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Studies of boar taint, with an emphasis on testicular function and development

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Contents

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Part I

Summary

Boar taint is an odour reminiscent of sweat, urine or faeces, perceived as off-flavour when meat from entire male swine is heated. The compounds that cause boar taint are androstenone and skatole/indole. Androstenone is secreted as a testis steroid, while skatole/indole originates in the large intestine. Environmental factors do not have much impact on androstenone levels. Castration is therefore used to eliminate the site of androstenone synthesis. This also influences skatole degradation, which is inhibited by androstenone in the liver. However, surgical castration is being phased out for animal welfare reasons, so that alternative ways of reducing boar taint are needed.

Heritability for androstenone levels is high, so that breeding for lower levels is possible. However, this will only be successful if unwanted side effects can be avoided. Side effects include lower levels of other sexual steroids and delayed sexual maturation in both boars and gilts. Breed differences in androstenone levels and sexual maturation have also been shown.

The principal aim of this thesis was to investigate the relationships between levels of androstenone and other testis steroids and testis development, including interactions between genotype, phenotype and breed differences.

Relationships between androgens, histomorphological phenotype and estimated breeding value for androstenone ($EBV_{androstenone}$) in Duroc were investigated. Functional pathway analysis of transcripts was carried out at different stages of pubertal testis development in Duroc. Breed response to luteinizing hormone/human chorionic gonadotropin (LH/hCG) stimulation was compared *in vivo* and *in vitro*.

The studies showed that the relative increase in androstenone was the same in Landrace and Duroc after LH/hCG stimulation *in vivo* and *in vitro*. Elimination of androstenone was slower in Duroc boars than in Landrace. Leydig cells from 3-week-old Duroc boars responded to lower LH doses and produced higher levels of testosterone than Leydig cells from Landrace. In both breeds, maximum levels for androstenone and oestradiol were achieved at a lower LH dose than maximum testosterone levels. The developmental study of Duroc showed that $EBV_{androstenone}$ was related to

pubertal androstenone levels and postnatal testosterone levels. Testis morphology was not related to pubertal $EBV_{androstenone}$ estimates. There were individual variations in steroid levels and histomorphological development. Changes in gene expression coincided with major histomorphological change in the testis. These were transcripts associated with specific growth factors, neurosteroids and morphological pathways. With pubertal onset, the expression of genes coding for neurological pathways and paracrine factors increased.

It is suggested that more testis development stages should be included in future studies to provide additional information for efforts to reduce androstenone through breeding. Studies of primary cultures of Leydig cells would be useful. The substantial individual variations and breed differences in the timing of morphological testis development should be taken into account. One relevant approach would be to conduct breeding trials over several generations. Single experiments may not reveal complex and unpredictable relationships between androstenone and other functions in the boar.

Sammendrag

Hanngriser utsondrer en urin- faecal-aktig kroppslukt som gjerne betegnes som rånelukt. Denne kan oppfattes som frastøtende når kjøtt fra hanngriser varmes opp. Lukten fra hanngriser kommer fra skatol /indol og androstenon. Skatol og indol blir dannet i tykktarmen. Nivåene av skatol og indol kan påvirkes av fôring og hygienisk standard, men det er også en betydelig arvelig komponent. Vekselvirkninger i leveren gjør at nedbrytingen av skatol påvirkes av androstenon.

Ettersom androstenon dannes sammen med steroidene i testiklene, har det vært vanlig å kastrere griser kirurgisk for å eliminere kjønnslukten. Kastrering ved kirurgisk inngrep fases ut grunnet dyrevernhensyn. Derfor er det etterspørsel etter bærekraftige alternativer for å redusere nivåene av androstenon.

Den høye arveligheten åpner for at nivåer for androstenon kan påvirkes gjennom avl, men slike tiltak kan først forventes når det blir mulig å unngå bieffekter av avlen. Slike bieffekter er lavere steroidnivåer og forsinket kjønnsmodning hos begge kjønn. I tillegg bidrar raseforsekjeller for både androstenonnivåer og kjønnsmodning til å komplisere problemstillingen.

Hovedformål med avhandlingen var å studere forholdet mellom androstenon, andre steroider med opphav i testikkelen og testikkelutvikling. Interaksjoner mellom genotype, fenotype og raseforskjeller var også inkludert. Studiene omfattet de ulike stadiene for kjønnsutviklingen hos Duroc hanngris sett i sammenheng med steroidogenese, histologiske funn og molekylære funksjoner. Spesifikk avlsverdi for androstenon ga grunnlag både for *in vivo* sammenligning mellom raser og for utviklingsstudier i Duroc som omfattet analyse av steroidnivåer, histologi og genekspresjonsprofiler. Effekt av stimulering med luteiniserende hormon/humant choriongonadotropin (LH/hCG) hos Duroc og Norsk Landsvin ble sammenlignet både *in vivo* og *in vitro*.

Spørsmål knyttet til androstenon ble undersøkt både *in vivo* og *in vitro*. Studiene viste at den relative økningen i androstenonnivå var lik hos de to rasene etter LH/hCG stimulering både *in vivo* og *in vitro.* Elimineringen av androstenon foregikk langsommere i Duroc råner enn i Norsk Landsvin. Videre viste et utviklingsstudie med Duroc at spesifikk avlsverdi for pubertal androstenon var

assosiert med testosteronnivåene hos spegris og androstenon nivåene i puberteten. Leydigceller fra 3 uker gamle Duroc griser responderte på lavere dose ved LH stimulering og med høyere testosteronnivåer enn Leydigceller fra Norsk Landsvin. I begge raser ble maksimumsnivåer for androstenon og østradiol oppnådd ved lavere LH dose enn for testosteron. Individuelle forskjeller mellom råner ble funnet både for steroidnivåer og morfologisk testikkelutvikling. Clustre med genuttrykk med endring over tid i puberteten, inneholdt ulike morfologiske pathways, vekstfaktorer og parakrine faktorer som kunne knyttes til spesifikke stadier i pubertetsutviklingen hos Duroc. Ved pubertetens begynnelse ble det funnet økt gen-ekspresjon for pathways og gener som koder for dannelse av nerver i testikkelen.

Effekter av avl for lavere androstenon nivåer er vanskelige å forutsi. Komplekse sammenhenger ligger bak reguleringen av dette steroidet. Heller ikke underliggende årsaker til raseforskjeller, kan forklares ved enkle mekanismer. Resultatene i avhandlingen har bidratt til å belyse denne kompleksiteten. En konklusjon er at ytterligere studier er nødvendige for å finne ut hvilke konsekvenser det vil få om det gjennomføres avlstiltak for reduksjon av androstenon. I denne sammenheng vil studier av primærkulturer med Leydig celler være et egnet verktøy.

En relevant tilnærming vil være å gjennomføre kontrollerte avlsforsøk over flere generasjoner, dette fordi isolerte, eksperimentelle forsøk ikke fanger opp kompleksiteten i hvordan androstenon er forbundet med andre kroppsfunksjoner. Likeledes bør det i framtidige studier tas hensyn til at kjønnsutviklingen er en dynamisk prosess som strekker seg over en lang tidsperiode. Det kan derfor være uheldig at seleksjon basert på fenotyper knyttes til en bestemt alder.

Abbreviations and synonyms

andien	androstadienol/5,16-androstadien3β-ol/ndienB
androstadienone	4,16-androstadien-3-one
androstenedione	4-androstenendione
androstenol	5α-androst-16-en3-a-ol/3a-androstenol/3β-androstenol
androstenone	5α-androst-16-en-3-one
3BHSD	HSD 3β-hydroxysteroid dehydrogenase
cholesterol	3β-cholest-5-en-3-ol
CYB5	cytochrome b-5/cholesterol side-chain cleavage enzyme, cholesterol desmolase/cytochrome P450scc
CYP2A6	cytochrome P4502A6
CYP2E1	cytochrome P4502E1
CYP11A1	cytochrome P450, family 11, subfamily A, polypeptide 1
CYP17	cytochrome P450 17A1/cytochrome P450-C17 steroid 17-alpha-hydroxylase/17,20 lyase steroid 17-alpha-monooxygenase/17,20 desmolaseP450C17
CYP19	P450arom CYP19
CYP21	21-hydroxylase
CYP51	Sterol 14α-Demethylase Cytochrome P450 Lanosterol 14α-demethylase
dehydroepiandrosterone	DHEA
deoxycorticosterone	21-hydroxyprogesterone/DOC
deoxycortisol	11-deoxycortisol/cortexolone
DHCR24	3 β-hydroxysteroid-Δ 24 reductase
EBV	estimated breeding value
EGF	epidermal growth factor
EPS15	epidermal growth factor receptor pathway substrate 15
estradiol	17β-oestradiol/oestradiol/E2
estron	oestrone/E1
GnRH	gonadotropin-releasing hormone
HPG	hyopthalamic-pituitary-gonadal axis
HSD20B	20βHSD/20β-hydroxysteroid dehydrogenase
hCG	human chorionic gonadotropin
HSA	homo sapines
HSD17B	17β-hydroxysteroid dehydrogenase
HSD3B	3β-hydroxysteroid dehydrogenase/3BHSD-isomerase
IGF	insulin-like growth factor
LH	luteinizing hormone
MAPK	mitogen-activated protein kinase
MAS	meiosis activating sterols
OH-pregnenolone	17-hydroxypregnenolone
OH-progesterone	17a-hydroxy-4-pregnene-3,20dione/17OHP 17-hydroxyprogesterone /17-OH progesterone
Pregnenolone	3β-hydroxypregn-5-en-20-one
Progesterone	pregn-4-ene-3,20-dione
qRT-PCR	quantitative real-time PCR
skatole	3-methyl indole/3MI
SSC	sus scrofa
STAR	steroidogenic acute regulatory protein
SULT2A1/SULT2B1	hydroxysteroid sulfotransferase
TGF	transforming growth factor
TGFBR	transforming growth factor beta receptor

List of papers

Paper I

I. C. Oskam, S. Lervik, H. Tajet, E. Dahl, E. Ropstad, Ø. Andresen. Differences in testosterone, androstenone, and skatole levels in plasma and fat between pubertal purebred Duroc and Landrace boars in response to human chorionic gonadotropin stimulation.
Theriogenology, 74 (2010) 1088–1098

Paper II

S. Lervik, K. von Krogh, C. Karlsson, I. Olsaker, Ø. Andresen, E. Dahl, S. Verhaegen, E. Ropstad. Steroidogenesis in primary cultures of neonatal porcine Leydig cells from Duroc and Norwegian Landrace breeds. Theriogenology 76 (2011) 1058–1069

Paper III

S. Lervik, I. Oskam, A. Krogenaes, Ø. Andresen, E. Dahl, H.A. Haga, H. Tajet, I. Olsaker, E. Ropstad. Androstenone and testosterone levels and testicular morphology of Duroc boars related to estimated breeding value for androstenone. Theriogenology 2013, 79:986-994.

Paper IV

S. Lervik, A. B. Kristoffersen, L. N. Conley, I. C. Oskam, J. Hedegaard, E. Ropstad, I. Olsaker. Gene expression during testis development in Duroc boars.
Submitted to Animal

Background

1.1. Boar taint

Boar taint is released as an odour reminiscent of manure, sweat or urine when pork from some pubertal and adult male pigs is heated. Two main compounds, androstenone and skatole, are responsible for the unpleasant odour. The steroid pheromone androstenone has its origin in the testis, while skatole is produced in the large intestine. Carcasses with an off-flavour due to these lipophilic compounds are undesirable (Bonneau, 1982; Bonneau et al., 2000; Zamaratskaia and Squires, 2009), though consumer perception of boar taint varies (Weiler et al., 2000). Exposure may increase odour sensitivity (Morlein et al., 2013), but tolerance also has a genetic component (Wysocki and Beauchamp, 1984). Gender differences in perception have been found (Meier-Dinkel et al., 2013).

Surgical castration of piglets has traditionally been used to avoid boar taint. The method efficiently eliminates the site of androstenone synthesis in the boar (Katkov and Gower, 1968). Castration also reduces levels of skatole, the other main component of boar taint (Babol et al., 1999; Doran et al., 2004). The reason is that androstenone inhibits skatole degradation in the liver (Tambyrajah et al., 2004). More skatole is therefore broken down in boars with low levels of androstenone.

Currently, approximately 125 million male piglets per year are castrated in Europe (Fredriksen et al., 2009). A declaration on a voluntary end to surgical castration of boars in the European Union has been endorsed by a number of actors in the European pork industry. If alternative solutions to surgical castration can be found, the EU partners aim to eliminate the practice by 2018. Plans to prohibit castration in Norway from 2009 could not be implemented since no alternative solutions to the problem of boar taint had been found (Stenevik and Mejdell, 2011). Sustainable alternative solutions to surgical castration would be beneficial for pig breeders.

1.2. Boar taint compounds

1.2.1. Androstenone

The steroid pheromone androstenone is synthesised in the interstitial Leydig cells of the testis. From there, it circulates via the blood to the submaxillary gland. Some fractions are a sulfoconjugated form which is more water-soluble, facilitating blood transport (Sinclair et al., 2005b). The submaxillary gland secretes a bioactive form of androstenone (Katkov et al., 1972; Reed et al., 1974). Saliva composition may be influenced by other steroid intermediates and the fraction of androstenone bound to the protein pheromaxein in the submaxillary gland (Booth et al., 1973; Booth and von Glos, 1991). Correlation has been found between levels of androstenone in plasma, fat and the submaxillary gland (Booth et al., 1986).

Androstenone mediates sexual signals between boars and sows (Booth, 1975; Reed et al., 1974)}. The most described effect of androstenone-related behaviour is the standing reflex. This is a reflexive calmness of the sow in oestrus, ready for mating (Pedersen, 2007). The reflex is used for detection of oestrus in artificial insemination (Melrose et al., 1971; Reed et al., 1974). The pheromone androstenone also affects reproduction in other ways. Such are stimulation for pubertal onset, litter size, farrowing rate and non-return rate (Am-in et al., 2010). Territorial effects of urine-mediated androstenone have also been described (McGlone and Morrow, 1988).

Excess androstenone is temporarily stored in fatty tissue. Androstenone levels in both fat and plasma decrease if testis secretion is interrupted. In the liver, androstenone is degraded to soluble molecules before urinary clearance (Zamaratskaia and Squires, 2009).

1.2.2. Synthesis and degradation of androstenone

Steroidogenesis is induced when cholesterol is transported into the inner mitochondrial membrane (Prinz, 2007). Cholesterol transport is assisted by the key mediator steroidogenic acute regulatory protein (STAR) (Stocco and Clark, 1997).

Two main classes of proteins are involved as steroidogenic enzymes. One is cytochrome P450 (CYP), a family of haeme-containing proteins. The other is hydroxysteroid dehydrogenases (HSD). These have many tissue- and step-specific isoforms (Payne and Hales, 2004). The first steroidogenic step in the mitochondria is that CYP11A cleaves off cholesterol side chains to form pregnenolone and progesterone. These are the main substrates for overall steroidogenesis, and pregnenolone is the most important substrate for androstenone synthesis (Figure 1.). The levels of these two precursors have been shown to be correlated with androstenone in fat (Louveau et al., 1991).

The pathway with the highest yield of androstenone is called delta 5 synthesis (Booth, 1975). In the delta 5 pathway, pregnenolone is the substrate for androstenone (Katkov and Gower, 1970). Andien B synthetase including CYB5 converts pregnenolone into andien (androstadienol) by hydrogen shift via formation of a free alkoxy radical on a methyl group. The next intermediate stage is oxidation to androstadienone by 3β-hydroxysteroid dehydrogenase (HSD3B). In the final step, androstenone is formed by steroid 5α-reductase (SRD5A) (Brooks and Pearson, 1986; Nicolau-Solano et al., 2006).

Alternatively, androstenone can be synthesised from progesterone via the delta 4 pathway. In this case, the intermediates are OH-progesterone and androstadienone (Figure 1). Intermediates from the delta 4 and delta 5 pathways do not interact during synthesis of androstenone (Booth, 1975; Cooke, 1996; Hebert and Cooke, 1992).

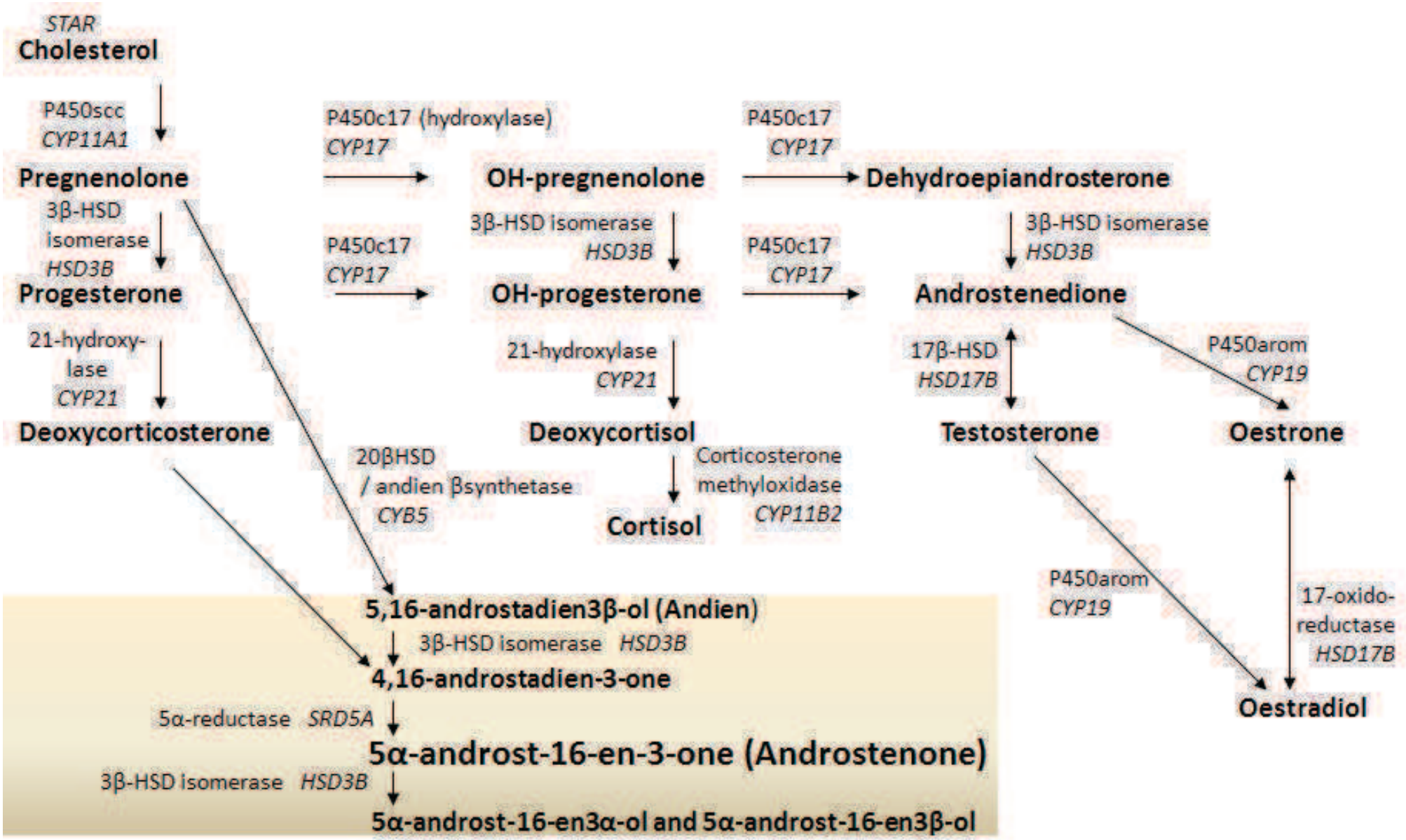

Figure 1. Pathways for synthesis of androstenone in the boar testis (highlighted) as described by (Brooks and Pearson, 1986; Gower, 1972) adjusted from (Payne and Hales, 2004). The figure shows the most important delta 5 pathway from pregnenolone and the delta 4 pathway from progesterone and steroidogenesis.

Androstenone degradation pathways may differ depending on age. Before the age of 12 weeks, output from the 3-alpha pathway to androstenol was found to dominate over that from the 3-beta pathway, whereas the 3-beta pathway was dominant in boars of 17 weeks and over (Gower, 1972).

The levels of androstenone in plasma depend on clearance in the liver and deposition in fat. Degradation of androstenone takes place mainly in the liver. The first step is hydrogenisation, which increases steroid polarity. This is followed by sulfoconjugation with hydroxysteroid sulfotransferase (SULT2A1, SULT2B1) (Moe et al., 2007a; Sinclair et al., 2005b). Sulfoconjugation takes place in both testes and liver tissue with variable impacts on androstenone clearance and accumulation in fat (Zamaratskaia et al., 2007). Two isoforms are created by sulfoconjugation. The degradation product 3b-androstenol dominates in the testis, whereas 3a-androstenol dominates liver output (Brophy and Gower, 1972; Hurden et al., 1979; Sinclair et al., 2005b). Conjugates formed by liver glucuronidation (phase II reactions) account for 68% of the conjugated androstenone fraction (Sinclair et al., 2005a). Degradation in the liver influences peripheral plasma levels of androstenone stored in fat tissue (Sinclair et al., 2005a).

1.2.3. Synthesis and degradation of skatole and indole

Skatole accumulated in fatty tissue contributes to boar taint. Skatole acts as a natural anti-pathogen, inhibiting overgrowth of the intestinal gram-negative flora (Yokoyama and Carlson, 1979). The gut flora is also the site where skatole formation takes place. The first step is dependent on the availability of the amino acid L-tryptophan and indolepyruvate-producing microorganisms containing tryptophanase. Various intestinal microbe species convert indolepyruvate to another boar taint compound, indole. Alternatively,

indolepyruvate may be decarboxylated via indolacetate to form skatole (3-methyl indole) (Deslandes et al., 2001; Yokoyama and Carlson, 1979).

After absorption of skatole and indole in the intestinal tract, these compounds are either transported to fatty tissue for storage or to the liver for degradation. Levels of stored skatole depend partly on the rate of degradation in the liver (Lin et al., 2004). Two phases of skatole degradation have been identified. First it is hydroxylated and methylated by cytochrome P4502A6 (CYP2A6), cytochrome P4502E1 (CYP2E1) and aldehyde oxidasis (Diaz and Squires, 2000a; Diaz and Squires, 2000b). The second phase is glucuronidation and sulfation (SULTA1) (Baek et al., 1997; Diaz and Squires, 2003). The degradation of skatole can be affected by inhibition of cytochrome P4502E1 (CYP2E1) (Babol et al., 1999; Doran et al., 2004). When CYP2E1 activity in the liver is low, more skatole is accumulated in the fatty tissue (Babol et al., 1998). Skatole metabolism in the liver is correlated with synthesis of androstenone in the testis (Babol et al., 1999). Androstenone can influence skatole degradation through an inhibitory effect on the promoter site of hepatic CYP2E1 (Tambyrajah et al., 2004). When boars are castrated, this effect is weakened (Tambyrajah et al., 2004).

2. Methods of reducing boar taint

A great deal of work has been done to identify the steroidogenic steps of the androstenone pathway, with the aim of finding ways of reducing androstenone levels without disrupting overall steroidogenesis (Dufort et al., 2001; Gray and Squires, 2013a; Gray and Squires, 2013b; Tambyrajah et al., 2004). However, more basic research is needed to find practical solutions.

Several factors have been studied to identify practical approaches to influence boar taint at different levels. Oestradiol has been shown both to influence androstenone and skatole levels (Zamaratskaia et al., 2005b) - and suppresses androstenone and sexual development (At-Taras et al., 2006; Echternkamp et al., 1969). However, commercial application of oestradiol in swine cannot be recommended because of the risk of residues in meat for human consumption.

Conflicting results have been obtained as regards a diurnal rhythm in androstenone production, with some studies finding signs of this (Andresen, 1975; Claus and Gimenez, 1977) while other authors doubted that light-dark cycles had any effect (Bonneau et al., 1987b). Interventions using light have therefore not been used in practice.

Social interactions and the effects on androstenone of grouping are complex. Studies of whether or not social effects have an impact on boar taint have produced diverging results (Giersing et al., 2000; Zamaratskaia et al., 2005c). The effects of regrouping boars on androstenone production have been evaluated. Raising pigs in stable groups in a farrow- to finish (FTF) management system has been suggested to reduce androstenone levels in fat. However, no beneficial effect of FTF management on skatole levels has been found (Fredriksen et al., 2006).

So far, most studies have focused on ways of interrupting the overall synthesis of skatole, indole and androstenone in order to reduce boar taint. These methods are described further below.

2.1. Feeding regimes

The level of skatole synthesis depends on the nutritional availability of tryptophan and the composition of the intestinal flora. Various dietary components can prevent

excessive growth of the intestinal gram-negative flora (Yokoyama and Carlson, 1979). For example, carbohydrates such as raw potato starch can lower skatole levels (Zamaratskaia et al., 2005a; Zamaratskaia et al., 2005d). Chicory inulin and sugar beet also support the colon flora and reduce skatole output (Rideout et al., 2004; Whittington et al., 2004).

Most studies have shown that androstenone levels are not affected by diet, as reviewed by Zamaratskaia (Zamaratskaia and Squires, 2009). However, new data have shown that adding chicory to the diet might result in some lowering of androstenone levels (Rasmussen et al., 2012).

2.2. Surgical castration

Surgical castration of piglets has traditionally been used to avoid boar taint (Prunier et al., 2006). The method efficiently eliminates the site of androstenone synthesis in the boar (Katkov and Gower, 1968). Castration also reduces levels of skatole, the other main component of boar taint (Babol et al., 1999; Doran et al., 2004). Analgesia is used in only 3 % of the castrations performed in EU countries (Fredriksen et al., 2009).

Local anaesthesia was made mandatory for all castration of boars in Norway in 2002. In the Netherlands, local anaesthesia has been used in organic pig production since 2007. In Sweden, farmers are offered courses where they can learn proper use of anaesthesia and standard procedures for handling and castrating piglets. Governmental grants are also available to farmers in Sweden to cover the costs of using anaesthesia during surgical castration.

Signs of inflammation after surgical castration might occur in the inguinal cave or associated with the scrotal site of incision. The complications most often seen after surgical castration are poorer overall condition and herniation and abscesses, the latter two at low

frequencies (Fredriksen et al., 2009). Wound healing is better after postnatal castration than after castration in week 3. *post partum* (Heinritzi et al., 2006). Additionally, wounds heal better in piglets given a short-term analgesic during surgical treatment (Sutherland et al., 2010). The short-term immune response is not influenced by use of analgesia during surgical castration (Sutherland et al., 2010).

In Norway, local treatment with lidocaine is commonly used for castration of piglets (Haga and Ranheim, 2005; Ranheim et al., 2005). For older animals, azaperone sedation combined with analgesia using ketamine or epidural injection has been common veterinary practice. In addition, the perioperative use of non-steroidal anti-inflammatory drugs (NSAIDs) is recommended. NSAIDs increase stability, drinking and eating behaviour after castration (von Borell et al., 2009). Meloxicam and ketoprofen are the NSAIDs most commonly used after castration of piglets in Norway (Fosse et al., 2008; Fosse et al., 2011a; Fosse et al., 2011b).

2.3. Immunological castration

Immunisation against GnRH is an alternative to surgical castration. Antibodies against GnRH efficiently reduce the output of gonadotropins from the pituitary gland (Zamaratskaia et al., 2008b), resulting in degeneration of the testes and reducing/ceasing the secretion of steroids, including androstenone (Brunius et al., 2011; Thompson, 2000). Immunologically castrated boars show higher food efficiency and weight gain than control groups (Dunshea et al., 2011).

In Norway the slaughter industry has strict routines to avoid tainted meat from immunologically castrated boars. If meat proves to be tainted after immunological

castration, the farmer bears the economic risk. Immunological castration has also been evaluated as logistically difficult by users (Fredriksen B. and Nafstad O., 2012).

The Norwegian slaughter industry recommends administering two injections with an interval of at least 28 days. The second injection must be given at least 28 days before slaughter. Controls are needed to confirm a decrease in testis size. A third injection may be given if necessary.

At the slaughterhouse, carcasses with an androstenone level of more than 1 µg/g androstenone in the neck fat are rejected for human consumption. However, more efficient techniques and sensory devices need to be developed. Rapid detection methods for measuring the components of boar taint are needed for use at slaughter plants (Haugen et al., 2012).

2.4. Sperm sexing

Sperm sexing and the use of X sperm only for insemination is a theoretical possibility. However, the costs are high and the technique can therefore only be recommended for very large production units (Tuyttens et al., 2012).

2.5. Early slaughter

Early slaughter of prepubertal animals is used in some countries to prevent the development of high levels of skatole and androstenone. However, skatole and androstenone levels above the tolerated cut-off levels can be found in fat from boars at 110 days of age or live weight of 75 kg (Aldal et al., 2005) and in 85 kg carcasses (Neupert et al., 1995). Because of individual variations in androstenone levels (Andresen, 1976; Bonneau et al., 1987a), tolerated levels of androstenone are likely to be exceeded in some boars.

Additionally at 50 kg live weight, considerable skatole concentrations may be found in the back fat (> 0.15 μg/g) (Hansen et al., 2005).

However, approximately 25 million European boars are raised without castration. The incidence of castration is lowest in Ireland and the United Kingdom (Fredriksen et al., 2009). Carcass weights in the Norwegian and EU markets are on average 90–100 kg. This is higher than in the UK and Ireland, where pigs are slaughtered at a market weight of 75 kg (Rault et al., 2011).

In addition to boar taint, the problem of aggression between pigs must be solved when raising entire males. There is risk that mounting and agonistic behaviour by more mature individuals result in injury (Giersing et al., 2000). More research is required to find solutions such as breeding for a temperament that will reduce behavioural problems in entire boars (von Borell et al., 2009).

2.6. Breeding for low androstenone

2.6.1. Breeding and side effects

There is a significant genetic component in variations in androstenone levels in boars. The boar taint phenotype is a quantitative trait measured for instance as the pheromone levels in fatty tissue and the length of the bulbourethral gland (Tajet et al., 2006). Studies have produced heritability estimates for androstenone levels ranging from 0.25 to 0.88 (Sellier, 1998; Sellier et al., 2000; Varona et al., 2005). It is therefore likely that breeding for lower levels of androstenone will have some success.

However, breeding for lower androsterone levels is associated with unwanted side effects, and this approach will not be efficient before such problems are solved. Low levels of androstenone are associated with delayed onset of puberty and changes in levels of other

sexual steroids (Willeke et al., 1987). An approach that circumvents the association between reproductive development and lower levels of androstenone is needed.

The high genetic correlation between levels of different steroids poses a significant challenge when breeding for low androstenone levels. Genetic correlation between plasma levels of androstenone and the testicular steroids oestronesulfate, oestradiol and testosterone ranges from 0.80 to 0.95 (Grindflek et al., 2011b). Research has therefore been undertaken to find key factors determining the properties of genes involved in sexual steroids and androstenone (Figure 1.) (Grindflek et al., 2011a; Quintanilla et al., 2003; Robic et al., 2008; Zamaratskaia and Squires, 2009).

The genetic correlation between androstenone in plasma and fat and other sexual steroids are higher than 0.8 (Grindflek et al., 2011b). This indicates that there should be a influence on other steroids if androstenone levels in fat are decreased through breeding. There is also a close association between phenotypic levels of androstenone and the other sexual steroids. Lines bred for low androstenone phenotype were found to have lower levels of oestradiol and testosterone (Willeke et al., 1987). Testis development is initiated and maintained by stimulation from pituitary gonadotropins. Balanced feedback and response from testis steroids is important for the timing of pubertal onset. The delicate balance between these processes is influenced by breeding for lower levels of androstenone. Unfortunately, the levels of androstenone and indicators for sexual maturation are correlated (Bonneau and Russeil, 1985; Xue et al., 1996). Delayed sexual maturation in the sow has been found as a side effect of breeding for low androstenone (Sellier and Bonneau, 1988; Willeke et al., 1987). It is very important to consider pubertal onset in this context, since pubertal onset in the boar is correlated with pubertal development of the sow (Schinckel et al., 1983; Sellier et al., 2000). Delayed onset of puberty would prolong

reproductive intervals of sows. Another reported side effect of breeding for low androstenone levels is a decrease in testis weight in the boar (Sellier and Bonneau, 1988; Willeke et al., 1987).

Several approaches have been tried to avoid the negative association between sexual development and androstenone described by Sellier and Bonneau (1988) and Willeke et al (1987). The status of pubertal development in the boar was indicated by characteristics of the bulbourethral gland. Its length was suggested as an indicator of increased levels of steroids, including androstenone, secreted from the pubertal boar testis (Bonneau and Russeil, 1985). Since then, other studies have been based on the genetic correlation between length of the bulbourethral gland and androstenone, which has been used in a selection index breeding against androstenone. However, these breeding studies did not succeed in reducing androstenone levels (Sellier et al., 2000). This can possibly be explained by the high genetic correlation between androstenone levels and sexual maturation (0.50) (Sellier et al., 2000; Bonneau and Sellier, 1986; Sellier and Bonneau, 1988).

Interestingly, experimental breeding of swine for lower cholesterol levels has previously been attempted with a view to lowering the incidence of human cardiovascular disease (Wise et al., 1993; Young et al., 1993). However, side effects were observed in the third generation of lines bred for low cholesterol. These were comparable with the side effects in the third generation of lines of boars bred for low levels of androstenone. In both cases, levels of other steroids that are essential for reproduction were affected. Furthermore, female fertility traits such as ovulation rates and litter size are influenced both by breeding for low cholesterol (Wise et al., 1993; Young et al., 1993) and by breeding for low androstenone (Sellier and Bonneau, 1988; Willeke et al., 1987).

2.6.2. Understanding androstenone

Studying the side effects of breeding for lower androstenone levels is difficult since the molecular functions associated with androstenone are complex. Androstenone is possibly the product of several different genes acting together (Zamaratskaia and Squires, 2009). More target genes have been studied (Duijvesteijn et al., 2010; Grindflek et al., 2010; Robic et al., 2011; Robic et al., 2008).

2.6.3. QTL analysis

Mapping of chromosomal regions associated with androstenone levels (that is quantitative trait loci, or QTL) might improve our understanding of the low androstenone phenotype. QTL regions contain genes involved in the metabolism of several testis steroids/androgens, not just androstenone (Duijvesteijn et al., 2010, Grindflek et al., 2011b). However, one particular region on chromosome 6 was identified as associated with androstenone levels in plasma at week 20–22 of age (Grindflek et al., 2011b).

Interestingly, Quintanilla et al (2003) (Quintanilla et al., 2003) showed that different loci could be related to androstenone at different stages of development. This raises the question of whether the results of studies of testicular genes in mid-pubertal boars, i.e. at slaughter age, can be extrapolated to other stages of testis development.

2.6.4. Functional pathway analysis

A promising method for gaining information on molecular functions during testis development is analysis of global gene expression. Knowledge of functional pathways can be expected to improve understanding of the most important changes during sexual development in the boar. Microarrays have been used in large-scale gene expression studies comparing extreme high and low boar taint phenotypes at mid-puberty (Moe et al., 2007b).

Sequencing of mRNA is another possible approach that become more widely used in recent years as the cost of sequencing has dropped.

3. Testis development

3.1. Development in the juvenile boar

During the perinatal weeks, the Leydig cells are well developed (Lejeune et al., 1998; Van Straten and Wensing, 1978). A peak in volume and organelle activity can be found at 14 days *post partum* (Lunstra et al., 1986). The perinatal proliferation of Leydig cells results in a high capacity for androstenone synthesis (Lundstrom et al., 1978; Sinclair et al., 2001b). Perinatal profiles for other steroids including oestradiol also show high levels (Tan and Raeside, 1980). The perinatal role of oestradiol has so far not been clearly identified (Mutembei et al., 2005), But studies on the bioactivator of oestradiol CYP19 have indicated that oestradiol has age-specific roles/functions (Conley et al., 1996; Haeussler et al., 2007; Mutembei et al., 2005).

Testosterone levels are also high in the perinatal period (Schwarzenberger et al., 1993), possibly indicating an anabolic role (Kuhn, 2002). Androgen receptors activated in the muscles stimulate growth (Claus and Weiler, 1994). In addition, oestradiol stimulates insulin-like growth factor I, which leads to an increase in growth hormone receptors in the liver (Claus and Weiler, 1994).

Conversely, growth factors have also been shown to have an effect on testosterone (Sordoillet et al., 1991; Sordoillet et al., 1992). Growth factors interact with gonadotropins and testosterone in the boar testis (Chuzel et al., 1996; Lejeune et al., 1996; Sordoillet et al., 1991; Sordoillet et al., 1992). It has also been suggested that thyroid hormones play an important role in testis development (Mendis-Handagama and Siril Ariyaratne, 2005; Syed et al., 1985; Vihko and Huhtaniemi, 1989). However the role of thyroid hormone in the

developing boar testis is not yet fully understood (Klobucar et al., 2003). A negative relationship has been found between thyroxin (T4) and androstenone (Zamaratskaia et al., 2004). On the other hand, breeding against androstenone failed to show any effect on triiodothyronine (T3) (Sellier et al., 2000).

Attempts to predict androstenone levels in pubertal boars by LH/hCG stimulation of postnatal piglets have not been successful. No relationship was found between postnatal basal and pubertal androstenone levels (Sinclair et al., 2001a). If a relationship between juvenile testis activity and the pubertal developing testis could be found, it would be of great interest in studies related to prediction of androstenone in boars.

3.2. Development in the pubertal boar

Morphological regression follows after the juvenile period of high steroidogenic and proliferative testis activity. From 40 until 120 days of age, cell size declines and androgenic secretion is low. After this, elevated pubertal steroidogenic and hypothalamic pituitary activity coincides with an increase in the volume of individual Leydig cells. The morphological development of the testis is a result of the interplay between gonadotropins, LH, follicle-stimulating hormone (FSH) and steroids (Lunstra et al., 1986). At this age, Leydig cell volume is important for the density of LH receptors (Peyrat et al., 1981). Furthermore, the Leydig cell LH receptors show higher binding affinity in pubertal boars than in immature boars (Tripepi et al., 2000).

Elevated levels of FSH coincide with a pubertal wave of Sertoli cell development. The length of the seminiferous tubules also increases (Franca et al., 2000; Vergouwen et al., 1991). When the lumen of the seminiferous tubules have developed sufficiently, the relative fraction of Leydig interstitial cells decreases (Dierichs and Wrobel, 1973). The onset of

puberty is reviewed by Franca (2005). This can be recognised by the presence of the first spermatozoa in the lumen of the seminiferous tubules (2005) (Franca et al., 2005). The molecular mechanisms related to the onset of puberty in the boar testis have not been described systematically.

4. Breed differences

4.1. High levels of androstenone in Duroc boars

Androstenone levels are considerably higher in Duroc boars than in Yorkshire, Landrace or Hampshire boars (Squires and Lou, 1995; Xue et al., 1996). This is unfortunate given that demand for Duroc is increasing due to its meat quality. This is an important issue in Norway since Duroc is preferred as paternal line in crossbreeds. The current commercial hybrid for production of pork in Norway consists of 25 % Landrace, 25% Yorkshire and 50% Duroc. The high levels of androstenone in Duroc make it important to give priority to studies comparing breed differences related to steroidogenesis and sexual development.

4.2. Onset of puberty

Levels of FSH differ considerably between breeds (Ford et al., 2001). This is important since FSH is associated with mitosis of the Sertoli cells, which are involved in the pubertal development of seminiferous compartments (Franca et al., 2005). The gonadal pituitary feedback effects also appear to differ between breeds (Wise et al., 1996). Other breed-related mechanisms have been demonstrated for proliferation within the tubular compartment in swine embryos. The fetal androgens depend on the enzyme CYP17 for tubular development, which also shows inter-breed differences (Kaminski et al., 1999).

For Norwegian crossbreeds, development of seminiferous compartments took place at approximately 125–146 days of age (Oskam et al., 2008). This is somewhat later than

found in Large White x Landrace crossbreeds (Florcruz and Lapwood, 1978) and in Landrace x Duroc boars, where the onset of puberty was indicated between day 100 and 130 (Allrich et al., 1983). The Meishan boar is known for early maturation, and sperm can be found as early as 75 days (Harayama et al., 1991). Further breed differences are reviewed by Ford (Ford et al., 2006).

The complexity of breed differences must also be considered for skatole levels. It has been suggested that they are related to pubertal onset (Babol et al., 2004). Peak skatole secretion occurs at different ages in different breeds (Babol et al., 2004). Breed differences are therefore of special interest when considering the onset of puberty in the boar.

4.3. In vitro assessment of breed differences

Studies of boar taint have previously been carried out using *in vitro* models based on isolated hepatocyte cultures, making it possible to demonstrate breed differences. When hepatocytes from androstenone phenotyped Duroc and Landrace boars were compared, breed differences were found in the expression of genes for enzymes involved in degradation of androstenone in the liver (Moe et al., 2007a).

This shows that *in vitro* analysis of cells from different breeds can be used to identify potential breed differences. Hence, Leydig cell cultures may be useful for comparing breed-specific traits related to androstenone.

Objectives

Heritability for androstenone levels is high (Sellier, 1998; Sellier et al., 2000; Varona et al., 2005). However, progress in breeding for lower androstenone levels will only be successful when unwanted side effects can be reduced or avoided. The complexity of unwanted side effects has often been illustrated by the high correlation between testis steroids, androstenone and sexual development. The close association between steroids and sexual development has been shown as genetic correlation between androstenone levels, testis weight and the size of the bulbourethral gland (Sellier et al., 2000; Bonneau and Sellier, 1986; Sellier and Bonneau, 1988). High phenotypic correlation has also been found between androstenone levels and bulbourethral gland length and weight (Bonneau and Russeil, 1985; Xue et al., 1996).

Breeding for lower levels of androstenone influences levels of steroids that are essential for reproduction (Willeke et al., 1987; Grindflek et al., 2011a; Grindflek et al., 2011b). This has caused detrimental effects on sexual maturation and fecundity (Sellier and Bonneau, 1988; Willeke et al., 1987). A complicating factor is that there are significant breed differences in both androstenone levels and the onset of puberty (Oskam et al., 2008; Florcruz and Lapwood, 1978; Harayama et al., 1991).

Principal aim of the study

The principal aim of the thesis was to investigate interactions between androstenone and other steroids occurring in the testis and their role in testis development, including interactions between genotype and phenotype and breed differences.

Secondary aims

The secondary aims of the thesis were to:

- Compare responses to *in vivo* and *in vitro* stimulation with LH/hCG in purebred Duroc and Landrace boars (Papers I and II).
- Investigate the relationship between androgens, histomorphological phenotype and $EBV_{androstenone}$ in Duroc (Paper I and Paper III).
- Study functional pathways of transcripts at different stages of pubertal testis development in Duroc (Paper III and Paper IV).

Results, summary of Papers I, II, III and IV

Paper I

I. C. Oskam, S. Lervik, H. Tajet, E. Dahl, E. Ropstad, Ø. Andresen. Differences in testosterone, androstenone, and skatole levels in plasma and fat between pubertal purebred Duroc and Landrace boars in response to human chorionic gonadotropin stimulation.

Theriogenology, 74 (2010) 1088–1098

Concentrations of the boar taint compounds androstenone and skatole in plasma and fat, together with those of testosterone in plasma, were investigated in pubertal purebred Duroc and Landrace boars following stimulation with human chorionic gonadotropin (hCG). Initial levels of androstenone and testosterone were higher in Duroc than in Landrace boars. Duroc boars, which were approximately ten days older than the Landrace boars, also showed a more advanced stage of spermatogenesis than Landrace boars. However, skatole levels were highest in Landrace boars. Following stimulation with hCG, the largest relative increases in testosterone, androstenone and skatole concentrations were measured in Landrace boars. The level of androstenone in fat three days after hCG stimulation exceeded 1 ug/g fat in all stimulated boars. The decreases in plasma levels of androstenone and testosterone on days 2 and 3 after hCG stimulation were more pronounced in Landrace than Duroc boars. However, unlike the plasma androstenone and testosterone levels, the plasma concentrations of skatole did not decrease on days 2 and 3 following stimulation, but remained elevated on day 3. These results indicate that the lower levels of testicular steroids in Landrace boars than in Duroc boars were not due to lower production capacity, but more likely to faster elimination of steroids in Landrace boars. The parameters age, live weight,

and testicular development did not significantly contribute to the variation in fat androstenone. Data from this study and previous reports on candidate genes related to androstenone biosynthesis and metabolism suggest that selection against factors associated with boar taint remains a possible solution to the problem of boar taint in the swine industry.

Paper II

S. Lervik, K. von Krogh, C. Karlsson, I. Olsaker, Ø. Andresen, E. Dahl, S. Verhaegen, E. Ropstad. Steroidogenesis in primary cultures of neonatal porcine Leydig cells from Duroc and Norwegian Landrace breeds. Theriogenology 76 (2011) 1058–1069

The steroidogenic activity of primary Leydig cells derived from neonatal purebred Duroc and Norwegian Landrace boars was investigated *in vitro* to identify any breed differences. Concentrations of testosterone, oestradiol, androstenone, cortisol and progesterone in the medium were determined. To explore underlying mechanisms, the cellular expression of a suite of genes relevant in steroidogenesis was measured using reverse transcription and quantitative PCR (RT-qPCR). Basal steroid concentrations indicated higher steroid levels in unstimulated Duroc cells. Stimulation of the cells with LH increased steroid hormone secretion significantly in both breeds in a dose-dependent manner. Testosterone and androstenone concentrations increased approximately 50- and 15-fold, respectively. Concentrations of oestradiol, cortisol and progesterone also increased, but to a lesser extent. At maximal LH stimulation, absolute steroid concentrations were higher in Duroc. However, the relative increase in hormone concentrations was significantly lower for oestradiol, progesterone and cortisol in Duroc cells than in Landrace cells when compared to

basal levels. LH exposure was associated with a general up-regulation of mRNA levels for steroidogenic genes, stronger in Duroc than in Landrace. This was in agreement with the higher absolute concentrations of steroid hormones measured in culture medium from the LH-stimulated Duroc Leydig cells, but was not consistent with the fact that the relative increase in hormone production was lower in Duroc than in Landrace Leydig cells for some hormones. It was concluded that differences between Norwegian Landrace and Duroc in steroid hormone concentrations and gene expression are complex and cannot be explained by a simple mechanism of action.

Paper III

S. Lervik, I. C. Oskam, A. Krogenæs, O. Andresen, E. Dahl, H. A. Haga, H. Tajet, I. Olsaker, E. Ropstad. Androstenone and testosterone levels and testicular morphology of Duroc boars related to estimated breeding value for androstenone. Theriogenology 2013, 79:986-994.

Androstenone and testosterone levels in Duroc boars selected on the basis of $EBV_{androstenone}$ were followed from birth to sexual maturity. Their breeding value for androstenone had been estimated based on androstenone levels in 1202 Duroc boars 24 weeks of age. Testosterone and androstenone levels were recorded at 1 and 3 weeks in two male siblings (n=35) and followed from 12 to 27 weeks in a third sibling (n=16). Histomorphology was performed at 12, 16, 20 and 27 weeks to determine sexual maturity status.

$EBV_{androstenone}$ was positively related to plasma androstenone at 12-27 weeks of age and to plasma testosterone levels in 1- and 3-week-old animals. $EBV_{androstenone}$ was not found to be related to testis morphology. The concentration of fat androstenone was positively

correlated to the percentage of immature seminiferous tubules and negatively correlated to the percentage of mature seminiferous tubules at 20 weeks. Testosterone in plasma showed no relationship with testis morphology. Most individuals reached puberty at 20 weeks of age, which indicates that Duroc mature later than crossbreeds. The results indicated that breeding value based on the single trait boar taint parameter $EBV_{androstenone}$ is not related to testicular development in the boar.

Paper IV

S. Lervik, A. B. Kristoffersen, L. N. Conley, I. C. Oskam, J. Hedegaard, E. Ropstad, I. Olsaker. Gene expression during testis development in Duroc boars. Submitted to Animal.

Androstenone is a steroid pheromone occurring in the pubertal Leydig cells. Breeding against androstenone can decrease pheromone odour in swine meat but appears to cause unwanted side effects such as delayed onset of puberty. To study causality, global gene expression in developing boar testes at 12, 16, 20 and 27 weeks was investigated using a porcine cDNA microarray. A previous publication describes the morphological status and androgenic levels in the same individuals. For this paper, associations between pathways of expressed genes were studied in the boar testes. Possible interactions with the known morphological development status, age and increased levels of steroids are discussed.

Nine clusters of genes with significant differential expression were found in the analysed testis samples. DAVID® pathway analysis identified 49 functional charts. In prepubertal testis tissue renewal and cell respiration were prominent together with indications of increased endocytocis possibly associated with the EGFR-pathway substrate *EPS15* and Sortilin. E-cadherines might be associated to onset of pubertal development in

the boar testis. Genes coding for $Ca^{(2+)}$ channel occurred at prepuberty. Together with expression of cAMP-dependent protein kinase regulators *PRKAR1A*, *PRKAR2B* and the PPAR pathway regulating lipid metabolism - prepubertal activity was indicated.

At common pubertal timing, genes coding for neuro steroids and pathways that could be associated to nerve development occurred concomitantly with increased steroidogenesis and histomorphological maturation. Genes in redox pathways also changed. This suggests that there is a developmental-specific period of neuromorphogenesis.

Increased expression of meiotic pathways agreed with onset of puberty. With elevated steroidogenesis (week 16-27), there was an increase in the expression of genes in MAPK-pathway, *STAR* and its analogue *STARD6.* Several growth factors were found increasing differently towards the mature testis as follows. *TGFB2* showed pubertal increase and may also be influenced by the common *FSTL3 increase* at weeks 16-27. Later, *IFNG* expression increased at week 20-27. Further on towards mature testis - *PDGFA* and the receptor *TGFBR2* increased. *GHRH* also increased towards the mature testis however remained lower expressed than the reference. Interaction between MAPK, *STAR* and growth factors might be suggested.

In conclusion, pathways for neurogenesis, morphological pathways and several transcripts for growth factors, with known modulating effects on steroidogenesis and gonadotropins in humans and rodents, act at specific ages and developmental stages in the boar testis. The age dependency and complexity shown for development-specific testis transcripts should be considered in marker-assisted selection and when parameters for breeding values are established.

General discussion

1. Methodological considerations

The thesis is based on well-established methods for steroid assays, histomorphology, reverse transcription quantitative PCR (RT-qPCR) and microarray analysis using porcine cDNA. Material and methods are described in the relevant sections of Papers I-IV. Some methodological issues of concern and modifications are discussed below.

Supplementary comments to the material and methods sections of Papers I- IV

1.1 Group size

The small litter size of Duroc pigs caused some problems as regards the design of the studies for Papers III and IV. There were too few male offspring in some litters both *post partum* and at three weeks of age (Figure 3.).

Some of the litters used in the study included only two male siblings or a single male offspring. To ensure adequate statistical power, this was compensated for by including three boars from reserve litters *post partum* and a further three at three weeks of age. The reserve litters were from different sows to the sixteen in the pubertal study. In all, fifty-one boars were used in the study. The reserve litters from which the extra boars were chosen were those with the closest match of estimated EBV for androstenone and time of birth (Figure 3.).

In postnatal piglets, there was a limited amount of fat tissue for measurement of androstenone, and it was only possible to quantify the amount in seven of nineteen samples. Adding three non-siblings both after birth and at three weeks of age was not expected to be a source of bias in the study for Paper III. However, a general drawback was that taking samples from different siblings in week 1 and week 3 meant that it was not possible to look at how steroid levels changed during this period in the same individuals.

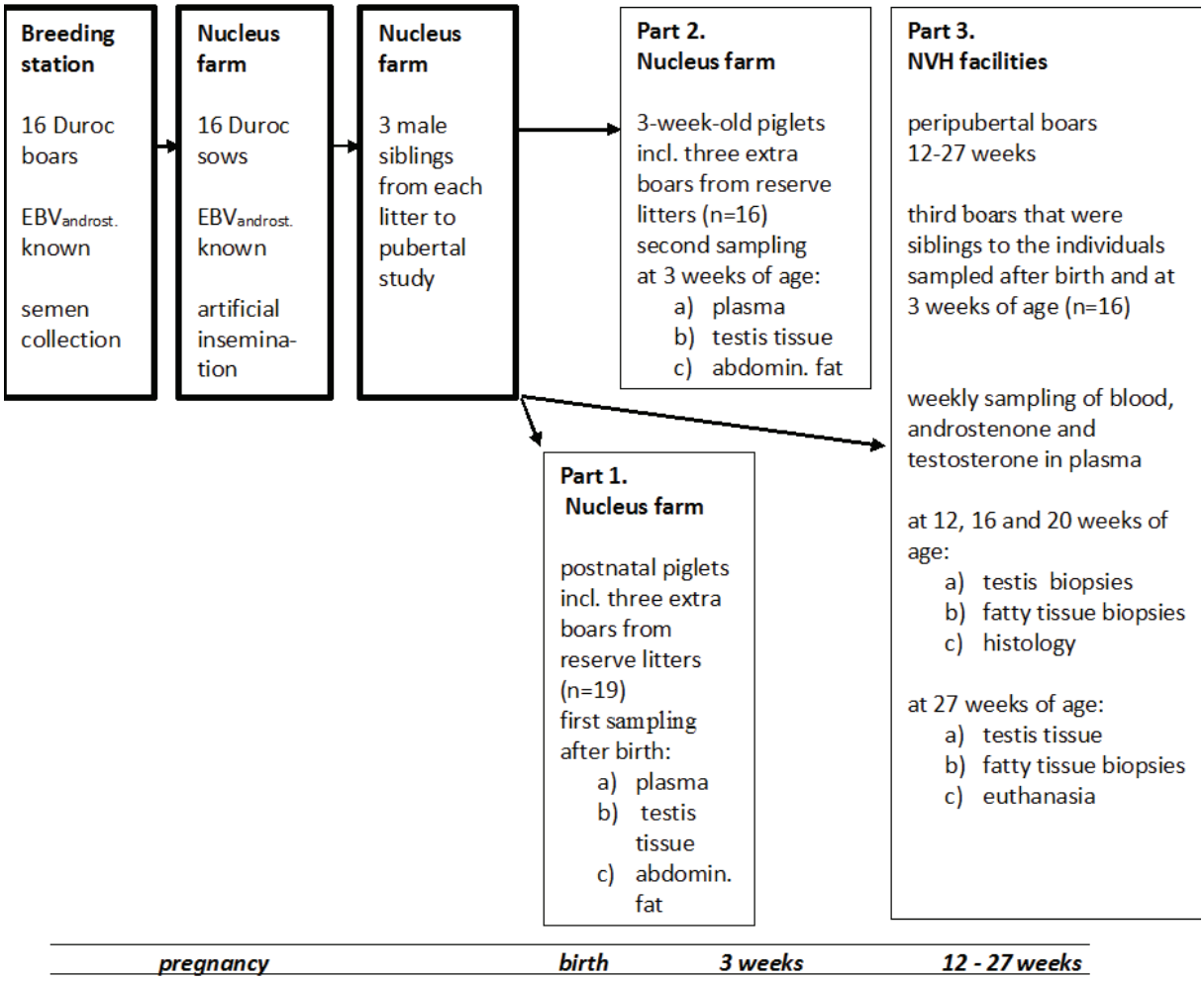

Figure 3. Samples collected and workflow for studies for Papers III and IV. The first series of samples were collected from one sibling per litter after birth. The second series of samples were collected from a second sibling boar at 3 weeks of age. Later, the third series of samples were taken from the third male siblings when the boars were 12–27 weeks old. There were too few boars (n<3) in some of the sixteen litters. Three boars from six reserve litters were therefore included in the first series of samples, and three more in the second series.

Single-trait breeding values specific for androstenone ($EBV_{androstenone}$) served as estimates for the androstenone phenotype. When $EBV_{androstenone}$ was calculated, commonly used boar taint index factors, i.e. skatole, indole and length of the bulbourethral gland, were excluded (Tajet et al., 2006). $EBV_{androstenone}$ was thereby weighted separately to reduce the effect of possible confounders. The Norsvin database, which held data on 1202 Duroc boars,

served as a reliable resource for calculation of variance components for estimation of androstenone breeding values (Tajet et al., 2006).

Paper IV reports that no association was found between the range of androstenone levels measured in fat (Paper III) and gene expression. This may be explained by high individual variation in androstenone levels (Paper III). Thus, a study involving a larger number of individuals might make it possible to analyse high versus low androstenone phenotypes.

The gene clusters identified in Paper IV did not involve significant pathways that could be related to steroidogenesis. Instead, the clustered genes and functional pathways were analysed in relation to the time intervals for developmental stages of the testis. This meant that the group size was up to twice as large, and it improved the study by increasing its statistical power. Steroid levels changed significantly over time (Paper III). However, no pathways coding for steroidogenesis changed between the four time points observed. Unfortunately, the limited budget did not allow any further increase in the number of samples or additional methods.

1.2. Sampling age

The choice of age for sampling Leydig cells was supported by other studies of porcine Leydig cells taken from 3-week-old piglets (Franca et al., 2000; Vanstraaten and Wensing, 1978). At this early age, there is a peak in steroidogenesis and morphological change in the Leydig cells (Choi et al., 2009; Franca et al., 2000; Schwarzenberger et al., 1993). This intervention delayed the on-farm routine castration date by two weeks.

The original intention was to discuss the results of the *in vitro* study (Paper II) in relation to the *in vivo* results from Paper III, since samples were taken from three-week-old

piglets in both cases. Information on $EBV_{androstenone}$ levels in the litters used for the *in vitro* analysis in Paper II would have been useful. However, for practical reasons, $EBV_{androstenone}$ for these litters was not available.

With the present model, the *in vitro* study in Paper II did not show whether there were development-dependent breed differences at three weeks of age. This cannot be excluded as a source of bias. The 10-day age difference between the Duroc and Landrace boars used in the study for Paper I is a possible source of bias. This difference may have influenced the breed comparison in Paper I.

Staining for *HSD3B* in Leydig cells was used to verify the protocol used for isolation of Leydig cells. The testis material used for verification was sampled at different intervals from those used in Paper II. However, the protocol was considered to be reliable since no modifications were made to the procedure.

The first samples around puberty were taken at 12 weeks, since low testicular activity can be expected between 3 and 12 weeks of age (Dierichs and Wrobel, 1973; Franca et al., 2000) (Papers III and IV). Blood sampling at weekly intervals made it possible to monitor variation in testicular steroid secretion both within and between animals throughout sexual maturation (Andresen, 1976; Bonneau et al., 1987a). Blood samples in weeks 12–27 were taken in the morning in order to minimise eventual variations related to the circadian rhythm (Andresen, 1975; Claus and Gimenez, 1977).

Histomorphological samples were taken at around week 20 since other studies of crossbreeds have shown a pubertal development peak at this age (Florcruz and Lapwood, 1978; Oskam et al., 2008).

Unfortunately, tissue samples for isolation of mRNA were not collected during sampling for Paper I. Adding one more sampling time point at commercial slaughter age (100

kg live weight) in the studies for Papers III and IV might have been useful, since others have found steroidogenic-related variations in testis transcripts from Duroc at this weight (Moe et al., 2007b).

1.3. Anaesthesia

To minimise residuals and ensure that the meat could still be used for human consumption, a commercial anaesthetic was used on the nucleus breeding farms (Papers II and III). This anaesthetic used has been evaluated for use in young piglets in several studies and been found satisfactory (Fosse et al., 2011b; Haga and Ranheim, 2005; Ranheim et al., 2005).

Lidocaine without adrenalin (Haukeland Hospital Pharmacy, Bergen, Norway), was chosen as a local anaesthetic to exclude the possibility of side effects of adrenalin on gene expression. It was injected into the inguinal string to prevent a build up of pressure in the testis. Application in the inguinal string to give a neurological block also helped to reduce differences in the indivdual response to anaesthesia.

Anaesthetising pubertal and adult swine can be difficult. For the purposes of this study, the main benefits of sevoflurane vaporised in oxygen (SevoFlo, Abbott Scandinavia AB, Sweden) were full immobility and analgesia in the adult boar (Paper III and IV). Gas anaesthesia is rapidly reversible. Together with application of an α2 adrenergic receptor antagonist atipamezol (antisedan® Pfizer Animal Health, New York US), a shorter recovery time, feed uptake and lower stress response was ensured. This was preferable to the effects of neuroleptic anaesthesia (Rintisch et al., 2012). Epidural anaesthesia (Swindle and Smith, 2013) was not considered to be an option because of the increased risk of trauma and prolonged recovery time, especially as the boars gained weight.

1.4. Stimulation with luteinizing hormone/human chorionic gonadotropin (LH/hCG)

The studies described in Papers I and II were based on recordings of endocrine profiles activated by artificial stimulation with LH/hCG. LH/hCG stimulation has previously been shown to be a valuable tool for studies of boar taint (Zamaratskaia et al., 2007; Zamaratskaia et al., 2008a). In many previous studies, androstenone was evaluated after maximum LH/hCG stimulation, and correlations between the elevated levels of different steroids were shown to be high (Carlström et al., 1975; Lundstrom et al., 1978). However, peak response, measured as the effect of artificial LH/hCG stimulation, does not necessarily mirror the normal steroidogenic scenario. Normally, LH is secreted as pulses that differ in size throughout puberty (Bonneau et al., 1987b). Maximum artificial LH/hCG stimulation may result in more prolonged and higher LH/hCG levels than normal pulses, ranges or thresholds.

1.5. Reverse transcription and quantitative PCR (RT-qPCR)

Genes for the study of Leydig cells (Paper II) were chosen for primer design based on the results of the microarray experiments performed in 2008. This was somewhat premature since analysis of the microarray data was still in progress. Other genes might have been selected after the ANOVA, cluster and pathway analyses were completed.

Of the six housekeeping genes tested, *ACTB* and *cyclophilinA* (Duvigneau et al., 2005) were found to be the most stable and were selected as reference genes for the RT-qPCR analyses for Papers II and IV. Reference genes with constant expression in the experimental samples serve as a measure of the amount of input mRNA. The expression of other genes in the experiment is adjusted according to the level of reference gene expression (Wong and Medrano, 2005, Silver et al., 2006). There are several software packages on the market that can be used to evaluate which reference genes are most stable. They use pairwise

comparison of all genes to exclude the housekeeping genes with most variation. The software choice of GeNorm (Vandesompele et al., 2002) was satisfactory, and based on experience at our own laboratory (Lewandowska-Sabat et al., 2013; Zimmer et al., 2011).

1.6. Expression profiling in the microarray experiment

Noise in microarray analysis is mainly of biological or technical origin. Variable cellular composition of testis samples and individual variation must be taken into account as biological noise in the study described in Paper IV. A variety of factors contribute to technical noise, including sample preparation, gene-specific dye bias for intensity of Cy3 and Cy5, cross-hybridisation and the properties of the array (Aris et al., 2004). Noise caused by cross-hybridisation is difficult to handle when dealing with cDNA microarrays. Uncertainty arises because closely related mRNA variants may be very similar in sequences even though they differ in length and content. As a result, specific cDNA may bind to more than one probe on the array during hybridisation. Splice variants of the same gene may bind to the same probe. Other splice variants may lack the sequences present in the array probe and do not bind at all. Related genes that differ from one another may also bind to the same probe. In addition, the analysis of microarray data is limited to the probes present on the array.

To handle biological and technical noise from the microarray, standardised steps for microarray data processing were performed using a Bioconductor software package (Gentleman et al., 2004; Smyth, 2004; Smyth and Speed, 2003). The huge toptable output that was obtained showed > 31,000 genes at a significance level of 0.01 that needed further processing. ANOVA analysis was chosen. ANOVA strengthened the output by picking approximately 17 % of the most significant genes, but did not result in any noteworthy change in the number of steroidogenic associated probes.

There were many probe sequences that matched unknown genes based on the annotation in 2008. The information about the swine genome was also incomplete in 2008. Therefore the significantly regulated genes in our experiment were reannotated with blast against human genes (HSA) and swine genes (SSC) in April 2014 (Paper IV). Thereby the HSA fraction of annotated genes increased from 73 % to 80 % from the renewed blast. Furthermore, 85 % genes were annotated with official HSA and/or SSC gene symbols in total. The increased feed into HSA pathway analysis in DAVID (Database for Annotation, Visualization and Integrated Discovery) v. 6.7 (Huang et al., 2009a; Huang et al., 2009b) resulted in an increase from 36 (2009) to 49 biochemical pathways with functional annotation in Kyoto Encyclopedia of Genes and Genomes (KEGG, Kanehisa Laboratories, Kyoto University, University of Tokyo, Japan) (2014).

The microarray experiment was set up as a reference design study. An efficient reference sample should represent most genes in the samples. The expression of cDNA on the microarray is analysed relative to its expression in the reference sample. One drawback is that the results show relative expression, which is not standardised to a level of no expression/zero point. However, in other ways measuring relative expression is a benefit of reference design. Variation in technical noise can be calculated and avoided.

The reference sample used in the porcine microarray (Paper IV) consisted of a mixture of mRNA extracted from testis samples at different ages. However, the size of the biopsies limited the total amount of mRNA available. Consequently, the amount of mRNA for both samples and reference samples for the microarray was restricted. In particular, there was only a small amount of biopsy tissue that could be used for isolation of mRNA from the 12-week-old boars. The reference sample therefore contained less mRNA from 12-week-old

boars than from other age groups. It is not known whether the differences in the amount of mRNA available between different sampling time points might lead to bias.

There was also a lack of reference material for measuring relative RT-qPCR expression for verification. However, not all authors support the idea of fixed procedures for verification of microarray data (Rockett and Hellmann, 2004). Studies support the reliability of microarray data in general since agreement can be found between different microarray platforms (Mecham et al., 2004; Petersen et al., 2005).

Another advantage of reference design is that a cluster analysis can be performed using the output. This would be more difficult with a balanced block design or a loop design where not all samples are compared with the same reference sample. The design was not chosen with a view to economising on the amount of mRNA needed.

Cluster and pathway analysis of microarray data is often used to make biological sense of the large numbers of genes represented on the microarray. However, pathway analysis tools for livestock are practically non-existent. Pathway analysis of farm animal genes must therefore be based on database resources developed for humans and based on human (or rodent) gene IDs. Analyses of livestock genes are thus based on the assumption that genes with similar sequences have the same function as that described for humans and the swine is not a man. Nevertheless, -human pathway analysis tools are the best available option for analysis of large amounts of swine transcripts such as are found in microarray data.

2. Relevance of the findings

2.1. Breed differences and estimated breeding value for androstenone ($EBV_{androstenone}$)

Lower androstenone levels are achievable by breeding (Sellier, 1998; Sellier et al., 2000; Varona et al., 2005). Paper I showed that androstenone levels in fat were related to

$EBV_{androstenone}$ before stimulation with hCG in Duroc. Duroc boars also showed higher basal androgen levels than Landrace (Papers I and II). In Landrace, $EBV_{androstenone}$ was related to androstenone levels measured after hCG stimulation. Interestingly, there was a relationship between $EBV_{androstenone}$ and the high basal androstenone levels in Duroc, but not between $EBV_{androstenone}$ and the lower basal levels in Landrace (Paper I).

In the study for Paper I, $EBV_{androstenone}$ was measured in mid-puberty, in other words, when boars reached slaughter age and a live weight of 100 kg. The fact that there was an age difference of 10 days between the Duroc and Landrace boars used in this study could be an important reason for the observed breed differences.

In addition, the onset of puberty is later in Duroc than in Landrace. Basal endocrine differences between paternal and maternal lines might be age dependent and/or specific to a particular developmental stage. Reproductive phenotypes might be another complicating factor in comparisons of $EBV_{androstenone}$ between breeds. There are differences between the overall breeding goals for the two breeds. By taking these into consideration, it may be possible to improve understanding of why $EBV_{androstenone}$ is related to androstenone before hCG stimulation in Duroc boars but after hCG stimuli in Landrace boars of slaughter weight. Since 1992, anabolic traits such as daily gain and efficient feed conversion have been weighted more heavily in the breeding goal for Norwegian Duroc than in the breeding goal for Norwegian Landrace. Conversely, maternal reproductive traits such as litter size and maternal ability have been more heavily weighted in the Landrace than in the Duroc breeding goal. Thus, breed differences in the response to LH/hCG stimulation might be due to differences in sexual phenotype or to the two breeds having reached different developmental stages.

Breed differences related to development and androstenone are complex (Paper I and II). It is important to be aware that if samples are taken i.e. at slaughter weight (100 kg), the results for $EBV_{androstenone}$ might only be valid for this specific state of sexual maturation (Paper IV). Individual variations for maturity, as found in studies for Paper III and Paper IV, can also be expected at this age. Onset of puberty is complex and highly affected by individual variations (Paper III and IV). It would therefore be useful to include more stages of pubertal development in future studies of breed differences.

2.2. Genes related to steroidogenesis

The *in vitro* study described in Paper II showed that steroidogenic genes were more upregulated in Duroc than in Landrace Leydig cell cultures. This corresponded with higher levels of steroids measured in the medium from Duroc cells. The highest gene expression found in Duroc did however not correspond with the relative response in steroid levels to LH stimulation. This was lower in Duroc than in Landrace for some steroids, suggesting that the underlying mechanisms for breed differences are complex.

RT-qPCR showed the highest expression and the largest breed difference in gene expression for steroid acute regulatory protein *(STAR)*. The upregulation of the *STAR* gene was also LH dose-dependent in Leydig cells from both Landrace and Duroc boars. *STAR* mediates cholesterol transport in the Leydig cells, which is a rate-limiting step at the start of steroidogenesis (Manna and Stocco, 2005). Further on the CYP17A1 enzyme functions as -lyase and –hydroxylase catalyse for several steroidogenic intermediates. Given the role of *STAR*, the Leydig cell model used in these studies cannot give an answer to the question of whether breed differences in *STAR* are linked to higher expression of *CYP17A1* in Duroc than

in Landrace. Increasing amount of *CYP17A1* products provides substrates to the quantitative breed differences in levels of oestradiol, cortisol and testosterone.

Moreover, after LH stimulation in 3-week-old Leydig cells, no significant breed or dose-response differences were found in expression data for CYP11A1, which encodes the cholesterol side-chain cleavage which catalyses the first step of androstenone and steroid synthesis (Figure 1 and Paper II). Breed differences in the expression of *STAR* and *CYP11A1* have previously been reported in pubertal boars (Moe et al., 2007b). A further relevant question is whether the differences in *CYP11A1* expression are age-related. Developmental studies in goat testis can support age differences for *CYP11A1* (Faucette et al., 2014). In contrast, *CYP11A1* was not among the significant differential expressed genes over time in the pubertal study of Duroc testis (Paper IV).

Data from Paper II may indicate that breed differences in the expression of *HSD17B4* and *HSD17B1* are associated with testis development. These genes play a part in the synthesis of oestradiol, which has also been suggested to play a role in testis development (At-Taras et al., 2006). The fact that higher sensitivity to LH was observed in Duroc than in Landrace is of interest for further studies of breed differences.

Cluster of genes with increased expression of *STAR* and the *STAR* homologue *STARD6* described by Soccio et al (Soccio et al., 2002), were coinciding with increased pubertal steroidogenesis (Paper III and Table 1 cluster one, and Supplementary Table S1, Paper IV). These are probably linked to increased cholesterol demand for steroidogenesis in the pubertal boar. However, conflicting evidence was provided by the observed changes in the regulation of the cholesterol-related *DHCR24* and *CYP51A1* genes. *DHCR24* and *CYP51A1* did not show increased expression to the reference when higher levels of steroids were measured in week 16 (cluster one, Paper IV). This may indicate that the two cholesterol-

related genes have more functions than supporting the lipid transfer of *STARD6* at steroidogenesis. These genes increased in expression below the reference sample during puberty to become close to the reference in the testis of mature 27-week-old boars (Table 1, cluster eight, and Supplementary Table S1, Paper IV). Based on the reference design does the reference sample not represent a real zero point of expression. Silence of these genes can therefore be misinterpreted. It cannot be excluded that the increase in cholesterol related genes below the reference sample are associated to increased steroidogenesis during puberty with different threshold level than i.e. *STAR*. In the testis of mature boars, Sertoli cell proliferation has come to an end and their cytoskeleton is established, permitting testis fluid flow carrying mature spermatozoa (Franca et al., 2005). Therefore, the increased expression of cholesterol related transcripts in the mature boar testis may also be associated with meiosis-activating sterols (MAS). MAS show highest levels in the adult testis. The relevance for MAS in sperm of many mammals has been suggested previously (Keber et al., 2013). The change in regulation found in cholesterol-related intermediates in the testis of mature boars could be associated to both spermatogenesis and steroidogenesis. This underlines the complexity of cholesterol-related questions in the pubertal boar.

However, there was another *DHCR24* probe in cluster associated to prepubertal expression and decreased regulation thereafter (Table 1 cluster four, and Supplementary Table S1, Paper IV). This is not in accordance with the time when pubertal steroid levels rose including the increased expression of *STAR* and *STARD6* from 16 weeks of age. The complexity of steroidogenesis and pubertal development is illustrated by several issues associated to cholesterol. Different roles for *STAR* and *STARD6* can be thought of in the pubertal boar testis. Conclusion during puberty on differential expression over time for cholesterol related genes are not simple.

Further associations with *STAR* and *STARD6* might be suggested since the gene showed activity in cluster with morphological MAPK pathway. In the gene clusters from data in Paper IV, it can be speculated if more growth factors modulate *STAR* in the pubertal testis of Duroc boars (Table 2, Paper IV). Assumptions must however be taken carefully since these genes might not act analogous in swine as shown in other mammals.

2.3. Testis growth

Studies of the same individuals for Papers III and IV showed that various growth factors and pathways related to morphology coincided with the milestones of pubertal development. In Paper III, $EBV_{androstenone}$ was found not to be related to pubertal integral/area under the curve of pubertal plasma testosterone levels. It was also concluded that $EBV_{androstenone}$ was not related to the morphological development of seminiferous tubules and gametogenesis (Paper III). It has been suggested that growth factors and morphological pathways affect modulation of STAR in other mammals (Table 2, Paper IV). It would be of interest to study $EBV_{androstenone}$ further to find out whether there is any relationship to STAR or morphological elements.

Paper III and IV together indicated a testosterone increase coinciding with the increased expression pathways related to morphological development (Table 1, cluster one, Paper IV). The anabolic effects of testosterone are well documented (Claus and Weiler, 1994; Kuhn, 2002). The pathway analysis also indicated pubertal onset coincided with increased gene expression in pathways activity indicating development changes for nerves (Paper III and Table 1, Paper IV). The complex secondary sexual phenotype of the boar, including muscle growth and agonistic behaviour, is influenced by testosterone. Studies in humans show pubertal neurogenesis is stimulated by testosterone (Sisk and Foster, 2004).

Data from Paper IV supports pubertal changes in genes associated to nerves. Expression of genes in the neurotrophin pathway at puberty (cluster two, Paper IV), further supports development of nerves in the boar testis might be associated with onset of puberty.

Paper IV concluded that a number of growth factors and pathways related to tissue development act at specific developmental stages in the pubertal Duroc testis. It is therefore possible that there is an influence between these and testosterone in the postnatal boar and $EBV_{androstenone}$ in the pubertal boar (Paper III). The morphological pathways and growth factors changed from one stage of development to another (Paper IV). Interactions between a particular growth factor, interstitial cells and the HPG axis at an early stage of development may change at later stage of development, so that the effects on growth, development and reproductive traits are also different. Dividing puberty into a larger number of clearly defined stages might improve our understanding of the onset of puberty.

2.4. Biological relevance of the findings

LH stimulation of steroidogenesis in Leydig cells (Paper II) showed that androstenone synthesis was stimulated at lower levels of LH (0.025 ng/mL) than testosterone synthesis (0.05 ng/mL). A maximum level seen as saturated plateau was also reached at lower LH doses for androstenone (0.05 ng/mL) and oestradiol (0.025 ng/mL) than for testosterone (0.5 ng/mL). This might indicate that testosterone has other tuners than androstenone. If so, androstenone might exert its effects as a pheromone independent of testosterone.

In LH-stimulated Leydig cells, the increase in hormone concentration in response to the highest LH levels was approximately 15-fold for androstenone and 50-fold for testosterone. A high correlation between androstenone and testosterone was found in medium from Landrace Leydig cells, but not in Duroc (Paper II). In Duroc, oestradiol and

testosterone were negatively correlated (borderline) and a low dose-response threshold for increase in oestradiol was found. In contrast, Landrace Leydig cells showed a positive correlation between oestradiol and testosterone and a higher dose-response threshold (Paper II). It was not determined whether these breed differences were associated with aromatase inhibition. Aromatase is the enzyme that converts androgens encoded by CYP19 to oestradiol. Another study showed elevated testosterone levels after porcine Leydig cells were treated with oestradiol inhibitor (At-Taras et al., 2008). Mechanisms associated with the synthesis of oestradiol may help to explain steroidogenic breed differences in Leydig cells.

In vivo experiments indicated that there are breed differences in the ratio between plasma testosterone and androstenone. As was found *in vitro*, *in vivo* relative androstenone secretion did not differ between breeds after stimulation with hCG. However, significantly higher testosterone concentrations were found in Landrace than in Duroc on the first day after hCG stimulation *in vivo* (Paper I). On the other hand, the elimination of both androstenone and testosterone from plasma was slower in Duroc than in Landrace. Taken together, the results of Papers I and II indicate that these breed differences were not caused by differences in relative androstenone secretion after LH/hCG stimulation. Paper I does not show whether or not breed differences in testosterone secretion were associated with prolonged elimination of androstenone. The results indicate that breed differences in steroid hormone secretion and gene expression are complex and difficult to explain by a simple mechanism.

With increased expression in the maturing testis (cluster two), $Ca^{(2+)}$ related pathway genes were coding for enzyme properties related to oxidative phosphorylation (Paper IV). In the prepubertal testis, unspecific modulation by $Ca^{(2+)}$ voltage channels was suggested to be

more prominent (Paper IV). When steroids are bound to receptors, they act as transcription factors binding to specific DNA elements that are gene promoters. Thus, transcription is activated or repressed, influencing protein synthesis (Cato et al., 1988). Transcription has a time cost. A peak in genomic transcription normally occurs several hours after steroid exposure. However, synergism between many steroid response cascades does not last longer than a few minutes. Rapid steroid responses have been reviewed (Baulieu and Robel, 1995; Foradori et al., 2008; Moriarty et al., 2006). A rapid intracellular rise in $Ca^{(2+)}$ results in modulation within seconds via $Ca^{(2+)}$ gates and membrane flexibility. This occurs in response to steroid hormones (Baulieu and Robel, 1995; Moriarty et al., 2006), and second messenger pathways can be activated directly. Secondary transcription can be activated by changes in levels for $Ca^{(2+)}$- in example when activating calmodulin. Pubertal signalling via calmodulin was relevant by its significant presence as a pathway gene in both the neurogenesis related neurotrophin signalling pathway and the spermatogenesis associated meiotic pathways (pubertal cluster two, Table 1. Paper IV).

Phosphorylation reactions are another example of fast-reacting pathway elements found to show increased expression at the same time as increased steroidogenesis at puberty (Paper III and IV). In these reactions, kinase pathways such as the $Ca^{(2+)}$-dependent P-kinase pathways SRC-tyrosin and ERK act on MAPK kinase pathways. These reactions modulate androgens when receptors become phosphorylated (Cabeza et al., 2004; Estrada et al., 2003; Wehling et al., 2006; Simoncini and Genazzani, 2003). This is a further illustration of the complex cascades of action associated with the events of increased steroidogenesis and onset of puberty in the boar testis.

A fast steroidogenic response may be more beneficial in a mating situation than awaiting slow transcription. Studies of rodents support the idea that reflexive steroidogenic

release has benefits in the mating situation (Nyby, 2008). It is not clear whether fast pheromonic response also has benefits during mating in the boar. However, an androstenone increase has been found after mating (Andresen, 1976). The studies of pubertal Duroc boars revealed similarities in timing between the MAPK pathway (Paper IV) and increased steroidogenesis (Paper III). MAPK are phosphorylation reactions that have a wide range of biological functions and flexibility (Raman et al., 2007). Among other things, it has been suggested that the MAPK complex is involved in an old conserved mechanism for pheromonic secretion (Jones, Jr. and Bennett, 2011). However, a study of the MAPK14 gene, could not detect causality for a QTL region involved in levels of androstenone in fat (Robic et al., 2011). Neither was this MAPK14 gene amongst the MAPK pathway genes showing differentially regulation over time in pubertal Duroc boars (Paper IV). More specifically designed studies than discussed here are needed to speculate whether fast phosphorylation reactions might contribute to an understanding of the mechanisms underlying phosphorylation reactions and androstenone production.

$EBV_{androstenone}$ was related to postnatal testosterone secretion (Paper III). Since testosterone has an effect on anabolism, the possibility that breeding for low androstenone has an effect on essential breeding goals in Duroc cannot be excluded. There may be effects on feed conversion and daily gain in young boars from lines bred for high and low levels of androstenone. Unexpected effects might occur if there is a coupling between anabolic testosterone in the young boar and pubertal $EBV_{androstenone}$ (Paper III). For example, agonistic behaviour could be influenced, since androgen levels might influence prevalence of aggression (Giersing et al., 2000). Further studies will be needed to reveal any such effects.

Steroid levels excreted by the developing testis vary greatly, both between individuals and over time (Paper III). Sexual steroids, especially testosterone, trigger growth.

However, steroid variability was not reflected in body weight increase and testis expansion (Paper III), indicating that these traits are more rigidly regulated. Paper IV concludes that pathways and transcripts for growth factors are differentially regulated over time, corroborating the suggestion that these factors contribute to steady growth and weight increase.

2.5. Suggestions for further research on breeding against androstenone

This thesis suggests that developmental phenotypes should be used in studies of breeding for lower androstenone. A useful approach would be to breed for different androstenone phenotypes through several generations. Following this with *in vitro* modelling with Leydig cells, as discussed in Paper II, might provide a suitable research model. Paper III showed the association between pubertal $EBV_{androstenone}$ and postnatal testosterone. Studies of Leydig cells could provide useful information when addressing further questions related to breed differences and developmental stages. Additionally, studies of interactions between LH/hCG, steroids and growth factors in cocultures with more types of testis cells, for example seminiferous tubule cells and Sertoli cells, could provide valuable information.

The results discussed in Paper IV indicate that functional pathways and transcripts associated with growth are expressed at specific stages of testis maturation. Puberty should be further divided into a larger number of clearly defined development phases to improve understanding of the possible associations between androstenone and sexual maturation.

Conclusions

The results discussed in this thesis indicate that the underlying causes of breed differences between Landrace and Duroc related to androstenone cannot be explained by

single factors. These differences are partly associated with differences in the timing of sexual development in the two breeds.

Steroid secretion increased in response to LH/hCG stimulation in both Duroc and Landrace, but androstenone was eliminated more slowly in Duroc. The two breeds showed differences in the LH threshold for maximal steroid levels, indicating that Duroc Leydig cells required less gonadotropin for maximal stimulation. Comparison of gene expression in Duroc and Landrace also indicated the expression of several steroidogenic genes in Leydig cells showed a greater response to LH stimulation in Duroc than in Landrace.

No association was found between testis morphology and pubertal $EBV_{androstenone}$ estimates. Individual variation in the timing of morphological testis development should be considered. However, $EBV_{androstenone}$ was related to androstenone levels in the pubertal boar and testosterone levels in the postnatal boar.

Clusters of transcripts including functional pathways associated with testis development were found to change in expression over time. There were changes both at the stage when pubertal steroid secretion increases and when there is major histomorphological change in the testis. Together with previous studies, this indicates that the effects of breeding for low androstenone might be unpredictable. By dividing testis development into more stages, it should be possible to obtain more information on pubertal traits and the effects of reducing androstenone by breeding.

Further research

The studies indicate that it is not possible to predict all effects of breeding for lower levels of androstenone. Even if breeding for low androstenone levels is successful, the side effects are complex and should be studied in controlled breeding studies covering several

generations. Additionally, *in vitro* studies of testis tissue are highly recommended as efficient models for studies of mechanistic questions related to breed differences and testicular development.

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Differences in testosterone, androstenone, and skatole levels in plasma and fat between pubertal purebred Duroc and Landrace boars in response to human chorionic gonadotrophin stimulation

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Abstract

The concentrations of the boar taint compounds androstenone and skatole in plasma and fat, together with those of testosterone in plasma, were investigated in pubertal purebred Duroc and Landrace boars following stimulation with human chorionic gonadotrophin (hCG). Higher initial levels of androstenone and testosterone were found in Duroc than Landrace boars. Duroc boars, which were approximately ten days older than the Landrace boars, also showed a more advanced stage of spermatogenesis than Landrace boars. While Landrace boars had the highest skatole levels. Following stimulation with hCG the relative increases in testosterone, androstenone, and skatole concentrations were highest in Landrace boars. The level of androstenone in fat three days after hCG stimulation exceeded 1 μg/g fat in all stimulated boars. The decreases in plasma levels of androstenone and testosterone on Days 2 and 3 after hCG stimulation were more pronounced in Landrace than Duroc boars. However, unlike the plasma androstenone and testosterone levels, the plasma concentrations of skatole did not decrease on Days 2 and 3 following stimulation, but remained elevated on Day 3. These results indicate that the lower levels of testicular steroids in Landrace boars compared with Duroc boars was not due to a lower production capacity, but more likely to a faster dissapearance of steroids in Landrace boars. In the present study, age, live weight, and testicular development did not significantly contribute to the variation in fat androstenone. The present data and previous reports on candidate genes related to androstenone biosynthesis and metabolism suggests that future selection against factors associated with boar taint remains a possible solution for the problem of boar taint in the swine industry.

Keywords: Boar; Boar taint; Androstenone; Skatole; Testosterone; Sexual maturation

1. Introduction

The restricted use of intact males in the production of pork is due to an offensive odour, known as boar taint, associated with heated fat from boars. Boar taint develops in connection with the onset of testicular activity. Two substances, androstenone (5α-androst-16-ene-3-one) and skatole (3-methyl-indole) are regarded as the main contributors to boar taint [1–3]. Androstenone is a steroid that acts as a pheromone, and is produced and released together with androgens in the testes [4–6]. Skatole is formed from tryptophan by bacteria in the colon [7]. Both androstenone and skatole are transported in the peripheral plasma, and both are lipophilic, and accumulate in the adipose tissue. Cur-

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rently, surgical castration of male piglets is the most usual method used to prevent boar taint [8], but this operation does not comply with good animal welfare principles. In addition, by removing the major source of anabolic steroids, the advantages of entire boar production upon growth and leanness are compromised.

As with androgens, breed and age differences have also been observed regarding the levels of 16-androstene steroids in pigs [9–11]. Duroc pigs normally have considerably higher androstenone levels than Yorkshire and Landrace varieties [10,12], while Large White breeds generally show higher levels than Landrace breeds [13]. Genetics greatly affect fat androstenone levels [9,14], and the heritabilities of androstenone level in fat are reported to range from 0.25 to 0.87 [9,13,15], meaning that in the pig population different individuals have a high or low potential for particular androstenone levels. Gene expression studies in the testes and livers of Duroc and Landrace boars with extremely high and extremely low levels of androstenone in fat have been performed, and a number of genes related to the biosynthesis and metabolism of androstenone have been identified [16,17]. Genetic aspects of boar taint have been reviewed by Robic et al [18].

When approaching puberty, androstenone levels are known to increase in parallel with the increase in testosterone levels [4,11,19,20]. Thus, in addition to the genetic effects and breed differences associated with androstenone levels, breed differences in sexual maturation and underlying changes in androstenone biosynthesis during Leydig cell development must also be taken into consideration.

Levels of androgens and androstenone are expressions of the steroidogenic capacity of the individual Leydig cells and their total number per testis [21,22]. However, hepatic metabolism may also affect the peripheral plasma level of androstenone, and thus the accumulation of this steroid in adipose tissue [23,24].

Skatole levels in fat are also higher in uncastrated males than in females or castrated males. However, as skatole levels in the intestines are similar in male and female pigs [25], different levels in fat might be due to sex-dependent differences in hepatic metabolism and clearance [26]. *In vitro* studies have shown that the metabolism of skatole by cytochrome P450IIE1 is induced by skatole and the induction is blocked by androstenone [27,28], indicating that androstenone may play an important role in the regulation of skatole levels.

Skatole levels are more affected by diet and environmental factors than androstenone [29–31]. In addition, genetic factors affect the accumulation of skatole in fat and breed differences in skatole levels have been reported [32]. There is a positive genetic correlation between levels of skatole and androstenone in fat. This means that boars with a genetic disposition towards high androstenone levels will often also be disposed towards high skatole levels [9].

Molecular genetic studies have demonstrated genetic polymorphisms in enzymes involved in androstenone production [33]. Studies on polymorphisms in candidate genes have indicated the possibility of being able to reduce boar taint without having a severe impact on the levels of steroids involved in reproductive functions [34]. Based on the genetic variability for boar taint and the promising development of genetic markers for the compounds involved, the potential for selection of taint-free uncastrated male boars appears to be promising [35]. One factor that is essential with regard to the possible selection against high levels of androstenone is the preservation of normal androgen production in order for the fertilization capacity of the adult entire male to be maintained. Thus, in addition to understanding the androgen and androstenone profiles in plasma and adipose tissue, more knowledge is needed about the steroidogenic potential of Leydig cells in uncastrated male pigs.

The primary objective of the present study was to obtain more information on differences in levels of boar taint compounds between purebred Duroc and Landrace boars belonging to the population of breeding animals. To sharpen breed differences the pigs were treated with a standard dose of human chorionic gonadotrophin (hCG), and thereafter the levels of androstenone and testosterone in plasma and in fat were monitored for 3 days. The effects of testicular steroids on indolic compounds were studied by measuring skatole and indole levels. DNA flow cytometry (FCM) of testicular tissue was performed to obtain insight into the progression of spermatogenesis, as a measurement of testicular development. The second objective of this study was to investigate the effect of a variety of variables, including the heritability of androstenone levels, on the variation in androstenone levels in adipose tissue following hCG stimulation.

2. Materials and methods

2.1. Animals and sampling

Purebred Duroc (n = 60) and Landrace (n = 67) male pigs were collected from nucleus farms at approx-

imately 25 kg live weight and transported to NORSVIN's boar test station. The animals were included in The Norwegian Pig Breeders' (Norsvins') operative breeding program and belonged to the population from which breeding animals are selected. At the test station the animals were kept grouped, together with other pigs, with a total of 12 pigs per pen, and the different breeds were housed separately. Each boar's estimated breeding value (EBV) for androstenone deposition in fat was calculated based on androstenone levels. The major information source was phenotypes of related boars, either half-siblings or half-siblings of their sires (1728 for Landrace and 1202 for Duroc respectively) [9]. The pigs were fed a commercial diet on an *ad libitum* basis and had free access to water. After the pigs had completed their participation in the breeding programme at 100 kg live weight, the current experiment was performed. On Day 0, prior to hCG stimulation, blood and adipose tissue were sampled. Briefly, blood was sampled by venapuncture of the jugular vein into 10 ml lithium heparin sample tubes, which were stored on ice for subsequent centrifugation, and the separation and storage of plasma at −20 °C for later analysis. After disinfection and local anaesthesia, an incision of approximately 2 cm was made 8–10 cm lateral to the midline in the neck region and a piece of adipose tissue removed. The tissue was placed in a cryovial on dry ice and stored at −80 °C for later analysis of boar taint compounds. The skin wound was sutured within 5 min. Immediately after, the boars were treated with hCG (Pregnyl®, N.V Organon, Oss, The Netherlands, 30 IU per kg live weight) intravenously through an ear vein to stimulate testicular steroid production [36–42]. Blood samples were collected at the same time each day for three consecutive days following stimulation. After slaughter, on Day 3 following stimulation, adipose tissue from the abdominal region was sampled and stored at −80 °C for later analysis for androstenone, skatole, and indole. Following removal, the testes were trimmed of excessive connective tissue, the epididymis, and *vas deferens*. One testis from each boar was cut longitudinally and a piece of testis tissue of approximately 5 mm^3 removed from the area between the tunica albuginea and mediastinum, and placed into a cryo vial on dry ice for storage at −70 °C and later DNA FCM analysis.

The experiment was carried out in compliance with the provisions enforced by the National Animal Research Authority. Approval of the experimental protocol: 2005/23090.

2.2. Chemical analyses

Androstenone concentrations in plasma were measured by a time-resolved fluoroimmunoassay for plasma samples described by Tuomola [43], which was modified by replacing the standard curve with standards diluted in zero androstenone plasma and using antiserum produced and characterized by Andresen [44]. The standard curve ranged from 0 to 100 ng/ml. Assay sensitivity was 2 ng/ml, corresponding to 95% binding of the labelled steroid. Inter-assay variation coefficients were 10.8% (3.8 ng/ml) and 3.5% (28.6 ng/ml) respectively.

Testosterone concentrations in plasma were determined by a solid-phase radioimmunoassay kit Coat-a-Count^R, Total Testosterone, (Siemens Medical Solutions Diagnostic Europe Ltd., Eschborn, Germany) according to the manufacturer's instructions. The standard curve ranged from 0 ng/ml to 16 ng/ml. Assay sensitivity was 0.1 ng/ml, corresponding to 95% binding of the labelled hormone. Inter-assay variation coefficients were 3.5% (6.3 ng/ml) and 7.5% (11.9 ng/ml) respectively.

Fat androstenone was measured by a time resolved fluoroimmunoassay [43] using the aforementioned antiserum [44]. Standard curve ranged from 0.1 μg/g fat to 10 μg/g fat. Assay sensitivity was 0.04 μg/g, corresponding to 95% binding of labeled steroid. Interassay coefficients of variation for samples with 0.27, 0.63, and 3.36 μg androstenone/g fat were 10.4%, 6.7%, and 7.6%, respectively.

Skatole and indole concentrations in extracted fat were determined by HPLC using fluorescence detection, according to a method developed by Gibis [45]. Fat samples were melted in a microwave oven for 4 min at 350W. The tubes were centrifuged (3000 g, 15 s) and water was removed by pipette and discarded. After heating to 55 °C, samples were spiked with internal standard (2-methyl-indole, 0.2 μg/g fat). 200 μl methanol was added to the samples, incubated for 5 min at 55 °C, mixed with a vortex mixer for 30 s and centrifuged (3000 g, 30 s). After freezing, the methanol phase was removed by pipette. This procedure was repeated. Mobile phase A was added to the samples up to 1 ml, cooled down and frozen and consequently filtered through a 0.22 μm-filter.

Plasma samples for the determination of skatole were prepared as described by Tuomola et al [46]. In brief, a 1.0 ml plasma sample spiked with 50 μl of internal standard (2-methyl-indole, 0.1 μg/ml in methanol) was extracted with 3 ml of diethylether, centrifuged (3000 g, 15 s), and frozen. The ether phase was decanted into a tube containing 1.0 ml mobile phase A,

and the ether removed by evaporating at 40 °C. A 100 μl aliquot of the resulting sample was analyzed by HPLC.

HPLC equipment: Agilent Technologies (www.agilent.com/chem/1100), Agilent 1100 Series. The columns used for the separation were: ZORBAX Eclipse XDB-C18, 4.6 × 150 mm, 3.5 micron with a precolumn Eclipse XDB-C18, 4.6 × 12.5 mm, 5 micron. Running conditions were: column temperature 40 °C, fluorescence detection at Ex = 270 nm, Em = 350 nm, injection volume = 50 μl, flow rate = 1 ml/min and isocratic elution of the components with a mobile phase consisting of 60% 0.02 M acetic acid, 25% acetonitrile, and 15% 2-propanol. Detection limit was 0.005 μg/ml for both skatole and indole. Intra-assay % CV for indole was 4.7%, mean = 0.0223 μg/ml (n = 8), and for skatole 1.3%, mean = 0.0235 μg/ml (n = 8).

2.3. Preparation of testicular cells and quantification by DNA flow cytometric analysis

The ploidy of isolated germ cells from the seminiferous tubules was analyzed by FCM, using the method described by Evenson et al [47], with minor boar-specific adjustments. In brief, the frozen testis tissue was placed into a 60 mm Petri dish containing 2 ml TNE buffer (0.15 M NaCl, 1 mM Tris-HCL, 1mM disodium ethylenediaminetetraacetic acid (EDTA), pH 7.4). The tissue was thawed at room temperature and minced with curved surgical scissors to liberate cells. Cell suspensions were gently transferred into the test tubes with a glass transfer pipette and tissue fragments were allowed to settle for about 2 min. The supernatant was filtered through a 40 μm nylon mesh and testicular cell samples were analyzed instantly. Chromatographically-purified acridine orange (AO) (Polyscience Inc., Warrington, Pennsylvania) was used for differential staining of DNA and RNA. The intensity of red and green staining was measured in a Coulter EPICS XL flow cytometer (Beckman Could Ltd., Luton, England) equipped with a 15 mW argon laser, excitation at 488 nm. Green fluorescence was detected using a 505–545 nm band pass (BP) filter (FL1), and red fluorescence detected using a 660–900 nm BP filter (FL4). Measurements were recorded 2.5 min after the acid pre-treatment. Fluorescence data were acquired on 5000 cells/sample at a flow rate of 100–200 cells/s. The percentage of the haploid (1n), diploid (2n) and tetraploid (4n) compartments were analyzed using EXPO32^{TM} ADC (Beckman Coulter version 1.1c). The percentages of round, elongating, and elongated spermatids were summarized as total haploids in order to evaluate the spermatogenetic progress in the pigs.

2.4. Statistical analysis

Statistical analyses were performed using JMP7 (SAS Institute, Inc, Cary, NC, USA). The frequency distributions of investigated variables were tested for normality by the Shapiro-Wilk's test. Log transformation of the dependent variables fat and plasma androstenone, plasma testosterone, and fat skatole gave a better fit to the normal distribution and were used in multivariate analyses. The Wilcoxon two-sample test was used to assess differences between mean values of steroids, skatole, indole, body weight, and age. Individual differences in steroid or skatole concentrations were analysed by Student's *t*-test.

The elimination of plasma androstenone and testosterone was analysed in multivariate mixed models using Proc Mixed in SAS [48]. Explanatory variables were breed, day of sampling, and the interaction of these variables. Measurements within the same boar were correlated; this was accounted for by using a first order autoregressive correlation structure. Other correlation structures were also modelled, but the first order autoregressive correlation resulted in the best model fit (Schwartze's Bayesian criterion was closest to zero).

The breed differences in number of haploid testicular cells (1ntot), the relative proportions of haploid versus diploid, and the log transformed ratio between haploid versus tetraploid cell populations (log 1n:4n) were investigated with General Linear Models (GLM). Predictor variables for cell populations and their ratios were breed, body weight, and age.

Sources of variation in fat androstenone were investigated prior to hCG stimulation and 3 days after hCG stimulation. Best fit models (GLM) were developed based on the predictor variables breed, age, body weight, testicular cell composition, plasma testosterone, estimated breeding value (EBV) for androstenone, and possible interactions. The predictor variable EBV was nested within breed.

3. Results

3.1. Weight and age

Although Duroc boars were on average ten days older than Landrace boars at slaughter [age of Duroc boars: mean (SE) 168 (1.2) and age of Landrace boars: mean (SE) 158 (1.0) days], the mean (SE) live body

weight was slightly higher in Landrace than in Duroc boars [Landrace: 110 (0.44); Duroc: 107 (0.41) kg].

3.2. Testosterone, androstenone, and skatole concentrations in plasma

The initial mean (SE) plasma testosterone concentration in Duroc boars [4.4 (0.43) ng/ml] was significantly higher than in Landrace [2.4 (0.40) ng/ml] (Fig. 1A). For both breeds the highest mean testosterone concentration was measured one day after stimulation. However, both the actual mean concentration and the increase in mean testosterone was significantly higher in Landrace boars than in Duroc (Fig. 1A). The mean level in Duroc boars was increased by a factor of 5.7, compared with 13.3 in Landrace. Over the following two days plasma testosterone concentrations decreased in both breeds towards those levels observed before stimulation. The elimination of testosterone was slower in Duroc boars than in Landrace. In mixed models, taking account of repeated measurements, log plasma testosterone was affected by day of sampling ($P < 0.001$), breed ($P = 0.06$), and the interaction between breed and day of sampling ($P < 0.001$).

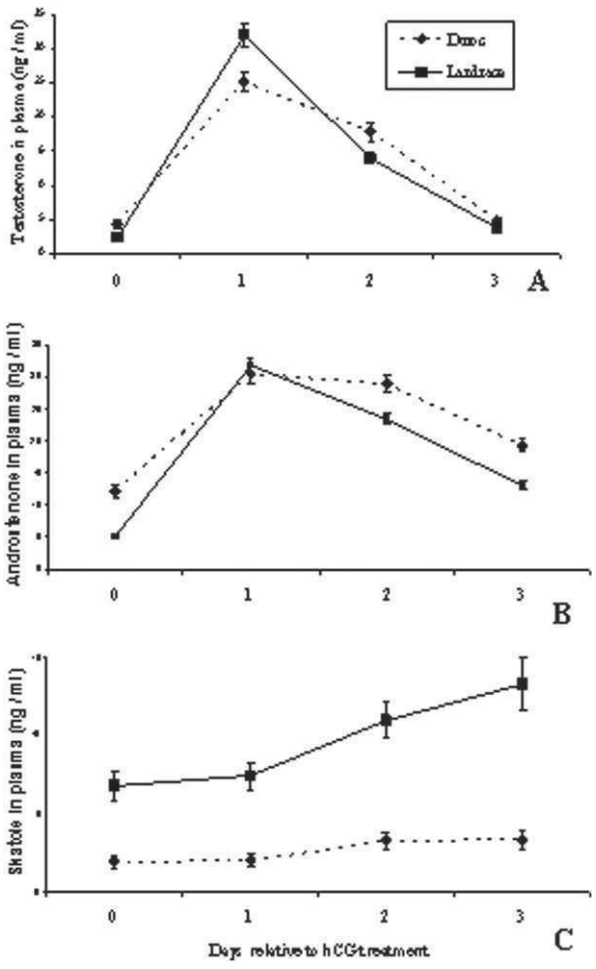

Figure 1. Testosterone (A), androstenone (B), and skatole (C) in plasma in Duroc (n = 60) and Landrace (n = 67) boars following stimulation with human chorionic gonadotrophin (hCG). hCG was given i.v. immediately after sampling on Day 0.

Prior to hCG stimulation, the mean (SE) plasma androstenone concentration was significantly higher in Duroc boars [12.1 (1.1) ng/ml] than in Landrace [5.2 (0.5) ng/ml]. All animals responded to the hCG stimulation with a significant increase in plasma androstenone concentrations on Day 1, but the mean peak concentrations did not differ significantly between breeds (Fig. 1B). However, the increase in mean plasma androstenone after hCG stimulation was considerably higher for Landrace boars than for Duroc. The level in Duroc boars was increased by a factor of 2.5, while a 6.1 fold increase was observed in Landrace. On Days 2 and 3 after hCG stimulation the mean (SE) plasma androstenone concentrations decreased in both breeds, but on Day 3 the plasma androstenone levels were still substantially higher than the levels before stimulation (Fig. 1B) The elimination of androstenone was slower in Duroc than Landrace boars (Fig. 1B). In mixed models, taking account of repeated measurements, log plasma androstenone concentrations were significantly affected by day of sampling ($P < 0.001$), breed ($P < 0.01$), and the interaction between breed and day of sampling ($P < 0.001$).

The mean (SE) plasma androstenone/testosterone ratio on Day 1 after hCG stimulation was significantly higher for Duroc than Landrace boars [3.4 (0.29) versus 3.2 (0.27), respectively], indicating a breed-specific difference in the ratio between plasma testosterone and androstenone.

On Day 0 the mean (SE) plasma concentration of skatole was more than three times higher in Landrace [6.7 (0.8) ng/ml] than Duroc boars [1.94 (0.8) ng/ml]. Three days after hCG stimulation, the mean plasma skatole concentrations had increased 1.7-fold in Duroc and 2-fold in Landrace boars (Fig. 1C).

3.3. Androstenone, skatole and indole skatole concentrations in fat

The mean initial level of androstenone in fat was 3.2-fold higher in Duroc than Landrace boars (Fig. 2A). For both breeds, there was a significant increase in fat androstenone concentrations 3 days after hCG stimulation. In Duroc boars, the mean (SE) level increased from 2.48 (0.23) μg/g to 6.58 (0.29) μg/g. In Landrace

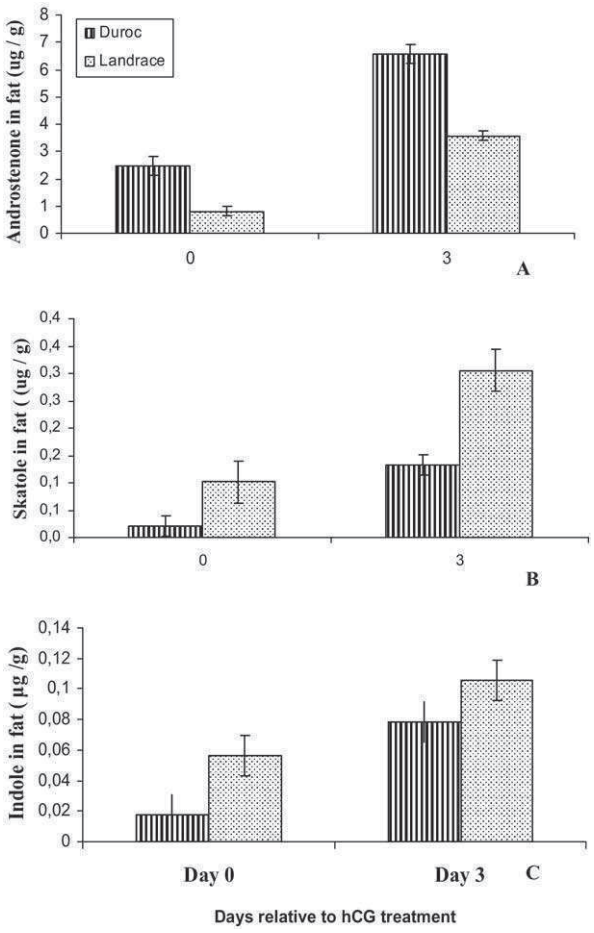

Figure 2. Androstenone (A) Duroc (n = 54) and Landrace (n = 64), skatole (B), and indole (C) (D; n = 16, L; n = 32), in fat following stimulation with human chorionic gonadotrophin (hCG).

boars, the mean (SE) level increased from 0.78 (0.20) μg/g to 3.56 (0.27) μg/g. The difference in fat concentrations before and after hCG, as well as the maximal response to hCG stimulation, was significantly greater in Duroc than Landrace boars (Fig. 2A). The mean level was increased by a factor of 2.6 in Duroc, and by a factor of 4.6 in Landrace boars. Three days after hCG stimulation, all boars had fat androstenone levels higher than 1 μg/g.

The initial levels of skatole in fat were higher in Landrace than Duroc boars (Fig. 2B). In both breeds, there was a significant increase in skatole concentrations in fat following hCG stimulation (Fig. 2B). In Duroc boars, the mean (SE) level of skatole in fat increased from 0.02 (0.05) μg/g before stimulation to 0.10 (0.03) μg/g at Day 3, and in Landrace boars, the mean (SE) level increased from 0.13 (0.03) μg/g to 0.31 (0.03) μg/g. The increase was significantly higher in Landrace boars than in Duroc (P = 0.002).

Indole levels in fat followed the same trend as skatole levels in fat: both breeds showed a significant increase in indole concentration following hCG stimulation (Fig. 2C). Although no significant difference between the breeds was observed (P = 0.07 for both), it should be noted that the number of observations included for skatole and indole levels before hCG stimulation was n = 16 for Duroc and n = 32 for Landrace respectively, due to the limited fat volume available for analysis.

3.4. Sexual maturation evaluated by DNA flow cytometry

Quantitative evaluation of testicular cells by the application of DNA FCM showed activated testicular activity in both breeds based on the presence of all ploidy compartments, including the haploid testicular cells (Table 1). However, significant differences were observed between the two breeds concerning progress through spermatogenesis. Univariate analyses showed significantly higher relative percentages of the total number of spermatids in Duroc boars, of which the higher percentage of elongating/elongated spermatids was most prominent. Furthermore, the germ cells showed that transformation of spermatogonia (2n) to primary spermatocytes (4n) and round spermatids (1n), as well as the overall conversion of diploid (2n) to haploid spermatogonia, was reduced in Landrace boars compared with Duroc boars. Additionally, the Duroc boars exhibited significantly higher ratios of haploid and diploid populations, as well as for the haploid and tetraploid populations (Table 1).

When correcting for body weight and age, the number of haploid testicular cells (1ntot), the relative pro-

Table 1
Relative fractions (mean (SE)) of the various testis subpopulations from entire male boars (Duroc and Landrace) At slaughter.

	elongating/ed	round	1n tot	2n	4n	1n:2n	1n:4n
Duroc (n = 54)	18.9[a] (1.2)	42.9[a] (1.4)	61.9[a] (1.8)	23.0[a] (1.4)	10.0[a] (0.8)	3.3[a] (0.2)	8.9[a] (1.0)
Landrace (n = 60)	14.7[b] (1.0)	41.2[a] (1.3)	55.9[b] (1.7)	26.7[b] (1.3)	13.2[b] (0.7)	2.5[b] (0.2)	5.3[b] (0.5)

[a,b] P < 0.05; Wilcoxon 2- sample test.

Table 2
Variation in Log fat androstenone in Landrace and Duroc boars on Day 0 (prior to hCG stimulation) and Day 3 (three days after hCG stimulation). Parameter estimates with standard errors (SE) and ANOVA table with sums of squares explained (SS), degrees of freedom (df), and residual SS, As well as the df values for the best fit regression models.

Term	Day 0; R^2-model = 0.57					Day 3; R^2-model = 0.48				
	Estimate	SE	df	SS	P-value	Estimate	SE	df	SS	P-value
Intercept	−0.52	0.09	1		<0.0001	1.09	0.08	1		<0.0001
Breed. e.g. (Duroc - Landrace)	0.49	0.07	1	23.3	<0.0001	0.27	0.04	1	7.3	<0.0001
Log plasma testosterone	0.58	0.08		27.4	<0.0001	0.31	0.06		4.8	<0.0001
Estimated Breeding Value (Duroc)	1.08	0.41	2	3.8	0.0088	0.26	0.23	2	1.2	0.2612
Estimated Breeding Value (Landrace)	0.39	0.43			0.3664	0.60	0.25			0.0169
Breed*log plasma testosterone	0.13	0.08	1	1.3	0.0995	0.11	0.06	1	0.6	0.0506
Residual			112	54.0				114	18.6	

portions of haploid versus diploid, and the log transformed ratio between haploid versus tetraploid log 1n:4n cell populations were significantly higher in Duroc boars.

3.5. Variations in fat androstenone concentrations

Breed and log plasma testosterone were the main variables explaining variation in log fat androstenone concentrations. Age, but not body weight, contributed significantly positively to log fat androstenone prior to hCG stimulation, but not afterwards. A significant positive relationship was found between estimated breeding value (EBV) in Duroc boars prior to hCG stimulation (Day 0) and after hCG stimulation in Landrace boars (Day 3; Table 2). Testicular development, as indicated by cell population characteristics, did not explain the additional variation in log fat androstenone when taking into account the aforementioned variables.

4. Discussion

In the present study, differences between purebred Duroc and Landrace boars were studied with respect to levels of boar taint-compounds and testosterone before and after hCG stimulation and in relation to development in the pubertal testis. To our knowledge these kinds of comparisons between purebred pig breeds have not been carried out previously.

4.1. Initial levels of steroids

Duroc boars revealed higher levels of androstenone in plasma and in fat than Landrace boars, as well as higher plasma levels of testosterone. This confirms previous results of studies done on different animals of the same population of animals [9,34]. With respect to levels of androstenone in different breeds, these results concur with those reported by Xue et al [10] and Squires and Lou [12]. The mean percentage fat in Duroc and Landrace boars reported by NORSVIN's testing station was 33.2% and 27.1% respectively. A larger distribution volume for androstenone in Duroc boars than in Landrace will obviously result in a lower concentration, and that higher concentrations of androstenone were found in fat in Duroc boars strengthens the view that there are probably substantial differences in production and/or elimination of androstenone between the two breeds.

4.2. Age and sexual maturation

Duroc animals were at slaughter, on average 10 days older than Landrace boars and showed a more advanced stage of spermatogenesis as analyzed by the accurate DNA FCM method, than the Landrace boars. In all likelyhood this difference also existed before stimulation, 72 h earlier. DNA FCM showed the appearance of round spermatids as a result of completion of the first meiosis and start of a full spermatogenetic cycle at the onset of puberty for all males of both breeds. However, the significantly lower percentage of elongated/elongating spermatids in Landrace boars suggests a less-advanced spermiogenesis in this breed at time of slaughter. Furthermore, fewer mature germ cells were observed in Landrace boars compared with the total number of haploid cells observed in Duroc boars. Additionally, the germ cell populations differed significantly between the two breeds following correction for age and body weight. Accordingly, with regard to differences in initial androstenone and testosterone levels, differences in testicular development must also be considered and it is possible that the differences in initial steroid levels between the two breeds may be partly explained by a more advanced testicular development in Duroc boars. In practice, there is no weight difference between the breeds and therefore, in a system where Duroc and Landrace boars are raised for pork production and slaughtered at the

same live weight, boar taint due to androstenone will be more problematic in Duroc boars than in Landrace boars.

4.3. Initial levels of indolic compounds

The significantly higher levels of skatole in plasma and in fat before stimulation in Landrace boars than Duroc reflect results previously reported by Tajet et al [9], in which skatole in Landrace was higher than in Duroc boars. Similar results have also been reported from Denmark [49,50]. However, Squires and Lou [12] found similar levels of skatole equivalents in the two breeds. It is well-known that both diet and environment affect skatole levels [7]. These factors were identical for both breeds in the present study. It is likely that differences in the rate of skatole metabolism in the liver is a main factor explaining different levels in Duroc and Landrace boars [51], with cytochrome P4502E1 (CYP2E1) and P4502A (CYP2A) as the most prominent enzymes for skatole metabolism [26,52–54], although differences in the intestinal content of indolic compounds between very different pig breeds (Jinhua and Landrace) have been reported [55]. Androstenone appears to block the induction of CYP2E1 and thus reduce the metabolism of skatole [27,28]. However, higher levels of skatole and, simultaneously, lower levels of androstenone in Landrace than Duroc boars, indicate breed differences in the regulation of skatole levels. Nevertheless, previous studies on breed differences in skatole metabolism have given unequivocal results. Doran et al [56] found a negative correlation between skatole level in fat and hepatic microsomal cytochrome CYP2E1content in Large White boars, but not in Meishan boars. Moe et al [34] found significant associations between single nucleotide polymorphisms (SNPs) and halotypes within CYP2E1 and levels of skatole and indole in Duroc and Landrace boars. However, the extent of variation in skatole and indole levels in fat that could be explained by CYP2E1, differed between the two breeds. In a study on hCG stimulation of Duroc and Landrace boars from different animals in the same population group of animals as in the present study, breed-related differences in the hepatic activities of CYP2E1 or CYP2A could not be detected in unstimulated animals [57].

4.4. Levels of steroids following hCG stimulation

Human chorionic gonadotrophin is a homologue analogue of luteinizing hormone (LH), binding to and activating the same LH receptor, and exerting similar effects on Leydig cell maturation and steroidogenesis [37,58,59]. In the present study, and similar to previous studies [36,38–42], intravenous hCG administration stimulated steroidogenesis, causing a peak in the plasma levels of testosterone and androstenone as well as in the androstenone levels in fat. As previously reported by Malmfors [42] and Claus and Alsing [41], maximum plasma levels for testosterone and androstenone were observed approximately 24 h after stimulation.

As the relative increases in steroids following hCG injection were much higher in Landrace than Duroc boars, it appears that the lower steroid levels found in Landrace boars under normal circumstances are not due to a lower production capacity in the testes, but more probably a result of either a lower stimulation of the testes by gonadotropic hormones or a faster disappearance of steroids.

The animals were slaughtered on Day 3 following stimulation, as maximum levels of androstenone in fat appear to be reached about 72 h following stimulation [42]. At slaughter all boars had androstenone levels higher than 1 μg/g fat, showing that all animals produced sufficient androstenone to cause an unpleasant odour and off-flavour in pork products. The considerable increase in fat levels of androstenone at slaughter indicates a similar increase in the level of free androstenone in plasma, rather than sulphated androstenone, as this compound is not expected to be transferred to adipose tissue [60].

The results of the present study revealed a significant positive relationship between EBV after hCG stimulation in Landrace boars. As the initiation of spermatogenesis requires only LH [61], the observed delay in spermatogenesis, as well as the higher response to hCG stimulation in Landrace boars, may indicate a gonadotrophin-dependent breed difference. The findings reported in Table 2 could imply that phenotypic selection of boars based on 5α-androstenone might be best performed before hCG-stimulation in Duroc boars, but after stimulation in Landrace boars.

The decrease in plasma steroid levels was slower for androstenone than for testosterone in both pig breeds. Carlström et al [39] also found differences in half-lives of testosterone and androstenone decaying from hCG induced levels, reporting half-lives of approximately 1 day and 1.8 days respectively. Lower plasma testosterone and androstenone levels were found in Landrace boars compared with Duroc boars 48 and 72 h after stimulation. Thus the balance between synthesis and elimination of the steroids was different between the breeds with a more rapid disappearance of the plasma

steroids in Landrace boars. The levels of androstenone in adipose tissue are mainly determined by the balance between production rate in the testes and elimination of the steroid [18,62]. Studies on hepatic metabolism of androstenone have been reported [24,63,64]. Nicolau-Solano et al [65,66] found a significant negative relationship between the expression of hepatic 3β-hydroxsteroiddehydrogenase (3β-HSD) protein and the level of androstenone in backfat. They concluded that the regulation of hepatic 3β-HSD activity may be a factor that influences the deposition of androstenone in pig adipose tissue, and found evidence to suggest that hepatic 3β-HSD protein in pigs is under the control of sex hormones. Chen et al [67] also found low 3β-HSD gene expression in the liver from boars with high androstenone levels in fat. In a study on polymorphisms in the porcine hepatic 3β-HSD gene [68], polymorphisms in the 3β-HSD 5′-flanking region was identified. Although these polymorphisms were breed-dependent, they were not associated with androstenone levels in fat. Moe et al [23] found a difference between Landrace and Duroc boars in gene expression of enzymes involved in both phase I and phase II metabolism of androstenone, with the expression of 3β-HSD being decreased in Landrace boars with extremely high concentrations of androstenone, while in Duroc boars with extremely high concentrations of androstenone the expression of hepatic sulphotransferase 2B1 was decreased. These studies, along with the results of the present study, suggest that hepatic androstenone metabolism may have a decisive role in regulating androstenone deposition in adipose tissue. On this basis, it should also be noted that adipose tissue is a major site for metabolism of sex steroids [69]. However, the extent to which the metabolism of androstenone in adipose tissue might have affected our results remains to be studied.

4.5. Indolic compounds following hCG-stimulation

The continued elevation of plasma skatole levels three days after stimulation indicates a lasting effect of increased androstenone/testicular steroid levels on skatole levels. Following hCG stimulation, Chen and co-authors [40] found a significant increase in the levels of indole in plasma and fat, but in contrast to the present study, no significant increase in skatole levels. Changes in indolic compounds could be due to changes in intestinal production as discussed by Chen et al [40], and/or by a decrease in the activities of metabolizing enzymes through the inhibitory effect of androstenone on skatole metabolism in the liver [27,28]. Different results have been reported regarding activities of CYP2E1 and CYP2A in the liver following hCG stimulation. Zamaratskaia et al [26] did not find any differences in enzyme activities although hCG administration caused a significant increase in fat androstenone levels. In contrast, studies on the hepatic activities of CYP2E1 and CYP2A in boars from the present study indicated that hCG stimulation can suppress hepatic CYP2E1 and CYP2A activities probably through an increase in the levels of testicular steroids [57]. However between-breed variations in skatole levels in fat were not found to be related to the activities of these enzymes.

The results of the present study revealed significant differences in the levels of boar taint substances between pubertal purebred elite Landrace and Duroc boars. The response to hCG stimulation and the elimination of these compounds varies between the two breeds. Differences in hepatic metabolism may be an important explanatory factor. Selection against factors associated with boar taint remains a possible solution for the problem of boar taint in the swine industry.

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Steroidogenesis in primary cultures of neonatal porcine Leydig cells from Duroc and Norwegian Landrace breeds

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Abstract

Breed differences in steroidogenic activity between primary Leydig cells derived from neonatal purebred Duroc and Norwegian Landrace boars were investigated *in vitro*. Concentrations of testosterone, estradiol, androstenone, cortisol and progesterone produced into the medium were determined. To explore underlying mechanisms the cellular expression of a suite of genes relevant in steroidogenesis was measured using reverse transcription and quantitative PCR (RT-qPCR). Basal steroid concentrations indicated a larger production capacity for steroids in unstimulated Duroc cells. Stimulation of the cells with LH increased steroid hormone secretion significantly in both breeds in a dose dependent manner. Testosterone and androstenone concentrations increased approximately 50- and 15-fold, respectively, whereas concentrations of estradiol, cortisol and progesterone increased to a lesser extent. At levels of maximal LH stimulation, absolute steroid concentrations were higher in Duroc. However, the relative increase in hormone concentrations was significantly lower in Duroc cells for estradiol, progesterone and cortisol when compared to basal levels. LH exposure was associated with a general up-regulation of mRNA levels for steroidogenic genes, stronger in Duroc than in Norwegian Landrace. This was in agreement with the higher absolute concentrations of steroid hormones measured in culture medium from the LH-stimulated Duroc Leydig cells, but did not concur with the fact that the relative increase in hormone production was lower in Duroc than in Norwegian Landrace Leydig cells for some hormones. It was concluded that breed differences in steroid hormone concentrations and gene expression between Norwegian Landrace and Duroc are complex and cannot be explained by a simple mechanism of action.

Keywords: Pig breeds; LH; hCG; Steroidogenesis; Gene expression; Androstenone; Estradiol; Boar; Leydig cells *in vitro*

1. Introduction

In intact boars, levels of testicular steroids reflect the steroidogenic capacity of the individual Leydig cells and their total number per testis, although hepatic metabolism may also affect the peripheral plasma steroid concentrations [1,2].

The pig testis has been described as "the most versatile steroid producing organ known" [3]. Dynamic changes occur in the pig testis during the neonatal period. Increases of Leydig cell volume and Sertoli cell proliferation take place during the first month after birth [4]. In the neonatal pig, predominantly between two and three weeks of age, most of the testicular volume is made up of Leydig cells [5]. Syntheses of androgens, androstenone (5α-androst-16-ene-3-one), and estrogens occur in Leydig cells, so that these cells require a

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number of steroidogenic enzymes. The expression and presence of steroidogenic enzymes have been well addressed in the domestic pig testis [6–11]. Estrogens are synthesized from aromatization of androgens through the action of cytochrome P450 aromatase (CYP19). In fetal pig testis, CYP19 is present in Leydig cells and/or gonocytes [10,12,13]. Differential expression of other steroidogenic enzymes has also been reported during fetal and postnatal development in the pig testis [11,14,15].

Biosynthesis of androgens and 16 unsaturated steroids has been described in neonatal porcine testicular preparations [16]. The peak of estrogen concentrations occurs during neonatal development, between one and three weeks of age, then transiently decreases and remains at low level until pubertal development [17]. Changes in the serum levels of free androgens and of conjugated steroids also show similar patterns to those of estrogens during the postnatal development of male pigs [18,19]. Androstenone is a testicular steroid with special interest in the boar. It accumulates in adipose tissue and constitutes a major component of the boar taint, an unpleasant odor and flavor, limiting the use of intact males in the production of pork [20].

Breed and age differences have emerged regarding the levels of steroids in pigs. Duroc pigs normally have considerably higher androstenone levels than Yorkshire and Norwegian Landrace varieties, while Large White breeds generally show higher levels than Landrace breeds [21–23]. Genetic variation greatly affects fat androstenone levels [24,25]. In the pig population different individuals have a high or low potential for particular androstenone levels. Gene expression studies have been performed in the testes and livers of Duroc and Norwegian Landrace boars with extremely high and low levels of androstenone in fat [26–28, 35]. A recent genome wide single nucleotide polymorphism (SNP) association analysis revealed several areas of the genome responsible for variation of androstenone levels in intact Duroc boars. These regions contain both old and new candidate genes [29]. Thus, in addition to understanding the sex steroid and androstenone profiles in plasma and adipose tissue, more knowledge is needed about the steroidogenic potential of Leydig cells and the underlying mechanisms for breed differences.

The primary objective of the present study was to obtain more information on differences in steroidogenic activity between purebred Duroc and Norwegian Landrace boars by investigating steroid secretion from primary neonatal porcine Leydig cells *in vitro*. In order to get a better picture of breed differences, cells were exposed to increasing doses of LH. The breed differences in basal and LH-stimulated hormone secretion were investigated by measuring concentrations of testosterone, estradiol, androstenone, cortisol, and progesterone and the expression of a suite of genes relevant in steroidogenesis. Primary neonatal porcine Leydig cells provide a powerful tool to study the physiology and genetics of porcine steroidogenesis and also could possibly be used as a model for adult steroidogenesis.

2. Materials and methods

2.1. Collection of porcine testicular tissue

Testis tissue was obtained in Norwegian breeding farms from male offspring in approximately 21-day-old litters. Gonads were collected at three different occasions from a total of 29 Duroc and 29 Norwegian Landrace piglets. Due to litter size, the donors came from 10 Duroc litters, and seven Norwegian Landrace litters. Local anesthesia, a total of 2 mL 1% lidocaine without adrenalin (Haukeland Hospital Pharmacy, Bergen, Norway), was given subcutaneously on both sides of the scrotum and in the inguinal string. The skin was washed and then disinfected with 70% ethanol. Standard surgical procedure for castration of male piglets was followed on both sides. After intervention, piglets received a single treatment (6 mg/kg body weight i.m.) with ketoprofen (Romefen Vet; Merial GmbH, Hallbergmoos, Germany).

Extracted testes were left encapsulated and stored in Dulbecco's modified Eagles medium and Ham's modified medium F12 (DMEM/F12) mixture (1:1) supplemented with 1.2 mg/mL sodium bicarbonate and 15 mM HEPES buffer with L-glutamine and pyridoxine HCL pH 7.4 (Invitrogen, Paisley, UK) in presence of penicillin/streptomycin/neomycin (10 mL per 500 mL medium; Invitrogen). The material was transported on ice (2 h maximum). The experiment was carried out in compliance with the provisions enforced by the National Animal Research Authority.

2.2. Isolation and purification of porcine leydig cells

Isolation, subsequent purification, and culture of porcine Leydig cells were adapted from the protocol described by Lejeune et al. [30]. Testes were decapsulated, the tissue chopped with scissors, and digested with 0.5 mg/mL collagenase/dispase (Vibrio alginolyticus/Bacillus polymyxa, Roche Neuss, Düsseldorf, Germany) in DMEM/F12 medium at 34°C under agitation. Digested tissue was collected after 45, 90, and 120 min

and filtered through a nylon mesh. The cell suspension from each time point was centrifuged at 250 X g for 10 min, and the pellet resuspended in 50 mL DMEM/F12 medium. After two sedimentations at unit gravity (5 and 15 min respectively) the supernatants were centrifuged at 250 X g for10 min. The final pellet was resuspended in DMEM/F12 and kept at 4ºC. All samples from each breed were pooled. This resulted in two pooled populations from Duroc and three from Norwegian Landrace which were collected on the different sampling days.

Leydig cells were purified by centrifugation through a discontinous Percoll gradient. Percoll (Sigma-Aldrich, St. Louis, MO, USA) was made iso-osmotic by adding 1 volume of 10× Ham's F-10 (Biological Industries, Kibbutz Beit-Haemek, Israel) to 9 volumes of Percoll. This 90% Percoll was further diluted with DMEM/F12 to generate 60, 34, 26, and 21% Percoll solutions. These were layered to form the gradient [30]. About $10^8-1.5\times10^8$ cells from the pooled samples, in 5 mL of DMEM/F12, were added to each gradient and centrifuged at 1250 X g for 30 min at 4 °C. The enriched Leydig cell fraction was harvested from the 34% layer, washed, filtered, and counted in a hemacytometer (Superior, Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany).

2.3. 3 beta-hydroxysteroid dehydrogenase (HSD3B) staining for assessment of cell population purity

To assess cell identity, one round with cytochemical staining for HSD3B was performed on cultured cells. Due to reduction of tetrazolium by the enzyme, the Leydig cells turn blue and the color change is considered specific for Leydig cells in the testis [31]. Isolation of Leydig cells was performed as described above. The cells (300 000 cells/mL) were incubated with a solution containing 0.2 mg/mL nitro blue tetrazolium (Sigma-Aldrich), 0.12 mg/mL 5-androstane-3β-ol-one (Sigma-Aldrich) and 1 mg/mL NAD+ (Sigma-Aldrich) in 0.05 mol/L PBS, pH 7.4 at 34°C for 90 min. Upon development of the blue formazan deposits, the abundance of HSD3B-positive cells was determined with a hemocytometer.

2.4. Cell plating and addition of test compounds

The cell suspension was adjusted to 300 000 cells/mL and cells plated in 24-well plates (Primaria; BD Bioscience, Franklin Lakes, NJ, USA) in DMEM/F12 supplemented with 5 mL ITS and Premix (Invitrogen) and 12.5 mL NuSerum (BD Bioscience) in 500 mL medium. Cells were incubated under 5% CO2 at 34°C. After 72 h medium was refreshed and cells exposed to recombinant porcine LH (tuenre.pLH.ig; Tucker Endocrine Research Institute, Atlanta, GA, USA) at different concentrations (0.025, 0.05, 0.25, 0.5, and 2.5 ng/mL). Controls were included with no LH exposure (medium blank). After 48 h, medium was collected and stored at −20°C prior to hormone analysis. Plates with cells were stored at −80°C until harvest for RNA extraction. Each exposure was performed in triplicates and carried out as independent Leydig cell isolations.

2.5. Cell viability assay

Cell viability was estimated using the AlamarBlue (Invitrogen) assay after collecting aliquots for hormone assays. Each well received 1 mL fresh medium containing 10% AlamarBlue (Invitrogen). Plates were incubated for 1 h, before a 100 μL sample from each well was transferred in duplicates to a fresh 96-well ELISA plate (Falcon, Franklin Lakes, NJ, USA) and absorbance read in a Victor3TM spectrophotometer (Perkin Elmer, Shelton, CT, USA) at 570 nm and 600 nm. Viability was expressed as percentage of control (medium blank).

2.6. Hormone quantification

Cell medium levels of testosterone, estradiol, and cortisol were measured by using Coat-A-Count (Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA) solid phase radioimmunoassay kits (RIA). All hormone kits were used according to manufacturer instructions although modified by replacing the standards with fresh standard curves prepared in medium from the same batch as the medium used for the Leydig cell cultures. The assays were validated for use in cell culture medium by demonstrating parallelism between dilution in medium and the standard curve, and by recovery of the unlabeled ligand. No further modifications of the standard procedures were needed. Samples were measured in duplicate.

The sensitivity of the testosterone assay was 0.1 ng/mL, corresponding to 95% binding of the labeled hormone. The standard curve ranged from 0–20 ng/mL and the inter assay coefficients of variation were 10.2% (0.86 ng/mL) and 7.5% (11.89 ng/mL), respectively.

For estradiol, the assay sensitivity was 20 pg/mL. The standard curve range was 0–4000 pg/mL. The inter assay variation coefficients were 7.9% (154.3 pg/mL) and 10.9% (1397 pg/mL), respectively.

The cortisol assay sensitivity was 3 ng/mL, with standard curve range 0–500 ng/mL and inter assay

variation coefficients of 9.8% (57.7 ng/mL) and 7.3% (210.2 ng/mL), respectively.

Progesterone concentrations were analyzed by the Spectria Progesterone radioimmunoassay kit (Orion Diagnostica, Espoo, Finland) modified by using standards diluted in zero cell medium. The sensitivity of the progesterone assay was 0.8 ng/mL, the standard curve range was 0–40 ng/mL and the inter assay variation coefficients were 6.5% (3.87 ng/mL) and 6.1% (12.8 ng/mL), respectively.

Androstenone concentration in cell medium was measured by a time-resolved fluoroimmunoassay for serum samples [32] modified by replacing the standard curve with standards diluted in cell medium. The assay was validated to use in cell culture medium by demonstrating parallelism between dilution in medium and the standard curve, and by recovery of the unlabeled ligand. The antiserum has been described by Andresen [33]. Assay sensitivity was 2 ng/mL and the standard curve ranged from 0–100 ng/mL. Inter assay variation coefficients were 11.8% (4.3 ng/mL) and 9.2% (20.1 ng/mL).

2.7. RNA isolation

Following removal of media by suction, Leydig cells were lysed by addition of RLT Lysis Buffer (Qiagen Ltd., Crawley, West Sussex, UK) directly to each well of the culture plate. Triplicates from each plate were pooled and transferred to a Qia shredder column (Qiagen) and centrifuged. Total RNA was isolated using RNeasy Mini kit (Qiagen) as described by the manufacturer. Samples were eluted in 55 μL RNase Free Water (Qiagen) and stored at −75°C until further use. Each sample was treated with DNase I (Invitrogen) for 10 min followed by inactivation at 65°C for 5 min. RNA concentration was measured using a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA) and the RNA quality was checked by Agilent 2100 Bioanalyzer using RNA 6000 Nano LabChip kit (Agilent Technologies Inc, Santa Clara CA, USA).

2.8. Reverse transcription and quantitative PCR (RT-qPCR)

cDNA synthesis and quantitative polymerase chain reaction (qPCR) were performed using SuperScript III Platinum Two-Step RT-qPCR Kit with SYBR Green (Invitrogen) according to the manufacturers protocol. Initially, the assay was optimized with respect to the concentration of cDNA and annealing temperature. The products of each primer pair were analyzed by agarose gel electrophoresis for single bands of the predicted size. Amplification efficiency was also investigated and found to be nearly 100% for all primer pairs. cDNA was produced from 600 ng total RNA in a total volume of 20 μL. The reactions were performed by DNA Engine Tetrad Thermal Cycler (MJ Research, Waltham, MA, USA) during 10 min at 25°C, 50 min at 42°C, and 5 min at 85°C. The resulting cDNA was further diluted with sterile water.

Quantitative PCR reactions were run in a DNA Engine Thermal Cycler with Chromo 4 Real-Time Detector (MJ Research) operated by the Opticon Monitor 3 software (Bio-Rad Laboratories, Hercules, CA, USA) with uracil DNA glycosylase (UDG) incubation for 2 min at 50°C, enzyme activation for 2 min at 95°C, followed by 40 cycles of 95°C for 15 sec, 62°C for 30 sec and 72°C for 30 sec, and finally a melting curve from 65°C to 90°C read for 1 sec every 0.3°C. Each qPCR reaction contained 10 ng cDNA, 200 nM of each primer, and kit reagents in a total volume of 25 μL. Both cDNA and qPCR reactions were set up as technical duplicates. For each RNA sample a control with no added reverse transcriptase was included to check for genomic DNA contamination. For each primer pair a negative control with no added template and a positive control (Duroc, DMSO control) were included.

A set of 6 housekeeping genes were tested for suitability as reference genes for the qPCR assays. Porcine primers for these genes were derived from Duvigneau et al. [34] (*ACTB*, *HPRT*, *PPIA,* and *GAPDH*) or designed using PrimerExpress version 1.5 (Applied Biosystems, Carlsbad, CA, USA) (*PGK1,* accession number AY677198, forward: TGCCATCCCAAGCATCAA and reverse: GCCTAGGTGGCTCATAAGAACAA and *S18,* accession number NR_002170, forward: AGGGCCTCGAAAGAGTCCTG and reverse: ACATCCAAGGAAGGCAGCAG). According to GeNorm software analysis (Primer Design Ltd., Southampton, UK) *ACTB*, *HPRT,* and *PPIA* showed best stability (M value < 0.5) and were chosen as reference genes for normalization in the present experimental material.

Genes were selected based on data from microarray analysis of prepubertal (manuscript in preparation) and pubertal [35] porcine testis, and from genes known to be involved in the steroidogenesis of Leydig cells. Porcine primers were designed using PrimerExpress version 1.5 (Applied Biosystems). The primers were checked for specificity using nucleotideBLAST and primerBLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The list of selected genes with primer sequences are given in Table 1.

Table 1
Porcine primers designed and used in this work.

Gene symbol, and name	Accession Number	Primers 5′–3′
AKR1C4, aldo-keto reductase family 1, member C4	NM_001123075	F: TGCCAATCACGATGAAGCCT R: CGCAGGTCCACCGTATCAAA
CYB5A, cytochrome b5 type A (microsomal)	NM_001001770	F: TCAAAGATTGCCAAGCCTTCG R: ACAACCAGTGCTGAGATGGCTG
CYP11A, cytochrome P450, family 11, subfamily A, polypeptide 1	NM_214427	F: CACCCCATCTCCGTGACC R: GCATAGACGGCCACTTGTACC
CYP17A1, cytochrome P450 17A1	NM_214428	F: AGCCAAGACGAACGCAGAA R: CCCCAAAGATGTCCGCAAC
CYP19A1, cytochrome P450 19A1	NM_214429	F: AAAGCACCCCCAGGTTGAA R: CCACCACTTCGAGTTTTTGCA
CYP21A2, cytochrome P450, family 21, subfamily A, polypepide 2	NM_214433	F: CCATAGAGAACAGGGACCACCT R: TAGTCCAGCATGTCCCTCCAC
CYP51, cytochrome P450, family 51, subfamily A, polypeptide 1	NM_214432	F: TATGTGCCATTTGGAGCTGG R: CGAAGCATAGTGGACCAAATTG
FTL, ferritin, light polypeptide	AY610290	F: TTCCTGGATGAGGAGGTGAAGC R: CTTTCGAAGAGGTACTCGCCCA
HSD3B1, hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1	NM_001004049	F: GGAGGAAGCCAAGCAGAAAA R: TTTTCAGCGCCTCCTTGTG
HSD17B1, hydroxysteroid (17-beta) dehydrogenase 1	BP144707	F: TCGGGTCGCATATTGGTGA R: GCGCAGTAAACAGCGTTGAA
HSD17B4, hydroxysteroid (17-beta) dehydrogenase 4	NM_214306	F: TTGCCATGAGAGTTGTGAGGAA R: GTCTTACAAGGGCTCCAAGGG
INSL3, insulin-like 3 (Leydig cell)	NM_213970	F: GAGGACGGGCGAGCTGT R: ACTGGCCATCAGCCCATG
MGST1, microsomal glutathione S-transferase 1	NM_214300	F: GAACGTGTACGAAGAGCCCACC R: TGGCCGTAGAGAGATCTGGACC
NR5A1, nuclear receptor subfamily 5, group A, member 1 (*SF1*)	NM_214179	F: GCCAGGAGTTCGTCTGCCT R: GTTCGCCTTCTCCTGAGCG
ST5AR2, steroid 5-alpha-reductase 2	NM_213988	F: ATCGGCTATGCCTTGGCCA R: AAGCTCGCAGCCCAAGGAA
STAR, steroidogenic acute regulatory protein	NM_213755	F: AGAGCTTGTGGAGCGCATG R: CATGGGTGATGACTGTGTCTTTTC
ST8SIA5, ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 5	EV996704	F: TCCGCGTCAAGTACGTGCTG R: AGCCAGTAGCGCGACACGTT

F, forward; R, reverse.

2.9. Statistical analysis

Data were analyzed by JMP 7 software (SAS Institute Inc., Cary, NC, USA). The frequency distribution of investigated variables was tested for normality by Shapiro-Wilk test. Log transformation of the dependent variables androstenone, testosterone, progesterone, and cortisol gave a better fit to the normal distribution and were used in statistical analyses.

Differences between mean log hormone concentrations were assessed with Tukey HSD (Honestly Significant Difference) test. Cell viability differences between LH exposed cells and unexposed controls were also analyzed using this test.

General linear mixed models were used to assess dose-response relationships and fold change in hormone concentrations. Measured hormone concentrations (estradiol) or log-transformed hormone concentrations were dependent variables. Independent variables were experiment (N = 3), modeled as a random effect, and LH concentration in culture medium modeled as a fixed effect. Correlation analysis (Spearman correlation coefficient, r_s) was used to investigate the relationship between hormone concentrations.

Quantitaive PCR raw data generated by the Opticon Monitor 3 software (Bio-Rad Laboratories) were imported to Excel 2003 (Microsoft Office, Redmond, WA, USA) and all genes were normalized to the mean of the three reference genes (ACTB, HPRT, PPIA) in each sample. The $2^{-\Delta\Delta Ct}$ [36] method was used to visualize the fold changes in gene expression.

The log2 transformed fold change values ($\Delta\Delta Ct*(-1)$) were used for statistical testing using the Wilcoxon signed rank test. Gene expression data were available for three

Table 2
Concentration of androstenone, testosterone, estradiol, progesterone, and cortisol in cell culture medium of unstimulated primary Leydig cells from neonatal (21 days of age) Duroc and Norwegian (N.) Landrace piglets.

Steroid (ng/mL)	N. Landrace	Duroc
Androstenone	10.4 ± 1.2[a]	13.2 ± 0.9[b]
Testosterone	3.7 ± 0.5[a]	5.9 ± 1.7[b]
Estradiol	0.5 ± 0.06[a]	1.2 ± 0.10[b]
Progesterone	0.2 ± 0.04*	0.3 ± 0.04*
Cortisol	0.3 ± 0.2[a]	1.1 ± 0.54[b]

Data are mean ± SEM.
[a,b] Differences between mean values differ significantly ($P < 0.05$).
* Borderline significant difference ($P = 0.05$).

concentrations of LH (e.g., 0.25, 0.5, and 2.5 ng/mL). Dose-response relationships were assessed by general linear mixed models as described above. In addition, all data for single genes were pooled to assess an overall effect of LH exposure on gene expression. P values ≤ 0.05 were considered statistically significant.

3. Results

3.1. Leydig cell purity and viability

Staining for HSD3B activity, a marker for Leydig cells, showed 80% positive cells in the fraction used. No adverse effect of LH stimulation on cell viability was found by the AlamarBlue assay (Invitrogen).

3.2. Hormone production in Duroc and Norwegian Landrace Leydig cells

The mean basal concentration of all hormones was significantly greater in Duroc than in Norwegian Landrace Leydig cells (Table 2; Fig. 1) except progesterone which was borderline significantly greater in Duroc cells ($P = 0.05$).

The mean concentration of all hormones increased significantly in both breeds as a result of increasing concentration of LH in the culture medium (Fig. 1; $P < 0.05$). The increase in hormone concentration in response to the highest LH levels was approximately 15-fold for androstenone and 50-fold for testosterone when compared with unstimulated cells (Fig. 1A and B). Lower increments (approximately 3–10 fold) were found for estradiol, progesterone, and cortisol (Fig. 1C and E).

Absolute mean steroid concentrations were higher in Duroc at levels of maximal LH stimulation (Fig. 1). However, when including the three highest LH concentrations and expressing fold change in hormone concentrations relative to the mean of unstimulated cells, the increase in mean hormone concentrations was significantly lower from Duroc cells for estradiol, progesterone, and cortisol. Although the increase in testosterone was significantly higher from Duroc Leydig cells than from Norwegian Landrace cells, the increase in androstenone was not meaningfully different between breeds.

Depending on breed and LH concentration in the culture medium androstenone, testosterone, and cortisol reached plateau levels in response to increasing LH concentration in the culture medium. The plateau level was reached at a lower LH concentration for androstenone than for testosterone in both breeds (0.05 vs. 0.5 ng LH/mL, respectively; Fig. 1A and B).

An apparent breed difference in the response to LH was observed for estradiol in that Duroc cells showed a maximal estradiol concentration already at the lowest LH concentration (0.025 ng LH/mL culture medium), whereas estradiol production by Norwegian Landrace cells reached a plateau at a LH concentration of 0.25 ng/mL (Fig. 1C). Progesterone was the only hormone where no apparent plateau level was observed (Fig. 1D).

A negative relationship was found between androstenone and testosterone in unstimulated cells from both breeds ($r_s = -0.60$ [$N = 9$; $P = 0.09$] and $r_s = -0.52$ [$N = 12$; $P = 0.08$] in Duroc and Norwegian Landrace, respectively).

Breed differences in hormone secretion in response to high LH concentrations were indicated by correlation analysis. When including the three highest LH concentrations, the correlation coefficients (r_s) between androstenone and testosterone were 0.20 ($N = 60$; NS) and 0.82 ($N = 60$; $P < 0.001$) in Duroc and Norwegian Landrace cells, respectively.

There was a borderline significant negative correlation between testosterone and estradiol ($r_s = -0.38$; $P = 0.06$; $N = 60$) in Duroc cells, whereas the correlation coefficient was significantly positive in Norwegian Landrace ($r_s = 0.69$; $P < 0.0001$; $N = 60$). Estradiol and androstenone was significantly positively correlated in both breeds.

3.3. Gene expression in LH stimulated Duroc and Norwegian Landrace Leydig cells

LH exposure of Leydig cells from both Duroc and Norwegian Landrace resulted in a general upregulation of mRNA levels for most genes analyzed (Table 3).

In Duroc Leydig cells, the upregulation was significant only for *STAR*, *CYP11A1* (at two LH doses), and *MGST1* (Table 3B), whereas for Norwegian Landrace

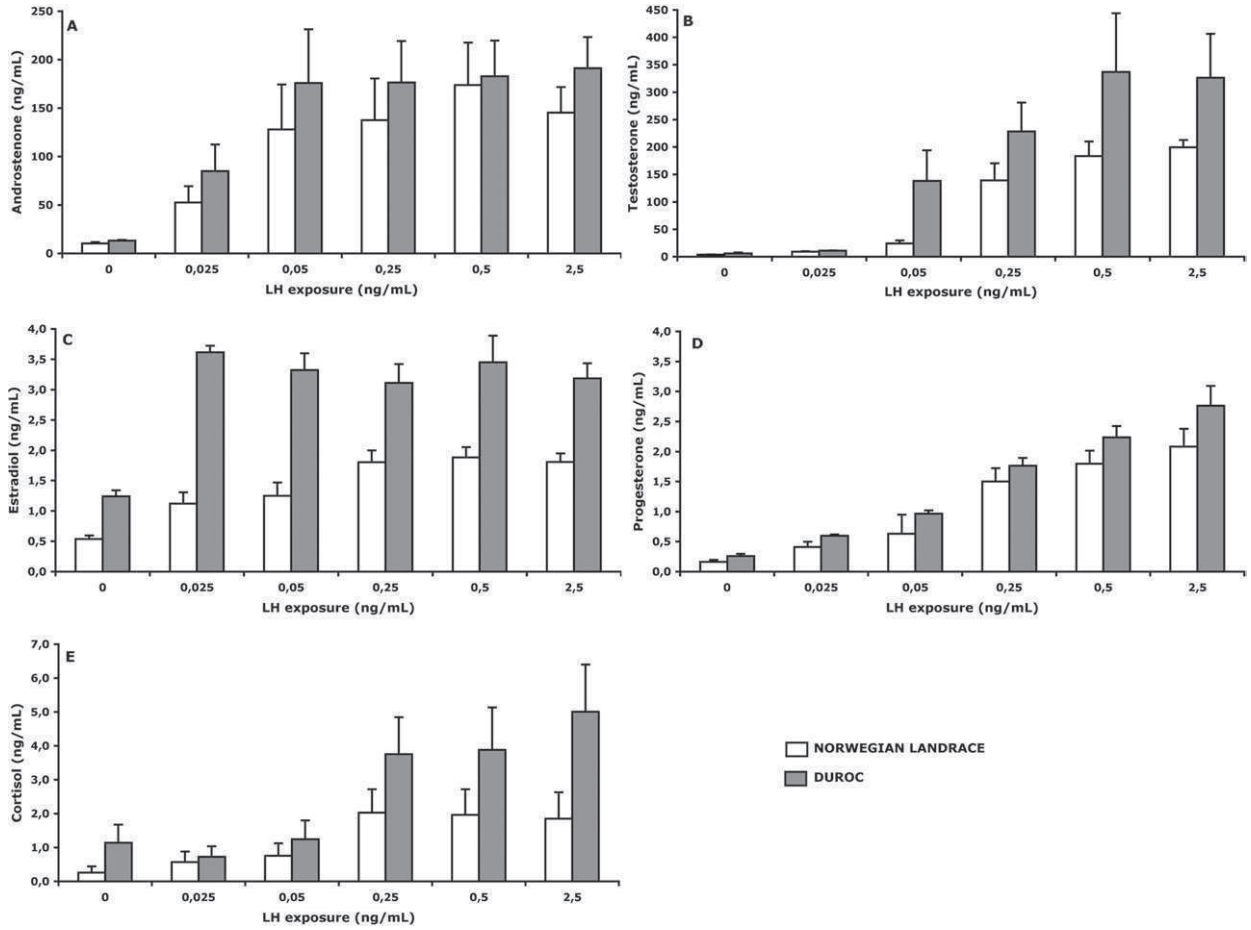

Fig. 1. Mean + SEM concentrations of (A) androstenone, (B) testosterone, (C) estradiol, (D) progesterone, and (E) cortisol in cell culture medium of unstimulated and LH stimulated primary Leydig cells from neonatal (21 days of age) Duroc and Norwegian Landrace piglets. LH concentration range, 0.025–2.5 ng/mL culture medium.

Leydig cells the upregulation was significant at all three LH doses for *STAR*, *CYP17A1*, *CYP11A1*, *HSD17B4*, *FTL*, *AKR1C4,* and *MGST1* and at one or two doses for *CYP51*, *CYB5,* and *CYP21* (Table 3A).

The most prominent alteration in expression was that of *STAR*, with a 30-fold upregulation in Duroc and a 17-fold upregulation in Norwegian Landrace relative to respective controls at the highest LH dose. *STAR* also showed a significant LH-dose dependent increase in expression in both breeds. In Duroc dose-responses were also significant for *CYP51* and *CYP21*, although the levels of expression were not significantly altered in comparison with control. In Norwegian Landrace *CYP21* and *FTL* displayed significant positive dose-responses, while *CYB5* had a significant negative dose-response.

Only the expression of *HSD3B* was significantly downregulated as a result of LH exposure. This was observed in cells from both breeds, although in Norwegian Landrace only for the two highest LH doses. However, as opposed to Duroc, Norwegian Landrace *HSD3B* expression decreased in a dose-dependent manner.

When pooling results from all LH exposures (Fig. 2A and B), the alteration in gene expression compared with control was more pronounced than reported for individual LH doses in both breeds. The expression of most genes was significantly upregulated compared with control ($P \leq 0.05$). However, *HSD3B* expression was significantly downregulated ($P < 0.05$), while the expression of *ST5AR2*, *HSD17B1,* and *ST8SIA5* showed no regulation.

When investigating LH-exposed cells and comparing Duroc versus Norwegian Landrace, expression of *STAR*, *CYP17A1*, *HSD17B4, CYP21,* and *HSD17B1* ($P < 0.05$) were significantly higher, while that of *ST8SIA* ($P < 0.05$) was significantly lower in Duroc (Fig. 2C).

Table 3
Gene expression in Norwegian Landrace and Duroc Leydig cells exposed to three different concentrations of LH relative to expression in control cells.

Gene	0.25 ng LH/mL	P	0.5 ng LH/mL	P	2.5 ng LH/mL	P	Dose response
A. Norwegian Landrace							
STAR	7.49 ± 2.25	0.02	11.73 ± 3.81	0.02	17.35 ± 4.15	0.01	*
CYP17A1	6.10 ± 2.13	0.03	6.43 ± 2.69	0.05	6.05 ± 2.47	0.05	NS
CYP51	2.90 ± 1.17	0.14	3.76 ± 1.07	0.04	4.59 ± 1.78	0.06	NS
CYP19A1	2.71 ± 1.49	0.30	2.88 ± 1.54	0.25	2.86 ± 1.41	0.21	NS
CYB5	3.00 ± 0.23	0.01	2.29 ± 0.16	0.01	1.56 ± 0.32	0.27	†
CYP11A1	2.31 ± 0.20	0.01	2.68 ± 0.30	0.01	2.62 ± 0.36	0.02	NS
INSL3	3.17 ± 1.01	0.07	2.69 ± 1.25	0.21	1.50 ± 0.72	0.73	NS
HSD17B4	1.94 ± 0.27	0.05	2.25 ± 0.16	0.01	2.27 ± 0.16	0.01	NS
CYP21	1.22 ± 0.12	0.19	1.72 ± 0.22	0.05	2.69 ± 0.11	0.00	†
FTL	1.53 ± 0.30	0.01	1.82 ± 0.19	0.03	1.98 ± 0.17	0.02	*
NR5A1	1.59 ± 0.30	0.14	1.58 ± 0.19	0.06	1.48 ± 0.26	0.17	NS
AKR1C4	1.48 ± 0.06	0.01	1.63 ± 0.08	0.01	1.36 ± 0.09	0.04	NS
MGST1	1.43 ± 0.02	0.00	1.50 ± 0.08	0.02	1.29 ± 0.04	0.02	NS
ST5AR2	0.95 ± 0.19	0.70	1.04 ± 0.20	0.10	0.95 ± 0.16	0.71	NS
HDS17B1	0.99 ± 0.09	0.88	0.76 ± 0.08	0.11	1.06 ± 0.14	0.55	NS
ST8SIA5	1.01 ± 0.05	0.91	0.93 ± 0.06	0.34	0.82 ± 0.14	0.91	NS
HSD3B	0.71 ± 0.06	0.06	0.60 ± 0.07	0.05	0.44 ± 0.05	0.02	*
B. Duroc							
STAR	11.58 ± 0.65	0.02	16.06 ± 1.59	0.02	30.39 ± 1.98	0.01	†
CYP17A1	11.39 ± 3.61	0.09	16.40 ± 5.31	0.08	16.87 ± 6.50	0.09	NS
CYP51	3.35 ± 0.62	0.10	4.12 ± 0.81	0.09	6.06 ± 1.18	0.07	*
CYP19A1	4.86 ± 3.90	0.51	4.47 ± 3.15	0.42	8.63 ± 6.90	0.37	NS
CYB5	3.53 ± 0.89	0.13	3.12 ± 0.87	0.16	2.02 ± 0.40	0.18	NS
CYP11A1	2.20 ± 0.04	0.01	2.29 ± 0.35	0.12	2.90 ± 0.00	0.00	NS
INSL3	3.33 ± 1.05	0.18	2.46 ± 0.86	0.26	1.47 ± 0.86	0.84	NS
HSD17B4	3.36 ± 1.19	0.12	3.52 ± 0.70	0.10	4.52 ± 2.08	0.22	NS
CYP21	1.16 ± 0.11	0.37	1.56 ± 0.20	0.19	2.56 ± 0.76	0.21	*
FTL	1.46 ± 0.43	0.47	1.49 ± 0.43	0.46	1.55 ± 0.43	0.39	NS
NR5A1	1.55 ± 0.08	0.08	1.72 ± 0.54	0.37	1.83 ± 0.21	0.12	NS
AKR1C4	1.49 ± 0.38	0.40	1.49 ± 0.10	0.10	1.56 ± 0.46	0.41	NS
MGST1	1.56 ± 0.02	0.02	1.45 ± 0.03	0.04	1.70 ± 0.05	0.04	NS
ST5AR2	0.98 ± 0.02	0.57	2.05 ± 0.56	0.25	1.26 ± 0.40	0.68	NS
HSD17B1	0.90 ± 0.08	0.43	1.02 ± 0.23	0.97	0.87 ± 0.08	0.35	NS
ST8SIA5	0.84 ± 0.19	0.55	0.17 ± 0.29	0.63	0.70 ± 0.04	0.10	NS
HSD3B	0.48 ± 0.01	0.01	0.43 ± 0.02	0.03	0.49 ± 0.01	0.01	NS

Data are presented as mean fold change ± SEM (three repeats). Expression of the genes was normalized to the mean expression of *HPRT*, *PPIA*, and *ACTB*. Each exposure was performed in triplicate and carried out from three independent Leydig cell isolations. P-values were calculated from log2-transformed ΔΔC(t)-values.

* Significant (P value ≤ 0,05)

† Highly significant (P value ≤ 0,001)

4. Discussion

The present study is, to our knowledge, the first to compare hormone secretion in primary neonatal Leydig cells between pig breeds. The observed breed differences in basal steroid concentrations between Duroc and Norwegian Landrace Leydig cells indicated a larger production capacity for steroids in unstimulated Duroc cells. Before LH stimulation, a higher concentration of all measured steroids was found in the medium from Duroc Leydig cells compared with Norwegian Landrace cells. Basal testosterone levels from primary cultures of porcine Leydig cells have been reported previously [37,38], at levels which would compare with concentrations measured in the present study (Table 2). Basal androstenone levels do not seem to have been reported previously.

Differences in basal steroid concentrations between Norwegian Landrace and Duroc are also reported *in vivo*. Results from the same populations of boars as in the present study, showed higher plasma concentrations of testosterone and androstenone in Duroc boars than in Norwegian Landrace [23,25,39].

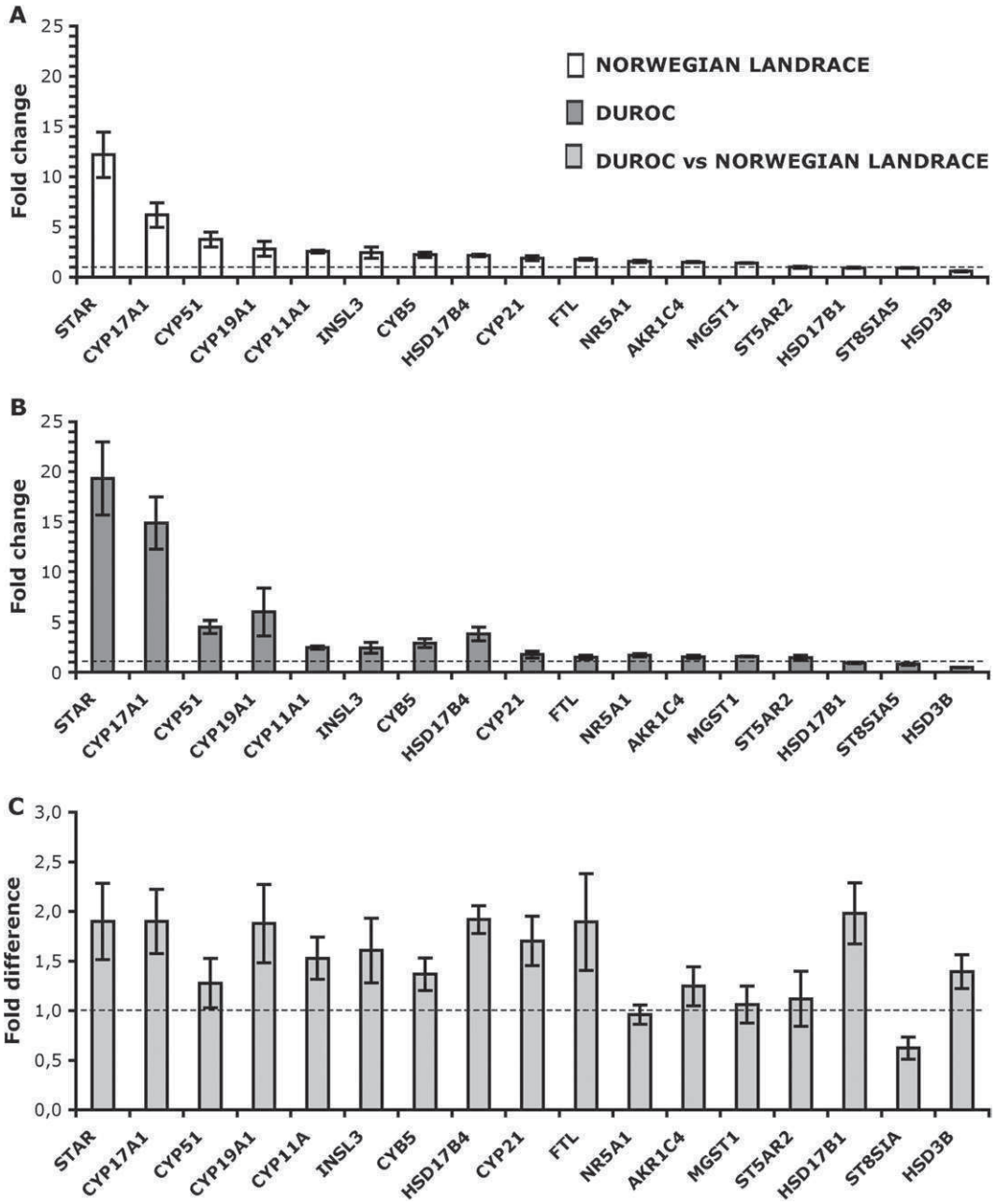

Fig. 2. The effect of LH exposure on gene expression in (A) Norwegian Landrace and (B and C) Duroc Leydig cells. The data are pooled values from three doses of LH exposure presented as mean fold change ± SEM (two replicates for Duroc and three for Norwegian Norwegian Landrace) relative to control (A and B) or to LH exposed Norwegian Landrace cells (C; set to 1, dotted line). When compared with unexposed controls the alteration in gene expression was significant in both breeds ($P \leq 0.05$) for all genes investigated except *ST5AR2*, *HSD17B1,* and *ST8SIA*. When Duroc was compared with Norwegian Landrace cells, differences in expression were significant for *STAR*, *CYP17A1*, *HSD17B4*, *CYP21*, *HSD17B1* and *ST8SIA* ($P < 0.05$).

The results demonstrated that LH exposure significantly increased steroid hormone secretion in neonatal porcine Leydig cells. Androstenone and testosterone concentrations increased 15- and 50-fold, respectively, whereas lower increments were observed for estradiol, progesterone, and cortisol.

The action of LH on steroidogenesis in Leydig cells is caused by binding and activation of the LH receptor

[40]. Previously, Leydig cells from different species, particularly in pigs, cultured *in vitro*, have demonstrated a high capacity for steroid synthesis [37,38]. These findings also concur with *in vivo* studies reporting increased plasma steroid concentrations following administration of hCG [39,41–44].

There were apparent differences in the way Leydig cells from Duroc and Norwegian Landrace responded to LH stimulation. Although absolute mean steroid concentrations were higher in Duroc cells at levels of maximal LH stimulation (i.e., the three highest LH concentrations; Fig. 1), the relative increase in hormone concentrations was significantly lower in Duroc cells for several measured steroids (estradiol, progesterone, and cortisol) when basal steroid concentrations were accounted for. The present data also suggested qualitative differences between Duroc and Norwegian Landrace in the steroid response to LH stimulation. This was particularly evident for estradiol secretion which increased at a much lower LH concentration in Duroc than in Norwegian Landrace cells (Fig 1C). Also, there was a breed difference in the relative amounts of steroids produced in response to LH, as indicated by breed differences in correlation coefficients between steroid hormone concentrations.

The fact that neonatal Leydig cells used in the present study had a high steroid production would indicate that they could possibly be used as a model for steroidogenesis in adult cells. However, further studies seem indicated to validate such a model, because in boars, neonatal and prepubertal Leydig cells do not survive into adulthood [5,45,46]. It has been documented that neonatal Leydig cells have higher amounts of LH receptors than pubertal and adult Leydig cells [1]. Also, size and volume of Leydig cells vary at different ages, affecting LH-hCG responsiveness and thereby also steroid secretion [2,5]. Furthermore, it was reported that steroid levels after hCG stimulation in prepubertal boars (30 kg body weight) could not predict steroid levels in the same individuals at puberty (85 kg body weight) [47]. However, at puberty (e.g., 75 days of age), when the same population of Leydig cells were present as in adult boars, steroid levels in older boars were successfully predicted [48].

There was a similar trend in the gene expression pattern after LH stimulation in both breeds (Table 3; Fig. 2A and B), although in general, the change in expression of genes encoding enzymes in the steroidogenesis was stronger in Duroc than in Norwegian Landrace. This is in agreement with the higher concentrations of steroid hormones measured in culture medium from the LH-stimulated Duroc Leydig cells in the present study (Fig. 1), but does not concur with the fact that the relative increase in hormone production was lower for several hormones in Duroc than in Norwegian Landrace cells.

The final enzymatic steps in estradiol production are catalyzed by either aromatase, encoded by *CYP19A1*, or hydroxysteroid (17 beta) dehydrogenase 1, encoded by *HSD17B1,* from testosterone or estrone, respectively. mRNA levels of *CYP19A1* were elevated, although not significantly, in both breeds after LH stimulation. Although not significant, there was a trend for higher levels in Duroc. At the highest LH doses (0.5 and 2.5 ng/mL), the expression of *CYP19A1* increased from around 4.5-fold to 8.6, but no increase in estradiol production was observed in this interval, which might indicate that Duroc cells had reached maximum estradiol production.

In both breeds, the expression of *STAR* was most altered by LH stimulation. After LH release the steroidogenic acute regulatory protein (STAR) mediates an acute transfer of cholesterol, which is the key substrate for all steroidogenesis, to the inner mitochondrial membrane, where the cholesterol side-chain cleavage enzyme (encoded by *CYP11A1*) resides. This enzyme catalyzes the conversion of cholesterol to pregnenolone and is regarded the first rate-limiting step of steroidogenesis. While the relative increase in mRNA levels of *STAR* was higher in Duroc cells than in Norwegian Landrace cells after LH stimulation, no significant differences in *CYP11A1* expression was seen between breeds.

After LH stimulation the transcription of the *CYP17A1* gene increased significantly in both breeds. This gene codes for cytochrome P450 17A1 (CYP17A1), an enzyme with both hydroxylase and lyase activity, which is involved in several steps in the steroidogenesis. An increase in this enzyme's activity would lead to more substrates necessary for production of cortisol, testosterone, and estradiol. In fact, it is with these three hormones we see the biggest differences in production level between breeds. Duroc cells produced higher amounts of these three hormones after LH stimulation and had a significantly higher expression of *CYP17A1* than Norwegian Landrace when combining all concentrations of LH in the comparison.

The HSD3B enzyme converts pregnenolone to progesterone. The mRNA level of *HSB3B* was significantly downregulated after LH stimulation in both breeds. However, the sequence of only one isoform of porcine *HSDB3* (*HSD3B1*) is available at present. Comparing with other species (human, mouse) our

primers correspond to several isoforms. The levels of progesterone did not decrease after LH stimulation. One possible explanation could be that there are several isoforms of *HSD3B* in porcine Leydig cells and the observed regulation of mRNA does not reflect the correct isoform responsible for the conversion of pregnenolone. Another option may be that the half-life of HSD3B is of such a length that the incubation time in this study did not affect the enzyme population. This should be investigated in future studies.

In conclusion, neonatal Duroc Leydig cells produced more hormones and altered gene expression to a higher degree than neonatal Norwegian Landrace Leydig cells after LH stimulation. The results suggested that breed differences in steroid hormone secretion and gene expression between Norwegian Landrace and Duroc Leydig cells are complex and cannot be explained by a simple mechanism of action. The hormone production plateau for testosterone, estradiol, androstenone, and cortisol, reached at higher concentrations of LH, is most likely related to mechanisms or events occurring downstream in the steroidogenesis pathway.

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Androstenone and testosterone levels and testicular morphology of Duroc boars related to estimated breeding value for androstenone

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ABSTRACT

Androstenone and testosterone levels in Duroc boars with an estimated breeding value for androstenone ($EBV_{androstenone}$) were followed in the period from birth to sexual maturity. The breeding value for androstenone had been estimated based on androstenone levels in 1202 Duroc boars at an age of 24 weeks. Testosterone and androstenone levels in plasma were recorded in 19 boars at 1 week of age and in their 15 respective litter-siblings at 3 weeks of age. Between 12 and 27 weeks of age, plasma levels were recorded weekly in a third set of 16 litter-siblings. In the last group, histomorphology was performed at 12, 16, 20, and 27 weeks of age to determine sexual maturity status. The $EBV_{androstenone}$ was positively related to plasma androstenone in animals 12 to 27 weeks of age and to plasma testosterone levels in 1- and 3-week-old animals. The $EBV_{androstenone}$ was not related to testis morphology. The concentration of fat androstenone was positively correlated to the percentage of immature seminiferous tubules and negatively correlated to the percentage of mature seminiferous tubules at 20 weeks. Testosterone in plasma showed no relationship with testis morphology. Most individuals reached puberty at 20 weeks of age, which indicates that Duroc mature later than crossbred boars. The results indicated that breeding value based on the single trait boar taint parameter $EBV_{androstenone}$ was not related to testicular development.

1. Introduction

Approximately 100 million pigs are castrated [1,2] in Europe every year (European Food Safety Authority, 2004) [3]. In Norway, a ban on castration was introduced in 2009. However, the implementation was postponed. Castration can now only be performed by veterinarians and anesthesia is mandatory. Castration is carried out to avoid the unpleasant odor (and flavor) known as boar taint, which develops when meat from some intact boars is heated. The pheromone androstenone (5α-androst-16-en-3-one) and the tryptophan metabolite skatole are the main factors responsible for this characteristic off-flavor. Androstenone is produced and secreted by the Leydig cells, together with other steroids. It is lipophilic and accumulates in adipose tissue [4,5].

There is significant testicular steroidogenic activity in newborn male pigs [6,7], and testicular steroid levels reach a peak at 2 to 4 weeks of age. From 2 to 5 months, low levels have been reported followed by marked increases in older animals [8,9]. Wide individual variation in levels of both androstenone and testosterone has been reported in pubertal boars [10,11]. There have been few studies of androstenone levels in newborn and prepubertal boars and on the correlations between levels in young and older

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animals. Early prediction of androstenone levels in mature animals could be useful in efforts to reduce the boar taint problem. Elimination of young individuals with high androstenone levels would be beneficial, especially for breeding animals.

The development of Leydig cells reaches a maximum at a few weeks postpartum. This is followed by regression and then by renewed development in the pubertal animal [8,12]. Histomorphometrical studies of testes from boars with varying fat androstenone levels showed that peripubertal fat androstenone concentration was positively associated with the number and size of Leydig cells [13]. The fraction of Leydig cells relative to other testis tissue has been found to reach a maximum proportion of four to one at 12 to 17 days of age [14]. Opposite distribution of Leydig cells of one to four is present in testis tissue of mature animals where seminiferous tubules are the predominant structures [8].

The heritability of androstenone levels in fat is high [15,16], and knowledge of the genetic component of boar taint has greatly increased in recent years [17–19]. Breeding against androstenone therefore appears to be an interesting approach to reducing the boar taint problem. However, there are indications that breeding against androstenone might affect reproductive traits negatively [20,21].

Purebred Duroc boars have gained use in commercial crossbreeds because of their desirable meat quality traits. However, the use of Duroc boars also results in undesirably high levels of androstenone. The proportion of Duroc boars with undesirably high concentrations of androstenone is considerably higher than for other purebreds at slaughter age [5,22]. Profound steroidogenic differences between Duroc and Landrace boars are also evident in neonatal Leydig cells [23], underscoring the need for more knowledge on the effect of breeding on testis development in Duroc boars.

Androstenone measurements in fat from 1202 Duroc boars from the breeding nucleus in Norway made it possible to determine the estimated breeding values for androstenone ($EBV_{androstenone}$) [16]. The aim of this study was to investigate the relationship between the $EBV_{androstenone}$ and phenotypic traits, such as steroid profiles and testicular morphology during development. The study was conducted in Duroc boars because they have high androstenone levels and thus also a very significant boar taint problem [5,22]. The possibility of a relationship between levels of androstenone and sexual maturation was also investigated. In addition, neonatal piglets were studied to find out whether hormone levels at this early age could be used to predict testosterone and/or androstenone levels at mature age. Ages well known for high steroidogenic and proliferative activity in testis [24] were chosen to gain further knowledge on possible relationships to $EBV_{androstenone}$.

2. Materials and methods

The experiment was conducted in agreement with the provisions enforced by the National Animal Research Authority.

2.1. Animals and $EBV_{androstenone}$

The study used 51 Duroc male piglets originating from 16 litters born on a Norsvin breeding nucleus farm. The litters were selected on the basis of $EBV_{androstenone}$ [16]. The breeding values for sires, dams, and their offspring were calculated based on androstenone levels measured in subcutaneous neck fat from a total of 1202 Duroc boars with extensive pedigree information. The fat samples were collected immediately after slaughter, at an average age of 170 days (24 weeks). The model used for estimation of $EBV_{androstenone}$ calculated variance components [16,25].

Three male siblings were selected randomly from each of the 16 litters at three different ages: (1) Week 1, in the first week of life, before 3 days of age and preferably on the day of birth; (2) Week 3, at 3 weeks of age; and (3) Week 5, at weaning at approximately 5 weeks of age. Siblings selected in Week 1 and Week 3 were euthanized after collection of tissues and blood. The group selected in Week 5 was transported to facilities at the Norwegian School of Veterinary Science at 8 weeks of age. From 12 to 27 weeks of age, this group of 16 boars was used in a longitudinal study of sexual development. They were kept in five 11-m^2 pens with concrete floors covered in sawdust and fed commercially.

2.2. Sampling of neonatal boars (Week 1 and Week 3)

Blood and mesenterial fat were sampled from 19 neonatal boars in Week 1 and in 15 of their litter-siblings at 3 weeks of age. Blood was collected from the cranial vena cava accessed in Vacutainers (Leuven, Belgium) containing heparin. Samples were centrifuged at 3000 rpm for 10 minutes. After separation of blood plasma, samples were stored at −20 °C until analyzed. After blood sampling, animals were premedicated by administering 80 μg/kg medetomidine (Domitor, Orion Corporation, Turku, Finland) intramuscularly.

Anesthesia was induced and maintained using sevoflurane (SevoFlo, Abbott Scandinavia AB, Sweden) vaporized in oxygen and air administered through a mask. During anesthesia, testes were surgically removed following standard procedures. After castration while still under anesthesia, the boars were euthanized by intravenous injection of saturated potassium chloride, after which mesenterial fat was collected and stored on ice (<3 hours) and subsequently transferred to storage at −20 °C.

2.3. Sampling during the longitudinal study

In the period from 12 to 27 weeks of age, blood samples were collected and body weights recorded once a week, before 11:00 AM. At four time points (12, 16, 20, and 27 weeks of age), the boars went through a surgical procedure to collect testis and fat tissues. Premedication and anesthesia were in principle as described for neonatal boars, endotracheal intubation was performed and anesthesia maintained by sevoflurane vaporized in oxygen and air. A venous and an arterial catheter were placed and a 10-mL/kg/h infusion of a balanced polyionic electrolyte solution (Ringers acetate, Fresenius Kabi, Oslo, Norway)

was administered. The boars were connected to a multiparameter anesthetic monitor (S-5; Datex-Ohmeda, Helsinki, Finland) for monitoring of end-tidal carbon dioxide and sevoflurane, arterial oxygen saturation, electrocardiography, invasive arterial blood pressure, and body temperature.

2.4. Assessment of testicular diameter

Testis diameter was measured during anesthesia. The animal was positioned in dorsal recumbency with fixed back limbs. A carpenters caliper was used to read the width at the widest horizontal axis. The maximum length of each testis was also measured.

2.5. Testis and adipose tissue

A sample of approximately 0.3 to 2.0 cm subcutaneous adipose tissue was surgically removed from the neck area and the incision closed with 0:2 Ethicon (Johnson & Johnson, Lifescan Norway) and wound enclosure silicon spray (Carex Health Brands, Sioux Falls, SD, USA). Tissue samples were processed as described for the neonatal piglets.

Fifteen animals were included for histological evaluation of testis development and spermatogenic activity. Testis tissue biopsies were collected from each animal at 12, 16, 20, and 27 weeks of age. The scrotum was surgically prepared. After a 0.5-cm incision through the scrotum and tunica vaginalis, biopsies were collected with a 14-gauge 9-cm needle (Quick-Core; Cook, Bjaeverskov, Denmark) inserted 2.5 cm into the testicular parenchyma in various angles to the longitudinal axis of the testis.

After the surgery was complete, still during anesthesia, 45,000 IU procaine penicillin (Penovet; Boehringer Ingelheim, Copenhagen, Denmark) and 3 mg/kg ketoprofen (Romefen; Merial SAS, Lyon, France) was given intramuscularly. At the end of the session, 40 μg/kg atipamezolhydrochloride (Antisedan; Orion Corporation, Turku, Finland) was administered intravenously and 40 μg/kg intramuscularly to reverse the pharmacological effects of medetomidine and shorten the postoperative convalescence. At the end of the last session of the longitudinal experiment (27 weeks), the boars were euthanized as described for the neonatal boars.

2.6. Steroid analyses

Testosterone concentrations in plasma were determined using a solid-phase radioimmunoassay kit, Coat-a-Count, Total Testosterone (Siemens Medical Solutions Diagnostic Europe Ltd., Eschborn, Germany), according to the manufacturer's instructions. The standard curve ranged from 0 to 6 ng/mL. Assay sensitivity was 0.1 ng/mL, corresponding to 95% binding of the labeled hormone. Interassay coefficients of variation were 3.5% (6.3 ng/mL) and 7.5% (11.9 ng/mL).

Androstenone concentrations in plasma were measured by a time-resolved fluoroimmunoassay for serum samples as described by Tuomola et al. [26], modified by replacing the standard curve with standards diluted in zero androstenone plasma and by using antiserum produced and characterized by Andresen [27]. The standard curve ranged from 0 to 100 ng/mL. Assay sensitivity was 2 ng/mL, corresponding to 95% binding of the labeled hormone. Interassay coefficients of variation were 10.8% (3.8 ng/mL) and 3.5% (28.6 ng/mL).

Fat androstenone was measured by time-resolved fluoroimmunoassay as described by Tuomola et al. [26], modified by using antiserum produced and characterized by Andresen [27]. The standard curve ranged from 0.1 μg/g to 10 μg/g androstenone fat. Assay sensitivity was 0.04 μg/g, corresponding to 95% binding of the labeled hormone. Interassay coefficients of variation for samples containing 0.27, 0.63, and 3.36 μg/g androstenone fat were 10.4%, 6.7%, and 7.6%, respectively. Specificity of assay was assessed by measuring cross-reactivity of related hormones. Accuracy was evaluated by linearity spiking of serial dilutions of fat samples with known concentrations.

2.7. Histological evaluation of testes

Testis tissue sections of 6 μm were fixed with Bouin's solution, prepared and stained with hematoxylin and eosin. Samples were scored as described by Oskam et al. [28] with some modifications (Table 1). Each tissue section was scored with respect to the general structure of the testis and the presence of lumina and of round and elongating spermatids. The specific types of germ cells were identified according to their morphological characteristics. If possible, 150 randomly selected seminiferous tubules per section were studied by light microscopy (magnification, 250-fold) and scored according to the criteria summarized in Table 1. Eighteen sections contained fewer than 150 tubules, and in these cases all the tubules were scored. Briefly, tissue sections with disorganized epithelium and little or no evidence of lumen formation were scored as category I, and sections in which more than 50% of the tubules contained two generations of spermatogonia and primarily round spermatids as category II. Sections in which more than 50% of the tubules showed a full spermatogenetic cycle were scored as category III. The frequencies of the three categories in each section were used to classify the animal's state of sexual maturity.

If more than 90% of the seminiferous tubules in a section were scored as category I and/or less than 10% of them were scored as category III, the animal was characterized as immature. If more than 20% of the seminiferous tubules were scored as category II and/or less than 70% as category III, the animal was considered to be transitional. Mature animals were those with more than 80% of tubules in category III.

2.8. Statistical analyses

Statistical analyses were performed using JMP 9.0 software (SAS Institute, Inc., Cary, NC, USA). Because individual animals were sampled repeatedly between 12 and 27 weeks of age and not independent, the areas under the androstenone and testosterone curves, androstenone (AUC) and testosterone (AUC), respectively, were estimated according to the trapezium rule (http://en.wikipedia.org/wiki/Trapezoidal_rule). Samples collected in the neonatal period (Week 1 and Week 3) consisted of, respectively, 13

Table 1
Criteria used to characterize testicular spermatogenesis and stages of sexual maturation by histological evaluation.

Category	Criteria to quantify the level of spermatogenesis in seminiferous tubules in entire male pigs
I	Disorganized germinal epithelium with a closed and/or hardly open visible lumen. The presence of spermatogonia and only a few (<10%) primary spermatocytes.
II	Organized germinal epithelium with a visible open lumen and the presence of spermatogonia, two generations of spermatocytes, and one generation of spermatids; round spermatids.
III	The presence of spermatogonia, two generations of spermatocytes, and two generations of spermatids; round spermatids and elongating and/or elongated spermatids.
Sexual stage	**Histological evaluation**
Immature	>90% of the seminiferous tubules scored as category I and/or < 10% scored as category III
Transitional period	>20% of the seminiferous tubules scored as category II and/or < 70% scored as category III
Mature	>80% of the seminiferous tubules scored as category III

and 12 single individual samples, and samples collected from Week 12 to Week 27 were repeated samples from a group of 16 individuals.

Generalized linear models were established for the outcome variables: plasma testosterone and androstenone at 1 and 3 weeks of age, androstenone(AUC), and testosterone(AUC). The following explanatory variables and interactions were investigated: plasma androstenone and testosterone, week of age, androstenone(AUC), testosterone(AUC), body weight, testicular diameter, and $EBV_{androstenone}$. When testosterone was the outcome variable, androstenone was used as an explanatory variable and *vice versa*. Variables with a P value <0.10 were maintained in the final models. The $EBV_{androstenone}$ estimates were used as explanatory variables for the variation in androstenone(AUC) and testosterone(AUC) (Week 12–27) and for neonatal plasma androstenone and testosterone (Week 1 and 3).

The variables androstenone(AUC), testosterone(AUC), and fat androstenone (12–27 weeks of age) were log-transformed before analysis to obtain a satisfactory fit to the normal distribution (Shapiro–Wilk test).

Boars were categorized as high or low phenotype according to their fat androstenone concentration at 27 weeks of age. A discriminatory concentration greater than 3.5 μg/g and less than 3.0 μg/g was used.

Correlation analysis (Spearman's correlation coefficient, r_s) was used to investigate the relationship between studied end points. P values ≤0.05 were regarded as statistically significant.

3. Results

There was a significant difference in mean (SEM) plasma androstenone from 13.3 (2.0) ng/mL in piglets sampled at birth to 25.6 (2.8) ng/mL and in their siblings sampled at 3 weeks ($P < 0.002$). Similarly, mean fat androstenone concentration increased from 0.06 (0.02) to 2.8 (0.4) μg/g ($P < 0.001$). Mean plasma testosterone decreased in the same period from 2.6 (0.2) to 1.5 (0.2) ng/mL ($P < 0.001$; Fig. 1).

From 12 to 27 weeks, mean (SEM) live weight increased from 36.6 kg (1.3) to 126.2 kg (5.4). In this period there was a linear relationship between body weight and week of age (body weight [kg] = 6.8 [week of age] − 51.2; $R^2 = 0.94$; $P < 0.001$). Mean (SEM) testicular diameter increased from 33.8 mm (0.9) to 70.7 mm (4.2) in this period. Testicular diameter also increased linearly with age (testicular diameter [mm] = 2.8 [week of age] + 1.1; $R^2 = 0.87$; $P < 0.001$).

In the period between 12 and 27 weeks of age, the mean of androstenone and testosterone concentrations in plasma increased with age. There was considerable individual variation in measured concentrations of both steroids (Fig. 2). Individuals ranging at high levels of androstenone contributed more to the mean level than the average shown by the median steroid concentrations lower than the mean values for the whole study period. When week of age was taken into account, live body weight and testicular diameter had no significant effect on measured steroid

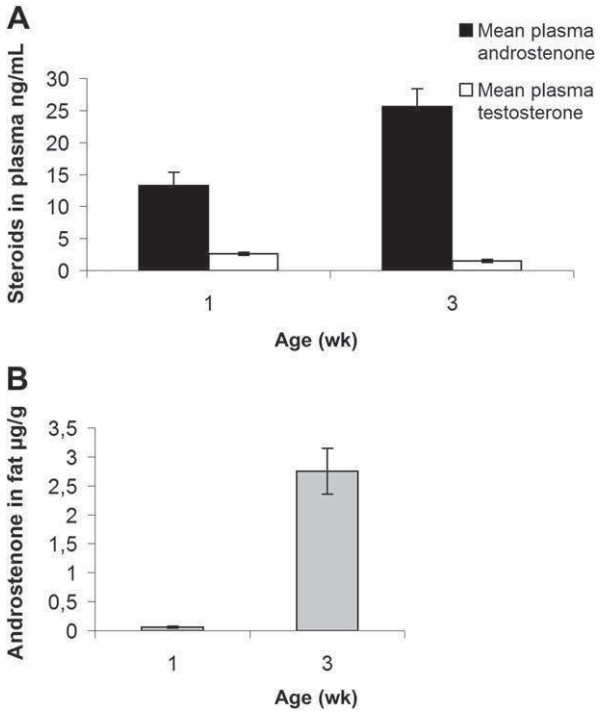

Fig. 1. (A) Mean (± SEM) plasma androstenone and testosterone levels in 19 newborn Duroc boars (Week 1) and in 15 of their litter-siblings at 3 weeks of age (Week 3). (B) The mean (± SEM) mesenterial fat androstenone in eight newborn Duroc boars (Week 1) and in 15 of their litter-siblings at 3 weeks of age (Week 3).

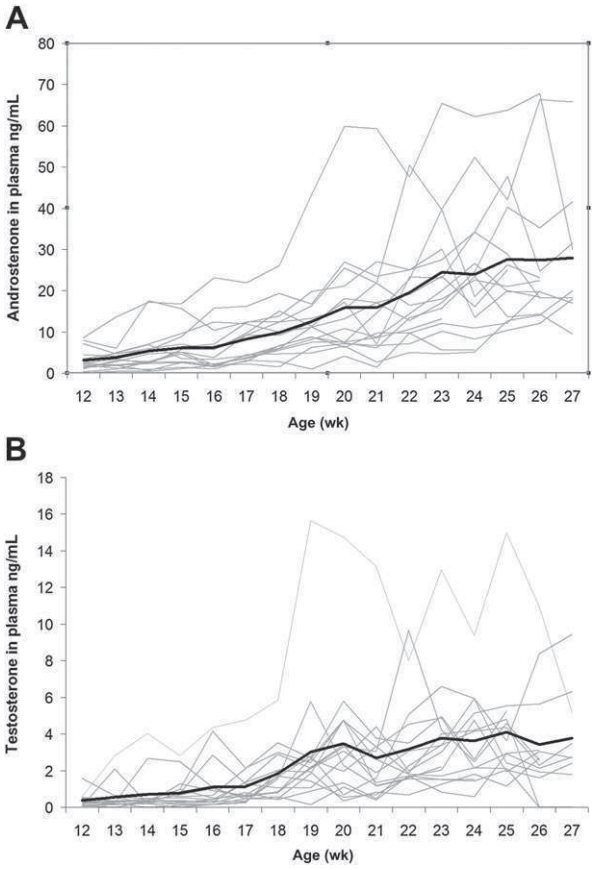

Fig. 2. Individual plasma concentrations of androstenone (A) and testosterone (B) in 16 Duroc boars. Bold solid line shows the mean concentrations.

concentrations or on testosterone log(AUC) and androstenone log(AUC).

In neonatal piglets, the models explained 47% and 41% of the variation in plasma testosterone and androstenone, respectively (Table 2). The models predicted a significant positive relationship between $EBV_{androstenone}$ and plasma testosterone, but not between $EBV_{androstenone}$ and plasma androstenone. Week of age contributed most to the variation in neonatal plasma androstenone and testosterone, but testosterone also made a significant positive contribution to androstenone variation, and *vice versa* (Table 2).

Within weeks, except in Weeks 1 and 19, there was a significant positive correlation between plasma androstenone and testosterone. The correlation coefficients ranged between 0.45 and 0.95, and were the highest between 21 and 25 weeks of age. The mean (SEM) fat concentrations of androstenone in Week 12, 16, 20, and 27 are shown in Figure 3. There was significant positive correlation between plasma androstenone and fat androstenone, with correlation coefficients in the same range as for plasma androstenone and testosterone.

For samples collected between 11 and 27 weeks of age, the outcome variable androstenone log(AUC) was significantly positively influenced by testosterone log(AUC) and $EBV_{androstenone}$ (Table 2). A similar model with testosterone log(AUC) as the dependent variable, but with androstenone log(AUC) and $EBV_{androstenone}$ as explanatory variables, explained less variation (48% vs. 87%, respectively) and there was no significant contribution from $EBV_{androstenone}$ (Table 2).

When the 27-week-old boars were separated into high and low phenotypes (>3.5 μg/g = high fat androstenone; <3.0 μg/g = low fat androstenone), differences in the weekly recorded mean plasma androstenone and testosterone could be traced back in time to Weeks 12 (androstenone) and 18 (testosterone), respectively (Fig. 4).

3.1. Changes in germinal epithelium between the four time points investigated (12, 16, 20, and 27 weeks)

Most individuals became sexually mature between 12 and 27 weeks of age (Fig. 5). At 12 weeks of age, all individuals were immature, and 4 weeks later three of 14 animals had reached the transitional stage and the rest were still immature. At 20 weeks of age, 11 individuals were categorized as transitional, only two were still immature, and one was sexually mature. At 27 weeks of age, 12 animals were mature, one was transitional, and one was intermediate between transitional and mature. There was a marked decrease in the percentage of category I seminiferous tubules and an increase in the percentage of category III tubules with increasing age. The percentage of category II tubuli peaked at 20 weeks. Figure 5 shows typical histological views of the three different maturation stages: immature, transitional, and mature.

There was a significant negative relationship between fat androstenone and sexual maturation at 20 weeks of age. This was indicated by a significant positive correlation between fat androstenone and the percentage of category I tubules (r_s = 0.67; P = 0.01) and a negative correlation between fat androstenone and the percentage of category III tubules (r_s= 0.61; P = 0.02). The $EBV_{androstenone}$ and plasma testosterone were not significantly correlated with testicular morphology.

4. Discussion

The $EBV_{androstenone}$ [16] was related to plasma testosterone levels in the neonatal Duroc siblings, but not to plasma androstenone levels. The relationship between the $EBV_{androstenone}$ and testosterone was strong in the newborn piglets and in their 3-week-old siblings. This relationship indicates that breeding to reduce androstenone levels on the basis of the $EBV_{androstenone}$ could influence neonatal testosterone levels in Duroc boars. Another possible outcome could be that testosterone-mediated functions would be affected.

Higher testosterone levels in boars sampled at Week 1 than in their siblings in Week 3 has not been found in previous studies, but different study designs must be taken into consideration [6,29,30]. In the present study siblings were sampled, and in previous studies, the same individuals had been sampled repeatedly.

The neonatal increase in plasma androstenone was associated with a substantial increase in fat androstenone levels. The physiological effect of this increase is intriguing and could be involved in pheromonic actions and social

Table 2
Parameter estimates with standard errors (SE) and ANOVA tables with sums of squares (SS), degrees of freedom (*df*), for regression models explaining variation in the dependent variables plasma testosterone and androstenone (upper panel) and in the area under the curve (AUC) for androstenone and testosterone (lower panel).

Response	Term	*df*	Estimate	SE	SS	R^2-model	P
Weeks of age: 1 and 3							
Plasma testosterone, ng/mL	Intercept		1.51	0.33		0.47	<0.0001[a]
	Plasma androstenone, ng/mL	1	0.04	0.01	4.14		0.0196[a]
	Week of age	1	0.77	0.17	14.21		<0.0001[a]
	$EBV_{androstenone}$	1	0.90	0.41	3.31		0.0351[a]
	Residual	30			20.41		
Plasma androstenone, ng/mL	Intercept		9.54	4.53		0.41	0.0436[a]
	Plasma testosterone, ng/mL		4.61	1.87	521		0.0196[a]
	Week of age		−8.65	1.90	1779		<0.0001[a]
	$EBV_{androstenone}$		−2.48	4.90	22		0.617
	Residual	30	—	—	2567		
Weeks of age: 12 to 27							
Androstenone, log(AUC)	Intercept		2.66	0.66	—	0.87	0.0014[a]
	Testosterone, log(AUC)	1	0.85	0.12	3.71		<0.0001[a]
	$EBV_{androstenone}$	1	0.56	0.22	0.49		0.0245[a]
	Residual	13	—	—	0.97		
Testosterone, log(AUC)	Intercept		4.44	0.39	—	0.48	<0.0001[a]
	Androstenone, log(AUC)	1	0.55	0.20	5.10		0.0184[a]
	$EBV_{androstenone}$	1	0.12	0.44	0.02		0.7937
	Residual	13	—	—	1.22		

[a] Independent variables were estimated breeding value ($EBV_{androstenone}$), week of age, plasma androstenone or testosterone in newborn piglet blood, sampled either at birth (Week 1) or at 3 weeks of age (upper panel), or $EBV_{androstenone}$ and log transformed (log(AUC)) for plasma androstenone or plasma testosterone in piglet blood sampled once weekly between 12 and 27 weeks of age (lower panel).

interplay between siblings and or parents. Further research must clarify possible behavioral responses of androstenone in the young piglet.

There was little evidence of a direct link between neonatal and pubertal androstenone levels in the present data. This finding agrees with the results of other studies, in which early neonatal and prepubertal testicular steroid secretion were compared after treatment with chorionic gonadotrophin [31,32]. The large variation between individuals and variability over time for levels of androstenone [10,33] and testosterone [6,30] was in accordance with previous studies.

However, when the 27-week-old boars were separated into high and low phenotypes, differences in mean plasma androstenone could be traced back in time to Week 12. This might indicate that the $EBV_{androstenone}$ mirrors high and low prepubertal phenotypes and the $EBV_{androstenone}$ does influence the phenotype more than environmental factors.

The high levels of testosterone in plasma in Week 1 were not associated with plasma androstenone. The opposite was found in pubertal boars, with testosterone as a strong predictor for androstenone secretion (AUC estimates). The effects of gonadotrophins might contribute to these differences. Differences between the testicular sensitivity of neonatal and adult boars to luteinizing hormone (LH) have been found [9]. Additionally, França et al. (2000) recorded higher levels of follicle stimulating hormone (FSH) in plasma in the first week of age than at puberty [8]. A stronger relationship between prepubertal androstenone and testosterone levels in plasma was indicated in the older animals. The weaker relationship between plasma levels of androstenone and testosterone before sexual maturation might be explained by the great variation in plasma testosterone concentrations before puberty [11].

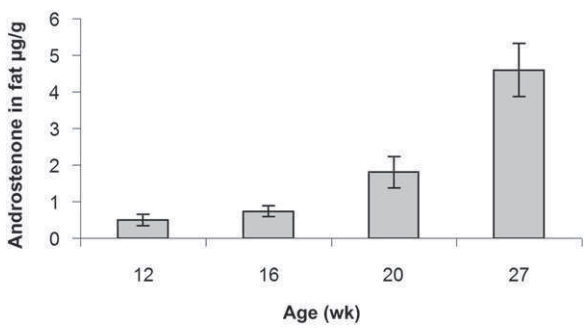

Fig. 3. Mean (± SEM) fat androstenone concentration in 16 pubertal Duroc boars.

As expected, a strong relationship between plasma androstenone and the $EBV_{androstenone}$ was found at approximately the age of puberty, because the $EBV_{androstenone}$ is based on measurements at slaughter (100 kg dressed weight), at approximately 24 weeks of age. However, most of the samples from the 16 boars in the present study were collected repeatedly, before Week 24, at Weeks 12, 16, and 20. The indication is that there might be a potential for prepubertal prediction of adult androstenone concentrations.

4.1. Development of testis morphology

In this study, most individuals were classified as transitional at 20 weeks of age, indicating that they had reached the onset of puberty, and most were fully mature by 27 weeks of age. Clear breed differences in testicular maturation and onset of puberty have been reported. These are explained by a combination of factors, including genetic variation. In crossbred pigs, Landrace-Duroc × Landrace-Yorkshire of

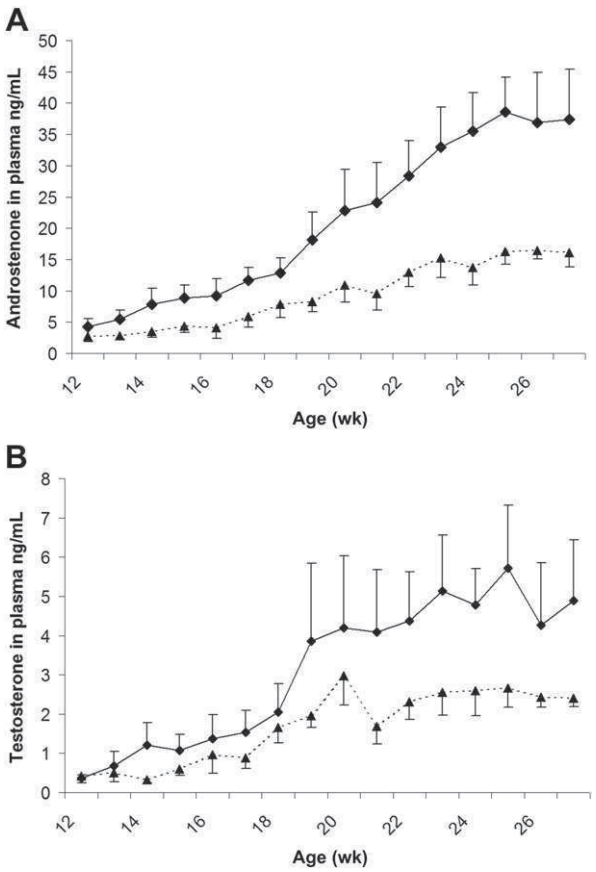

Fig. 4. Mean (± SEM) plasma androstenone (A) and plasma testosterone (B) from high phenotype (fat androstenone >3.5 μg/g; squares) and low phenotype (fat androstenone <3.0 μg/g; triangles) at 27 weeks of age.

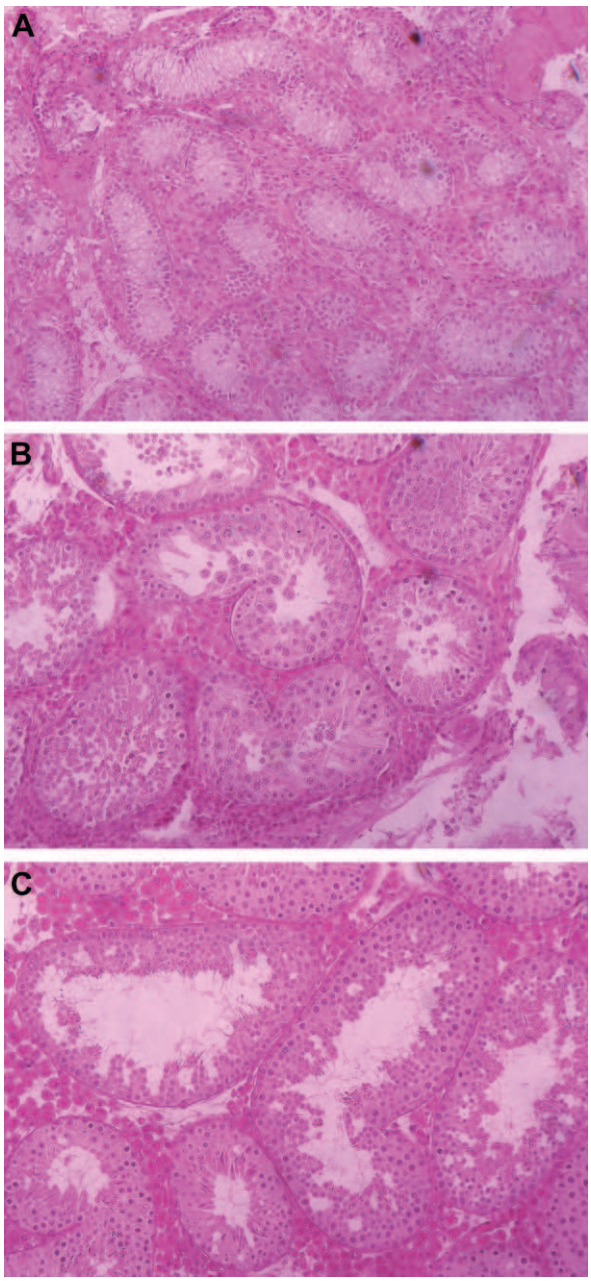

Fig. 5. Photomicrographs of cross-sections of seminiferous tubules at different maturation stages (A) immature, (B) transitional, and (C) mature.

125 to 146 days of age, the percentages of animals characterized as immature, transitional, and fully mature were 18%, 42%, and 40% respectively [28], which indicates earlier maturation of the testes than in the present studies. Very early testicular development has been reported in Chinese Meishan boars, with the first spermatozoa present in the tubules as early as 75 days of age and morphologically normal spermatozoa at approximately 120 days [34]. França et al. (2000) followed Sertoli cell, germ cell, and Leydig cell proliferation from birth to adulthood in Piau pigs and correlated this with plasma levels of testosterone. They showed a dramatic increase in germ cells per cross-section of seminiferous tubules from 120 to 150 days of age, simultaneously with a substantial increase in testosterone level, indicating the onset of puberty [8]. In crossbred Landrace × Duroc boars, Allrich et al. (1983) reported a marked increase in volume and diameter of seminiferous tubules, indicating the onset of puberty, at between 100 and 130 days [35].

No correlation between testis morphology and the $EBV_{androstenone}$ was found in the present study. This might indicate that breeding for lower levels of androstenone in Duroc boars does not influence sexual maturation. Nevertheless, the negative relationship between fat androstenone and morphology at 20 weeks of age indicates a link between androstenone and development of the seminiferous tubules. In other studies, no relationship has been reported between fat androstenone and morphology during sexual maturation [13,34]. Several authors have pointed out that breeding for low androstenone could result in problems because of a delayed onset of puberty in female offspring [20,21].

There was no correlation between measures of testis morphology and testosterone in plasma. Testosterone levels increased mainly from Week 13 to 18 and were more stable in Week 19 to 27 which is in accordance with previous reports [6,30]. The same time period for increased

levels of testosterone and onset of maturation might indicate an association, even though this was not supported by the present data.

4.2. Conclusions

The results suggest a strong genetic influence on androstenone concentrations in Duroc boars and indicate that environmental factors have less effect on the androstenone phenotype. The drive from the $EBV_{androstenone}$ as an internal and not an environmental factor was further supported by the presented curves of high and low phenotypes categorized by the $EBV_{androstenone}$. These data indicate that breeding to reduce androstenone levels on the basis of the pubertal $EBV_{androstenone}$ might alter testosterone levels in newborn boars. This could in turn influence testosterone-mediated functions such as development and differentiation of reproductive tissue and secondary sexual phenotype. Despite the strong relationship between the $EBV_{androstenone}$ and androstenone phenotype, there was no significant relationship with testicular morphology.

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Gene expression during testis development in Duroc boars

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Running title:

Gene expression in pubertal boar testes

Abstract

Androstenone is a steroid pheromone occurring in the pubertal Leydig cells. Breeding against androstenone can decrease pheromone odour in swine meat but appears to cause unwanted side effects such as delayed onset of puberty. To study causality, global gene expression in developing boar testes at 12, 16, 20 and 27 weeks was investigated using a porcine cDNA microarray. A previous publication describes the morphological status and androgenic levels in the same individuals. For this paper, associations between pathways of expressed genes were studied in the boar testes. Possible interactions with the known morphological development status, age and increased levels of steroids are discussed.

Nine clusters of genes with significant differential expression were found in the analysed testis samples. DAVID® pathway analysis identified 49 functional charts. In prepubertal testis tissue renewal and cell respiration were prominent together with indications of increased endocytocis possibly associated with the EGFR-pathway substrate *EPS15* and Sortilin. E-cadherines might be associated to onset of pubertal development in the boar testis. Genes coding for $Ca^{(2+)}$ channel occurred at prepuberty. Together with expression of cAMP-dependent protein kinase regulators *PRKAR1A*, *PRKAR2B* and the PPAR pathway regulating lipid metabolism - prepubertal activity was indicated.

At common pubertal timing, genes coding for neuro steroids and pathways that could be associated to nerve development occurred concomitantly with increased steroidogenesis and histomorphological maturation. Genes in redox pathways also changed. This suggests that there is a developmental-specific period of neuromorphogenesis.

Increased expression of meiotic pathways agreed with onset of puberty. With elevated steroidogenesis (week 16-27), there was an increase in the expression of genes in MAPK-pathway, *STAR* and its analogue *STARD6.* Several growth factors were found increasing differently towards the mature testis as follows. *TGFB2* showed pubertal increase and may also be influenced by the common *FSTL3 increase* at weeks 16-27. Later, *IFNG* expression increased at week 20-27. Further on towards mature testis - *PDGFA* and the receptor *TGFBR2* increased. *GHRH* also increased towards the mature testis however remained lower expressed than the reference. Interaction between MAPK, *STAR* and growth factors might be suggested.

In conclusion, pathways for neurogenesis, morphological pathways and several transcripts for growth factors, with known modulating effects on steroidogenesis and gonadotropins in humans and rodents, act at specific ages and developmental stages in the boar testis. The age dependency and complexity shown for development-specific testis transcripts should be considered in marker-assisted selection and when parameters for breeding values are established.

Keywords

Boar testis, testis development, pubertal, gene expression, pathway analysis,

Implications

High heritability for the boar off-odour androstenone opens for breeding towards lower levels. Breeding will first be successful when side effects of breeding as delayed sexual maturation are understood. This analysis of global gene expression indicates that stages of the developing testis should be specifically addressed. Specific stages of puberty showed expression of complex morphological signals and movements. Sampling for breeding against androstenone at slaughter (100 kg) can limit to the specific gene expression state of sexual maturation at this age. It can be suggested to include more stages of pubertal development in future breeding studies for lower androstenone levels.

Introduction

Testicular growth and development is a complex and strictly regulated process. The hypothalamic-pituitary-gonadal axis stimulates testis development and testicular steroidogenesis in a cascade of precisely timed events (Lejeune *et al.*, 1998). In the prepubertal boar, the testis remains morphologically silent until the animal is approximately 12 weeks old. Thereafter pubertal cell proliferative movements begin. Early stages of germ cells and interstitial cells develop from week 13 to week 16 (Franca *et al.*, 2000).

Maturation of testis cells is supported by complex interactions between the seminiferous tubules, Sertoli cells, germ cells, Leydig cells and their receptors (Avelar *et al.*, 2010, Revelli *et al.*, 1998). After the establishment of adequate populations of interstitial Sertoli and Leydig cells, a marked increase in steroid secretion can be expected around 16-18 weeks of age (Florcruz and Lapwood, 1978). Moreover, steroids stimulate the onset and maintenance of germ cell differentiation and maturation of first spermatids week 18-24 of age (Franca *et al.*, 2005, Oskam *et al.*, 2008). This development results from the interplay between gonadotropins and steroidogenic actions. Growth factors are also involved (Khan *et al.*, 1992, Le Roy *et al.*, 1999). However, most investigations of the role of growth factors in testis development have been performed *in vitro* on rodent testis samples. Their conclusions are not necessarily valid for boar testes (Klobucar *et al.*, 2003). Little information is available on the impact of growth factors on the development of the boar testes.

The onset of puberty in the boar is linked to the differentiation and expansion of the seminiferous tubules. A wave of Sertoli cell development and proliferation coincides with expansion of the seminiferous tubules (Franca *et al.*, 2000) involving

growth factor signals and complex interstitial cell to cell communication (Mendis-Handagama and Siril Ariyaratne, 2005, Vihko and Huhtaniemi, 1989). The differentiation status of the tubules is correlated to testis size, testis weight and body weight (Schinckel *et al.*, 1984). Though, the detailed interplay between growth and onset of puberty is not well understood.

So far, descriptions of molecular functions during the pubertal development of the boar testis are rare. Analysis of global gene expression during different stages of boar testis development might improve our understanding of interactions at molecular level during puberty.

The aim of this study was to investigate the molecular processes in the developing boar testis. It included analysis of differentially regulated transcripts expressed during the pubertal development of testes with known morphological development status.

Analysis of global gene expression was followed by cluster and pathway analysis based on transcripts differentially regulated at four time points with known androgen levels and morphological maturation status during puberty.

Material and methods

The experiment was conducted in agreement with the provisions enforced by the Norwegian Animal Research Authority.

Animals and samples

The study used n=16 Duroc boars from a Norsvin breeding nucleus farm. Selection of animals, management and sampling have been described previously

(Lervik *et al.*, 2013). The litters were selected based on estimated breeding values for androstenone in subcutaneous fat at 24 weeks.

Individual records of testosterone and androstenone in plasma and androstenone in fat at four time points (12, 16, 20 and 27 weeks of age) are available together with the expression data in the NCBI Gene Expression Omnibus (GEO) database [www.ncbi.nlm.nih.gov/geo] (Edgar *et al.*, 2002) with the accession number GSE39322 (array 39 to array 99). In Supplementary Table S1 GSM links to the individual GEO data is given. More data, including mean androgen levels, has been published previously (Lervik et al., 2013).

The boars went through a surgical procedure to collect testis tissue at four time points (12, 16, 20 and 27 weeks of age). Eleven samples were processed from week 12 biopsies. Thereafter fifteen samples were processed at weeks 16, 20 and 27. At the end of the last surgery (week 27), the boars were sacrificed after the sampling procedure was completed. The protocol for anaesthesia, sampling and evaluation of morphological development has been described previously.

Testis tissue was sampled by needle biopsy. The scrotum was surgically prepared. A 0.5 cm incision was made through the scrotum and tunica vaginalis, and biopsies were collected with a 14 gauge 9 cm needle (Quick-CoreRCook, Bjaeverskov, Denmark) inserted 2.5 cm into the testicular parenchyma at various angles to the longitudinal axis of the testis. The testis tissue was snap frozen in liquid nitrogen and stored at –70° C.

Criteria for categorization of testis maturation between 12, 16, 20 and 27 weeks

Detailed method description of morphological criteria for sexual maturation status has been given previously (Lervik et al., 2013). Individual maturation status at four time points (12, 16, 20 and 27 weeks of age) is given in Figure 1.

Isolation of mRNA

Frozen individual testis tissue samples were placed in phenol/guanidine isothiocyanate (TRIzol® Invitrogen) and homogenized with a mixer mill (Retsch NM300, Haan, Germany) using 5 mm diameter tungsten beads. Total RNA was extracted from the homogenates using RNeasy 96 Universal Tissue Kit (Qiagen Ltd., Crawley, West Sussex, UK) according to the producer's protocol. The RNA was eluted and stored in RNase free water (Qiagen) at -78 °C.
RNA quantity was measured using a NanoDrop ND-1000 spectrophotometer (Thermo Fischer Scientific, Wyman St Waltham, USA) and RNA quality was evaluated using Agilent RNA 6000 Nano LabChip on the Agilent 2100 Bioanalyzer (Agilent Technologies Inc, Santa Clara CA USA). DNase treatment was not performed since a test treatment revealed no difference in quality or quantity between DNase-treated and untreated RNA.

DNA microarrays

A porcine cDNA microarray with 26,877 EST fragments printed in duplicate (accession no. GPL6173 in the NCBI Gene Expression Omnibus (GEO) [www.ncbi.nlm.nih.gov/geo] was used for the expression analyses, with dual channel detection.

A total of 56 arrays were used in a common reference design, in which each sample was co-hybridized with a common reference sample. The reference material

consisted of pooled RNA from testis tissue samples from a total of n=40 Duroc animals aged between 0 and 27 weeks old (siblings of the test animals) that displayed below-average values for androstenone, in addition to one unrelated Duroc boar with a low skatol level, sampled at 69 kg live weight.

Alexa Fluor-labelled cDNA was synthesized from 10-20 µg of total-RNA using SuperScript Indirect cDNA Labelling System (Invitrogen Corporation, Carlsbad, CA, USA). The reference samples were labelled with Alexa-555, purified, mixed and divided into aliquots. The individual samples were labelled with Alexa-647. Subsequently each of the labelled samples was co-hybridized with an aliquot of the labelled reference sample and a hybridization blocker consisting of polydA and Yeast tRNA (Invitrogen) to porcine cDNA microarrays using a Discovery XT hybridization station (Ventana Discovery Systems /Roche Tissue diagnostics, Tucson, Arizona, USA).

Following hybridization, washing and drying, the slides were scanned in a ScanArray Express HT system (version 3.0, PerkinElmer, Inc. Massachusetts, USA) and the resulting images were analysed using GenePix Pro (version 6.0.1.27, Molecular Devices).

The expression data and additional details of the array experiments have been deposited in the GEO database [www.ncbi.nlm.nih.gov/geo] with the accession number GSE39322. Probes were annotated in April 2014 by BLASTN against the nr Entrez databases of human (HSA) and of domestic pig (*Sus scrofa* – SSC gene sequences. For mapped entries, the different NCBI gene IDs were fed to Ingenuity software (Ingenuity Systems Inc., Redwood City, CA) from where the official gene symbols were used as abbreviations. Official abbreviations of HSA and SSC gene names were also given from blast result.

Statistical analysis of the microarray data

Pre-processing, normalization and statistical analysis were carried out in the R computing environment (version 2.4.1 for Windows, REF:www.R-project.org) using the Linear Models for Microarray Analysis packages (Limma, version 2.16.4) (Smyth, 2004), which is part of the Bioconductor project (Gentleman *et al.*, 2004). Log_2 transformed ratios of Alexa-647 to Alexa-555 (not background corrected) were normalized within-slide using the printtip-loess method with default parameters and normalized between arrays using the scale method in Limma (Smyth and Speed, 2003).

A regression model was used to identify potentially significant differential regulation of genes over time using FDR-adjusted $P<0.05$. ANOVA analysis, both univariate and multivariate, was performed based on two variables: age (four levels = [ages]) and androstenone phenotype (two levels: high and low = [androstenone]). The P-values were adjusted by false discovery rate (FDR) (Benjamini and Hochberg, 1995). Arrays from n=3 individuals listed in GEO Accession: GSE39322 were excluded from the ANOVA analysis due to skeletal disorder (numbers=54, 70, 82, 98), melanoma (numbers=60, 76, 93) and testis abnormality (numbers=64, 80, 86, 100).

The identified genes with significant different change in expression over time [ages], and by mixed model [ages and androstenone] by ANOVA were hierarchically clustered in Cluster (Gene Cluster 3.0) by average linkage and modified Pearson equation for uncentred correlation similarity metric. For graphical display TreeView (Eisen Lab, Berkeley, CA, USA http://rana.lbl.gov/manuals/ClusterTreeView.pdf) (Eisen *et al.*, 1998) was used conveying tree clustering of the rows of genes grouped

together with a mathematical similarity of changes in regulation. By visual inspection of the ThreeView results the genes were grouped into nine clusters. The genes from these nine gene clusters were analysed for functional networks and pathways using DAVID (Database for Annotation, Visualization and Integrated Discovery) v. 6.7 (Huang *et al.*, 2009a, Huang *et al.*, 2009b). Functional annotations to biochemical pathways were selected as Kyoto Encyclopedia of Genes and Genomes (KEGG, Kanehisa Laboratories, Kyoto University, University of Tokyo, Japan) in order to reveal the most represented pathways in the clusters. The significance level of the annotation analyses was set to $P<0.1$.

Since pathway analysis tools for livestock are practically non-existent, pathway analysis was based on database resources developed for humans and based on human gene IDs.

In addition to cluster and pathway analysis, a regression model was used to search for possible association between androstenone levels, and the mean gene regulation slopes over time for each of the functional clusters selected for further study. FDR adjusted P-values were calculated.

An extra analysis was carried out for genes possibly related to phenotypic androstenone levels. For this, samples were ranked according to the androstenone levels in fat measured in the sampled animals at 27 weeks of age.

Results

Histomorphological developmental status of the testes is given in Figure 1. Individual androgen levels at the timepoints of sampling are listed in GEO Accession: GSE39322. Direct link to individual data is also given as GSM links in Supplementary Table S1. More data on the boars can be found in Lervik et al., (2013). All testes

evaluated for this study were immature at 12 weeks. At 16 weeks of age, transitional stage of maturation was found in 3 of 12 morphology slices and the other boars testes were immature. When boars were 20 weeks old, 11 (of 12) testes had developed to transitional maturation status. At the end of the study, with 27 weeks, 7 testis were mature, 4 (of 12) were very close to mature and 1 testis had remained in sexual transitional stage.

The ANOVA analysis of all 26,877 porcine cDNA probes identified 4,659 genes with significant changes between different ages. In addition, the multivariate analysis using both age and androstenone levels identified 2,407 genes. When duplicates from these two steps were discarded ([ages] and [ages and androstenone]), the sum of genes with significant changes was a total of 4,748 (Supplementary Table S1, http://tiny.cc/SupplementaryTableS1). Cluster analysis of these 4,748 genes, followed by visual inspection in TreeView (Eisen Lab) resulted in nine clusters of genes displaying differences over time (Figure 1).

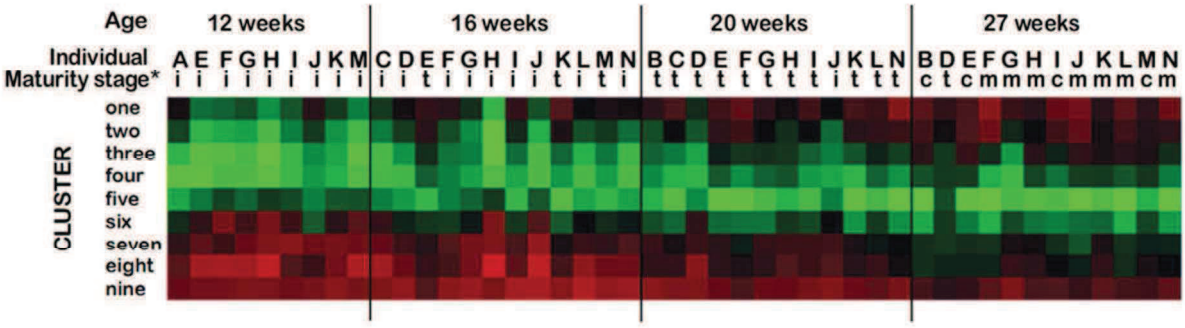

Figure 1. Heat map of calculated means of the expression levels of all transcripts in each of the nine identified clusters for each individual at the different ages. Visualized in Tree View. A-N: individual boars. Green: downregulated, Red: upregulated.

*By histomorphology: i=immature, t=transition, c=close to mature, m=mature.

Official HSA and/or SSC gene identities (IDs) were identified for 4,031 (85 %) of the clustered genes. Out of these, the number of HSA gene IDs which could be used further in DAVID pathway analysis were 3,788 (80 %) (Supplementary Table S1).

In cluster one (767 genes) gene expression was low - compared to the reference in week 12. The expression level was higher in week 16, and continued to increase up to week 27. In cluster two (1,281 genes), expression was low in week 12 and 16 and then increased relative to the reference sample in week 20 and 27. In cluster three (362 genes), the expression remained low until week 20 and then increased to the level of the reference sample in week 27. In cluster four and five, the genes were expressed to a lower degree than the reference sample trough all ages. Cluster four (251 genes) was increasing over time and cluster five (158 genes) was decreasing over time. In cluster six (1,339 genes), the expression was slightly higher than the reference level in week 12 and decreased from week 16 onwards. Gene expression in cluster seven (233 genes), decreased relative to the reference sample during week 20-27. Cluster eight (162 genes) contained genes with a higher expression level than the reference in week 12 and 16, which dropped to slightly below the reference level in week 27. The expression level of the genes in cluster eight was higher than for cluster seven at week 20, but they showed a similar pattern: a tendency for decrease in expression to become similar to the reference in week 27. The genes in cluster nine (195 genes), displayed higher expression than in the reference sample - but without any obvious trend of change over time.

Based on the HSA gene IDs 49 functional KEGG annotated pathways were identified by DAVID in the nine clusters (Table 1). The results are based on the assumption that the function of homologous genes in pigs and humans are the same in both species. This may not always be true.

Table 1. *Identified pathways with genes in the nine clusters of transcripts with significant differential expression in boar testis during pubertal development.*

Pathway names in David/KEGG	count	P-value	official gene names
Cluster one: HSA genes lower than reference week 12 increasing week 16-27			
HSA 05014:Amyotrophic lateral sclerosis	7	0.01	*GRIN2C, BCL2, BAX, RAC1, NEFH, PPP3CC, NEFM*
HSA 05200:Pathways in cancer	20	0.02	*DVL2, MSH6, CKS1B, AR, RXRB, MMP9, FZD5, CTNNA3, RBX1, GLI1, TGFB2, CTNNA2, PRKCB, CCNE2, BAX, BCL2, RAC1, RALB, PIK3CA, FGF1*
HSA 04670:Leukocyte transendothelial migration	10	0.02	*CLDN4, ACTN4, MMP9, RAC1, PIK3CA, MYL12B, MYL12A, CTNNA3, PRKCB, CTNNA2*
HSA 05210:Colorectal cancer	8	0.02	*DVL2, MSH6, BCL2, BAX, RAC1, PIK3CA, FZD5, TGFB2*
HSA 03010:Ribosome	8	0.03	*RPSA, RPS16, RPS3A, RPL34, RPS15, RPL15, RPL22L1, RPS5*
HSA 04114:Oocyte meiosis	9	0.03	*CCNE2, CDK1, AR, ADCY7, RPS6KA1, PPP3CC, CDC20, PPP1CC, RBX1*
HSA 04530:Tight junction	10	0.04	*CLDN4, ACTN4, MAGI1, MRAS, MYH2, MYL12B, MYL12A, CTNNA3, PRKCB, CTNNA2*
HSA 05010:Alzheimer's disease	11	0.05	*UQCRC2, NDUFS4, PSEN1, APH1A, GRIN2C, MAPT, SDHC, PPP3CC, NDUFC1, APBB1, ATP5G3*
HSA 00561:Glycerolipid metabolism	5	0.07	*AKR1A1, PPAP2C, ALDH2, DGKH, PPAP2B*
HSA 04010:MAPK signaling pathway	15	0.07	*IL1R1, MRAS, CACNB3, DDIT3, TGFB2, PRKCB, RPS6KA1, MAP3K2,*

			MAPT, RAC1, MAPK8IP2, PPP3CC, FGF1, CD14, MAP2K5
HSA 04115:p53 signaling pathway	6	0.08	*CCNE2, CDK1, CD82, BAX, SHISA5, ATR,*
HSA 04540:Gap junction	7	0.08	*CDK1, ADCY7, TUBB2B, MAP3K2, TUBA4A, PRKCB, MAP2K5*
HSA 00250:Alanine, aspartate and glutamate metabolism	4	0.09	*ASPA, ABAT, CPS1, GPT2*
Cluster two: HSA genes lower than reference week 12-16 increasing week 20-27			
HSA 00510:N-Glycan biosynthesis	8	0.01	*STT3B, TUSC3, MAN1A2, DPM1, DPM3, DOLPP1, MAN1C1, DDOST*
HSA 00190:Oxidative phosphorylation	14	0.01	*TCIRG1, COX11, COX7A2, NDUFA9, COX8A, NDUFA10, UQCRQ, COX5B, NDUFA11, ATP6V1C1, SDHB, NDUFV1, ATP5A1, ATP6V0A2*
HSA 04260:Cardiac muscle contraction	9	0.04	*FXYD2, CACNA2D1, COX7A2, ATP1B3, COX8A, CACNG2, UQCRQ, COX5B, SLC9A1*
HSA 00534:Heparan sulfate biosynthesis	5	0.04	*NDST1, HS6ST2, HS6ST1, HS3ST3B1, GLCE*
HSA 04110:Cell cycle	12	0.04	*YWHAZ, GSK3B, GADD45G, YWHAB, YWHAQ, TTK, CDK6, SMAD2, ANAPC11, ANAPC7, MAD2L2, YWHAE*
HSA 04114:Oocyte meiosis	11	0.04	*PLCZ1, ADCY4, YWHAZ, PPP2R5D, YWHAB, YWHAQ, ANAPC11, ANAPC7, MAD2L2, YWHAE, CALM1*
HSA 04610:Complement and coagulation cascades	8	0.05	*C8A, KNG1, A2M, C5, SERPING1, SERPINA1, CFI, C8G*
HSA 05010:Alzheimer's disease	14	0.05	*COX7A2, NDUFA9, COX8A, BAD, NDUFA10, COX5B, UQCRQ, NCSTN, SDHB, NDUFV1, GSK3B,*

			SDHB, NDUFV1, GSK3B, BACE2, ATP5A1, CALM1
HSA 04722:Neurotrophin signaling pathway	11	0.08	*YWHAZ, CAMK4, MAP2K2, GSK3B, YWHAB, YWHAQ, BAD, YWHAE, ARHGDIA, CALM1, AKT2*
HSA 00982:Drug metabolism	7	0.08	*CYP3A4, MGST3, GSTM3, GSTM4, MAOB, GSTO2, GSTP1*
HSA 00480:Glutathione metabolism	6	0.09	*MGST3, GGT5, GSTM3, GSTM4, GSTO2, GSTP1*
HSA 04360:Axon guidance	11	0.10	*NCK2, GNAI3, CFL2, SEMA3F, GSK3B, EFNB1, DPYSL5, EFNA5, ROBO2, NFATC4, PAK1*
Cluster three: HSA genes lower than reference until increased expression week 27			
HAS 04130:SNARE interactions in vesicular transport	4	0.03	*STX8, STX18, STX16, VAMP4*
HSA 05322:Systemic lupus erythematosus	5	0.09	*HIST1H2BD, HIST2H2BE, HIST2H2BF, HIST1H2AK, HLA-DRA*
Cluster four: HSA genes lower than reference sample and increasing over time			
HSA 03010:Ribosome	4	0.09	*RPL18A, RPL36AP51, FAU, UBA52*
HSA 00730:Thiamine metabolism	2	0.09	*TPK1, MTMR1*
Cluster five: HSA genes lower than reference sample and decreasing over time			
HSA 03320:PPAR signaling pathway	5	0.00	*CD36, FADS2, FABP1, SCP2, ANGPTL4*
Cluster six: HSA genes decrease beginning week 16 continuing decrease to week 27			
HSA 03010:Ribosome	42	0.00	*RPL18, RPS2P17, RPL19, RPL14, RPL13, RPL15, RPL27A, RPL17P7, RPL35, RPL36, RPL22L1, RPS3, MRPL13, RPL32, RPL7, RPL6, RPL12P2, RPL9, RPL34, RPL8, RPL3, RPL26L1, RPL10, RPL5, RPL7A, RPL10A, RPS20, RPL4, RPS23, RPS16P10, RPL35A, RPL26, RPS8P8, RPL23A, RPS4X, RPL21P16, RPS5, RPS8, RPS7, RPL22, RPL37A, RPS10,*

			RPL6P19, RPS11, RPL17P36
HSA 00071:Fatty acid metabolism	8	0.01	*CYP4A11, ALDH7A1, ADH1C, ADH5, ACAT1, ALDH3A2, ALDH9A1, HADHB*
HSA 00010:Glycolysis / Gluconeogenesis	10	0.01	*ALDH7A1, ADH1C, ADH5, ENO3, PGK1, GAPDH, ALDH3A2, ALDH9A1, PDHB, ENO1*
HSA 00650:Butanoate metabolism	7	0.02	*ALDH7A1, OXCT1, ACSM2A, ACAT1, ALDH3A2, ALDH9A1, PDHB*
HSA 00053:Ascorbate and aldarate metabolism	5	0.02	*ALDH7A1, UGDH, ALDH3A2, UGT2B7, ALDH9A1*
HSA 04610:Complement and coagulation cascades	10	0.02	*KNG1, C1QB, CR1, A2M, C4A, C3, KLKB1, CD46, TFPI, C1R*
HSA 00980:Metabolism of xenobiotics by cytochrome P450	9	0.03	*CYP3A4, GSTA1, GSTA2, GSTM3, ADH1C, ADH5, AKR1C1, UGT2B7, MGST1*
HSA 00982:Drug metabolism	9	0.03	*CYP3A4, GSTA1, GSTA2, GSTM3, FMO1, ADH1C, ADH5, UGT2B7, MGST1*
HSA 03018:RNA degradation	8	0.06	*DIS3, PARN, EXOSC9, DCP1A, EDC3, ENO3, HSPD1, ENO1*
HSA 00620:Pyruvate metabolism	6	0.09	*ALDH7A1, GLO1, ACAT1, ALDH3A2, ALDH9A1, PDHB*
Cluster seven: HSA genes higher than reference week 12-16 then decreasing			
HSA 04662:B cell receptor signaling pathway	4	0.04	*NFAT5, MALT1, CD79A, PIK3R3*
HSA 05412:Arrhythmogenic right ventricular cardiomyopathy (ARVC)	4	0.04	*RYR2, SGCD, CACNA1S, CACNA2D4*
HSA 04260:Cardiac muscle contraction	4	0.05	*RYR2, COX5A, CACNA1S, CACNA2D4*
HSA 05410:Hypertrophic cardiomyopathy (HCM)	4	0.06	*RYR2, SGCD, CACNA1S, CACNA2D4*
HSA 05414:Dilated cardiomyopathy	4	0.07	*RYR2, SGCD, CACNA1S, CACNA2D4*

HSA 04930:Type II diabetes mellitus	3	0.09	*PIK3R3, PRKCE, CACNA1B*
Cluster eight: HSA genes higher expression level but similar pattern to cluster seven			
HSA 03010:Ribosome	6	0.00	*RPL7, RPL3, RPL27A, RPL10, RPL6P19, RPS24*
HSA 04210:Apoptosis	4	0.03	*IRAK2, MYD88, ENDOD1, IKBKB*
Cluster nine: HSA genes higher than the reference sample - indifferent over time			
HSA 04710:Circadian rhythm	3	0.01	*NR1D1, CRY1, CLOCK*

Significance level was set to P=0.1.

The HSA genes that decreased in expression compared to the reference sample in the prepubertal stages from week 16 were associated with pathways of normal cell respiration such as the citric acid cycle and glycolysis and fatty-acid metabolism (Table 1). In addition, cAMP-dependent protein kinase regulator *PRKAR2B* and HSA and SSC annotated *PRKAR1A* decreased from week 20 and later (Table 1, Supplementary Table S1). SSC annotated epidermal growth factor receptor pathway substrate 15 (*EPS15)* decreased in expression from week 20 and so on. In this cluster were also the SSC mapped EGF-like-domains *EGFL6* and HSA *EGFL7*, HSA mapped protein carrier for LDL/cholesterol from plasma into cells and sortilin-related receptor (*SORL1*). Other genes whose expression decreased over time beginning week 16 included transcripts coding for growth and growth inhibitor factors, such as HSA mapped growth hormone receptor (*GHR*). Suppressor family genes SSC inhibitor of growth family members (*ING2,* and *ING3)* were also decreasing in expression at puberty (week 16-27). Other suppressor of growth family genes were decreasing over time to become downregulated to the reference later in week 27 (*ING4, ING1*). In contrast, SSC growth hormone releasing hormone (*GHRH*) was increasing over to slightly lower expression than the reference at week 27.

Expression of the cell adhesion molecule SSC cadherin 1, type 1, Epithelial (E)-cadherin (*CDH1*) was higher than the reference at the prepubertal and early pubertal stage (week 12-20), and showed decrease in expression below the reference at week 27 (Supplementary Table S1). Additionally, the PPAR pathway decreased in expression over time below the reference (cluster five, Table 1).

Some HSA Calcium$^{(2+)}$ voltage channel genes were expressed higher than the reference as “KEGG cardiac pathways” at early puberty (cluster seven, decreasing week 20-27). When these prepubertal pathways were decreasing, several other “cardiac” related pathways genes showed increasing expression in week 20 and 27. Some of the latter pubertal pathway genes were coding for -c oxidase subunits (*COX*-) in oxidative phosphorylation enzymes.

Pathways associated to nerves were highly present in pubertal clusters (-one and -two, Table 1.) Cell-protective metabolism pathway coding for redox and glutathione metabolism changed in expression at puberty both at 16 (decreasing cluster six) and 20-27 (increasing cluster two) weeks. Different redox genes occurred in the pathways at prepuberty and puberty. Amongst prepubertal expressed redox pathway genes were *ADH1C* and *ADH5* - two isomerase members of the alcohol dehydrogenase family genes coding for genes which metabolize hydroxysteroids and more.

The proliferative MAPK pathway showed pubertal increase in expression compared to the reference sample (16-27 week). Increased steroidogenesis was observed at this time (Lervik *et al.*, 2013). Some of the MAPK pathway genes were present in other cluster one pathways (cluster one, Table 1). Amongst these were calcineurin (*PPP3CC*), a calcium-dependent calmodulin-stimulated protein-phosphatase involved in the downstream regulation of dopaminergic signal

transduction together with glutamate receptor *GRIN2C.* The *GRIN2C* gene is also known for synaptic long term potentiation. Other MAPK pathway genes were shared with morphological pathways. Amongst these were *RAC1*, *MAP2K5* and *RPS6KA1* genes which have previously been suggested for association to morphogenesis of nerves. The Schwann cell growth factor HSA neuregulin1 (*NRG1*), known to interact with MAPK, was also in cluster one.

Other genes of interest were in pubertal clusters, but not included in the 49 pathways. HSA follicle stimulating hormone, beta polypeptide (*FSHB*), HSA transforming growth factor beta2 (*TGFB2),* HSA follistatin-like 3 secreted glycoprotein (*FSTL3*) and the neuropeptide HSA somatostatin (*SST*) increased in expression week 16-27. Furthermore were genes coding for SSC mapped steroidogenic acute regulatory protein (*STAR*) and the STAR analogue *STAR*-functional related lipid transfer domain containing6 (*STARD6*) expressed in cluster one. Growth factors with suggested effect on *STAR* are listed in Table 2.

24-dehydrocholesterol reductase (*DHCR24*) and lanosterol 14alpha-demethylase (*CYP51A1*), known to be related to cholesterol, occurred in cluster with genes downregulated to the reference sample whose expression increases towards week 27 in mature boars (Supplementary Table S1).

In parallel with the histomorphological transition of the testis and maturation of germ cells, expression of meiotic pathways increased to reference sample level in weeks 16-27 and 20-27 (cluster one and -two). The MAPK pathway genes *RPS6K51* and *PP3CC* (the latter mentioned above) were in the earliest meiotic pathway (cluster one). Different genes occurred in the meiotic pathway with increased expression four weeks later (cluster two, Table 1). Pathways involved in testis junction formations

Table 2. *Growth associated factors acting as STAR regulators in mammals.*

Factor	Property (species) References	Associated genes found in cluster
Thyroid hormone	stimulating STAR in Leydig cells (mouse) (Manna et al., 2001b)	*THRAP3* cluster three (HSA) *TRHDE* cluster seven (HSA and SSC)
Prolactin	Inhibiting STAR in Leydig cells (mouse) (Manna et al., 2001a)	*PRLR* cluster two (HSA) *PRL* cluster two (HSA and SSC)
IGF-I and IGF-II	stimulating STAR in Leydig cells (mouse, rat) (Manna et al., 2006a) (Manna et al., 2006b) (Lin et al., 1998b)	*IGFBP3* cluster two (SSC and HSA) *IGFBP1* cluster three (SSC and HSA) *IGF1R* cluster three (HSA and SSC) *IGFBP2* cluster two (HSA and SSC) *IGFBP6* cluster two (HSA and SSC) *IGFBP3* cluster six (HSA and SSC) *IGF1R* cluster seven (HSA)
EGF/TGFA	stimulating STAR in Leydig cells (rat) (Millena et al., 2004)	EGF substrate *EPS15* in cluster seven (SSC and HSA) EGF substrate *EPS8* in cluster five (SSC and HSA)
TGFB	inhibiting STAR in ovarian thecal cells (human) (Attia et al., 2000)	*TGFB2* cluster one (HSA) *TGFBR2* cluster eight (HSA)
interferon-gamma	inhibiting STAR in Leydig cells (rat) (Lin et al., 1998a)	*IFNG* cluster two (HSA and SSC)

also showed increased expression at puberty. Tight junction and gap junction pathway occurred in cluster one.

SSC platelet-derived growth factor alpha polypeptide (*PDGFA*) was expressed in cluster three, showing most increased expression in the mature testis. Amongst genes decreasing during puberty was HSA platelet derived growth factor D (*PDGFD*) (downregulated to reference week 16-27). SSC platelet derived growth factor C (*PDGFC*) showed decreasing expression lower than the reference over time (Supplementary Table S1).

Some SSC spermatogenesis associated transcripts (*SPATA*-) were spread in the clusters which increased to reference sample from puberty and onwards (cluster one, -two and -three). HSA gamma-aminobutyric acid A receptor beta showed increased expression in the pubertal clusters one (*GABRB2*) and two (*GABRB3*) (Supplementary Table S1).

Three probes represented a follicle-stimulating hormone beta polypeptide regulator called SMAD specific E3 ubiquitin protein ligase 2 (*SMURF2*). Expression of these genes increased and decreased compared to the reference in the mature testis (Supplementary Table S1). HSA transforming growth factor B receptorII (*TGFBR2)*, showed increased expression slightly lower than the reference sample (Supplementary Table S1 and Table 2).

No genes showed significant differences between high and low androstenone levels in the ANOVA analysis. Neither did observed differential expression of genes over time show association with androstenone levels. When the testis samples were ranged according to androstenone levels in fat at 27 weeks of age, a search for significant genes related to the androstenone level also gave a negative result.

Discussion

The gene profiles in the nine clusters changed from 12 to 27 weeks, in other words before, during and after onset of puberty. The identified KEGG pathways could be associated with the timing of histomorphological development of the testis, spermatogenesis and increased steroidogenesis, as investigated in the same animals. Analyses of porcine genes were thus based on the assumption that genes with similar sequences have the same function as that described for humans. Based on this, precaution must be taken that genes similar in sequence might not have the same functions in swine as described for humans or rodents in DAVID/KEGG. Nevertheless, human pathway analysis tools are the best available option for analysis of large amounts of porcine transcripts such as are found in microarray data.

Age- and development-specific functional pathways and genes coding for growth signalling could be related to KEGG pathways mapped as HSA organ morphogenesis before and after the onset of pubertal development. Several of the suggested functions is supported by the mapping to SSC annotations (Supplementary Table S1). The observed prepubertal and pubertal pathways and genes expressed, were supported by steroidogenic and morphological data (Lervik et al., 2013).

Pathways associated with morphogenesis during prepuberty

Pathways known to be directly associated with reproductive development were still less active in the testis tissue from the 12-week-old boars than in the reference material. At this age the histomorphological movements towards puberty were not observed in the tissue (Lervik et al., 2013), in concordance with other studies (Franca *et al.*, 2000, Vandalem *et al.*, 1986). The genes that were significantly upregulated compared to the reference sample during week 12 were associated with pathways for metabolism showing

stable normal cell respiration. However, the pathways with consistently higher or lower expression than in the reference sample during the other time points were not associated with general basal cell metabolism. This might indicate an elevated prepubertal metabolism linked to the upcoming pubertal changes in the testis. Expression of HSA sortilin (*SORL1*) was also higher than in the reference sample in weeks 12 and 16, decreasing in week 20 and further. With the assumption and precaution that the function of the gene is similar in humans and swine - this might support higher level of endocytic activity as suggested by Jacobsen et al (Jacobsen *et al.*, 1996). Additionally expression of *EPS15* was higher than the reference value during week 12, with decrease from week 16 onward. The involvement of *EPS15* suggests that the EGF receptor might be directed into an endocytic pathway, as suggested in other species (de Melker *et al.*, 2004). High amount of c-AMP are required for EGF activation (Evaul and Hammes, 2008). High c-AMP activity might be supported by the protein kinase regulatory subunits (*PRKAR1A, PRKAR2B*) found in the prepubertal clusters (Supplementary Table S1). This might contribute to intensity and cell respiration indicated in the prepubertal testis. An explanation can also be looked for in the PPAR pathway, with decreasing expression over time below the reference (cluster five, Table 1). This pathway was previously suggested to regulate Sertoli cell metabolism in the rat (Regueira et al., 2014). If the *EPS15* gene and PPAR pathway genes work similar in swine as in rodents and humans, these might contribute to metabolism in the prepubertal testis.

Notable is the expression of $Ca^{(2+)}$ voltage channel genes before maturation. Moreover, the “cardiac pathways”, highlighted the involvement of $Ca^{(2+)}$ voltage channels at early puberty (expression decreased week 20-27). The molecular functions as identified here by DAVID are still not well characterized. However, cAMP and $Ca^{(2+)}$ pathways are involved in most LH-induced steroidogenesis in Leydig cells (Abdou *et al.*, 2013). The

$Ca^{(2+)}$-associated pathway would therefore have been expected to appear in cluster one- or two with genes whose expression increased during puberty, and not in the prepubertal cluster seven. However, a pubertal change occurred in pathways with increased expression to the reference in the maturing testis (week 20-27). Then, several Ca related "Cardiac pathway" genes were coding for enzyme properties related to oxidative phosphorylation. The data indicates modulation by $Ca^{(2+)}$ voltage channels might be more prominent in the prepubertal testis compared to the mature testis.

At beginning of sexual maturity (week 12-20) the increased expression of the SSC mapped specific epithelial (E)-cadherine gene (*CDH1*) might indicate a link to sexual development. During development of the sow, E-cadherines express regulatory characteristics by establishing the morphology of ovaries (Kirkup *et al.*, 2000). The present data might suggest that E-cadherines may play a role also during early development of the boar testis.

Pathways associated with morphogenesis during sexual maturation

There was a shift in functional pathways related to organ morphogenesis after the onset of pubertal development. Beginning in week 16, there was increasing expression of genes in the MAPK pathway associated with tissue development. Interestingly, this upregulation coincided with the pubertal increase in steroid production in these boars. MAPK pathways are commonly associated with cell growth and differentiation. The upregulation of MAPK was seen some weeks earlier than the most complex morphological restructuring, that is the transformation of testis tissue into reproductive tissue. The effect of MAPK in the testis tissue could be to stimulate general growth, which is the role of MAPK in a large number of other tissues. However, the potential of MAPK to regulate mitochondrial steroid acute regulatory protein (*STAR*) has been discussed in rodents and

humans (Gyles *et al.*, 2001, Manna and Stocco, 2011). Functions might act analogic in the Swine. The STAR protein is a cholesterol trafficker known to regulate the quantitative rate-limiting step in steroidogenesis (Christenson and Strauss, 2000). The *STARD6* gene has been regarded as coding for a STAR analogue cholesterol transport mediator (Bose *et al.*, 2008). The increased expression of *STAR* and *STARD6* in the testis from week 16 onwards occurred simultaneously as MAPK and might indicate its functional involvement. Also in the same cluster (-one), was *TGFB2* (Table 2). These have been related to *STAR* in studies of human and rodent Leydig cells (Attia et al., 2000, Lin et al., 1998a). However based on the present results, it can be speculated if more growth factors modulate STAR in the pubertal developing testis of Duroc boars (Table 2). Assumptions must be taken carefully. These genes might not act analogous in swine Leydig cells, as shown in rodents and humans. However, modulation of androgen-stimulated crosstalk by MAPK has a role during organogenesis (Raman *et al.*, 2007). The present study indicates that MAPKs play a complex role in the development of the boar testis.

When puberty developed (week 20- onwards), there was an increase in protective redox/detox support pathways “glutathione and drug metabolism” (cluster two). These have a protective role during increased steroidogenesis. However, a potent redox/detox battery is also essential for the vitality and renewal of neurons during development (Aspberg and Tottmar, 1992, Kagias *et al.*, 2012). Little has been reported on neuronal development in the testis tissue during pubertal development in the boar. However, relevance is supported from studies in humans and rats (Gerendai et al., 2005). The high proportion of pathways associated to nerves in which expression increased at 16-27 and 20-27 weeks coincided in time with both increased steroidiogenesis and histomorphological maturation. Increased expression of the dopamine activated pathway gene *calcineurin* and glutamate receptor *GRIN2C* which is associated with dopamine

function, in the same cluster (-one), might support increased neuronal puberty signalling. A gene coding for the neuropeptide somatostatin, was also in earliest pubertal cluster (-one, increased week16-27) with possible roles both as endocrine regulator and at morphogenesis. In brain tissues, it has been shown that pubertal neural networking is driven by steroids (Sisk and Foster, 2004). The increased expression of neuromorphogenic pathways and changes in the properties of redox pathways at puberty, might suggest pubertal influence on the innervation and maturation of neurons in the testis of the Duroc boar.

A gene coding for the HSA neuropeptide hormone somatostatin (*SST*) was found at a specific age, as its expression increased at about the same time as steroidogenesis (week 16-27). It has previously been suggested that SST stimulates testosterone levels (Vasankari et al., 1995). Effect of SST might be similar to that in rats, where stimulation of steroidogenesis was shown in juvenile animals - but not in adults (Gerendai *et al.*, 1996). The data supports the conclusion that *SST* increases action when steroid levels rise at beginning of puberty.

A gene coding for HSA gamma-aminobutyric acid A receptor, beta (*GABRB2*) was also in this pubertal cluster one (week 16-27, Table 1). In addition, *GABRB3* increased expression to the reference at week 20-27. The timing of the *GABRB* increase in expression is in agreement with the individual onset of morphological transition and gametogenesis observed in the present boar testis material. Location and function of gamma-aminobutyric acidA receptor has previously been related to spermatogenesis in mice and humans (Kanbara *et al.*, 2011, Kanbara *et al.*, 2010). It is therefore possible that *GABRB2* has a function also in boar gametogenesis. Indication of maturing gametogenesis was supported in the pubertal clusters (-one, -two and -three) by the presence of various SSC mapped spermatogenesis associated transcripts (*SPATA*-).

Together with the histomorphological status, the relevance of HSA mapped *GABRB* transcripts for spermatogenesis in boar testis might be supported.

Interestingly, the histomorphological data was supporting testis maturation coinciding with the meiotic pathways which could be related to spermatogenesis in cluster one and two (16-27 and 20-27 weeks). Amongst the genes in the meiotic cluster which occurred first (16. week), the protein kinase A signaling *PPP3CC* gene could be associated with nerves (Amyotrophic lateral sclerosis and Alzheimers pathways, Table 1). Four weeks later, the androgen signalling calmodulin *(CALM1)* was amongst the pathway genes occurring both in this meiotic pathway (20 weeks) and in the neurotrophin signalling pathway in the same cluster. *CALM1* might have more functions as a common factor for developing nerves by “neurotrophin” pathway in addition to spermatogenesis in the “meiotic” pathway.

The sexually mature boar

Platelet-derived growth factors are key factors for gonad development in mammals (Basciani et al., 2010, Schmahl et al., 2008). Interestingly, the *PDGFA* expression occurred in mature testis. Lack of *PDGFA* has been associated with Leydig cell loss and impaired spermatogenesis in mice (Gnessi et al., 2000). The data might suggest association for *PDGFA* to reproductive functions in the mature testis of the boar.

Cholesterol-related genes, represented by 24-dehydrocholesterol reductase (*DHCR24*) and cytochrome P450 family 51A1 (*CYP51A1*), displayed increased expression lower than the reference sample over time. The highest mean levels below reference sample were seen in mature testis. Expression of cholesterol transcripts in the mature boar testis may be associated with meiosis-activating sterols (MAS). MAS show high levels in the adult testis and sperm of many mammals (Keber et al., 2013). Therefore, it

could be that increased expression found in cholesterol-related intermediates in the testis of mature boars was related to both spermatogenesis and steroidogenesis. Additionally, another probe showed *DHCR24* in cluster associated to prepubertal expression and decreased regulation thereafter (Supplementary Table S1). A complex role of cholesterol for steroidogenesis and testis function might be considered. Based on this analysis of differential gene expression over time, conclusion for action of cholesterol related genes were not simple.

Probes expressing SMAD specific E3 ubiquitin protein ligase 2 (*SMURF2*), a follicle-stimulating hormone beta polypeptide *FSHB* regulator, described by Tran (Tran *et al.*, 2011), showed increasing and decreasing expression relative to the reference sample in the mature testis (clusters three and eight, Supplementary Table S1). In addition to regulating *FSHB,* in the mouse, *SMURF2* interacts with *TGFBR2* (Dragovic *et al.*, 2007). In the mature testis, *SMURF2* increased (cluster three) in expression. This might indicate an association with transforming growth factor B receptor II (*TGFBR2)*, which showed a slightly lower increase in expression at the same time (cluster four, Table 2). Caution must be taken at interpretation, since the *SMURF2* gene function can vary between mouse and swine. Additionally, there was another *SMURF2* probe in the array showing decreased expression to the reference sample in adult testis (cluster eight). However, if the interactions of *SMURF2* are analogue in swine and mouse, these genes may contribute to completion of testis development in the boar. The change in *TGFBR2* regulation, found at 27 weeks of age, may contribute to completion or to the overall stability of the mature testis.

Additionally, HSA *TGFB2* and *FSHB* were upregulated in cluster one increasing over time. More functions are probably involved, since TGFB1–B3 is essential for the development of both germ and Sertoli cells (Miles *et al.*, 2013). In rats, these genes are

suggested to be age-dependent, since studies have shown that TGFB1–B3 influences germ cell survival at the beginning of spermatogenesis and around puberty (Dias *et al.*, 2009). Another regulator of testis aging and size, *FSTL3,* working through *TGFBR2* (Oldknow *et al.*, 2013), was also upregulated in the pubertal cluster one (increased expression 16-27 week). This might be associated with the expression of *TGFB2*, in the same cluster (-one, increasing week 16-27). *TGFB* is also able to inhibit STAR (Table 2). Steroidogenesis in Leydig cells might be affected by *TGFB*.

Together, the interactions of morphological pathways, growth factors and neurosteroids in the present data suggest functional pathways and transcripts associated with growth are expressed at specific stages of testis maturation.

Androstenone levels and gene expression

No significant association was observed between levels of the steroid pheromone androstenone and differential expression of genes with the model in this study. This may be due to individual variation both in gene expression levels and in androstenone levels during pubertal development. Generally, there is also a high quantitative threshold needed for detection of differential gene expression in a microarray assay compared to other methods. Studies of mice have also demonstrated the difficulties involved in modelling expression of testis steroids and their respective receptors during development (Willems *et al.*, 2010).

In conclusion, the present study showed that growth pathways, neurosteroids and growth factors, known to have modulating effects on steroidogenesis and gonadotropins, act at specific age and developmental stages of the boar testis. The developmental specificity of transcripts must be considered in order to determine the suitable age for

measurement of androstenone levels if breeding for lower androstenone. Specifically this is important in breeding programmes where for instance only mid-pubertal samples from the slaughter line are tested. This study illustrates the high complexity of pubertal development.

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