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Relationship between carcass weight, adipose tissue androstenone level and expression of the hepatic 3β-hydroxysteroid dehydrogenase in entire commercial pigs

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Boar taint is a major meat-quality defect in pigs and is due to excessive accumulation of skatole and androstenone in adipose tissue. The present work investigated the relationship between carcass weight, levels of skatole and androstenone in adipose tissue, and expression of the hepatic androstenone-metabolising enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD), in 22 entire male and 22 entire female crossbred pigs (Large White (40%) × Landrace (40%) × Duroc (20%)). Animals of each gender were divided into two subgroups (11 pigs in each subgroup): (i) conventional weight (carcass weight 59 to 77 kg) and (ii) heavy weight (carcass weight 84 to 95 kg). No relationship between carcass weight and adipose tissue skatole level was found for entire male pigs (r² = 0.013, P > 0.05). There was a significant negative relationship between carcass weight and expression of the hepatic 3β-HSD protein (r² = 0.502, P < 0.001) and a significant negative relationship between 3β-HSD protein expression and androstenone level in adipose tissue (r² = 0.24, P < 0.05) in entire males. No relationship was found between carcass weight and 3β-HSD protein expression in female pigs (r² = 0.001, P > 0.05). 3β-HSD expression was 59% higher in conventional-weight male pigs when compared with heavy-weight animals (P < 0.05) and 36% higher in heavy-weight females when compared with heavy-weight males (P < 0.05). It is concluded that an increase in slaughter weight of entire commercial crossbred Large White pigs is accompanied by inhibition of expression of the hepatic 3β-HSD protein, which might result in a reduced rate of hepatic androstenone clearance with its subsequent accumulation in adipose tissue. It is suggested that regulation of pig hepatic 3β-HSD expression is under the control of sex hormones.

Keywords: *androstenone, boar taint, 3β-hydroxysteroid dehydrogenase, pigs*

Introduction

Raising entire male pigs has a number of production, environmental and animal welfare benefits, when compared with the production of castrated animals. Entire male pigs have a better feed conversion rate, higher growth rate and lean percentage, which reduce production costs (Fowler *et al.*, 1981; Andersson *et al.*, 1997). In addition, entire male pigs excrete less nitrogen when compared with castrates, as they have a better efficiency for nitrogen retention and therefore such a production system is more environmentally friendly (Walstra, 1974). Furthermore, meat from entire male pigs has a lower fat content and higher level of unsaturated fatty acids (Wood and Enser, 1982; Barton-Gade, 1987), which are of direct benefit for human health.

Therefore, production of entire male pigs would be beneficial from an economic point of view and would also be environmentally friendly, which enhances the 'ethical quality' of pork.

However, there are a number of drawbacks, such as higher frequency of social stress displayed as fighting and aggressive behaviour, associated with the production of entire male pigs when compared with entire pigs. Aggressive behaviour can lead to carcass downgrading because of skin lesions (Warriss and Brown, 1985). The softer fat of entire males may also cause problems during processing and may also be more prone to rancidity problems (Bonneau and Squires, 2001).

A critical point in the production of entire male pigs is slaughter weight. On one hand, production of heavy carcasses is desirable, since it reduces the total animal production cost and hence improves pork market competitiveness. On the other hand, an increase in slaughter weight

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can result in an increased risk of boar taint. Therefore, one of the challenges for the pig industry is to produce heavy-weight entire male pigs with a reduced incidence of boar taint.

Boar taint is one of the major meat-quality defects in pigs, which is perceived as an unpleasant odour and flavour in pork and pork products during cooking (Gower, 1972; Lundstrom and Bonneau, 1996). Boar taint may lead to a poor eating experience with the subsequent negative economic repercussion for the meat industry. Boar taint occurs at puberty in some entire male pigs due to the excessive accumulation of mainly two compounds, androstenone and skatole, in adipose tissue (Lundstrom and Bonneau, 1996). However, results in the literature regarding the relationship between carcass weight and skatole and androstenone deposition are inconsistent. Thus, positive correlations ($P < 0.05$) between carcass weight and skatole and/or androstenone levels have been reported by Mortensen *et al.* (1986), Walstra *et al.* (1999) and Sinclair *et al.* (2001). The other authors did not find a significant relationship ($P > 0.05$) between skatole and androstenone levels and carcass weight (Hennessy *et al.*, 1995).

A European Union-wide study on boar taint (Matthews *et al.*, 2000) defined skatole as a major contributor to the perception of boar taint in fresh meat and recommendations were given to optimise production factors to reduce skatole level as a short-term solution to boar taint (Bonneau *et al.*, 2000). However, our previous work has suggested that accumulation of androstenone might be a primary event in boar taint development, since high levels of androstenone can inhibit the expression of the hepatic skatole-metabolising enzyme CYP2E1 and thus reduce the rate of hepatic skatole clearance (Doran *et al.*, 2002a). The reason for the high androstenone accumulation in some pigs is not clear. Excessive androstenone levels can be attributed, at least partially, to defective expression and activity of the hepatic androstenone-metabolising enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD) (Doran *et al.*, 2004; Nicolau-Solano *et al.*, 2006). The mechanism regulating 3β-HSD expression in pig liver remains unknown. Since the incidence of boar taint increases in puberty and coincides with changes in hormonal status (Babol *et al.*, 2004; Zamaratskaia *et al.*, 2004), sex hormones might be one of the factors regulating the expression of pig hepatic 3β-HSD.

This study was designed with two aims: (i) to investigate the relationship between carcass weight, the level of adipose tissue androstenone and skatole and the expression of hepatic 3β-HSD in entire commercial Large White crossbred pigs and (ii) to establish whether the expression of hepatic 3β-HSD depends on the hormonal status of the animals (as determined by gender and slaughter weight).

Material and methods

Animals
In all, 44 intact commercial crossbred pigs (Large White (40%) $\times$ Landrace (40%) $\times$ Duroc (20%)) from different litters were used in this study. All the animals were reared in the same farm and under the same commercial husbandry practices. Individuals were allocated to different experimental groups (11 animals in each group) depending on gender and carcass weight immediately after slaughter. The groups were defined as: (1) conventional-weight males, (2) heavy-weight males, (3) conventional-weight females and (4) heavy-weight females. The average carcass weight with the standard errors for means for experimental groups 1, 2, 3 and 4 were: 70 ± 1.8, 91 ± 1.0, 73 ± 1.4 and 98 ± 4.5 kg, respectively. Carcasses over 80 kg were considered as heavy-weight pigs. The UK market average carcass weight is 74 kg as defined by the Scientific Panel for Animal Health and Welfare (2004).

Animals were reared on a commercial standard pelleted diet (ABN, Peterborough, UK), which consisted of growing and finishing diets. The growing diet was fed *ad libitum* to pigs up to 12 weeks of age. This diet contained 5% vegetable oil, 18.5% crude protein, 1.20% lysine and 3.3% crude fibre. After 12 weeks, the pigs were transferred to the finishing diet, which was fed until slaughter at 22 to 26 weeks of age. The finishing diet contained 3.5% vegetable oil, 16.5% crude protein, 1.25% lysine and 3.8% crude fibre.

Pigs were slaughtered in the EU-approved abattoir of the Department of Clinical Veterinary Science, University of Bristol, Bristol, UK, in compliance with regulations for humane care and slaughter.

Sample collection
Samples of liver and subcutaneous fat were collected within 5 to 10 min after exsanguination. For enzyme expression measurement, liver samples were snap-frozen in liquid nitrogen and stored at $-80°C$ until assayed. Samples of subcutaneous fat for skatole and androstenone analyses were obtained from the region of the last cervical vertebra and kept frozen at $-20°C$ until analysed.

Isolation of microsomes
3β-HSD is an endoplasmic reticulum-associated enzyme. Therefore, analyses of 3β-HSD protein expression were performed on isolated pig liver microsomes. The microsomes were isolated by differential centrifugation, as described previously (Doran *et al.*, 2002b). The resulting pellet was re-suspended at a protein concentration of about 20 mg/ml in a medium containing 50 mmol/l Tris-HCl, 10 mmol/l KH_2PO_4, 0.1 mmol/l EDTA, 20% glycerol and inhibitors of proteolytic enzymes antipain + pepstatin + leupeptin (1 μg/ml) (Sigma, Dorset, UK).

Protein levels in isolated microsomes were determined by the Bradford method (Bradford, 1976) using bovine serum albumin (fraction V) as a standard.

Analysis of 3β-HSD protein expression
Expression of 3β-HSD protein in isolated pig liver microsomes was determined by Western blot. The optimum

amount of protein required for the analysis has been determined in our previous work and was 6 μg per well (Nicolau-Solano et al., 2006). The microsomal proteins were separated by SDS-PAGE, electroblotted onto a nitrocellulose membrane, pore size 0.45 μm (Bio-Rad, Hertfordshire, UK) at a constant voltage of 100 V for 1 h and probed with rabbit anti-porcine 3β-HSD antibody for 1.5 h. The antibody was produced against a synthetic peptide corresponding to amino acids 357 to 370 of the porcine 3β-HSD protein (GenBank accession no. NP 001004049). After probing with the primary antibody, the nitrocellulose membrane was re-probed with a commercial secondary antibody (horse-radish peroxidase linked donkey anti-rabbit Ig (Amersham, Buckinghamshire, UK) for 1 h. The complex 3β-HSD-primary antibody-secondary antibody was visualised using an enhanced chemiluminescence detection system (Amersham). The films were scanned and the intensity of the corresponding bands was quantified using the Image-Quant programme (Molecular Dynamics, Buckinghamshire, England).

Analysis of androstenone in adipose tissue

Androstenone level in adipose tissue was measured by high-resolution gas chromatography (HRGC) using a modification of the method of De Brabander and Verbeke (1986). In this procedure, 0.4 g of fat was saponified for 1 h at 60°C in toluene and KOH in methanol with 5α-androstane-3, 17-dione (Sigma) as an internal standard. After cooling, aqueous methanol was added and the samples were extracted four times with petroleum-ether (boiling point 40°C to 60°C) /diethyl ether (1:1 vol/vol). The pooled upper layers were reduced in volume under nitrogen before derivatisation using Tri-Sil reagent (Pierce, Cheshire, UK). The samples as tri-methyl silyl-derivatives were analysed using a Fisons 8000 series gas chromatograph (Fisons Instruments, Crawley, Sussex, England) in hot splitless/split mode (20:1), equipped with a Sil8 capillary column (25 m × 0.25 mm, internal diameter) (Chrompak Ltd, Middelburg, The Netherlands), with helium as the carrier gas and using a flame ionisation detector. All analyses were performed in duplicate and the variation between duplicates was less than 10%.

Analysis of skatole in adipose tissue

Adipose tissue skatole level was analysed using the simultaneous distillation/extraction procedure, with 5-methylindole as an internal standard, as described by Whittington et al. (2004). Analysis was carried out using a Fisons 8000 Series HRGC, equipped with a CP57Wax capillary column (50 m × 0.32 mm internal diameter) using helium as the carrier gas, and a nitrogen–phosphorus-specific flame-ionisation detector. The linearity of the response was verified using a calibration cocktail containing known amounts of indole, skatole and 5-methylindole. All analyses were performed in duplicate and the variation between duplicates was less than 10%.

Statistics

Statistical analysis of hepatic 3β-HSD was by the general linear model (Minitab, Release 14) with gender and weight as factors and including the interaction term. Paired comparisons between mean values for the significant interaction were assessed post hoc using the Tukey test at the 0.05 level. The relationships between variables (carcass weight, levels of androstenone and skatole in adipose tissue, and hepatic 3β-HSD) were investigated using simple linear regression analysis.
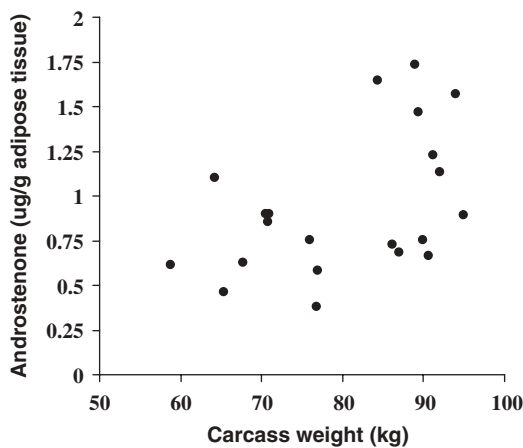
Results

Relationship between carcass weight and levels of skatole and androstenone in adipose tissue of entire male pigs

The relationship between carcass weight and the levels of skatole and androstenone in adipose tissue was investigated in conventional- and heavy-weight males (experimental groups 1 and 2). Figure 1 shows that an increase in carcass weight from 59 to 95 kg was not accompanied by changes in adipose tissue skatole level. The relationship between these two parameters was not significant ($r^2 = 0.013$, $P > 0.05$). The average value of adipose tissue skatole for these animals was 0.076 ± 0.018 μg/g of fat. Two out of 22 entire males (9%) had a skatole level over 0.2 μg/g of adipose tissue, which is the commonly used threshold value associated with boar taint (Bonneau et al., 1992). The percentage of male pigs with low (up to 0.01 μg/g), medium (0.01 to 0.2 μg/g) and high (more than 0.2 μg/g) skatole in adipose tissue within each weight group was 80%, 10% and 10%, respectively, for pigs under 80 kg carcass weight; and 90%, 0% and 10%, respectively, for pigs over 80 kg carcass weight.

Regarding the carcass weight and adipose tissue androstenone (Figure 2), although the relationship was significant ($P < 0.05$), the r^2 value was low (0.22). There was a large amount of scatter and the nature of the
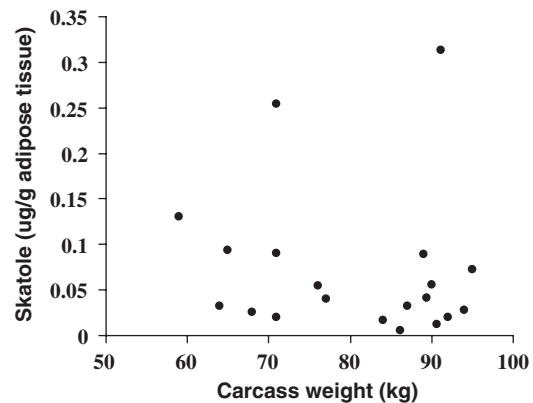
Figure 1 Relationship between carcass weight and skatole level in subcutaneous adipose tissue of entire male pigs. Each point represents an average of duplicate measurements for an individual pig. The duplicates varied by less than 10%.

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Figure 2 Relationship between carcass weight and androstenone level in subcutaneous adipose tissue of entire male pigs. Each point represents an average of duplicate measurements for an individual pig. The duplicates varied by less than 10%.

Figure 3 Relationship between 3β-hydroxysteroid dehydrogenase (3β-HSD) protein expression in isolated pig liver microsomes and the level of androstenone in adipose tissue in entire male pigs. Each point represents an average of duplicate measurements for an individual pig. The duplicates varied by less than 10%.

relationship between these two parameters is unclear. The average value for androstenone level was $0.919 \pm 0.084\,\mu g/g$ of adipose tissue. In the population studied, 59% of the pigs had androstenone levels between 0.5 and $1.0\,\mu g/g$ of fat, and 32% of the animals showed androstenone levels above $1.0\,\mu g/g$ of fat, which is the commonly used threshold androstenone value for boar taint (Desmoulin *et al.*, 1982). The percentage of male pigs with low (0 to $0.5\,\mu g/g$), medium (0.5 to $1.0\,\mu g/g$) and high (more than $1.0\,\mu g/g$) androstenone in adipose tissue within each weight group was: 18%, 73% and 9%, respectively, for pigs under 80 kg carcass weight and 0%, 45% and 55%, respectively, for pigs over 80 kg carcass weight.

*Gender and weight-related changes in expression of the hepatic 3β-HSD protein*
Figure 3 shows that there was a significant negative relationship between adipose tissue androstenone and expression of the hepatic androstenone-metabolising enzyme 3β-HSD ($r^2 = 0.24$, $P < 0.05$).

To investigate the effect of the hormonal status on the expression of hepatic 3β-HSD, animals of different gender and weight were compared. A significant gender $\times$ weight interaction (F $(1.40) = 17.57$, $P < 0.001$) was found. *Post hoc* comparisons are shown in Figure 4 and indicate no significant differences in the levels of 3β-HSD protein expression between females of both weights and conventional-weight males. However, there was a significant difference between these three groups and the heavy-weight males. The expression of 3β-HSD was more than 40% lower for the heavy-weight males compared with the conventional-weight males. In contrast, heavy-weight females had more than 35% higher 3β-HSD protein expression when compared with heavy-weight males.

The relationship between hepatic 3β-HSD protein expression and carcass weight for male and female pigs is presented in Figure 5a and b, respectively. There was a
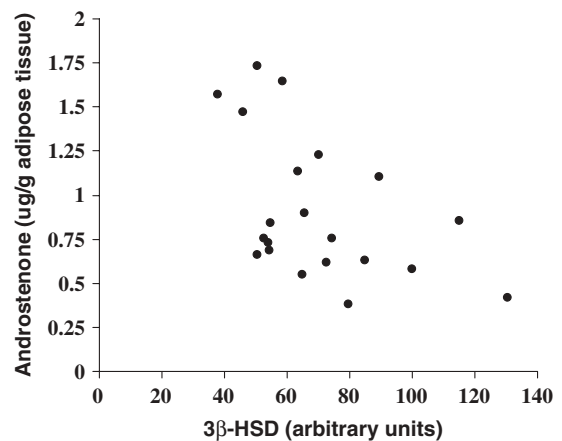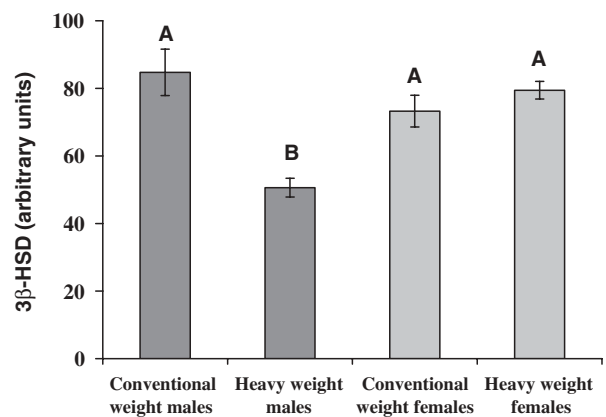
Figure 4 Expression of 3β-hydroxysteroid dehydrogenase (3β-HSD) protein in isolate microsomes from liver of conventional and heavy-weight male and female pigs. Each bar represents a mean value for 11 animals. Measurements for each animal had been done in duplicates. The error bars represent standard error for means. Bars with different superscripts differ significantly. One sample from a particular pig (the reference sample) was present on every blot and the 3β-HSD protein content of this sample was taken as 100 arbitrary units throughout. The amount of 3β-HSD in other samples on the blot was expressed as a percentage of the reference sample.

significant negative correlation between these two parameters for males ($r^2 = 0.502$, $P < 0.001$; Figure 5a), while no significant relationship was found for females ($r^2 = 0.001$, $P > 0.05$; Figure 5b).

Discussion

Until recently it has been generally accepted that the main reason for excessive accumulation of androstenone in adipose tissue is its overproduction in the testis (Forland *et al.*, 1980; Schinckel *et al.*, 1984; Oonk *et al.*, 1995). However, during the last few years it has been shown that adipose

(a)

(b)
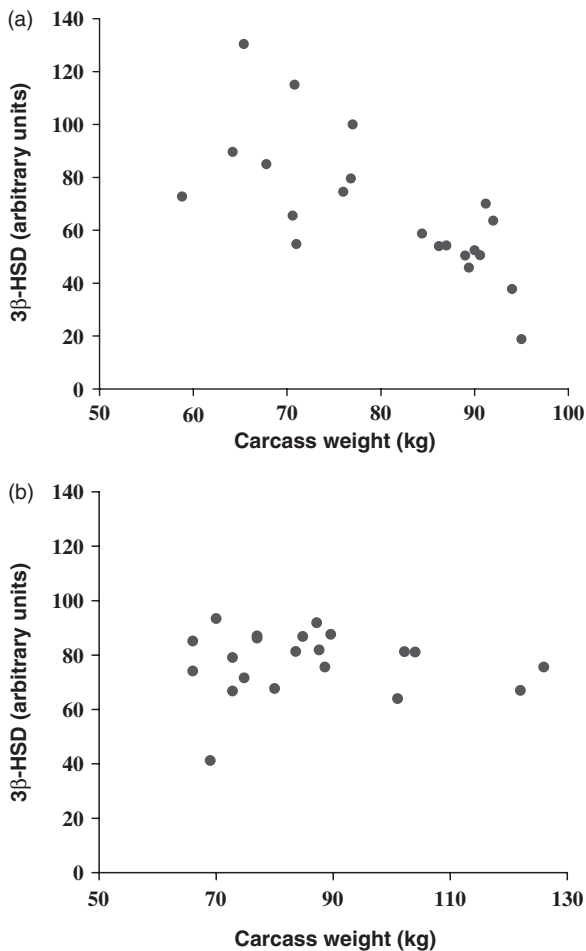
Figure 5 (**a**) Relationship between 3β-hydroxysteroid dehydrogenase (3β-HSD) protein expression in microsomes isolated from liver of entire male pigs and carcass weight. Each point represents an average of duplicate measurements for an individual pig. The duplicates varied by less than 10%. (**b**) Relationship between 3β-hydroxysteroid dehydrogenase (3β-HSD) protein expression in microsomes isolated from liver of entire female pigs and carcass weight. Each point represents an average of duplicate measurements for an individual pig. The duplicates varied by less than 10%.

tissue androstenone accumulation also depends on the rate of its clearance in the liver (Babol *et al.*, 1999; Doran *et al.*, 2004; Sinclair *et al.*, 2005). 3β-HSD is the key enzyme involved in androstenone metabolism in pig liver (Doran *et al.*, 2002b; Sinclair *et al.*, 2005). Animals with a high androstenone level have low 3β-HSD activity, mRNA and protein levels (Doran *et al.*, 2004; Nicolau-Solano *et al.*, 2006).

In the present study, the expression of hepatic 3β-HSD was negatively related to carcass weight of entire male pigs and to androstenone level in subcutaneous adipose tissue. The r^2 value (0.24) for the relationship between 3β-HSD expression and androstenone level in the present study was lower when compared with the r^2 value (0.64) in our previous study on Meishan and Large White crossbreeds. This could be due to using animals of different breeds and weights in these two studies. It is possible that the mechanisms regulating 3β-HSD expression and

androstenone deposition are breed specific. Our earlier work showed that there is a breed-specific regulation of deposition of the other boar taint compound, skatole (Doran *et al.*, 2002b).

The low expression of hepatic 3β-HSD in heavy-weight pigs observed in this study suggests that the rate of androstenone degradation in the liver of these pigs was lower when compared with the conventional-weight animals. A reduced rate of hepatic androstenone catabolism via 3β-HSD could be one of the possible explanations for an increase in the level of androstenone in adipose tissue with increasing carcass weight, as reported by Bonneau (1987), Walstra *et al.* (1999) and Sinclair *et al.* (2001). However, there are also a number of publications that did not find any correlation between backfat androstenone and carcass weight (LeDenmat *et al.*, 1993; Neupert *et al.*, 1995). The present study has also investigated the relationship between androstenone and carcass weight and has shown that although the relationship between these two parameters was significant ($P < 0.05$), the r^2 value of 0.22 indicates that only 22% of the variation in androstenone level can be explained by association with carcass weight.

Average levels of both androstenone and skatole in adipose tissue from entire males (0.919 and 0.076 μg/g, respectively) were below the commonly used respective thresholds for tainted meat (1.0 and 0.2 μg/g, respectively) (Desmoulin *et al.*, 1982; Mortensen *et al.*, 1986; Bonneau *et al.*, 1992). In the present study, around 32% of the entire male pigs showed androstenone levels above the commonly used threshold for boar taint perception. These results are in agreement with the data of the international study where over 4000 fat samples from entire males from six European countries were analysed (Walstra *et al.*, 1999). The percentage of pigs with medium androstenone level (0.5 to 1.0 μg/g) was higher in the present study compared with data for the UK pigs in the European study (59% *v.* 30.9%). This might be due to differences in the average carcass weight between pigs used in these two studies (80 and 65 kg, respectively). The results for skatole in the present work show that only 9% of entire males had a level of skatole above the threshold for boar taint. These results are not consistent with the finding of the international study, which reported that around 15% of entire males have skatole level above the threshold (Walstra *et al.*, 1999). This discrepancy between the results may be due a number of factors such as the use of different techniques for skatole measurement. In the present work, skatole level was detected by HRGC, whereas in the international study a colorimetric assay was used. The colorimetric assay detects not only skatole but the sum of skatole and skatole-related compounds, such as indole (Mortensen *et al.*, 1986). The other explanation for variation between our results and the data of the international study could be that different genotypes and production systems were used.

The fact that in our study the percentage of pigs with androstenone level exceeding the commonly used threshold for boar taint was higher than the percentage of animals

with elevated skatole level (35% *v.* 9%) might indicate that androstenone accumulation might be a primary event in the development of boar taint. This is consistent with our previous finding in cell culture experiments that androstenone inhibits the expression of the skatole-metabolising enzyme CYP2E1 in pig liver, which leads to a reduction of the rate of hepatic skatole clearance with subsequent skatole accumulation in adipose tissue (Doran *et al.*, 2002a). Gillberg *et al.* (2006) demonstrated that sex steroids can also affect the expression of another hepatic skatole-metabolising enzyme, CYP2A. In their study, the castration of mature minipig boars resulted in a significant increase in the hepatic CYP2A mRNA, protein and enzyme activity. The mechanism of androstenone action on CYP2E1 expression has been investigated in our previous study and it established that the inhibitory effect of androstenone is mediated via inhibition of binding of the transcription factor COUP-TF1 to the pig CYP2E1 promoter (Tambyrajah *et al.*, 2004).

Not only CYP2E1 expression but also CYP2E1 activity can be modulated by androstenone. Zamaratskaia *et al.* (2007) demonstrated a decrease in CYP2E1 catalytic activity in the presence of micromolar concentration of androstenone in microsomes isolated from liver of male pigs. The CYP2E1 activity in this system was also inhibited by another testicular steroid, 17β-oestradiol, but not by testosterone. It is presently unknown whether the effect of 17β-oestradiol on CYP2E1 activity is mediated via modulation of CYP2E1 expression or whether CYP2E1 expression can be regulated by sex steroids other than androstenone.

As it has been mentioned above, an excessive accumulation of androstenone in pig adipose tissue is related to a defective expression of the hepatic 3β-HSD and, as a consequence, to a low rate of hepatic androstenone metabolism. The mechanism regulating the expression of pig hepatic 3β-HSD is not clear. Studies on 3β-HSD in various tissues in rats and mice established that 3β-HSD expression in these species is under the control of sex steroids (Naville *et al.*, 1991; Soma *et al.*, 2005). The present study has shown that the expression of hepatic 3β-HSD protein significantly differed between animals of different genders (conventional-weight males and females). Moreover, in the present study we have also observed a 59% decrease in 3β-HSD protein expression in heavy-weight males (92 kg mean carcass weight) when compared with conventional-weight males (70 kg mean carcass weight). The onset of puberty is thought to be linked to the weight and the age of the pigs (Einarsson *et al.*, 1979; Schinckel *et al.*, 1984; Prunier *et al.*, 1987). Generally, the pubertal development of boars occurs around the age of 5 to 6 months (Claus *et al.*, 1994). The carcass weight of Large White male pigs at this age is around 70 kg (Dede, 1983), which corresponds to the carcass weight of animals in the conventional group of the present study. Since the animals in the present work were from the same cross-breed, were reared under the same husbandry system and were fed with the same diet, the weight of the animals was taken as a simplified indicator of sexual maturity and hence as the hormonal status of the pigs. Therefore, the weight-dependent variation in 3β-HSD protein level might also be considered as an indication of hormonal regulation of 3β-HSD expression. Further experiments are currently being carried out in our group to establish the effect of individual sex steroids on 3β-HSD expression using cell culture as a model system.

In conclusion, the present study demonstrates that an increase in the slaughter weight of entire commercial crossbred Large White male pigs is accompanied by an inhibition of hepatic 3β-HSD protein expression. The expression of pig hepatic 3β-HSD protein depends on the gender and weight of animals, which suggests that 3β-HSD expression might be under the control of sex hormones. Low expression of 3β-HSD in heavy-weight pigs was accompanied by an increased level of androstenone in subcutaneous adipose tissue, which might be due to a reduced rate of androstenone clearance in liver of these animals.

Acknowledgments

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