Early Immunocastration of Male Pigs

Effects on Physiology, Performance and Behaviour

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Doctoral Thesis
Swedish University of Agricultural Sciences
Uppsala 2011

Acta Universitatis Agriculturae Sueciae
2011:84

Cover: Effects of early immunocastration
on physiology, performance and behaviour.
(Illustration: C. Brunius)

ISSN 1652-6880
ISBN 978-91-576-7628-3

Print: SLU Service/Repro, Uppsala 2011

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Abstract

The aim of this study was to investigate the effects of prepubertal or early pubertal vaccination against GnRH using Improvac® (Pfizer Ltd.) on boar taint, reproductive organs, anabolic hormones, cytochrome P450 enzymes, performance and behaviour. Crossbred male pigs (n=192) were randomly allocated to four groups: one group surgically castrated without anaesthesia, a second group receiving early vaccination (at ages 10 and 14 weeks), a third standard vaccinated group (at ages 16 and 20 weeks), and a fourth group of entire male pigs.

After the second injection, antibody titres increased rapidly and testicular steroids decreased to the low levels of castrates. Reproductive organs were small in vaccinated pigs and smaller after early vaccination. Spermatogenesis and steroidogenesis were disrupted with a more severe, possibly irreversible effect after early vaccination. Oestradiol was suppressed for castrated and vaccinated pigs. IGF-1 was lowest for castrates and highest for entire male pigs, with vaccinated pigs at an intermediate level.

Hepatic CYP450 mRNA expression was highest for castrated and early vaccinated pigs, and lowest for entire male pigs, suggesting suppression at the transcriptional level of CYP1A2, CYP2A and CYP2E1 by testicular steroids. This did not correspond exactly with protein expression and activities, suggesting other regulations for some CYP450s. The levels of skatole and androstenone in adipose tissue were low in castrated and vaccinated pigs, whereas entire male pigs had elevated levels.

Daily weight gain and feed conversion did not differ between groups and income per carcass did not differ between castrated or vaccinated pigs. The income was lower for entire male pigs, due to the additional cost for boar taint analyses and reduced payment for tainted carcasses. After vaccination, the frequency of interactions, both problematic and non-problematic, decreased from the levels of entire males to those of surgically castrated pigs.

Under these conditions, early vaccination with Improvac can be used to control boar taint, testicular function and behaviour with unaffected profitability. Thus, the flexibility of vaccination may be extended, implying advantages in terms of animal welfare and sustainability.

Keywords: Early immunocastration, male pigs, boar taint, androstenone, skatole, testicular function, hormones, cytochrome P450, performance, behaviour.

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Dedication

To my family – past, present and future.

To know that one knows what one knows,
and to know that one doesn't know what one doesn't,
there lies true wisdom

Confucius

The more you know the less you understand.

Lao Tzu

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I **Brunius, C.** and Zamaratskaia, G. A modified high performance liquid chromatographic method for simultaneous quantification of skatole and indole in porcine plasma (submitted).

II **Brunius, C.,** Zamaratskaia, G., Andersson, K., Chen, G., Norrby, M., Madej, A. and Lundström, K. Early vaccination with Improvac® - effects on boar taint, hormones and reproductive organs. *Vaccine* (accepted).

III Einarsson, S., **Brunius, C.,** Wallgren, M., Lundström, K., Andersson, K., Zamaratskaia, G. and Rodriguez-Martinez, H. (2011) Effects of early vaccination with Improvac® on the development and function of reproductive organs of male pigs. *Animal Reproduction Science* 127(1-2), 50-55.

IV **Brunius, C.,** Rasmussen, K. M., Lacoutière, H., Andersson, K., Ekstrand, B and Zamaratskaia, G. (2011) Expression and activities of hepatic cytochrome P450 (CYP1A, CYP2A, and CYP2E1) in entire and castrated male pigs. *Animal* (in press, doi:10.1017/S1751731111001674).

V Andersson, K., **Brunius, C.,** Zamaratskaia, G. and Lundström, K. (2011). Early vaccination with Improvac® - effects on performance and behaviour of male pigs. *Animal* (in press, doi:10.1017/S1751731111001200).

Papers III-V are reproduced with the permission of the publishers.

The contribution of Carl Brunius to the papers included in this thesis was as follows:

I Participated in the planning of the experimental work, solely performed the laboratory work, evaluated the results and was responsible for manuscript preparation.

II Participated in the planning of the experimental work. Participated in the collection of samples and performed some laboratory work. Was responsible for statistical evaluation and interpretation of the results and for manuscript preparation.

III Participated in the collection of samples, was responsible for analysis of steroids and participated in manuscript preparation.

IV Participated in the collection of samples, performed some laboratory work. Was responsible for statistical evaluation and interpretation of the results and for manuscript preparation.

V Participated in statistical evaluation and interpretation of the results and in manuscript preparation.

Abbreviations

COH	Coumarin 7-hydroxylase
CYP	Cytochrome P450
EIA	Enzyme immunoassay
EROD	7-Ethoxyresorufin O-deethylase
FSH	Follicle stimulating hormone
GnRH	Gonadotropin releasing hormone
GnRHR	GnRH receptor
HPG	Hypothalamic-pituitary-gonadal
HPLC	High performance liquid chromatography
IGF-1	Insulin-like growth factor 1
LH	Luteinising hormone
LS	Least squares
LW	Live weight
MROD	7-Methoxyresorufin O-demethylase
PCR	Polymerase chain reaction
PNPH	p-Nitrophenol hydroxylase
RIA	Radioimmunoassay
SE	Standard error of the mean

1 Implications

Immunisation of male pigs against gonadotropin-releasing hormone (GnRH) using a commercial vaccine (Improvac®, Pfizer Ltd.) is an available alternative to surgical castration of male pigs. Standard vaccination involves two injections at least 4 weeks apart, with the second injection up to a maximum of 10 weeks before slaughter.

This study has shown for the first time that an earlier than recommended immunocastration gave results comparable to surgical castration and standard vaccination regarding boar taint, behaviour, performance and income per carcass. This implies that the time span of vaccination can potentially be extended and the flexibility of vaccination increased for farmers that find standard vaccination impractical. With a flexible vaccination schedule, there are potential benefits for both animal welfare and sustainability: (i) surgical castration is avoided; (ii) immunisation can be timed to the sexual maturity of the pigs, effectively controlling problematic boar behaviour and; (iii) vaccinated pigs have a higher anabolic potential than surgically castrated pigs.

Earlier vaccination is a potential alternative to the currently recommended vaccination schedule. However, early vaccination needs to be more extensively studied under different practical conditions before coming to general conclusions and recommendations.

2 Introduction

Since the domestication of mammals, humans have sought to modulate their reproductive systems to avoid uncontrolled reproduction, to control problematic behaviour and, in the case of livestock, to obtain production benefits in terms of increased growth, weight and/or fattening and the suppression of oppressive odours. This control has historically been achieved through physical castration, mostly of males, either by surgery, elastration or emasculation.

2.1 HPG axis

After the discovery of the hypothalamic-pituitary-gonadal (HPG) axis and its role in regulation of the reproductive system (Fig. 1), control has been sought through agonistic or antagonistic interactions using hormonal analogues or immunisation.

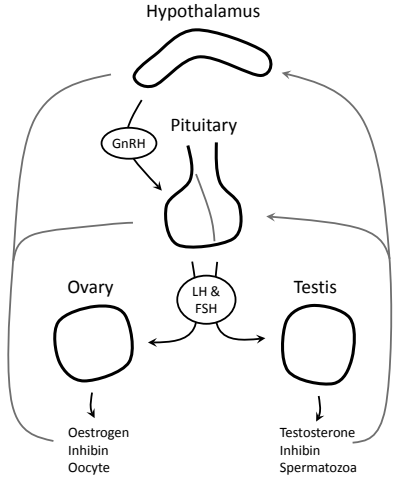

Figure 1. The hypothalamic-pituitary-gonadal axis with key hormones.

The hormonal cascade in the HPG axis is initiated by GnRH, sometimes referred to as luteinising hormone releasing hormone (LHRH), a neuropeptide conserved across all species of mammals, which was first identified in the mid 1960s in bovine hypothalamus (Schally & Bowers, 1964). Schally's group was also first to propose the amino acid sequence (Matsuo *et al.*, 1971). It is a linear decapeptide (Fig. 2) enzymatically produced from the prepro-GnRH precursor in hypothalamic neurons and secreted in a pulsatile manner into the hypophysial portal bloodstream (Thompson Jr, 2000; Millar, 2005; Schneider *et al.*, 2006). After transportation to the gonadotrope cells of the anterior pituitary gland, it binds to the GnRH receptor (GnRHR) which in turn triggers the production of the gonadotropic hormones; luteinising hormone (LH) and follicle stimulating hormone (FSH) (Pawson & McNeilly, 2005). In the female, LH then triggers ovulation and maintains corpus luteum function, while FSH initiates growth of ovarian follicles and subsequent oestrogen synthesis. In the male, LH stimulates Leydig cell production of testosterone, while FSH stimulates spermatogenesis in the seminiferous tubuli. FSH is in both sexes also responsible for the release of inhibin, which together with gonadal hormones imposes feedback regulation of the HPG axis. Whereas the regulation of LH secretion is strongly governed by the GnRH-GnRHR interaction, the regulation of FSH seems to be dependent also on other releasing factors (Li *et al.*, 1998; Padmanabhan & McNeilly, 2001; Schneider *et al.*, 2006).

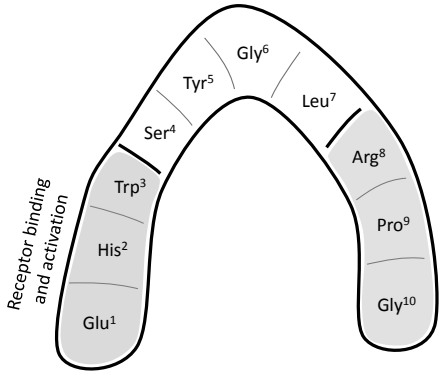

Figure 2. Peptide structure of GnRH in the folded conformation adopted for receptor binding. Both COOH- and NH_2-terminal domains are highly conserved between natural isoforms, indicating importance for receptor binding and activation. Substitution of Gly^6 with D-amino acid enhances activity. D-amino acid substitution of amino acids 1-3, which govern receptor binding and activation, is frequent in GnRH antagonists. Adapted from Millar (2005).

Recently, the neuropeptide kisspeptin was discovered to trigger the release of GnRH through binding with GPR54 receptors on the GnRH neurons. Although the mechanisms of kisspeptin and HPG axis regulation are not yet fully

explained, the influence of kisspeptin/GPR54 has been amply reviewed, for example by Dungan *et al.* (2006), Smith *et al.* (2006) and Clarke (2011). The kisspeptin pathway can also help explain the negative feedback regulation of gonadal steroids on GnRH release, because whereas GnRH neurons seemingly do not express sex steroid receptors, kisspeptin neurons do. Thus, negative feedback on GnRH secretion by steroids seems to be regulated upstream.

Many potential targets exist for the purpose of HPG axis modulation. These targets range from GnRH via gonadotropins to gonadal steroids and even gametes and conceptus. Even kisspeptin has been targeted for research purposes (Kinoshita *et al.*, 2005). The main target has however been GnRH due to its pivotal role in governing the HPG hormonal cascade. The focus in this thesis is on GnRH suppression through prepubertal or early pubertal immunisation. Other targets for immunocontraceptive or immunoneutering purposes have been reviewed elsewhere (D'Occhio, 1993; Meeusen *et al.*, 2007).

2.2 Modulation of GnRH function

So far, the main target for control of sexual development and function has been GnRH since it is the key hormone governing reproductive function through the HPG axis. Owing to the conservation of the peptide structure of GnRH, modulation would thus regulate reproductive function in all mammals. Modulation of GnRH function is applied for either of two main purposes: pro-fertility or anti-fertility. An improvement of fertility of domestic animals is mostly obtained through short-term or low dosage administration of GnRH agonists, thereby stimulating luteinisation and progesterone production (Peters, 2005). Anti-fertility methods, on the other hand, are based on reversible suppression of the HPG axis. Long-term fertility control is of interest in humans, domestic and companion animals and wildlife, especially when some consequences of physical castration, such as permanent sterility, trauma, production setback and increased mortality are undesirable. Suppression is achieved by either: (i) chronic administration of GnRH agonists; (ii) administration of GnRH antagonists or; (iii) immunisation against GnRH (Fraser, 1982; Herbert & Trigg, 2005).

GnRH agonists are GnRH analogues i.e. natural or synthetic peptides bearing structural similarities to mammalian GnRH (Fig. 2), which interact with GnRHR to produce and secrete gonadotropins. The research and development of agonists has focused on improving receptor-binding and activation and consists largely of substituting L-amino acids with D-isomers (Padula, 2005; Schneider *et al.*, 2006). Short-term and/or low level dosage of

an agonist results in a surge of gonadotropins, leading to enhanced gonadal function. Long-term or chronic administration however, after an initial surge of gonadotropins ("flare" effect), causes a down-regulation of GnRHR and desensitisation of gonadotrope cells, in general leading to suppression of gonadal activity (Pawson & McNeilly, 2005).

GnRH antagonists are also GnRH analogues, which however do not induce gonadotropin release. Instead they block the receptors for access by endogenous GnRH, while not inducing receptor activity themselves (Padula, 2005; Schneider *et al.*, 2006). The use of earlier generations of GnRH antagonists was associated with significant histamine release. However, later generations have been developed to avoid this side-effect (Padula, 2005).

Immunisation against GnRH requires a sufficient amount of antibodies against GnRH present in the hypophysial portal bloodstream, leading to the neutralisation of free GnRH before reaching the receptors in the pituitary (Thompson Jr, 2000). Immunisation can be either active or passive, where in active immunisation the subject is injected with an antigen, against which the body develops antibodies. These antibodies later bind to and neutralise endogenous GnRH if the antigen is structurally similar enough to GnRH. In passive immunisation, however, the subject is injected with readymade antibodies raised elsewhere. While passive immunisation is more selective and reproducible, it also requires large volumes of antisera and does not produce prolonged immunological effects and is therefore of little practical use, especially in livestock (van der Lende *et al.*, 1993).

Generating antibodies against GnRH faces two distinct problems: (i) GnRH is a self-peptide and as such is not naturally immunogenic; (ii) GnRH in itself is too small to elicit an immunological response. Antigens must therefore be constructed from GnRH or an analogue conjugated to an immunogenic carrier. The immune system develops antibodies against the conjugate and is thereby deceived into recognising endogenous GnRH as foreign (Thompson Jr, 2000; Herbert & Trigg, 2005).

The first developed vaccines used GnRH adsorbed or bound to carriers, such as polyvinylpyrrolidone, bovine serum albumin or glutaraldehyde (Thompson Jr, 2000). Later vaccines have in general focused on synthetic analogues conjugated to carrier proteins, such as bacterial toxoids or ovalbumin (Ferro & Stimson, 1998; Meeusen *et al.*, 2007). Normally, peptide-carrier conjugates are adjuvanted to improve the immunogenic effect. The more effective the adjuvant, the larger is the provoked immunologic response, with a longer lasting effect. However, this normally also results in increased local tissue response or even damage and may not be possible from a practical or legislative perspective. On the other hand, with a less prominent immune

system response comes the need of booster injections, to guarantee immunisation or prolonged immunisation (Thompson Jr, 2000; Herbert & Trigg, 2005).

There are three main areas of interest for the suppression of GnRH function in animal production: (i) to reduce problematic (aggressive and sexual) behaviour and increase intramuscular marbling in bulls; (ii) to prevent oestrous behaviour and fertility in heifers and; (iii) to control boar taint and problematic behaviour in male pigs (Bonneau & Enright, 1995). Lately, the use of GnRH modulation using analogues has also received attention for the purpose of increasing fertility in livestock, albeit with varied results (Bartolome *et al.*, 2005; Peters, 2005; Brüssow *et al.*, 2011). Although of enormous interest in terms of fertility, production and economy, this particular usage will not be further discussed.

There is an obvious advantage in using immunisation rather than administration of analogues for livestock management in terms of residual components in the meat (EC, 1996). Hormonal analogues are administered continuously for maintained effect, whereas vaccines after injection (several weeks before slaughter) are degraded almost instantaneously. Using vaccination, there is thus no risk of hormonally active or disrupting agents remaining in the final meat or milk products.

2.3 Modulation of GnRH function in cattle

Bulls

Although intact bulls grow more rapidly and have higher feed efficiency than castrated animals, castration of bulls is often performed to control behaviour and enhance the palatability of the meat, through increased marbling and tenderness. Treatment with GnRH antagonists seems to be an effective means of controlling testosterone production in bulls (Jiménez-Severiano *et al.*, 2007), whereas application of GnRH agonists result in increased testicular activity and testosterone concentration, contrary to the general mechanism previously described (Adams, 2005; Jiménez-Severiano *et al.*, 2007).

Immunisation against GnRH has been shown to reduce spermatogenesis and steroidogenesis in bulls, resulting in the advantages of castrates discussed above (Price *et al.*, 2003). Moreover, immunised bulls do not suffer from the disadvantages of diminished growth rate and feed conversion to the same extent as castrated animals. However, immunisation against GnRH still remains impractical due to the large variation in individual response to vaccination (Adams, 2005). Undoubtedly, this variation results to a large degree from individual differences in immunocompetence, but vaccine

formulation in terms of antigen, carrier and adjuvant is also a large contributor. Novel vaccines may improve immunogenic response and increase the potential for GnRH immunisation treatment as an alternative to castration (Theubet *et al.*, 2010).

Heifers

Sexually mature heifers show oestrous behaviour and may also become accidentally pregnant. These potential managerial issues may be addressed through modulation of GnRH function. Treatment of heifers with GnRH agonists, contrary to bulls, may result in effective control of the entire HPG axis through the use of long-lasting depot formulations (D'Occhio *et al.*, 2000; 2002). Depot formulations of hormonally active substances in livestock may however constitute a problem, especially in the EU (EC, 1996). Vaccines have suffered from the previously described variation in individual response. However, promising results on the use of recombinant vaccines against GnRH may increase the potential of immunisation for control of heifer fertility (Stevens *et al.*, 2005; Geary *et al.*, 2006).

2.4 Modulation of GnRH function in male pigs

2.4.1 Boar taint and castration

Surgical castration of male piglets is still common practice to avoid boar taint and to reduce problematic behaviours associated with sexual maturity of the boar (Fredriksen *et al.*, 2009; Lundström *et al.*, 2009). Boar taint is an offensive odour pronounced upon heating of meat products from entire male pigs and is caused mainly by two compounds: androstenone, a steroid synthesised in the Leydig cells of the testes analogously to testosterone (Fig. 1) and; skatole, which is bacterially produced from tryptophan in the large intestine (Zamaratskaia & Squires, 2009). Androstenone, although structurally similar to testicular steroid hormones, possesses no hormonal activity but acts as a pheromone. After synthesis, it is absorbed into the blood, after which some of the circulating androstenone is excreted via the salivary glands and, together with other androst-16-ene steroids, induces the mating stance of the sow (Signoret, 1970; Dorries *et al.*, 1995). Skatole, on the other hand, is to the greater part excreted directly via faeces, although a portion gets absorbed into the blood. After circulation, skatole is metabolised in the liver by cytochrome P450 (CYP450) isoforms 1A2, 2A and 2E1 and subsequently excreted via urine (Fig. 3) (Matal *et al.*, 2009; Zamaratskaia & Squires, 2009).

Androstenone and skatole do not account for all cases of boar taint reported by sensory analysis. Other substances have therefore been suggested to be

important in boar taint. One of these substances, although not as important as androstenone and skatole is indole, a structural analogue to skatole. It is usually measured simultaneously with skatole (Haugen *et al.*, 2011). p-Cresol has also been mentioned as a potential candidate. However, its contribution to boar taint is likely minimal (unpublished results).

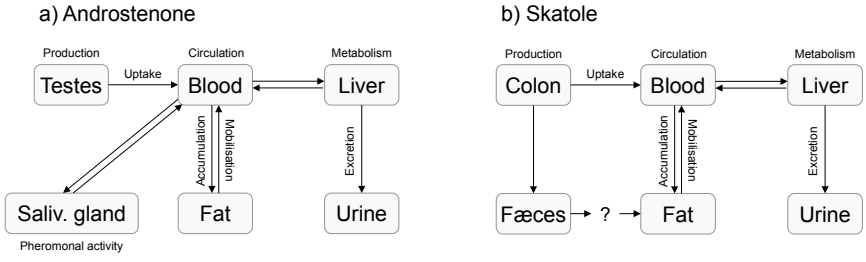

Figure 3. Dynamic aspects of a) androstenone and b) skatole partitioning in the pig. Partitioning dynamics are similar for the two substances, except that: androstenone is excreted from the salivary glands and induces the mating stance in the sow; skatole is partly excreted via faeces, and may under poor management be reabsorbed.

Owing to the lipophilic character of androstenone, skatole and indole, deposition from the blood into the adipose tissue can occur (Fig. 3). The extent of deposition determines whether or not meat is tainted. Although sensory evaluation is the only true measure of taint, threshold values for instrumental measurements of androstenone and skatole concentrations in fat are often employed. There is no international consensus on threshold values, but levels of 0.5-1 µg/g fat for androstenone and 0.2-0.25 µg/g fat for skatole are usually reported in the literature (Walstra *et al.*, 1999).

The level of skatole in fat is determined by the relationship between formation/absorption on one hand and metabolism on the other. The role of gender in the regulation of CYP450 enzyme activities is well established and the metabolism of skatole by CYP1A2, 2A and 2E1 is generally slower in the boar compared to barrows and gilts. It is likely that the gender-related differences are due to the presence of testicular steroids affecting these enzymes. Androstenone and oestrogens which are produced in increased concentrations in the boar testis (Raeside *et al.*, 2006) were suggested to be particularly important in this modulation (Zamaratskaia *et al.*, 2007; Zamaratskaia *et al.*, 2011). Studies on the interaction between testicular steroids and selected CYP450 have shown an effect both *in vitro* (Doran *et al.*, 2002; Zamaratskaia *et al.*, 2007; Chen *et al.*, 2008; Rasmussen *et al.*, 2011c) and *in vivo* (Whittington *et al.*, 2004; Kojima *et al.*, 2008; Zamaratskaia *et al.*,

2008b; Zamaratskaia *et al.*, 2009). Castration thus results in the absence of androstenone formation through the removal of the testes and a subsequent increase in skatole metabolism.

It is generally believed that dominance and sexual and aggressive behaviours of the boar are related to high circulating levels of testosterone (Signoret, 1976; Ellis, 1986; Giersing *et al.*, 2000). After castration, testosterone is only produced in limited amounts in the adrenal gland and aggressive and sexual behaviours are as a consequence reduced (Cronin *et al.*, 2003; Rydhmer *et al.*, 2010).

Animal welfare and sustainability

There are animal welfare issues to consider in raising both barrows and boars. On one hand, the castration procedure is normally performed without the use of anaesthesia or analgesia and most often within the first 7 days of life. This is undoubtedly associated with pain, discomfort and stress, but also an increased risk of infection and mortality (EFSA, 2004; Prunier *et al.*, 2006; Llamas Moya *et al.*, 2008; Fredriksen *et al.*, 2009). On the other hand, boars have in general increased aggressive and sexual behaviour, problems often overlooked in the literature on raising entire males. In feral pigs, boars seem to be divided into dominant, mobile individuals and more sedentary, subordinate individuals (Saunders & Kay, 1991; Hampton *et al.*, 2004). Thus, animal welfare consequences of problematic behaviour related to high testosterone levels are in feral pigs naturally minimised. In a confined space, such as a production site, individuals have no practical means of avoiding conflicts and it is likely that high testosterone and its associated problematic behaviour would negatively affect all three states of animal welfare (physical, mental and telos/naturalness).

Even if raising entire male pigs implies disadvantages in terms of animal welfare, there are some important advantages for sustainability. Entire males generally exhibit increased feed efficiency, leaner carcasses and higher nitrogen retention, through the increased deposition of protein compared to barrows (Xue *et al.*, 1997; Bonneau, 1998; Lundström *et al.*, 2009). Moreover, there are some indications that surgical castration may affect the long-term welfare of pigs in terms of increased morbidity and mortality compared to boars (Tielen, 1974; de Kruijf & Welling, 1988; Strøm, 1996; Lessard *et al.*, 2002), which should also presumably be reflected in decreased productivity.

Public and political concern about the negative impact of castration on animal welfare is continuously increasing. In Norway, this concern resulted in a ban on surgical castration of male piglets without anaesthesia in 2002 and a full ban on surgical castration effective from 2009 although this was later

postponed due to the difficulties in finding viable alternatives. At present, no new date is set for the full ban (Bente Fredriksen, personal communication). In Switzerland, corresponding dates are 2010 and 2015 for the partial and full ban, respectively. In the EU, this concern was embodied in a declaration signed in 2010 by several actors in the European pig sector "committed to a plan to voluntarily end surgical castration of pigs in Europe by 1 January 2018. As a first step, from 1 January 2012, surgical castration of pigs will be performed with prolonged analgesia and/or anaesthesia, if carried out" (EC, 2010). It remains to be seen whether this declaration will practically affect pig management.

Alternatives to surgical castration.

An overview of the entire range of alternatives to surgical castration without anaesthesia is beyond the scope of this thesis. This was reviewed by EFSA (2004) and later updated by von Borell *et al.* (2009). However, a few of the more currently viable alternatives include: (i) raising entire male pigs; (ii) surgical castration with anaesthesia and/or analgesia or; (iii) immunisation against GnRH, so-called immunocastration. Still, genetic selection, although not presently a viable option due to problems with fertility and productivity, represents the long-term, sustainable target.

Raising entire male pigs for meat production is associated with production benefits, but also problematic behaviours (as described above). This type of production may be combined with various techniques or approaches, such as: (i) slaughtering at lower age/weight to reduce the risk of boar taint; (ii) sorting out tainted carcasses at the slaughter line and/or; (iii) learning to live with boar taint through cultural adaptation or accepting a market gap consisting of consumers sensitive to boar taint. Apart from the aspects of raising boars already discussed, to date there exists no satisfactory online method to detect tainted carcasses. Various analytical techniques for research purposes have been developed (Haugen *et al.*, 2011), but unfortunately they either require advanced sample preparation or are too technically demanding for routine use in an abattoir environment. At present, only a small portion of male pigs are raised intact and only in certain markets, such as the UK, Ireland and some specialty products from southern Europe (Fredriksen *et al.*, 2009).

Surgical castration with the use of anaesthesia and/or analgesia is performed to various degrees in some European countries. In Sweden, key actors in the pig sectors have signed an agreement which implores farmers to perform castration in combination with nonsteroidal anti-inflammatory drug (NSAID) administration (SvDHV, 2011). This has in a recent report to the Swedish Board of Agriculture been shown to have only limited effect in suppressing

pain and discomfort in piglets, whereas a combination of local anaestetics and NSAID produces a better effect (Hansson *et al.*, 2010; 2011). In Sweden, the right to administer such substances has been extended to animal keepers from previously being restricted to veterinarians (Jordbruksverket, 2011). This procedure has thus been facilitated. It should be noted that the administration of anaesthesia and/or analgesia also results in extra workload/time/cost and involves extra handling of the piglets causing increased stress (Hansson *et al.*, 2011). In Norway and Switzerland, as previously mentioned, practically all male piglets are castrated under anaesthesia, and in Denmark and Germany castration in combination with NSAID was initiated in 2009. In the Netherlands, castration is performed under CO_2 anaesthesia although not by law, but after an industry agreement. This procedure has been questioned due to the high level of stress in the early induction phase (Mühlbauer *et al.*, 2010) and cannot be deemed a suitable method from at least the point of view of Swedish animal welfare legislation (Hansson *et al.*, 2011).

2.4.2 Agonistic GnRH modulation

Androstenone production is governed by LH secretion and GnRH release patterns (Fig. 4). Long-acting GnRH agonist administration to mature or pubertal boars was shown to diminish androstenone concentration (Xue *et al.*, 1994; Schneider *et al.*, 1998; Kauffold *et al.*, 2010). However, in some cases the variation in individual response limits this approach from a commercial perspective. Again, the use of depot formulations of hormonally active compounds may limit its commercial use in livestock in some markets (EC, 1996).

2.4.3 Immunocastration

Immunisation against various antigens has been studied. Of the potential targets for immunisation, GnRH seems the most viable candidate (Oonk *et al.*, 1998; Dunshea *et al.*, 2001; Jaros *et al.*, 2005; Zamaratskaia *et al.*, 2008a; Fang *et al.*, 2010; Turkstra *et al.*, 2011).

A plausible general approach for antigen formulation would be a modification of amino acids 1-3 i.e. the region responsible for binding and activation of the GnRH receptor (Fig. 2). This would render the antigen hormonally inactive, but still effectively result in specific antibodies against GnRH. Such a peptide could be obtained by deletion of the first amino acid in the sequence, which would render the antigen virtually intact, while effectively hindering receptor binding and activation (McNamara, 1988; 2009; 2010). Other approaches include polymerisation of the GnRH (or analogue) sequence (Oonk *et al.*, 1998) or recombinant GnRH fusion antigens (Fang *et al.*, 2010).

Immunocastration of male pigs has been performed commercially since 1998 in Australia and New Zealand using Improvac (Pfizer Inc., formerly CSL Ltd). The said vaccine was in 2009 approved for use in the EU. The mode of action is through active immunisation against GnRH and subsequent control over the HPG axis (Fig. 1 and 4). Immunocastrated pigs are in effect boars until successfully immunised, normally 4-6 weeks before slaughter, and thus have the production benefits of the boar discussed above. After immunisation, the reduced levels of anabolic hormones might however negatively affect performance and therefore lead to increased costs. Such negative effects have not been noticed following standard vaccination (Dunshea *et al.*, 2001; Cronin *et al.*, 2003; Zamaratskaia *et al.*, 2008a). Immunising at an earlier stage might however result in lower growth rate and fatter carcasses.

The control over testicular function obtained after immunisation decreases the problematic behaviours of the boar. The painful incision of the standard castration procedure with its associated increased risk of infections is also avoided. Immunocastration thus has a large potential for increased animal welfare. Disadvantages of this procedure include an increased workload/time/ cost compare to entire male pigs, a perceived sense of unnaturalness associated with the modulation (or manipulation) of a "normal" or "natural" hormonal pathway and the risk of accidental self-injection, which after two injections would result in temporary suppression of the HPG axis and associated symptoms also in humans. Accidental self-injections are effectively avoided through the use of a safety vaccinator, "which has a dual safety system providing both a needle guard and a mechanism to prevent accidental operation of the trigger" (EMA, 2010). Nevertheless, vaccine injections should not be performed after an accidental first self-injection or by pregnant women.

The exact structure of Improvac has not been divulged; however, it consists of a synthetic peptide GnRH analogue conjugated to a diphteria toxoid carrier protein and is delivered in an aqueous dextran-based adjuvant (EMA, 2010). The manufacturer has shown that their vaccine antigen is hormonally inactive (Clarke *et al.*, 2008) yet effectively produces specific antibodies against GnRH. The vaccine is given subcutaneously as two doses of 2 mL at least 4 weeks apart with the first injection after age 8 weeks and the second 4-6 weeks before slaughter although the risk of boar taint is minimal as much as 10 weeks after the second injection according to the manufacturer (EMA, 2010).

What distinguishes Improvac from most of the non-commercialised vaccines is its high efficacy although other vaccine formulations have shown promising results (Fang *et al.*, 2010; Turkstra *et al.*, 2011).

Vaccination with Improvac results in active immunisation against GnRH and a subsequent decrease in LH and FSH secretion and testicular function,

including androstenone synthesis (Dunshea *et al.*, 2001) (Fig. 4). Later studies have confirmed the efficacy of this vaccine formulation (Jaros *et al.*, 2005; Zamaratskaia *et al.*, 2008a; Pauly *et al.*, 2009).

Immunisation against GnRH was shown to increase the activity of hepatic CYP1A, 2A and 2E1 (Zamaratskaia *et al.*, 2009) from the lower levels of entire male pigs similarly to surgical castration (Gillberg *et al.*, 2006; Zamaratskaia *et al.*, 2006). The increased activity is most likely related to a decrease in androstenone and oestradiol concentrations. The inhibition of the skatole metabolising system by these steroids is decreased and skatole concentrations are reduced (Zamaratskaia & Squires, 2009).

The vaccine is thus as efficient in controlling fertility, behaviour and boar taint as surgical castration, for which e.g. chryptorchidism must be considered.

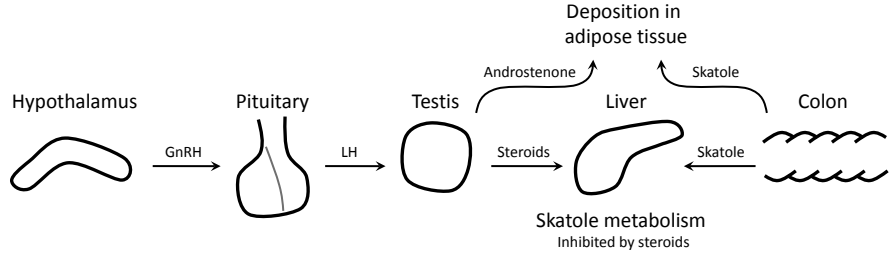

Figure 4. Relationship between the HPG axis, androstenone production and skatole metabolism. In the boar, production of testicular steroids, including androstenone, in the HPG axis inhibits hepatic skatole metabolism. Androstenone and skatole are therefore deposited in the adipose tissue. In the immunocastrate, the lack of free circulating GnRH arrests testicular steroid production including androstenone. Skatole metabolism is increased due to the lack of inhibition.

The effects of immunisation are supposedly transient and a return of functionality is initialised after antibody titres have decreased below a threshold level (Hilbe *et al.*, 2006; Claus *et al.*, 2008). In fact, the manufacturer indicates effective control over boar taint up to 10 weeks after the second injection. However, it has been shown that the effects of Improvac can be present at least 22 weeks after the second injection (Zamaratskaia *et al.*, 2008c). Moreover, the long-term effect of immunocastration, or even the reversibility, may be questioned if the procedure were to take place at a lower age i.e. if testicular development was hindered at a crucial developmental stage (Setty, 1979; Sofikitis *et al.*, 2008).

Although, as mentioned, Improvac is approved for use in many markets, the practical use of this vaccine has been limited. There is at present no routine in Sweden or other EU member states excluding Belgium for how to treat meat from vaccinated animals in the chain from abattoir to consumer. In Belgium, however, the large retailer Colrouyt has adopted a policy where meat from

male pigs is exclusively from vaccinated pigs. Moreover, organic producers in Sweden have expressed a desire to use immunocastration (Lars Hellbom, KRAV, personal communication). However, this is at present not perceived as compatible with the EU Regulation on ecological production (EC, 2007; KRAV, 2011).

2.5 Adverse effects of GnRH function modulation

As already stated, suppression of GnRH function leads to suppression of the entire HPG axis. Such therapies thus provoke effects related to low levels of gonadal hormones. In female mammals, this translates into symptoms normally related to menopause in humans, such as decreased libido, mood alterations and osteoporosis. In the male, effects include increased risk of obesity, diabetes, cardiovascular diseases and osteoporosis (Saylor *et al.*, 2009). Whether these effects are adverse or not depends on the application and the time-scale of the therapy. In human GnRH therapy for treatment of endometriosis (Batzer, 2006) or cancer (Chengalvala *et al.*, 2003; Montagnani Marelli *et al.*, 2006), most notably prostate cancer (Huhtaniemi *et al.*, 2009), these effects must be considered adverse. However, for immunocastration of male pigs, such effects as decreased libido and mood alterations are in fact desired.

Moreover, it is known that GnRH receptors are also present in many extra-pituitary tissues, e.g. breasts, gonads, heart and central nervous system (Ramakrishnappa *et al.*, 2005; Rispoli & Nett, 2005). GnRH is thus not only the pivotal hormone regulating reproduction, but seems to also be a neurotransmitter with physiological effects throughout the central nervous system. Suppression of GnRH function, either by analogues or immunisation, can thus potentially alter important physiological functions other than reproductive function (Kirkpatrick *et al.*, 2011).

3 Aims

This is the first study to investigate prepubertal or early pubertal immunisation of male pigs against GnRH. The rationale was created from the combination of the potential long-term effects of immunological castration together with a desire among some farmers to avoid group treatment of older and heavier pigs (Einarsson, 2006). An aim was also to discuss animal welfare and sustainability aspects of immunocastration, in particular the early vaccination schedule. Aspects that have been investigated include:

- antibody response
- size and function of reproductive organs
- hormones with anabolic effects
- regulation of skatole metabolising enzymes
- boar taint substances
- performance and carcass quality
- behaviour and
- long-term effects of early vaccination.

4 Materials and methods

4.1 Experimental design (II-V)

A total of 192 crossbred male pigs (Swedish Yorkshire dams × Swedish Landrace or Swedish Yorkshire sires) from 40 litters were used in this study, comprising two identical trials each with 96 pigs. The study was performed at Funbo-Lövsta Research Station, SLU, Uppsala, in accordance with Swedish regulations for use of pigs. The sires used were randomly selected from sires available for artificial insemination.

Piglets within litter were in each trial randomly allocated to four equally sized groups at birth. In the first group, piglets were surgically castrated without anaesthesia before the age of one week. Pigs in the second, early vaccination, group were vaccinated with Improvac (Pfizer Ltd., Sandwich, Kent, UK, 2 mL per injection) when aged 10 weeks (72.5 ± 7.2 days (mean ± standard deviation); live weight (LW) 28.9 ± 7.1 kg) and 14 weeks (100.0 ± 7.1 days; LW 47.3 ± 9.9 kg). Pigs in the third, standard vaccination, group were vaccinated when aged 16 weeks (114.0 ± 7.1 days; LW 58.9 ± 11.3 kg) and 20 weeks (142.0 ± 7.1 days; LW 86.2 ± 14.4 kg). The injections were given just behind and below the base of the ear (200 µg GnRH analogue-protein conjugate/mL). Pigs in the fourth, entire male, group were kept intact throughout the study. No placebo substance was administered.

In Papers III and IV, a smaller subset of the population consisting of 8 individuals per treatment group from the first trial was investigated.

The growing/finishing period started when the pigs were at an age of 72.3 ± 7.0 days and had an initial LW of 29.3 ± 7.4 kg. Each pen held 8 pigs from only one treatment group. All pigs were fed restrictedly with the same commercial diet (12.4 MJ ME per kg, digestible CP 13.5%) twice a day according to the standard feeding regimen for growing/finishing pigs in Sweden (Andersson *et al.*, 1997). Pigs were weighed individually at the start of the study then fortnightly until their final weighing one day prior to slaughter.

Feed consumption was recorded on a daily basis and feed conversion ratio was calculated pen-wise. During the growing/finishing period, two surgically castrated, two early vaccinated and one entire male pig died or had to be euthanised for reasons not related to the study.

Blood samples were taken by jugular venipuncture from all pigs on four occasions: before the first Improvac injection of both vaccination regimens i.e. at 10 and 16 weeks of age (72.8 ± 7.1 and 114.8 ± 7.1 days, respectively), thereafter at an age of 22 weeks (156.8 ± 7.1 days) and one day prior to slaughter (25 weeks, 177.0 ± 8.3 days). To obtain plasma, blood samples were collected into heparinised vacutainer tubes, separated by centrifugation at 2000 *g* and stored at –80°C until analysis. To obtain serum, blood samples were collected in vacutainer tubes without heparin, separated by centrifugation at 2000 *g* and stored at –20°C until determination of antibodies against GnRH.

Slaughter was performed in a commercial Swedish abattoir according to standard procedure on two occasions per pen at an average age of 25 weeks (178.0 ± 8.3 days) and an average LW of 119.3 ± 10.2 kg with the four heaviest pigs slaughtered on the first occasion. All pigs were mixed with unfamiliar pigs during transport (350 km) and lairage, to simulate normal transport and slaughter conditions. Before cooling, carcass weight was recorded and lean meat content was evaluated with a Hennessy Grading Probe. The amount of abdominal fat was recorded.

Samples of adipose tissue were taken from the neck region of the carcass and kept at –20°C until analysis. Testes and bulbourethral glands were removed and dissected from extraneous tissue. The lengths of both bulbourethral glands were measured to record the average length, and testes were weighed as pairs. Details of sampling of proximal and distal testes and cauda epididymal content are described in Paper III. Additionally, skin lesions were visually recorded at slaughter by one observer using a 6-point scale (0: no visible skin damage; 5: very highly damaged skin). These records were used to classify the pigs into two groups: without skin lesions or with skin lesions. Pigs with only a few light skin lesions (score 0 or 1) were classified as ‘without’. Vaccination schedules and sampling are graphically represented in Fig. 5.

Daily lean meat growth from start of the experiment to slaughter was calculated using a formula of our own invention: % lean × (carcass weight - initial weight × 0.72) / days in experiment, with the value 0.72 representing a hypothetical dressing percentage at start. The income per carcass was calculated on the basis of carcass weight and estimated lean meat content, according to prices from the Swedish co-operative slaughterhouse, contract note November 2010. For entire male pigs, payment was reduced owing to

boar taint analysis and tainted carcasses according to standard procedure. Prices were converted from SEK into EUR (1 SEK = 0.11 EUR).

The study was approved by the local Ethics Committee on Animal Research, Uppsala, Sweden, ensuring compliance with EC Directive 86/609/EEC for animal experiments.

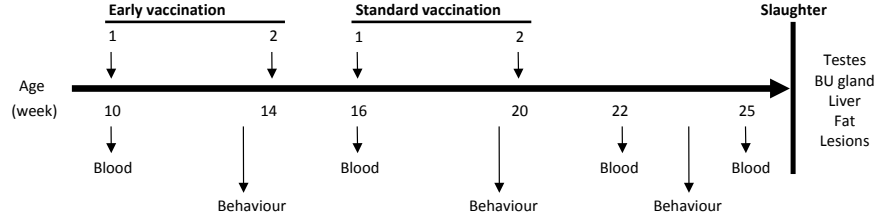

Figure 5. Time schedule for experimental design and sampling.

4.2 Biochemical analyses (I-IV)

Skatole in plasma was quantitated by HPLC as described by Zamaratskaia *et al*. (2004a), modified and validated by Brunius and Zamaratskaia (Paper I). Androstenone, skatole and indole concentrations in fat were measured using HPLC as described by Chen *et al*. (2006). Antibodies against GnRH were measured by Frontage Laboratories Co., Ltd., Shanghai, P.R. China, using an in-house validated ELISA method (SHAM-043-R0).

Testosterone concentration in plasma was measured using a commercial RIA kit (TKTT, Diagnostic Products Corporation, Los Angeles, CA, USA), according to the manufacturer's instructions. Insulin-like growth factor 1 (IGF-1) concentration in plasma was measured using a commercial EIA kit (DSL-10-2800, Diagnostic System Laboratories, Webster, TX, USA) in accordance with the manufacturer's instructions. Oestradiol-17β concentration in plasma was measured using a commercial EIA human salivary kit (1-3207, Salimetrics, State College, PA, USA), through an adaptation of the manufacturer's protocol (Paper II).

Chemical analyses were performed in duplicate, except oestradiol and IGF-1 analyses for the surgically castrated and vaccinated groups, for which one analysis per sample was performed. Surgically castrated pigs were excluded from anti-GnRH analyses since it is known that they are at the same low level as entire male pigs (Zamaratskaia *et al.*, 2008a).

4.3 Testicular histology and spermatozoal morphology (III)

Testes samples were paraffin-embedded and conventionally sectioned and stained with haematoxylin and eosin for histological analysis. The tubular diameter in each specimen was measured manually using an ocular micrometer when examining the slides at 100× magnification.

At the semen laboratory, sperm morphology was evaluated by experienced laboratory assistants in wet formol saline-fixed preparations (Bane, 1961) and in air-dried smears stained with carbolfuchsin-eosin according to the method described by Williams (1920) and modified by Lagerlöf (1934). In the wet smears, 200 spermatozoa, when possible, were checked under a phase contrast microscope (1000× magnification). All abnormalities on any given spermatozoon were counted and the overall frequencies were classified according to Bane (1961). For a more detailed examination of the sperm head, when possible, 500 spermatozoa were checked in each stained smear under a light microscope at a magnification of 1000×. Sperm head morphology was classified according to Lagerlöf (1934). The morphological abnormalities were expressed as a percentage of the total number of counted spermatozoa.

4.4 CYP1A, 2A and 2E1 (IV)

Microsomal preparation

Microsomes were prepared by a calcium aggregation method using TRIS-EDTA homogenisation buffer (Rasmussen *et al.*, 2011a). Protein concentrations in the microsomes were assayed with a commercially available kit (Bio-Rad Laboratories Inc., Hercules, CA, USA) according to the manufacturer's instructions. Microsomes were diluted to a protein concentration of 4 mg/mL and stored at –80°C until use within 1 month of preparations.

Real-time RT-PCR

Isolation of mRNA, reverse transcription and PCR were performed as described by Rasmussen *et al*. (2011d). Relative mRNA expression was calculated from the obtained Ct values and normalised against mRNA expression of GAPDH. The average expression in the group of entire male pigs was arbitrarily set to 1.

Western blot

Western blotting was performed according to Rasmussen *et al.* (2011b). The antibodies used were all raised against a human epitope but have been shown

to be specific against porcine cytochromes (Rasmussen *et al.*, 2011d). Relative protein expression was quantified using ImageJ (version 1.43u, NIH, USA) and expressed relative to the average of the group of entire male pigs.

Microsomal enzyme activity

The CYP-catalyzed activities of the following enzymes were measured: 7-ethoxyresorufin O-deethylase (EROD; CYP1A1/1A2); 7-methoxyresorufin O-demethylase (MROD, CYP1A2); coumarin 7-hydroxylase (COH; CYP2A) and; p-nitrophenol hydroxylase (PNPH; CYP2A/2E1). EROD and MROD activities were measured as described by Zamaratskaia and Zlabek (2009), and COH activities as described by Zamaratskaia *et al.* (2009). The activity of PNPH was measured using a simplified HPLC-based method (Paper IV).

4.5 Behavioural analyses (V)

Activity behaviours and social interactions of pigs from all groups were studied by direct observations on three occasions per pen: at ~13 weeks (the week before the second injection of the early vaccinated pigs), at ~19 weeks (the week before the second injection of the standard vaccinated pigs), and at ~22 weeks (2-3 weeks before slaughter) (Fig. 5). The observations were recorded by one observer standing outside the pen. Observations did not start until the pigs were accustomed to and no longer seemed to pay attention to the observer. All observations were performed between 10:00 and 15:30 i.e. outside feeding time.

Nine observation rounds per pen were made in a consecutive pen order to distribute the observations of pens equally over time, and each pen was studied for a 10-min session per round. The observations consisted of two kinds of sampling and recording: instantaneous scan sampling of activity behaviours, and continuous recording of frequencies of social interactions (Fig. 6). Instantaneous scan samplings of activity behaviours were performed at the beginning and end of each observation round. Between these scan sampling observations, frequencies of social interactions were recorded for a total of 8 min. Thus, one observation day gave 18 instantaneous scan samples and a total of 72 min of social interactions per pen. The behavioural observations were performed over a total of 4 days per occasion.

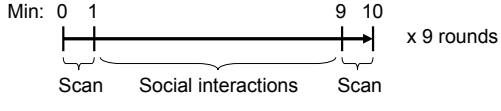

Figure 6. Time schedule of an observation round for behavioural analysis.

The definitions of the behaviour parameters are presented in Table 1 and 2. Contact during scan sampling included aggressive as well as non-aggressive behaviour. In the continuous frequency recording of social interactions, problematic (aggressive and mounting) and non-problematic (sniffing, pushing, crowding, manipulating pen mate and playing) interactions were recorded separately.

Table 1. *Definitions of behaviour parameters during scan sampling.*

Behaviour parameter	Definition
Resting	Lying or sitting down
Standing	Standing, walking or running
Eating	Head in the trough or waiting for feed beside the trough or drinking
Contact	Touching another pig in some way including mounting

Table 2. *Definitions of behaviour parameters during continuous recording.*

Behaviour parameter	Definition
Non-problematic	
Sniffing	One pig sniffing at another pig or nose to nose contact
Pushing	One pig pushing or nibbling or lifting another pig
Crowding	Two or more pigs pushing each other to reach feed or water
Manipulating pen mate	One pig has another pig's tail or ear in its mouth
Playing	One pig playing (jump, carry straw or run)
Problematic	
Aggressive	Two or more pigs fighting or giving or receiving head-knocks or bites
Mounting	One pig is mounting another pig

4.6 Statistical analyses

Data were analysed with SAS 9.2 (SAS Institute, Cary, NC, USA). For the analysis of oestradiol and IGF-1 in plasma, data were obtained and analysed only from the first trial. For all other analyses, data were combined from the two trials. In Papers III and IV, data were analysed for the smaller subset of the population, consisting of 8 individuals per treatment group from the first trial.

The effect of treatment on testes weight, bulbourethral gland length, indole, skatole and androstenone in fat and oestradiol and IGF-1 in plasma, performance, carcass quality and skin lesions recorded at slaughter was evaluated with the MIXED procedure. The model included the fixed factor of treatment (surgical castration, early vaccination and standard vaccination, and

entire males) and the random factors of trial, pen and litter. When analysing daily weight gain during suckling, surgically castrated pigs were compared with all entire pigs i.e. also pigs intended for vaccination. To evaluate the effects on variables measured repeatedly in plasma (testosterone and skatole) and serum (antibodies against GnRH), the model included the same factors as above with an additional repeated statement (by individual, unstructured covariance structure). Main effects at different time points were evaluated using the slice statement. The concentrations of skatole, testosterone, oestradiol and IGF-1 in plasma, antibodies against GnRH in serum and concentrations of indole, skatole and androstenone in fat were log-transformed prior to statistical analyses to normalise residual distributions.

The effect of treatment on mRNA and protein expression and enzymatic activity was evaluated using the MIXED procedure on rank-transformed data, with treatment as fixed factor. Pair-wise comparisons between treatments were obtained through the pdiff option. Spearman correlations between measured variables were calculated for all pigs.

Activity behaviours were recorded as the percentage of pigs performing a particular behaviour at each sampling occasion (9 rounds * 2 scan samplings) and were analysed as percent of time. The social behaviour was recorded as the total number of interactions performed per pen and hour at each sampling occasion (9 rounds * 8 minutes). Pen was the statistical unit for all behaviour analyses. These parameters were evaluated within each observation occasion with the MIXED procedure. The model included treatment as fixed factor and trial as random factor. For comparisons over time, analogous evaluation was performed within treatment, with occasion as fixed factor and trial as random factor. Activity behaviour data were transformed via ‘arcsine (square root)’ and social interactions via ‘square root’ before statistical analysis to normalise residual distributions.

The impact of treatment on skin lesions recorded at slaughter was tested as a logistic regression using a binomial distribution with a logit link function. These analyses were done with the GENMOD procedure and the model included the effect of treatment and trial.

5 Results

5.1 Antibodies

Titres of antibodies against GnRH were detected only in low concentrations in serum from entire male pigs (Fig. 7). For both groups of vaccinated pigs, antibodies were at the same concentration as for entire male pigs until the second vaccine injection. After the second injection, the concentration of antibodies increased rapidly and then gradually decayed. All vaccinated pigs had increased concentrations of antibodies at the end of the trial.

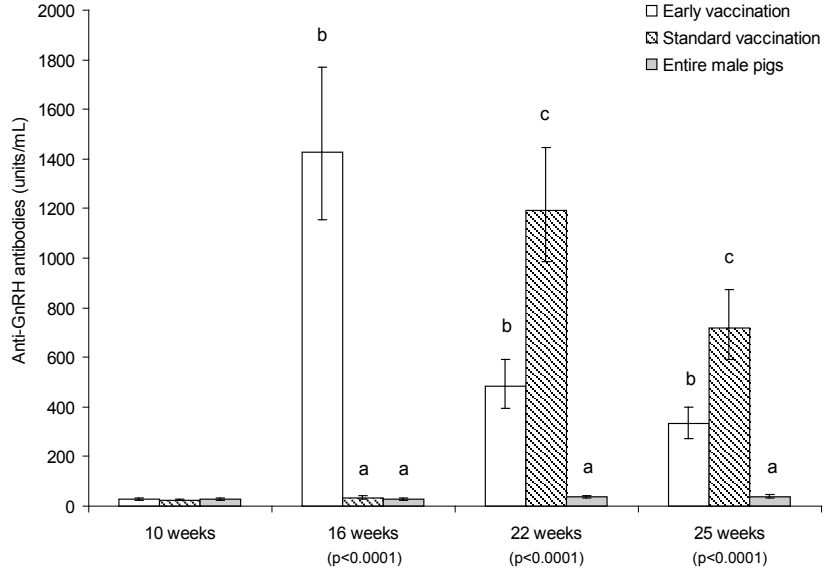

Figure 7. Titres of antibodies against GnRH in serum of surgically castrated, early and standard vaccinated and entire male pigs. The second vaccine injection occurred at 14 weeks for early vaccinated and 20 weeks for standard vaccinated pigs, respectively. Data are presented as LS means ± SE after back-transformation to original scale. Means with different letters within sampling occasion differ at $p<0.05$.

5.2 Reproductive organs

Vaccination resulted in lower testes weight and bulbourethral gland length compared with entire male pigs, with early vaccination showing a larger reduction of both testes weight and bulbourethral gland length (Table 3). Surgically castrated pigs had the smallest bulbourethral glands. Reproductive organs showed an overlap of distributions for standard vaccinated and entire male pigs, whereas the distributions for early vaccinated and entire male pigs were almost completely separated (Paper II Fig. 3).

Table 3. *Paired testes weight and average bulbourethral gland length of surgically castrated, early and standard vaccinated and entire male pigs at time of slaughter.*

	Surgical castration	Early vaccination	Standard vaccination	Entire male pigs	p-value
Testes pair (g)	N/A	74 ± 17^{a}	226 ± 16^{b}	539 ± 17^{c}	<0.0001
Bulbourethral gland (cm)	5.2 ± 0.3^{a}	6.5 ± 0.3^{b}	8.2 ± 0.3^{c}	12.3 ± 0.3^{d}	<0.0001

Data are presented as LS means ± SE. Means with different superscripts within the row differ at $p<0.05$.

Testicular histology

Individual variation in testicular histology was present between control pigs, but their testicular tissue showed mostly normal appearance in relation to presence, size and distribution of typical eosinophilic Leydig cells (lc in Fig. 8a´-a´´) as well as seminiferous tubuli. Spermatogenesis was fully developed in all controls at age 25 weeks although some variation in size and degree of intact spermatogenesis was observed. That variation was, however, to be expected for the age of pigs.

In vaccinated male pigs, testicular histology was clearly affected. Compared with controls, tubular diameter was reduced by a mean of 18% in the standard vaccinated pigs and more than 38% in the early vaccinated pigs ($p<0.01$). Together with an apparent reduction in the size of the interstitium (compare Fig. 8a to 8b-c), such morphological differences were reflected in the significant reduction of testicular weight (Table 3). Vaccination with Improvac clearly disrupted the number and morphology of the interstitial Leydig cells in the standard vaccinated pigs and dramatically so in the early vaccinated animals (Fig. 8a-c´´). The Leydig cells lost their cytoplasmic eosinophilia, were fewer, and were represented by pycnotic-like nuclei, difficult to distinguish from the interstitial fibroblasts and endothelial cells. Spermatogenesis was also clearly affected in vaccinated pigs. In standard vaccinated pigs (Fig. 8b-b´´), changes ranged from mild disruption such as spermatocyte loss and decrease in the normal number of layers of germ cells to severe loss of germ cells including tubuli being Sertoli cell-only i.e. complete

disappearance of germ cells. In early vaccinated pigs, however, the changes were more dramatic with only 3 out of 8 specimens presenting tubuli with spermatocytes or round spermatids. Most specimens had shrunken tubuli with only Sertoli cells or a few spermatocytes (Fig. 8c-c´´).

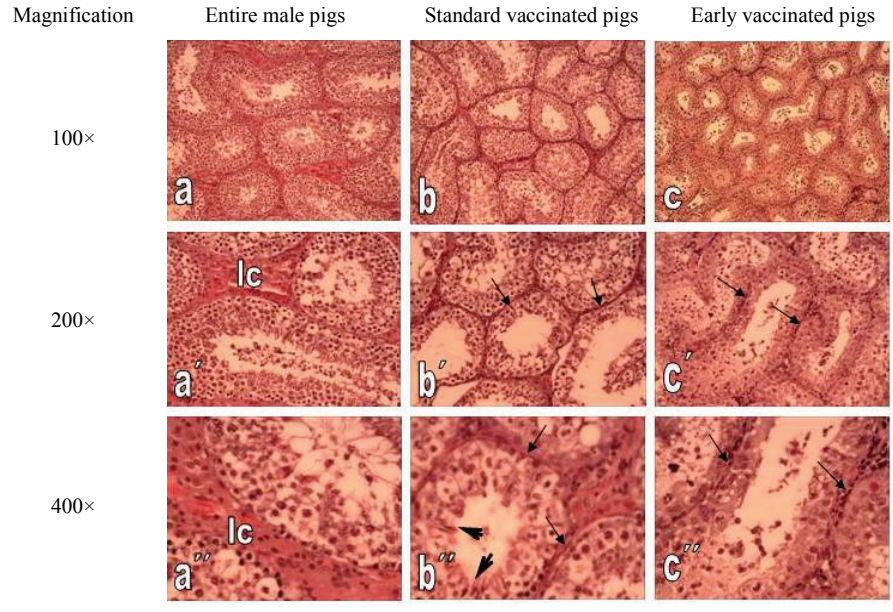

Figure 8. Light microphotographs of testicular tissue from entire male (Fig. a-a´´), standard vaccinated (Fig. b-b´´) and early vaccinated (Fig. c-c´´) pigs slaughtered at 25 weeks of age. Note the reduction in the size of the seminiferous tubules (Fig. a-c), and in the space of the interstitial tissue (arrows) containing the Leydig cells (lc) in the vaccinated pigs (Fig. a´-c´), and the number of layers in the epithelium (Fig. a´´-c´´), for vaccinated pigs compared to controls. While some standard vaccinated pigs still had tubuli with elongated spermatids (Fig. b´´ arrow heads), most tubuli in early vaccinated pigs had only Sertoli cells or spermatocytes left (Fig. c´´).

Spermatozoal morphology

The sperm morphology of controls was within normal limits for their age (Table 4). Among early vaccinated pigs, there were no spermatozoa collectable in 5 out of 8 animals. In the remaining 3 males, the number of spermatozoa collected was extremely reduced: less than 100 were counted after centrifugation of the fixed suspension. Another striking difference between controls and vaccinated pigs was the proportion of spermatozoa with normal morphology: only about 5% of spermatozoa in the vaccinated pigs were normal compared with more than 70% in the controls ($p<0.01$). Among the deviant spermatozoa, the dominant morphological abnormality was the proportion of immature spermatozoa carrying proximal cytoplasmic droplets ($p<0.001$).

Likewise, the proportions of spermatozoa depicting pathological sperm heads, or mid-piece defects were higher ($p<0.05$) in vaccinated pigs compared with controls. Differences were not seen between early and standard vaccinated pigs, but the number of early vaccinated pigs with enough spermatozoa for evaluation was too small to accurately compare these differences.

Table 4. *Effect of immunisation against GnRH on the morphology of spermatozoa collected from the cauda epididymides at slaughter.*

	Early vaccination	Standard vaccination	Entire male pigs	p-value
Number of animals	3[1]	8	8	
Sperm morphology (%)				
Abnormal head shapes	26.6 [b] ± 6.59	22.6 [b] ± 4.18	11.8 [a] ± 3.39	<0.05
Loose heads	1.7 [b] ± 0.48	3.2 [a] ± 1.34	3.7 [a] ± 0.93	<0.05
Acrosome spec defect	1.2 [b] ± 0.94	2.4 [a] ± 0.65	3.7 [a] ± 0.97	ns
Acrosome abnormality	1.7 [a] ± 0.53	2.6 [a] ± 0.66	2.3 [a] ± 0.54	ns
Proximal droplets	60.0 [b] ± 19.30	78.1 [b] ± 6.27	10.1 [a] ± 4.21	<0.001
Distal droplets	5.9 [b] ± 2.67	2.9 [b] ± 1.59	72.0 [a] ± 5.74	<0.001
Mid-piece defects	6.1 [b] ± 1.61	5.9 [ab] ± 2.95	1.3 [a] ± 0.24	<0.05
Simple bent tail	4.2 [a] ± 1.87	2.8 [a] ± 1.68	2.5 [a] ± 0.61	ns
Normal spermatozoa	5.3 [b] ± 3.02	5.0 [b] ± 1.67	71.1 [a] ± 6.27	<0.01
	(3-13)	(1-17)	(39-95)	

Data are presented as LS means ± SE. Means with different superscript within row differ at $p<0.05$.
[1] In early vaccinated pigs no spermatozoa were found in the cauda epididymides of 5 out of 8 males pigs, whereas in the other 3 male pigs the percentages of abnormalities are based on <100 spermatozoa.

5.3 Hormones

Plasma concentrations of testosterone were low in castrated pigs and high in entire males throughout the entire trial (Fig. 9). After the second vaccination, testosterone concentrations in both groups of vaccinated pigs decreased to that of surgically castrated pigs. Standard vaccinated pigs had reduced plasma testosterone levels already at 16 weeks i.e. at the time of their first injection, but not to the degree observed after the second injection.

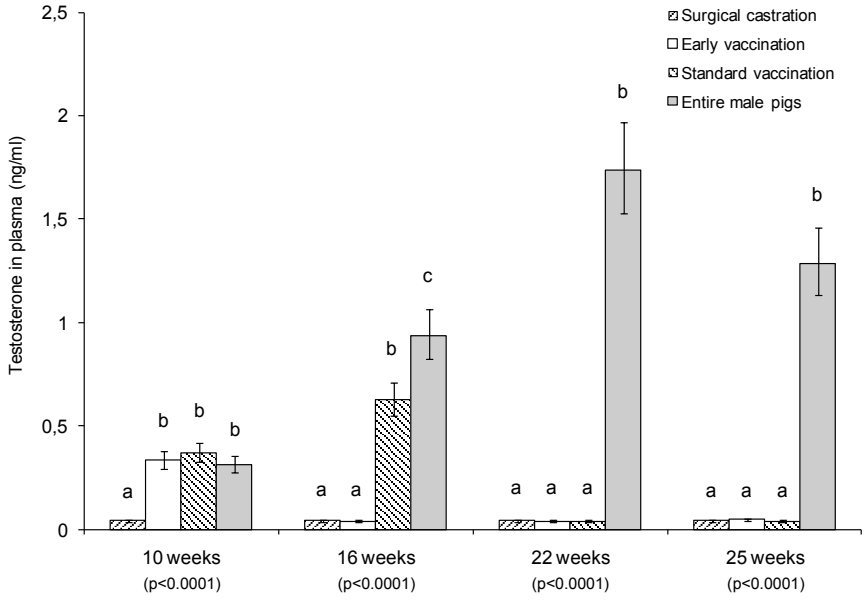

Figure 9. Testosterone levels in plasma of surgically castrated, early and standard vaccinated and entire male pigs. The second vaccine injection occurred at 14 weeks for early vaccinated and 20 weeks for standard vaccinated pigs, respectively. Data are presented as LS means ± SE after back-transformation to original scale. Means with different letters within sampling occasion differ at $p<0.05$.

Oestradiol levels in plasma one day prior to slaughter followed the same pattern as testosterone levels on the fourth sampling occasion, showing low levels in surgically castrated and both groups of vaccinated pigs and increased levels only in entire male pigs (Table 5).

IGF-1 levels in plasma one day prior to slaughter were higher in entire male than in surgically castrated pigs, with vaccinated pigs at an intermediate level (Table 5).

Table 5. *Oestradiol and IGF-1 concentrations in plasma of surgically castrated, early and standard vaccinated and entire male pigs at time of slaughter.*

	Surgical castration	Early vaccination	Standard vaccination	Entire male pigs	p-value
Oestradiol (pg/mL)	0.34^{a} (0.21-0.57)	0.37^{a} (0.22-0.63)	0.56^{a} (0.35-0.89)	4.31^{b} (2.69-6.90)	<0.0001
IGF-1 (ng/mL)	256^{a} (219-298)	317^{b} (272-370)	332^{b} (285-386)	459^{c} (394-534)	<0.0001

Data are presented as back-transformed LS means with confidence limits within parentheses.
Means with different superscripts within the row differ at $p<0.05$.

5.4 CYP1A, 2A and 2E1

Expression of mRNA

Treatment was significantly associated with mRNA expression of CYP1A2, 2A and 2E1 (Fig. 10). Compared with entire male pigs, mRNA expression was higher for the three isoforms in surgically castrated (CYP1A2 +81%, p=0.003; CYP2A +219%, p=0.000; and CYP2E1 +40%, p=0.003) and early vaccinated pigs (CYP1A2 +129%, p=0.000; CYP2A +205%, p=0.000; and CYP2E1 +57%, p=0.001). Expression in standard vaccinated pigs was higher only for CYP1A2 (+60%, p=0.009).

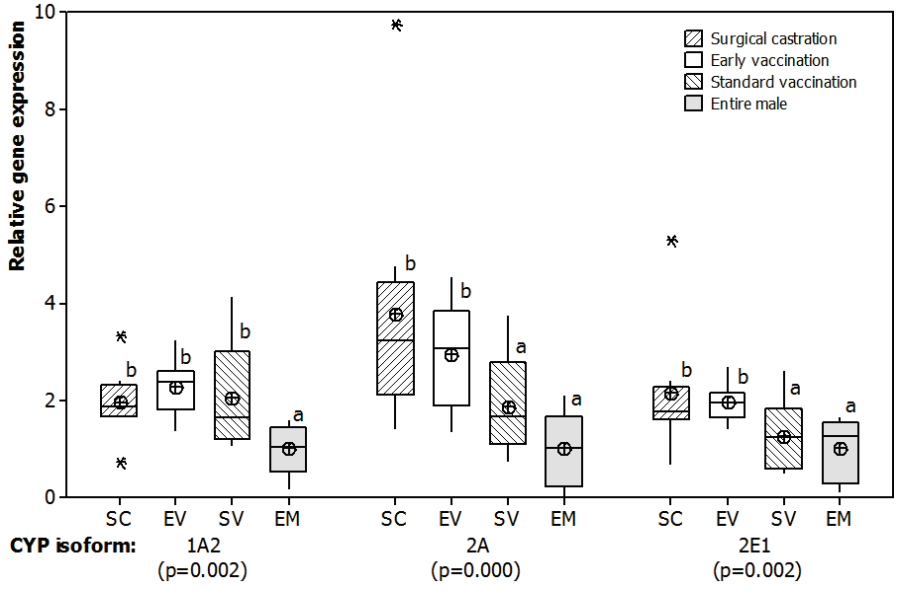

Figure 10. Hepatic mRNA expression of CYP1A2, 2A and 2E1 in surgically castrated (SC), early vaccinated (EV), standard vaccinated (SV) and entire male (EM) pigs. Data are presented as boxplots, with median and interquartile ranges (horizontal lines), mean value (⊕), and total range (extreme ends of vertical lines). Potential outliers (*) were not included in total range. Medians with different letters within each isoform differ (p<0.05) (pair-wise comparisons). The p-values in parentheses indicate the overall effect.

Protein expression

Protein expression differed between treatments for the different isoforms (Fig. 11). Compared with entire male pigs, CYP1A2 and 2A protein expressions were higher in surgically castrated (CYP1A2 +118%, p=0.032; CYP2A +109%, p=0.021) and early vaccinated pigs (CYP1A2 +131%, p=0.005; CYP2A +97%, p=0.020), whereas standard vaccinated pigs had higher

expression only of CYP1A2 (+79%, p=0.042) (Fig. 11). Large differences within treatments were observed in protein expression of all isoforms.

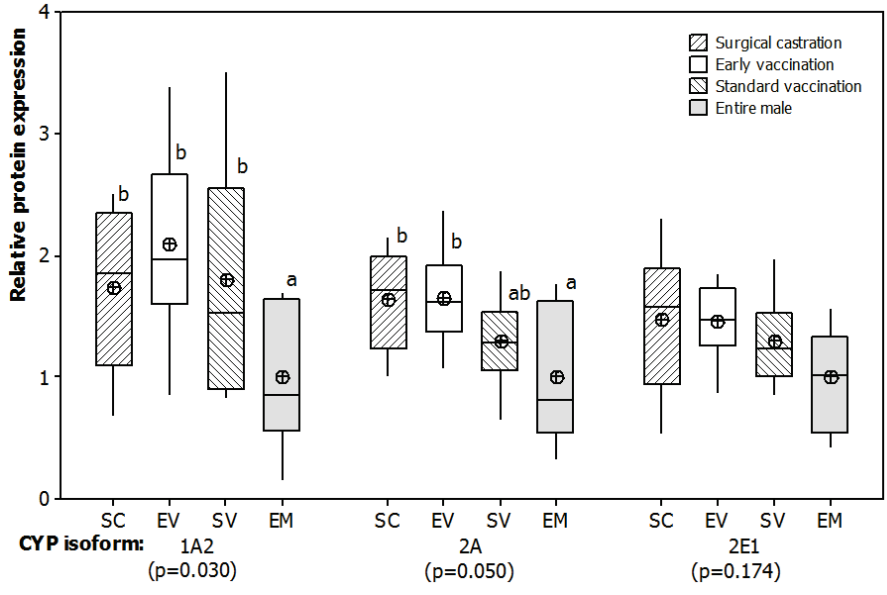

Figure 11. Protein expression of CYP1A2, 2A and 2E1 in microsomes from surgically castrated (SC), early vaccinated (EV), standard vaccinated (SV) and entire male (EM) pigs. Data are presented as boxplots, with median and interquartile ranges (horizontal lines), mean value (⊕), and total range (extreme ends of vertical lines). Medians with different letters within each isoform differ (p<0.05) (pair-wise comparisons). The p-values in parentheses indicate the overall effect.

Enzyme activity

Only CYP2A-dependent COH activity differed significantly between treatments (Table 6). Compared with entire male pigs, COH activity was 82% higher in surgically castrated pigs (p=0.026), 81% higher in early vaccinated pigs (p=0.006) and 45% higher in standard vaccinated pigs (p=0.043). Although the overall effect on the other isoforms was not significant, pair-wise comparisons showed higher EROD (+39%, p=0.037) and MROD (+72%, p=0.016) activities in early vaccinated pigs, compared with these in entire male pigs.

Table 6. *Effect of treatment on CYP450 enzyme activities in porcine microsomes.*

	Surgical castration	Early vaccination	Standard vaccination	Entire male pigs	p-value
EROD (CYP1A)	15.9[ab] (12.2-19.3)	16.5[b] (15.6-20.1)	17.1[ab] (14.8-22.3)	11.8[a] (8.7-15.8)	0.157
MROD (CYP1A2)	4.30[ab] (3.14-4.80)	5.42[b] (4.01-7.33)	4.68[ab] (1.46-5.89)	3.16[a] (2.50-4.33)	0.102
COH (CYP2A)	57.5[b] (28.6-77.2)	57.2[b] (36.2-123.1)	45.8[b] (40.0-54.8)	31.6[a] (4.8-38.5)	0.044
PNPH (CYP2E1)	64.4 (42.6-85.1)	51.2 (44.5-84.0)	49.4 (43.8-69.6)	51.1 34.0-55.9)	0.730

EROD, 7-ethoxyresorufin O-deethylase; MROD, 7-methoxyresorufin O-demethylase; COH, coumarin hydroxylase; PNPH, p-nitrophenol hydroxylase. Corresponding CYP450 isoform(s) to the enzyme activity measures are within parentheses. Data are presented as medians with interquartile ranges within parentheses (pmol/min/mg), n=8 per treatment group. Medians with different superscripts within each isoform differ ($p<0.05$).

Correlation between mRNA, protein expression and enzymatic activities

Expressions of mRNA and protein and enzyme activity were correlated for CYP1A and 2A (Table 7). For CYP2E1, a correlation was observed only between mRNA and protein expressions. Correlations were also observed between the isoforms at the levels of mRNA and protein expression and enzyme activity, respectively. Testosterone and oestradiol levels in plasma were negatively correlated with mRNA expression for all isoforms. Their correlations with protein expression and enzyme activities varied depending on isoform.

Table 7. *Spearman correlations between hormones in plasma, mRNA and protein expressions and activity of CYP1A, 2A and 2E1.*

	Testosterone	Oestradiol	PCR1A2	PCR2A	PCR2E1	WB1A2	WB2A	WB2E1	EROD	MROD	COH
Oestradiol	0.78***										
PCR1A2[a]	-0.60***	-0.44*									
PCR2A[a]	-0.52**	-0.57***	0.52**								
PCR2E1[a]	-0.43*	-0.47**	0.53**	0.90***							
WB1A2[b]	-0.49**	-0.43*	0.42*	0.30	0.34						
WB2A[b]	-0.36*	-0.19	0.34	0.58***	0.59***	0.56***					
WB2E1[b]	-0.27	-0.14	0.44*	0.29	0.40*	0.70***	0.39*				
EROD	-0.45**	-0.45*	0.18	0.15	0.12	0.28	0.04	-0.09			
MROD	-0.43*	-0.39*	0.46**	0.26	0.25	0.41*	0.16	0.24	0.75***		
COH	-0.48**	-0.37*	0.49**	0.37*	0.39*	0.42*	0.35*	0.26	0.58***	0.68***	
PNPH	-0.16	0.09	0.19	0.05	0.20	0.17	0.26	0.12	0.47**	0.55**	0.76***

[a] Expression of mRNA measured by PCR.

[b] Protein expression measured by Western blot.

EROD, 7-ethoxyresorufin O-deethylase, CYP1A; MROD, 7-methoxyresorufin O-demethylase CYP1A2; COH, coumarin hydroxylase, CYP2A; PNPH, p-nitrophenol hydroxylase CYP2E1. The correlation analysis was performed on the complete data set obtained by pooling data from all treatment groups (n = 32).

Significance level: * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

5.5 Boar taint substances

Androstenone, skatole and indole levels in fat from surgically castrated and vaccinated pigs were lower than those from entire male pigs (Table 8). Among the entire male pigs (n=47), 29 pigs had a fat androstenone concentration above 1 μg/g and 5 had a fat skatole concentration above 0.2 μg/g. Among these, 3 pigs had both androstenone and skatole concentrations above those limits, which were used as threshold values for sensory acceptance (Walstra *et al.*, 1999). A total of 31 (66%) entire male pigs were thus above threshold levels for boar taint. None of the pigs from the surgically castrated or vaccinated groups had androstenone or skatole concentration above those threshold values.

Table 8. *Boar taint compounds in fat of surgically castrated, early and standard vaccinated and entire male pigs at time of slaughter.*

	Surgical castration	Early vaccination	Standard vaccination	Entire male pigs	p-value
Androstenone (ng/g)	157[a] (84-295)	169[a] (90-318)	169[a] (90-317)	1092[b] (581-2052)	<0.0001
Skatole (ng/g)	24[a] (19-30)	25[a] (20-32)	26[a] (21-33)	68[b] (54-86)	<0.0001
Indole (ng/g)	6.8[a] (4.9-9.5)	6.9[a] (4.9-9.6)	6.1[a] (4.3-8.4)	15.0[b] (10.8-20.9)	<0.0001

Data are presented as back-transformed LS means with confidence limits within parentheses.
Means with different superscripts within the row differ at $p<0.05$.

No differences in plasma skatole concentrations were observed between the groups on the first two sampling occasions (Fig. 12). On the third occasion, surgically castrated and early vaccinated pigs had lower levels than standard vaccinated and entire male pigs. On the day before slaughter, the surgically castrated as well as both vaccinated groups had low concentrations of skatole in plasma, compared with entire male pigs.

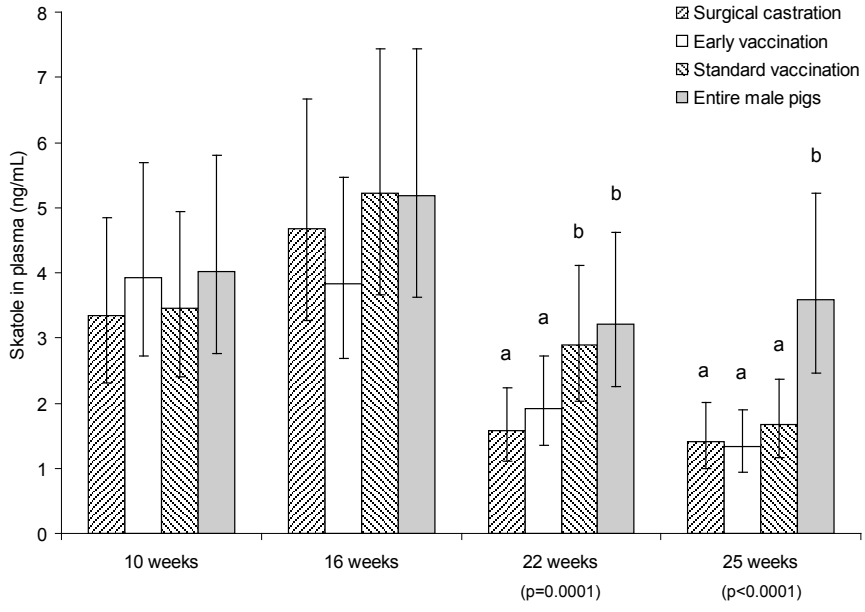

Figure 12. Skatole levels in plasma of surgically castrated, early and standard vaccinated and entire male pigs. The second vaccine injection occurred at 14 weeks for early vaccinated and 20 weeks for standard vaccinated pigs, respectively. Data are presented as LS means ± SE after back-transformation to original scale. Means with different letters within sampling occasion differ at $p<0.05$.

5.6 Performance and carcass quality

Daily weight gain and feed conversion ratio did not differ between the groups (Table 9). There was a possible effect of treatment on estimated lean meat content ($p=0.053$). Early vaccinated and surgically castrated pigs had lower values compared with entire male pigs ($p=0.014$ and 0.021, respectively), whereas standard vaccinated pigs did not differ from entire males. Dressing percentage was higher in early vaccinated and surgically castrated pigs than in standard vaccinated and entire male pigs ($p<0.001$). Average daily lean meat growth from start of the study to slaughter did not differ between the groups ($p=0.567$).

Table 9. *Performance and carcass quality for surgically castrated, early and standard vaccinated pigs and entire male pigs.*

	Surgical castration	Early vaccination	Standard vaccination	Entire male pigs	SE	p-value
No. of pigs	46	46	48	47		
Initial weight (kg)	30.9	28.9	29.0	29.0	2.2	0.894
Final weight (kg)	119.5	118.6	119.0	118.6	3.2	0.943
Daily weight gain (g)						
start to 2nd inj.[1]	825	831	825	812	68	0.749
2nd inj. to slaughter[1]	906	889	891	927	27	0.522
start to slaughter	859	853	850	854	49	0.960
Daily feed consumption (kg)	2.42	2.36	2.34	2.33	0.06	0.775
Feed conversion ratio	2.80	2.76	2.76	2.74	0.13	0.908
Age at slaughter (days)	177	178	178	178	2	0.891
Carcass weight (kg)	89.9	88.5	88.0	87.9	2.2	0.466
Estimated lean meat (%)	57.0^{a}	56.9^{a}	57.3^{ab}	57.7^{b}	0.5	0.053
Dressing percentage	75.1^{a}	74.6^{a}	74.0^{b}	74.1^{b}	0.3	<0.001
Abdominal fat (kg)	1.20^{a}	1.12^{b}	0.99^{c}	0.86^{d}	0.45	<0.001
Daily lean meat growth (g)	371	363	364	370	18	0.567
Carcass value (EUR)	116.2^{a}	113.6^{a}	114.3^{a}	95.3^{b}	3.4	<0.001

Data are presented as LS means. Means with different superscripts within the rows differ at p<0.05.
[1] 2nd injection of standard vaccinated pigs.

5.7 Behaviour

5.7.1 Activity behaviour

Treatment did not significantly affect the time that pigs spent resting and standing at any observation occasion (Table 10). Eating was affected by treatment, but the effect was not consistent over time. On the first observation occasion, surgically castrated pigs had less contact with each other than pigs in the other groups, who were all entire male pigs at that time ($p<0.05$). On the next observation occasion, when early vaccinated pigs had received their second injection they spent less time on contact compared with the two groups with entire male pigs ($p<0.01$), but a similar time to surgically castrated pigs ($p=0.587$). On the third observation occasion, standard vaccinated pigs had also been injected twice and spent as little time on contact as surgically and early vaccinated pigs ($p>0.65$), whereas entire male pigs continued to use approximately the same time on contact with each other as on the earlier observation occasions ($p>0.15$).

Table 10. *Percentage of day time used on scan sampling activity behaviours.*

	Surgical castration	Early vaccination	Standard vaccination	Entire male pigs	SE	p-value
No. of pens	6	6	6	6		
Before 2nd injection of early vaccinated pigs (age 13-14 weeks)						
Resting	54.8	39.5	51.6	50.0	0.5	0.127
Standing	35.0	40.0	32.6	31.7	0.6	0.463
Eating	8.0	14.6	9.0	11.3	0.1	0.057
Contact	2.2^{a}	5.9^{b}	6.8^{b}	7.0^{b}	0.2	0.037
Before 2nd injection of standard vaccinated pigs (age 19-20 weeks)						
Resting	59.3	57.8	58.5	54.8	0.7	0.749
Standing	36.1	38.6	29.7	33.2	0.9	0.398
Eating	4.2^{a}	3.4^{a}	9.0^{b}	7.8^{ab}	0.1	0.050
Contact	0.4^{a}	0.2^{a}	2.8^{b}	4.2^{b}	0.1	0.001
Before slaughter (age 22-23 weeks)						
Resting	58.0	59.1	57.3	58.4	0.7	0.964
Standing	37.6	38.3	38.9	34.3	1.0	0.813
Eating	4.2	2.1	3.6	3.1	0.1	0.386
Contact	0.2^{a}	0.5^{a}	0.2^{a}	5.2^{b}	0.2	0.011

Data are presented as LS means. Means with different superscript within row differ at $p<0.05$.
See Table 1 for definitions of behaviour parameters used at the scan sampling.

5.7.2 Social interactions

Non-problematic interactions:

On the first observation occasion, before the second injection of early vaccinated pigs (Fig. 5), surgically castrated pigs performed less sniffing ($p<0.002$) and pushing ($p<0.05$) than the other pigs (Table 11). On the second occasion, when early vaccinated but not standard vaccinated pigs had received their second injection, the frequencies of sniffing ($p<0.002$) and pushing ($p<0.001$) were lower for early vaccinated pigs than for standard vaccinated and entire male pigs, but were similar to those of castrates. On the third observation occasion, these behaviours were at the same level for standard vaccinated pigs as for surgically castrated and early vaccinated pigs. For entire male pigs, these frequencies were unaffected over time ($p>0.51$). The occurrences of crowding, manipulating pen mate and playing did not differ between groups.

Total interactions were higher for entire male than for surgically castrated pigs (Table 11), with a lower proportion of non-problematic behaviours already at 13 weeks of age (Fig. 13). This pattern was unaffected over time. For the vaccinated pigs, both non-problematic and total interactions decreased after

their second injection, from the levels of entire males to those of surgically castrated pigs. The proportion of non-problematic interactions increased to the higher level of surgically castrated pigs (Fig. 13). Although the early vaccinated pigs had received their second injection already at 14 weeks of age, the effect on activity remained until the end of the trial.

Table 11. *Frequency of observed social interactions per pen and hour.*

	Surgical castration	Early vaccination	Standard vaccination	Entire male pigs	SE	p-value
No. of pens	6	6	6	6		
Before 2nd injection of early vaccinated pigs (age 13-14 weeks)						
Non-problematic						
Sniffing	43.1[a]	65.4[b]	74.0[b]	74.3[b]	1.0	<0.001
Pushing	26.0[a]	44.5[b]	40.1[b]	52.7[b]	1.2	0.007
Crowding	0.3	1.1	0.5	1.1	0.0	0.465
Man. pen mate	4.1	8.3	6.7	5.4	0.1	0.054
Playing	4.1	6.1	3.4	5.3	0.8	0.682
Problematic						
Aggressive	7.3	12.8	11.9	12.0	1.0	0.250
Mounting	0.5[a]	5.9[b]	8.8[b]	6.6[b]	0.1	<0.001
Total	85.4[a]	144.1[b]	145.4[b]	157.4[b]	3.1	<0.001
Before 2nd injection of standard vaccinated pigs (age 19-20 weeks)						
Non-problematic						
Sniffing	38.9[a]	35.8[a]	56.2[b]	70.4[c]	0.5	<0.001
Pushing	22.0[a]	17.2[a]	36.8[b]	45.8[b]	0.3	<0.001
Crowding	1.8	0.5	1.4	1.1	0.1	0.387
Man. pen mate	2.2	1.3	4.6	2.9	0.1	0.312
Playing	0.5	0.1	1.0	0.8	0.1	0.189
Problematic						
Aggressive	4.3	3.7	7.1	7.9	0.6	0.177
Mounting	0[a]	0[a]	3.0[b]	8.0[c]	0.1	<0.001
Total	69.7[a]	58.6[a]	110.1[b]	136.9[b]	1.0	<0.001

	Surgical castration	Early vaccination	Standard vaccination	Entire male pigs	SE	p-value
Before slaughter (age 22-23 weeks)						
Non-problematic						
Sniffing	27.5[a]	23.3[a]	26.3[a]	82.8[b]	0.3	<0.001
Pushing	19.8[a]	16.2[a]	13.8[a]	44.9[b]	0.3	<0.001
Crowding	2.0	2.3	1.2	1.3	0.1	0.676
Man. pen mate	1.4	2.7	1.7	1.4	0.1	0.644
Playing	0.2	0	0	0	0.0	0.631
Problematic						
Aggressive	3.4[a]	2.7[a]	2.5[a]	8.0[b]	0.1	0.010
Mounting	0[a]	0[a]	0[a]	8.6[b]	0.1	<0.001
Total	54.3[a]	47.2[a]	45.5[a]	147.0[b]	0.5	<0.001

Data are presented as LS means. Means with different superscript within row differ at $p<0.05$.
See Table 2 for definitions of social interactions.

Problematic interactions:

Problematic interactions were higher for entire male pigs than for surgically castrated pigs (Fig. 13). For the vaccinated pigs, problematic interactions decreased after their second injection, from the levels of entire males to those of surgically castrated pigs. On the first observation occasion, mounting frequency was lower for surgically castrated pigs than for the other groups, which were all still entire male pigs ($p<0.001$; Table 11). On the next observation occasion, early vaccinated pigs had stopped the mounting behaviour completely. Although standard vaccinated pigs had not yet received their second injection, they had decreased their frequency of mounting to a lower level than entire male pigs ($p=0.008$). Before slaughter, mounting was also absent in standard vaccinated pigs, but was still present among the entire male pigs at the same level as previously.

After the second injection, the frequency of problematic behaviour decreased to the level of castrates, but no clear effect of vaccination on aggressive behaviour could be observed. However, on the third observation occasion, surgically castrated and vaccinated pigs performed significantly less aggressive behaviour than entire male pigs.

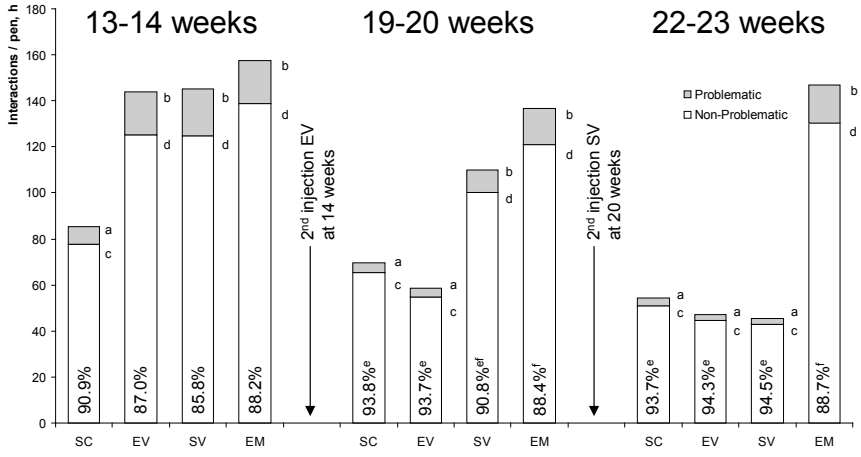

Figure 13. Problematic (a and b different at p<0.05) and non-problematic (c and d different at p<0.05) behaviours of surgically castrated (SC), early vaccinated (EV), standard vaccinated (SV) and entire male pigs (EM). The proportion of non-problematic behaviours is shown at the bottom of each bar, expressed as percentage of total behaviours (e and f different at p<0.05). Problematic interactions include aggression and mounting. Non-problematic interactions include sniffing, pushing, crowding, manipulating pen mate and playing.

5.8 Skin lesions

Treatment affected skin lesions recorded at slaughter (p<0.001), with more lesions observed on entire male pigs (65%) than in the other groups. The occurrence did not differ significantly between surgically castrated (14%), early (31%) and standard vaccinated pigs (25%).

Furthermore, the severity of skin lesions did not differ between early vaccinated (2.22 points) and standard vaccinated pigs (2.01 points; p=0.353). However, the vaccinated pigs had more severe skin lesions than surgically castrated pigs (1.58 points; p<0.049) but less severe than entire male pigs (3.01 points; p<0.001).

6 Discussion

Immunisation of male pigs against GnRH with the commercially available vaccine Improvac was proven effective for the control of boar taint over a range of conditions (Dunshea *et al.*, 2001; Jaros *et al.*, 2005; Zamaratskaia *et al.*, 2008a). The present study is however the first to investigate the effects of early vaccination (at a prepubertal or early pubertal stage) on antibody response, reproductive organ development, testicular secretory activities, skatole metabolism, boar taint levels, performance and behaviour.

It should however be noted that, technically, the early immunisation against GnRH used in this study constitutes an off-label usage and that the manufacturer indicates an effective control of boar taint only up to 10 weeks after the second vaccine injection.

6.1 Antibodies

For all vaccinated pigs, antibodies were at the end of the trial present at high levels. For standard vaccinated pigs, this is in agreement with previous findings (Dunshea *et al.*, 2001; Zamaratskaia *et al.*, 2008a). For early vaccinated pigs, these are novel results. The decay in antibody concentration seemingly follows an exponential curve (Fig.7), indicating that increased antibody titres would be present an appreciable time after 11 weeks. In accordance with the study by Zamaratskaia *et al.* (2008c), this strongly indicates that the effects of immunocastration on testicular function, hepatic skatole metabolism and boar taint can last longer than the 10 weeks indicated by the manufacturer.

6.2 Reproductive organs

In accordance with previous studies (Dunshea *et al.*, 2001; Zamaratskaia *et al.*, 2008a), testes and bulbourethral glands of vaccinated animals were diminished

in size, with a more pronounced reduction for early vaccinated pigs. Overlap in testes weight distributions between entire male and standard vaccinated pigs has been reported (Zamaratskaia *et al.*, 2008a; Schmoll *et al.*, 2009). Substantially less overlap was found for early vaccinated pigs. Thus, for early vaccination, testes weight or size has a larger potential as a practical tool to sort out accidentally non-immunised, therefore possibly tainted, pigs at slaughter. The decreased testicular size was also reflected in reduced testicular function, as was also previously found (Hilbe *et al.*, 2006; Einarsson *et al.*, 2009). The histological status of the testes of vaccinated pigs was dramatically affected, with major disruption of spermatogenesis, and a clear effect on the number and size of the Leydig cells. These effects were even more conspicuous among early vaccinated pigs. The morphology of the cauda epididymal spermatozoa indicated sexual maturity for several of the control male pigs but none for the vaccinated male pigs. No differences in sperm morphology could be found between early and standard vaccinated pigs. However, 5 of the 8 investigated early vaccinated pigs had no spermatozoa in the cauda epididymis and, of the other 3, fewer than 100 spermatozoa could be seen, suggesting a severe spermatogenic dysfunction. The more pronounced reduction in size and function of reproductive organs of early vaccinated pigs is most likely the result of a combination of an arrest of testicular development at an earlier age, thus smaller testes size, and a prolonged time of reduced testicular function.

6.3 Hormones

Circulating levels of testicular steroids were analysed as a measure of secretory activity. Testosterone levels are known to decrease within days of the second injection as part of standard vaccination – a result of inhibited secretory activity arising from reduced testicular function (Claus *et al.*, 2007; Zamaratskaia *et al.*, 2008a). In the present study, testosterone levels in vaccinated pigs were reduced to the levels of surgically castrated pigs two weeks after the second injection. The drastic reduction in testosterone concentrations in peripheral blood in vaccinated pigs suggests a hypo-function of the Leydig cells, presumably from a lack of GnRH production after vaccination. These effects oppose those observed when GnRH had been administered in a chronic pulsatile low-dose formulation in pre-pubertal male pigs (Dijkstra *et al.*, 1988). The effect of treatment on oestradiol and testosterone one day prior to slaughter was similar, with high levels observed only for entire male pigs.

In accordance with Metz and Claus (2003), decreased IGF-1 levels at slaughter were found in vaccinated pigs compared with entire males. This

expected decrease is related to the low level of oestradiol in vaccinated pigs. Claus *et al*. (2007) showed that IGF-1 levels decreased gradually and reached a stable level approximately 2-3 weeks after the second injection. In this study, no difference in IGF-1 was found between early and standard vaccination, indicating that it had decreased to a stable, sustained level after both vaccination regimens. Furthermore, vaccinated pigs had higher IGF-1 levels than surgically castrated pigs. This implies that vaccinated pigs have a higher anabolic potential than surgically castrated pigs also after immunisation and arrested testicular endocrine function although no significant difference in performance could be observed between these groups (see section 6.6). The observed difference in IGF-1 levels may arise, for example, from an increase in growth hormone releasing hormone after GnRH inhibition by vaccination (Claus & Weiler, 1994; Metz & Claus, 2003).

6.4 CYP1A, 2A and 2E1

Hepatic gene and protein expression, and enzyme activity of CYP1A, 2A and 2E1 were analysed to investigate the status of the skatole metabolising system. The only previous study investigating CYP450 after Improvac administration (Zamaratskaia *et al.*, 2009) focused on the enzymatic activities and did not attempt to relate the activities to either gene or protein expression. That study reported that the activities of CYP1A, 2A and 2E1 were higher in surgically castrated and vaccinated pigs compared with entire male pigs at normal slaughter age (24 weeks) and weight (LW 124 kg). In the present study, similar trends were observed for CYP1A and 2A, but not for CYP2E1. This discrepancy is surprising because Zamaratskaia *et al.* (2009) and the present study used pigs of same breed, and samples were taken at approximately the same age and live weight. The differences in activities were much lower compared with the differences in mRNA and protein expression of corresponding isoforms. The higher expressions of CYP450 mRNA found in surgically castrated and early vaccinated pigs imply that castration, whether by surgical or immunological means, up-regulates gene expression. These differences in gene expression of investigated CYP450 could be due to interactions between testicular steroids and proteins involved in transcriptional regulation of CYP450 genes. The pattern observed for protein expression of CYP1A2 and CYP2A was similar to that for gene expression. This relation in combination with strong correlations between gene and protein expression indicates that protein expression is regulated by the expression of corresponding genes. Interestingly, increased mRNA expression of CYP2E1 did not result in increased protein expression and activity. This implies that

porcine hepatic CYP2E1 is regulated by post-transcriptional stabilisation of the enzyme. Indeed, this is a common mechanism of regulation for CYP2E1 (Kocarek *et al.*, 2000).

In this study it was shown that mRNA and protein expression of the hepatic CYP1A2, 2A and 2E1 and their catalytic activities in early vaccinated pigs were similar to surgically castrated pigs. In standard vaccinated pigs, these parameters were more similar with those of entire male pigs. This enhanced modulation of skatole metabolising enzymes after early vaccination is probably because early vaccinated pigs had their testicular function suppressed 6 weeks earlier than standard vaccinated pigs. It was also strongly indicated that the testicular tissue never subsequently developed (as discussed under 6.8). Thus, a longer-term suppression of testicular steroid production resulted in similar CYP450 characteristics in early vaccinated and surgically castrated pigs. Interestingly, in this study, mRNA expression and protein expression and activities of these isoforms were similar in standard vaccinated and entire male pigs. Synthesis of testicular steroids in standard vaccinated pigs was blocked 4 weeks before liver samples were taken. It is likely that this was not sufficient time to completely remove the inhibitory effect of testicular steroids (as discussed also under 6.5).

6.5 Boar taint

Early vaccination results in a profile of skatole levels in plasma similar to that of surgically castrated pigs, with a higher concentration at the beginning of the trial and a subsequent decrease after 16 weeks of age. The decrease in plasma skatole is most likely due to increased hepatic metabolism and subsequent clearance resulting from the absence of testicular steroids discussed above (Zamaratskaia *et al.*, 2009). For standard vaccinated pigs, a decrease compared with entire male pigs was observed at the end of the trial, but not at age 22 weeks i.e. two weeks after their second injection. Thus it was shown that the decrease does not occur as quickly as that of testosterone. The observed sustained concentration is believed to arise from either of or the combination of two mechanisms: (i) addition of skatole to the plasma reservoir through depletion of skatole previously accumulated in the adipose tissue (Fig. 3b); (ii) a delay phase needed for skatole metabolism to adapt to the change in testicular steroid concentrations. Analogously to (ii), the *in vivo* increase in skatole concentration at puberty is delayed after the increase in circulating steroid hormone levels (Zamaratskaia *et al.*, 2004a; Zamaratskaia *et al.*, 2004b). For the analyses of skatole concentrations in plasma, the previous HPLC method (Zamaratskaia *et al.*, 2004a) was modified and validated

(Paper I), to exclude the use of acetonitrile in the mobile phase due to the global shortage in 2009 and its higher environmental impact compared to methanol.

Both skatole and indole concentrations in fat were lower in surgically castrated and vaccinated pigs compared with entire male pigs. Generally, skatole concentrations in plasma and adipose tissue are positively correlated, at least in mature male pigs (Zamaratskaia *et al.*, 2005). As discussed above, a reduction of skatole concentrations is likely due to increased expression and activity of cytochrome P450 owing to the absence of the CYP450 inhibitors androstenone and oestradiol (Doran *et al.*, 2002; Zamaratskaia *et al.*, 2007; Zamaratskaia *et al.*, 2008b). This would also explain an enhanced metabolism and low accumulation in fat of indole since porcine CYP2A and 2E1 are likely to be involved in indole metabolism (Gillam *et al.*, 2000; Zamaratskaia *et al.*, 2006). Concentrations of androstenone in fat from surgically castrated and vaccinated pigs varied from undetectable to low. Androstenone concentrations above the threshold value of 1 μg/g were detected only in fat from entire male pigs. Thus, both early and standard vaccination result in androstenone concentrations similar to those in surgically castrated pigs. Sensory evaluation of meat samples was not performed in the present study. However, a considerable reduction of androstenone, skatole and indole concentrations strongly implies that boar taint can be effectively prevented also by early vaccination against GnRH. This prevention does not appear to be dependent on vaccination regimen.

6.6 Performance and carcass quality

The effect of immunocastration on performance has in previous trials not been unambiguous (Millet *et al.*, 2011). In previous studies where pigs were vaccinated according to the manufacturer's recommendation (Zamaratskaia *et al.*, 2008a; Pauly *et al.*, 2009; Fàbrega *et al.*, 2010), an increase in growth rate after the second injection has been observed, due to a higher feed consumption. This has partly been explained by a change in behaviour (decreased aggression and mounting) after vaccination, due to lower levels of testosterone and oestrogens (Dunshea *et al.*, 2001; Cronin *et al.*, 2003). It has also been shown that oestrogens directly affect feed intake negatively (Bonavera *et al.*, 1994). Effects of behaviour on growth rate have also been found by Rydhmer *et al.* (2006), who reported that male pigs performing many mountings had a lower growth rate than male pigs not mounting. The discrepancy between earlier findings and the present study can probably be explained by a difference in feeding regime. In the present study, the feed intake was restricted whereas the

pigs in the reported studies were fed (*semi*) *ad libitum*. Thus, even with an increased interest in eating, the restricted feed supply could not allow the vaccinated pigs a higher feed intake and associated increase in growth rate. Moreover, all pigs were fed the same standard diet, excluding possible interactions between treatment and limiting factors (e.g. amino acids). Vaccinated pigs may therefore not have had the opportunity to take advantage of their higher IGF-1 levels compared to castrates.

In previous studies, standard vaccinated pigs had lean meat content in-between entire male pigs and castrates (Jaros *et al.*, 2005; Fàbrega *et al.*, 2010). The results from the present study, although not a perfect match to previous results, point in the same direction. Moreover, a longer time between the second vaccination and slaughter for early vaccinated compared with standard vaccinated pigs could account for the numerically lower, but not significantly different, lean content for early vaccinated compared to standard vaccinated pigs.

The higher dressing percentage in early vaccinated and surgically castrated pigs compared to standard vaccinated and entire male pigs are in line with results of Pauly *et al*. (2009). In contrast, Dunshea *et al*. (2001) and Zamaratskaia *et al*. (2008a) found that standard vaccination resulted in lower dressing percentage compared to entire male pigs, which has been interpreted mainly as depending on a higher feed intake and gut fill in the vaccinated pigs. The discrepancy between the different studies is probably an effect of the applied restricted feeding regime. The higher dressing percentage for early vaccinated pigs in this study can partly be explained by a lower size and weight of reproductive organs, although this increase is somewhat counterbalanced by a higher amount of interstitial abdominal fat (Table 9).

Since average daily lean meat growth did not differ between the groups, no differences in income per carcass were observed for surgically castrated or vaccinated pigs (Table 9). However, for entire male pigs the income was lower since the payment system considers the additional cost for boar taint analyses and reduced payment for tainted carcasses. A more comprehensive economic analysis, including costs related to the different treatments during production was not performed. Such a model would include e.g. labour costs for castration or vaccination and the cost of the vaccine. Another factor to consider would be the underestimation of lean meat percentage for entire male pigs compared with castrated and female pigs (Andersson *et al.*, 1995; Andersson *et al.*, 1997). It is likely that lean meat percentage also of vaccinated pigs is under-estimated analogously.

6.7 Behaviour

From activity analysis, it was evident that surgically castrated pigs exhibited lower activity than entire males already at the start of the growing/finishing period and until the end of the trial. This is in accordance with the studies by Baumgartner *et al.* (2010) and Rydhmer *et al.* (2010). Vaccination, whether early or standard, reduced the activity of the pigs after the second injection from the level of entire male pigs to that of surgically castrated pigs. The interaction analysis revealed that the frequencies of both non-problematic and problematic interactions were lower for surgically castrated than for entire male pigs. Moreover, the ratio of non-problematic interactions was higher in castrated pigs. Again, vaccinated pigs before their second injection behaved more like entire male pigs, whereas their behaviour was similar to that of castrates after the second injection. A change in behaviour in standard vaccinated pigs was observed already after their first injection. A corresponding reduction in testosterone was apparent already before their first injection, which may account for the behavioural shift. However, no satisfactory explanation can be provided for the decrease in testosterone.

Vaccination resulted in a reduction of skin lesions, both in occurrence and severity, compared with entire male pigs. This further strengthens the evidence that castration, regardless of method, suppresses problematic behaviours. However, it should be noted that, although behavioural studies at the end of the growing/finishing period indicated no difference in aggression and mounting between surgically castrated and vaccinated pigs, the severity of skin lesions on vaccinated pigs at slaughter indicates a possible difference compared to castrates in behaviour at mixing with unfamiliar pigs.

6.8 Long-term effect of immunocastration

The long-term effects of immunisation against GnRH using Improvac are still not sufficiently investigated. Testicular dysfunction as a consequence of vaccination against GnRH is largely considered to be reversible in a longer-term perspective. The general consensus is that return to functionality is attained after antibodies titres have decreased below a threshold value (Bonneau & Enright, 1995; Thompson Jr, 2000). In fact, Hilbe *et al*. (2006) and Claus *et al*. (2008) have reported a return of Leydig cell activity and testicular function, and in one case also of reproductive function (Hilbe *et al.*, 2006). However, the time-frame and mechanism of this return of functionality were not fully investigated. Zamaratskaia *et al*. (2008c) reported a long-term effect at least 22 weeks after the second injection following a standard vaccination schedule. Moreover, the assumption of reversibility is based on

immunocastration of pubertal or mature animals, who have not been subjected to e.g. testosterone deprivation at crucial maturational stages of testes development.

In this study, the clear relationship between antibody titres, disruption of testicular function, low levels of testosterone and skatole in circulating plasma at the time of slaughter and the control over behaviour provides evidence of the sustained effects of the vaccination, particularly early vaccination. Moreover, it is strongly indicated that early vaccination may have caused an irreversible disruption of the testicular structure and function. Spermatogenesis and Sertoli cell function depend on testosterone diffusing through the tubuli walls and into the testicular fluid that flows through the epididymis (Raeside *et al.*, 2006; Sofikitis *et al.*, 2008). A lack of testosterone during the maturational period may thus have permanently affected spermatogenesis (Huhtaniemi & Toppari, 1995; Roser, 2008; Sofikitis *et al.*, 2008). The evident numbers of Sertoli cell-only tubuli would suggest that these testes could not return to normality since most germs cells (including spermatogoniae) had been destroyed. Moreover, a high proportion of abnormalities in the few spermatozoa that reached the epididymal cauda was also evident, indicating that the low concentration of testosterone could not maintain the function of the epithelial target cells, thereby causing a diminished epididymal function and an impairment of sperm maturation (Setty, 1979).

Whether such irreversibility would be the case also for Leydig cells and the production of testicular steroids is not as certain. At slaughter, the Leydig cells were severely affected regarding both number and morphology. In order for steroid production to be recovered, antibodies against GnRH would first have to be decreased below threshold concentration, gonadotrope cell secretion of LH resumed and Leydig cells regenerated. Furthermore, from a boar taint perspective, it would also require time after successful recapture of steroid synthesis for androstenone and skatole to be deposited in adipose tissue in sufficient amount to arise above threshold levels. Regardless of reversibility or not, it remains highly unlikely that testicular steroid synthesis should be resumed and boar taint present at normal slaughter weight/age in animals immunocastrated at a prepubertal or early pubertal stage.

6.9 Implications for animal welfare and sustainability

The behavioural studies clearly suggest that effective control of behaviour in vaccinated pigs was achieved when monitored 2 weeks after the second injection. However, it was shown that a decrease of testosterone levels occurs already within days after immunisation (Claus *et al.*, 2008). Thus, control of

behaviour is possibly obtained earlier than was observed in this study. The manufacturer's instructions for Improvac administration state that the two injections should be performed at least 4 weeks apart with the first injection after age 8 weeks. This means that the earliest time for the administration of the second injection would be at age 12 weeks. From that point on, potentially, a second injection could be administered at the first sight of problematic (aggressive of sexual) behaviours related to sexual maturation in order to curb these behaviours. This flexibility implies a two-fold advantage in terms of animal welfare: (i) surgical castration with associated increase in pain, discomfort, stress and risk of infection and mortality is avoided; (ii) the welfare problem of boar behaviour is minimised to the relatively brief interval between on one hand observed problematic behaviour and second vaccine injection and on the other hand the delay time until control of behaviour is achieved. Moreover, analogous advantages are implied for sustainability. The higher anabolic potential of boars compared to castrates is present until the time of the second injection, implicating higher lean content and feed efficiency and more efficient use of nitrogen. If the second injection comes as a response to problematic behaviour, control over behaviour may increase productivity (an important aspect of sustainability) compared to boars, since growth rate in general is negatively correlated with e.g. mountings (Rydhmer *et al.*, 2006). Moreover, vaccinated pigs still have a higher anabolic potential after the second vaccine injection than castrates, evident from the higher levels of IGF-1, although this was not manifested in productivity results in this study, possibly related to feeding strategy.

7 Conclusions

Taken together, these results strongly indicate that a prepubertal or early pubertal vaccination against GnRH causes disruption of testicular structure and function in pigs at least until slaughter at age 25 weeks. Vaccination resulted in a reduction in size of reproductive organs, which was more pronounced for early vaccination. The potential of testes size as a practical tool to identify accidentally non-immunised pigs at slaughter is thus enhanced for early vaccinated pigs. IGF-1 was higher in vaccinated than surgically castrated pigs, indicating a higher anabolic potential.

Both vaccination regimens produced a reduction in circulating levels of testicular steroids, leading to control over reproductive function and boar taint. Boar taint was effectively minimised through arrested testicular production of androstenone and increased skatole metabolism.

It was for the first time shown that early vaccination up-regulated mRNA and protein expressions of the CYP450 enzymes responsible for skatole metabolism. The degree of this up-regulation was similar in surgically castrated and early vaccinated pigs. The expressions of CYP1A2, 2A and 2E1 were not precisely reflected in enzyme activities, suggesting that the regulation of these isoforms is probably more complex and needs further investigations.

Early vaccination yielded production results similar to standard vaccination and surgical castration, although vaccinated pigs had a higher anabolic potential. Income per carcass was similar for vaccinated and surgically castrated pigs. Furthermore, it was shown that early vaccination may be associated with animal welfare improvements over standard vaccination and entire male production as problematic aggressive and sexual behaviours are minimised, and over surgical castration since the incision is avoided.

There was no indication of return of testicular function for any of the vaccinated pigs at slaughter. These novel results put in question the reversibility of the effects of vaccination at a prepubertal or early pubertal stage on at least reproductive function. This implies for the first time that the

long-term effect of vaccination against GnRH may depend on the timing of the second injection in relation to the pig's developmental status.

Under these experimental conditions, early vaccination with Improvac can be used as an alternative to the recommended schedule with maintained control of boar taint and testicular secretory activity, without affecting income per carcass. This implies the possibility of a more flexible vaccination schedule and advantages in terms of animal welfare and sustainability. Early vaccination should, however, be tested using different breeds and feeding strategies before a final recommendation can be given.

8 Indications for future research

Management of immunised pigs

It is known that performance differs between boars and barrows and that feeding strategy affects performance and productivity of pigs (Xue *et al.*, 1997). Immunocastration also clearly affects performance although there seems to be conflicting results in the literature (Millet *et al.*, 2011). To better evaluate the effects of immunocastration on performance and carcass quality, having bearing on economy and sustainability, managerial aspects such as growth curves and nutrient requirements need to be further investigated.

The long-term effect of vaccination

Early vaccination under these conditions effectively controls boar taint and behaviour with maintained profitability. However, the long-term effects and mechanisms thereof have not been sufficiently investigated. Experiments need to be conducted under different managerial conditions (breed, feeding, etc) in order to come to any general conclusions and recommendations. Furthermore, the relationship between long-term effect and timing of the immunisation, in relation to the pig's developmental status deserves further investigation.

Skatole metabolism

Immunocastration controls boar taint through an arrested testicular synthesis of androstenone and increased metabolism of skatole. However, the metabolism of skatole has not been fully explained and needs further investigation. Expression and activity of skatole metabolising enzymes are normally performed on simplified systems, such as microsomes or 2D hepatocyte cultures. It is known that these systems do not accurately represent the tissue of interest, in this case the liver. They are, for example, not capable of expressing the microdifferentiation of the natural tissue. Moreover, it is not likely that they accurately represent the way metabolites (or indeed any metabolism regulating

substance) are presented to the involved enzymes. An investigation of 3D cell/tissue culture techniques could provide valuable insights into the metabolism, and regulation thereof, of endogenous or exogenous compounds. This could be realised through spheroid cell cultures or more advanced tissue engineering involving cooperation with, for example, material or polymer scientists and microbiologists. Such knowledge could be used in the field of boar taint as well as studies on toxicology, biocompatibility or screening of metabolically active substances or drugs without the use of laboratory animals.

Methods for accurate, specific and highly sensitive detection of metabolites would have to be developed accordingly, preferentially through the use of metabolomics techniques or targeted LC/MS^n.

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10 Acknowledgements

Financial support

The Swedish Board of Agriculture and SLU Dept of Food Science provided financial support. Pfizer Ltd. provided Improvac vaccine and additional financial support for extended sampling and analyses.

Supervisors

Galia Zamaratskaia, Kerstin Lundström and Andrzej Madej. You have been fantastic. The best one could hope for. Thanks for having the courage to employ a vegetarian to meat science and thank you for all your support, guidance, trust and friendship. You have shown me so many fascinating aspects of animal science. A special thanks to my main supervisor Galina for always having time, for being a wonderful scientific role-model as well as a good friend and for the insight into the virtues of laziness. Kristina Andersson has been my supervisor in all but name. You've also been a fantastic friend. We've all made quite a team together.

Co-workers and co-authors

Eleonor Palmér, my industry mentor, has shown me friendship and been a great partner in discussion and helped me to focus on the practical use of science. Thanks to our Danish colleagues Martin Krøyer Rasmussen and Bo Ekstrand for a fantastic collaboration, nice discussions and a good time together. Let's keep doing this in the future. Gang Chen helped with steroid analyses. Mattias Norrby developed the oestradiol-method and was a good discussion partner. Jing Li was a fantastic resource in HPLC analysis, performing long series of analyses always with a smile. Jana Tomànkovà, Tatiana Borisova, Isabelle Herbin and Hélène Lacoutière provided help with HPLC method development and CYP studies. Heriberto Rodriguez-Martinez, Stig Einarsson and Margareta Wallgren are the veterinarians with expert knowledge on testicular histology and spermatozoal morphology. All the staff

at Funbo-Lövsta research station, especially Ulla Schmidt and Eva Norling, took excellent care of the animals. Rolf Grahm, Gunilla Jacobsson, Johanna Zingmark, Eleonor, Galina, Kristina, Eva and especially Ulla all helped with sampling during slaughter. A big thank you also to John-Erik Haugen for a great collaboration on our review paper on androstenone and skatole analyses.

Proofreading

Thanks to everybody who helped me with suggestions on the thesis: Galina, Kerstin, Andrzej, Kristina, Eleonor, my aunt Margareta and my sambo Karolina. Steve Scott Robson has corrected the language and in the meantime acquainted me with some of the niceties of the English language. Steve and the team at Pfizer (led by Michael Pearce) are also gratefully acknowledged for suggestions and feedback on Papers II-V

TA Staff

Thanks to the awfully nice and friendly people that actually make things work around here: Margaretha Wijkström, Carina Nylander, Christina Nilsson and Lotta Wall. A special thanks to Lotta for being such a good friend.

Past and present colleagues

Pernilla and Jinfeng, my former roommates, with whom I've talked about science, philosophy, life in general and stuff. Thanks for making every day a nice one. Lucia, my new roommate, has unfortunately only seen me in "thesis writing mode". Sorry about that. Tyler and Honza, you are really cool people. Keep doing what you do. The little time I had with Maria was fun. It's nice and refreshing with people who see things differently. Good luck at Lövsta. Thank you Åsa for your warmth and your will and efforts to make everyone feel comfortable. You are one of the friendliest and most thoughtful persons I have known. I would have liked to get to know Erik a bit more, but I think he's happier working with sports and training and such. Thanks to Xin Li, Matti, Anna, Hanna, AnnaLotta, Liane, Carolin and all other PhD students for making "institutionalised" life more fun.

Friends

I am thankful to all my friends for the time we have had together and for not making me feel bad about the time we didn't have. Thank you Jonas, Tomas, Leo, Ulf and Ulf and all the other fantastic Täby/Cookiehead boys for all the music and good times. Mattias N has been one of my best friends since when we were students at KTH. Karin, Stina, Mattias L, Emma and Peter, my good friends from the time at the polymer department. Federico and Chiara, my

friends from a life-changing year in Italy. I also made some excellent friends, Henrik, Patrik and Johan, when I was a teacher in högstadiet. Thanks to everybody in Orphei Drängar for singing with me. Tuesday evening rehearsals and concerts are a real energy boost. Thanks to you and to all those I may have forgotten to mention.

Family

My family are the most important people in my life. You are my context. Without you I am not complete. To my fantastic and loving parents, Gustaf and Carin. I have inherited the joy of learning and interest in everything under the sun from you dad. You are the standard against which I compare myself. A part of me is missing. I will miss you always. To my brothers, Gustaf and Johan, and their families. I am fortunate to know the deep bond of brothers. A simple thank you is not enough to tell you how blessed I am for having you. To Karolina. We share life, love, friendship, attitude but not temperament. I love you more with each passing day. Thank you for making me part of your fantastic family and for starting a new one with me. To our sons, Ossian and Vide. You are the greatest gift. I love you all the way to the moon and back. In fact, so much that I sometimes think my chest is going to explode. To my future family, children, grandchildren and who-knows-what. I cannot wait to get to know you and to love you all.