0)	12.5(11.0-15.0)	*NS*
Play/Jumping	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)	*NS*
Forage/Grazing	7.5(6.0-9.0)	9.0(7.0-9.5)	10.0(8.0-10.5)	9.0(7.0-10.0)	10.0(8.0-11.0)	*NS*
Chewing	5.5(4.0-6.0)	4.0(2.0-6.0)	5.0(4.0-6.5)	4.0(3.0-5.0)	4.0(2.5-7.0)	*NS*
Suckling	0.5(0.0-1.0)	0.5(0.0-1.0)	0.5(0.0-1.0)	0.0(0.0-1.0)	0.0(0.0-1.0)	*NS*
Sniffing	1.5(0.0-2.0)	1.5(1.0-2.5)	1.0(1.0-2.5)	2.0(1.0-2.0)	2.0(0.5-3.0)	*NS*
Vocalizing	1.0(0.0-2.0)	0.0(0.0-1.0)	1.0(0.0-1.5)	1.0(0.0-2.0)	0.0(0.0-1.0)	*NS*

[1]Median frequency (Q1-Q3). [2](amended p-value due to Bonferroni correction P<0.01), NS not significant. Different letters indicate significant differences between treatments.

Table 8 Differences in the frequency of behaviours expressed by entire males (C), rubber ring castration (RR), local anaesthesia (LA), female (F) and immunocastration treatment (Vac 2) measured for 10 days at 13 weeks of age during 3rd period of ewe-lamb bonding observations. Data are medians with Q1 and Q3.

Behaviour	**C[1]**	**RR[1]**	**LA[1]**	**F[1]**	**Vac 2[1]**	**P-Value[2]**
Walking	1.5(0.0-3.0)	2.0(1.0-2.0)	2.0(1.0-2.0)	1.0(1.0-2.0)	1.0(0.0-2.0)	*NS*
Standing	19.5(17.0-21.0)	18.5(18.0-20.0)	19.0(16.5-20.0)	18.0(16.5-20.5)	20.0(17.0-22.0)	*NS*
Lying	9.0(8.0-11.0)	9.0(8.5-11.0)	10.0(8.0-11.0)	10.0(8.5-12.0)	8.5(4.5-10.0)	*NS*
Running	0.5(0.0-1.0)	0.0(0.0-0.5)	0.0(0.0-1.0)	0.0(0.0-1.0)	0.0(0.0-1.0)	*NS*

Inactive/sleeping	7.5(5.0-9.0)	5.093.5-7.0)	6.0(5.2-8.7)	5.5(4.0-7.5)	5.0(0.0-11.0)	*NS*
Play/Jumping	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)	*NS*
Forage/Grazing	14.5(12.0-16.0)	16.5(13.5-17.5)	15.0(12.5-16.0)	15.0(12.5-17.5)	15.0(13.5-17.5)	*NS*
Chewing	7.0(6.0-8.0)	8.0(6.5-9.5)	6.6(6.0-9.0)	8.0(6.4-9.0)	8.0(5.0-10.5)	*NS*
Suckling	0.0(0.0-1.0)	0.0(0.0-1.0)	0.0(0.0-0.7)	0.0(0.0-0.5)	0.0(0.0-0.0)	*NS*
Sniffing	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-1.0)	0.0(0.0-0.0)	0.0(0.0-0.5)	*NS*
Vocalizing	1.0(0.0-1.0)	0.0(0.0-1.0)	1.0(0.2-2.0)	1.0(0.0-1.5)	1.0(0.0-1.0)	*NS*

[1]Median frequency (Q1-Q3). [2](amended p-value due to Bonferroni correction P<0.01), NS not significant. Different letters indicate significant differences between treatments.

5.5.2 Stress responses of lambs

Overall 13 single behavioural measures were tested during the isolation test, 18 during the surprise test and 23 during the unfamiliar human test. Most of the behavioural measures did not differ significantly between treatments (Table 9-15) with the exception of the frequency with which area 2 of the testing apparatus (where the feeder and unfamiliar person were located) was entered during the unfamiliar human test. C lambs entered area 2 of the testing apparatus with a lower frequency than to RR, F and Vac2.

F lambs differed significantly from C lambs in the severity of fear score in all of the stressful tests. Moreover, the female group was also significantly more fearful than lambs castrated with the use of local anaesthesia (table 9) in the surprise test. With regard to the unfamiliar human test the fear score of C rams was noted to be smaller in comparison to all other treatments with the exception of the RR group (table 9).

Table 9 Differences in recorded fear score expressed by entire males (C), rubber ring castration (RR), local anaesthesia (LA), female (F) and immunocastration treatment (Vac 2) measured during 3 different stressful situations on a scale from 1 (minimum possible fear) to 4 (maximum possible fear) at ~ 14 weeks of age after each stress test has finished. Data are medians with Q1 and Q3.

Test type	C[1]	RR[1]	LA[1]	F[1]	Vac 2[1]	P-Value[2]
Isolation test	1.5(1.0-2.0)a	2.0(2.0-3.7)ab	2.0(1.2-3.0)ab	3.0(2.2-3.7)b	2.2(2.0-3.0)ab	**P=0.01**
Surprise test	2.0(1.0-2.0)a	3.0(2.0-3.0)ab	2.0(2.0-2.0)a	3.0(2.0-4.0)b	2.5(1.7-3.0)ab	**P=0.002**
Unfamiliar Human test	1.0(1.0-2.5)a	2.5(2.0-3.0)ab	2.5(2.0-2.8)ab	3.0(2.0-4.0)b	3.0(2.5-4.0)b	**P=0.01**

[1]Median frequency (Q1-Q3). [2](amended p-value due to Bonferroni correction P<0.01), NS not significant. Different letters indicate significant differences between treatments.

Isolation test results

Table 10 Differences in recorded frequency or duration (measured in seconds) of anxiety measurements expressed by entire males (C), rubber ring castration (RR), local anaesthesia (LA), female (F) and immunocastration treatment (Vac 2) during 4 min isolation form conspecifics test, conducted at ~ 14 weeks of age. Data are medians with Q1 and Q3.

Anxiety Measurements	C[1,*]	RR[1,*]	LA[1,*]	F[1,*]	Vac 2[1,*]	P-Value[2]
Walking*	97.9(80.2-114.5)	100.8(88.4-120.3)	109.4(96.0-126.7)	99.4(86.1-123.2)	89.7(61.7-98.0)	*NS*
Standing*	141.0(126.9-161.8)	140.4(120.2-153.4)	133.4(115.2-147.3)	141.8(117.7-155.3)	152.2(142.5-179.3)	*NS*
Vocalizing	4.0(0.0-6.5)	2.0(0.0-9.7)	6.090.0-9.5)	6.0(1.5-20.5)	1.5(0.0-7.5)	*NS*

Jumping/climbing on gates	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-1.0)	0.0(0.0-010)	*NS*
Sniffing Exploring Test Pen	18.0(16.7-23.0)	23.0(14.5-26.2)	22.0(20.0-23.7)	19.5(15.0-23.5)	18.0(12.0-23.5)	*NS*
Frequency of times entering area 1,2 or 3	11.5(9.0-19.0)	12.0(9.2-14.0)	12.0(9.2-20.5)	12.5(8.5-15.0	9.5(8.5-14.5)	*NS*
	10.5(7.0-14.0)	**14.0(11.5-16.7)**	**15.0(13.2-15.7)**	**13.5(11.0-16.0)**	**14.5(12.5-18.5)**	***NS***
	3.5(1.0-5.0)	**6.0(4.0-7.7)**	**4.0(3.0-6.7)**	**4.5(3.0-7.5)**	**4.5(3.0-7.5)**	***NS***
***Duration of time spend in area (measured in s) 1,2 or 3**	**125.1(99.5-189.9)**	**82.8(70.7-112.2)**	**102.2(84.1-142.3**	**103.0(78.2-141.1)**	**101.3(80.6-123.2)**	***NS***
	85.1(47.2-95.7)	**103.6(76.5-137.7)**	**76.5(64.9-91.4)**	**88.0(65.8-111.9)**	**92.8(77.5-118.2)**	***NS***
	29.3(3.5-54.0)	**27.7(21.2-56.8)**	**40.8(14.3-69.4)**	**36.5(17.7-52.7)**	**37.6(18.8-58.1)**	***NS***

[1]Median frequency (Q1-Q3). or * Median duration measured in seconds (Q1-Q3) for walking and standing behaviour. [2](amended p-value due to Bonferroni correction P<0.01), NS not significant. Different letters indicate significant differences between treatments.

Table 11 Differences in latency of time to perform particular behaviours (measured in seconds) expressed by entire males (C), rubber ring castration (RR), local anaesthesia (LA), female (F) and immunocastration treatment (Vac 2) during 4 min isolation form conspecifics test at ~ 14 weeks of age. Data are medians with Q1 and Q3.

Anxiety Measurement	**C[1]**	**RR[1]**	**LA[1]**	**F[1]**	**Vac 2[1]**	**P-Value[2]**
Time to enter the pen	5.0(1.2-17.5)	2.0(2.0-11.0)	3.0(1.5-4.5)	2.5(2.0-10.0)	3.0(2.0-9.0)	*NS*
Vocalizing	53.4(0.0-82.1)	42.2(0.0-74.7)	21.4(0.0-91.6)	34.9(13.2-60.1)	10.6(0.0-79.1)	*NS*
Jumping/climbing on gates	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-20.1)	0.0(0.0-114.1)	*NS*
Sniffing Exploring Test Pen	4.4(2.9-5.7)	3.4(3.0-6.1)	4.1(3.2-6.9)	4.5(3.3-6.1)	4.1(3.6-6.7)	*NS*

[1]Median latency of time (Q1-Q3) measured in seconds. [2](amended p-value due to Bonferroni correction P<0.01), NS not significant. Different letters indicate significant differences between treatments.

Unfamiliar object test results

Table 12 Differences in recorded frequency or duration (measured in seconds) of anxiety measurements expressed by entire males (C), rubber ring castration (RR), local anaesthesia (LA), female (F) and immunocastration treatment (Vac 2) during 4 min unfamiliar object test, conducted at ~ 14 weeks of age. Data are medians with Q1 and Q3.

Anxiety Measurements	**C[1,*]**	**RR[1,*]**	**LA[1,*]**	**F[1,*]**	**Vac 2[1,*]**	**P-Value[2]**
Walking*	89.4(80.3-99.7)	77.8(58.8-106.2)	74.0(63.6-91.3)	89.4(65.6-98.8)	73.4(62.4-98.4)	*NS*
Standing*	150.4(140.4-161.0)	164.4(136.1-183.9)	164.6(150.8-176.8)	149,8(142.6-175.3)	168.2(143.3-180.7)	*NS*
Vocalizing	2.0(0.0-14.2)	4.0(0.2-5.7)	1.0(0.0-7.5)	4.0(1.0-10.5)	2.5(0.0-7.0)	*NS*
Jumping/climbing on gates	0.0(0.0-0.0)	0.0(0.0-1.0)	0.0(0.0-1.0)	0.0(0.0-0.5)	0.0(0.0-0.0)	*NS*
Sniffing Exploring Test Pen	11.0(7.7-13.0)	13.0(10.0-16.7)	15.0(10.7-18.7)	14.0(10.0-16.0)	13.5(10.5-17.0)	*NS*
Number of times entering area 1,2 or 3	8.0(6.0-17.0)	9.0(6.2-15.5)	12.0(8.5-16.7)	13.0(9.5-16.5)	10.5(8.0-17.0)	*NS*
	29.4(10.9-51.0)	**25.6(11.4-48.4)**	**37.0(21.7-56.2**)	**35.3(19.7-73.5)**	**55.8(27.6-83.1)**	***NS***
	4.0(1.0-5.2)	**2.0(1.0-3.7)**	**4.0(2.2-5.0)**	**4.0(1.7-7.2)**	**4.0(2.0-8.5)**	***NS***
***Duration of time spend in area 1,2 or 3**	**151.9(118.6-193.8)**	**154.6(111.1-182.6)**	**140.2(95.5-152.1)**	**116.3(77.3-150.4)**	**85.2(45.3-131.3)**	***NS***
	17.0(9.7-20.0)	**11.0(9.0-16.5)**	**14.0(9.0-15.7)**	**12.0(7.0-18.7)**	**6.5(6.0-9.0)**	***NS***
	55.6(26.7-89.0)	**63.4(38.7-91.3)**	**59.6(40.3-88.0)**	**79.0(40.4-99.9)**	**73.8(57.6-114.1)**	***NS***
Sniffing/touching	3.0(1.0-5,2)	4.0(2.2-5.0)	5.0(3.2-8.0)	3.5(2.5-6.5)	2.5(1.5-6.5)	***NS***

ball						
Looking at the direction of ball	4.0(3.5-5.0)	5.0(3.2-10.2)	3.0(2.0-7.2)	4.0(3.0-6.0)	4.5(2.0-7.0)	*NS*
Butting ball	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)	*NS*

[1]Median frequency (Q1-Q3). or * Median duration measured in seconds (Q1-Q3) for walking and standing behaviour. [2](amended p-value due to Bonferroni correction P<0.01), NS not significant. Different letters indicate significant differences between treatments.

Table 13 Differences in latency of time to perform particular behaviours (measured in seconds) expressed by entire males (C), rubber ring castration (RR), local anaesthesia (LA), female (F) and immunocastration treatment (Vac 2) during 4 min isolation form conspecifics test at ~ 14 weeks of age. Data are medians with Q1 and Q3.

Anxiety Measurements	**C[1]**	**RR[1]**	**LA[1]**	**F[1]**	**Vac 2[1]**	**P-Value[2]**
Time to enter the pen	9.0(3.0-135.0)	25.5(8.0-172.0)	9.5(2.5-116.0)	9.0(5.0-301.0)	35.0(7.2-301.8)	*NS*
Vocalizing	23.2(0.0-113.3)	29.5(2.2-88.3)	31.0(0.0-47.9)	17.5(6.8-44.8)	17.6(0.0-38.0)	*NS*
Jumping/climbing on gates	0.0(0.0-0.0)	0.0(0.0-56.6)	0.0(0.0-131.5)	0.0(0.0-11.7)	0.0(0.0-0.0)	*NS*
Sniffing Exploring Test Pen	7.0(6.2-17.5)	6.6(5.2-15.3)	13.6(6.2-17.4)	8.8(6.8-21.1)	6.8(5.1-10.3)	*NS*
Sniffing/touching ball	85.5(74.1-92.7)	76.3(73.8-85.9)	75.2(72.7-87.4)	80.1(73.9-97.8)	84.3(76.5-96.)	*NS*
Looking at the direction of ball	76.1(69.8-100.2)	70.8(69.1-74.9)	70.0(67.4-79.2)	72.3(68.6-78.0)	74.8(70.5-88.1)	*NS*

Butting ball	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)	*NS*

[1]Median latency of time (Q1-Q3) measured in seconds. [2](amended p-value due to Bonferroni correction P<0.01), NS not significant. Different letters indicate significant differences between treatments.

Unfamiliar Human test results

Table 14 Differences in recorded frequency or duration (measured in seconds) of anxiety measurements expressed by entire males (C), rubber ring castration (RR), local anaesthesia (LA), female (F) and immunocastration treatment (Vac 2) during 4 min unfamiliar human test, conducted at ~ 14 weeks of age. Data are medians with Q1 and Q3.

Anxiety Measurements	**C[1,*]**	**RR[1,*]**	**LA[1,*]**	**F[1,*]**	**Vac 2[1,*]**	**P-Value[2]**
Walking*	32.5(25.6-39.6)	40.3(29.7-77.6)	35.2(23.0-52.6)	49.1(32.4-75.0)	37.2(22.4-46.3)	*NS*
Standing*	87.8(79.4-97.6)	116.4(68.1-141.2)	90.7(64.7-105.1)	88.9(80.1-137.9)	97.2(85.8-112.2)	*NS*
Vocalizing	0.0(0.0-0.2)	0.0(0.0-6.0)	0.0(0.0-0.0)	0.0(0.0-1.5)	0.0(0.0-0.5)	*NS*
Jumping/climbing on gates	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)	*NS*
Sniffing Exploring Test Pen	4.0(2.7-6.2)	7.0(5.2-9.0)	3.5(3.0-5.0)	5.0(3.0-9.0)	5.0(2.5-7.0)	*NS*
Frequency of entering area 1,2 or 3	**6.0(5.0-9.5)**	**8.0(5.0-13.7)**	**10.0(7.2-11.7)**	**7.5(5.0-15.0)**	**7.5(4.0-10.0)**	**NS**
	11.0(8.5-13.0)a	**22.0(16.2-22.7)b**	**14.0(13.0-20.2)c**	**15.5(13.0-25.0)ac**	**16.0(13.5-24.0)ac**	**P=0.01**
	2.0(0.703.5)	**6.0(1.0-9.7)**	**3.0(1.2-10.2)**	**4.5(3.0-10.0)**	**7.5(2.0-12.0)**	**NS**

***Duration of time spend in area 1,2 or 3**	**48.9(27.7-55.9)**	**59.7(25.3-113.3)**	**44.3(16.8-80.1)**	**36.5(17.5-99.2)**	**32.2(20.0-56.2)**	***NS***
	211.4(201.9-303.7)	**281.7(235.8-328.5)**	**287.2(220.5-311.9)**	**278.0(209.3-337.8)**	**258.8(205.8-284.8)**	***NS***
	12.6(5.7-31.1)	**54.1(6.4-121.8)**	**12.8(10.4-34.5)**	**34.3(14.0-90.6)**	**44.4(5.8-85.4)**	***NS***
Sniffing/licking feeder	7.0(4.7-10.5)	5.0(2.2-5.00	7.0(5.0-9.0)	5.5(4.0-8.0)	8.0(6.0-10.0)	*NS*
Sniffing/touching human	4.0(3.0-4.0)	1.0(0.0-2.7)	3.0(1.0-4.0)	2.0(0.0-4.0)	3.5(0.5-4.5)	*NS*
Looking at the direction of human	4.0(1.5-5.2)	10.0(2.5-13.0)	3.0(1.0-5.0)	4.5(1.5-6.5)	5.5(1.5-7.5)	*NS*
Duration of Feeding	124.7(95.4-136.8)	56.1(24.3-118.0)	119.5(93.3-141.0)	97.3(14.5-125.7)	109.5(88.8-132.2)	*NS*

[1]Median frequency (Q1-Q3). or * Median duration measured in seconds (Q1-Q3) for walking and standing behaviour. [2](amended p-value due to Bonferroni correction P<0.01), NS not significant. Different letters indicate significant differences between treatments.

Table 15 Differences in latency of time to perform particular behaviours (measured in seconds) expressed by entire males (C), rubber ring castration (RR), local anaesthesia (LA), female (F) and immunocastration treatment (Vac 2) during 4 min unfamiliar human test, conducted at ~ 14 weeks of age. Data are medians with Q1 and Q3.

Anxiety Measurements	**C[1]**	**RR[1]**	**LA[1]**	**F[1]**	**Vac 2[1]**	**P-Value[2]**
Time to enter the	4.0(2.0-30.0)	9.5(4.0-37.0)	6.0(3.0-180.0)	8.0(3.7-27.5)	14.0(3.0-92.5)	*NS*

pen						
Time to exit the pen	74.7(26.1-167.6)	151.3(43.7-289.1)	89.2(47.0-253.7)	154.5(40.6-303.1)	93.7(35.4-200.3)	*NS*
Vocalizing	0.0(0.0-6.1)	0.0(0.0-0.8)	0.0(0.0-0.0)	0.0(0.0-32.6)	0.0(0.0-19.7)	*NS*
Jumping/climbing on gates	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)	*NS*
Sniffing Exploring Test Pen	16.0(12.2-26.2)	19.3(14.2-24.8)	25.1(10.2-50.4)	21.3(8.2-44.6)	15.9(7.7-42.2)	*NS*
Sniffing/touching trough	8.2(5.3-19.4)	5.1(3.9-7.8)	7.9(5.5-18.9)	7.1(4.9-10.0)	7.1(5.6-23.0)	*NS*
Sniffing/touching human	182.2(135.0-193.5)	79.8(0.0(191.3)	172.6(106.6-196.2)	78.0(0.0-185.7)	179.2(37.5-197.2)	*NS*
Looking at the direction of human	55.9(39.8-70.0)	56.2(54.2-63.2)	62.2(57.8-64.3)	59.0(53.8-73.7)	59.3(53.4-69.7)	*NS*
Butting Human	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0 .0)	*NS*
Feeding	77.3(73.9-82.6)	78.0(18.5-129.6)	83.2(73.1-105.0)	76.4(34.5-108.1)	74.0(66.5-80.4)	*NS*

[1]Median latency of time (Q1-Q3) measured in seconds. [2](amended p-value due to Bonferroni correction P<0.01), NS not significant. Different letters indicate significant differences between treatments.

5.5.3 Cortisol concentrations

Analysis of the plasma cortisol concentration pre and post the surprise test did not reveal any significant differences between treatments (table 16 below). There were no significant differences between cortisol levels pre and post-test within particular treatments.

Table 16 Differences in the plasma cortisol concentration (measured ng/ml) recorded for entire males (C), rubber ring castration (RR), local anaesthesia (LA), female (F) and immunocastration treatment (Vac 2). The measurement was taken just before (pre-test cortisol results) and 15 min after (post-test cortisol results) unfamiliar object test at ~ 14 weeks of age. Data are medians with Q1 and Q3.

Plasma Cortisol Concentration	C[1]	RR[1]	LA[1]	F[1]	Vac 2[1]	P-Value[2]
Pre-test	11.0(8.7-20.6)	13.9(6.4-22.5)	15.3(12.6-28.9)	14.3(7.5-19.6)	19.0(14.2-33.3)	NS
Post-test	16.5(11.5-36.7)	18.9(13.0-28.3)	12.1(8.9-25.4)	13.0(3.4-21.9)	11.3(5.8-20.1)	NS

[1]Median plasma cortisol concentration (Q1-Q3) measured in ng/ml. [2](amended p-value due to Bonferroni correction $P<0.01$), NS not significant. Different letters indicate significant differences between treatments.

5.5.4 Expression of aggressive behaviours

Control lambs showed a significantly higher frequency of sexual behaviours during the food competition test than all other lamb groups (Table 17, **P=0.01).** However, there were no significant differences between treatments in the expression of the other types of behaviours (maintenance behaviours, performed aggression, returned aggression, total number of aggressive encounters and avoidance/escape type behaviours; Table 17).

Table 17 Differences in the frequency of aggressive behaviours expressed by entire males (C), rubber ring castration (RR), local anaesthesia (LA), female (F), and immunocastration (Vac 2) treatment, recorded for 30 minutes during feed competition test at ~24 weeks of age. Data are medians with Q1 and Q3.

Aggressive Behaviours	C[1]	RR[1]	LA[1]	F[1]	Vac 2[1]	P-Value[2]
Maintenance Behaviours	20.0(19.0-31.5)	19.0(16.5-25.5)	21.0(13.5-24.0)	22.0(17.5-31.0)	17.0(15.5-25.0)	*NS*
Performed Aggression	4.0(2.7-7.2)	7.0(2.7-19.0)	5.0(1.2-10.2)	9.0(3.5-18.5)	5.0(2.5-9.5)	*NS*
Returned Aggression	0.0(0.0-1.0)	1.0(0.0-2.0)	0.0(0.0-0.7)	0.0(0.0-2.0)	1.0(0.0-1.0)	*NS*
Total Aggression	7.0(5.2-15.5)	9.0(3.2-23.2)	5.0(1.2-11.2)	10.5(6.5-23.0)	6.5(3.0-11.0)	*NS*
Sexual Behaviours	1.0(0.0-8.2)a	0.0(0.0-0.0)b	0.0(0.0-0.0)b	0.0(0.0-0.0)b	0.0(0.0-0.5)b	**P=0.01**
Avoidance/Escape Behaviours	3.0(1.7-6.0)	7.0(2.5-20.2)	7.0(3.2-9.5)	3.0(1.5-8.5)	4.5(2.4-10.0)	*NS*

[1]Median frequency (Q1-Q3). [2](amended p-value due to Bonferroni correction P<0.01), NS not significant. Different letters indicate significant differences between treatments.

5.6 Discussion

Results of this study suggests that castration, and lamb sex, had no impact on lamb spontaneous behaviours and spatial proximity. This suggests that early experience of pain (either acute or chronic) or presence or absence of testosterone, does not influence the behaviour of the pre-weaning lamb. There was however some influence of testosterone exposure on the development and expression of social behaviours (aggression) and stress responses later on in life. Entire males appear to be different from all other groups in terms of their expression of fearfulness, and sexual behaviour in competition tests, but the other treatment groups do not differ from one another. Treatment tended to affect ewe-lamb spatial relationships in the period immediately after birth (days 1-11; Period 1) but there were no significant differences between treatments in periods 2 and 3. Immediately after birth entire male lambs (C group) that had not been castrated were further from their mothers than other groups. Although it has to be noted that the Vac2 lambs were not handled equally to C lambs at that stage they did not show this difference in behaviour so the results should be treated with caution. Moreover, treatment had some impact on stress responses after weaning. C lambs were consistently scored as less fearful than F lambs in all fear eliciting conditions. However, there were no significant differences between treatments in other recorded anxiety measures. C lambs had a significantly greater frequency of expressing sexual behaviours than all other treatments during the feed competition test which may be related to dominance hierarchy at this stage of life.

Differences in the emotional reaction to fear eliciting stimuli between the sexes, entire males and wethers have been found before (Bouissou & Vandenheede, 1993b; Bouissou & Vandenheede, 1995; Bouissou & Vandenheede, 1996). Wethers were found to be more fearful than rams in the various test situations i.e. the surprise test and the presence of unfamiliar human test (Bouissou & Vandenheede, 1996). Ewes treated with testosterone have been also found to be less fearful (Bouissou & Vandenheede, 1993a). Similarly, exogenous treatment of cows revealed a reduced fear response in comparison

to not treated individuals (Boissy and Bouissou 1994). Bouissou & Vandenheede (1996) have concluded that androgens may be responsible for observed differences in fearfulness between males and females. It was also established that ewes treated with androgens are less fearful than untreated entire males (Bouissou & Vandenheede, 1996). In this study it has been shown that the fear score given to the lambs during three fearful situations was consistently lower in entire males in contrast with the female group, indicating reduced fear in male lambs. Moreover, C lambs were the only group with physiological levels of testosterone which may suggest that the presence of circulating testosterone played a role in fear related responses. This is consistent with previously reported findings. Furthermore, the frequency of entry into the area of the testing pen where the feeder, and unfamiliar person were present was lower for the C group in comparison to RR, Vac 2 and F lambs. This may indicate that the feeding pattern for the C lambs was less interrupted. It is also possible that C rams were less afraid of the unfamiliar human and therefore took their time to finish feeding without the need to step back and forward or engage in interaction with the unfamiliar human (looking, sniffing, licking the human). Although there was no difference in total time spent in the area by each treatment.

Results of the cortisol analysis did not reveal any significant differences between treatments. This finding is consistent with results of all other anxiety measurements which were analysed in this study. However, it must be noted that due to technical difficulties not all of the taken samples could have been analysed. Therefore, direct comparisons with other studies were not possible. For example, findings reported by Bouissou & Vandenheede (1996) showed that castrated rams had higher cortisol concentration than entire rams before the surprise test.

Presence of circulating testosterone in the blood may also have an impact on the expression of social behaviours such as aggression or dominance hierarchy. It has been previously reported that steroid hormones have an important impact on the development and occurrence of aggressive behaviours which appear to be under the control of androgens (Bouissou, 1995). Frequent aggressive encounters and fights are a form of

hierarchy establishment which lead to securing the reproductive success of dominant individuals (Zuk, 1991; Iwasa and Pomiankowki, 1994; Jacobs, 1996). Moreover, dominant rams are able to disturb the sexual activity of subordinate rams (Lindsay et al., 1976; Synnott and Fulkerson, 1984; Tilbrook et al., 1987; Ungerfeldnd González-Pensado, 2008a). Reduction or elimination of circulating testosterone may be a means to achieve timidity in animals. In this study C lambs were found to express a significantly greater frequency of sexual behaviours (e.g. attempt to mount, low stretch and nudging that sum up into one category of sexual behaviours) during the feed competition test than any other treatment. Such behaviours may be related to testosterone levels or indicate that C rams could have been dominant individuals in the flock (Ungerfeld and González-Pensado, 2008b). Because other treatment groups could only have a residual level of testosterone, sexual behaviours were not seen in those groups at higher frequencies or they were not present at all. In this study occurrence of sexual behaviours during the food competition test may be associated with establishment of hierarchy which could be a form of aggression. Dominant rams would mount more frequently than subordinate animals and because this form of behaviour was only seen in group C it can be argued that other groups were more timid in comparison to entire rams. It is perhaps due to the fact that only entire males would use this behaviour, and thus only seen in this group's hierarchy establishment whereas other groups might be using other behaviours to determine hierarchy. Although there was no direct comparison between dominant and subordinate males done in this study and we can only assume that C rams were the dominant group. In this thesis expression of sexual behaviours which were correlated with testosterone levels were observed in C lambs only (Chapter 4). Other groups with significantly reduced levels of circulating testosterone were not seen to perform sexual behaviours at all (RR group) or the frequency of such behaviours was reduced (SSC, Vac 2-6 groups). C rams were also observed to have greater scrotal circumference, testes consistency and a larger size of the seminiferous tubules in comparison to immunized and short scrotum lambs (Chapter 4). This is consistent with previous findings, which show that males with greater body weight, scrotal circumference, and semen production

will also express sexual behaviour at earlier ages of life (Ungerfeld and González-Pensado, 2008b).

It has to be noted that it is crucial to make a distinction between a statistically significant result in an experiment and a biologically meaningful outcome. Statistical significance at $p<0.05$ means that the null hypothesis, that there is no difference between the treatments, is rejected. Statistically it can be expected that there is a 95% chance that there will be a difference between treatments. However, this does not account for the most important question that needs to be asked. Is statistical (static) difference large enough to make a biological difference, which is really the bottom line? In this study the expression of sexual behaviour by C rams during feed competition test was linked with the establishment of dominance hierarchy and related to a form of aggression. In practicality it is possible to achieve statistical difference but it does not mean that it should be interpreted as a meaningful biological finding. It is important to remember that biological occurrences are dynamic but are often treated as steady state phenomena. There is also tendency to look for large differences, which are easier to link to the conditions compared and assume that small differences are not important. In conclusion, it is hard to interpret biological events and careful consideration should be given when doing so. It is easy to draw wrong conclusions because even relevant data may not be seen as significant in indiscriminate datasets and vice versa. Analysis of biological events should always go through experimental validation in order to determine their biological relevance.

A second area of interest of this study was to evaluate the impact of sex and treatment on development and maintenance of ewe-lamb behaviours. The spatial relationship between a lamb and its dam, and the range of lamb's behaviours were measured, for example, the frequency of suckling behaviour and occurrence of vocal communications. It has been previously shown that maternal care is a crucial component of lamb survival. During the neonatal period maternal care is associated with the formation of a bond between the ewe and its lamb (Pickup and Dwyer, 2011). Later, maternal care is allied with behaviours such as seeking lambs, keeping a close relationship, communication with

lambs and cooperation during suckling (Pickup and Dwyer, 2011). It has also previously been shown that the maintenance of ewe-lamb distance is very closely associated with lamb survival (Dwyer and Lawrence, 2005). Because lambs and their dams are usually moved onto pasture shortly after birth it is crucial for the lambs to keep a close distance to their mothers as the flock may move during the grazing period. To keep an appropriate distance with the ewe, lambs and their mothers need to recognize each other which may be accomplished by following and approaching behaviour as well as use of vocalizations and olfactory signals. The ewe also plays a part in the maintenance of the spatial relationship with the lamb and should respond to lamb suckling and communication attempts to maintain the bond and behavioural synchrony (Pickup and Dwyer, 2011). Results of this study did not indicate any differences between treatments in expressed frequencies of suckling attempts, vocalizations and spatial relationship with the ewe in period 2 and 3. However it was observed that C lambs tended to have a greater spatial relationship with the ewe in comparison to F and Vac 2 lambs in the first period (just after birth). This may indicate that treatment and sex had no effect or minimal effect on bond formation just after birth which would lead in the end to no or minimal differences between treatments in the exclusive bond with the ewe manifested by a good spatial relationship, communication and suckling behaviour in all tested groups. There are a few possibilities allowing to explain this outcome. First of all, the acute pain experienced by the lambs in the RR group had disappeared and was not affecting their behaviour. Furthermore, the chronic pain or discomfort that the RR and LA lambs might be experiencing through the presence of scrotal lesions are not enough to affect their behaviour or the chronic pain was not present at the time of assessment. Moreover, it is possible that lamb sex has no effect on the ewe-lamb relationship although there are some papers that suggest that the mother-offspring conflict may be different with males and females, and with the social organisation of sheep it might to be expected for ewe lambs to be closer to their mothers than male lambs. Nonetheless male lambs might be more demanding of maternal milk than ewe lambs. It is also possible that the sample of animals used in the study was not sufficient enough to detect specific differences between treatments. Furthermore, it is probable that the mule ewes that took

part in this study were exceptionally good mothers with a high maternal instinct, which could have acted as a mitigating factor minimizing impact of early pain exposure, handling or sex on bond formation and maintenance later on in life. It has been shown by Futro et al. (2015, article in press) that ewes are able to distinguish different levels of pain experienced by their lambs and direct more maternal attention toward those with the most severe reactions. Thus there were no differences found between treatments. Previously reported findings showed some differences in spatial relationship with the ewe between entire males and females (Dwyer et al., 2003). Hernandez et al. (2009) found that litter size and sex will have an impact on ewe-lamb and separation from the ewe up to four weeks after birth. On the other hand, the study investigating ewe–lamb behaviour in a hill and lowland breed of sheep have shown no effect of sex on the ewe lamb relationship (Dwyer and Lawrence, 1999). The fact that the Vac 2 rams tended to keep a closer spatial relationship with the ewe in period 1 of the observations was a surprising result as those lambs have not been treated until the second period of field observations when the primary vaccination with anti-GnRF vaccine has been administered. It is possible that the impact of environmental factors has changed lambs' behaviour. It has been shown that lambs with lower thermal sensitivity will keep closer to their dam (Held et al., 2010). On the second day of the field observations new born lambs experienced an unexpected thermal stimulus (low temperature and snow fall) as the weather conditions were severe for a few days (figure 7 c). Some of the lambs had to be brought back to the shed and placed in the "hot box" as they were showing symptoms of hypothermia. This seems to suggest that the Vac2 group was disproportionately affected. All lambs were balanced across the treatments over the lambing window. There is no explanation why it would it only be the Vac 2 treatment that was affected most by the weather change. It is possible that this was a random effect and with a small sample size it had influenced the results in an unexpected way. All lambs recovered very quickly and were brought back to the grazing field to join the ewes and their siblings the next day. There is evidence of the small effect of handling on C lambs' behaviour (Futro et al., 2015, article in press) although it is unlikely to explain the data differences between C and Vac2 lambs because C lambs were handled and the Vac2 lambs were

not. However, this event had no effect on the spatial proximity later in life as there were no significant differences found in spatial relationship seen between lambs and their dams in period 2 and 3 of the field observations.

5.7 Conclusion

Neonate lambs are often subjected to painful husbandry procedures, which could have long term effects on their behavioural and physiological responses later in life. The first objective of this study was to evaluate long term effects of the castration method including immunization against GnRF and sex on the expression of stress responses and social behaviours (i.e. aggression). The influence of early pain and testosterone exposure on lambs' behaviour later on in life was investigated. It was hypothesised that immunocastrated lambs will show similar levels of activity as entire males before the primary vaccination is administered. Moreover, it was assumed that immunocastrated males will resemble castrated lambs and/or female lambs in the levels of aggressive behaviour and stress responses when faced with three fear eliciting situations. It was also hypothesized that immunized lambs should form the bond with the ewe without disturbance or with minimal impact (related to environmental factors and individual characteristic of specific animals) equally to C. Results of this study indicate that there was no long term impact of early pain exposure associated with the castration procedure on the lambs' behaviour. Treatment did not affect the behavioural patterns of lambs in this study. There was also no effect of prior exposure of testosterone on the lambs' behavioural demeanour. This study has shown that observed changes in behavioural patterns associated with fear were related to current circulating levels of testosterone rather than prior exposure. Equally observed sexual behaviour during the feed competition test was associated with current testosterone levels rather than prior exposure to testosterone. Formation of ewe-lamb and spatial relationship seems to be affected by sex accordingly to the type of social organization in sheep species.

In conclusion treatment and lamb sex had some impact on the maintenance of ewe-lamb distance. Entire male lambs were consistently less fearful than female lambs during all tested fear eliciting situations. C lambs were also observed to express reduced fear

reactions compared to all other groups during the unfamiliar human test. There were no long term effects of early pain on lamb behavioural responses. The new method of castration for ram lambs with the use of anti- GnRF vaccine had no adverse effects on the expression of lambs' social behaviours, stress responses and ewe-lamb bonding.

Chapter 6 The effects of Immunocastration with a novel anti-GnRF vaccine developed by Zoetis on aggression, growth rate, carcass and meat quality characteristics in ram lambs

Abstract

The impact of an anti-GnRF vaccine on carcass and meat quality characteristics as well as expression of aggressive behaviours and growth rate in mule x terminal sire (Texel or Suffolk) lambs was evaluated in this study. Growth rate was measured over a period of 3 years. In the first year of the study 20 lambs per treatment were allocated to one of 5 treatments: handled only (control, C), castrated using conventional rubber rings (RR), short-scrotum castration (SSC) and rubber rings castration combined with Burdizzo (COM) and 1mmunocastration group (Vac1 – vaccinated at 6 weeks of age by s.c. injection in the neck). On the second year of the study the following treatments were formed entire males - control group (C), castrated using conventional rubber rings (RR), castrated using conventional rubber rings with use of local anaesthesia (LA), immunized rams with 0.5ml of anti-GnRF vaccine at 6, 12 and 22 weeks of age (Vac2), n=12 per treatment and the female group (F) n=24. On the third year of the study 14 rams per treatment group were allocated as follows: positive controls (C) – lambs were handled only; negative controls (RR) – lambs were castrated using standard rubber rings; Vac 3 - Lambs immunized at 10 and 16 weeks of age; Vac 4 - Lambs immunized at 10 and 20 weeks of age; Vac 5 - Lambs immunized at 12 and 18 weeks of age; Vac 6 - Lambs immunized at 12 and 22 weeks of age. In total 256 lambs took part in the study. All selected lambs were balanced for maternal parity and sire breed. Growth rate was measured every four weeks until the day of slaughter. Aggressive behaviours were evaluated during a feed competition test. Following food deprivation overnight, lambs were placed in groups of three in a 2x2m pen, offered a small amount of food and allowed to compete for it for 0.5 h. Behaviours were recorded and scored by a single observer in Observer XT 11.5. Carcass and meat quality characteristics such as finish weight, carcass weight, joint tenderness (shear force), ultimate pH and colour were assessed. Repeated measures REML statistical tests were carried out for normally distributed data (growth rate, live/finish weight, carcass weight, joint weight etc.) and post hoc two sample t-tests used to detect significant differences between treatments. Non parametric data was analysed by the Kruskal-Wallis and Mann-Whitney test. Bonferroni correction was considered to account for multiple testing. The treatment did

not significantly affect growth rate but there was an effect of time on weight gain throughout the whole study (P<0.001). Rubber ring castrated lambs were observed to express a significantly greater number of 'returned aggression' (butting or pushing back behaviour when attacked by other lambs) in comparison to all other treatments in the feed competition test (median frequency: C=0.0; RR=1.5; Vac3=1.0; Vac4=0.0; Vac5=0.0; Vac6=0.0; P=0.006). There were no differences between treatments in finish weight, carcass weight, joint tenderness, ultimate pH and colour. However, there was a tendency for the RR group to have a lower finish weight in comparison to C, Vac5 and Vac6 treatment (median weight: C=53.5; RR=47.2; Vac3=51.5; Vac4=52.7; Vac5=53.5; Vac6=53.7; P=0.07). There was also a tendency for Vac 4 treatment to have greater decrease of carcass pH 45 minutes after kill than the C, RR and Vac5 group (median frequency: C=6.6; RR=6.6; Vac3=6.6; Vac4=6.1; Vac5=6.5; Vac6=6.6; P=0.08). There were no negative effects of Immunocastration on growth rate, carcass and meat quality characteristics therefore it was concluded that this method is a good alternative to traditional castration techniques for ram lambs.

Key Words: Immunocastration, Carcass and Meat Quality Characteristics

6.1 Introduction

Castration of male livestock is used globally to achieve major production advantages, in particular ease of management by reduction of sexual and aggressive behaviours, improved feed efficiency leading to enhanced carcass and meat quality (Huxsoll et al., 1998; Bouissou et al., 2001; Price et al., 2003; Stookey and Watts, 2004). However, castration is painful and distressing and may lead to post-castration complications (i.e. haemorrhage, clostridia infection, trapping of the urethra or rudimentary teats, ineffective castration, chronic pain etc.) depending on the technique which was used and the environmental factors (housing conditions, weather etc.). This may result in economic losses. Therefore, immunocastration has been proposed as a new, potentially pain-free, method of castration that leads to a reduction in serum testosterone concentration, suppression of the development of the testes and elimination of unwanted behaviours in male livestock species (for example aggression, fighting and inappropriate breeding) (Adams & Adams, 1992; Hoskinson et al., 1990; Robertson, Fraser, Innes, & Jones, 1982; Ülker et al., 2005; Ülker, Küçük et al., 2009; Ülker, Yılmaz et al., 2009). Immunization has also been proposed in the past as a method for fertility control which could be applied later on in life (Robertson et al., 1979; Adams and Adams, 1992; Bonneau and Enright, 1995) as intact males have been found to have better average daily growth (ADG) and improved feed conversation (Field, 1971; Seideman et al., 1982; Lee et al., 1990). Some studies also suggest that there is improvement in feed efficiency and carcass conformation in animals immunized against GnRH (Thompson, 2000). This is very important as at the present time there is demand for products with specific qualities like appropriate tenderness or texture. Features such as, for example, appearance, flavour, texture or tenderness are often perceived as most important factors for high meat quality (Aaslyng, 2009; Maltin et al., 2003; Risvik, 1994). Moreover, consumption of lamb meat has been increasing since the time of the Second World War (Conington, 2010). Ever since that time production of lambs for meat has been growing. Consumption of meat has been growing in other part of Europe and the USA since the 1950s as well as Britain due to the growth of the economy and prosperity and has increased by approximately 50% in the last 70 years (Aaslyng, 2009; Breadsworth and

Bryman, 2004; Horowitz, 2006). Meat has become one of the most important food items in western countries (Holm and Mohl, 2000; Twigg, 1984). Alongside the increasing demand for meat production attitudes towards meat have also been changing. The drive for leaner meat has led to a stronger acceptance of castration procedures therefore most EU countries allow for castration of male stock (EC, 2001). However, public opinion is also interested in mitigation of pain caused by husbandry practices, therefore immunization is believed to be a good alternative to traditional techniques of physical castration. Immunocastration may be also potentially a more economical way of fertility control as it may be applied later on in life which would allow animals to grow and have feed conversion at the rate equal to entire males. There is evidence that castrated animals may grow slower and their feed conversion is poorer. This means they need to stay longer on the farm before slaughter which may be less economical. Consequently, there are potential benefits from raising entire males and late immunization would allow for the capture of production advantages, for example improved meat quality, and control unwanted behaviours by late vaccine administration. Immunocastration has already been successfully implemented in cattle, goats and pigs where improved feed efficiency, carcass conformation (Thompson 2000; Amatayakul-Chantler et al., 2013) and quality were found (Schanbacher, 1982). The products like Vaxtrate® for bucks, Improvac® for pigs and Bopriva® for cattle are currently present on the market. However, there is little or no information on the effects of immunization in sheep species. The objective of this study was to determine the effects of an anti-GnRF vaccine on the expression of aggressive behaviours, growth rate, carcass and meat quality characteristics (meat tenderness, ultimate pH and colour) in mule x terminal sire (Texel or Suffolk) lambs.

Chapter 6 Hypothesis

1. Carcass conformation and quality may be different in rams castrated with different castration techniques.

2. Immunocastration improves carcass conformation and meat quality in immunized animals in comparison to physically castrated and control males.

3. Growth rates may be different in rams castrated with different castration techniques in comparison to entire male lambs.

Chapter 6 Objectives

1. Evaluation of the 4 different vaccination regimes to determine most practical, economic and efficient way of anti-GnRF vaccine administration.
2. Assessment of the impact of an anti-GnRF vaccine on the expression of aggressive behaviours and growth rate.
3. Evaluation of the impact of an anti-GnRF vaccine on the carcass and meat quality characteristics (meat tenderness, ultimate pH and colour).

6.2 Materials and methods

6.2.1 Housing and Management

General husbandry procedures are described in section 2.3 of Chapter 2 where specific details of housing and management (section 2.3.2), allocation and implementation of treatments (section 2.3.3, see also table 1), time of weaning (section 2.3.4) and "the end point" (section 2.3.5) are defined. The study was conducted on the Woodhouselee farm (property of SRUC) Edinburgh, Scotland between April 2011 and December 2013 for the measurment of growth rate. The expression of aggressive behaviours was carried out in October 2013. The carcass and meat quality parameters were collected in December 2013 and analysed in January 2014. Each year a specific group of animals was allocated to each treatment group. The number of animals and the treatment groups were different each year throughout the whole study period therefore they will be described separately.

6.2.2 Animals

In the first year of the study 20 (mule x terminal sire (Texel or Suffolk) lambs per treatment were selected balanced for maternal parity and sire breed (see table 1 in Chapter 2). In the second year of the study 48 ram lambs and 24 female lambs (mule x terminal sire Texel or Suffolk) took part in the study. In the third year of the study 14

rams per treatment group (mule x terminal sire (Texel or Suffolk) were selected. Allocation to specific group and treatments applied are described in chapter 2 section 2.3.3 as well as chapter 4 section 4.2.2.

Lambs in group RR and LA were castrated between 24 and 48 hours after birth with or without anaesthesia. Lambs were kept in small pens (approx. 1.5 x 1.5 m) with their mother and siblings. C were similarly handled, but not castrated and Vac 1-6 lambs were untreated at this time and immunized as described above. At least 2 hours after castration/handling all lambs were weighed, double-tagged with colour coded EID tags and tail docked with rubber rings after administration of a local anaesthetic. The day following treatment, lambs and their mothers were moved out to graze in a paddock. Female lambs took part only in the growth rate measurements in this study.

6.3 Datasets Description

6.3.1 Immunization procedure

The general technique of the immunization procedure is described in of Chapter 4. Specific immunization regimes for particular years of the study are described in chapter 4 indicating intervals between primary and booster vaccinations as well as the number of booster vaccinations.

6.3.2 Growth rate

In the first year of the study (2011) lambs were weighed at birth (within 24 hours of delivery), and at weekly intervals until 8 weeks of age and at 4 weekly intervals thereafter until the end of the study. In the second year of the study (2012) lambs were weighed at birth (within 24 hours of delivery), and at weekly intervals until 10 weeks of age and at 4 weekly intervals thereafter until the end point. In the third year of the study (2013) lambs were weighed at birth (within 24 hours of delivery), and at 4 week intervals.

6.3.3 Assessment of aggressive behaviours expression

Assessment of aggressive behaviour was only carried out in year 3 of the study. At approximately 5.5 months old lambs were tested in a food competition test. Aggressive interactions and displacement were recorded for 30 minutes. Each treatment groups had 4 replicates, balanced across time, days and test pens. One day before testing animals were brought into the shed and segregated in to 4 home pens accordingly to their group (6 lambs per pen). Lambs from different groups were not kept together. All animals had access to hay and water ad libitum during the night but solid food was not present. Three testing pens (2m x 2m) were prepared on the opposite side to the pens in which animals were kept overnight. During the testing sessions three lambs from each group were placed in a testing pen. The order of the treatment groups being placed in particular test pen at a particular time was randomized across all treatments with use of R statistical programme to avoid any bias. Individuals were marked with spray marker on their backs so they could be individually identified. A small amount of concentrate food (an equivalent quantity for each group – approximately 0.5 kg) was placed in a feed trough for each trio. Animals were allowed to compete for access to food for 30 min, and interactions captured on digital video. Recordings were made with a Canon XM2 3CCD Digital Video Camcorder (Canon Inc, Japan) placed on a tripod and directed towards the pen in such way to allow for full vision of the pen. Recordings were then scored using the Observer XT 9 (Noldus Information Technology®) program by a single observer. Table 1 indicates the recorded measurements, which were grouped into 5 categories: maintenance behaviours, performed aggression, returned aggression, sexual behaviours and escape avoidance behaviours. The total number of aggressive encounters was also analysed by adding together behaviours of performed aggression, returned aggression and sexual behaviours categories.

Table 1 Aggressive behaviours Ethogram

Behaviour/Posture	Description
Maintenance behaviours	
Walking/standing	Lamb stands on the ground with all four legs or moves

	forward in a slow motion.
Lying	Ventral (sternal) recumbence with the legs tucked in and the head up or down, either round to one side or directly in front.
Feeding	Lamb has head down in the feeder or up. Chewing movements are visible for at least 3 s.
Exploring feeder	Lamb head is directed towards the feeder, lamb nose is within 5 cm of the feeder with or without physical contact, licking the feeder was also included.
Ruminating	Lamb is standing, walking or lying with its head up with visible chewing movement.
Not eating	Lamb is standing, walking or lying and does not perform any other activity.
Performed Aggression	
Head Butting	A blow with the forehead directed at another ram.
Displacing	Forcing another animal to leave its place of resting or feeding with or without physical contact.
Threat	A blow with the forehead directed at another ram without physical contact.
Pushing	Pressing and or pushing body against body, head to head pushing, sometimes followed by head to neck pushing.
Returned aggression	
Butting back	A blow with the forehead directed at another ram as a consequence of pushing or butting performed by another lamb.
Pushing back	Pressing and or pushing body against body, head to head pushing, sometimes followed by head to neck pushing as a consequence of pushing or butting performed by another lamb.

Sexual Behaviours	
Nudging	Combination of kick (forefeet used in a pawing motion while standing parallel to ewe), rubbing (the ram rubs head and shoulders along or under ewe's flank.
Low stretch	Neck being held horizontal to the ground with the muzzle forward and raised. Head can be turned through 90° as well, with or without running tongue in or out.
Attempt to mount	Lamb is attempting to mount (set of back and forward movements while standing in front of or side of rump) jumps on the back of the lamb with or without firm contact of ram brisket with rump.
Avoidance/escape behaviours	
Climbing/jumping on gates	Lamb climbs on gates with its fore legs or jumps on it.
Withdrawal	Lamb is changing its place of presence, resting or feeding after or without physical contact with another lamb usual seen after displacement.
Avoidance	Lamb is avoiding physical contact with another lamb by changing its place of resting or feeding usually seen after threat.
Escape	Lamb manages to leave testing pen or attempts to leave by series of movements trying to escape underneath the gates.
Vocalizing	High pitched bleats.

6.3.4 Assessment of carcass conformation and meat quality characteristics

The measurements were carried out on the third year of the thesis.

6.3.4.1 Slaughter of lambs

At 8 months of age lambs were transported to the Bio Support Unit at the University of Nottingham, Sutton Bonington Campus to undergo commercial slaughter. The following meat quality measures were taken: Finish weight (prior to slaughter), hot carcass weight (HCW) at slaughter, MLC carcass evaluation, and pH of the carcass in situ 45 minutes after kill. Longissimus dorsi (LD) loin muscle was used in the assessment of meat quality parameters. Ultimate pH of the LD, meat colour and shear force were measured.

Lambs were transported 4 days before slaughter. Hay and water were present continually. Lambs were also given 1/3 kg of concentrated feed per animal 2 x a day. Sixteen animals were killed every day for 5 days. Lambs were brought to slaughter in groups of four. Two animals were killed at any one time to avoid separation anxiety. Kill order was randomized across treatment groups to avoid any bias. Live (finish) weight was taken from each animal on the day of the slaughter. Each lamb was identified individually prior to slaughter. After stunning, ear tags were checked again and a kill number was noted. After dressing each carcass was marked with appropriate kill number on the right back leg (see figure 1a) and the ears with ear tags were left attached to the carcass. The weight of each carcass following dressing was measured and recorded. Temperature and pH of left LD was measured in situ by Hannah® instruments Ltd testo-205 probe in three sections across the loin (see figure 1b) 45 min after dressing. The probe was calibrated every 2 hours in Hannah pH buffers 4.01 and 7.01. Carcasses were chilled for 24 h. The temperature was monitored continuously by Hannah® instruments Ltd temperature probe. The left LD loin was harvested from each carcass on the next day after kill, vacuum packed and left for maturing in the chiller in the vacuum bags for 7 days. After maturing all loins were frozen in -20 degrees Celsius before further analysis. Each loin was weighed before freezing.

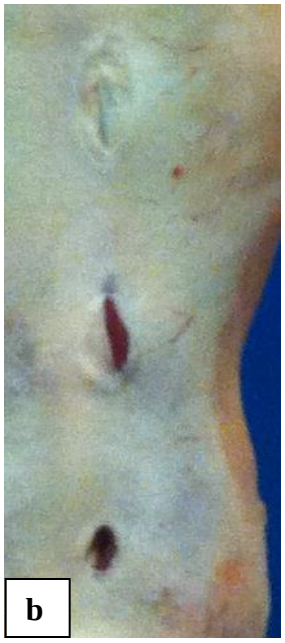


Figure 1 a, b: 1a - Traceability of carcasses was assured by leaving ears with ear tags attached which allowed for cross checking ear tag numbers with kill number and by marking each carcass with the number on the right back leg. 1b - Measurement of pH and temperature 45 min after kill across left LD loin.

6.3.4.3 Conformation and fatness grading

Classification of meat is a very important factor for the producers because it provides them with crucial information concerning the value of the product and its suitability for a particular market: retail, catering, processing (DEFRA Livestock knowledge transfer, 2001). Classification is associated with the market price of the final product. The price is calculated on the basis of a particular classification category. It also gives the farmers essential information regarding premium payments, and indicates which product is most attractive at market and will give best return (DEFRA Livestock knowledge transfer, 2001). Carcass grading varies across the UK but the general principles remain the same. The carcass is graded according to its muscularity and the amount of fat. Carcasses are graded to give a prediction of the amount of meat which they will yield. In the UK, sheep grading is carried out by independent graders supplied by the Meat and Livestock Commission (MLC). Fat class is assessed by classifying the fat from 1 (very thin) to 5 (very fat). Saleable meat yield is assessed by classifying the muscularity of the carcass

from E (heavily muscled) through to P (very lightly muscled). Table 2 below represents an example of the classification grid for sheep. Combinations of conformation and fat grading attract different enhanced payments or penalties relative to the basic price (B) as indicated in the Table 2.

Table 2 Sheep carcass classification grid (DEFRA Livestock knowledge transfer, 2001)

Conformation/ Fitness	**Fat classes increasing fatness**						
	1	**2**	**3L**	**3H**	**4L**	**4H**	**5**
E	**B**	**+10**	**+10**	**B**	-10	-25	-35
U	**B**	**+5**	**+5**	**B**	-10	-25	-35
R	**B**	**B**	**B**	**B**	-10	-25	-35
O	-20	-5	-5	-5	-15	-25	-35
P	-25	-25	-25	-25	-25	-25	-35

In this study the MLC grading system described above was used to determine carcass fatness and fitness (conformation). Carcasses were graded 24 h after kill by a skilled slaughter man who was blinded to the nature of the treatments. To allow for the statistical analysis of the probable beneficial for the farmers' economic value of different treatments in relation to enhanced payments or penalties relative to basic price, each combination of conformation and fat grading was given a score. The scores were as follows: Basic price was given a score of 0 as it was neither enhancing nor reducing the final price of the carcass. All other combinations were given a score as indicated in the table 2 by the penalty or premium i.e. E2 – score of +10; O1 – score of -20 etc. Each combination was also presented as a percentage in relation to the total number of lambs in the particular group. The percentage of specific carcass fatness and fitness classes in relation to total number of lambs in a particular treatment was also shown.

6.3.4.4 Limitations to the accuracy and reliability of the carcass assessment performed in the study

As mentioned in the previous paragraph carcass grading in this study was conducted by a skilled slaughter man. The grader was blinded to the nature of the treatment of particular sheep and the order of kill. The reliability of the slaughter man in conducting the grading was not checked which may have influenced results of the study. It is

possible that the grading of the carcasses conducted in this study would be more accurate if the reliability of the slaughter man was assessed during the course of grading the carcases. The grading was done accordingly to the MLC leaflet containing images of the carcasses assigned to the particular sheep carcass classification grid (see table 2). To grade each carcass slaughter man had to compare it to the classification grid in the MLC leaflet. It was believed that due to slaughter man experience (over 30 years) and the guidelines given in the MLC leaflet, grades given to the carcasses will be accurate. It was also believed that because all of the carcasses were scored by the same slaughter man, who was blind to lamb treatment that any inconsistencies in assessment would be unlikely to be biased towards a particular treatment group. The main intention was for the sheep to undergo commercial slaughter accordingly to the UK standards. The objective was to investigate possible cost/benefits of carcase characteristics shown by particular treatment groups during commercial slaughter. In the UK sheep are being graded on the line by MLC grader or slaughter man during the process of slaughter, in an identical procedure to that conducted in this study. Thus, although there may have been inconsistencies in grading by the slaughterman they would have been unlikely to influence the results and would be consistent with normal on farm practice.

6.3.4.5 Shear Force, ultimate pH, temperature and meat colour measurement

In January 2014 the meat quality measures were conducted at SRUC CT scanning unit, Edinburgh, Scotland. LD loins were removed from the freezer and left overnight in the chiller in +3 degrees Celsius. Temperature was monitored. The weight of defrosted loins was noted. Ultimate pH and temperature of each loin matured was measured by Hannah testo-205 probe in three sections across the loin (figure 2) similarly to the measurements taken in situ. A fresh cut surface was open for each loin and left for 10 minutes to bloom so the muscle colour measurement could be taken using a Konica® Minolta 410 chromometer. (Konica Minolta Sensing Europe B.V.). Lightness (L*), redness (a*) and yellowness (b*) values were determined (figure 3).

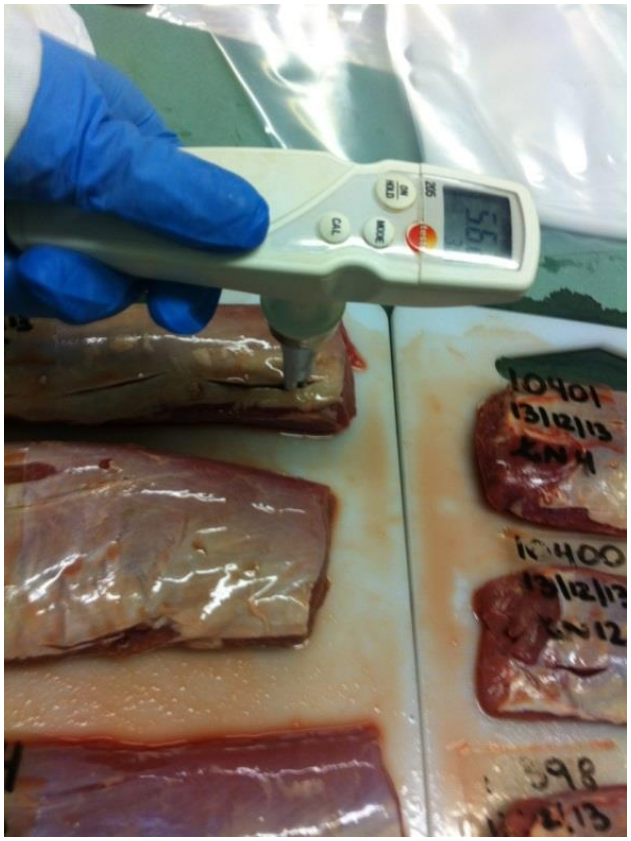


Figure 2 Measurement of ultimate pH and temperature of loins by Hannah testo-205 probe in three sections across the loin.

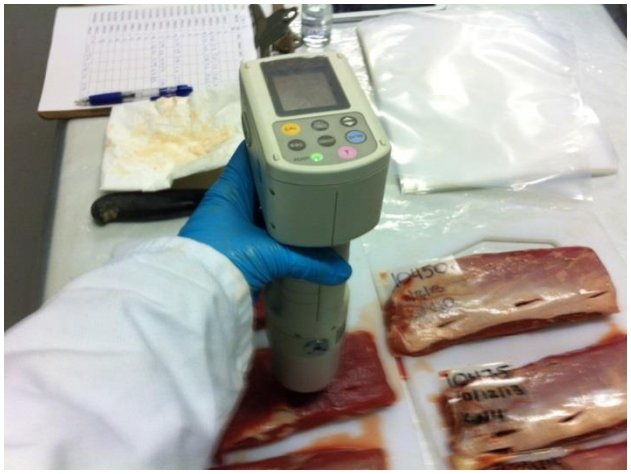
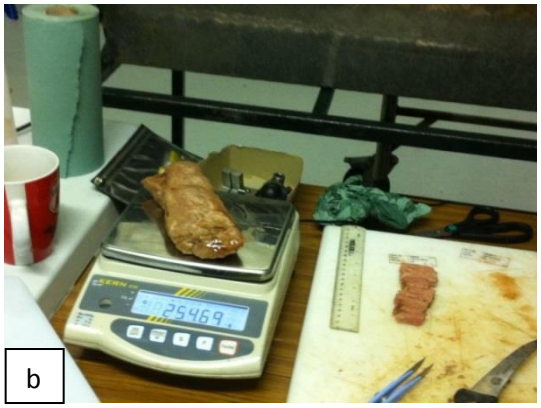


Figure 3 Measurement of Lightness (L*), redness (a*) and yellowness (b*) of meat by Konica Minolta 410 chromometer.

The Mirintz tenderometer (MacFarlane and Marer, 1966) protocol was used to determine shear force. Each loin was cooked by placing it in a polythene bag and submerging it in a water bath set to 80 degrees Celsius (figure 4 a). The temperature of each loin was monitored continuously with a Hannah® instruments Ltd temperature probe until it reached 75ºC when the sample was removed and placed in ice/water for at least 10 minutes. Cooked loins were stored in +3 degrees Celsius overnight. The day following cooking, loin samples were weighed (figure 4 b) and trimmed to a weight of 100g (figure 4 c). Ten sub-samples of 10 mm by 10 mm cross-section orthogonal to muscle fibre orientation were harvested from each loin (figure 4 c, e). Shear force was measured (figure 4 d, f) by recording a force in Newtons (N – is a measure of force, 1 N = 1 kg·m/s2) by which the tenderometer compressed the loin of particular individual. Shear force values were then related to the following tenderness levels (Bickerstaffe et al., 2001): < 5.0 kgF indicates very tender meat, 5 - 7.9 kgF indicates tender meat, 8 - 10.9 kgF indicates acceptable meat, 11 - 14.9 kgF indicates tough meat, > 15 kgF indicates very tough meat.


a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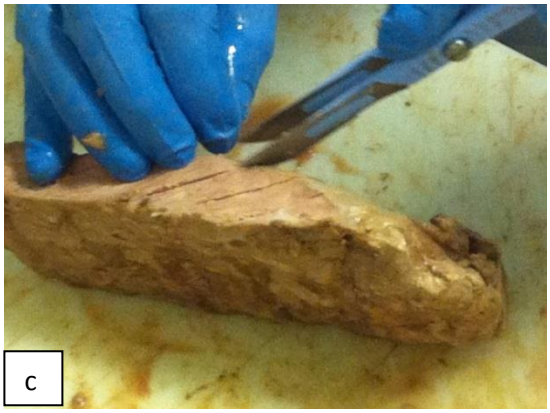


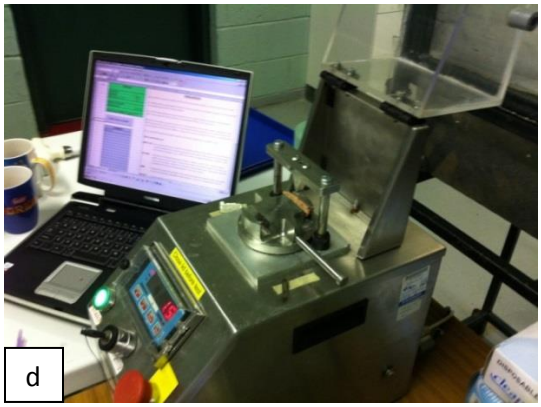


Figure 4a,b,c,d,e,f process of shear force measurement from the moment of loin cooking (a), measuring post cooking weight (b), cutting transverse sections of the loin (c) and preparing it for measurement (e), measuring shear force with Mirintz tenderometer (d, f)

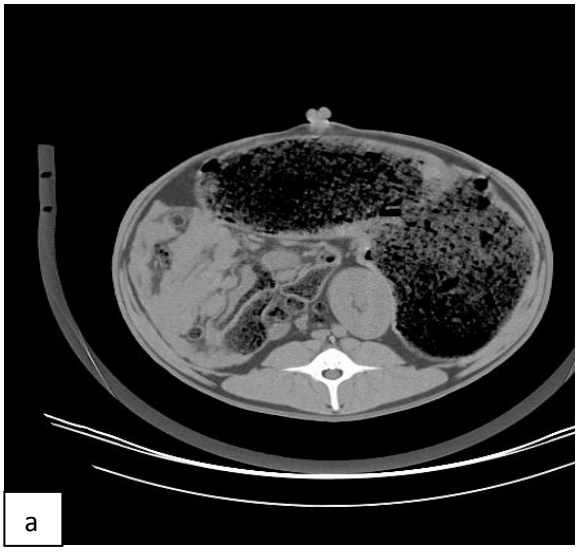
6.3.4.6 Cooking loss

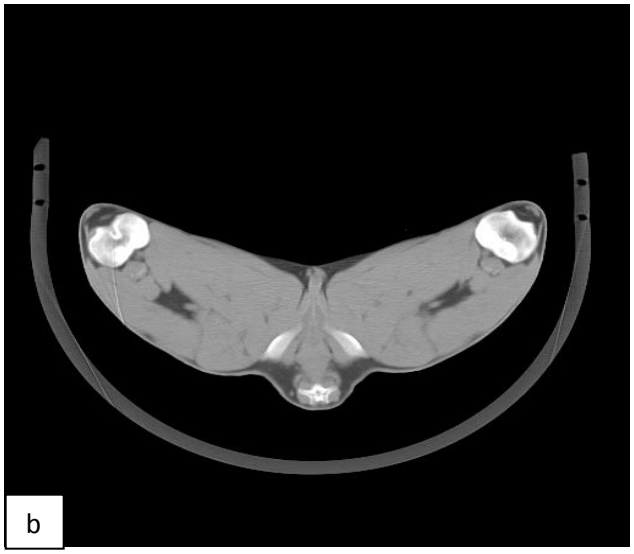
The cooking loss was calculated as amount of weight loss was expressed as a percentage of the original sample weight.

6.4 Prediction of carcass quality in live rams - CT scanning

In early October 2011 a pilot study of Computed Tomography (CT) scanning at the SRUC CT scanning Unit, Edinburgh, Scotland was carried out to predict carcass quality in live animals as an alternative to traditional post slaughter measurements. Forty lambs took part in the study (10 each of control, rubber ring, short scrotum and Immunocastration (Vac 1) group balanced for body weight) to determine body composition.

The selected group of animals was brought to the scanning unit in the morning and placed in the preparation pens. Animals were not fed in the evening before the scanning day. Water was available ad libitum. Each animal was handled individually and a sedative drug Rompun® (active ingredient xylazine at a dose rate of 0.1-0.2mg/kg body weight) was given 15 minutes before the scan took place. When there were signs that the sedative was working, the animal was placed on the cradle and taken to the scanning room. Lambs were CT scanned in three positions: at the level of LV5 (lumbar vertebrae 5, figure 5 a), TV8 (Thoracic vertebrae 8, figure 5 c) and Ischium (hip figure 5 b). Prediction of fat, muscle and bone tissue was made for each animal to calculate the amount of tissue and carcass quality. When the scan was finished each lamb was placed in a resting pen where food and water were provided.





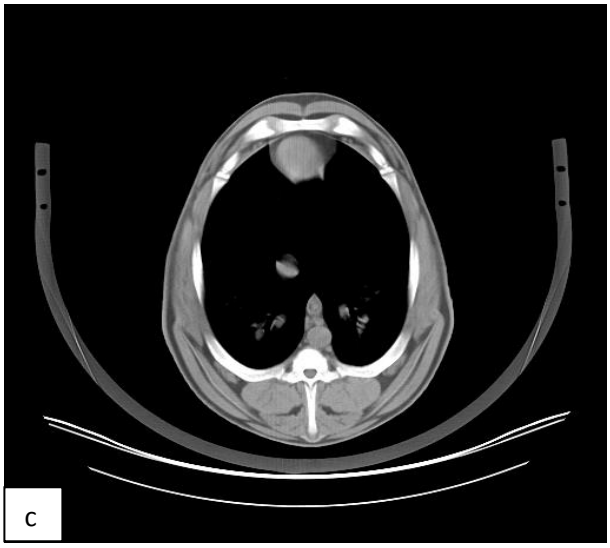


Figure 5 a,b,c CT scans of one of the study rams in three positions: LV5 (lumbar vertebrae 5, figure 5 a), TV8 (Thoracic vertebrae 8, figure 5 c) and Ischium (hip figure 5 b)

6.5 Statistical analysis

The distribution of data was analysed in Minitab statistical package 16th edition (Minitab, Inc, State Collage, PA). The Anderson-Darling test was used to test the distribution of the data. It was impossible to normalize the behavioural data therefore it was decided that Kruskal-Wallis and Mann-Whitney non parametric tests should be used to determine significant differences between treatment groups using Genstat 14th edition Genstat (GenStat® 14th edition, Lawes Agricultural Trust, VSN International Ltd, Oxford, UK). All non-parametric data was analysed this way. The Bonfrerroni correction was also applied to account for the multiple testing. In practice, to correct for multiple comparisons the critical p-value of 0.05 has to be divided by the number of comparisons (treatment groups of the particular study). In this study due to different number of treatment groups in particular experiments the new critical p-value will be as follows; a) for experiments with 6 treatment groups new p – value would be $P<0.008$; b) for experiments with 5 treatment groups new p – value would be $P<0.01$; c) for experiments with 4 treatment groups new p – value would be $P<0.0125$. Normally distributed data (weight gain in growth rate measurement as well as carcass weight and live weight of lambs just before slaughter) was analysed with use of Repeated Measures

and REML tests in Genstat 14th edition Genstat (GenStat® 14th edition, Lawes Agricultural Trust, VSN International Ltd, Oxford, UK. Two sample T-tests were carried out to determine significant differences between treatment groups.

6.6 Results

6.5.1 Growth rate

In the studies of 2011 and 2013, the treatment did not significantly affect growth rate throughout the study however there was an effect of time on weight gain (2011: Wald statistic=56220.23, F=4324.63, P<0.001; 2013: Wald statistic=15897.06, F=1987.13, P<0.001). In 2012 there were significant treatment, time and treatment*time interactions in the growth rate of lambs (see Table 3 below). There were no effects of ewe parity, birth weight or sire breed on growth rate.

Table 3 Differences between treatments in growth rate

Repeated measures Effect of:	**Wald statistic**	**F - statistic**	**n.d.f**	**d.d.f**	**P - Value**
Time	48678.12	3477.01	14	884.6	**P<0.001**
Group	9.35	2.34	4	67.2	**P=0.064**
Time * Group	350.34	6.37	55	884.8	**P<0.001**

Post hoc analysis revealed that the growth rate was different between the groups at 16 weeks of age (see table 4 and figure 6). F group had a lower increase in growth than C and Vac 2 group when lambs were 16 and 20 weeks old. C rams were observed to have greater growth at 24 weeks of age in comparison to F lambs. There were no differences in weight gain between C and Vac 2 rams in any of the measured time points. This indicates that there was no negative effect of the three dose regime immunization (at 6, 12 and 22 weeks of age) on weight gain in vaccinated animals. C rams also had different weight gain to the RR and LA group when lambs were 20, 28 and 16, 20, 28 weeks old respectively. In summary, it can be argued that C lambs were observed to be heavier than F, RR and LA lambs, and Vac 2 were intermediate between C and other groups.

Table 4 Differences in growth rate between control (C), rubber ring (RR), female (F), local anaesthesia (LA) and immunicatration (Vac 2) treatment groups recorded monthly until 28 weeks of age. Data are Means with standard errors.

Age in Weeks	C[1]	F[1]	RR[1]	LA[1]	Vac2[1]	P-Value[2]
16	36.5(2.5)a	32.6(2.8)b	33.1(3.6)a	33.6(2.5)b	35.1(2.7)a	**P=0.006**
20	41.7(3.5)a	34.6(3.8)b	35.9(4.6)b	36.5(3.2)b	38.8(3.5)a	**P<0.001**
24	49.3(5.4)a	42.4(3.4)b	42.9(3.3)a	43.7(4.2)a	45.6(5.3)a	**P=0.001**
28	59.8(7.4)a	Group already slaughtered at this stage	50.0(5.1)b	52.2(5.4)b	55.1(6.1)a	**P=0.004**

[1]Mean values with standard error SE. [2](amended p-value due to Bonferroni correction P<0.01), NS not significant. Different letters indicate significant differences between treatments .

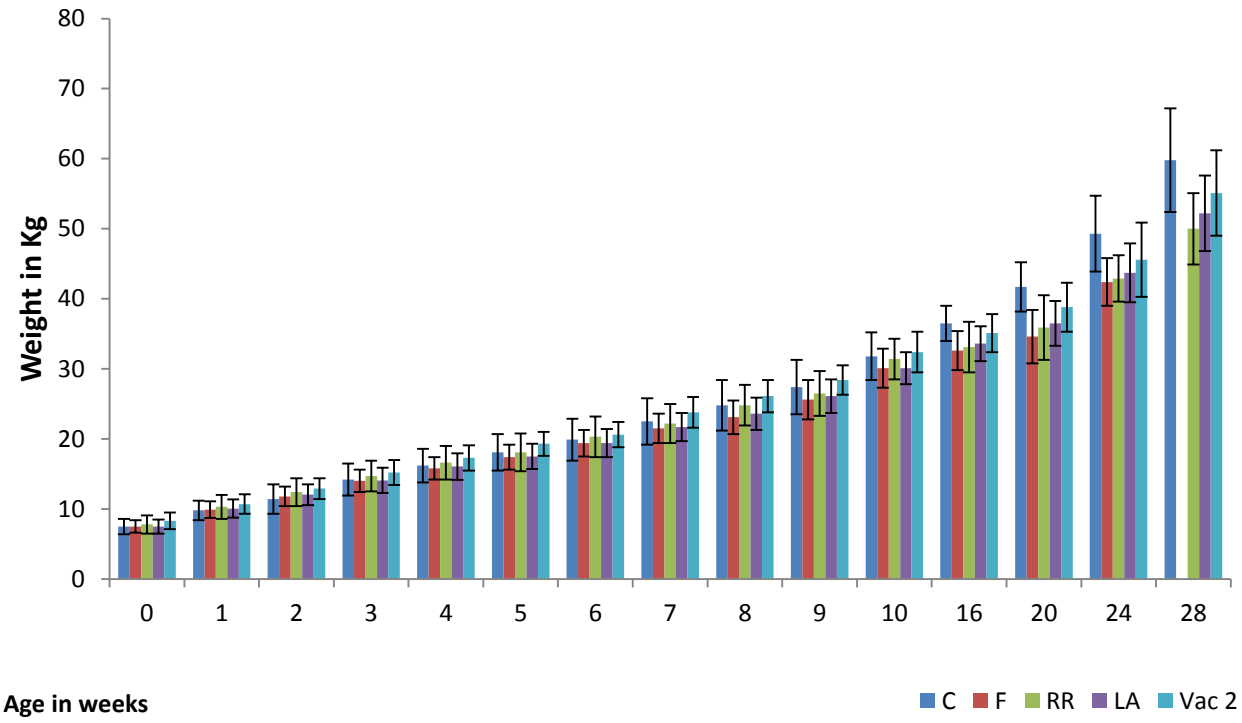


Figure 6 Differences in growth rate measured in Kg between control (C), female (F), rubber ring (RR), local anaesthesia (LA) and immunocastration (Vac 2) treatment, over the period of 28 weeks of age (from the moment of birth until slaughter during the study of 2012). Data are Means with standard errors.

6.5.2 Assessment of carcass quality parameters (finish weight, carcass weight, carcass pH and temperature 45 min after kill, carcass fatness and fitness)

Overall no significant differences between treatments in the carcass quality parameters were found except for dressing percentage (see table 5). There was a tendency for the RR rams to have a lower finishing weight in comparison to C, Vac5 and Vac 6 rams. There was also a tendency for the Vac 4 rams to show a quicker drop in the average pH parameter in comparison to C, RR and Vac 5 treatment. Moreover, all of the assessed carcasses 24h following slaughter were in majority classed accordingly to the sheep classification grid within the acceptable by the retailers (see table 2 for details) classes

for conformation and fatness as well (table 7, figure 7.8 and 9). There were no significant differences between treatments in the probable economic value of different treatments in relation to enhanced payments or penalties relative to basic price of each combination of conformation and fat grading's $H = 1.75$, $DF = 5$, $P = 0.88$.

Table 5 Differences in the meat quality parameters recorded at the time of slaughter (when lambs were 32 weeks of age) for entire males (C), rubber ring castration (RR), and immunocastration (Vac 3-6) treatment. Data are medians with Q1 and Q3 or Means with standard error for the finish weight, carcase weight and dressing %.

Meat Quality measures	C[1,*]	RR[1,*]	Vac3[1,*]	Vac4[1,*]	Vac5[1,*]	Vac6[1,*]	P-Value[2]
Finish weight*	53.5(1.92)a	47.50(1.40)b	51.1(1.31)ab	51.3(1.95)ab	53.2(1.36)a	53.5(1.63)a	P=0.07
Carcass* weight	26.5(0.95)	23.0(0.83)	25.0(0.84)	24.5(1.12)	24.9(0.76)	25.1(0.95)	**NS**
Dressing Percentage*	49.6(1.09)	48.5(0.56)	48.9(0.77)	47.5(0.81)	46.7(0.55)	46.8(0.65)	P=0.04
Average Carcass pH	6.6(6.5-6.8)a	6.6(6.5-6.6)a	6.6(6.4-6.8)ab	6.1(6.3-6.2)b	6.5(6.5-6.7)a	6.6(6.4-6.7)ab	P=0.08
Average Carcass Temperature	36.4(35.6-37.1)	35.5(35.1=36.4)	36.3(33.7-37.3)	36.5(34.1-37.9)	35.9(35.1-37.0)	35.8(33.6-37.3)	**NS**
Carcass Fitness	2.0(2.0-2.2)	2.0(2.0-4.0)	3.0(2.0-3.0)	3.0(2.0-3.0)	3.0(2.0-3.0)	2.0(2.0-3.0)	**NS**
Carcass Fatness	3.0(2.0-3.0)	2.0(2.0-4.0)	3.0(3.0-4.0)	3.0(3.0-4.0)	3.0(3.0-4.0)	3.0(2.0-4.0)	**NS**

[1]Median (Q1-Q3) or * Mean values (standard error). [2](amended p-value due to Bonferroni correction P<0.008), NS not significant. Different letters indicate significant differences between treatments Dressing percentage of Carcass (weight/finish weight) is presented as %.

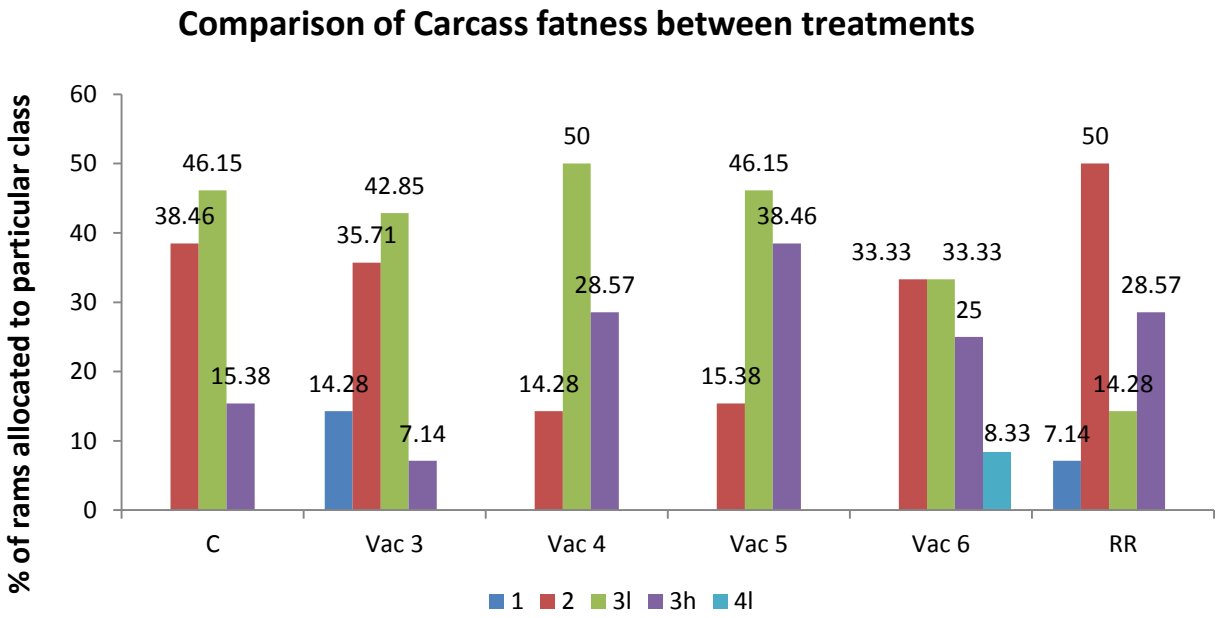


Figure 7 Comparison of percentage of rams allocated to specific class of carcass fatness between treatments. The percentage was calculated on the basis of sample size in each group.

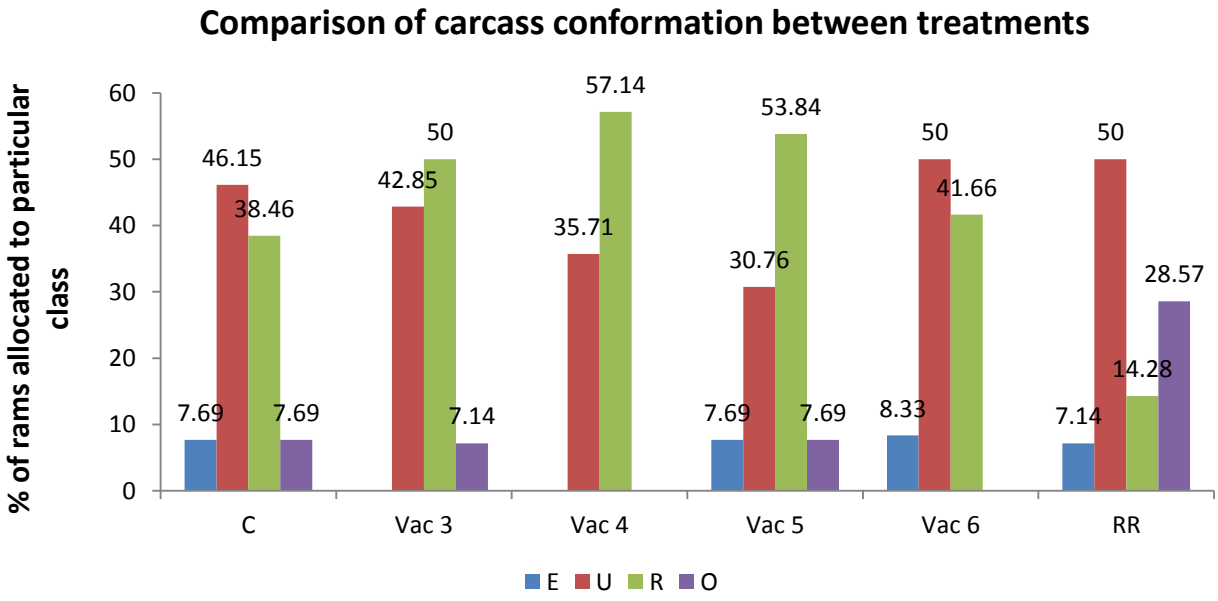


Figure 8 Comparison of percentage of rams allocated to specific class of carcass

conformation between treatments. The percentage was calculated on the basis of sample size in each group.

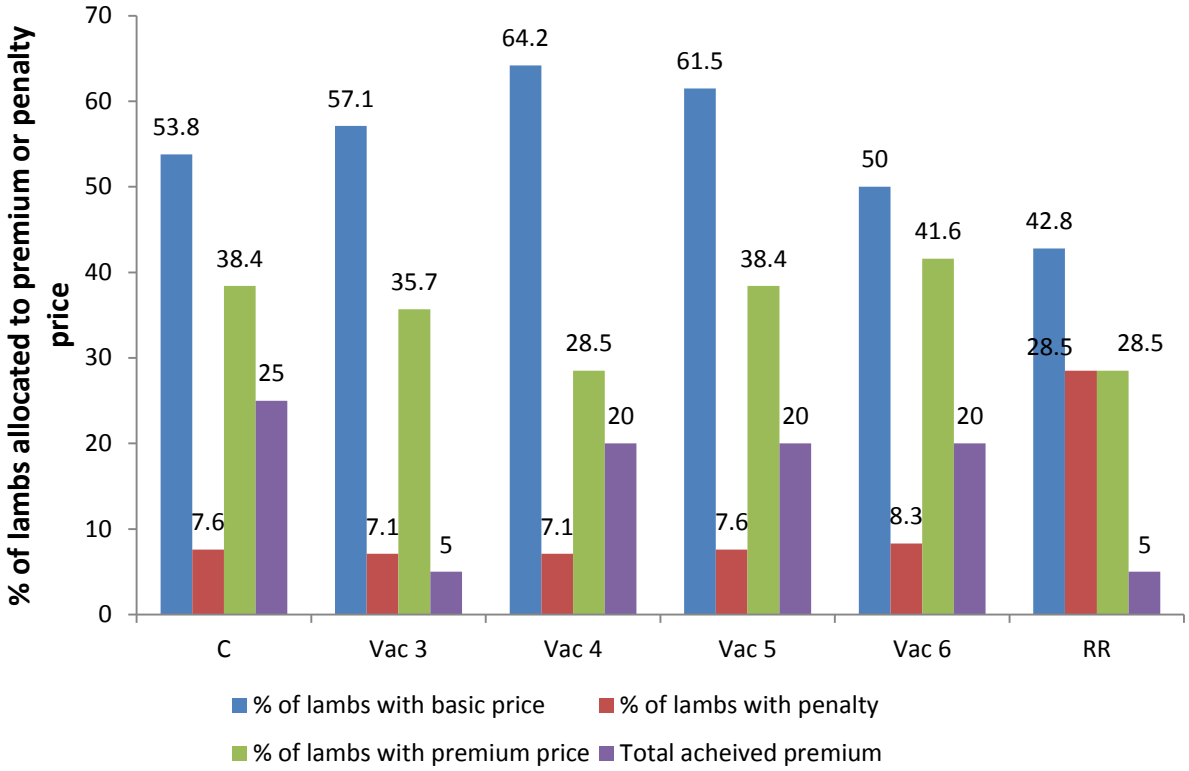


Figure 9 Estimation of the relative cost benefits or losses for each treatment group with indication of the proportion of carcasses that could be penalised vs those that could receive premiums. The percentage was calculated on the basis of sample size in each group

6.5.3 Assessment of meat quality parameters (Shear Force, ultimate pH, temperature, cooked weight, pre and post freezing weight and colour measurement of longissimus dorsi)

There were no significant differences in the measured meat quality parameters between treatment groups (table 6). However, it has to be noted that shear force variation within particular treatment groups was relatively large (especially in RR group) therefore a bigger sample size might be needed to detect differences between groups.

Table 6 Differences in the meat quality parameters recorded after slaughter for entire males (C), rubber ring castration (RR), and immunocastration (Vac 3-6) treatment. Data are medians with Q1 and Q3.

Meat Quality Parameters	C[1]	RR[1]	Vac3[1]	Vac4[1]	Vac5[1]	Vac6[1]	P-Value[2]
Shear force	4.8(4.4-6.0)	4.8(4.1-6.5)	5.0(4.3-6.0)	4.6(4.3-6.3)	4.9(4.1-5.9)	4.9(4.2-5.4)	**NS**
Ultimate pH	5.5(5.5-5.6)	5.5(5.5-5.6)	5.6(5.5-5.6)	5.5(5.5-5.6)	5.5(5.5-5.6)	5.5(5.5-5.6)	**NS**
Temperature	3.0(2.6-3.9)	3.5(2.6-5.6)	4.3(2.6-6.3)	3.6(2.7-3.9)	3.2(2.3-4.7)	4.0(3.5-5.2)	**NS**
Colour L	41.5(40.1-43.3)	41.2(40.9-42.0)	41.5(39.8-43.2)	41.6(40.3-42.3)	41.4(40.1-42.1)	41.3(40.4-41.7)	**NS**
Colour a	23.2(22.8-24.0)	24.0(23.2-24.1)	23.6(23.2-24.3)	23.3(22.8-24.1)	24.0(23.8-24.4)	23.5(23.2-24.2)	**NS**
Colour b	7.8(7.2-7.9)	7.7(7.4-8.0)	7.8(7.4-8.1)	7.6(7.2-8.3)	7.8(7.6-8.1)	8.0(7.1-8.3)	**NS**
Cooked weight	254.4(225.1-307.1)	233.6(214.1-273.6)	253.7(230.3-281.5)	260.1(237.3-285.3)	257.4(234.2-276.2)	266.1(226.4-300.0)	**NS**
Pre-freezing weight	377.4(311.9-405.1)	314.6(282.8-373.2)	346.7(303.3-367.4)	356.8(332.2-382.7)	369.2(315.2-285.2)	363.9(312.3-389.5)	**NS**

Post-freezing weight	380.4(314.3-407.6)	315.9(284.6-376.7)	348.1(305.3-371.1)	357.9(335.5-384.6)	365.2(315.7-378.8)	366.6(313.6-390.8)	**NS**
Cooking loss in %	28.3(25.8-28.7)	27.5(24.6-28.6)	26.9(24.2-28.9)	28.9(24.3-30.1)	26.3(25.7-28.3)	26.9(24.0-30.4)	**NS**

[1]Median values (Q1-Q3). [2](amended p-value due to Bonferroni correction P<0.008), NS not significant. Different letters indicate significant differences between treatments.

6.5.4 Expression of Aggressive behaviours

Overall there were no significant differences between treatments in the expression of aggressive behaviours with the exception of returned aggression (see table 7, figure 10 and 11 below). RR lambs were observed to react with greater aggression when attacked by other lambs during the test compared to other treatment groups. This result was expressed in the increased frequency of 'butting back' behaviour when analysed on its own (see figure 10). There was also a tendency for the C lambs to express higher frequency of maintenance behaviours than Vac 5 group.

Table 7 Differences in the frequency of aggressive behaviours expressed by entire males (C), rubber ring castration (RR), and immunocastration (Vac 3-6) treatment, recorded for 30 minutes at ~24 weeks of age during feed competition test. Data are medians with Q1 and Q3.

Aggressive Behaviours	C[1]	RR[1]	Vac3[1]	Vac4[1]	Vac5[1]	Vac6[1]	P-Value[2]
Maintenance Behaviours	33.5(21.5-48.5)a	23.0(19.5-27.5)a	24.5(19.0-28.0)a	23.5(15.5-29.5)a	17.0(12.0-23.0)b	23.0(17.5-31.0)a	P=0.04
Performed Aggression	6.0(4.5-9.5)	11.5(9.0-16.5)	8.5(5.0-20.5)	7.5(5.0-11.5)	10.0(6.5-13.0)	9.5(7.5-14.0)	*NS*
Returned Aggression	0.0(0.0-0.0)a	1.5(0.5-3.5)b	1.0(0.0-1.0)a	0.0(0.0-1.0)a	0.0(0.0-1.0)a	0.0(0.0-0.0)a	**P=0.006**
Total Aggression	7.0(5.5-10.0)	15.0(10.5-21.0)	11.5(6.0-24.5)	9.0(5.5-13.0)	11.5(7.0-18.0)	9.5(8.0-14.5)	*NS*
Sexual Behaviours	0.0(0.0-1.5)	0.0(0.0-0.5)	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)	*NS*
Avoidance/ Escape Behaviours	6.0(4.0-7.0)	5.5(2.5-9.5)	6.0(2.5-8.5)	2.5(1.5-6.0)	6.0(2.5-7.0)	3.0(2.0-4.0)	*NS*

[1]Median frequency (Q1-Q3). [2](amended p-value due to Bonferroni correction P<0.008), NS not significant. Different letters indicate significant differences between treatments.

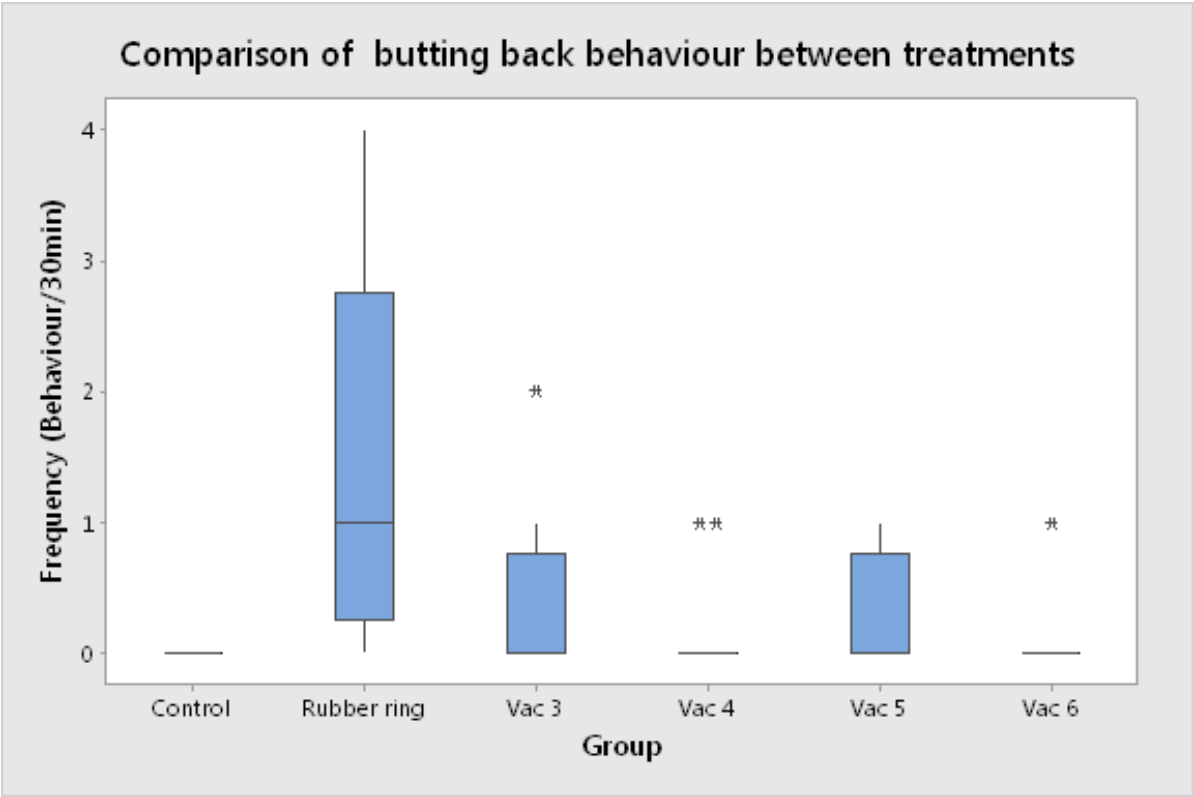


Figure 10 Box plot of butting back behaviour measured for control (C), rubber ring castration (RR) and immunocastration treatment (Vac 3-6). Parameter was recorded during 30 min feed competition test. Data are medians with Q1 and Q3. Whiskers represent upper and lower extreme. *outlier/single data point.

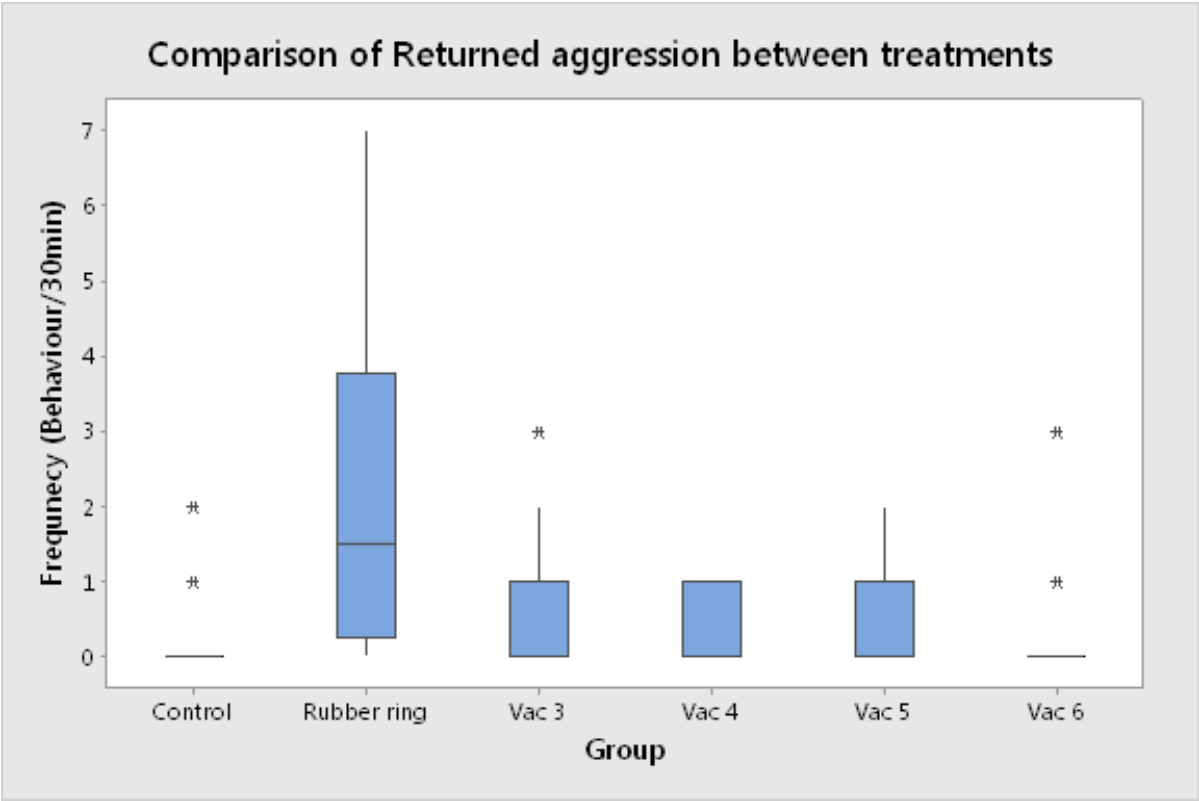


Figure 11 Box plot of returned aggression measured for control (C), rubber ring castration (RR) and immunocastration treatment (Vac 3-6). Parameter was recorded during 30 min feed competition test. Data are medians with Q1 and Q3. Whiskers represent upper and lower extreme. *outlier/single data point.

6.5.5 Assessment of carcass quality parameters in live rams by use of CT scanning procedure

The preliminary study on the use of CT scanning as a means to predict carcass quality in live animals showed that RR lambs achieved a better killing out % (total tissue weight divided by live weight) than all other treatments (see table 8). This may have been related to the lower weight of RR rams at the time of assessment. However, it has to be noted that rams were kept for too long due to experimental protocol therefore weights of other treatments exceeded desired in UK slaughter weight of 40 kg which have led to a poorer Killing Out Percentage (KO%).

Results of this study show that CT scanning may be useful technique in prediction of slaughter qualities in live animals. Because this pilot study was done in the first year of the thesis, the results could not be directly compared to the final carcase grades carried out in the third year of the thesis as they were derived from different set of lambs.

Table 8 Differences in the predicted carcase quality parameters estimated for control (C), immunocastration (Vac 2), rubber ring (RR) and short scrotum treatment (SSC) at the age of ~ 24 weeks in live animals. Data are Medians (Q1-Q3) or Mean values (with standard error) for live weight parameter.

Posture	C[1,*]	Vac 2[1,*]	RR[1,*]	SSC[1,*]	P-Value[2]
Killing Out %	4.5(4.4-4.6)a	4.5(4.5-4.6)a	4.7(4.5-4.8)b	4.5(4.4-4.5)a	**P= 0.01**
Predicted carcass fat	4.0(3.0-5.0)	3.6(2.9-4.0)	4.1(2.9-4.6)	3.7(3.3-41)	**NS**
Predicted carcass muscle	13.1(12.9-14.3)	13.4(12.5-14.6)	13.2(11.7-13,3)	12.6(11.8-13.7)	**NS**
Predicted carcass bone	4.4(4.0-4.5)	4.0(3.8-4.5)	3.9(3.8-4.1)	4.1(3.0-4.2)	**NS**
Total tissue weight	21.6(19.9-23.3)	21.2(19.6-24.1)	21.3(18.6-22.1)	20.5(19.4-22.1)	**NS**
Live Weight*	47.3(2.40)	47.0(1.66)	43.7(1.43)	46.4(2.11)	**NS**

[1]Median (Q1-Q3) or * Mean values (with standard error) for live weight. [2](amended p-value due to Bonferroni correction P<0.01), NS not significant. Different letters indicate significant differences between treatments .

6.6 Discussion

Analyses have revealed that there were no negative effects of immunization on growth rates, carcass and meat quality characteristics. Moreover, increased aggressive reaction of RR lambs in comparison to the C and Vac 3-6 group during the feed competition test have also shown no negative impacts of immunization on the behaviour of entire or vaccinated lambs in comparison to the rubber ring castration method. The results reported here are consistent with previous studies investigating similar products in cattle and pigs where no negative effects on the expression of aggressive behaviours, growth rate, and carcass and meat quality have been reported (Adams et al., 1996; Amatayakul-Chantler et al., 2012; Amatayakul-Chantler et al., 2013; Fabrega et al., 2010).

The results of the study reported here showed no negative impact of immunization on the growth of lambs. Growth rate during the first and third year of the study did not significantly differ. On the second year of the study, differences between treatments in growth were noted from approximately 16 weeks of age until the end point of the study (28 weeks of age). This difference was mainly caused by the F group which had a reduced weight gain in comparison to C and Vac 2 groups. This result is consistent with earlier findings describing a slower growth rate in female lambs in comparison to male lambs (Sanudo et al., 1998). C rams also had a greater weight gain compared to RR and LA group when lambs were 20, 28 and 16, 20, 28 weeks old respectively which may be related to the lower testosterone concentration in physically castrated groups in comparison to entire males. Entire males have been found before to have increased growth in comparison to steers (Huxsoll et al., 1998) and better feed conversations (Bonneau, 1998) in cattle. Similarly, in earlier studies of cattle investigating the effect of immunocastration on Bos Taurus bulls found no effect on performance (Adams and Adams, 1992; Huxoll et al., 1998) and reduced growth rate when compared to entire bulls (Adams et al., 1993; Cook et al., 2000).

Following slaughter measurements of carcass conformation, fatness and weight were taken as well as pH and temperature 45 min after kill. Analysis showed no significant

differences between treatment groups in any of the measured parameters. However, there was a tendency for the RR rams to have a lower finishing weight in comparison to C, Vac5 and Vac 6 rams. This outcome is consistent with the previous findings showing better feed conversion and average growth rate in entire males (Amatayakul-Chantler et al., 2012; Bonneau, 1998). It is possible that delayed vaccination allowed Vac 5 and Vac 6 rams to grow at a rate similar to C rams and perhaps a larger sample size would allow the detection of significant differences between treatments.

All treatments have shown very good conformation and fatness classes. Therefore all groups could have been allocated to 'suitable' from the retailer's point of view classes of carcass conformation and fatness (Supermarket Requirements in UK are as follows: 16 – 21.5 Kg for the carcass weight, U & R class for the conformation and 2/3L for fatness, UK Butcher requirements are: 20 – 24 Kg of carcass weight, U & R for conformation and 3L/3H for fatness class (data taken from QMS- Quality Meat Scotland website) even though the live weight at the time of slaughter exceeded expected finish weights due to the experimental protocol. On the other hand, the farmer's perspective e.g. in terms of the value of carcasses had to be taken into account and the proportion of carcasses that were penalised vs those that received premiums was calculated across all treatment groups. This was very important because it would be the farmers who would have to pay for the use of the immunization method. Thus evaluation of potential benefits of immunization was a vital part of this study. Results have shown that overall there were no differences between treatments in the calculated across treatments payment penalties vs payment premiums. However, there was a tendency for RR rams to have greater variability between particular individuals. Although results did not significantly differ between treatments, RR lambs seemed to have greater % of lambs with penalty payments and a smaller % of lambs with premium payments in comparison to other treatments. The total premium payment which could have been achieved after slaughter of RR lambs in this study accordingly to the EUROP scale was also lower in comparison with other groups.

Carcass conformation in the EU grading system is a predictor of sellable meat yield. It represents market requirements for specific carcass characteristics and allows the quantification of these characteristics in order to evaluate the financial value of the carcass. According to this classification and the estimated premium prices for sheep in the classification grid (table 2) the majority of lambs in this study fitted within the class of good economical return. There were also no significant differences between treatments in meat quality measures although Vac 4 rams tended to show a quicker drop in the average pH parameter in comparison to C, RR and Vac 5 treatments. This result may be beneficial in the process of meat maturing. It has been shown that increased ultimate pH affected meat tenderness and as a result the meat was more tender (Purchas, 1990). In the study presented here all of the treatments were observed to have median shear force of < 5.0 or equal 5.0 kgF indicating very tender meat. This result may be a consequence of the low ultimate pH level recorded for each treatment and the young age of animals. It was described by Devine et al. (1992) that young animals (7 months of age) and animals with low ultimate pH tended to be tender. There is no information in the literature on the effects of immunization in older rams. It is perhaps due to the fact that lambs, unlike cattle, are slaughtered earlier in their life therefore direct comparison of the meat quality, especially toughness of muscles in young and older animals, are not possible. However, it has been shown previously in cattle that with increasing age some of the muscles may lose their toughness (Shorthose & Harris, 1990) therefore the meat quality of older animals may be lower which may result in decreased loin tenderness causing a significant impact on consumers' perception of meat quality.

There was no negative impact of immunization on the expression of aggressive behaviour. Only RR lambs were observed to express increased frequency of aggression when attacked by other lambs during the feed competition test. It is also possible that RR lambs as smaller and presumably subordinate flock members will fight more frequently for resources compared to other lambs. Therefore, when attacked, pushed or displaced from the feeder they might have had expressed an exaggerated reaction which may have been manifested in a higher frequency of returned aggression ('butting back'

behaviour) similarly to the greater frequency of returned aggression which was observed in this study. In earlier studies dominant individuals have been found to have greater body weight (Ungerfeld and González-Pensado, 2008b). Aggressive encounters and fights may be a manifestation of a dominance hierarchy establishment which should secure access to desired assets, for example, females (Zuk, 1991; Iwasa and Pomiankowki, 1994; Jacobs, 1996) or feed. Ruiz-de-la-Torre and Manteca (2010) suggested that the effect of circulating testosterone on aggressive behaviour depends on the context and presence of testosterone which may increase the tendency for establishment of hierarchy in the group rather than expression of aggressiveness. C rams in this study may already have had a very well established hierarchy at the time of the test. Therefore, they did not have to fight for resources at the same rate as lambs in the RR group. On the other hand, the studies described before by Fabrega et al. (2010) investigating the impact of an anti-GnRF vaccine, Improvac, on pigs' behaviour found increased frequencies of aggression at the feeder in entire males in comparison to castrated males, immunized males and females. However, this was only seen just before slaughter. There were no differences between treatments in expression of aggressive behaviour until 25 weeks of age.

This technique may be more expensive than the use of rubber rings. However, it was believed to be potentially more economical as animals may have a better feed conversion and growth rate. In the light of increased interest of public opinion in the reduction of pain during husbandry procedures, such as castration, and significantly increased demand for leaner meat it can be argued that people may be willing to pay more to achieve the desired product. There are reports that consumers are willing to pay more for more tender meat (Boleman et al., 1997; Huffman et al., 1996), which may indicate that farmers will be willing to use a more expensive method as it will allow achieving the desired outcome and perhaps add to the farm economy.

Evaluation of the CT scanning technique as a method for carcass qualities estimation in live animals has shown promising outcomes. The method was able to distinguish difference between treatments in killing out percentage (KO %) between treatments. CT

can be used to predict the growth rates, the total muscle, bone and fat yield or estimate the value of the animals before slaughter. It also allows the farmers to spot outstanding rams in the flock which could benefit the owner in the breed improvement. CT is widely used for: estimation of weight for fat, muscle and bone in the carcass; estimation of % of fat, muscle and bone in the carcass; estimation of Killing Out Percentage (KO% - total tissue weight / live weight); prediction of ratio of muscle to bone and muscle to fat in the carcass; distribution of muscle in the carcass. It is possible that RR rams may have grown slower due to castration therefore at the time of assessment their live weight was lower and killing out % better although lack of a significant effect on live weight makes this rather a difficult argument.

6.7 Conclusion

Due to the increased interest of public opinion in the reduction of pain and distress in castrated males, scientist have been faced with a very challenging task, to find a method of castration which would be less painful but would have a beneficial impact on management and meat quality at the same time. The new method should also potentially mitigate other factors related to castration, for example, poorer growth rate. The objective of this study was to determine the effects of the castration technique including immunization with an anti-GnRF vaccine on the expression of aggressive behaviours, growth rate, carcass and meat quality characteristics (meat tenderness, ultimate pH and colour) in mule x terminal sire (Texel or Suffolk) lambs. The use of a CT scanning technique to predict carcass and meat characteristics was also assessed. Furthermore, evaluation of the impact of the castration treatment on the premium payments vs premium penalties accordingly to the EUROP carcass grading system was carried out to estimate economical cost/benefits. Investigation of the efficiency of the CT scanning technique as a method for evaluation of carcass qualities revealed that this method was able to detect significant differences between treatments in Killing out Percentage (KO%). The CT scanning method used in this study proved to be a good technique for carcass evaluation in live animals. Results of this study revealed that the treatment had no impact on the expression of aggressive behaviours, growth rate, carcass and meat

characteristics. There was a tendency for RR rams to grow slower and have smaller live weight and carcass weight. Analysed data have shown that in this study immunization did not improve meat quality as well. The estimated value of carcasses carried out across the treatments revealed that RR lambs were observed to have a greater proportion of carcasses that were penalised vs those that received premiums. The total premium payment which could have been achieved after slaughter of RR lambs was also lower than other groups. Although this finding did not significantly differ between treatments and according to this classification and the estimated premium prices for sheep in the classification grid, the majority of lambs in this study fitted within the class of good economic return. It is possible that a greater sample size would allow for more accurate investigation therefore further studies on a larger scale would be recommended. There were also no significant differences between treatments in meat quality measures although Vac 4 rams tended to show a quicker drop in the average pH parameter in comparison to C, RR and Vac 5 treatments. On the basis of the evaluated data immunization was found to be a neutral method having no negative and no positive effect on the growth rate, carcass quality and conformation. The desired outcome of the immunization group showing better results in growth, conformation and loin parameters than RR treatment was not observed. Immunization at weaning did not significantly affect the measured parameters as well. Thus it is possible that weaning had minimal or no effect on the effectiveness of the vaccination. RR rams were found to express significantly higher frequencies of returned aggression during the feed competition test than all other groups. On the basis of the evaluation of potential benefits of immunization on the carcass characteristics, meat quality, growth, expression of aggressive behaviours, estimated economic return and the absence of negative effects of immunization it was concluded that it is a good alternative for traditional castration techniques.

Chapter 7 General discussion, implications for the future and conclusions

7.1 Introduction

The overall objective of this study was to determine efficacy of new anti-GnRF vaccine, developed by Zoetis, as a more welfare friendly method of castration for ram lambs. The impact of the vaccine on the various aspects of lambs' behavioural responses, physiology and productivity was investigated. The work was divided into four main areas: i) to assess whether there is a negative impact of immunization on behavioural expression and emotionality of lambs in comparison to other castration techniques (i.e. development and severity of expressed pain related behaviours (Chapter 3), ewe-lamb bonding, anxiety, aggression, blood cortisol concentration pre and post fearful experience (Chapter 5)); ii) to understand how vaccination against GnRF may affect physiological processes of the rams and to evaluate efficacy of the vaccine in achieving sterility (i.e. plasma testosterone concentration levels, scrotal circumference, testes consistency, histology of the testes, expression of courtship and sexual behaviours; Chapter 4); iii) to assess the effects of immunization on lambs' productivity (growth rate, carcass conformation and meat quality; Chapter 6); iv) to evaluate the most efficient vaccination regime which would best fit on farm husbandry procedures and prolong effective immunity in immunized lambs (combining data from Chapters 4-6).

Castration of male animals is used globally to achieve ease of management (reduction or elimination of unwanted behaviours for example inbreeding, indiscriminate breeding, and aggression) and improvement of productivity (enhanced carcass conformation and meat quality). In the UK male rams are in the majority castrated with the use of rubber rings, (DEFRA Farm Survey, 2005) which is quick and efficient, however causes the most severe distress and pain responses in comparison to other commonly used methods (i.e. short scrotum castration, Burdizzo castration, see chapter 1 for more details). The broader aim of this study was to provide practical advice on the most efficient, useful and economic castration technique which could be applied in current UK and global sheep production systems, minimising animal distress during and after the procedure as well as enhancing productivity. Moreover, results of the studies presented here provide an opportunity for the farmers to use knowledge of animal behaviour to improve well-

being of their own flocks. For example, behaviours presented in Chapter 3 and measurement methods of pain related behaviours, expression of aggression, sexual and courtship behaviours.

In addition to the main objective of this study, application of Qualitative Behavioural Assessment (QBA) as an alternative assessment technique to quantitative (i.e. ethogram-based) pain recognition and evaluation methods was investigated. The purpose of this chapter is to provide an overview of the results from the studies reported in particular sections of this thesis and discuss how they relate to the scientific literature. Furthermore, this chapter will highlight the areas which require further research and provide key recommendations and implications for the future.

7.2 Summary of the results

7.2.1 Impact of different castration techniques on the expression of pain related behaviours, restlessness, lesion formation and time of healing

The first scientific question that was investigated regarded the issue of pain and distress that follows traditional physical castration methods: rubber ring, short scrotum or rubber ring combined with the Burdizzo, (which has been previously reported: Molony et al., 1993; Kent et al., 1995; Lester et al., 1996; Molony & Kent, 1997; Thornton & Waterman-Pearson, 1999; Kent et al., 2000; Thornton & Waterman-Pearson, 2002; Molony et al., 2002; Kent et al., 2004; Molony et al., 2011) compared to immunisation. The intention was to investigate whether alternative immunization methods would result in a reduction of distress and pain related behaviours in rams or even be a pain free technique. Behavioural as well as physical (i.e. time of lesion formation, time to heal the lesion etc.) indicators were used during the assessment of different techniques (for details see chapter 3). Table 1 below represents a summary of the results of the methods' evaluation (the overall evaluation of the results presented here is based on the results shown in chapters 3-6 of the thesis. The table is not a representation of the actual presented earlier P-values of particular measurements. This table is an overall evaluation of specific castration treatment effective in achieving a reduction in fertility).

Table 1 Overall evaluation of the treatment effect on particular measurements recorded during the study period

Measured Parameter	**Overall effect of treatment on expression of pain related behaviours, lesion formation, lesion severity, time to heal and testes shedding**									
	C	**COM**	**RR**	**SSC**	**Vac 1**	**Vac 2**	**Vac3**	**Vac 4**	**Vac 5**	**Vac 6**
Frequency of pain related behaviours	**0**	**NSD**	**↑↑↑**	**↑↑↑**	**NSD**	**NI**	**NI**	**NI**	**NI**	**NI**
Frequency of restlessness	**0**	**NSD**	**↑↑↑**	**↑↑**	**↑**	**NI**	**NI**	**NI**	**NI**	**NI**
Lesion formation	**0**	**↑**	**↑**	**↑**	**NSD**	**NSD**	**NSD**	**NSD**	**NSD**	**NSD**
Presence of a lesion	**0**	**↑**	**↑**	**↑**	**NSD**	**NSD**	**NSD**	**NSD**	**NSD**	**NSD**
Time of lesion formation (in weeks)	**0**	**0**	**1**	**1**	**NSD**	**NSD**	**NSD**	**NSD**	**NSD**	**NSD**
Time to heal lesion completely (in weeks)	**0**	**6**	**>8**	**5**	**NSD**	**NSD**	**NSD**	**NSD**	**NSD**	**NSD**
Lesion severity (7weeks after procedure)	**0**	**NP**	**↑↑↑**	**NP**	**NSD**	**NSD**	**NSD**	**NSD**	**NSD**	**NSD**
Lesion size (7 weeks after procedure)	**0**	**NP**	**↑↑↑**	**NP**	**NSD**	**NSD**	**NSD**	**NSD**	**NSD**	**NSD**

Time to shed testicles/scrotum sac* (in days)	**0**	**↑↑**	**↑↑↑**	**↑***	**N/A**	**N/A**	**N/A**	**N/A**	**N/A**	**N/A**
Overall effect of treatment on the levels of circulation testosterone, scrotal measures, histology of the testes and immunity period										
	C	**COM**	**RR**	**SSC**	**Vac 1**	**Vac 2**	**Vac3**	**Vac 4**	**Vac 5**	**Vac 6**
Reduction in testosterone concentration	**0**	**N/A**	**N/A**	**↓↓**	**↓**	**↓↓↓**	**↓↓**	**↓↓↓**	**↓↓**	**↓↓↓**
Reduction of scrotal circumference	**0**	**N/A**	**N/A**	**NI**	**↓**	**↓↓↓**	**↓↓↓**	**↓↓↓**	**↓↓**	**↓↓↓**
Reduction of testes consistency	**0**	**N/A**	**N/A**	**NI**	**↓↓**	**↓↓↓**	**↓↓↓**	**↓↓↓**	**↓↓**	**↓↓↓**
Testicular measures post slaughter	**0**	**N/A**	**N/A**	**↓↓**	**↓/NP**	**↓**	**NI**	**NI**	**NI**	**NI**
Tubules count (per slide)	**0**	**N/A**	**N/A**	**↑↑**	**↑↑**	**↑↑↑**	**NI**	**NI**	**NI**	**NI**
Tubules size (per slide)	**0**	**N/A**	**N/A**	**↓**	**↓↓**	**↓↓↓**	**NI**	**NI**	**NI**	**NI**
Period of immunity	**N/A**	**N/A**	**N/A**	**NI**	**~12**	**~20**	**~16**	**~16**	**~16**	**~16**

(in weeks)										
Overall effect of treatment on expression of sexual/courtship behaviours										
	C	**COM**	**RR**	**SSC**	**Vac 1**	**Vac 2**	**Vac3**	**Vac 4**	**Vac 5**	**Vac 6**
Flehmen	**0**	**NI**	**↓↓↓**	**↓↓**	**↓↓**	**↓↓↓**	**↓↓**	**↓↓↓**	**↓↓**	**↓↓↓**
Nudge	**0**	**NI**	**↓↓↓**	**↓↓**	**↓↓**	**↓↓↓**	**↓↓**	**↓↓↓**	**↓↓**	**↓↓**
Low stretch	**0**	**NI**	**↓↓↓**	**↓↓**	**↓↓**	**↓↓↓**	**↓↓**	**↓↓↓**	**↓↓**	**↓↓↓**
Mount attempts	**0**	**NI**	**↓↓↓**	**↓↓**	**↓↓**	**↓↓↓**	**↓↓**	**↓↓↓**	**↓↓**	**↓↓**
Sniff/nose	**0**	**NI**	**↓↓↓**	**↓↓**	**NSD**	**↓↓↓**	**↓↓↓**	**↓↓↓**	**↓↓↓**	**↓↓↓**
Mounting Movements	**0**	**NI**	**↓↓↓**	**NSD**	**NSD**	**↓↓↓**	**NSD**	**NSD**	**NSD**	**NSD**
Overall effect of treatment on expression of escape attempts, vocalizations, aggression										
Escape attempts	**0**	**NI**	**↑↑**	**↑↑**	**↑**	**↑↑**	**↑**	**↑↑**	**↑↑↑**	**↑↑↑**
Vocalizations	**0**	**NI**	**↑↑↑**	**↑↑**	**↑↑**	**↑↑↑**	**↑↑↑**	**↑↑↑**	**↑↑↑**	**↑↑↑**
Received aggression	**0**	**NI**	**↑↑↑**	**↑**	**↑↑**	**↑↑↑**	**↑↑↑**	**↑↑**	**↑↑↑**	**↑↑↑**
Overall effect of treatment on ewe's perception of rams										
	C	**COM**	**RR**	**SSC**	**Vac 1**	**Vac 2**	**Vac3**	**Vac 4**	**Vac 5**	**Vac 6**
Head turning	**0**	**NI**	**↓↓↓**	**↓↓**	**↓↓**	**↓↓↓**	**NI**	**NI**	**NI**	**NI**
Aggression	**0**	**NI**	**↑↑↑**	**↑↑**	**↑↑**	**↑↑**	**NI**	**NI**	**NI**	**NI**
Avoiding	**0**	**NI**	**↓↓↓**	**↓↓**	**↓↓**	**NP**	**NI**	**NI**	**NI**	**NI**
Circulating	**0**	**NI**	**↓↓↓**	**↓↓**	**↓↓**	**NP**	**NI**	**NI**	**NI**	**NI**

Crouch	**0**	**NI**	**↓↓↓**	**↓↓↓**	**↓↓**	**↓↓**	**NI**	**NI**	**NI**	**NI**
Follow male	**0**	**NI**	**↑↑↑**	**↑↑**	**↑↑**	**↑↑**	**NI**	**NI**	**NI**	**NI**
Moving away	**0**	**NI**	**↓↓↓**	**↓↓↓**	**↓↓**	**↓↓↓**	**NI**	**NI**	**NI**	**NI**
Received aggression	**0**	**NI**	**↓↓↓**	**↓↓↓**	**↓↓**	**↓↓↓**	**NI**	**NI**	**NI**	**NI**
Standing firm	**0**	**NI**	**↓↓↓**	**↓↓**	**↓↓**	**↓↓↓**	**NI**	**NI**	**NI**	**NI**
Wagging tail	**0**	**NI**	**↓↓↓**	**↓↓**	**↓↓**	**↓↓↓**	**NI**	**NI**	**NI**	**NI**

Legend:

- NI – not investigated.
- N/A – not applicable.
- NP – not present.
- NSD – no significant difference in relation to reference "0" level.
- 0 – reference level of particular measurement (all other measurements presented in the table were always related to basic reference level), entire males (C), were the reference level to which other treatments were compared.
- The arrows represent increase ↑, ↑↑, ↑↑↑ or decrease ↓, ↓↓, ↓↓↓ of recorded frequencies of behaviours or other measures i.e. testosterone concentration, tubules counts and size etc. The number of presented arrows indicates the level of increase or decrease in relation to basic control level of recorded measurement.

The RR method was found to be the most painful castration technique investigated in this study followed by SSC and COM treatments. Moreover, this study investigated, for the first time, the impact of immunization on the lambs' emotionality and the expression of pain related behaviours. There is no or very little information in the literature regarding this issue. Immunization did not significantly affect lamb behaviour. There were no significant differences between immunized rams and entire males with the exception of restlessness behaviour. In contrast, when physical castration techniques were compared to the entire male group a significant increase in the frequency of performed pain related behaviours and restlessness was seen. The RR group expressed the highest median frequency followed by SSC treatment. This agrees with previously reported findings of Molony et al. (2002, 2011) and Kent et al. (2000) describing the impact of different castration techniques on male livestock wellbeing. Further analysis of the effect of the castration technique on the chronic pain indicators (times to form a lesion, size of the lesion, lesion severity, time to heal the lesion) have shown that immunization was the least painful method of fertility control. There was no negative effect of immunization on the vaccination site. In this study administration of the vaccine did not cause formation of any kind of lesions. Similar outcomes have been reported by Amatayakul-Chantler et al. (2013) who described no systemic or localized adverse effect of anti-GnRF vaccine Bopriva after product administration in cattle. Ghoneim et al. (2012) also noted no site reaction to immunization in camels. In contrast the RR, SSC and COM castration techniques have been shown to cause chronic pain due to the presence of lesions which vary in size and severity. In particular RR treatment caused prolonged chronic pain inducing larger, more severe and longer lasting lesions than other evaluated physical castration methods, which is consistent with the reports of Thornton & Waterman-Pearson (1999) and Kent et al. (2000).

Evaluation of the study results has also revealed that COM treatment was particularly difficult to assess. On the one hand, the technique caused immediate formation of the

lesion lasting up to 6 weeks which may be a proxy for chronic pain (Kent et al., 2000). On the other hand, the behavioural data did not show significant differences in the median frequencies of pain related behaviours and restlessness between the C and COM group. It is possible that this was caused by long periods of immobility which may have been a form of pain expression. Moreover, it can be argued that similar results in behavioural and physiological data seen in RR and SSC group are related to the fact that the procedure of castration in these two methods is almost the same. Arterial blood supply and venous drainage is stopped by rubber ring application (Kent et al., 2004) which leads to ischaemic pain and the necrosis of the tissues. Although with lower tissue damage in the case of the SSC treatment (the rubber ring is applied on the scrotum sac only and the testicles are pushed back into the abdominal cavity), and therefore this technique was somewhat less painful than the RR method. The procedure of the COM castration technique, however, is very different in comparison to the RR and SSC techniques as it involves crushing of spermatic cords and perhaps a different mechanism of pain sensation. Therefore, it was difficult to establish if this method is in fact less painful or the pain and distress is manifested in a different way. Therefore, an additional assessment technique, QBA, has been applied in this study. The intention was to investigate if QBA would be able to allow for a more accurate comparison and better understanding of how a specific castration treatment may affect rams' emotionality. QBA was applied for the first time to evaluate levels of distress in castrated animals. Video data of castrated lambs were assessed by blinded observers using a Free Choice Profiling method (the details of the QBA technique principles are described in chapter 3).

Analysis of the QBA results showed that the observers were able to detect behavioural differences between treatments and allocate lambs to appropriate groups associated with mild or severe pain. Although direct comparison with the previous findings reported in the literature cannot be made, as QBA was used for the first time in evaluation of pain and distress in castrated lambs, observers using this technique have been found in the past to reach a high level of agreement in their assessment of sheep behavioural

expression (Pythian et al., 2013) which was also found in the present study. The QBA technique, as with quantitative assessment methods, also defined the RR group as most painful and the SSC as the second most painful castration method. It also clearly identified the difference between lambs that were handled only and rams experiencing pain after castration. With regard to the COM treatment it was clearly associated by observers with calm/comfortable, lethargic/tired, low arousal and negative welfare behavioural scores. This means that the observers identified that lambs from this treatment did not show restlessness but they have not associated this behaviour with positive welfare in contrast to the C group lambs. The combination of quantitative and qualitative techniques gave a better understanding of the impact of the COM castration method on lamb behavioural expression. There was also a very strong correlation between qualitative and quantitative assessments, which may act as a validation for the use of QBA in pain assessment. This is consistent with earlier findings. Use of QBA has been supported by other studies of behavioural expression of animals showing good correlation of QBA with traditional ethogram based behavioural assessment (Napolitano et al., 2008; Minero et al., 2009) or physiological indicators (Stockman et al., 2011). Rutherford et al. (2012) presented high sensitivity of QBA to behavioural expression of pigs in an open field test carried out under the influence of the anti-anxiety drug azaperone or neutral saline solution. Napolitano et al. (2012) have reported a good correlation between QBA and frequencies of flight attempts and vocalizations in buffaloes. Stockman et al. (2012) have shown good correlation of QBA and plasma lactate concentration in dairy cattle before slaughter. Moreover many studies have shown good intra- and inter-observer reliability (Wemelsfelder et al., 2001, 2009 a, b; Rousing and Wemelsfelder, 2006; Walker et al., 2010) adding to the value of QBA, and further supporting the use of this method as a reliable welfare/distress assessment technique which could be applied in the field or experimental setting.

7.2.1.1 Summary of the key findings

1. The anti-GnRH vaccine tested in this trial may have caused less pain than any of the physical castration methods.

2. The vaccine was safe to use in animals as young as 6 weeks of age as there was no site reaction to the active agent of the vaccine throughout the study period.
3. The rubber ring castration was found to be the most severe castration method causing highest levels of acute as well as chronic pain.
4. Evaluation of quantitative and qualitative methods of pain recognition and assessment has shown that both methods were successful in recognizing lambs' suffering, pain and distress.
5. Qualitative Behavioural Assessment (QBA) allowed for a more robust assessment of the combined method of castration by evaluation of subtle responses. Combining both types of assessment would be recommended as it provided stronger analysis of emotional state of lambs in this study allowing for more accurate conclusions in relation to inflicted levels of pain by particular castration treatment.

7.2.2 Impact of different castration techniques or handling and gender on behavioural expression of rams related to ewe-lamb bonding, anxiety and aggressive behaviours

Overall the results have shown that castration method, handling procedure or gender had no effect on the development of an ewe-lamb bond. There was however a tendency for the C group to have a greater spatial relationship with the ewes during the first period of observations (first 10 days following the birth) in comparison to F and Vac 2 lambs. This result was unexpected as it was hypothesized that the Vac 2 group will resemble behaviour of C lambs due to fact that at the time of observations this group of lambs have not yet been immunized. There was a dramatic change in the weather conditions on the second day of the observations when lambs were placed on the grazing paddock. The snow fall and drop in the ambient temperature caused hypothermia in some of the lambs and it was necessary to bring them back into the shed for one day as there was a concern regarding their health. It is possible that the mothers of lambs that were affected by the changing weather conditions altered their behavioural pattern and were recorded to stay at closer proximity to their offspring than other ewes of less or not affected at all lambs. However, all lambs were balanced across the treatments over the lambing window,

therefore why the Vac2 group might have been disproportionately affected by the weather cannot be explained. It is possible that this was a random effect and with a small sample size it had influenced the results in an unexpected way. It is also possible that ewes are able to distinguish between different levels of pain or distress experienced by their lambs and direct more maternal attention toward those with the most severe reactions (Futro et al., 2015; Hild et al., 2011). Furthermore, it has been found previously that maternal care is expressed during lactation by a close spatial distance of the ewe to its offspring (Pickup and Dwyer, 2011). Moreover, maintenance of ewe-lamb distance was also very closely associated with lamb survival (Dwyer and Lawrence, 2005). Therefore, it can be argued that the tendency for F and Vac 2 groups to have a closer spatial relationship with the ewes was caused by a change in maternal behaviour of the ewes after a distressful event. This is also further supported by the fact that there were no differences between treatments in the observed spatial relationship with the ewes during periods 2 and 3 of the study observations. Another plausible explanation might be that F lambs are different from males as ewes might treat female and male lambs differently.

Further analysis of the impact of early pain or testosterone exposure on the development and expression of social behaviours (aggression) and stress responses later on in life have shown some differences between treatments. C rams were consistently less fearful than F lambs in all fear eliciting conditions. However, there were no significant differences between treatments in other recorded anxiety measures. There was no difference between treatments in plasma cortisol concentrations just before and after the surprise test which was found to be in contrast with previous findings reported by Bouissou & Vandenheede (1996) where castrated males had higher cortisol concentrations than entire rams before the surprise test. However, technical problems with the plasma samples analysis, the very small sample size and also high individual variation are possibly the cause of the lack of significance. C rams were also shown to have significantly greater frequency of sexual behaviours than all other treatments during the feed competition test which may have been related to dominance hierarchy

behaviours. Ungerfeld and González-Pensado (2008b) have reported previously that dominant males may express sexual behaviour at earlier ages of life.

With regard to analysis of the expression of aggressive behaviours, no negative impact of immunization on the occurrence of such behaviours was recorded in this study. This is consistent with the findings of Fabrega et al. (2010) who also reported no adverse impact of immunization on the occurrence of aggressive encounters in pigs. Fabrega et al. (2010) have shown that only entire males were more aggressive at the feeder in contrast with castrated males, immunized males and females. In this study the RR treatment was observed to show significantly increased frequencies of returned aggression during the feed competition test in comparison to entire and immunized males. Manifestation of this behaviour was associated with a tendency of the RR group to have a lower weight. Greater body mass has been previously associated with dominance status (Ungerfeld and González-Pensado, 2008b) and a greater frequency of fighting could be a technique to achieve a desired resource, for example feed, by subordinate animals. It is possible that C rams in this study may already have had a very well established hierarchy at the time of the test. Therefore, they did not have to fight for resources at the same rate as lambs in the RR group. On the other hand, Ruiz-de-la-Torre and Manteca (2010) suggested that the effect of circulating testosterone on aggressive behaviour depends on the context and presence of testosterone which may increase the tendency for an early establishment of hierarchy in the group rather than expression of aggressiveness. Table 5 in Chapter 6 summarises the impact of different castration techniques on the expression of aggressive behaviours during the feed competition test.

7.2.2.1 Summary of the key findings

1. There was no long term impact of early pain exposure associated with the castration procedure on the lambs' behaviour. Treatment did not affect the behavioural patterns of lambs.

2. There was no effect of prior exposure of testosterone on the lambs' behavioural demeanour. Observed changes in behavioural patterns associated with fear were related to current circulating levels of testosterone rather than prior exposure.
3. Observed sexual behaviour during the feed competition test was associated with current testosterone levels rather than prior exposure to testosterone.
4. Formation of ewe-lamb bond and spatial relationship seemed to be affected by sex accordingly to the type of social organization in sheep species.
5. Entire male lambs were consistently less fearful than female lambs during all tested fear eliciting situations. C lambs were also observed to express reduced fear reactions compared to all other groups during the unfamiliar human test.

7.2.3 Assessment of different vaccination regimes effectiveness in reduction of fertility parameters (testosterone concentration, scrotal measures, testes consistency, testes histology and expression of sexual behaviour)

Overall results have shown that the vaccination with the product provided by Zoetis was effective in achieving sterility in rams. Throughout the whole study there was a significant reduction of plasma testosterone concentration, testicular development and occurrence of reproductive behaviours. Although there are differences between specific vaccination regimes and their effectiveness, all of the tested vaccination protocols were sufficient in suppression of the ram's reproductive functions (for the period of at least 12 weeks). This outcome agrees with the previously reported findings describing suppression of circulating testosterone concentrations to the levels of less than 5ng/ml after anti-GnRF vaccination (Amatayakul-Chantler et al., 2012; Amatayakul-Chantler et al., 2013; Janett et al., 2012), reduction of testicular size and consistency (Kiyma et al., 2000; Ülker, et al., 2009; Ülker et al., 2005) as well as decreased expression of courtship and sexual behaviours in the presence of oestrus females (Ghoneim et al., 2012; Kiyma et al., 2000; Parthasarathy et al., 2002; Turkstra et al., 2005; Janett et al., 2009) compared to entire males. Changes in testes histology, and reduced seminiferous tubules diameter have also been reported (Chapter 4) and is consistent with other studies

investigating the impact of immunization on histology of the testes in pigs (Einarsson et al., 2009; Fang et al., 2010), bucks (Ülker, et al., 2009) and rams (Kiyma et al., 2000; Ülker, Kanter, Gökdal, de Avila, & Reeves, 2001; Ülker et al., 2009; Ülker et al., 2005). Table 1 represents the degree of impact of particular types of vaccine administration on various reproductive parameters which were measured throughout the present study. Evaluation of the most effective vaccination was done at the end point of each study protocol. Ewes' perception of rams from different treatments during expression of sexual behaviours observations was also investigated.

Analysis of the results revealed differences in the immunity period between immunized groups. It has been shown that immunity will last longer if the vaccination is administered at the later stages of life and/or when the interval between primary and booster vaccination is longer. This technique of vaccine administration allowed for the use of only 2 doses of the vaccine to achieve the desired effect of prolonged immunity. This was also reported before by Amatayakul-Chantler et al. (2012) describing suppression of testosterone up to 15 weeks after administration of the vaccine in Bos Taurus bulls. Analysis of the study data has also shown that the rams have residual testosterone levels until approximately 3 – 4.5 months of age therefore vaccination at earlier stages of life is not needed. Primary vaccinations given at approximately the time of weaning was found to be the most effective and practical method of vaccine administration. This is very important from the practical and economical point of view because immunization could potentially fit into normal husbandry practices of lowland as well as hill flock systems, saving a lot of time and effort by being practical and economical at the same time. Farmers would not have to gather animals additionally for the purpose of the vaccination. Gatherings are time consuming and usually require more staff to be present which leads to further costs and add additional stress to the lambs. Therefore, the possibility of the vaccination being administered during normal husbandry practices could potentially lead to better acceptance of the product. This study has also taken the farmers' perspective (e.g. in terms of value of carcasses) into account. The proportion of carcasses that would be penalised vs those that would receive

premiums during the slaughter process at the current values for sheep was calculated across all treatment groups. This was very important because it would be the farmers who would have to pay for use of the immunization method to achieve societal benefits of improved welfare. Thus evaluation of potential benefits of immunization was a vital part of this study. Although results did not significantly differ between treatments, RR lambs tended to have a greater % of lambs with penalty payments and a smaller % of lambs with premium payments in comparison to other treatments. The total premium payment which could have been achieved after slaughter of RR lambs in this study accordingly to the EUROP scale was also lower in comparison with other groups.

Overall there was a significant reduction in expression of courtship and sexual behaviours in all immunized rams although there were specific differences recorded for each vaccination regime. This was related to the time of primary and booster vaccination administration which had an impact on the immunity period. The C group consistently had a greater frequency of sexual and courtship behaviours throughout the whole study and reduced frequencies of escape attempts, received aggression and vocalizations in comparison to RR and Vac 1-6 treatments (Table 1).

Analysis of the behavioural pattern of ewes during the expression of courtship and sexual behaviours test with males from different treatments has also shown significant differences (see Chapter 4 for more details). This study reported for the first time the impact of the immunization on the ewes' perception of rams' attractiveness (Table 1). Ewes expressed a significantly greater frequency of reproductive behaviours i.e. standing firm, head turning and wagging tail in the presence of C rams. Entire males were also observed to be more aggressive during the test than other treatments. When RR and immunized rams were presented, ewes expressed an increased frequency of aggression and 'follow male' behaviour which might indicate frequency of attempts to initiate the contact that could lead to escalation of courtship and sexual behaviours. Results indicate that ewes were able to discriminate between entire, castrated or immunized rams and altered their behavioural pattern accordingly during the sexual behaviour test.

7.2.3.1 Summary of the key findings

1. The anti-GnRH vaccine was effective in achieving sterility by reduction of circulating testosterone leading to altered testes consistency, scrotal circumference, testicular histology and suppression of reproductive behaviours (mounting attempts, flehmen, low stretch, nudging and male follow).
2. An extended interval between primary and booster vaccination prolonged the immunity period
3. Evaluation of testosterone concentrations after administration of particular vaccination regime have shown that testosterone levels remained suppressed for at least 12 weeks (vaccine administration at 6 and 12 weeks of age), 20 weeks (after vaccination at 6,12,22 weeks of age) and until 28-32 weeks of age (vaccine administration at 10 and 16; 10 and 20; 12 and 18; 12 and 22 weeks of age).
4. Ewes' perception of male attractiveness was influenced by immunization. Ewes were more aggressive towards immunized or physically castrated males in comparison to entire rams and expressed more mating behaviours like standing firm while rams were attempting to mount, head turning and wagging tail behaviour in the presence of entire males

7.2.4 Impact of new anti-GnRF vaccine for ram lambs on growth rate, carcass and meat characteristics

There were no negative effects of immunization with the anti-GnRF vaccine on growth rate, carcass and meat quality of rams, which is consistent with the previous studies investigating the use of similar product to control fertility in cattle and pigs (Adams et al., 1996; Amatayakul-Chantler et al., 2012; Amatayakul-Chantler et al., 2013; Faberga et al., 2010). The growth rate was affected by gender and treatment. C rams were shown to have greater weight than RR and LA rams on the second year of the study at later stages of life. Huxsoll et al. (1998) also found increased growth of entire males in comparison to steers. The female group had reduced weight gain in comparison to C and Vac 2 treatments. This agrees with earlier findings describing slower growth rate of female lambs in comparison to males (Saňudo et al., 1998). This finding is related to the

effect of circulating testosterone. Entire males have been shown to have greater body mass than castrated males and females because they were exposed to the beneficial impact of testosterone on the growth throughout maturing in contrast with wethers and the female group. On the last year of the study when carcass and meat quality measures were collected it was found that RR rams tended to have a lower finished weight than entire and immunized rams but there was no difference in carcass weight between treatments after dressing. A similar outcome was reported by Amatayakul-Chantler et al. (2012) presenting no adverse impact on growth rate of a similar product for fertility control in cattle. Table 11 and 12 in Chapter 6 represent summary results of the impact of immunization on carcass and meat quality characteristics. Overall results have shown no differences in meat and carcass quality measures. Therefore, it was concluded that immunization had no negative impact on such parameters. However, Vac 4 rams tended to show a quicker drop in the average pH parameter in comparison to C, RR and Vac 5 treatments, which is very important factor in the process of meat tenderness. It is possible that the sample size was not sufficient to detect significant differences between treatments due to high within treatment variability and perhaps a larger sample size would allow a greater degree of statistical confidence in this result. Thus conducting a larger study would be recommended to allow for in depth evaluation of the immunization impact on the productivity of male lambs.

7.2.4.1 Summary of the key findings

1. Treatment had some impact on the expression of aggressive behaviours and growth rate.
2. There were no significant differences between treatments in meat quality measures.
3. Immunization was found to be a neutral method having no negative and no positive effect on the growth rate, carcass quality and conformation.
4. On the basis of the evaluation of potential benefits of immunization on the carcass characteristics, meat quality, growth, expression of aggressive behaviours, estimated economic return and the absence of negative effects of

immunization it was concluded that it is a good alternative for traditional castration techniques.

7.2.5 Selection of optimal vaccination regime from the tested protocols

One of the objectives of this thesis was to investigate different vaccination regimes to estimate which of the studies protocols was most efficient in reduction of plasma testosterone concentration and scrotal measures, practicality of administration, ease of management (reduction of fighting and expression of sexual behaviours) enhancement of growth rate, improved carcase conformation and meat characteristics. Although all of the tested vaccination regimes were more welfare friendly, successful in reduction of fertility, and there was no significant impact on the carcase characteristics and growth, two of the tested protocols were observed to give better results in terms of practicality and feasibility of on farm use. Table 1 represents an overall summary of the measured parameters. On the basis of the results shown in table 1, estimation was made to recommend most sufficient vaccination regime. However, it has to be noted that this is only estimation and more detailed analysis should be carried out for the commercial reasons before any of the protocols is recommended to the farmers as best. It has to be also noted that the results shown in this thesis give the farmers a bit of flexibility and it is commercially possible to recommend more than one vaccination regime as all of tested protocols were successful but differed in the time of immunity period.

In experiment 1 carried out on the first year of the study rams were vaccinated at 6 and 12 weeks of age. The immunity period has lasted for 12 weeks and there was no significant effect on the growth rate.

Analysis of the plasma testosterone concentration have shown that levels of testosterone up to 12 weeks of age are minimal therefore primary vaccination before 10 – 12 weeks of age may be perceived as waste of resources. On the other hand, the immunity lasted until 24 weeks of age, which should be sufficient time for rams to achieve desired slaughter weight without need to separate male and female stock to avoid indiscriminate breeding. Rams that did not achieve the desired weight would have to be separated or re-

vaccinated which may not be economical as additional time, staff and financial resources could be needed.

Experiment 2, carried out in the second year of the study, tested a 3 dose regime protocol for the vaccination at 6, 12, and 22 weeks of age. The immunity period lasted until the slaughter end point, which would allow for keeping all stock together until slaughter but it required additional gathering for the 3rd vaccination and an economical cost associated with an additional vaccine dose.

Experiment 3, carried out in the third year of the study, tested 4 different vaccination regimes. Rams were vaccinated primarily at 10 or 12 weeks of age. The booster vaccination was administered at 16, 18, 20 or 22 weeks of age, either a 6 or 10 week interval between vaccinations. The immunity period was prolonged and lasted until slaughter date. The expression of sexual behaviours, testosterone concentration and scrotal measures were reduced in all proposed vaccination dates. There was no significant impact on growth rate and carcase characteristics. However, there was a tendency for Vac 4 to have quicker drop in the pH after slaughter, which may be beneficial in the process of meat production leading to better tenderness of meat. In my opinion the Vac 4 and Vac 6 vaccination protocols were the best immunisation regimes. I took into account flexibility of use, reduction in scrotal measures, expression of sexual/aggressive behaviours, testosterone concentration, time of immunity period, practicality and economy of administration (i.e. number of booster vaccinations), possible economical return after slaughter. In comparison to control treatment Vac 4 and Vac 6 groups showed better reductions in testosterone concentration and scrotal measures as well as expression of courtship and sexual behaviours. The vaccination achieved a longer period of immunity (until the time of slaughter) in comparison to Vac 1 treatment with only one booster vaccination. Vac 2 achieved even more elongated period of immunity than Vac 4 and Vac 6 treatments, nonetheless 2 booster vaccinations were required. It has to be noted however that some of the analysed results were not significant i.e. growth rate, carcase conformation and meat quality and due to the small

sample size it is possible that different vaccination protocol will be more effective and practical in a commercial trial.

However, it must be noted that all of the presented administration techniques were successful in achieving sterility and had no negative impact on behavioural expression and productivity of male lambs. Overall, findings have shown that immunization was less painful or even possibly a pain free method of castration. It was also believed that immunization may be a more economical technique of fertility control if the growth benefit is increased in comparison to the cost of the vaccine. RR method is cheap and practical (requires only one gathering) therefore it is favoured by farmers, although RR might also lead to increased lamb losses. The literature review revealed that in the recent years public opinion has been increasingly interested in the alleviation of pain during husbandry procedures (chapter 1). The demand for leaner meat has been growing as well (EC, 2001). In the light of these findings it can be argued that people may be willing to pay more to achieve a required product. There are reports that consumers are willing to pay more for the desirable more tender meat (Boleman et al., 1997; Huffman et al., 1996). Conversely, the complete farm-level economics of immunization use was not studied here. There is a need for further study in this area. Larger studies on a commercial scale including for example cost/benefits modelling, and an on farm trial of the product could give a better indication of farmers' intentions and willingness to use this technique. Conduction of the large scale commercial trial taking into account all of the steps of meat production "from farm to fork" type of trial could also possibly answer if the consumers would be willing to buy what might be a more expensive product to achieve the welfare benefits of reducing pain on the farm.

7.2.6 Analysis of the key limitations of the study

7.2.6.1 Measurement of pain related behaviours and postures

The analysis of the QBA results in relation to the assessment of influence of the castration technique on the rams' behaviours with regard to the combined method was difficult. In this study the quality of inactivity shown by the combined group suggested

that he combined treatment was significantly different than control group. However it is possible that this outcome can not be attributed to the expression of pain related behaviours. Therefore, interpreting the QBA results as a single pain assessment protocol proved to be very difficult. Nonetheless, combining both qualitative and quantitative assessments outcomes as well as presenting findings already shown in literature, indicating that combined method of castration is less painful than other traditional castration methods such as Burdizzo and rubber ring but do induce considerable levels of pain (i.e. Kent et al., 1995) allowed for understanding and analysis of the achieved results.

7.2.6.2Measurement of chronic pain indicators

Chronic pain in this study was measured by comparison of formation, severity and time to heal of castration lesion. It is possible that the results would be more detailed and accurate if the reaction to mechanical stimulation (by palpation) would also be measured later on in life until rams were slaughtered.

7.2.6.3 Recordings of aggressive encounters, sexual and courtship behaviours frequency

In the second year of the study 4 rams had a very characteristic coat which was unique in comparison to the rest of the sheep in the flock. The analysis of the recorded video-clips with the rams' behaviours was analysed later on in the study period by one observer blinded to the treatment. It is possible that the observer was biased and not blinded in relation to the analysis of the behaviours of 4 mentioned above reams. However, due to fact that these 4 rams were part of different treatment groups it is very unlikely that the overall outcome of the study was affected. Results from each particular individual did not influence the final results of the specific studies. Therefore, data derived form those specific individuals was included in the final analysis.

7.2.6.4 Power and sample size evaluation

The sample size used in any study is determined by the need to have sufficient statistical power. Larger sample sizes generally lead to increased precision when estimating unknown parameters. Sample size can be determined by choosing the appropriate

number of observations or replicates to include in a statistical sample. Sample sizes may be chosen in several different ways:

1. experience – by using items that are readily available or convenient to collect. There is a danger however that sample sizes, may result in wide confidence intervals or risks of errors in statistical hypothesis testing.
2. using a target variance for an estimate to be derived from the sample eventually obtained
3. using a target for the power of a statistical test to be applied once the sample is collected.

In this study sample sizes in all of the observations/experiments were chosen based on experience. For example, literature review was carried out to determine ethogram of pain related, aggressive, courtship and sexual, fear anxiety and ewe-lamb bonding behaviours. There was variability between studies in the number of animals used to create appropriate sample size. For instance, when selecting appropriate sample size for the observation of pain related postures and behaviours the number of individuals in an experimental group ranged from n=6 to n=24. In some cases, there were very little or no information in the literature that could have been related to specific studies, i.e. the impact of immunization on the expression of pain related behaviours and postures or the impact of immunization on the expression of courtship and sexual behaviours. Most of found references were related to other species i.e. cattle and pigs but not sheep. Therefore, it has to be noted that the power calculation was based on the literature for the pain related behaviours, but no data was analysed to allow estimating some of the later parameters, or those were not considered in the initial protocol development on the first year of the study.

For the development and expression of agonistic courtship and sexual behaviours pilot study was conducted in the first year of the thesis testing 8 rams from control (C), rubber ring (RR), short scrotum (SSC) and immunocastration (Vac 1) group. Results have shown that this sample size was appropriate to achieve significant difference between treatments.

It is possible that for some of the studies this method of sample size selection may have not been sufficient (i.e. study of the impact of immunization on carcase characteristic and meat quality as well as observations of anxiety/fear reactions during three fearful situations). However, it has to be noted that it is perfectly possible to have a situation in which a power calculation based on knowledge before the experiment gave a power of 20% but when the experimenter still went ahead, it might get a statistically significant difference. Therefore, the question to be asked if there was no statistically significant difference is whether this is because there is not a meaningful difference or whether it is simply that the experiment was too small. The way to answer that question is to produce confidence intervals for the differences between treatment means. If the statistically significant differences can be observed, then zero will not lie within the interval. If a statistically significant difference is not present, then zero will lie in the interval. The key question is how wide those confidence intervals are? If they are comparatively narrow then it can be assured that the true difference is not that big. Nevertheless, if the confidence interval is wide then the data is consistent with both small and large differences. Hence it can not to be concluded either way. In this study it was concluded that there was no statistical evidence of a difference because statistically significant difference for certain comparisons have not been found. Nonetheless, it has to be noted that “no evidence of a difference" and "evidence of no difference" are two very different things. If an experiment is too small then the outcome will be “no evidence of a difference" but it will not be “evidence of no difference".

After consultation with the statistician it was decided that confidence intervals should be calculated to determine how appropriate the sample sizes were in selected studies where no statistically significant difference between treatment groups was noted. Confidence interval (CI) is an estimate of a population parameter. It is calculated from the observations. How frequently the observed interval contains the parameter is determined by the confidence level or confidence coefficient.

For the parametric data (i.e. live weight, carcase weight) ANOVA in Minitab statistical package 17th edition (Minitab, Inc, State Collage, PA). was carried out to compare CI

between treatment groups. Then Tukey pairwise comparisons were conducted to calculate Standard error of difference (SED) and Least significant difference (LSD). The Tukey test was selected to account for multiple comparisons which were not possible with use of Fisher test. Log 10 transformation was conducted to normalize non-parametric data and achieve approximately normal distribution to estimate the 95% CI. The interpretation of the results was as follows:

If the individual CIs did not overlap then it was certain that there is indeed evidence of a difference between the treatments. However, if the individual confidence intervals do overlap (as in analysis shown below see table 2-11), there may still be evidence of a difference. For example, if hypothetically variance is 8 and n=8 then SED = sqrt(8/8)=1. So Trt A has CI of mean A +/- 2 and Trt B has CI of mean B +/- 2 SED = sqrt(8/8+8/8)=sqrt(2)=1.414

So Trt A - Trt B has CI of mean A - mean B +/- 2(1.414)

= mean A - mean B +/- 2.818

If mean A =8, mean B = 5 and CI for A is (6,10) and B is (3,7), so they overlap but CI for A-B = (8-5) +/- 2.818 = (0.182, 5.818) so the interval does not include zero, and thus gives a statistically significant difference at the 5% level.

Following parameters were taken into consideration during confidence intervals analysis: live weight, carcase weight, shear force and expression of aggressive behaviours conducted on the 2nd and 3rd year of the thesis.

Results of calculated CI are shown inn the tables 2-11 below.

Overall analysis has shown that individual CIs did not overlap which is an evidence of no difference between the treatments. Further analysis of the outputs described in tables 2-11 revealed that zero did not lie within the interval and the CI was comparatively narrow. The p-values were greater than the significance level (0.05), therefore there is not enough evidence to reject the null hypothesis that the population means are all equal. Grouping information from the Tukey Pairwise Comparisons Method has also shown that there were no significant differences between treatment groups. Therefore it can be assured that the true difference between analysed treatments with regards to specific parameters is not that big hence the statistically significant differences could not been

observed. It is possible that increasing the number of animals during analysis of these specific measurements would allow for the difference between treatments to be bigger, the power of the test to be stronger and the statistically significant difference to be more easily observed.

Results shown in table 4 and 5 indicate that there is significant difference between RR and C treatments in expressed total aggression (summation of butting, butting back, push, push back and threat behaviours). The output of the table 4 is displaying that the interval does not include zero, and the p-value is lower than the significance level (0.05), therefore there is enough evidence to reject the null hypothesis that the population means are all equal. Grouping information from the Tukey Pairwise Comparisons Method has also shown significant difference between C and RR group. Thus a shown result gives a statistically significant difference at the 5% level.

Table 2 Differences in expression of total aggression (summation of butting, butting back, displacing and threat behaviours) between control (C), rubber ring (RR), female (F), local anaesthesia (LA) and immunicatration (Vac 2) treatment groups recorded for 30 minutes during feed competition test at ~24 weeks of age. Data are log 10 transformed means with 95% CI.

Group	N	Mean	StDev	95% CI
[a]C	9	-0.0395	0.1099	(-0.1522, 0.0732)
[a]F	24	0.0239	0.1784	(-0.0451, 0.0929)
[a]LA	11	-0.0242	0.1567	(-0.1262, 0.0777)
[a]RR	11	0.0313	0.2128	(-0.0706, 0.1333)
[a]Vac 2	12	-0.0453	0.1499	(-0.1429, 0.0523)

Note that analysis was carried out with use of One Way ANOVA, the P-Value=0.66, test statistic F=0.60, pooled StDev=0.169150. If the p-value is greater than the significance level (0.05), there is not enough evidence to reject the null hypothesis that the population means are all equal. The test may have not enough power to detect a difference that is practically significant. [a] Grouping information using the Tukey Pairwise Comparisons Method and 95% confidence, groups that do not share a letter are significantly different. N-sample size.

Table 3 (Tukey Simultaneous Tests for Differences of Means) Differences in expression of total aggression (summation of butting, butting back, displacing and threat behaviours) between control (C), rubber ring (RR), female (F), local anaesthesia (LA) and immunicastration (Vac 2) treatment groups recorded for 30 minutes during feed competition test at ~24 weeks of age. Data are log 10 transformed means with 95% CI.

[1]Difference of Levels	[2]Difference of Means	[3]SED	[4]95% CI	[5]T-Value	[6]Adjusted P-Value
F - C	0.0634	0.0661	(-0.1222, 0.2490)	0.96	0.872
LA - C	0.0153	0.0760	(-0.1981, 0.2287)	0.20	1.000
RR - C	0.0709	0.0760	(-0.1426, 0.2843)	0.93	0.883
Vac 2 - C	-0.0058	0.0746	(-0.2152, 0.2036)	-0.08	1.000
LA - F	-0.0481	0.0616	(-0.2210, 0.1248)	-0.78	0.935
RR - F	0.0074	0.0616	(-0.1654, 0.1803)	0.12	1.000
Vac 2 - F	-0.0692	0.0598	(-0.2371, 0.0986)	-1.16	0.775

RR - LA	0.0556	0.0721	(-0.1469, 0.2580)	0.77	0.938
Vac 2 - LA	-0.0211	0.0706	(-0.2193, 0.1771)	-0.30	0.998
Vac 2 - RR	-0.0767	0.0706	(-0.2749, 0.1215)	-1.09	0.813

Please Note: [1]Multiple comparisons to assess differences in group means. [2] Tests for differences of means, use the confidence intervals to determine likely ranges for the differences and to determine whether the differences are practically significant. [3] Standard Error of Difference. [4] the 95% simultaneous confidence level, indicates that there is 95% confidence that all the confidence intervals contain the true differences. Confidence intervals that do not contain zero indicate a mean difference that is statistically significant. [5] tests statistic. [6] adjusted p-value. Individual confidence level = 99.33%. This result indicates that there is 99.33% confidence that each individual interval contains the true difference between a specific pair of group means. The individual confidence levels for each comparison produce the 95% simultaneous confidence level for all comparisons.

Table 4 Differences in expression of total aggression (summation of butting, butting back, push, push back and threat behaviours) between control (C), rubber ring (RR), and immunicastration (Vac 3-6) treatment groups recorded for 30 minutes during feed competition test at ~24 weeks of age. Data are log 10 transformed means with 95% CI.

Group	N	Mean	StDev	95% CI
[a] C	12	0.8153	0.2505	(0.6683, 0.9624)
[b] RR	12	1.1790	0.2297	(1.0319, 1.3261)
[a,b] Vac 3	12	1.0096	0.3262	(0.8626, 1.1567)
[a,b] Vac 4	12	0.9027	0.2372	(0.7556, 1.0497)
[a,b] Vac 5	12	0.9889	0.2205	(0.8418, 1.1360)
[a,b] Vac 6	12	1.0455	0.2530	(0.8984, 1.1926)

Note that analysis was carried out with use of One Way ANOVA, the P-Value=0.02, test statistic F=2.85, pooled StDev=. 0.255202. If the p-value is greater than the significance level (0.05), there is not enough evidence to reject the null hypothesis that the population means are all equal. The test may have not enough power to detect a difference that is practically significant. [a,b] Grouping information using the Tukey Pairwise Comparisons Method and 95% confidence, groups that do not share a letter are significantly different. N- sample size.

Table 5 (Tukey Simultaneous Tests for Differences of Means) Differences in expression of total aggression (summation of butting, butting back, push, push back and threat behaviours) between control (C), rubber ring (RR) and immunocastration 3-6 (Vac 3-6) treatment groups recorded for 30 minutes during feed competition test at ~24 weeks of age. Data are log 10 transformed means with 95% CI.

[1]Difference of Levels	[2]Difference of Means	[3]SE of Difference	[4]95% CI	[5]T-Value	[6]Adjusted P-Value
RR - C	0.364	0.104	**(0.058, 0.669)**	3.49	**0.011**
Vac 3 - C	0.194	0.104	(-0.111, 0.500)	1.86	0.433
Vac 4 - C	0.087	0.104	(-0.218, 0.393)	0.84	0.959
Vac 5 - C	0.174	0.104	(-0.132, 0.479)	1.67	0.559
Vac 6 - C	0.230	0.104	(-0.076, 0.536)	2.21	0.248

Vac 3 - RR	-0.169	0.104	(-0.475, 0.136)	-1.63	0.585
Vac 4 - RR	-0.276	0.104	(-0.582, 0.029)	-2.65	0.099
Vac 5 - RR	-0.190	0.104	(-0.496, 0.116)	-1.82	0.458
Vac 6 - RR	-0.133	0.104	(-0.439, 0.172)	-1.28	0.794
Vac 4 - Vac 3	-0.107	0.104	(-0.413, 0.199)	-1.03	0.907
Vac 5 - Vac 3	-0.021	0.104	(-0.326, 0.285)	-0.20	1.000
Vac 6 - Vac 3	0.036	0.104	(-0.270, 0.342)	0.34	0.999
Vac 5 - Vac 4	0.086	0.104	(-0.219, 0.392)	0.83	0.961
Vac 6 - Vac 4	0.143	0.104	(-0.163, 0.449)	1.37	0.744
Vac 6 - Vac 5	0.057	0.104	(-0.249, 0.362)	0.54	0.994

Please Note: [1]Multiple comparisons to assess differences in group means. [2] Tests for differences of means, use the confidence intervals to determine likely ranges for the differences and to determine whether the differences are practically significant. [3] Standard Error of Difference. [4] the 95% simultaneous confidence level, indicates that there is 95% confidence that all the confidence intervals contain the true differences. Confidence intervals that do not contain zero indicate a mean difference that is statistically significant. [5] tests statistic. [6] adjusted p-value. Individual confidence level = 99.54%. This result indicates that there is 99.54% confidence that each individual interval contains the true difference between a specific pair of group means. The individual confidence levels for each comparison produce the 95% simultaneous confidence level for all comparisons.

Table 6 Differences in live weight between control (C), rubber ring (RR), and immunicatration (Vac 3-6) treatment groups recorded on the day of the slaughter at ~ 32 weeks of age. Data are means with 95% CI.

Group	N	Mean	StDev	95% CI
[a] C	13	53.58	6.92	(50.32, 56.83)
[a] RR	14	47.50	5.23	(44.36, 50.64)
[a] Vac 3	14	51.11	4.90	(47.97, 54.24)
[a] Vac 4	14	51.36	7.29	(48.22, 54.49)
[a] Vac 5	13	53.27	4.87	(50.01, 56.52)
[a] Vac 6	12	53.50	5.63	(50.11, 56.89)

Note that analysis was carried out with use of One Way ANOVA, the P-Value=0.07, test statistic F=2.13, pooled StDev= 5.89094. If the p-value is greater than the significance level (0.05), there is not enough evidence to reject the null hypothesis that the population means are all equal. The test may have not enough power to detect a difference that is practically significant. [a] Grouping information using the Tukey Pairwise Comparisons Method and 95% confidence, groups that do not share a letter are significantly different. N-sample size.

Table 7 (Tukey Simultaneous Tests for Differences of Means results) Differences in live weight between control (C), rubber ring (RR), and immunicastration (Vac 3-6) treatment groups recorded on the day of the slaughter at ~ 32 weeks of age. Data are means with 95% CI.

[1]Difference of Levels	[2]Difference of Means	[3]SE of Difference	[4]95% CI	[5]T-Value	[6]Adjusted P-Value
RR - C	-6.08	2.27	(-12.72, 0.57)	-2.68	0.092
Vac 3 - C	-2.47	2.27	(-9.11, 4.17)	-1.09	0.884
Vac 4 - C	-2.22	2.27	(-8.86, 4.42)	-0.98	0.923
Vac 5 - C	-0.31	2.31	(-7.07, 6.46)	-0.13	1.000
Vac 6 - C	-0.08	2.36	(-6.98, 6.83)	-0.03	1.000
Vac 3 - RR	3.61	2.23	(-2.91, 10.13)	1.62	0.588
Vac 4 - RR	3.86	2.23	(-2.66, 10.38)	1.73	0.515

Vac 5 - RR	5.77	2.27	(-0.87, 12.41)	2.54	0.125
Vac 6 - RR	6.00	2.32	(-0.78, 12.78)	2.59	0.113
Vac 4 - Vac 3	0.25	2.23	(-6.27, 6.77)	0.11	1.000
Vac 5 - Vac 3	2.16	2.27	(-4.48, 8.80)	0.95	0.931
Vac 6 - Vac 3	2.39	2.32	(-4.39, 9.18)	1.03	0.905
Vac 5 - Vac 4	1.91	2.27	(-4.73, 8.55)	0.84	0.958
Vac 6 - Vac 4	2.14	2.32	(-4.64, 8.93)	0.92	0.939
Vac 6 - Vac 5	0.23	2.36	(-6.67, 7.13)	0.10	1.000

Please Note: [1]Multiple comparisons to assess differences in group means. [2] Tests for differences of means, use the confidence intervals to determine likely ranges for the differences and to determine whether the differences are practically significant. [3] Standard Error of Difference. [4] the 95% simultaneous confidence level, indicates that there is 95% confidence that all the confidence intervals contain the true differences. Confidence intervals that do not contain zero indicate a mean difference that is statistically significant. [5] tests statistic. [6] adjusted p-value. Individual confidence level = 99.55%. This result indicates that there is 99.55% confidence that each individual interval contains the true difference between a specific pair of group means. The individual confidence levels for each comparison produce the 95% simultaneous confidence level for all comparisons.

Table 8 Differences in carcase weight between control (C), rubber ring (RR), and immunicastration (Vac 3-6) treatment groups recorded on the day of the slaughter at ~ 32 weeks of age. Data are means with 95% CI.

Group	N	Mean	StDev	95% CI
[a] C	13	26.538	3.424	(24.682, 28.395)
[a] RR	14	23.093	3.133	(21.304, 24.882)
[a] Vac 3	14	25.071	3.155	(23.282, 26.860)
[a] Vac 4	14	24.50	4.18	(22.71, 26.29)
[a] Vac 5	13	24.923	2.745	(23.066, 26.780)
[a] Vac 6	12	25.125	3.297	(23.193, 27.057)

Note that analysis was carried out with use of One Way ANOVA, the P-Value=0.20, test statistic F=1.48, pooled StDev= 3.35957 . If the p-value is greater than the significance level (0.05), there is not enough evidence to reject the null hypothesis that the population means are all equal. The test may have not enough power to detect a difference that is practically significant. [a] Grouping information using the Tukey Pairwise Comparisons Method and 95% confidence, groups that do not share a letter are significantly different. N-sample size.

Table 9 (Tukey Simultaneous Tests for Differences of Means) Differences in carcase weight between control (C), rubber ring (RR), and immunicastration (Vac 3-6) treatment groups recorded on the day of the slaughter at ~ 32 weeks of age. Data are means with 95% CI.

[1] Difference of Levels	[2] Difference of Means	[3] SE of Difference	[4] 95% CI	[5] T-Value	[6] Adjusted P-Value
RR - C	-3.45	1.29	(-7.23, 0.34)	-2.66	0.095
Vac 3 - C	-1.47	1.29	(-5.26, 2.32)	-1.13	0.866
Vac 4 - C	-2.04	1.29	(-5.83, 1.75)	-1.58	0.617
Vac 5 - C	-1.62	1.32	(-5.47, 2.24)	-1.23	0.823
Vac 6 - C	-1.41	1.34	(-5.35, 2.52)	-1.05	0.899
Vac 3 - RR	1.98	1.27	(-1.74, 5.70)	1.56	0.628
Vac 4 - RR	1.41	1.27	(-2.31, 5.12)	1.11	0.877

Vac 5 - RR	1.83	1.29	(-1.96, 5.62)	1.41	0.718
Vac 6 - RR	2.03	1.32	(-1.84, 5.90)	1.54	0.641
Vac 4 - Vac 3	-0.57	1.27	(-4.29, 3.15)	-0.45	0.998
Vac 5 - Vac 3	-0.15	1.29	(-3.94, 3.64)	-0.11	1.000
Vac 6 - Vac 3	0.05	1.32	(-3.82, 3.92)	0.04	1.000
Vac 5 - Vac 4	0.42	1.29	(-3.36, 4.21)	0.33	0.999
Vac 6 - Vac 4	0.63	1.32	(-3.24, 4.49)	0.47	0.997
Vac 6 - Vac 5	0.20	1.34	(-3.74, 4.14)	0.15	1.000

Please Note: [1]Multiple comparisons to assess differences in group means. [2] Tests for differences of means, use the confidence intervals to determine likely ranges for the differences and to determine whether the differences are practically significant. [3] Standard Error of Difference. [4] the 95% simultaneous confidence level, indicates that there is 95% confidence that all the confidence intervals contain the true differences. Confidence intervals that do not contain zero indicate a mean difference that is statistically significant. [5] tests statistic. [6] adjusted p-value. Individual confidence level = 99.55%. This result indicates that there is 99.55% confidence that each individual interval contains the true difference between a specific pair of group means. The individual confidence levels for each comparison produce the 95% simultaneous confidence level for all comparisons.

Table 10 Differences in shear force between control (C), rubber ring (RR), and immunicastration (Vac 3-6) treatment groups recorded in the course of estimation of Longissimus Dorsi muscle tenderness. Data are Log 10 transformed means with 95% CI.

Group	N	Mean	StDev	95% CI
[a] C	13	-0.1525	0.0525	(-0.1913, -0.1137)
[a] RR	14	-0.1530	0.0821	(-0.1904, -0.1157)
[a] Vac 3	14	-0. 1439	0.0700	(-0.1813, -0.1065)
[a] Vac 4	14	-0.1556	0.0705	(-0.1929, -0.1182)
[a] Vac 5	13	-0.1688	0.0823	(-0.2076, -0.1300)
[a] Vac 6	12	-0.1631	0.0558	(-0.2035, -0.1227)

Note that analysis was carried out with use of One Way ANOVA, the P-Value=0.95, test statistic F=0.21, pooled StDev= 0.0701755 . If the p-value is greater than the significance level (0.05), there is not enough evidence to reject the null hypothesis that the population means are all equal. The test may have not enough power to detect a difference that is practically significant. [a] Grouping information using the Tukey Pairwise Comparisons Method and 95% confidence, groups that do not share a letter are significantly different. N-sample size.

Table 11 (Tukey Simultaneous Tests for Differences of Means) Differences in shear force between control (C), rubber ring (RR), and immunicastration (Vac 3-6) treatment groups recorded in the course of estimation of Longissimus Dorsi muscle tenderness. Data are log 10 transformed means with 95% CI.

[1]Difference of Levels	[2]Difference of Means	[3]SE of Difference	[4]95% CI	[5]T-Value	[6]Adjusted P-Value
RR - C	-0.0005	0.0270	(-0.0797, 0.0786)	-0.02	1.000
Vac 3 - C	0.0086	0.0270	(-0.0705, 0.0877)	0.32	1.000
Vac 4 - C	-0.0031	0.0270	(-0.0822, 0.0761)	-0.11	1.000
Vac 5 - C	-0.0163	0.0275	(-0.0969, 0.0643)	-0.59	0.991
Vac 6 - C	-0.0106	0.0281	(-0.0928, 0.0716)	-0.38	0.999
Vac 3 - RR	0.0091	0.0265	(-0.0685, 0.0868)	0.34	0.999
Vac 4 - RR	-0.0025	0.0265	(-0.0802, 0.0751)	-0.10	1.000

Vac 5 - RR	-0.0158	0.0270	(-0.0949, 0.0633)	-0.58	0.992
Vac 6 - RR	-0.0101	0.0276	(-0.0909, 0.0708)	-0.36	0.999
Vac 4 - Vac 3	-0.0117	0.0265	(-0.0893, 0.0660)	-0.44	0.998
Vac 5 - Vac 3	-0.0249	0.0270	(-0.1040, 0.0542)	-0.92	0.940
Vac 6 - Vac 3	-0.0192	0.0276	(-0.1000, 0.0616)	-0.70	0.982
Vac 5 - Vac 4	-0.0132	0.0270	(-0.0924, 0.0659)	-0.49	0.996
Vac 6 - Vac 4	-0.0075	0.0276	(-0.0884, 0.0733)	-0.27	1.000
Vac 6 - Vac 5	0.0057	0.0281	(-0.0765, 0.0880)	0.20	1.000

Please Note: [1]Multiple comparisons to assess differences in group means. [2] Tests for differences of means, use the confidence intervals to determine likely ranges for the differences and to determine whether the differences are practically significant. [3] Standard Error of Difference. [4] the 95% simultaneous confidence level, indicates that there is 95% confidence that all the confidence intervals contain the true differences. Confidence intervals that do not contain zero indicate a mean difference that is statistically significant. [5] tests statistic. [6] adjusted p-value. Individual confidence level = 99.55%. This result indicates that there is 99.55% confidence that each individual interval contains the true difference between a specific pair of group means. The individual confidence levels for each comparison produce the 95% simultaneous confidence level for all comparisons.

7.2.6.5 Technical difficulties

7.2.6.6 Technical difficulties with the plasma cortisol concentration analysis by ELISA test

In this study technical difficulties with the ELISA test procedure did not allow for the analysis of all collected blood samples although the protocol has been fully followed. There are few possibilities explaining why ELISA test in this study did not go as planned. These include: plate, plate-wash instrumentation, buffers, and procedure itself including procedural error (human mistake). Plate-wash problems commonly imitate reagent issues.

Because the result is read in last step of the assay it is very hard to say what may have gone wrong during carrying out the steps of the planned procedure. After careful analysis of the particular assays outcomes, it was concluded that most likely cause of problems encountered in this study was caused by plate-washing instrument and/or procedural error.

On one occasion the entire plate had minimal signal. The expected colometric change throughout the plate was minimal. This means that most likely a procedural error has occurred or a key reagent was inappropriate. It is also possible that the labelled detector antibody was not added to the assay. It is probable that the enzyme was non-functional. Another option is that the substrate buffer was improper and severely inhibited the expected colour change.

Second problem encountered during the course of the study was inconsistent results across replicate samples and/or controls. This type of issue is one of the most difficult to deal with. It may be caused by the faulty plate-washer or a bad lot of plates or manufacturing issues. In addition to bad plates, the ability of the operator to pipette accurately has to be considered. It is important to check if pipette that is used is properly calibrated. Even small variations in pipetting across multiple reagents can produce results that will not pass quality control limits of variability.

It was also noted on few occasion that the controls have pass quality control criteria, but the samples themselves appeared to be challenging to interpret. It is likely due to procedural issues such preparing the wrong sample dilution, using the wrong buffer, or pipetting the incorrect amount.

The assay procedure has been repeated for three times on some occasions. Each time different problem was encountered, making collection of appropriate sample size for the analysis impossible. These issues impacted on the collected data. It is possible that collection of all results from every individual would allow observing significant differences between treatments. Nonetheless, it was decided to include results of this study in the thesis even though they were not significant (due to time and effort that was spent to conduct this study).

7.2.6.7 Carcase assessment

The Limitation with regard to the accuracy of the carcase assessment conducted by a skilled slaughter man during meat quality and carcase characteristics study were discussed in chapter 6.

7.3 Future work Recommendations

7.3.1 QBA

Implementation of QBA in the evaluation of behavioural expression of lambs following castration was found to be a very good method of pain recognition and assessment. QBA was able to distinguish between different castrations techniques and categorise lambs from different treatments according to their apparent pain expression. This allowed for a better understanding of behavioural expression, especially in the case of the COM treatment which was particularly difficult to assess. Results of this study have presented QBA as a new method of pain recognition. QBA offers a 'whole animal' approach that integrates the whole range of animal behavioural expressions (like body language) providing a valuable measure of emotional state (Rutherford et al., 2012). QBA could be potentially used to assess levels of pain during other husbandry practices in the farm setting (i.e. disbudding, dehorning, tail docking, mulesing) as well as after surgical

procedures which would allow for use of appropriate pain mitigation. There is a need for further QBA evaluation in the farm and other environments. This would allow for the investigation whether trained observers are able to detect different levels of pain and distress after application of husbandry or surgical procedures. QBA has potential to be a quick and economical technique which could be effectively applied in the field with only brief initial training.

7.3.2 CT scanning

Analysed results of the CT scanning implementation revealed that this method was able to distinguish difference between treatments in killing out % (live weight divided by total tissue weight). The sample size however was perhaps too small as there was higher than expected variation within particular treatments and it is possible that a larger sample size would possibly provide a greater degree of statistical confidence and detect significant differences between treatments. Nonetheless the outcome shown in this study was very encouraging. Thus conduction of further evaluation on the application of the CT scanning technique in the commercial size study of the effects of anti-GnRF immunisation on carcass measures would be recommended.

7.3.4 Outline of potential follow up studies

On farm commercial trial of the anti-GnRF vaccine for lamb rams

Background

The anti-GnRF vaccine has been shown to be effective and more welfare-friendly than other methods of castration in the studies presented in this thesis. The objectives of this proposed follow up study are as follows: A) to test the product in commercial farm setting which could give an indication of farmers' intentions and willingness to use this technique. Understanding of potential technical difficulties during product administration would be also measured. To assess feasibility of immunization use in a commercial farm setting, survey of the farmers experience during the use of the vaccine (including farmers' views on practicality, safety, easiness, time taken to immunize lambs and most importantly potential economic benefit of this method). Testosterone, scrotal

circumference and testes consistency data would also be collected. Along the site survey, trained assessors would be also asked to vaccinate rams on commercial farms together with farm staff. Gathered data on the time, practicality, ease of use and safety would be compared with the farmers experience; B) Assessment of cost/benefits modelling before trial starts to best fit most practical and economic vaccine administration regime.; C) Evaluation of carcase conformation and meat quality parameters on a commercial scale to investigate whether there are significant differences between treatments in the meat quality indicators.; D) To adjust cost/benefits model after meat quality data have been analysed and include in the model consumer willingness to buy meat from immunized sheep to reveal the true value of product to the farmers and set most efficient management of the stock. Consumer willingness to buy meat form immunized sheep could be assessed in a survey. Two groups of assessors would be proposed. First groups of assessors would take part in a choice preference survey, based purely on the price of the product (meat form immunized lambs) in comparison to other available products (meat form not immunized rams). Second group would take part in teste panel assessment. The origin of the product would be unknown to the assessors. The judgement would be based on the price of the product and taste values of particular products;

Cost/Benefits modelling

The general steps that will be included in the analysis are as follows: 1. List alternative castration techniques; 2. List all types of stakeholders that will be included in the study; 3. Select measurements and measure all cost/benefit elements; 4. Prediction of the outcome of cost and benefits over the period of time from the vaccination to the slaughter of animals; 5. Conversion of all costs and benefits into an economic value (currency); 6. Calculation of net present value of different castration options; 7. Presentation of the analysis; Evaluation of positive and negative consequences of immunization use (effects of immunization on technique users, effects on non-users or non-participants, external out of control effects, option value and other social benefits).

Three experimental groups will be formed:

1. Positive controls (C) – lambs were handled only

2. Negative controls (RR) – lambs were castrated using standard rubber rings

3. Immunocastration (Vac 1) – lambs were vaccinated at 10 and 20 weeks with the anti-GnRH vaccine.

Power/Sample size Analysis

Preliminary power/sample size analysis was carried out to investigate how many individuals should be allocated to the treatment group to detect significant differences between them. Results of the previously measured parameters such as shear force, live weight, carcase weight, average pH 45 min after slaughter and average ultimate pH of the longissimus dorsi loin were used to estimate appropriate sample size. Due to large differences between parameters included in the estimation literature review was also carried out to investigate similar studies reported in the past. This preliminary analysis would be discussed with a statistician before making any decision of how many animals or replicates of the study needs to be carried out.

Subsequent steps were followed to estimate appropriate sample size:

Power and sample size analysis was carried out in Minitab statistical package 17th edition (Minitab, Inc, State Collage, PA) with use of One Way ANOVA test. Estimation of the difference between the smallest and largest actual factor level means was done; Standard deviation was calculated on the basis of previous studies and literature review.

For the parameters such as live weight, carcase weight, average pH measured 45 min after slaughter, ultimate average pH, estimate of 25 animals per treatment group would be sufficient to detect significant differences between treatments. The evaluation was based on the largest sample size calculated for mentioned above parameters. The possibility of mortalities (in the events of i.e. bad weather, predation, external/internal parasites, general health problems etc.) was also considered and the sample size was made larger to account for unexpected events.

For the shear force analysis estimated sample size appropriate to detect significant differences between treatments was 250 animals in each treatment. Literature review has revealed that in the studies investigating carcase and meat quality characteristics used number of animals was in most cases very high.

Assessment of growth

Slaughter of lambs

Following meat quality measures will be taken: Live weight (prior to slaughter), hot carcass weight at slaughter, MLC carcase evaluation, and pH of the carcass 45 minutes after kill. Longissimus dorsi loin (LD) was used in the assessment of meat quality parameters. Ultimate pH of LD, meat colour and shear force was measured.

Conformation and fatness grading

Carcass conformation and fatness would be graded accordingly to the EU grading system to allow for quantifying specific carcase characteristics in order to evaluate financial value of the carcass.

Shear Force, ultimate pH, temperature and colour measurement

Final cost/benefits analysis

7.4 Conclusion

The data have shown that immunization against GnRF is a very good alternative to traditional physical castration techniques providing similar fertility control and behavioural management to physical castration, and productivity similar to entire males. Immunization was found to be less painful than other castration techniques. There was no negative impact on lambs' emotionality and behavioural expression related to ewe-lamb bonding, anxiety, stress responses and aggressive behaviours. Immunized groups have shown significant reductions in plasma testosterone concentration, testicular measures (circumference, consistency, histology) and occurrence of courtship and sexual behaviours in comparison to entire males. There were no adverse effects of vaccine administration on the injection site and immunization was found to be a safe technique which may be used in animals as young as 6 weeks of age. It was also shown that an

extended interval between primary and booster vaccination prolonged the immunity period and the primary vaccination given at weaning is the most effective and practical method of vaccine administration. Further analysis of carcass and meat quality measure revealed that there was no negative impact of immunization on carcass and meat quality characteristics as well. Findings reported here confirm that an anti-GnRH vaccine for ram lambs is effective, more welfare friendly than traditional physical methods of castration and an efficient method of fertility control.

7.5 Summary of thesis key conclusions

New anti GnRF vaccine developed by Zoetis for immunocastration of sheep may result in:

Improvements in the following behaviours and parameters:

a. Frequency and occurrence of acute and chronic pain behaviours and parameters (i.e. lesion formation)
b. No side effects of the immunization on the tissues
c. Fighting and aggression
d. Dominance and territorial behaviours
e. Reduced plasma testosterone concentration
f. Reduces scrotal measures (testes consistency and circumference which is very good and easy to spot indicator of vaccine efficacy)
g. Occurrence of sexual behaviours
h. Transformed Ewes perception of immunocastrated males
i. Indiscriminate breeding
j. Unwanted mating

No negative impact on the following behaviours and parameters:

k. Occurrence of fear and anxiety behaviours
l. Ewe-lamb bonding
m. Administration of the vaccine at the time of weaning

n. Growth rates
o. Carcase conformation and meat quality

Potential benefits for the farm management:

p. Easier management of the stock
q. More grazing management options (no need to separate sexes)
r. Less labour
s. Reduced amount of injuries (stronger economic value)
t. Flexibility of the vaccine administration time and interval between booster and primary vaccination to fit farm management practices

References

Aaslyng, M. D. 2009. Trends in meat consumption and the need for fresh meat and meat products of improved quality. Page 3 in Improving the sensory and nutritional quality of fresh meat. J. Kerry and D. Ledward, eds. Woodhead publishing limited.

Adams, T. E., and B. M. Adams. 1992. Feedlot performance of steers and bulls actively immunized against gonadotropin-releasing hormone. J. Anim. Sci. 70:1691.

Amatayakul-Chantler, S., Hoe, F., Jackson, J.A., Roca, R.O., Stegner, J.E., King, V., Howard, R., Lopez, E., Walker, J., 2013. Effects on performance and carcass and meat quality attributes following Immunocastration with the gonadotropin releasing factor vaccine Bopriva or surgical castration of Bos indicus bulls raised on pasture in Brazil. Meat Science 95 (2013) 78–84

Amatayakul-Chantler, S., Jackson, J. A., Stegner, J., King, V., Rubio, L. M. S., Howard, R., Lopez, E. and Walker, J., 2012. Immunocastration of Bos indicus × Brown Swiss bulls in feedlot with gonadotropin-releasing hormone vaccine Bopriva provides improved performance and meat quality. J ANIM SCI 2012, 90:3718-3728.

Archer, N., Johnston A M., Khalid M., 2004. Differences in the acute pain responses of two breeds of lambs following castration and tail docking with the rubber ring method. Animal Welfare 2004, 13:135-141.

Arimura, A., Sato, H., Kumasaka, T., Worobec, R.B., Debeljuk, L., Dunn, J., Schally, A.V., 1973. Production of antiserum to LH-releasing hormone LH-RH. associated with gonadal atrophy in rabbits: development of radioimmunoassays for LH-RH. Endocrinology 93, 1092–1103.

Azad N, Pitale S, Barnes WE, et al. 2003. Testosterone treatment enhances regional brain perfusion in hypogonadal men. J Clin Endocrinol Metab, 88:3064–8.

Boissy, A., Arnould, C., Chaillou, E., Desire, L., Duvaux-Ponter, C., Greiveldinger, L., Leterrier, C., Richard, S., Russel, S., Saint-Dizier, H., Meunier-Salaun, M.C., Valance, D., Veissier, I., 2007. Emotions and cognition: a new approach to animal welfare. Anim. Welfare 16, 37-43.

Bonneau, M., & Enright, W. (1995). Immunocastration in cattle and pigs. Livestock Production Science, 42(2–3), 193–200.

Bouissou, M. F., 1992. La relation homme-animal. Conse´quences et possibilite´s d'ame´lioration. INRA Productions Animales, 5, 303–318.

Breadsworth, A. and A. Bryman. 2004. Meat consumption and meat avoidance among young people: An 11-year longitudinal study. British food journal 106:313-327.

Brewer, M. S., L. G. Zhu, and F. K. McKeith. 2001. Marbling effects on quality characteristics of pork loin chops: consumer purchase intent, visual and sensory characteristics. Meat Science 59:153-163.

Broom, D.M, 1988. The scientific assessment of animal welfare. Applied Animal Behavioural Science 20 (1-2):5-19.

Broom, D.M, 1991. Concepts and measurments. J Anim. Sci69(10): 4167-4175.

Brown, B.W., Mattner, P.E., Carroll, P.A., Holland, E.J., Paull, D.R., Hoskinson, R.M., Rigby, R.D., 1994. Immunization of sheep against GnRH early in life: effects on reproductive function and hormones in rams. J. Reprod. Fertil. 101, 15–21.

Chaffaux, S., Crochot, C., De Haas, V., Deletang, F., 1985. Castration immunologique des mammiferes par immunisation active avec de la gonadoliberine. Rec. Med. Vet. 161, 133–145.

Conington, J 2010. Sheep breeding, production and conservation grazing in the UK – An example from Northern Europe. Proceedings 8th World Merino Conference, May 3-6, 2010, Rambouillet, France.

Cupps, P. T., 1991. Reproduction in Domestic Animals Fourth Edition. Academic Press. Inc, San Diego, California.

D'Occhio, M.J., 1993. Immunological suppression of reproductive functions in male and female mammals. Anim. Reprod. Sci. 33, 345–372.

David, E. Noakes, Timothy, J. Parkinson, Gary, C.W, England 2009. Vetwrinary Reproduction and Obstretics. Elsevier Limited 2009.

Diagram of ram reproductive tract - http://www2.dpi.qld.gov.au/sheep/8371.html Downloaded on the 30/4/12 at 13:15

Dijksterhuis, G.B., Heiser, W.J., 1995. The role of permutation tests in exploratory multivariate data analysis. Food Qual. Pref. 6, 263–270.

Dwyer, C. M., 2008. Individual variation in the expression of maternal behaviour: a review of the neuroendocrine mechanisms in the sheep. J. Neuroendocrinol. 20, 526–34.

Dwyer, C. M.,. 2008. The Welfare of Sheep. Springer Science Business Media B.V.

Dwyer, C.M., Lawrence, A.B, 2000. Maternal behaviour in domestic sheep (Ovis aries): constancy and change with maternal experience. Behaviour. 137, 1391–1413.

Dwyer, C.M., Lawrence, A.B., 1998. Variability in the expression of maternal behaviour in primiparous sheep: Effects of genotype and litter size. Appl. Anim. Behav. Sci. 58, 311–330.

Eales, F. A., Small, J., 1986. Practical Lambing; Longman, Harlow, UK.

Ewing, S. A., Lay Jr, D. C., Von Borell, E. 1999. Farm Animal Well-Being Stress Physiology, Animal Behaviour and Environmental Design.. Prentice Hall Inc, Upper Saddle Rivet, New Jersey

Fàbrega, E., Velarde, A., Cros, J., Gispert, M., Suárez, P., Tibau,J., Soler, J., 2010. Effect of vaccination against gonadotrophin-releasing hormone, using Improvac®, on growth performance, body composition, behaviour and acute phase proteins. Livestock Science 132 (2010) 53–59.

Fang, F., Li, H., Liu, Y., Zhang, Y., Tao, Y., Li, Y., Cao, H., Wang, S., Wang, L., Zhang, X., 2010. Active immunization with recombinant GnRH fusion protein in boars reduces both testicular development and mRNA expression levels of GnRH receptor in pituitary. Animal Reproduction Science 2010, 119:275-281.

FAWC, 2008. Report on the Implications of Castration and Tail Docking for the Welfare of Lambs.. Welfare Council, UK.

FAWC, 2009. Farm Animal Welfare in Great Britain: Past, Present and Future. FAWC, London, UK.

Field, R. A. (1971). Effect of castration on meat quality and quantity. Journal of Animal Immunological Aspects of Reproduction in Mammals. Butterworths, London, pp. 345–362.

Fisher, A.D., Paul, D.R., Lee, C., Atkinson, S.J., Colditz, I.G, 2007. New Research on Methods for Alleviating Pain in Farm Animals. CSIRO Livestock Industries, FD McMaster Laboratory, Locked Bag 1, Armidale, New South Wales, Australia.

Fisher, M,W., Bray, A.R., Johnstone, P.D., 2010. Implications of removing or altering the testicles of ram lambs on the financial returns from carcasses. New Zealand Journal of Agricultural Research 2, 135-143.

Fleming, P. A., Paisley, C.L., Barnes, A.L., Wemelsfelder, F.,2013. Application of Qualitative Behavioural Assessment to horses during an endurance ride. Applied Animal Behaviour Science 144 (2013) 80– 88.

Fraser, D. 2003. Assessing animal welfare at the farm and group level: the interplay of science and values. Anim Welf 12(4): 433-443.

Fraser, H.M., 1980. Inhibition of reproductive function by antibodies to LHRH. In: Hearn, J.P. _Ed..,Immunological Aspects of Reproduction and Fertility Control. University Press, Baltimore, MD, pp.143–171.

Fraser, H.M., 1986. LHRH immunoneutralization: basic studies and prospects for practical application. In: Talwar, G.P. _Ed.., Immunological Approaches to Contraception and Promotion of Fertility. Plenum, New York, pp. 125–141.

Fraser, H.M., Gunn, A., 1973. Effects of antibodies to luteinizing hormone-releasing hormone in the male rabbit and on the rat oestrous cycle. Nature London. 244, 160–161.
Fregonesi, J. A and Leaver, J. D., 2002. Influence of space allowance and milk yield level on behaviour, performance and health of diary cows housed in strawyard and cubicle system. Livestock Production Science 79 245-257.

Fuller, J.L., Easler, C.A., Banks, E.M., 1950. Formation of conditioned avoidance responses in young puppies. American Journal of Physiology 160, 462-6. In Gregory, N.G., Physiology and Behaviour of Animal Suffering 2004. Blackwell Publishing, The Universities Federation for Animal Welfare, UK.

Goddard, P.J., Fawcett, A.R., Macdonald, A.J. & Reid, H.W., 2000. The behavioural, physiological and immunological responses of lambs from two rearing systems and two genotypes to exposure to humans. Applied Animal Behaviour Science 66: 305–321.

Godfrey S.I., Wlakden-Brown, S. W., Martin, G.B., Speijers, E. J. 1996. Immunisation of goat bucks against GnRH to prevent seasonal reproductive and agonistic behaviour. Animal reproduction Science 44:41-54.4

Graham, M. J., Kent, J. E. & Molony, V. (1997). Effects of four analgesic treatments on the behavioural and cortisol responses of three week old lambs to tail docking. The Veterinary Journal 153, 87–97.

Grandin, T., 1987. Animal Handling. Veterinary Clinics of North America: Food Animal Practice, 3, 323-338.

Greenlee MW. 2000. Human cortical areas underlying the perception of optic flow: brain imaging studies. Int Rev Neurobiol, 44:269–92.

Gregory, N.G., Physiology and Behaviour of Animal Suffering 2004. Blackwell Publishing, The Universities Federation for Animal Welfare, UK.

Hafez, B.; Hafez, E.S.E.; 2000. Reproduction in Farm Animals 7th edition. Lippnicott Williams & Wilkins. USA.

Harlow, H.J., Thorne, E.T., Williams, E.S., Belden, E.L. & Gern, W.A., 1987. Adrenal responsiveness in domestic sheep (*Ovis aries*) to acute and chronic stressors as predicted by remote monitoring of cardiac frequency. *Canadian Journal of Zoology* 65: 2021–2027.

Harrison, R. 1964.Animal Machines - The new factory farming industry. Vincent Stuart Publishers Ltd., London.

Hemsworth, P.H., Barnett, J.L., Beveridge, L., Matthwes, L.R., 1995. The welfare of extensively managed diary cattle: A review. Applied Animal Behaviour Science 42 161-182.

Henderson, D.C., 1990. The Veterinary book for Sheep Farmers; Farming Press, Ipswich, UK.

Ho¨tzel, M.J., Caraty, A., Martin, G.B., 1997. Effects of nutrition on testicular growth in mature Merino rams actively immunized against GnRH. J. Reprod. Fertil. 110, 307–313. Hogg, J.T., 1984. Mating in bighorn sheep: multiple creative male strategies. Science 225, 526–529.

Holm, L. and M. Mohl. 2000. The role of meat in everyday food culture: an analysis of an interview study in Copenhagen. Appetite 34:277-283.

Horowitz, R. 2006. Putting meat on the American table: taste, technology, transformation. The Johns Hopkins University Press.

Hoskinson, R. M., R.D.G. Rigby, P. E. Mattner, V. L. Huynh, M. D'Occhio, A. Neish, T. E. Trigg, B. A. Moss, M. J. Lindsey, G. D. Coleman, and C. L. Schwartzkoff. 1990. Vaxstrateâ: An antireproductive vaccine for cattle. Aust. J. Biotech. 4(3):166-170.

Iwasa, Y., Pomiankowki, A., 1994. The evolution of mate preferences for multiple sexual ornaments. Evolution 48 (3),853–867.

Jacobs, L.F., 1996. Sexual selection and the brain. TREE 11, 82–86.

Janett, F., Lanker, U., Jörg, H., Hässig, M., Thun, R.., (2003). The castration of male lambs by immunization against GnRH. Klinik für Fortpflanzungsmedizin der Universität Zürich. Schweizer Archiv fur Tierheilkunde. 2003, 145(6):291-299.

Janetta, F, Geriga, T., Tschuorb, A.C., Amatayakul-Chantlerc, S., Walkerc, J., Howardc, R., Bollweina,d, H., Thuna, R., 2012. Vaccination against gonadotropin-releasing factor (GnRF) with opriva significantly decreases testicular development, serum testosterone levels and physical activity in pubertal bulls. Theriogenology 78 (2012) 182–188.

Jeffcoate, I.A., Lucas, J.M.S., Crighton, D.B., 1982. Effects of active immunization of ram lambs and bull calves against synthetic luteinizing hormone releasing hormone. Theriogenology 18, 65–77.

Kent, J E., Thrusfield, M V., Molony, V., Hoise, B D., Sheppard B W., 2004. Randomised controlled field trial of two new techniques for the castration and tail docking of lambs less than two days of age. Veterinary Record 2004, 154:193-200.

Kent, J.E., Jackson, R.E., Molony, V., Hosie, B.D., 2000. Effects of acute pain reduction methods on the chronic inflammatory lesions and behaviour of lambs castrated and tail docked with rubber rings at less than two days of age. The Veterinary Journal 160, 33–41.

Kent, J.E., Molony, V., Robertson, I.S., 1995. Comparison of the Burdizzo and rubber ring methods for castrating and tail docking lambs. The Veterinary Record 136, 192–196.

Kent, J. E., Molony, V. & Robertson, I. S., (1993). Changes in plasma concentrations of cortisol in lambs of three ages in the first three hours after three methods of castration and tail docking. Research in Veterinary Science 55, 246–51.

Kent, J. E., Molony, V., Hosie, B. & Sheppard, B., (1997). Assessment of chronic inflammatory pain after rubber ring castration of six week old lambs. Proceedings of the Sheep Veterinary Society 21, 93.

Kent, J. E., Molony, V. & Graham, M. J., (1998). Comparison of methods for the reduction of acute pain produced by rubber ring castration or tail docking of week old lambs. The Veterinary Journal 155, 39–51.

Kent, J. E., Molony, V., Jackson, R. E., & Hosie, B., (1999). Chronic inflammatory responses of lambs to rubber ring castration: are there any effects of age or size of lamb at treatment? British Society of Animal Science Occasional Publication 23, 160–2.

Kerdelhue´, B., Catlin, S., Kordon, C., Jutisz, M., 1976. Delayed effects of in vivo LHRH immunoneutralization on gonadotropins and prolactin secretion in the female rat. Endocrinology 98, 1539–1549.

Kiyma, Z., Adams, T. E., Hess, B. W., Riley, M. L., Murdoch, W. J., Moss, G. E., 2000.Gonadal function, sexual behaviour, feedlot performance, and carcass traits of ram lambs actively immunized against GnRH. Journal of Animal Science 2000, 78:2237-2243

Komesaroff, P.A., Esler, M., Clarke, I.J., Fullerton, M.J. & Funder, J.W., 1998. Effects of estrogen and estrous cycle on glucocorticoid and catecholamine responses to stress in sheep. *American Journal of Physiology* 275: E671–E678.

Lawrance, A.B., 2008. Applied animal behaviour science: Past, present and future prospects. Applied Animal Behaviour Science 115 1-24.

Lester, S.J., Mellor, D.J., Holmes, R.J., Ward, R.N., Stafford, K.J., 1996. Behavioural and cortisol responses of lambs to castration and tailing using different methods. New Zealand Veterinary Journal 44, 45–54.

Lester, S.J., Mellor, D.J., Ward, R.N., Holmes, R.J., 1991. Cortisol responses of young lambs to castration and tailing using different methods. New Zealand Veterinary Journal 39, 134-138.

Lindsay, D.R., Dunsmore, D.G., Williams, J.D., Syme, G.J., 1976. Audience effects on the mating behaviour of rams. Anim. Behav. 24, 818–821.

Lynch, J.J., Hinch, G.N., Adams, D.B., 1992. The behavior of sheep: biological principles and implications for production. CSIRO Division of Animal Production, Armidale, NSW, Australia.

Maltin, C., D. Balcerzak, R. Tilley, and M. Delday. 2003. Determinants of meat quality: tenderness. Proceedings of the Nutrition Society 62:337-347.

McLachlan, R.I., Wreford, N.G., de Kretser, D.M., Robertson, D.M., 1995. The effects of recombinant follicle-stimulating hormone on the restoration of spermatogenesis in the gonadotropin-releasing hormoneimmunizedadult rat. Endocrinology 136, 4035–4043.

Mellema, S.C., Marcus, D. G., Wechsler, B., Thueer, S., Steiner, A., 2006. Influence of local anaesthesia on pain and distress induced by two bloodless castration methods in young lambs. The Veterinary Journal 172: 274–283

Mellor, D.J and K. J. Stafford., 2001. Integrating, practical, regulatory and ethical strategies for enhancing for farm animal welfare. Austral Vet J 79(11): 762-768.

Mellor, D.J., Murray, L., (1989a). Effects of tail docking and castration on behaviour and plasma cortisol concentrations in young lambs. Research in Veterinary Science 46, 387–391.

Mellor, D.J., Murray, L., (1989b). Changes in the cortisol responses of lambs to tail docking, castration and ACTH injection during the first seven days after birth. Research Veterinary Science 46, 392–5.

Mellor, D. J., Molony, V., & Robertson, I. S. (1991). Effects of castration on behaviour and plasma cortisol concentrations in young lambs, kids and calves. Research in Veterinary Science 51, 149–54.

Mellor, D.J., Stafford, K.J., 1999. Assessing and minimising the distress caused by painful husbandry procedures in ruminants. Farm Animal Practice 9, 436–446.

Mendl, M., Burnam, O.H.P., Parker, R.M.A., Paul, E.S., 2009. Cognitive bias as an indicator of animal emotion and welfare: emerging evidence and underlying mechanisms. Appl. Anim. Behev. Sci. 118. 161-181.

Millet, S., Gielken, S K., De Brobander, D., Jenssens, G P J., 2011. Consideration on the performance of immunocastrated male pigs. The Animal Consortium 2011, 5:7 1119-1123.

Molony, V., Kent, J. , Viñuela-Fernández,I., Anderson, C. , Dwyer, C M., 2011.Pain in lambs castrated at two days with novel smaller and tighter rubber rings compared with conventional rubber rings and after local anaesthetic treatment. The Veterinary Journal Available online 16 December 2011.

Molony, V., Kent, J., McKendrick, I J., 2002. Validation of a method for assessment of an acute pain in lambs. Applied Animal Behaviour Science 76: 215-238.

Molony, V., Kent, J.E., 1997. Assessment of acute pain in farm animals using behavioural and physiological measurements. Journal of Animal Science 75, 266–272.

Molony, V., Kent, J.E., Hosie, B.D., Graham, M.J., 1997. Reduction in pain suffered by lambs at castration. Vet. J. 153, 205–213.

Molony, V., Kent, J.E., Robertson, I.S., 1993. Behavioural responses of lambs of three ages in the first three hours after three methods of castration and tail docking. Research in Veterinary Science 55,236–245

Molony, V., Kent, J.E., Robertson, I.S., 1995. Assessment of acute and chronic pain after different methods of castration of calves. Applied Animal Behaviour Science 46, 33–48.

Napolitano, F., De Rosa, G., Grasso, F., Wmelsfelder, F., 2012. Qualitative behavioural assessment of diary buffalos (Bubalus Bubalis). Applied Animal Behaviour Science 141, 91-100.

Price, E.O., Borgwardt, R., Blackshaw, J.K., Blackshaw, A., Dally, M.R., Erhard, H., 1994. Effect of early experience on the sexual performance of yearling rams. Appl. Anim. Behav. Sci. 42, 41–48.

Price, J., Nolan, A M., 2001. Analgesia of new born lambs before castration and tail docking with rubber rings. Veterinary Record 2001, 149, 321-324.

Raisen, A. H., Zilbert, D. E., 1975. Behavioural consequences of variations in early sensory environment. In Gregory, N.G., Physiology and Behaviour of Animal Suffering 2004. Blackwell Publishing, The Universities Federation for Animal Welfare, UK.

Risvik, E. 1994. Sensory Properties and Preferences. Meat Science 36:67-77.

Robertson, I. S., H. M. Fraser, G. M. Innes, and A. S. Jones. 1982. Effect of immunological castration on sexual and production characteristics of male cattle. Vet. Rec. 111:529.

Robertson, I. S., J. C. Wilson, and H. M. Fraser. 1979. Immunological castration in male cattle. Vet. Rec. 105:556.

Rutherford, K.M.D., Donald, R.D., Lawrence, A.B., Wemelsfelder, F., 2012. Qualitative Behavioural Assessment of emotionality in pigs. Applied Animal Behaviour Science 139 218-224.

Saňudo, C., Sierra, I., Oleta, J. L., Martin, L., Campo, M. M., Santolaria, P., Wood, J. D., & Nute, G. R. (1998). In¯uence of weaning on carcass quality, fatty acid composition and meat quality in intensive lamb production systems. Animal Science, 66, 175±187.

Schanbacher, B.D., 1982. Responses of ram lambs to active immunization against testosterone and luteinizing hormone-releasing hormone. Am. J. Physiol. 242, E201–E205.

Schanbacher, B.D., 1984. Active immunization against LHRH in the male. In: Crighton, D.B. Ed.,Science, 32(5), 849–858.

Seideman, S. C., Cross, H. R., Oltjen, R. R., & Schanbacher, B. D. (1982). Utilization of the intact male for red meat production: a review. Journal of Animal Science, 55(4), 826–840.

Seksel, K., 2007. How Pain Affects Animals. Proceedings - Australian Animal Welfare Strategy Science Summit on Pain and Pain management, May 2007.

Skinner, J.D., Booth, W.D., Rowson, L.E.A., Karg, H., 1968. The post-natal development of the reproductive tract of the Suffolk ream, and changes in the gonadotrophin content of the pituitary. Journal of Reproduction and Fertility, 16, 463-477.

Stamp Dawkins, M.; 2008 The Science of Animal Suffering; Ethology 114(2008) 937-945 therapy. British Journal of Occupational Therapy. 49: 83-86.

Steiner A , Janett F., (2013). Castration in the bull calf and ram lamb. Nutztierklinik, Vetsuisse-Fakultät Universität Bern. Schweizer Archiv fur Tierheilkunde. 2013, 155(11):603-611.

Synnott, A.L., Fulkerson, W.J., 1984. Influence of social interaction between rams on their serving capacity. Appl. Anim. Ethol. 11, 283–289.

Thompson, D.L. Jr., 2000. Immunization against GnRH in male species (comparative aspects). Animal Reproduction Science 2000, 60-61:459-469.

Thornton, P D., Waterman-Pearson, A E., 1999. Quantification of the pain and distress responses to castration in young lambs. Research in Veterinary Science 1999, 66:107-118.

Thornton, P D., Waterman-Pearson, A E., 2002. Behavioural responses to castration in lambs. Animal Welfare 2002, 11:203-212.

Tilbrook, A.J., Cameron, A.W.N., Lindsay, D.R., 1987. The influence of ram mating preferences and social interaction between rams on the proportion of ewes mated at field joining. Appl. Anim. Behav. Sci. 18,173–184.

Tillbrook, A,J.; 1987b. The influence of factors associated with oestrus on the sexual attractiveness of ewes to rams. Applied animals Behaviour Science 17, 117-28.

Tillbrook, A.J.; 1987a, Physical and behavioural factors affecting the sexual attractiveness of the ewe. Applied Animal Behaviour Science 17, 109-15.

Twigg, J. 1984. Vegetarianism and the meanings of meat. Page 18 in The sociology of food and eating. A. Murcott, ed. Gower Publishing.

Ungerfeld, R., González-Pensado, S.P., 2008a. Social dominance and courtship and mating behavior in rams in non-competitive and competitive pen tests. Reprod. Domest. Anim. 44, 44–47.

Ungerfeld, R., González-Pensado, S.P., 2008b. Social rank affects reproductive development in male lambs. Anim. Reprod. Sci. 109, 161–171.

Wemelsfelder, F., Hunter, T E.A.,. Mendl, M T., Lawrence, A B., 2001. Assessing the 'whole animal': a free choice profiling approach. Animal Behaviour2001, 62, 209-220

Wemelsfelder, F., Hunter, T E.A.,. Mendl, M T., Lawrence, A B., 2000. The spontaneous qualitative assessment of behavioural expressions in pigs: first explorations of a novel methodology for interpretative animal welfare measurement. Appl. Anim. Behev. Sci. 67, 193-215.

Wemelsfelder, F., Nevison, I., Lawrence, A B., 2009. Effect of perceived environmental background on qualitative assessment of pig behaviour. Animal Behaviour 78 477-484.

William O. Reece, 1997. Physiology of Domestic Animals. Williams & Wilkins. A Waverly Company, USA

Wood, G.N., Molony, V., Fleetwood-Walker, S.M., Hodgson, J.C., Mellor, D.J., 1991. Effects of local anaesthesia and intravenous naloxone on the changes in behaviour and plasma concentrations of cortisol produced by castration and tail docking with tight rubber rings in young lambs. Research in Veterinary Science 51, 193–199.

Zoetis website – Information about Bopriva vaccine for Immunocastration of beef cattle. http://www.zoetis.co.nz/products/Bopriva

Zuk, M., 1991. Sexual ornaments as animal signals. TREE 6, 228–23

Annex 1

Figures 1-9 represent QBA results of the assessments carried out by specific observers. Figures show examples of the word charts of the observers. The axes (1 and 2) of these word charts reflect the first two principal axes of the consensus profile, and indicate which terms used by particular observer best correlates with those axes. For each observer all shown terms correlate strongly with the axes of the consensus profile (r values between 0.5 and 0.9), and thus describe these axes reliably. The axes of consensus profile defined by each observer provided important outline for the description of lambs' behavioural expression. Note, that table 9 shown in chapter 3 represents all off the descriptors provided by the observers and indicates which terms of their vocabulary showed the highest positive and negative correlation with the Axis 1 and 2. In conclusion shown in here Word Charts represent the ability of untrained observers (which were allowed to generate their own terms) to display good semantic agreement in the way they have used their own descriptors as coherent outline for the description of lambs behavioural expression.

Figure 10 is showing lamb plots of GPA consensus profile for all lambs that were scored in the study.

Because it was impossible to show raw QBA data scores for each observer table 1a, b is showing example of QBA scores provided by Miguel. Given results are scores provided by Miguel on a visual analogue scale, a line of 12.5 cm (125 mm) ranging from "minimum" (left end of the scale) to "maximum" (right end of the scale) used in this study to score behavioural expression of lambs. Note that results are given in mm.

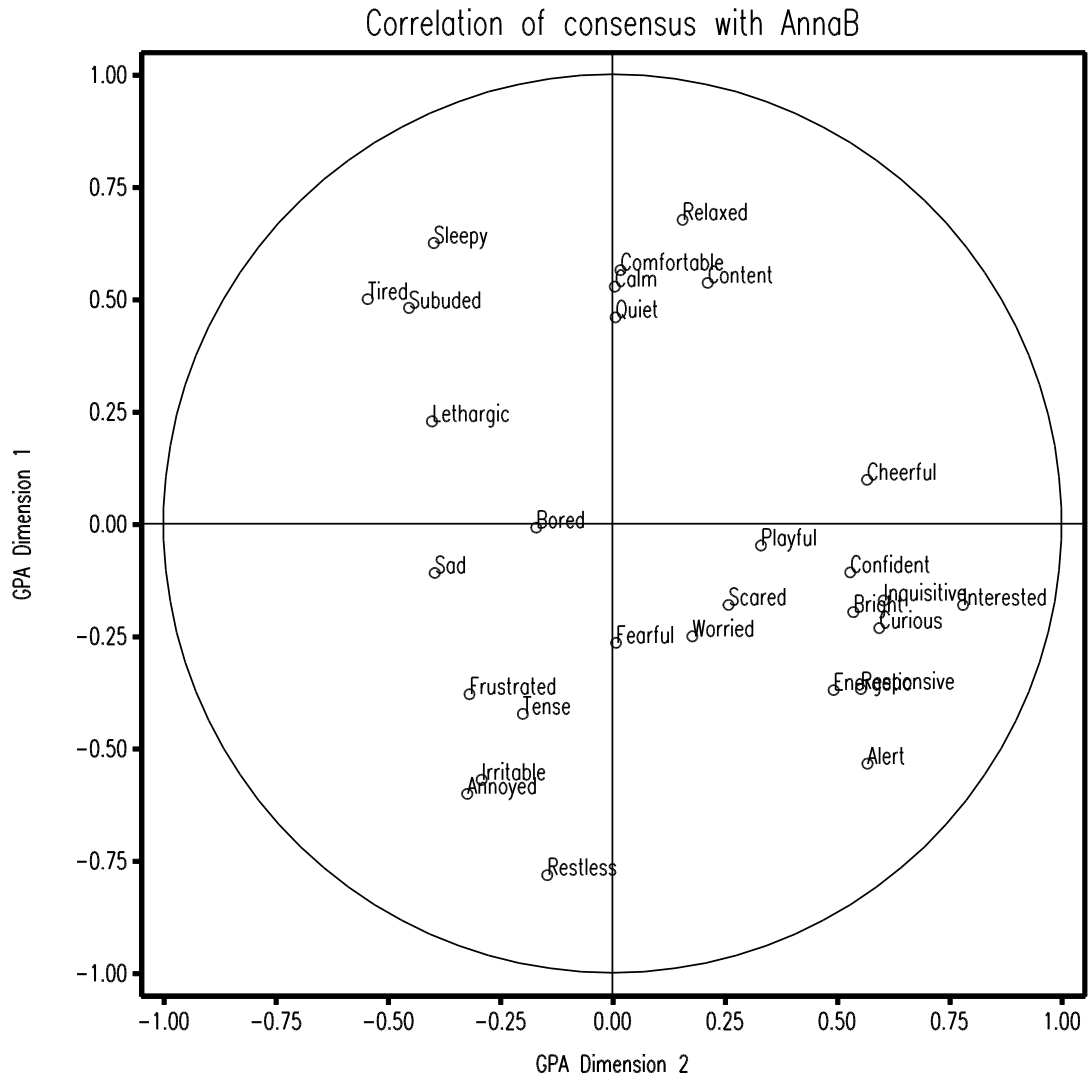


Figure 1 Word chart of observations carried out by Anna B. Axes reflect correlation of observers terms with axes 1 and 2 of consensus profile.

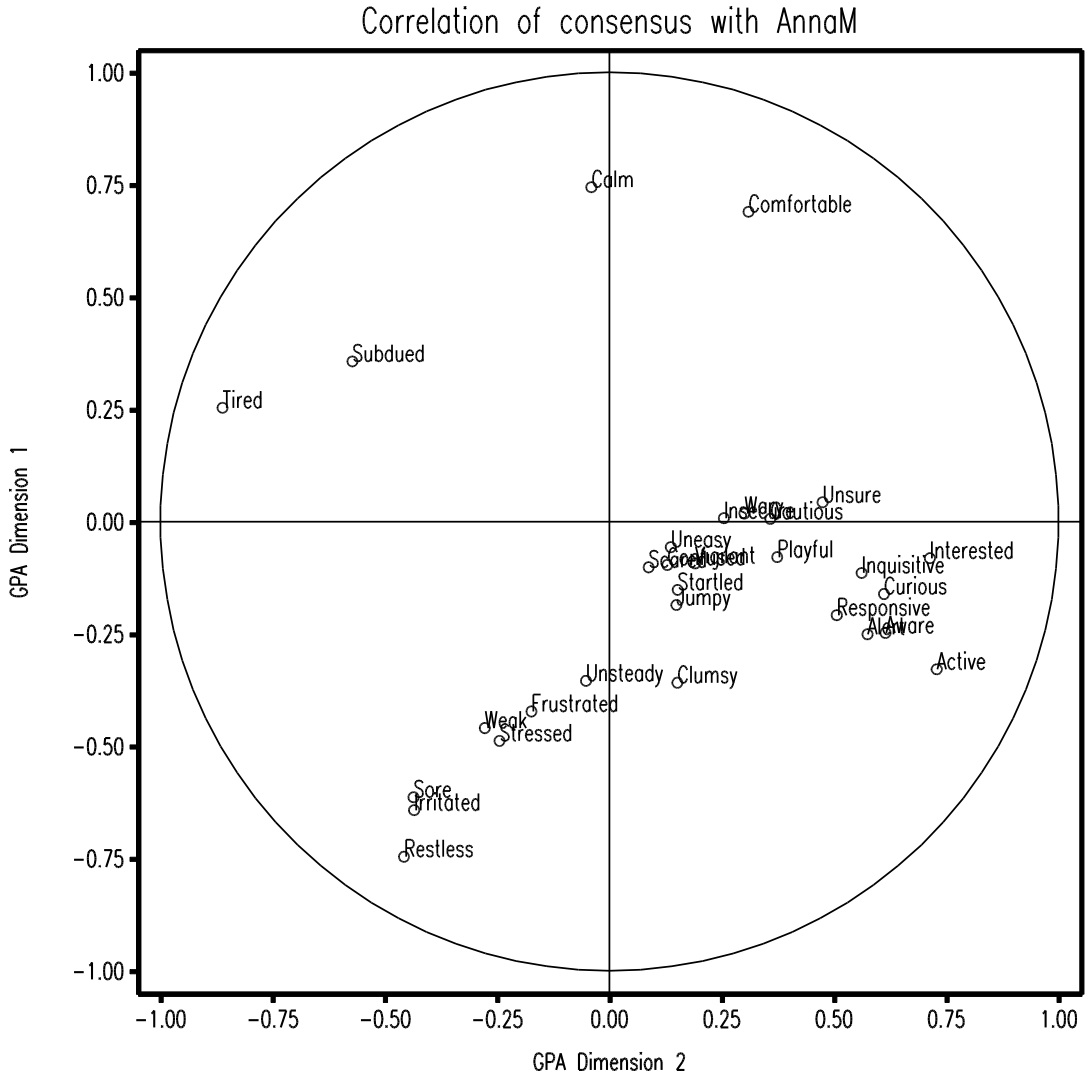


Figure 2 Word chart of observations carried out by Anna M. Axes reflect correlation of observers terms with axes 1 and 2 of consensus profile.

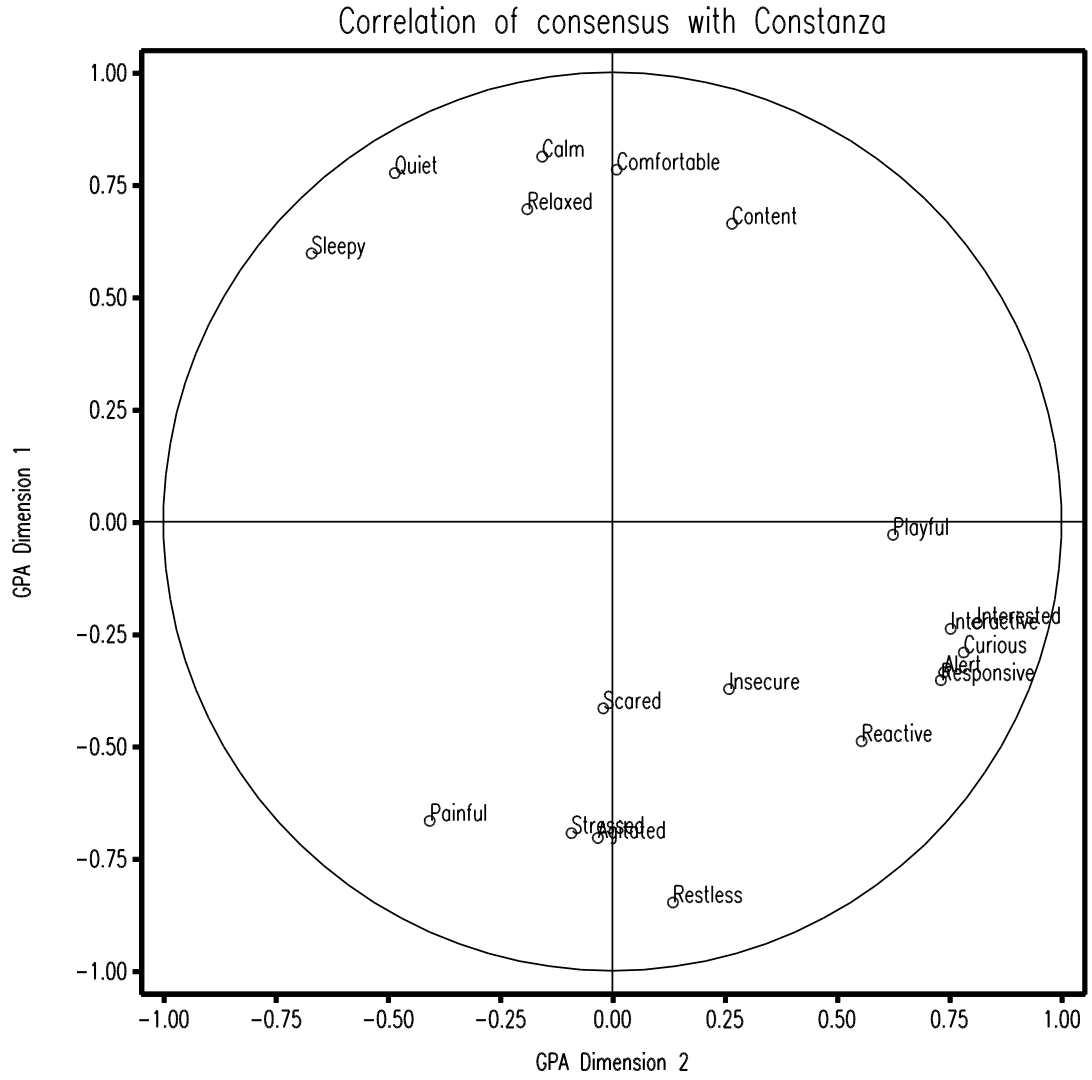


Figure 3 Word chart of observations carried out by Constanza. Axes reflect correlation of observer's terms with axes 1 and 2 of consensus profile.

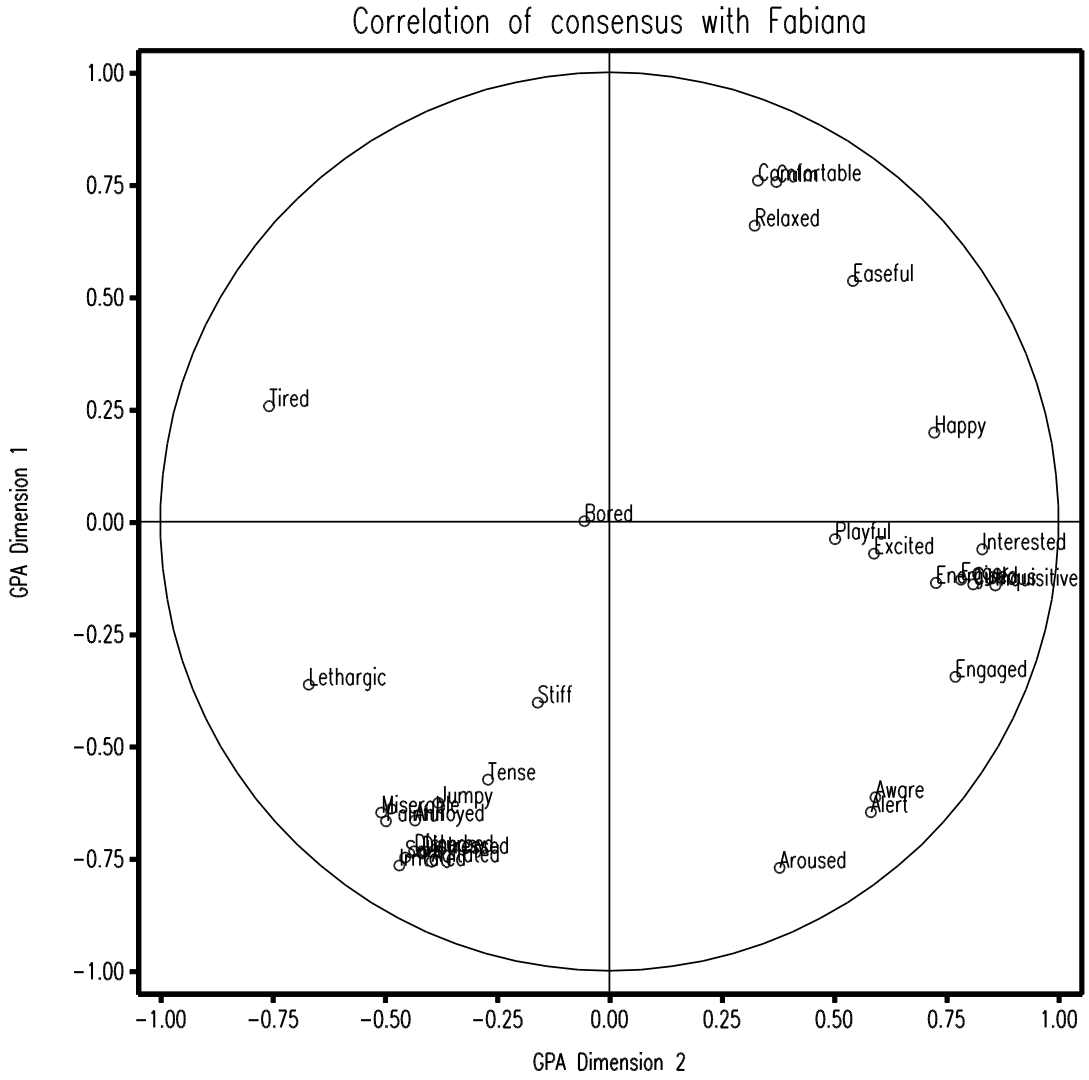


Figure 4 Word chart of observations carried out by Fabiana. Axes reflect correlation of observers terms with axes 1 and 2 of consensus profile.

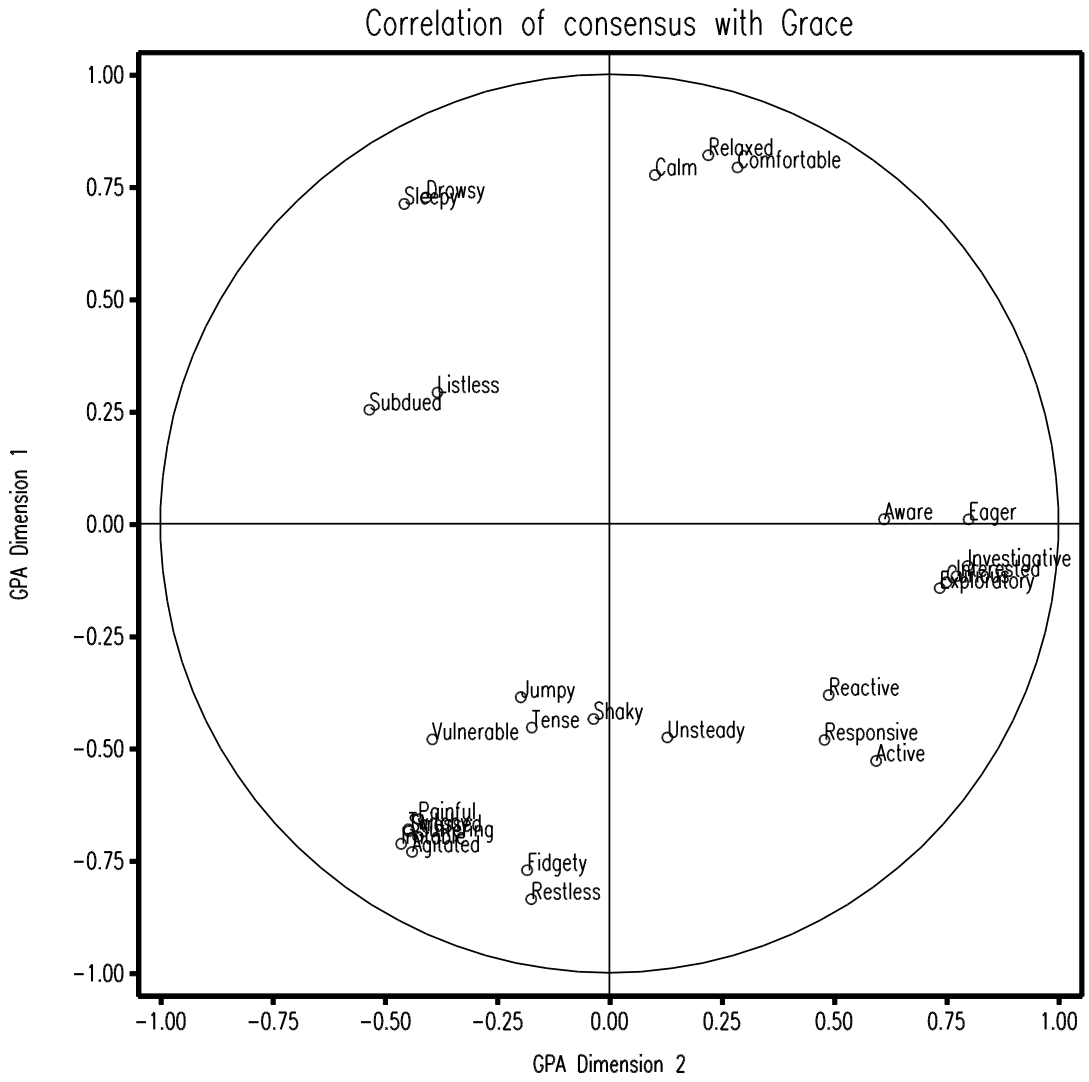


Figure 5 Word chart of observations carried out by Grace. Axes reflect correlation of observers terms with axes 1 and 2 of consensus profile.

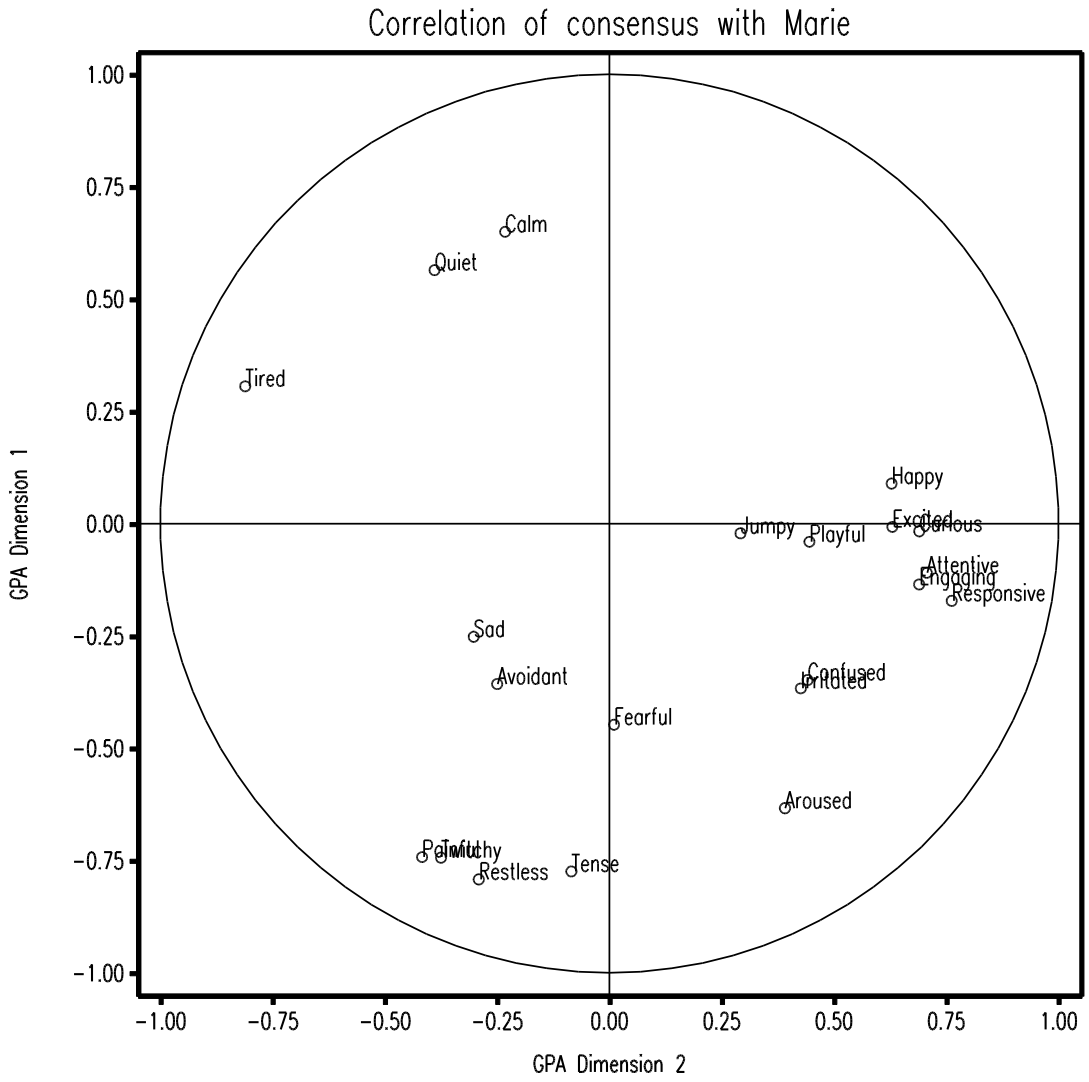


Figure 6 Word chart of observations carried out by Marie. Axes reflect correlation of observers terms with axes 1 and 2 of consensus profile.

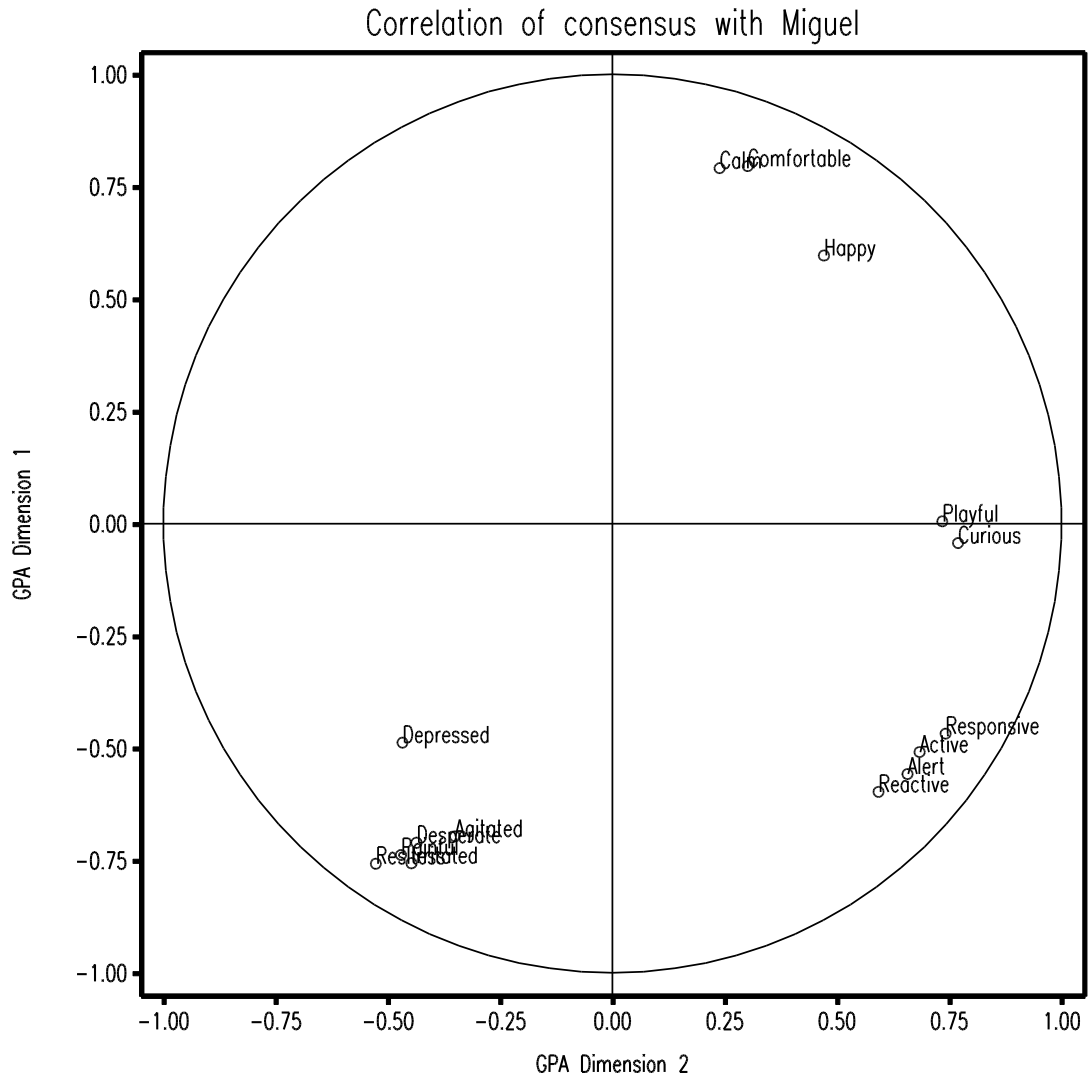


Figure 7 Word chart of observations carried out by Miguel. Axes reflect correlation of observers terms with axes 1 and 2 of consensus profile.

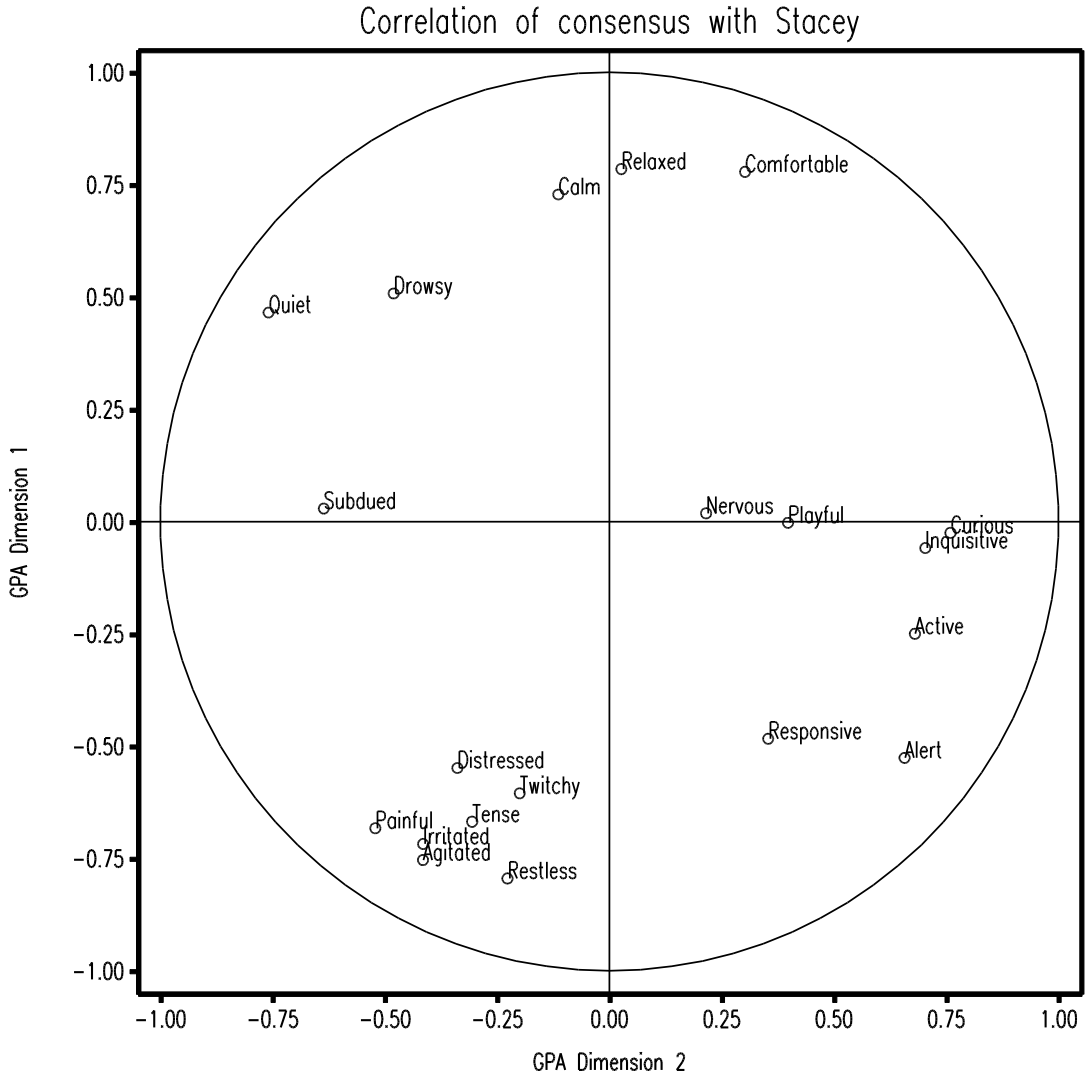


Figure 8 Word chart of observations carried out by Stacey. Axes reflect correlation of observers' terms with axes 1 and 2 of consensus profile.

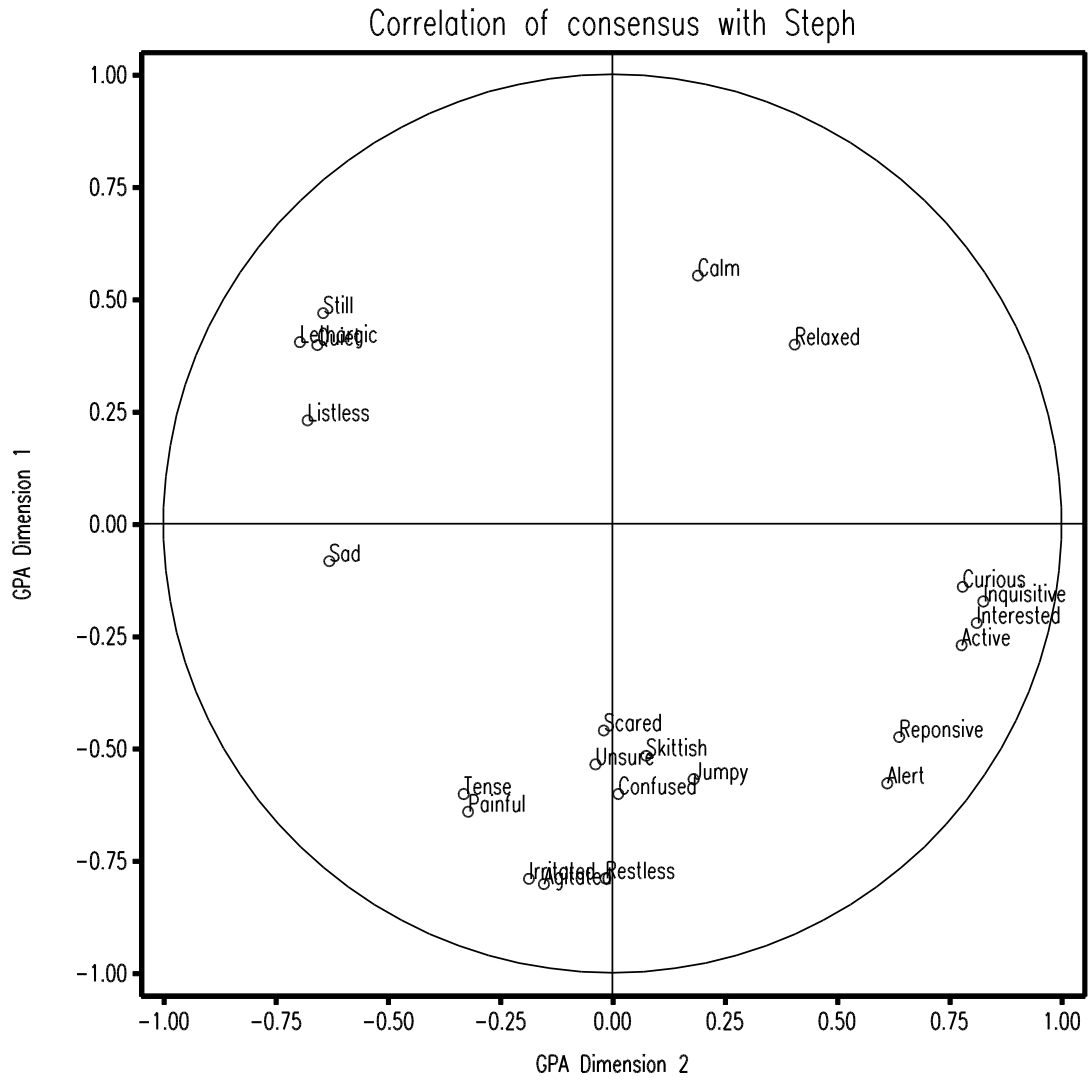


Figure 9 Word chart of observations carried out by Steph. Axes reflect correlation of observers' terms with axes 1 and 2 of consensus profile.

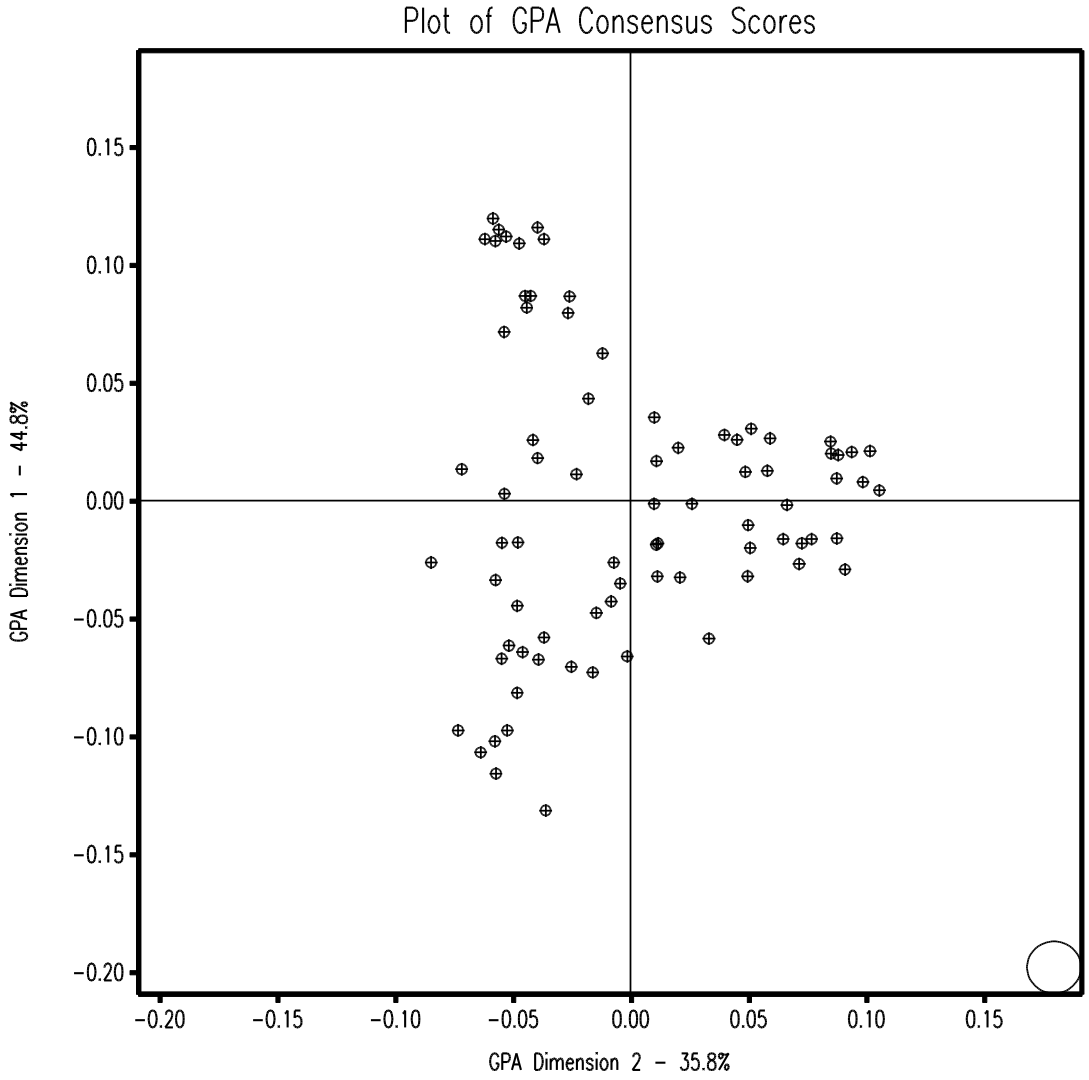


Figure 10 Lamb plots of GPA consensus profile. Axes reflect GPA scaling-values for relative sample (lambs) distance on Axes 1 and 2 of the consensus profile. Dots represent individual lambs. The circle in the right bottom corner reflects the standard error for each lamb's position in the plot. In this study Axis 1(dimension 1) explains 44.8 % of the variation between lambs. Axis 2 (dimension 2) explains 35.8 % of the variation.

Table 1a Example of QBA scores provided by Miguel. Given results are scores provided by Miguel on a visual analogue scale a line of 12.5 cm (125 mm) ranging from “minimum” (left end of the scale) to “maximum” (right end of the scale) used in this study to score behavioural expression of lambs. Note that results are given in mm.

Subject	Active	Curious	Irritated	Responsive	Playful	Agitated	Painful	Alert
Clip1.1control	91	94	3	98	65	42	1	82
Clip1.2combined	1	1	1	8	1	1	1	18
Clip1.3rr	35	1	105	66	1	117	116	88
Clip1.4ss	14	0	92	8	0	84	93	31
Clip1.5control	99	8	0	80	24	3	0	82
Clip1.6combined	3	2	1	14	1	1	0	38
Clip1.7ss	0	0	4	0	0	5	19	4
Clip1.8control	47	15	0	40	6	0	0	79
Clip1.9rr	57	0	120	54	0	112	121	91
Clip1.10ss	80	0	100	56	0	100	100	73
Clip1.11combined	0	0	0	0	0	0	0	0
Clip1.12control	91	86	2	87	84	0	0	88
Clip1.13rr	82	0	105	55	1	94	97	86
Clip1.14rr	106	0	122	75	0	118	100	74
Clip1.15combined	115	117	8	90	22	1	22	96
Clip1.16control	84	77	0	68	48	0	0	81
Clip1.17combined	0	0	0	0	0	0	0	0
Clip1.18ss	58	5	122	25	0	82	84	13
Clip1.19ss	59	82	3	83	5	0	3	74
Clip1.20control	59	60	7	75	5	16	0	80
Clip1.21rr	78	10	91	59	0	76	86	80
Clip1.22ss	90	96	5	77	75	0	4	84
Clip1.23combined	0	0	0	0	0	0	5	0

Clip1.24control	98	62	29	83	0	74	0	87
Clip1.25ss	0	0	0	0	0	0	0	0
Clip1.26combined	45	0	80	60	0	61	73	25
Clip1.27rr	79	10	85	86	0	74	83	17
Clip2.28control	82	76	0	82	83	6	0	85
Clip2.29rr	19	7	82	15	0	43	77	32
Clip2.30combined	7	0	0	16	0	5	0	51
Clip2.31ss	0	0	6	0	0	0	15	0
Clip2.32control	40	20	0	31	10	0	7	76
Clip2.33ss	72	9	71	43	0	31	60	72
Clip2.34rr	6	0	69	7	0	47	47	18
Clip2.35combined	9	0	61	13	0	43	74	9
Clip2.36ss	0	0	0	0	0	0	0	0
Clip2.37rr	6	0	82	0	0	90	90	0
Clip2.38rr	18	0	77	46	0	80	88	68
Clip2.39combined	17	3	74	12	0	39	61	25
Clip2.40control	54	39	5	76	28	0	0	85
Clip2.41control	79	85	4	77	60	3	0	85
Clip2.42combined	0	0	0	0	0	0	0	5
Clip2.43combined	4	6	0	6	2	0	0	42
Clip2.44ss	80	90	0	78	64	1	1	84
Clip2.45rr	78	0	87	47	0	92	83	88
Clip2.46control	78	39	70	73	1	58	50	72
Clip2.47ss	62	18	17	46	12	4	32	58
Clip2.48rr	70	0	115	52	0	10	107	88
Clip2.49combined	74	100	0	86	78	0	0	82
Clip2.50control	52	15	0	62	15	0	0	70
Clip2.51rr	55	32	0	58	4	0	0	75

Clip2.52rr	21	0	64	54	0	0	74	75
Clip2.53ss	24	0	0	62	5	0	7	70
Clip3.54combined	4	2	0	6	0	0	0	15
Clip3.55control	85	93	0	89	80	0	0	93
Clip3.56ss	15	0	38	31	0	40	44	51
Clip3.57ss	42	15	64	27	10	43	74	77
Clip3.58combined	0	0	0	0	0	0	0	0
Clip3.59rr	14	1	58	18	0	41	47	38
Clip3.60control	61	29	4	52	3	0	0	73
Clip3.61rr	18	16	60	39	2	53	49	71
Clip3.62ss	80	78	1	79	39	1	0	82
Clip3.63combined	33	14	20	31	6	2	14	60
Clip3.64ss	96	104	0	72	87	4	0	93
Clip3.65control	19	4	0	22	0	0	0	34
Clip3.66combined	39	34	0	50	35	0	0	68
Clip3.67rr	33	3	30	42	0	24	37	60
Clip3.68ss	14	0	40	33	0	10	35	62
Clip3.69ss	8	0	9	16	0	0	4	35
Clip3.70control	40	28	0	44	30	0	0	72
Clip3.71combined	6	5	7	0	0	0	0	12
Clip3.72rr	10	0	37	32	0	41	55	60
Clip3.73ss	0	0	0	0	0	0	0	0
Clip3.74control	29	16	31	49	5	22	46	75
Clip3.75rr	28	0	23	48	0	57	21	79
Clip3.76rr	68	0	65	20	0	68	77	81
Clip3.77combined	0	0	7	0	0	0	10	0
Clip3.78control	74	68	0	75	44	0	0	90
Clip3.79control	37	35	0	49	12	0	0	73

Table 1b Example of scores provided by Miguel QBA. Given results are scores provided by Miguel on a visual analogue scale a line of 12.5 cm (125 mm) ranging from “minimum” (left end of the scale) to “maximum” (right end of the scale) used in this study to score behavioural expression of lambs. Note that results are given in mm.

Subject	Depressed	Reactive	Comfortable	Desperate	Calm	Happy	Restless
Clip1.1control	1	73	112	0	102	108	0
Clip1.2combined	0	0	123	1	124	112	1
Clip1.3rr	76	78	1	107	1	0	112
Clip1.4ss	42	12	5	47	23	0	111
Clip1.5control	0	72	102	5	113	119	2
Clip1.6combined	2	9	118	0	119	109	1
Clip1.7ss	12	2	53	3	81	62	5
Clip1.8control	0	51	94	0	102	112	2
Clip1.9rr	94	64	0	125	0	0	124
Clip1.10ss	44	69	0	85	16	0	108
Clip1.11combined	0	0	125	0	125	125	0
Clip1.12control	1	83	86	0	95	103	1
Clip1.13rr	12	58	6	70	0	0	93
Clip1.14rr	18	68	1	116	0	0	102
Clip1.15combined	0	55	66	5	68	64	6
Clip1.16control	0	69	84	0	76	75	0
Clip1.17combined	0	0	125	0	120	110	0
Clip1.18ss	30	22	0	105	0	0	104
Clip1.19ss	0	60	54	1	93	14	0
Clip1.20control	0	35	58	0	60	39	0

Clip1.21rr	16	54	0	70	72	0	100
Clip1.22ss	0	74	86	0	82	84	0
Clip1.23combined	4	0	107	0	125	39	0
Clip1.24control	0	90	23	10	17	16	1
Clip1.25ss	0	0	125	0	125	90	0
Clip1.26combined	33	64	13	88	70	0	100
Clip1.27rr	52	42	0	85	0	0	89
Clip2.28control	0	49	90	0	78	94	0
Clip2.29rr	55	41	10	58	0	9	82
Clip2.30combined	0	59	76	4	40	20	4
Clip2.31ss	14	0	78	0	91	18	6
Clip2.32control	0	60	68	0	81	73	0
Clip2.33ss	9	62	0	34	72	0	88
Clip2.34rr	17	56	0	16	12	0	79
Clip2.35combined	66	11	14	44	66	0	82
Clip2.36ss	0	5	111	0	115	100	1
Clip2.37rr	90	7	5	74	1	0	102
Clip2.38rr	48	74	0	93	0	0	112
Clip2.39combined	51	5	3	29	7	0	78
Clip2.40control	0	70	70	0	83	66	1
Clip2.41control	0	75	69	0	75	78	1
Clip2.42combined	0	0	73	0	82	36	0
Clip2.43combined	0	0	77	0	100	10	0
Clip2.44ss	0	70	85	1	86	80	0
Clip2.45rr	51	65	0	77	0	0	101
Clip2.46control	5	64	16	0	57	16	24
Clip2.47ss	0	62	20	0	48	11	30
Clip2.48rr	47	62	0	107	0	0	124

Clip2.49combined	0	75	71	0	76	84	0
Clip2.50control	9	60	67	0	70	61	0
Clip2.51rr	0	26	56	0	36	54	3
Clip2.52rr	0	62	10	54	11	0	38
Clip2.53ss	0	70	20	0	16	20	26
Clip3.54combined	0	0	74	0	98	66	0
Clip3.55control	1	68	76	0	66	94	0
Clip3.56ss	11	17	3	18	24	0	64
Clip3.57ss	0	51	3	49	6	0	72
Clip3.58combined	0	0	125	0	125	84	0
Clip3.59rr	15	33	10	21	16	0	75
Clip3.60control	0	25	74	1	70	66	0
Clip3.61rr	13	57	12	33	5	0	88
Clip3.62ss	0	48	73	0	65	59	0
Clip3.63combined	7	33	11	3	22	3	57
Clip3.64ss	0	74	76	0	50	98	3
Clip3.65control	0	53	50	0	40	29	5
Clip3.66combined	0	64	47	0	30	45	0
Clip3.67rr	3	43	0	14	9	0	64
Clip3.68ss	0	67	1	13	3	0	65
Clip3.69ss	0	0	29	5	11	0	33
Clip3.70control	0	45	82	0	65	64	0
Clip3.71combined	6	17	81	0	82	46	2
Clip3.72rr	13	28	6	67	5	0	75
Clip3.73ss	0	0	125	0	125	78	0
Clip3.74control	0	64	7	58	38	6	60
Clip3.75rr	0	54	0	70	5	0	70
Clip3.76rr	0	78	0	84	0	0	85

Clip3.77combined	10	0	10	0	31	0	19
Clip3.78control	0	88	82	1	70	63	0
Clip3.79control	0	48	65	0	69	70	0

Table 2 Example of the data used to calculate Spearman Rank Correlation of QBA dimensions with quantitative measurements (data correlated with QBA is 2 min frequency data of pain related behaviours and postures extracted form 2 min video-clips used by the observers during QBA scoring)

Group	QBA Clip	dim 1	dim 2	Easing Quarters	Foot stamping/kicking	Teat Seeking	Head Turning	Jump	Trembling Shaking	waggin g tail	Active pain
Control	1	-0.02	0.077	0	0	1	0	0	2	12	15
Combined	2	0.087	-0.05	0	0	0	0	0	0	0	0
Rubber ring	3	-0.08	-0.05	5	3	0	0	0	0	0	8
Short scrotum	4	-0.1	-0.05	4	19	0	4	0	0	0	27
Control	5	0.019	0.088	0	0	1	0	0	0	0	1
Combined	6	0.071	-0.05	1	0	0	0	0	0	0	1
Short scrotum	7	0.082	-0.04	3	0	0	3	0	0	0	6
Control	8	0.025	0.085	0	0	1	0	0	2	2	5
Rubber ring	9	-0.12	-0.06	7	11	0	0	0	0	0	19
Short scrotum	10	-0.1	-0.06	7	1	0	2	0	1	1	15
Combined	11	0.111	-0.06	0	0	0	0	0	0	0	0
Control	12	0.021	0.102	2	0	1	0	0	0	0	3

Rubber ring	13	-0.06	0.033	1	7	0	0	0	0	1	9
Rubber ring	14	-0.13	-0.04	4	10	0	0	0	0	1	15
Combined	15	-0.03	0.05	1	2	0	0	0	0	4	7
Control	16	0.02	0.085	0	0	0	0	0	0	0	0
Combined	17	0.115	-0.06	0	0	0	0	0	0	0	0
Short scrotum	18	-0.07	-0.06	5	8	0	2	0	0	0	15
Short scrotum	19	0.026	0.045	5	0	0	0	0	0	4	9
Control	20	-0.01	0.05	1	0	0	0	0	1	2	4
Rubber ring	21	-0.04	-0	7	2	0	1	0	0	0	11
Short scrotum	22	-0.03	0.091	2	1	2	1	1	3	5	17
Combined	23	0.12	-0.06	0	0	0	0	0	0	0	0
Control	24	-0.03	0.071	0	0	2	0	0	0	3	5
Short scrotum	25	0.111	-0.04	3	0	0	0	0	0	0	3
Combined	26	-0.05	-0.05	7	5	0	0	0	0	1	14
Rubber ring	27	-0.07	-0	5	2	0	0	0	0	0	7
Control	28	0.004	0.106	1	0	1	0	0	3	10	15
Rubber ring	29	-	-	2	3	0	1	0	0	4	10

		0.06	0.05								
Combined	30	0.043	-0.02	1	1	0	1	0	1	3	7
Short scrotum	31	0.112	-0.05	15	1	0	0	1	0	6	25
Control	32	0.026	0.059	1	0	0	0	0	0	0	1
Short scrotum	33	-0.07	-0.02	5	3	2	4	0	0	6	21
Rubber ring	34	-0.02	-0.06	3	1	0	0	0	0	0	5
Combined	35	0.026	-0.04	1	0	0	0	0	0	0	1
Short scrotum	36	0.109	-0.05	0	0	0	1	0	0	0	1
Rubber ring	37	-0.03	-0.09	4	6	0	0	0	0	0	10
Rubber ring	38	-0.1	-0.07	2	11	0	1	0	0	2	16
Combined	39	-0.03	-0.06	8	1	0	0	0	0	0	10
Control	40	0.009	0.087	0	0	2	0	0	0	2	4
Control	41	-0.02	0.073	3	0	1	0	0	3	5	12
Combined	42	0.079	-0.03	1	0	0	0	0	0	0	2
Combined	43	0.087	-0.04	0	0	0	0	0	0	0	0
Short scrotum	44	-0	0.026	5	2	0	0	0	0	0	7
Rubber ring	45	-0.03	0.011	5	5	0	1	0	0	0	11

Control	46	-0.02	0.051	1	1	0	0	0	2	15	19
Short scrotum	47	-0.02	0.065	5	0	0	0	1	1	1	8
Rubber ring	48	-0.11	-0.06	4	6	0	0	0	0	2	12
Combined	49	0.008	0.099	3	0	0	0	0	1	1	5
Control	50	0.012	0.049	1	0	0	1	0	0	2	4
Rubber ring	51	-0.02	-0.012	7	0	0	0	0	0	0	8
Rubber ring	52	-0.02	-0.011	0	2	0	0	0	0	1	3
Short scrotum	53	0.017	0.011	5	0	0	1	0	2	1	9
Combined	54	0.062	-0.01	1	0	0	0	0	0	0	1
Control	55	0.021	0.094	0	0	2	0	0	0	1	3
Short scrotum	56	-0.02	-0.05	3	5	0	1	0	0	0	9
Short scrotum	57	-0.07	-0.03	3	1	0	0	0	1	7	12
Combined	58	-0.11	-0.06	0	0	0	0	0	0	0	0
Rubber ring	59	0.003	-0.05	0	0	0	0	0	0	0	0
Control	60	0.022	0.02	0	0	0	0	0	0	2	2
Rubber ring	61	-0.06	-0.05	5	2	0	0	0	0	1	9
Short	62	-0	0.01	3	0	0	0	0	0	2	5

scrotum											
Combined	63	-0.03	-0.01	7	0	0	0	0	0	0	9
Short scrotum	64	-0.02	0.087	5	0	0	1	2	2	0	10
Control	65	0.035	0.01	1	0	0	0	1	2	0	4
Combined	66	0.013	0.058	1	0	0	0	0	0	0	1
Rubber ring	67	-0.04	-0.01	5	2	0	2	0	0	1	10
Short scrotum	68	-0.06	-0.04	7	5	0	1	0	0	0	14
Short scrotum	69	0.011	-0.02	7	0	0	0	0	0	0	7
Control	70	0.03	0.051	0	0	2	0	0	1	4	7
Combined	71	0.086	-0.03	1	0	0	2	0	0	0	3
Rubber ring	72	0.018	-0.04	7	1	0	1	0	0	1	10
Short scrotum	73	0.116	-0.04	0	0	0	0	0	0	0	0
Control	74	-0.03	0.021	6	0	0	1	0	1	0	8
Rubber ring	75	-0.05	-0.02	9	5	0	4	0	0	0	18
Rubber ring	76	-0.07	-0.04	9	6	0	0	0	0	1	18
Combined	77	0.013	-0.07	4	2	0	0	0	0	0	6
Control	78	-0	0.066	0	0	0	0	0	0	0	0

Control	79	0.02 8	0.04	0	0	0	0	0	0	4	4

Table 3 Example of the data used to calculate Spearman Rank Correlation of QBA dimensions with quantitative measurements (data correlated with QBA is 2 min duration data of pain related behaviours and postures extracted form 2 min video-clips used by the observers during QBA scoring)

Group	QBA Clip	dim1	dim2	Ventral lying	Normal Standing	Abnormal Standing	Normal Lying	Lateral Lying	Dog Siting	Restlessness
Control	1	-0.016	0.077	0	0	86.9	0	0	0	1
Combined	2	0.087	-0.045	80.19	0	0	0	0	0	2
Rubber ring	3	-0.082	-0.048	109	0	0	0	0	0	3
Short scrotum	4	-0.098	-0.052	120	0	0	0	0	0	1
Control	5	0.019	0.088	0	0	120	0	0	0	1
Combined	6	0.071	-0.054	120	0	0	0	0	0	1
Short scrotum	7	0.082	-0.044	120	0	0	0	0	0	1
Control	8	0.025	0.085	0	8.85	111.15	0	0	0	2
Rubber ring	9	-0.116	-0.057	0	0	2.98	0	114	3	2
Short scrotum	10	-0.102	-0.058	74.81	0	7.98	0	0.32	37	6
Combined	11	0.111	-0.062	120	0	0	0	0	0	1
Control	12	0.021	0.102	0	0	84.07	0	0	0	1
Rubber ring	13	-0.059	0.033	33.83	0	51.1	0	27.4	0	7
Rubber ring	14	-0.132	-0.036	0	0	11.04	0	104.1	0	3
Combined	15	-0.032	0.05	17.38	0	38.96	0	0	0	3
Control	16	0.02	0.085	34.49	0	85.51	0	0	0	2
Combined	17	0.115	-0.056	120	0	0	0	0	0	1
Short	18	-0.067	-0.055	106.85	0	13.15	0	0	0	2

scrotum										
Short scrotum	19	0.026	0.045	0	120	0	0	0	0	1
Control	20	-0.011	0.05	0	109.43	0	0	0	0	1
Rubber ring	21	-0.035	-0.004	75.39	0	42.41	0	0	2.2	3
Short scrotum	22	-0.029	0.091	11.54	0	90.52	0	0	18	2
Combined	23	0.12	-0.058	120	0	0	0	0	0	1
Control	24	-0.027	0.071	0	103.46	0	0	0	0	2
Short scrotum	25	0.111	-0.037	120	0	0	0	0	0	1
Combined	26	-0.045	-0.048	114.28	0	0	0	0	5.7	2
Rubber ring	27	-0.066	-0.001	54.96	0	36.3	0	28.74	0	6
Control	28	0.004	0.106	0	69.58	38.48	0	0	0	2
Rubber ring	29	-0.064	-0.046	55.1	0	34.43	0	2.26	0	7
Combined	30	0.043	-0.018	94.03	0	25.97	0	0	0	2
Short scrotum	31	0.112	-0.053	82.3	0	35.05	0	0	2.4	4
Control	32	0.026	0.059	30.11	79.53	0	0	0	0	4
Short scrotum	33	-0.073	-0.016	28.56	0	50.13	0	34.56	6.7	4
Rubber ring	34	-0.018	-0.055	84.86	0	29.86	0	0	5.3	4
Combined	35	0.026	-0.041	17.78	0	27.59	0	0	0	2
Short scrotum	36	0.109	-0.047	120	0	0	0	0	0	1
Rubber ring	37	-0.026	-0.085	120	0	0	0	0	0	1
Rubber ring	38	-0.098	-0.073	0	0	0	0	120	0	1
Combined	39	-0.034	-0.057	0	0	6.32	0	112.6	1	2
Control	40	0.009	0.087	0	0	81.14	0	0	0	1
Control	41	-0.018	0.073	0	0	120	0	0	0	1
Combined	42	0.079	-0.027	116.71	0	0	0	0	3.3	1

Combined	43	0.087	-0.042	0	0	0	0	0	0	0
Short scrotum	44	-0.001	0.026	87.58	0	32.42	0	0	0	2
Rubber ring	45	-0.032	0.011	101.53	0	18.47	0	0	0	3
Control	46	-0.02	0.051	0	95.5	24.5	0	0	0	2
Short scrotum	47	-0.016	0.065	10.24	0	78.51	0	0	0	4
Rubber ring	48	-0.107	-0.064	0	0	6.11	0	59.51	0	4
Combined	49	0.008	0.099	0	35.18	32.33	0	52.49	0	3
Control	50	0.012	0.049	0	23.47	96.53	0	0	0	4
Rubber ring	51	-0.018	0.012	67.23	0	26.87	0	0	2.1	6
Rubber ring	52	-0.019	0.011	10.25	0	29.28	0	0	0	4
Short scrotum	53	0.017	0.011	63.1	0	47.56	0	0	0	3
Combined	54	0.062	-0.012	120	0	0	0	0	0	1
Control	55	0.021	0.094	0	111.46	0	0	0	0	2
Short scrotum	56	-0.018	-0.048	102.55	0	17.45	0	0	0	2
Short scrotum	57	-0.071	-0.025	10.24	0	57.52	0	0	0	3
Combined	58	0.11	-0.057	0	0	0	120	0	0	1
Rubber ring	59	0.003	-0.054	0	0	0	0	31.25	0	1
Control	60	0.022	0.02	0	13.34	31.65	0	0	0	2
Rubber ring	61	-0.062	-0.052	59.62	0	34.74	0	0	2.1	2
Short scrotum	62	-0.001	0.01	20.15	0	33.74	0	0	0	3
Combined	63	-0.026	-0.007	57.93	0	58.01	0	0	4.1	4
Short scrotum	64	-0.016	0.087	9.85	15.71	94.44	0	0	0	6
Control	65	0.035	0.01	0	48.08	0	0	0	0	1
Combined	66	0.013	0.058	0	110.9	9.1	0	0	0	2

Rubber ring	67	-0.043	-0.008	14.6	1.73	33.56	0	39.22	0	5
Short scrotum	68	-0.058	-0.037	97.35	0	10.95	0	0	12	4
Short scrotum	69	0.011	-0.023	120	0	0	0	0	0	1
Control	70	0.03	0.051	0	103.53	0	0	0	0	3
Combined	71	0.086	-0.026	120	0	0	0	0	0	1
Rubber ring	72	0.018	-0.039	120	0	0	0	0	0	1
Short scrotum	73	0.116	-0.039	120	0	0	0	0	0	1
Control	74	-0.033	0.021	18.14	0	101.86	0	0	0	2
Rubber ring	75	-0.048	-0.015	120	0	0	0	0	0	1
Rubber ring	76	-0.068	-0.039	39.67	0	30.54	0	43.56	6.2	5
Combined	77	0.013	-0.072	0	0	0	0	120	0	1
Control	78	-0.002	0.066	0	0	14.6	0	0	0	1
Control	79	0.028	0.04	0	23.69	0	0	0	0	2