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Exogenous influences on skatole formation in the pig

DISSERTATION

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ACTH	Adrenocorticotropic hormone
ALF	Alfalfa hay
ANOVA	Analysis of variance
BLE	German Federal Office for Agriculture and Food
BMELV	German Federal Ministry of Food, Agriculture and Consumer Protection
cm	Centimeter
CO2	Carbon Dioxide
CRH	Corticotropin-releasing hormone
CYP	Cytochrom P
d	Day
DM	Dry matter
ELISA	Enzyme-linked immunosorbent assay
EtOH	Ethanol
FLD	Fluorescence detection
FOS	Fructooligosaccharides
FSH	Follicle stimulating hormone
GnRH	Gondadotropin releasing hormone
hCG	Human chorionic gonadotropin
HSF	High Skatole Feces
HSF-S	High skatole feces superficial
HSF-D	High skatole feces deep
HST	Hydroxysteroid sulfotranferase
i.v.	Intra venous
IAA	Indole acetic acid
IPA	Indole pyruvic acid
IS	Internal standard
kg	Kilogram
LH	Luteinizing Hormone
MeOH	Methanol
Min	Minutes
MJ	Megajoule
ME	Metabolizing Energy
Ml	Milliliter
mm	Millimeter
mml	Millimole
n.d	Not defined
NaOH	Sodium hydroxide

NGO	non governmental organisation
nm	Nanometer
OATPs	organic anion-transporting peptides
hCG	Human chorionic gonadotropin
PCA	Principal component analysis
PT	Pre unloading time
PUFA	polyunsaturated fatty acids
PB	punch biopsy
PBG	punch biopsy group
Rcf	Relative centrifugal force
RPS	Raw potato starch
RYE	Rye grass hay
SB	Surgical biopsies
SBP	Sugar beet pulp
SCFA	Short chain fatty acid
SFA	Saturated fatty acid
SBG	Surgical biopsy group
T_A	Threshold androstenone
TRP	Tryptphane
T_S	Threshold skatole
TT	transport time
UGT	Uridin diphosphate glucuronyltransferase
UPLC	Ultra Performance Liquid Chromatography
v/v	Volume/volume
vs	Versus
µg	Microgram
µm	Micrometer
µmol	Micromol
°C	Degree Celsius

CHAPTER 1

GENERAL INTRODUCTION

1 GENERAL INTRODUCTION

1.1 CASTRATION OF MALE PIGLETS: THE DILEMMA BETWEEN ANIMAL WELFARE AND CONSUMER PROTECTION

Pig production in Europe is facing tremendous changes in the near future. So far, castration of male piglets without anesthesia until day 7 post natum is common legal practice in Europe. In a national declaration (Düsseldorfer Erklärung, 2008) German stakeholders took a stand to ban castration of conscious male piglets as soon as possible. In the year 2010 the European meat industry, retailers and NGOs agreed voluntarily to end castration of young male piglets by 1st of January of 2018. This agreed stand became part of an amendment of national animal welfare laws (TierSchG, 2013), so that castration of male piglet without anesthesia ends in Germany by the 1st of January, 2019. However, the available alternative methods to conventional castration are still under-developed and need further modification before adoption into practice (Von Borell et al., 2009).

The importance of the traditional castration of male piglets in most of the Europe countries is the prevention of sex specific off odor called the boar taint (Goltz, 1897). Even if the substances responsible for the off odor were unknown until the 20th century, it was well established since centuries, that castration was effective to avoid the occurrence of boar taint Irrespectively of different levels of daily gain and the duration of fattening period at the end of the 19th century, the amount of tainted carcasses from entire males was assumed to be about 20% at that time (Goltz, 1897).

About 70 years later, however, both Patterson (1968) and Vold (1970) identified the testicular steroid androstenone and tryptophan metabolite skatole respectively as the two different causative substances of the boar taint. Both compounds are lipophilic and thus accumulate in fat but differ enormously in consumers' perceptual experience. In this regards, for androstenone a specific anosmia has been shown for about 30% of the consumers in several studies; whereas skatole can be perceived by almost every consumer (Whittington et al., 2011; Weiler et al., 2000, 1998; Claus, 1979). In addition, both substances differ in their origin of formation, physiological impact, and the effects of management and environmental factors (Weiler & Wesoly, 2012; Wesoly & Weiler, 2012 a,b). In the upcoming section, the formation, metabolism of both androstenone and skatole will be discussed in detail basing on the review of Weiler & Wesoly (2012).

1.2 THE BOAR TAINT COMPOUND - ANDROSTENONE

1.2.1 PHYSIOLOGICAL ROLE OF ANDROSTENONE IN PIGS

Androstenone (5α-androst-16en-3one), one of the chemical compounds that constitute the pheromones in the boar, is characterized with its urine like odor. In addition to androstenone are the two musk like alcoholic metabolites 3α - and 3β-androstenole (5α-androst-16en-3α-ole and 5α-androst-16en-3β-ole). The structural difference between pheromone and hormone active steroids is the presence of a double bond at the C16 atom in the pheromonal active steroids, also known as Δ16 steroids. These steroids are either released via salivary foam during pre-mating behavior of boars or excreted with urine (Claus, 1979; Booth, 1982). The mixture of pheromonal active steroids affect various reproductive phenomena in sows but also plays an important role in communication between male and female individuals, as well as in intermale communication (Signoret, 1970; Claus, 1994).

Boar pheromones encode information about individuality and social rank between male pigs (Giersing et al., 2000) through differences in the overall level and an individual mixture of these three compounds (Katkov et al., 1972; Claus, 1979, Fischer et al., 2011). In mixed groups of unfamiliar gilts and barrows, topical application of androstenone reduced aggressive behavior compared to untreated control pointing to a function in establishing and preserving social hierarchy (Mc Glone & Morrow, 1988).

In addition, boar pheromones also influence several reproductive phenomena in female pigs. It is well established that the weaning to estrus interval is reduced if sows are in contact with boars. Boar pheromones play a major role in inducing this estrus behavior especially standing heat in sows (Signoret, 1970). Even if several stimuli (e.g., optic, acoustic) of a boar act together, boar pheromones are the main stimulus in this reaction (Signoret, 1970; Langendijk et al., 2003, 2005). Therefore application of “boar scent spray” has become a routine mechanism during artificial insemination of sows. In addition, boar pheromones can induce the release of oxytocin in sows during estrus (Mattioli et al., 1986; Claus & Schams, 1990), although the effect is more pronounced when a real boar is present (Langendijk et al., 2003, 2005). In addition to seminal estrogens, oxytocin release during mating hastens sperm transport in female genital tract to the site of fertilization (Claus & Schams, 1990; Langendijk et al., 2005)

Similarly, boar presence due of its pheromones is known to induce earlier puberty with higher prepuberal estrogen secretion in gilts, leading to significantly larger and heavier uteri at puberty (Booth, 1984; Mezger, 1994).

Due to their importance in reproduction, synthesis of boar pheromone is closely linked to testicular hormone synthesis. In consequence, spermatogenesis and libido are therefore always influenced in the same direction as the formation of pheromonal active steroids (see 1.2.2.2).

1.2.2 PATHWAY AND REGULATION OF ANDROSTENONE SYNTHESIS

1.2.2.1 SYNTHESIS OF ANDROSTENONE AND ANDROSTENOLES

Androstenone is synthesized in testicular Leydig cells and may be further reduced into the 3α- and 3β-androstenole. The musky smelling androstenoles predominate in the testes and the salivary glands, while androstenone exceeds concentrations of the androstenoles in adipose tissue several fold. The pathway of androstenone synthesis is given in Fig.1.1.

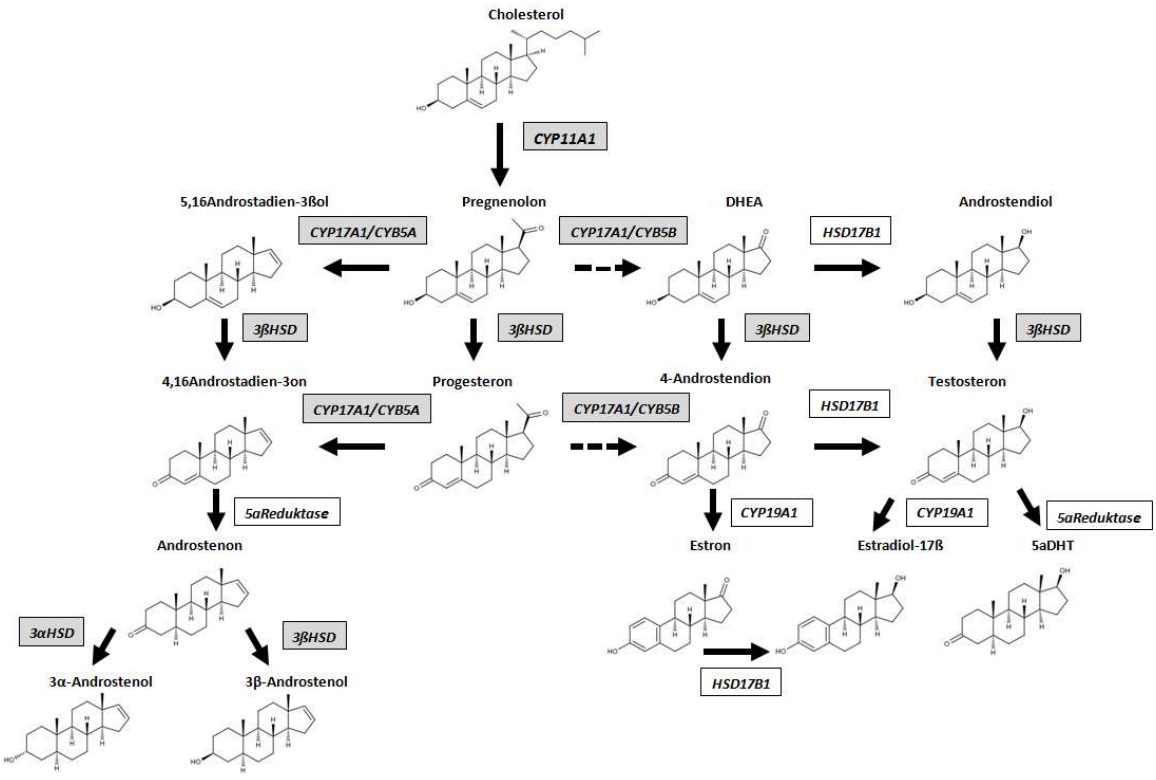

Fig.1.1: Pathway of androstenone synthesis (from Weiler & Wesoly, 2012)

Androstenone originates initially from cholesterol metabolism. Cholesterol is metabolized through the CYP11A1 enzyme into pregnenolone and via 3ß-HSD further to progesterone, the main precursors of hormone and pheromone synthesis (pregnenolone: Δ5-synthesis pathway, progesterone: Δ4-synthesis pathway).

From pregnolone and progesterone the acetyl group at C_{17} is substituted by the pheromone specific double bond at C_{16}. The precursors 5,16 androstadien-3ß-ole (Δ5-synthesis pathway) and 4,16 androstadien- 3-one (Δ4-synthesis pathway) are synthesized at this step. In the pathway specific for hormone synthesis, the acetyl group is substituted by a ketone group at C_{17} and DHEA and 4-androstadion are synthetized this way. The key enzyme regulating the hormone and pheromone synthesis is CYP17A1 with both, 17 hydroxylase and 17,20 lyase activity respectively. In addition, CYP17A1 also has a 16-ene-synthase activity essential for pheromone synthesis (Soucy et al., 2003). CYP17A1 enzymatic activity provides precursors for pheromone or hormone synthesis and is regulated through CYB5 isoenzymes. Pheromone synthesis is stimulated when CYP17A1 interacts with CYB5A, whereas hormone synthesis is catalyzed when CYP17A1 interacts with CYB5B (Billen & Squires, 2009).

1.2.2.2 REGULATION OF ANDROSTENONE AND ANDROSTENOLE SYNTHESIS PATHWAY

Formation of steroids in the Leydig cells is under hypothalamic control, which regulates hormone synthesis in testis, especially androgens and estrogens (see Fig.1.2). If concentrations of these hormones are low, the release of gonadotropins, FSH (Follicle Stimulating Hormone) and LH (Luteinizing Hormone), is stimulated by the hypothalamus through GnRH (Gondadotropin Releasing Hormone). FSH and LH in turn stimulate function in specific target cells; LH targets Leydig cells, while FSH targets Sertoli cells and spermatogonia. LH stimulates testicular steroid synthesis via the hormone dependent activity of the key enzymes CYP11A1, CYP17A1 and 3ßHSD, which controls hormone and pheromone synthesis (Lejeune et al., 1998; Robic et al., 2008; Lervik et al., 2011). Additionally the activity of these key enzymes can be stimulated by IGF-I, explaining in part the effect of nutrition on testicular activity (Chuzel et al., 1996).

The close relationship between LH and the synthesis of testicular steroid was proven by the application of hCG (Human Chorionic Gonadotropin) (Andresen, 1975; Claus & Alsing, 1976) and confirmed in more recent studies (Zamaratskaia et al., 2006; Oskam et al., 2010). After hCG injection, androstenone concentrations increase in blood within 90 minutes to reach a first maximal value, and a second peak can be measured after 24 - 28 hours (Andresen, 1975; Claus & Alsing, 1976). The first peak of this bimodal course is caused as a result of

rapid metabolism of available precursors like pregnenolon into testosterone or Δ16-steroids. The second peak occurs with delay and represents the de novo steroid synthesis (Hurden et al., 1984). Basal concentrations of androsterone and testosterone are measured in blood within three days after a single hCG application (Claus & Alsing, 1976).

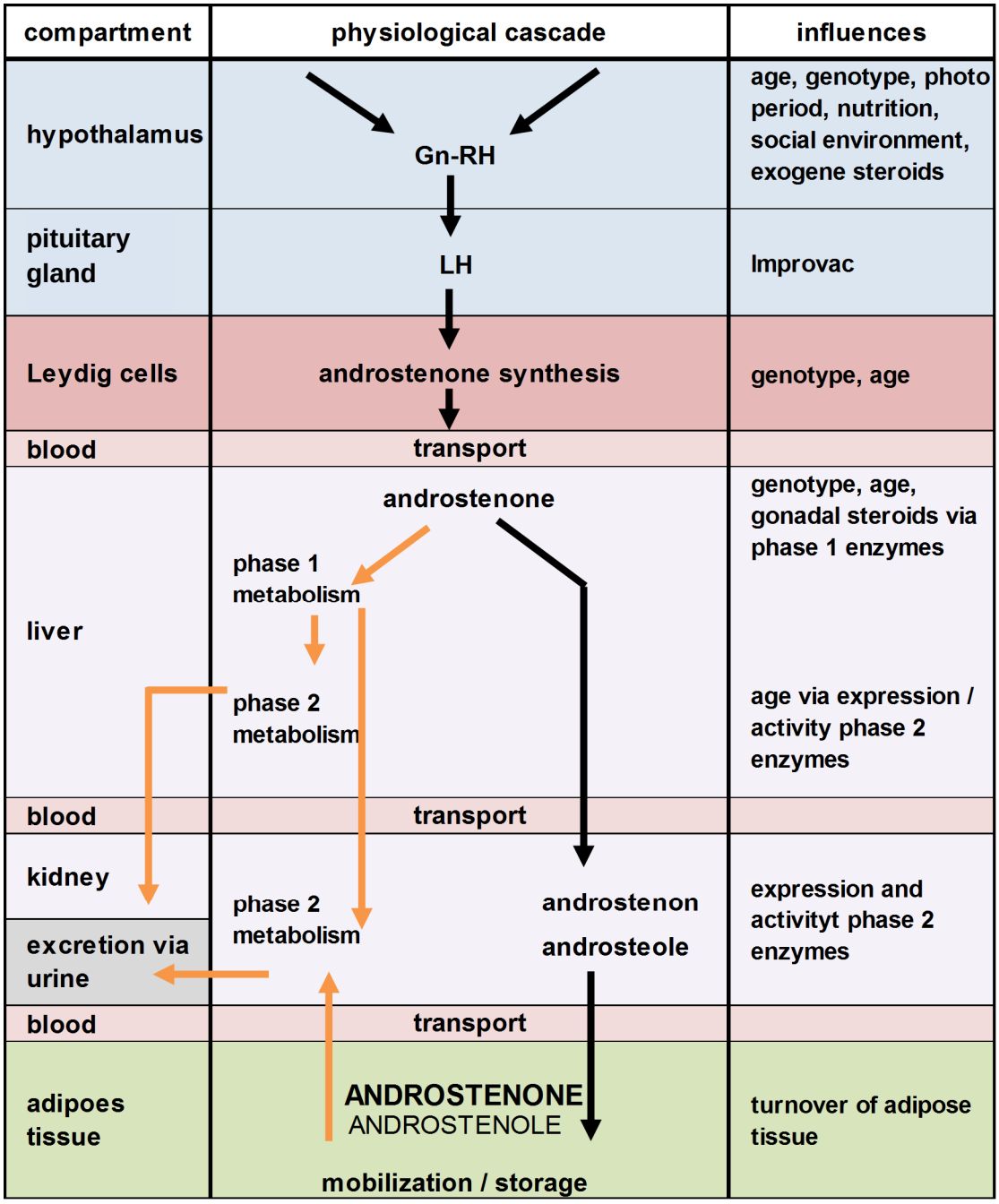

Fig.1.2: Cascade of physiological events leading to androstenone formation, further metabolism and accumulation of androstenone in adipose tissue (according to Weiler & Wesoly, 2012)

The predominant hormonal role of GnRH can be proven through repeated Improvac® injections that results in immunological inhibition of GnRH. In addition, the reduced LH secretion is reflected by lower androstenone and testosterone concentrations within four to eight days after a second Improvac® booster injection. This is shown in the 75% reduction of androstenone concentrations in blood from 1.48 ng/ml to 0.35 ng/ml; and this reduction is also strongly correlated to the testosterone blood level ($r=0.67$; $p<0.001$) (Claus et al., 2007).

Furthermore, high doses of GnRH analogs also induce an inhibition of LH secretion from pituitary gland (Xue et al., 1994; Schneider et al., 1998; Kauffold et al., 2010). Similarly, testicular hormone production is inhibited through application of feed-back active steroids (Daxenberger et al., 2001). Those treatments lead to reduced androstenone and testosterone synthesis accordingly (Xue et al., 1994, Schneider et al., 1998, Daxenberger et al., 2001).

1.2.3 DISTRIBUTION OF ANDROSTENONE AND ANDROSTENOLES IN DIFFERENT TISSUES AND ITS METABOLISM

1.2.3.1 SELECTIVE ACCUMULATION OF Δ 16 STEROIDS IN ADIPOSE TISSUE AND SALIVARY GLANDS

After formation in Leydig cells, a major percentage of Δ16 steroids are sulfate conjugated and in consequence more hydrophilic than the unconjugated steroids. The sulfate conjugated variants comprise the main fraction (up to 70%) of Δ16 steroids measurable in peripheral blood (Sinclair & Squires, 2005). Due to higher lipophilic properties, only unconjugated Δ16 steroids may accumulate in adipose tissue (Gower, 1972; Gower et al., 1997; Sinclair & Squires; 2005), despite that conjugated steroids can be transported actively into adipose tissue through OATPs (organic anion-transporting peptides) as described for human adipocytes (Valle et al., 2006). A similar mechanism for Δ16 steroids in pigs has been discussed, but has not been validated (Desnoyer, 2011). Unconjugated androstenone is more lipophilic than the two androstenoles. Accordingly, androstenone deposition into adipose tissue is favored almost selectively when testicular steroid production is increased as shown in Fig.1.3. Thus, in contrast to androstenoles, which predominate in testicular tissue but not in fat, 5-10 fold higher androstenone concentrations can be measured in fat than in the testes (Claus, 1975). Elevated androstenone concentrations in fat were measurable already 6-7 hours after hCG application in an individual boar (Claus & Alsing, 1976, Claus & Karg, 1976).

Androstenone concentrations in blood are relatively low compared to 10 fold higher levels in testes and 100 fold higher concentrations in fat (Claus et al., 1971; Claus, 1979; Sinclair &

Squires, 2005). The maximum detectable concentration of Δ16 steroids of up to 114 µg/g, however, can be found in salivary glands (Babol et al., 1995). Mainly 3α-androstenole predominates in salivary glands as it accumulates almost selectively through the porcine steroid binding protein pheromaxein (Booth, 1984; Booth & van Glos, 1991). In addition, the highest concenrations of pheromaxein are found in entire males (Babol et al., 1996); and androstenone concentrations are up to three fold higher in salivary gland tissue compared to adipose tissue.

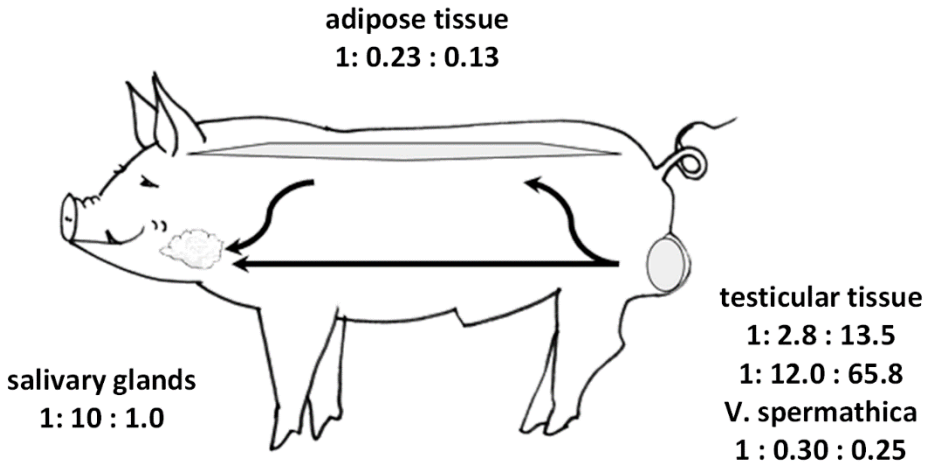

Fig.1.3: Relation between concentrations of androstenone, 3α-androstenole and 3β-androstenole in blood and tissues of boars (androstenone : 3α-androstenole : 3β-androstenole) (from Weiler & Wesoly, 2012).

Accumulation of androstenone in adipose tissue is as a result of the rate of diffusion from blood and its concomitant rate of release. However, the rate of diffusion into fat is mainly determined by the level of steroid synthesis in the testes.

The rate of release of androstenone from fat after castration is age dependent and differs between mature and pubertal boars. Claus (1976) reported a half-life of androstenone concentrations in fat of one week for young boars (100 kg LW) compared to 2.5 weeks in mature boars (140 - 240 kg LW). It may be assumed, however, that these differences can be explained by age specific turnover rates of adipose tissue possibly due to differences in fatty acid composition as discussed later (1.2.4.2). The rate of release is of importance because it defines the period between booster injection and before slaughter of vaccinated boars.

Within the carcass, androstenone concentrations reveal a moderate variability as reported for nine different ventral and dorsal subcutaneous fat samples, but concentrations tended to be slightly higher in flare fat (Claus, 1975).

1.2.3.2 METABOLISM AND EXCRETION OF ANDROSTENONE

Metabolism of androstenone can be divided into two steps, an oxidative phase (phase 1) and a conjugative phase (phase 2). The enzymes involved in phase 1 metabolism are well described in literature. Main key enzymes are identified to be 3α-hydroxysteroide hydrogenase and 3ß-hydroxysteroide hydrogenase (3α-HSD and 3ß-HSD) (Doran et al., 2004; Sinclair et al., 2005). The detailed mechanism of activity of 3α-HSD and 3ß-HSD involves replacing the ketone group of androstenone with a hydroxyl group, this reduction process results in the alcoholic variants 3α- and 3ß-androstenole (see Fig.1.1). First phase 1 metabolism steps occur already in testes resulting in the specific mixture of androstenone and androstenoles as described above. The main phase 1 metabolism occurs during the degradation of Δ16 steroids in the liver. An earlier study confirmed the importance of this step for androstenone metabolism since expression of 3α-HSD and 3ß-HSD in the liver, but not in testis correlated negatively with androstenone concentrations in fat (Nicolau-Solano et al., 2006).

The hydroxyl group at the Δ16 steroids is a prerequisite for the conjugation step in phase 2; as the main enzymes involved in androstenone phase 2 metabolism HST/SULT2A (hydroxysteroid sulfotranferase) and UGT (uridin diphosphate glucuronyltransferase) can only link sulfate or gylucuronic acid - group to a functional hydroxyl group (Sinclair & Squires, 2005; Moe et al., 2007; Zamaratskaia et al., 2012). This conjugation step increases the polarity of androstenoles and reduces the diffusion of the hydrophilic conjugates into adipose tissue. Sulfate conjugation of Δ16 steroids may also be regarded as a transport mechanism in blood, because a local activity of sulfatase has been shown for different target tissues, which results in the unconjugated androstenoles (Coughtrie et al., 1998). An *in vitro* study with Δ16 steroid incubation with Leydig and hepatic cells revealed that sulfate conjugation of 3β-androstenole is more relevant in Leydig cells (70%) than in hepatic cells (21%), whereas in hepatic cells 68% of incubated 3α- and 3ß-androstenole were conjugated with glucuronic acid in a process of glucuronidation (Sinclair et al., 2005). Glucuronidation is an important process for the excretion of Δ16 steroids, since Δ16 steroids are found in urine mainly conjugated to glucuronic acid (Gower, 1984).

1.2.4 FACTORS AFFECTING THE FORMATION AND STORAGE OF ANDROSTENONE IN FAT

1.2.4.1 INFLUENCE OF SEX, AGE, AND LIVE WEIGHT

Androstenone is synthesized almost exclusively in testicular tissue. Accordingly, only very low concentrations of androstenone originating from adrenal cortex can be measured in sows, gilts and barrows. Similarly, as described for steroid hormones, cryptorchidism leads to similar androstenone concentrations as observed in intact boars of the same age (Claus, 1979).

Changes in the level of androstenone in fat and blood can reflect the age related changes of testicular function along puberty. Synthesis of testicular steroids in young male piglets increases during the first 3 to 6 weeks of age, so that testosterone levels up to 2.5 ng/ml blood can be measured (Booth, 1975; Franca et al., 2000; Lanthier et al., 2006). The biological function of early increase in testicular hormone synthesis is not clear, but a role during sexual differentiation of behavior has been discussed (Ford, 1990). Also an effect on the endocrine balance between androgen and estrogen synthesis in mature animals (Kotula-Balak et al., 2012), or for sex specific growth promotion via the synthesis of anabolic active nortestosterone (Choi et al., 2009) have been suggested. This transient increase of testicular hormone synthesis in young piglets is accompanied accordingly by an elevated Δ16-steroid synthesis, mainly 3α-andostenole (Booth, 1975). However, systematic measurements in such young piglets are still scanty.

Along pubertal development, levels of testicular hormones increase in peripheral blood and reach transient maximum values at an age of about six to eight months depending on genotype. Thereafter, concentrations of testicular hormones decrease slightly to the mature level (Claus & Hoffmann, 1980; Franca et al., 2000). Accordingly, androstenone concentrations in adipose tissue increase, however with less day to day variations. Repeated adipose tissue biopsies in growing boars revealed androstenone concentrations above a threshold of 0.5 µg/g after 150 days of age and reached a maximum level after 250 days (Claus, 1975).

Consequently, endogenous and exogenous factors stimulating an earlier puberty hasten the increase in androstenone synthesis and in parallel, androstenone metabolizing phase 1 and phase 2 enzymes are affected. In a recent study, it was shown that the expression of 3ß-HSD was significantly lower in older and heavier boars than in younger and lighter male animals (Nicolau-Solano & Doran, 2008).

Also androstenone concentrations may vary considerably between carcasses of similar slaughter weights. A European-wide study using 4313 slaughtered boars and high variability of slaughter weights could only in part explain the observed elevated androstenone concentrations through higher slaughter weights (Walstra et al., 1999). Carcasses from the UK had the lowest slaughter weights in this study; and they were significantly more likely to reveal high androstenone concentrations than those from the Netherlands. On the other hand, Dutch boars had the lowest amount of androstenone tainted carcasses (>1 µg/g) but the highest carcass weights. These differences can be partly explained by the different genotypes used in the countries included in this study.

1.2.4.2 INFLUENCE OF GENOTYPE

Genetic selection against high androstenone levels seems to be favorable due to high heritability of $h^2 = 0.6$, and is applied in several breeding programs and has been reviewed recently (Frieden et al., 2012). The physiological mechanisms leading to the reduction of androstenone, however, have to be considered in detail to avoid negative side effects on sow fertility (Frieden et al., 2014).

Genetic influences on androstenone production, metabolism and accumulation in fat have been studied extensively in the past (Robic et al., 2008). In general, the genetic determination of the age at puberty is regarded as a main genetic mechanism, to explain differences in androstenone levels at slaughter weight. A high heritability for the trait androstenone level was demonstrated in an early selection study, leading to two lines with distinct differences in androstenone concentrations within 3 generations. A similar effect was also observed with the concentration of testosterone, even if this criterion was not included into the selection program (Willecke et al., 1980). Additionally, the turnover rate of adipose tissue may modify androstenone accumulation in fat since leaner breeds in general have both a higher fat turnover and lower androstenone concentrations (Metz & Dekker, 1981; Mörlein & Tholen, 2014).

A further promising approach for breeding programs is the identification of differences between genotypes in the activity of androstenone metabolizing enzymes (Moe et al., 2007) or differences in the enzyme pattern during steroid synthesis (Bai et al., 2014). Until such an approach can be included into breeding programs further research is needed.

1.2.4.3 INFLUENCE OF HOUSING AND SOCIAL ENVIRONMENT

It was shown in an earlier study that testicular steroid synthesis of boars was influenced if the animals were transferred into a new unfamiliar pen. Testosterone and androstenone levels in blood were about 3 fold higher after the transfer and remained elevated for 7 days (Claus & Alsing, 1976). The concentration of androstenone in adipose tissue was two fold elevated in a fat biopsy taken 15 h after the transfer. However, this study was carried out with individual animals and the impact of these factors under practical conditions has not been studied yet.

A similar effect was described when boars were penned close to estrous gilts (without mating). Androstenone and testosterone concentrations increased to maximum levels (two to three fold) within 90 minutes. When boars were allowed to mate however, concentrations further increased and reached maximum levels of 15 ng testosterone/ml blood plasma within 15 minutes and 13.3 ng androstenone/ml blood plasma after 90 minutes. In addition, the fat biopsy taken 4 days after mating also revealed increased androstenone concentrations (Claus & Alsing, 1976); however, even without mating, such an increase may be observed. It was shown that pigs kept in mixed groups of both sexes during fattening revealed significantly higher androstenone concentrations than boars raised in single sex groups at various ages (81 kg, 93 kg, and 110 kg). This effect was more pronounced in older and heavier animals (Patterson & Lightfoot, 1984). In contrast however, social isolation of boars significantly lowered androstenone concentrations in blood within 5 days (Claus, 1979). The social rank also affects the level of steroid synthesis. Boars with higher social rank had higher testosterone and androstenone concentrations compared to boars with a lower social rank (Giersing et al., 2000). This difference was obvious for animals with 85 kg as well as for heavier animals of 115 kg live weight. The effect was more pronounced for androstenone than for testosterone. If social ranks remains stable (constant litter groups from weaning to slaughter) the androstenone concentrations in boars tended to be lower than in groups mixed after weaning (Fredriksen et al., 2008).

1.2.4.4 INFLUENCE OF NUTRITION

Nutritional influences on androstenone are ascribed either to the effects of energy or to specific compounds in the ration affecting androstenone synthesis and metabolism. The overall effect of nutrition on androstenone, however, is less important than for skatole.

In contrast to gilts, the occurrence of puberty in boars is influenced by both, age and live weight, thus influencing also androstenone levels. A study with ad libitum fed boars compared to a restrictively fed control, slaughtered at same weight (125 kg) or same age

(180 days), revealed both, age and live weight influences on androstenone deposition in fat (Bonneau, 1987). However, live weight tended to have a more pronounced effect on this parameter in this study compared to age at puberty. This is in accordance with other findings, where high-energy nutrition increased IGF-I release and in consequence steroid synthesis (Claus et al., 1994; Chuzel et al., 1996). This positive relationship between steroid synthesis and growth intensity may counteract the strategy to intensify boar fattening to reach slaughter weight before puberty. However, in some studies with different energy contents in diets (11.7 vs 13.7 MJ ME/kg; Lundström et al., 1988 or 12.6 vs 13.8 MJ ME/kg; Neupert et al., 1995) no or only a slight tendency for higher androstenone concentrations in groups fed with higher energy content were obvious (12.6 MJ ME vs 13.8 MJ ME: 0.81 vs 1.37 µg androstenone/g fat; each 75 kg slaughter weight; Neupert et al., 1995).

A recent study, initially designed to check the nutritional effect of specific feed additives on skatole concentrations concomitantly lowered androstenone concentrations in fat. This effect was ascribed to changed enzyme activities (3ß-HSD) (Rasmussen et al., 2012). Similarly, the feeding of charcoal to reduce the recycling of androsteone in the entrohepathic circulation was effective to lower androstenone in fat (Jen & Squires, 2011). A promising approach was described in rats, feeding of papaya seed extract resulted in reduced cholesterol concentrations in testes (Lakshman & Changamma, 2013). As cholesterol is the main precursor of testicular steroid synthesis (see Fig.1.1), a reduction of the precursor cholesterol could result in decreased androstenone synthesis in some mammals, but this has not been investigated in pigs.

1.2.4.5 INFLUENCE OF PHOTOPERIOD

In male wild boars, photoperiod affects the activity of the central metabolic hormones, testicular function and feed intake (Weiler et al., 1996). In details, testicular function is increased with decreasing day length and inhibited with increasing day length. The seasonal course is typically bimodal with a second smaller increase in the month of May corresponding to a second mating season, as shown in samples from hunted wild boars (Treyer et al., 2012).

A different degree of seasonality can be observed in domestic pigs, which is mainly reflected by a reduced fertility in sows during the summer season (Claus & Weiler, 1985). Decreasing day length accelerates the beginning of puberty in boars and consequently leads to higher androstenone concentrations in fat at a given age (Claus & Weiler 1985, Andersson et al. 1998). However, these effects of seasonal variation or photoperiod are not confirmed in most studies, due to the effect genotype and other environmental factors (Walstra et al., 1999;

Neupert et al., 1995, Weiler et al., 1995). Seasonal variations of androstenone and skatole concentrations in blood plasma of a wild boar are illustrated in Fig.1.4. It is obvious that skatole formation is uncoupled from steroid formation in this animal model (Weiler et al., 1992; Claus, 1993). The seasonal course of androstenone is in accordance to the decreasing day length. This effect of season on androstenone was recently confirmed in domestic boars by Frieden et al. (2014).

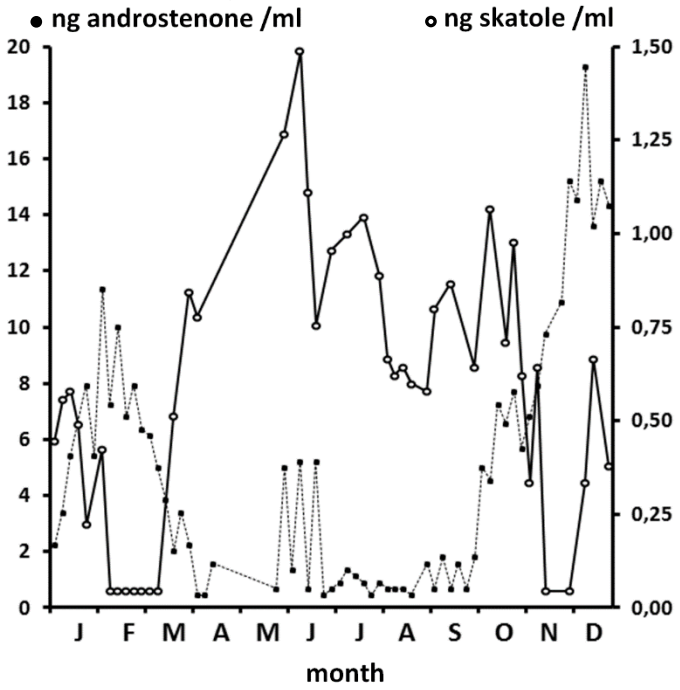

Fig.1.4: Seasonal changes of androstenone and skatole concentrations in male wild boar (from Weiler & Wesoly, 2012).

1.3 THE BOAR TAINT COMPOUND-SKATOLE

Skatole is a lipophilic compound stored in adipose tissue and contributes to off odors in boars. Skatole formation is located in the colon and compared to androstenone therefore not limited to the male sex neither to pigs. However, skatole concentrations in boar carcasses are higher than in gilts and barrows (Robic et al., 2008). The main reason for elevated skatole concentrations in boars is the interaction of steroids and skatole in liver metabolism (Kojima & Degawa, 2013; Rasmussen et al., 2013) leading to a reduced hepatic skatole degradation and a higher accumulation in fat.

For consumer perception of off odor of boar carcasses skatole is at least as relevant as androstenone. A specific anosmia, as described for androstenone has not been described for skatole so far. The fecal-like odor of skatole can be perceived by almost 100% of consumers and is mostly judged negative (Annor-Frempong et al., 1997; Weiler et al., 2000; Meier-Dinkel et al., 2015). A concentration threshold for both substances is mandatory to quantify and define the precise level for consumer rejections. However, the thresholds defined in earlier studies (0.5 µg androstenone/g fat and 0.25 µg skatole/g fat) are under review as new studies recommend a far lower threshold for skatole (0.1 µg/g - 0.2 µg/g) in boar carcasses (Lunde et al., 2010; Bonneau & Chevillion. 2012; Meier-Dinkel et al., 2013). Bonneau & Chevillion (2012) reported low consumer rejections in samples with very low skatole concentrations (0.04 - 0.05 µg/g) but high androstenone concentrations (2.1 - 3.4 µg/g). However, if skatole concentrations are high (>0.25 µ/g) samples are commonly scored negatively, irrespective of androstenone concentrations (Weiler et al., 2000). Disregarding variances of androstenone perception among consumer, lowering the risk of skatole tainted carcasses could be a promising approach to ensure quality safety in boar meat. Due to the origin of skatole formation in the colon, it may be predominately influenced by nutrition. At present, there is no feed additive capable of reducing skatole concentrations under practical conditions, considering economic limitations in form of feed conversion and daily gain. However, beside nutrition other exogenous factors are discussed controversially since decades, like the effect of soiling and transdermal skatole diffusion postulated in the early 90s (Hansen et al., 1994). Such an effect however, was not confirmed in later studies (Aluwe et al., 2011; Bekaert et al., 2012; Van Wagenberg et al., 2013).

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CHAPTER 2

2 OBJECTIVES

The general scope of this present thesis can be characterized as follows:

- to summarize latest knowledge of factors influencing skatole formation and to evaluate promising approaches to reduce skatole concentrations in fattened boars, especially with focus on nutritional factors (Chapter 2)

- to identify factors influencing boar taint compounds from farm to slaughter (Chapter 3)

- to investigate environmental factors influencing skatole concentrations while housing (Chapter 4)

CHAPTER 3

REVIEW

NUTRITIONAL INFLUENCES ON SKATOLE FORMATION AND SKATOLE METABOLISM IN THE PIG

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3 NUTRITIONAL INFLUENCES ON SKATOLE FORMATION AND SKATOLE METABOLISM IN THE PIG

3.1 ABSTRACT

Skatole is a tryptophan (TRP) metabolite with fecal odor. Together with the testicular steroid androstenone it is regarded as a main determinant of boar taint, even if elevated concentrations of skatole occur occasionally in gilts and barrows. Skatole concentrations in adipose tissue result from a complex process, which includes the availability of TRP and the presence of specialized bacteria in the gut in need of TRP for energy production, as well as absorption, transport and accumulation of skatole in adipose tissue. Several steps of this process are influenced by diet and specific feed compounds. In the present paper the current knowledge about physiological mechanisms of skatole dynamics is summarized. Additionally mechanisms are discussed, by which effective feeding strategies and feed additives exert their influence in the prevention of high skatole concentrations in adipose pig tissue. It was concluded that the most effective measures are those which influence several steps of skatole formation. Despite the numerous studies carried out in the field of skatole physiology, interesting aspects still need clarification, such as the effect of adipose tissue turnover. Reliable control of skatole accretion in fat of boars is one of the main prerequisites for pork production with entire males.

3.2 INTRODUCTION

Boar taint is one of the major problems occurring in meat production of entire male pigs [1]. The two main compounds contributing to boar taint are androstenone (5α-androst-16-ene-3one, [2,3]), a steroidal pheromone produced in the Leydig cells of the testis, and skatole (3-methyl-indole, [4]), a product of microbial degradation of tryptophan (TRP) with fecal odor [5]. Skatole can be found in several species including ruminants, where it is mainly formed in the rumen, and monogastrics, such as the pig, where it is mainly formed in the colon [6]. In the pig, skatole has gained considerable scientific interest and is discussed as one of the principal compounds contributing to the off-odor of meat from entire males, but elevated concentrations occur occasionally in females and barrows [7]. Whereas the physiology of androstenone formation has been studied extensively and environmental and genetic influences are known [8,9], the physiological mechanisms of skatole formation, absorption and its metabolism within the pig are less clear. While androstenone concentration is only moderately influenced by nutrition [9,10,11,12,13], various feeding strategies are known to influence the concentrations of skatole in adipose tissue. This topic has been the subject of several scientific reviews during the last decade [9,14,15]. As a lot of new studies are

available, it is the aim of the present paper to describe current knowledge about the mechanisms leading to skatole formation and accretion in adipose tissue of pigs. Based on these mechanisms, an overview on possible feeding strategies or nutritional manipulations of skatole formation is summarized and their efficiency discussed.

3.3 PHYSIOLOGY OF SKATOLE FORMATION AND DEPOSITION IN ADIPOSE TISSUE

3.3.1 BIOCHEMICAL PATHWAY AND MICROBIAL ACTIVITY

Skatole results from a multistep degradation of TRP by microbial activity, mainly in the hind gut of the pigs [16]. Analogously, many other metabolites such as indole are formed which may also contribute to off odor in pork [17].

The reaction mechanism of TRP degradation is limited under the anaerobic conditions of the intestinal tract to reductive processes at the 3-position of the TRP indole ring structure, yielding the terminal products skatole and indole [16]. Whereas many bacteria are able to metabolize tryptophan to indole and indole acetic acid (IAA), the key precursor of skatole, only a few specialized gut bacteria, mainly from the Clostridium and Bacteroides genera, can catalyze the steps from IAA to skatole [18,19,20]. It was estimated that these bacteria represent less than 0.01% of the total intestinal flora [21]. In case of skatole, intermediate products are indole-3-lactic acid and indole-3-pyruvic acid, resulting from the deamination of TRP, which is decarboxylated to IAA, and then further decarboxylated to skatole (see Fig.3.1, upper part) [16,20]. This metabolic pathway has been identified in Lactobacilllus sp. strain 11201 [22] but also in C. drakeii and C. skatologenesis using deuterium labeled TRP or IAA through in vitro studies with bacteria from swine manure [20]. In this study, adding TRP together with glucose reduced the amount of skatole and increased IAA with both types of Clostridia. Similarly, the addition of TRP to cell cultures rich in C. scatologenes did not necessarily increase skatole formation [23]. Such data suggest that the microbial skatole forming activity is reduced, if alternative energy substrates are available, as further discussed below (see Section 3.3).

In two more recent studies which compared the microbial community in the colon of Jinhua and Landrace pigs, C. aminophilum was also suggested to affect skatole production in the Jinhua breed [24]. The same authors published an in vitro study with porcine gut microbes, which points to a contribution of C. disporicum to skatole formation [25]. A summary of formation and metabolism of skatole and indole in the pig is given in Fig.3.1.

3.3.2 ANATOMICAL SITES OF SKATOLE FORMATION

TRP degradation to indole and skatole starts in the proximal part of the colon. In contrast to earlier studies [26,27], which already revealed elevated concentrations of indole in the distal part of the small intestine and the cecum, the majority of more recent studies point to a later onset of TRP degradation to indole and skatole [24,28,29]. In one of the feeding studies [28], only traces of free tryptophan were detectable (<100 µmol/kg digesta) in the stomach but increased in the small intestine. Dividing the small intestine into three parts, elevated concentrations above 200 µmol TRP/kg digesta were already found in the proximal part, increasing to maximum concentrations (about two-fold) in the middle part. In the distal part, TRP concentrations decreased to values comparable to those of the proximal part. No conversion of TRP to indole or skatole was measurable from the stomach to the distal part of the small intestine. Low concentrations of skatole and indole were found in the cecum, but maximum concentrations of IAA and IPA (indolic pyruvic acid) were observed, which decrease continuously along the colon [28]. Correspondingly, almost linearly increasing concentrations of skatole and indole are observed. Maximum concentrations of both compounds are found either in the distal colon or the rectum [14,24,28,29].

Absorption of skatole and indole occurs along the colon and both substances are transferred to the liver via the portal vein. Total daily absorption rates of skatole were estimated between 820 and 365 µmol skatole, depending on the diet [28]. The daily absorption of indole was reported to be about threefold higher and ranged between 2.999 and 929 µmol [28]. The concentrations of skatole in portal blood, peripheral blood and feces is highly correlated within individuals, but that of indole is not, suggesting that the amount of skatole absorbed is proportional to the amount produced [27,28]. Such correlations were less obvious in one study if several individuals were included or between animal correlations were calculated [27,28], as individual differences in metabolism of skatole seem to exist [30].

The direct infusion of skatole into the cecum of pigs via a cannula in the ileo-cecal junction in 4 h intervals for three days increased skatole concentration in feces only slightly and excretion rates via feces were below 5%. An absorption rate above 90% of the amount of skatole, which is already available in the proximal part of the colon, was discussed [31]. Under physiological conditions, however, only a small amount of the total skatole production is formed in the proximal part of the colon, as shown above [28].

Thus, in a similar experimental approach using TRP infusion into the cecum, an appearance rate in the portal vein of 70% of the skatole formed along the colon was calculated. In contrast to a former study [32], this percentage seemed to be independent on the total amount of skatole synthesized [14].

3.3.3 ORIGIN OF TRYPTOPHAN AS A PRECURSOR FOR SKATOLE SYNTHESIS

The origin of TRP needed for the microbial synthesis of skatole is controversial [24,27]. Whereas in ruminants, the addition of TRP to the diet regularly leads to increased skatole formation [33,34], the effect of TRP in the diet of the pig is less clear [35,36,37]. Diets with low prececal protein digestibility were reported to increase skatole production [5,14,38], whereas feed supplements with synthetic L-TRP below or above the requirements (1.0 g/kg DM to 1.91 g/kg DM) were not effective in systematically changing skatole concentrations in the feces of growing pigs [37]. It was postulated that free TRP is mainly absorbed in the small intestine and is therefore not available for microbial metabolism in the colon. Studies aiming to influence the rate of mitosis and apoptosis in the small intestine experimentally showed that gut cell debris is a major source of tryptophan for the microbial skatole formation [39,40].

Changes in diet may lead to a reorganization of intestinal mucosa and thereby increase the amount of cell debris, as most obvious after weaning in piglets [41]. A clear increase in skatole formation after weaning was clearly shown for piglets of different sex and weaning ages, which, however, was accompanied by an age dependent decrease in SULT1A1 (see Section 2.4; [30,42]). Feed additives (antibiotics and Chinese herbs), which reduced the number of pathogenic bacteria in the intestine were effective in reducing atrophy of villi in piglets after weaning [41]

The direct infusion of 4.9 mmol TRP into the cecum of pigs was effective in increasing skatole concentrations in portal blood. The increase started after 2 h, reached maximum values up to five-fold concentrations after 6 h to 10 h and decreased thereafter [14]. The total conversion of infused TRP to skatole was estimated, based on portal blood concentrations. It ranged between 26% for animals given a low fiber diet and 6% for animals given a high fiber diet. Most of the infused TRP was converted to indole (69% and 35% respectively). In animals fed with high fiber diet a higher amount of indole-propionic acid (IPA) was measured, compared with animals on a low fiber diet (16 vs. 7%) [14]. Thus a generally lower proportion of degradation products of TRP was observed if more fiber was fed simultaneously. Under such conditions, only 57% of the cecally-infused TRP was metabolized to one of the three compounds (skatole, indole, IPA), whereas in animals on a low fiber diet the total amount of infused TRP was degraded to one of the three substances. It was concluded that in the case of a high fiber diet, more TRP is used for the microbial synthesis of protein instead of microbial energy production via TRP degradation [14]. Other factors such as colon motility, fecal transit time, secretory rates, osmolality in the colon which are influenced by the physiological and psychological status of the host may also have a significant role [16]. The

transfer of skatole and indole from gut to blood is probably due to passive diffusion, as there is no skatole carrier protein known [43].

3.3.4 METABOLISM OF SKATOLE IN LIVER AND KIDNEY

Skatole and indole are transported by the portal vein (V. porta) to the liver, where most of the indole derivatives are metabolized by specific enzymes. Small amounts of indoles, which are absorbed in the distal colon or rectum, can bypass the liver and are transferred via the vena cava caudalis directly into the peripheral bloodstream [10,27]. The liver metabolism is highly effective and skatole concentrations of the portal vein may be reduced severely in the liver (up to 90%), as was obvious from parallel measurements in the hepatic vein [32,44]. Based on these data, a half-life for skatole in blood of one hour was calculated [44].

Even if skatole and indole are mainly degraded in the liver [45,46,47], a contribution of other organs, such as the kidneys, as similarly shown for androstenone degradation, may be assumed [48]. The knowledge on hepatic metabolism of skatole and indole has been reviewed recently [8,9]. In brief, hepatic degradation of indoles can be divided in two distinct steps: an oxidative step, phase 1 metabolism, and a conjugative step, phase 2 metabolism. Responsible enzymes are various cytochrome P450 isozymes, which are known to play a predominant role in drug and xenobiotic metabolism. Two specific enzymes, CYP2E1 and CYP2A were identified as major enzymes of the phase 1 metabolism of skatole [47,49]. A minor contribution of other P450 isozymes to the skatole phase 1 metabolism was discussed, but is less clear [50,51,52,53,54]. During phase 1 metabolism skatole is degraded to seven intermediate products (see Fig.3.1, lower part). The main enzymes of phase 2 metabolism, SULT1A1 (sulfotransferase) and UGT (uridine-di-phosphate-glucuronosyltransferase) further modify these seven compounds mainly by increasing their hydrophilic properties, adding either a sulfate or glucuronyl group to the molecules [44]. Phase 2 metabolism thus results in a variety of terminal products, where 6-sulfatoxy-skatole, sulfated or glucuronic conjugates of 5-hydroxy-3-methylindole and 3-hydroxy-3-methyloxindole are predominant [55,56,57]. During phase 2 metabolism, the water solubility of the skatole metabolites is increased, facilitating excretion via urine [11,58]. Enzymes of phase 2 metabolism are mainly located in the liver, but can also be found other tissues, such as kidneys and lungs [48]. The main products of phase 1 and phase 2 metabolism are shown in Fig.3.1 (lower part).

The activity of the phase 1 enzymes is modulated by several gonadal steroids. It was shown that the addition of physiological concentrations of androstenone led to a significant reduction in the activity of CYP2E1 and CYP2A in hepatic microsomes from pigs [47,53], while the

addition of 17ß- and 17a-estradiol only revealed such an inhibiting effect in supraphysiological concentrations [59]. Similarly, a higher enzyme activity of these two enzymes in the hepatic tissue of barrows was reported [60], compared to activity in tissue of boars. It was concluded that the lower skatole concentrations in adipose tissue of barrows were due to these differences. Higher levels of enzyme expression were reported for barrows and immunocastrates when compared to boars, but this high expression level did not always result in high enzyme activity, suggesting the influence of additional factors [50]. This hypothesis was further supported by an in vitro study [53], where gender specific effects of a pre-incubation with androstenone on the activity of CYP2E1 and CYP2A in liver microsomes were reported. In this study, enzymes derived from liver microsomes of female pigs were not inhibited by the pre-incubation with androstenone, up to 15 ng/ml, whereas the same enzymes from male liver samples exhibited a decreasing activity after the addition of 15 ng androstenone/ml or 0.5 ng estradiol/ml to the in vitro system. The addition of testosterone at a dose of 5 ng/ml was without effect in both genders. A slight increase (10%) in CYP2A activity was induced by adding 0.5 ng 17ß-estradiol/ml to liver microsomes from female pigs [53]. This effect of sex hormones on hepatic skatole degrading enzyme activity offers an explanation for the regularly reported higher concentrations of skatole in blood and tissue of boars compared to sows, gilts and barrows [7,45,61].

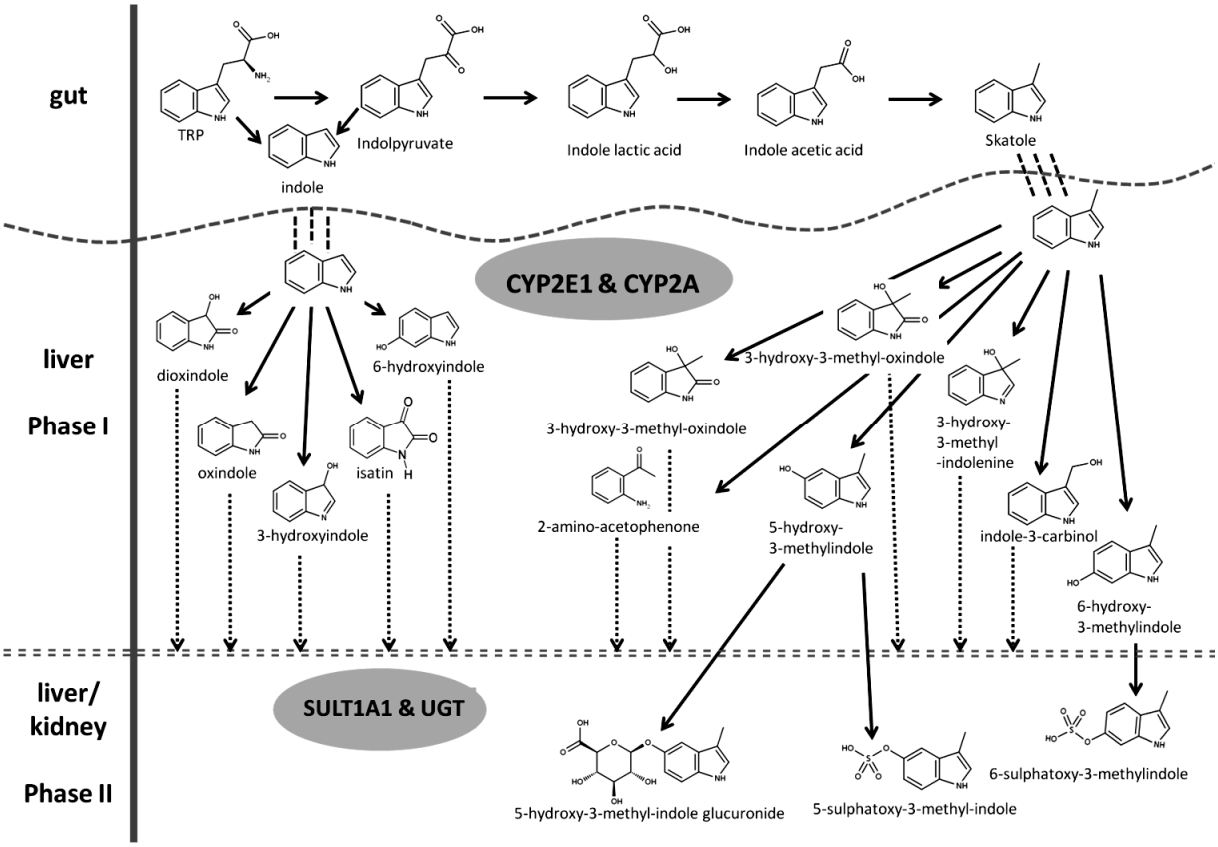

Fig.3.1: Formation of skatole (3-methyl-indole) and indole from TRP in the gut and further metabolism via phase 1 and phase 2 enzymes, (black arrows: known pathway; broken arrows: assumed pathway) [46,54,55,57,62].

3.3.5 ACCRETION OF SKATOLE IN ADIPOSE TISSUE

As a lipophilic substance, skatole is accumulated in adipose tissue if blood levels of skatole are elevated for a prolonged period. Similarly, skatole concentrations in adipose tissue drop within days, if skatole formation in the colon is reduced due to feeding measures, such as inulin supplementation as described below [10]. These changes occur more rapidly than in the case of the even more lipophilic steroid androstenone [10]. Adipose tissue differs within and between animals in its fatty acid turnover and in its stability according to its fatty acid composition. Main factors influencing fatty acid composition are the anatomical site (e.g., organ fat vs. subcutaneous or intramuscular fat), the overall degree of fatness of an individual or breed and the diet in monogastrics [63,64]. It is well established that leaner pigs have a lower ability to synthesize fatty acids combined with a greater mobilization, which results in adipose depots with more unsaturated lipids [65,66]. A relationship between skatole deposition and the turnover rate of adipose tissue has not yet been proven, but breed comparisons in skatole concentrations suggest such an influence. Lean breeds, such as the

Pietrain, revealed generally lower skatole and androstenone concentrations than the fatter Large White breed [67]. Similarly, the distribution of skatole within the carcass is in accordance with the amount of saturated fatty acids of the specific adipose tissue. Both are higher in flare than in belly fat, and higher in belly fat than in adipose tissue from the neck [68,69,70], suggesting again that adipose tissue with a higher amount of and lower turnover accumulates more skatole. Specific studies on fat turnover and skatole dynamics however need to be conducted.

3.3.6 SUMMARY OF THE PHYSIOLOGICAL MECHANISMS LEADING TO ELEVATED SKATOLE IN ADIPOSE TISSUE

The mechanisms leading to elevated concentrations of skatole in adipose tissue of pigs are summarized in the Fig.3.2. High concentrations of skatole require (A) a high amount of TRP with low prececal digestibility or cell debris for microbial degradation in the colon, (B) specialized microbes for skatole synthesis, (C) insufficient alternative energy sources for microbial activity, so that the metabolism of TRP to skatole occurs instead of the synthesis of bacterial protein, (D) a high absorption rate, such as in the case of a long transient time of digesta, (E) a reduced degradation of skatole in phase 1 metabolism of liver and phase 2 metabolism in liver and kidney and (F) the deposition in adipose tissue, which requires continuously high concentrations of skatole in peripheral blood and a low turnover of adipose tissue. This model allows a discussion of the mode of action of feed components on the complex mechanisms leading to a change of skatole concentrations in adipose tissue.

3.4 EFFECTS OF DIET AND FEED ADDITIVES ON SKATOLE FORMATION, ABSORPTION, METABOLISM AND ACCRETION IN ADIPOSE TISSUE OF PIGS

As shown in Fig.3.2, high or low concentrations of skatole in adipose tissue are the result of several independent steps, such as the formation, absorption, metabolism and accretion in adipose tissue. In the following section, feed components and feed additives which influence skatole dynamics are summarized and related to the physiological steps (Fig.3.2), contributing to skatole accumulation in adipose tissue. Feeding techniques, such as liquid feeding vs. dry feeding, exerted only minor effects on skatole formation [71,72]. Additionally, effects of feed intake behavior (feed intake rate, duration of meals) may explain some variability in skatole formation [73] but these aspects are not further discussed in this review.

3.4.1 EFFECTS OF DIET ON TRP AVAILABILITY IN THE COLON AND CONSEQUENCES FOR SKATOLE FORMATION (STEP A, FIG.3.2)

The effect of TRP availability was investigated by feeding diets with high and low prececal digestibility. It was obvious, as stated earlier, that diets with free TRP supplementation had only a low effect on skatole formation [37], whereas feeding animals using products with a 20% lower prececal digestibility of TRP (blood, meat and bone meal) than the control diet led to increasing skatole concentrations in adipose tissue [38]. In a series of studies, Claus and co-workers changed the TRP availability in the colon by influencing the mitosis and apoptosis rate in the small and large intestine [29,39,40,70,74,75]. These studies utilized an increased supply of energy and purines to increase mitosis and consequently apoptosis [39,40], and also the application of either coated butyrate [74] or butyrate-producing feed components, such as raw potato starch [29,70,75]. The increase of mitosis - as confirmed by histological evaluations - resulted in the postulated increase in skatole in feces, blood and adipose tissue, whereas the inhibition of apoptotic processes in the colon, as in a part of the studies confirmed by histochemical evaluation of the mucosa, led to low skatole concentrations in all compartments analyzed [29,70,74,75]. In contrast, the addition of coated butyrate in low amounts from 1.2 to 1.5% was without effect on the skatole formation in the colon and its accretion in adipose tissue as well [76,77].

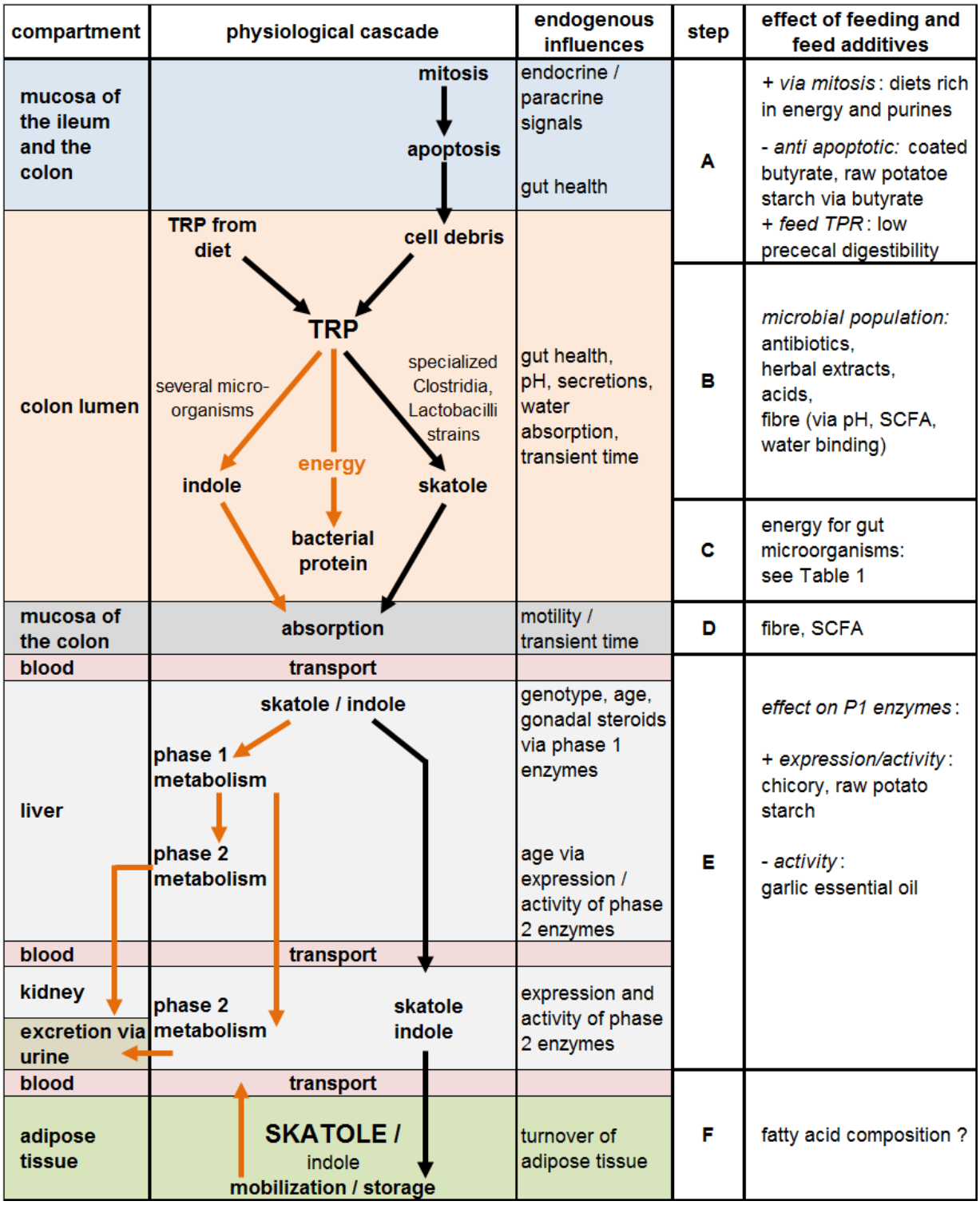

Fig.3.2: Cascade of physiological events leading to skatole formation, further metabolism and accumulation of skatole in adipose tissue. Steps correspond to Sections 3.1 (A) to 3.6 (F). The right column shows where distinct feeding influences exert their effects. Black arrows: steps leading to high skatole concentrations; brown arrows: skatole reducing or neutral conditions.

3.4.2 EFFECTS OF DIET OR FEED SUPPLEMENTS ON MICROBIAL POPULATION IN THE GUT AND CONSEQUENCES FOR SKATOLE PRODUCTION (STEP B, FIG.3.2)

As skatole is a product of microbial degradation, several strategies are documented to influence the microbial ecosystem in the gut of pigs to reduce skatole forming microbes. Such strategies include antimicrobial compounds or feed additives leading to changes in intestinal pH. It has been shown earlier that skatole-producing bacteria are favored under acidic conditions (pH 5.0), whereas indole-forming bacteria predominate if the pH was increased to 8.0 [5,18].

The most radical approaches to influence the microbial population within the gut are the applications of antibiotics such as Tylosin, Virginiamycin and Bacitracin and are mainly limited to studies during the late 90s [18,71,78,79]. Such rude treatments of the intestinal microbial ecosystem resulted in decreasing skatole concentrations in adipose tissue and/or feces if increasing concentrations of these substances were applied [18,71,78], whereas low doses were without effect on skatole in adipose tissue [79]. More recently, studies were published in which gut microbes were influenced via feeding of organic acids with weakly antibacterial properties (e.g., 1.0% formic acid, 0.85% benzoic acid, 0.85% sorbic acid, corresponding to 0.85% of pure acid) or components, which are metabolized by gut microbes to terminal SCFA [76,77]. Digesta samples for microbiological examinations were taken from proximal jejunum, colon descendens, and rectum. Pigs fed organic acids had significantly lower levels of coliforms, enterococci, and lactic acid-producing bacteria in all sampling sites of the gastro-intestinal tract. Formic acid had a stronger antibacterial effect on coliforms than benzoic or sorbic acids in the small intestine. It was concluded that supplementing diets with different organic acids reduces the number of coliforms, enterococci, and lactic acid-producing bacteria in the gastrointestinal tract. Even if there were no differences in levels of skatole or indole in colon descendens among pigs fed organic acid-supplemented diets, compared to a control diet, plasma skatole levels were significantly reduced in pigs fed diets containing formic acid or benzoic acid [76]. The levels of skatole in entire male pigs however were not reduced in a similar study [77].

The antimicrobial activity of selected plant extracts, such as Chinese medicinal herbs or feed additive derived from Sanguinaria canadensis, essential oil components from herbs and spices or tannin-rich preparations, may offer a strategy to reduce skatole formation [41,80,81,82]. The addition of tannin-rich extracts of several plants inhibits either microbial activity directly or by reducing the availability of proteins for bacterial metabolism. Such extracts were effective to reduce skatole in vitro [82], and in vivo in ewes [83]. A study with essential oil components from herbs and spices revealed that carvacrol, thymol, eugenol and

trans-cinnamaldehyde influence the microbial ecosystem and fermentation pattern in the gastrointestinal tract of pigs [81]. Under conditions of the jejunum, carvacrol and thymol showed very similar and non-selective antimicrobial properties. In cecal simulations, carvacrol, thymol and trans-cinnamaldehyde were equally effective while eugenol had an effect only on coliforms, but the effects of skatole, however, were not investigated. The addition of Chinese medicinal herbs to the diets of piglets after weaning had a clear effect on the bacterial population in the gut, reduced the occurrence of diarrhea and led to an increased villus height in the small intestine, suggesting reduced availability of cell debris. The effect on skatole formation was not measured [41].

A feed additive derived from Sanguinaria canadensis contains large amounts of sanguinarine, an alkaloid with known in vitro antimicrobial properties [84]. It was postulated that this alkaloid inhibits the activity of the amino acid, decarboxylase, which is involved in the synthesis of indole and skatole [85] and its application to prevent skatole formation has been claimed by a patent (EP 0581926, US 20030190344). Convincing studies with growing pigs, however, were not published. Approaches to control skatole formation by the addition of plant extracts may be attractive, as they reveal antimicrobial properties at very low concentrations [41,81] but need more systematic investigations. In contrast, the addition of different sources of fermentable carbohydrates to the diets of growing pigs led to reduced skatole formation or accretion in adipose tissue in several studies. These studies are summarized in Table 3.1. Easily fermentable carbohydrates are thought to exert two main effects: One is the change of intraluminal pH within the colon, which is assumed to influence the microbial ecosystem of the colon. The other is related to their function as a microbial energy source and further discussed in Section 3.3. Thus, in vitro results after the addition of 0.5 - 1.5% fructooligosaccharides (FOS), which revealed an increase in indole formation but not in skatole, were discussed as a result of a changed microbial ecosystem via feed-induced pH changes [86]. Similarly, differences in the intraluminal pH were reported after feeding oat-based diets versus barley-based diets [87]. Oat-based diets led to a higher pH and concomitantly higher indole concentrations when compared to the barley-based diets. As skatole concentrations were not changed by the treatments, it was postulated that indole-producing bacteria favored the higher pH, as similarly shown in in-vitro studies [5,18].

Even if the mechanisms were not clarified in detail, differences in the microbial populations were reported after the supplementation of diets of growing entire male pigs during the last weeks before slaughter with either chicory inulin (see Section 3.3) or potato starch in increasing amounts (0 to 15% in pellets). A reduction of enterobacteria counts in colon descendens and rectum was shown only for the inulin-containing diets with a tendency towards reduced levels of Enterococcus spp. in colon descendens and rectum. The

concentrations of skatole in the digesta were also reduced in the case of ≥6% of inulin supplementation, but not in the potato starch groups as further discussed below. The addition of raw potato starch in feed pellets was without effect on the microbial population [88].

The effect of a high protein diet may be associated with higher prevalence of the Clostridiaceae family and the Clostridium genus, which are predominantly proteolytic microbes. Furthermore, a reduced prevalence of the order Clostridiales, particularly the predominately saccharolytic microbes, especially butyrate producers, have to be expected (for review see [89]). Consequences for skatole formation, however, were not studied in detail but may favor skatole production.

3.4.3 Dietary Manipulations Resulting in Higher Energy Availability for Microbial Activity and Their Consequences for Skatole Formation (Step C, Fig.3.2)

Most of the studies which were effective in reducing skatole formation and deposition in adipose tissue increased energy availability and shifted microbial metabolism from proteolytic to saccharolytic. This feeding strategy was suggested in an early study in which inulin supplementation and bicarbonate were effective in significantly reducing skatole in pig feces. The decrease in adipose tissue was confirmed by two consecutive biopsies one week apart [10]. The importance of fiber as such an energy source was finally proven by the elegant experiment described in part already in Section 2.3 of this review, reporting differences in the conversion rate of TRP infused into the cecum to skatole, if animals were either on a diet with a low or high amount of fiber [14]. Such a diet influenced both the production of skatole in the hindgut and the absorption of skatole to the portal blood. With the low fiber diet, the hindgut bacteria transformed 26% of the infused tryptophan into skatole, resulting in a significant increase of skatole concentration in the portal blood, compared to 6% in the case of a high fiber diet. In the case of the high fiber diet, only 57% of the infused TRP was converted into indoles, whereas in animals on low fiber diets, the total amount was degraded to indoles [14]. It might be assumed that in the case of the high fiber diet, the remaining TRP was used for the synthesis of bacterial protein as similarly discussed after the application of fructooligosaccharides (FOS) to reduce skatole concentrations [86].

Feed supplementation for two weeks with 10% of either raw potato starch, FOS, such as inulin, lupines and barley hull meal was significantly effective in reducing skatole concentrations in daily blood samples of pigs, when compared to a control diet (barley, soya). The addition of either palm cake, coconut cake or sugar beet pulp at the same

concentration revealed no significant effect [14]. Since then, numerous studies have been carried out to evaluate the minimum dosage of such fiber additive to reduce skatole formation and thus the risk of skatole tainted pork. The most prominent fiber sources are either chicory inulin or raw potato starch (RPS) added to the diet during the last weeks before slaughter. Such studies are summarized in Table 3.1.

Diets supplemented with RPS proved to be effective in reducing skatole concentrations in hind-gut and adipose tissue, if at least 20% was added to the diet [29,75,90]. Reducing this amount of RPS to 10% of the diet, in contrast to blood, resulted in no significant reduction of skatole in adipose tissue being observed [18,91]. In contrast to the effect of raw potato starch powder, the addition of the same substance before pelleting did not influence skatole concentrations [88], since the process of gelatinisation at high temperatures increased prececal digestibility [92]. Therefore this application type was chosen as a control, when specific effects of raw potato starch on skatole formation were investigated [29,75].

Similar effects to those of RPS on skatole concentrations in fat or feces could be achieved by adding at least 6% inulin via the addition of chicory root extract to the diet [88,93]. However, adding 5% of a chicory extract, containing 66% inulin, had no effect [91]. It has to be kept in mind that these additives may exert their effect also via other mechanisms of skatole formation, e.g., via butyrate formation, as described previously for TRP availability (Section 3.1), or microbial population (Section 3.2). Additional effects on hepatic skatole metabolism are discussed later (Section 3.5). Four types of carbohydrate sources, sugar beet pulp (SBP), rye grass hay (RYE), alfalfa hay (ALF) and FOS were compared in their effects on the in vitro metabolism of L-tryptophan to skatole and indole by a mixed bacterial population from the large intestines of pigs [25]. Addition of SBP or FOS showed a significant inhibitory effect on skatole formation and relative production rate.

Table 3.1: Feed components used to influence skatole formation and accretion via fiber supplementation in recent studies and their effectiveness as influenced by the amount in the diet and duration of application. Amounts of feed components and inulin are given as percentage of the fresh matter (DM: dry matter; n.d.: not determined; +: significant decrease in skatole concentration ($p < 0.05$); (+): tendency for decrease in skatole concentration ($p < 0.10$); - no significant change in skatole concentration).

feed component	% in diet	% effective ingredient in diet	feeding duration (d)	decrease of skatole (p<0,05) in			[ref.]
				adipose tissue	blood	digesta/feces	
feed components with main effective ingredient inulin							
dried chicory roots	**10**	n.d.	**16**	**-**	**-**	n.d	[11]
	10-13.3	**3.6-4.7**	**7-14**	**-**	**-**	n.d	[99]
	25	**13.95**	**7**	**+**	**+**	n.d	[12]
			14	**+**	**+**	n.d	
			42	**+**	**+**	n.d	
crude chicory roots (25% DM)	**56.3**	**6.9**	**28**	**+**	**+**	n.d	
			42	**+**	**+**	n.d	
			63	**+**	**+**	n.d	
dried chicory root extract	**3**	**1.8**	**14**	**-**	n.d	n.d	[13]
		2.1	**28**	**-**	n.d	**-**	[92]
		2.1	**30**	**-**	n.d	**-**	[86]
	6	**3.6**	**14**	**+**	n.d	n.d	[13]
		4.2	**28**	**+**	n.d	**+**	[92]
		4.2	**30**	**+**	n.d	**+**	[86]
	9	**5.4**	**14**	**+**	n.d	n.d	[13]
		6.3	**28**	**+**	n.d	**+**	[92]
		6.3	**30**	**+**	n.d	**+**	[86]
Inulin	**16.3**	**15.5**	**42**	**+**	**+**	n.d	[12]
jerusalem artichoke	**8.1**	**4.2**	**7**	**-**	n.d	**+**	[102]
	12.2	**6.3**	**7**	**(+)**	n.d	**+**	
other feed components							
raw potato starch	**10**	n.d.	**28-42**	**-**	n.d	n.d	[90]
	10	n.d.	**14**	n.d.	**+**	n.d.	[18]
	20	n.d.	**14**	**+**	n.d	n.d	[89]
		n.d.	**14-21**	**+**	n.d	**+**	[74]
	30	n.d.	**14-21**	**+**	n.d	**+**	
lupine seeds	**10**	n.d.	**28-42**	**-**	n.d	n.d	[90]
	25	n.d.	**30**	**+**	n.d	**+**	[86]
sugar beet pulp	**10**	n.d	**28**	n.d	**+**	**-**	[28]
	15	n.d	**fattening period**	**-**	n.d	n.d	[103]
	20	n.d.	**30**	**+**	n.d	n.d	[98]

In contrast, the addition of both types of hay significantly increased skatole concentrations and the relative rate of skatole production after 24 h. The authors postulated that in the presence of fermentable carbohydrates these are used preferentially as an energy source by the intestinal bacteria, leading to a reduced protein catabolism [25,98]. In the case of a relatively higher fermentable RYE and ALF, the carbohydrate source is rapidly depleted (within 15 h), which in turn leads to a switch in the metabolism to proteolysis thereafter [25]. The availability of the slow fermentable SBP may provide a carbohydrate source for the bacteria over the whole investigation period of 24 h, preventing a metabolic switch to proteolysis of the skatole producing microbes. These results suggested that reduced concentration of skatole observed in the presence of SBP and FOS may be caused by an overall decreased rate of tryptophan degradation [25].

3.4.4 Effect of Feeding on the Absorption Rate of Skatole (Step D, Fig.3.2)

The effect of transient time on skatole absorption was more discussed than investigated. Positive correlations between dry matter content of feces and skatole concentrations in feces (µg/g DM) were reported for growing pigs [37]. It was discussed for other species that the formation of short-chain fatty acids, methane, CO_2 and other products from the bacterial metabolism of inulin may also contribute to increased peristalsis in the whole gastro intestinal tract, leading to changed conditions for bacterial fermentation and intestinal absorption in general [99]. Studies with the addition of complex clay minerals to the diet of pigs to bind skatole to prevent the absorption were published, but were not successful in all studies [91,100]. Similar approaches with more advanced additives, which should bind skatole and thus reduce the absorption rate, were carried out as in vitro studies, were not proven to be effective in vivo, but may be promising [101].

3.4.5 Effects of Feeding on Degradation of Skatole in the Liver and Kidney (Step E, Fig.3.2)

Various components of feed may either increase or inhibit the activity of skatole degrading enzymes of the phase 1 metabolism. The most impressive effect was obtained by adding garlic essential oil at a dose from zero to 2.15 g/kg to the feed. Concentrations of skatole and indole in adipose tissue increased after 57 days of feeding 2.15 g/kg garlic essential oil from 39.6 ng/g skatole and 35.8 ng/g indole in the control group to 1,001.5 ng/g skatole and 972.5 ng/g indole in the garlic supplemented group. It was suggested that the sulfur containing

compounds (allyl di- and tri-sulfides) had an inhibitory effect on the CYP-dependent skatole metabolism, thereby decreasing clearance of skatole and indole from the blood, since similar effects have been observed in other species [38,83,102]. Stimulatory effects of feed components on enzyme expression, as characterized by measuring either transcription through mRNAs or the enzyme activity, as characterized via enzymatic conversion rates, were reported for enzymes of the phase 1 metabolism in the liver [52,97]. For example, activity or expression of phase 1 enzymes were increased after the addition of dried chicory [52], sugar beet pulp [97] and potentially raw potato starch [90] to the diets. Similar effects were not described for any of the phase 2 enzymes [11]. Interesting differences between expression levels, protein concentrations and final enzyme activities were reported [52], since a 10% increase in mRNA for CYP1A2, CYP2A and CYP2E1 was measurable after addition of 10% dried chicory to diets. However, protein concentrations and enzyme activity were only higher for CYP1A2, and CYP2A in that experiment. A similar effect on the metabolism of androstenone after feeding chicory roots via an influence on hepatic 3ß-HSD (3ß-hydroxy steroid dehydrogenase) has been recently reported [11]. However, this feedstuff seems to exhibit a highly variable effect due to varying concentrations of inulin in the dried roots [94]. Linseed within a diet was also shown to reduce skatole concentrations in adipose tissue of pigs [103]. It was suggested by the authors that myristicin (a benzodioxole compound found in linseed) induces hepatic P450 enzymes such as CYP2E1, which in turn leads to an increased degradation of skatole [104].

3.4.6 EFFECTS OF FEEDING ON ACCUMULATION OF SKATOLE IN ADIPOSE TISSUE (STEP F, FIG.3.2)

Only a few studies with parallel monitoring of skatole concentrations in blood and adipose tissue biopsies were carried out [10]. Only very little data are available, which allow conclusions to be drawn about the effect of fatty acid composition of feed and adipose tissue on skatole in relation to tissue turnover rate. For example, feeding a linseed diet which provides an elevated amount of PUFA led to a lower skatole accretion. However, this result was not discussed with turnover phenomena [103]. A contribution of dietary manipulations on skatole concentrations via the influence of an increased fat turnover may be discussed and needs further investigation.

3.5 CONCLUSIONS

The reliable control of skatole accretion in the fat of boars is one of the main prerequisites for pork production with entire males. Skatole formation in the gut is the result of several independent conditions, leading to elevated TRP availability for specialized bacteria in need of this substrate for energy production. Even high skatole formation in the colon does not necessarily elevate skatole in adipose tissue, as absorption varies and the metabolism mainly in the liver may degrade sufficient amounts of skatole to ensure low adipose tissue concentrations. Only if the production and absorption exceed the ability of the liver to degrade skatole via mainly phase 1 enzymes, then skatole may accumulate in adipose tissue. This accumulation may be further modified by adipose tissue turnover; however, this aspect needs further clarification.

Nutritional effects on almost all steps of the process leading to skatole accumulation in adipose tissue have been described. The effectiveness of a feeding intervention to reduce skatole concentrations in adipose tissue is high, if the feed additive affects several steps of skatole synthesis and accumulation. Thus the most efficient feeding interventions are actually the addition of inulin or raw potato starch, which influence the microbial ecosystem, probably via the intestinal pH, the energy availability for bacteria and the TRP availability via possible anti-apoptotic effects. Inulin seems to be effective at lower doses than raw potato starch. The antimicrobial activity of selected plant extracts or essential oil components from herbs and spices may offer an additional strategy to reduce skatole formation.

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CHAPTER 4

PRE-SLAUGHTER CONDITIONS INFLUENCE SKATOLE AND ANDROSTENONE IN ADIPOSE TISSUE OF BOARS

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4 PRE-SLAUGHTER CONDITIONS INFLUENCE SKATOLE AND ANDROSTENONE IN ADIPOSE TISSUE OF BOARS

4.1 ABSTRACT

Boar taint in carcasses may vary between farms and abattoirs, although the underlying mechanisms are not yet fully understood. In the present study, 169 boars from three farms were split into two groups and slaughtered at two abattoirs. Duration of transport and the time between arrival at the abattoir and unloading (pre-unloading time) were recorded. During slaughter, blood, feces, and urine were collected to measure testosterone and cortisol levels. Carcasses were classified according to the number of skin lesions, and fat samples were taken to determine skatole, indole and androstenone levels. Androstenone in fat and testosterone in blood, feces, and urine were mainly influenced by the duration of transport. Skatole and indole concentrations were increased by both pre-unloading time and duration of transport, but were also related to skin lesions. Thus it is concluded that androstenone and skatole concentrations in fat are significantly modified by pre-slaughter conditions.

4.2 INTRODUCTION

The number of boar carcasses with sex-specific off-odor varies considerably between studies (Bonneau, 1998). Several studies reveal differences in the numbers of tainted carcasses between abattoirs, although limited analytical data and a lack of standardized detection methods made it difficult to determine the causes (Moerlein et al., 2012 and Prusa et al., 2011). Although the reasons for such differences could not be determined so far, pre-slaughter conditions, especially transport and handling of the animals, have been discussed.

Androstenone and skatole are regarded as the main compounds leading to the sex-specific off-odor of boar carcasses (Claus, Weiler, & Herzog, 1994). Origin and regulation of formation differ between those substances. Androstenone is formed in the Leydig cells of boar testes, and the rates of formation and release are under endocrine control (Claus et al., 1994). It has been reported that several exogenous and endogenous factors influence androstenone formation and accumulation in adipose tissue. In addition to breed, age, and photoperiod, stimuli such as social rank, inter-male aggression, sexual activity and in particular the transfer to a new and unknown pen lead to changes in androstenone concentrations in blood and adipose tissue (Andersson et al., 1998, Andersson et al., 1998, Claus and Alsing, 1976, Giersing et al., 2000 and Walstra et al., 1999).

Skatole and indole are microbial products of tryptophan degradation in the colon and their formation, resorption, and accumulation are mainly affected by nutritional factors and endocrine effects (for review see: Wesoly & Weiler, 2012). Earlier studies (Claus et al., 1994) also discussed, but did not conclusively prove, the effect of stress-related hormones on skatole and indole formation and accumulation. Quantification of hormones associated with stress like cortisol and cortisol metabolites in different physiological substrates such as blood, urine, and feces allows the monitoring of stressful conditions, because hormone concentrations in these substrates reflect the course of transient changes in adrenal activity. A stimulation of adrenal activity by a CRH (corticotropin-releasing hormone) injection in pigs led to an increase in urinary cortisol excretion after only 0.5 h. Maximum concentrations in urine were measured within 2 - 3 h (Hay, Meunier-Salaün, Brulaud, Monnier, & Mormède, 2000). An increase of cortisol metabolites in feces occurs later, as the diurnal pattern of cortisol secretion with high concentrations in blood in the morning is reflected with a delay of 12 - 36 h in fecal samples. Consequently, maximum concentrations in feces are found in samples collected during the night (Carlsson, Lyberg, Royo, & Hau, 2007). Similarly, measurements of testosterone in blood, urine, and feces allow monitoring changes in testicular activity (Palme, Fischer, Schildorfer, & Ismail, 1996).

The aim of this study was to compare skatole and androstenone concentrations in boar carcasses from boars raised on three different farms, which were slaughtered at two different abattoirs. Moreover, we sought to identify possible environmental factors and the underlying physiological mechanisms leading to differences in boar taint compounds by measuring the adrenal and testicular steroids in various substrates.

4.3 MATERIAL AND METHODS

4.3.1 EXPERIMENTAL STRATEGY

The experiment had been designed to evaluate differences in concentrations of androstenone and skatole in adipose tissue of boars slaughtered at two abattoirs of the same company. The two abattoirs that employed similar technical equipment for slaughter were similar in their technical equipment for slaughter but were assumed to differ in the amount of tainted boar carcasses according to human nose scoring at the slaughter line. In order to determine a possible contribution of different pre-slaughter conditions, animals from each farm were split into two groups, then transported to and slaughtered at the two abattoirs. As shown in Table 4.1, pre-slaughter conditions were evaluated for animals from three different farms. In order to analyze the physiological reactions of the boars during transport and pre-slaughter period, samples from different compartments (blood, feces, urine, and adipose tissue) were collected. These samples allow a determination of long-term parameters (measurements in liquid fat and feces), mid - term parameters (measurements in urine) and short term parameters (measurements in blood) of testicular and cortical activities.

Table 4.1: Number of boars from each farm sampled at the two abattoirs and the corresponding duration of transport from farm to abattoir (min) and pre-unloading time (min).

Farm	Slaughter plant	Tissue samples				Transportation time (min)	Pre-unloading time (min)
		adipose tissue	plasma	urine	feces		
A	1	29	28	19	16	60	480
	2	21	20	19	16	240	202
B	1	31	31	33	27	150	165
	2	33	32	25	22	270	93
C	1	28	27	28	20	300	260
	2	27	27	29	23	90	17

4.3.2 ANIMALS AND SAMPLING

A total of 207 boars from three farms with different genotypes (Farm: A Pietrain × BW Hybrids, Farms B and C: Duroc × Danbreed) were studied; each with a live weight of approximately 120 kg. All boars had been raised with commercial diets for growing boars, although feed composition and feeding regimen were not standardized between farms. The animals from each farm were delivered to the abattoirs on two consecutive days in separate vehicles, although animals from different pens of the same farm were mixed during transport. The duration of transport (transport time) and the time spent on the vehicle after arriving at the abattoir (pre-unloading time) were recorded. All animals had access to water after unloading during the standardized 60 min lairage period, before the animals were CO_2 stunned in a gondola-dip-lift system. After expulsion from the stunning pit, 27 - 38 animals per day were randomly fitted with ear tags to enable the identification of individual carcasses along the slaughter line. During exsanguination, 20 ml of blood from the jugular vein was collected into heparinized vials.

After scalding, dehairing, and flaming, the carcass was opened to remove the red offal. At this stage, distal colons and bladders from animals with ear tags were collected for further sampling of feces and urine. After cooling, each individual carcass was examined on one side for signs of fighting, especially skin lesions, and classified in a four-level system according to the following criteria: no skin lesions/side = score 0; 1 - 8 lesions/side = score 1; 8 - 25 lesions/side = score 2; and more than 25 lesions/side = score 3. From each tagged carcass an adipose tissue sample was taken from the neck area for further analysis. Due to mechanical influences, about 20% of the ear tags got lost at the slaughter line, so that samples could be obtained only from a reduced number of animals (maximum n = 169). Moreover, urine and feces could not be obtained from every animal due to empty bladders or colons. Details are provided in Table 4.1.

4.3.3 TREATMENT OF SAMPLES

Blood samples were centrifuged within 1 h after collection and plasma was stored on ice until freezing. Feces were collected manually from the rectum and distal colon and stored on ice. Urine samples were aspirated with a syringe from the bladder and stored immediately on ice. All samples were then transferred on-ice to the lab within 12 h and stored frozen at − 20°C until assayed.

4.3.4 DETERMINATION OF ANDROSTENONE IN ADIPOSE TISSUE

The androstenone concentrations in adipose tissue were measured after extraction with an in-house enzyme immunoassay as described earlier (Claus et al., 1997 and Weiler et al., 2013). In short, 50 µl of melted fat was added to 450 µl warm methanol (55°C). After mixing, samples were allowed to cool down to room temperature and centrifuged thereafter (200 rcf, 10 min, 4°C). 100 µl of the supernatant was diluted with 900 µl assay buffer and 100 µl was used for further enzyme immunological tests as described above. All samples were measured in duplicate. Precision was determined using spiked fat samples with a mean recovery rate of 96.4%. Intra-assay variance and inter-assay variance were determined with biological samples and was below 10% and below 14%, respectively. A threshold of 0.5 µg/g fat for tainted samples was chosen in accordance with previous studies (Walstra et al., 1999). In this study, concentrations of androstenone, skatole and indole in adipose tissue refer to g melted fat.

4.3.5 DETERMINATION OF SKATOLE AND INDOLE IN ADIPOSE TISSUE

Skatole and indole concentrations in adipose tissue were determined according to Dehnhard, Claus, Hillenbrand, and Herzog (1993), with slight modifications for UPLC systems. Samples of 100 µl liquid fat were pipetted in duplicate and the mass of each sample recorded (approximately 100 mg melted fat). One ml n-hexane and the internal standard 2-methyl-indole (IS; 30 ng/sample dissolved in 30 µl hexane) were added and mixed. Afterward, a solvent distribution was carried out against 1 ml acetonitrile: H_2O (4:1). The hexane layer was removed after centrifugation and the remaining sample was further analyzed with a Dionex UPLC system (pump: Ultimate 3000 RS; column oven: Ultimate 3000 RS Column Compartment; autosampler: Ultimate 3000 RS Autosampler WPS 3000 RS; Thermo Scientific, Karlsruhe, Germany) combined with a fluorescence detector FLD (Ultimate 3000 RS Fluorescence Detector FLD-3400RS). Separation of the analytes was performed with an Acclaim 120 C18 2.1 × 100 mm 3 µm column (Thermo Scientific, Karlsruhe, Germany). The mobile phase was 0.011 M acetic acid in H_2O: acetonitrile: 2-propanol (55:30:15). A volume of 5.0 µl was injected at a flow rate of 0.4 ml/min and detection was performed with excitation wavelength of 275 nm and emission wavelength of 352 nm.

Precision was determined by measuring skatole- and indole-spiked fat samples. Mean recovery rate for skatole and indole was 97.3% and 101.2%, respectively. Intra-assay variability and inter-assay variability were determined for skatole and indole with biological samples and were below 5% each.

4.3.6 DETERMINATION OF TESTOSTERONE CONCENTRATIONS IN PLASMA

Testosterone concentrations in plasma were determined in duplicate with a commercially available ELISA (EIA-1559, DRG Instrument GmbH, Marburg, Germany) which has been developed for testosterone measurements in human blood plasma. Measurements were carried out in pig plasma without modifications. Intra-assay variability and inter-assay variability were determined with pig plasma samples and was below 10% each. Precision was determined with samples of spiked pig plasma. The mean recovery rate was 110.5%.

4.3.7 DETERMINATION OF TESTOSTERONE CONCENTRATIONS IN URINE AND FECES

A radioimmunological analysis of testosterone concentrations in urine and fecal samples was carried out after extraction, as described below. The radioimmunoassay was carried out according to Claus, Schopper, & Wagner (1983). The antiserum had been raised in rabbits against testosterone-3CMO-BSA. Cross reactivity was 67% with 5αDHT, and below 2% for other tested steroids. In case of urine, aliquots of 10 µl urine were diluted with 100 µl aqua bidest and were extracted in duplicate with 3 ml of buthyl methyl ether. To compensate for procedural losses, the recovery rate was determined with ^{3}H-testosterone in each assay and was 94.1% in the average. Precision was determined with spiked pig urine samples and revealed a mean recovery rate of 82.9%. Intra-assay variation and inter-assay variation were determined with biological pig urine samples and was below 10% and below 15%, respectively.

Testosterone was extracted from feces in a two-step solvent distribution. Fecal samples of about 0.5 g each were first dissolved in 500 µl of water, then 4 ml methanol was added. After mixing the sample for 30 min, 3 ml petroleum ether was added for solvent distribution. After mixing and centrifugation, the petroleum ether was discharged, and an aliquot of 100 µl of the methanol/water fraction was further diluted with 600 µl water and extracted with 5 ml of 7:3 (v/v) petroleum ether/ethyl acetate. After incubation for 30 min and freezing, the supernatant was collected and evaporated. The residue was reconstituted with 100 µl phosphate buffer and the hormone concentrations determined in a radioimmunological analysis as described above. The average recovery rate for ^{3}H-testosterone from fecal samples was 50.2%. Intra-assay variability and inter-assay variability in fecal samples were below 5% each. Precision was determined with spiked fecal samples and revealed a mean recovery rate of 76.8%.

4.3.8 DETERMINATION OF CORTISOL IN URINE AND FECES

A radioimmunological analysis of cortisol concentrations in urine and fecal samples was performed after extraction. The radioimmunological determination was carried out according to Claus and Weiler (1996). The antiserum had been raised in rabbits against cortisol-21-HS-BSA (cortisol-21-hemisuccinate-bovine serum albumin) and revealed a cross-reactivity of 12.1% with corticosterone.

In the case of urine, aliquots of 2.5 µl diluted with 97.5 µl aqua bidest were extracted in duplicate with 2 ml of ethyl acetate. To establish procedural losses the recovery rate was determined with ^{3}H-cortisol in each assay and was 91.5% in the average. Precision was determined with spiked pig urine samples and revealed a mean recovery rate of 102%. Intra-assay variation and inter-assay variation were determined with biological samples and were below 5% and below 10%, respectively.

Cortisol was extracted from feces in a two-step solvent distribution. Fecal samples of about 0.5 g each were first dissolved in 500 µl of water, then 4 ml methanol was added. After mixing the sample for 30 min, 3 ml petroleum ether was added. After mixing and centrifugation the petroleum ether was discharged, and an aliquot of 100 µl of the methanol/water fraction was further diluted with 600 µl water and extracted with 5 ml of 7:3 (v/v) petroleum ether/ ethyl acetate. After incubation for 30 min and freezing, the supernatant was collected and evaporated. The residue was reconstituted with 100 µl phosphate buffer and the hormone concentrations determined in a radioimmunological analysis as described above. The mean recovery rate for ^{3}H cortisol from fecal samples was 50.2%. Precision was determined with spiked samples. The mean recovery rate of spiked cortisol was 76.8%. The intra-assay variability and inter-assay variability of biological samples were below 5% each.

4.3.9 DETERMINATION OF CREATININE IN URINE

To compensate for the masking effects of variable water supply during transport and resting period, creatinine concentrations were determined photometrically (PowerWaveX BIO-TEK ® Instruments Inc.) in urine samples after dilution and addition of a chromogenic substrate using a commercial kit (KIT-Nr. 553-172G, mti-diagnostics GmbH, Idstein, Germany) according to the Jaffé-method (Jaffe, 1886). In short, 15 µl diluted urine (1:10 up to 1:120 with H_2O, depending on the concentration) was added to 150 µl chromogenic substrate (8.73 mmol/l picric acid; 187.8 mmol/l NaOH; 7.5 mmol/l phosphate), mixed, and the extinction determined at 520 nm wavelength. Testosterone and cortisol concentrations in urine were

given as ng/mg creatinine to correct for possible dilution of urine depending on water intake (Crockett, Bowers, Sackett, & Bowden, 1993).

4.4 STATISTICAL ANALYSIS

Data was subject to an ANOVA based either on the original data (testosterone in feces, lesion scores) or on logarithmic transformation (all other parameters) after testing for normal distribution of the residuals. The ANOVA was carried out by SPSS (IBM®SPSS® Statistics 20) using the following two models.

Model 1 included farm and abattoir as fixed effects. The duration of transport from farm to abattoir (transport time) and the time spent in the vehicle until unloading (pre-unloading time) were included as covariates. Pearson correlations were calculated based on the residual values calculated with ANOVA model 1. To compensate for an unbalanced design, LS-means (± SEM) were calculated and differences between groups were tested for significance with Tukey-HSD. Level of significance was 5%. Coefficients of regression were calculated to quantify significant effects (estimated increase per hour). In case of log-transformed parameters, LS-means and estimated coefficients of regression are given as untransformed data.

As the principal component analysis (PCA) (see below) indicated a correlation between pre-unloading time and lesion score, differences between lesion score classes in skatole and indole were tested for significance by Kruskal-Wallis after combining scores 2 and 3 into one score group. LS-means were calculated with a second model for ANOVA, which included farm, abattoir, and lesion score class as fixed effects. Transport time was included as a covariate.

In addition, a PCA was performed to analyze interdependencies between investigated parameters based either on the original data (lesion score, transport time, pre-unloading time and testosterone concentrations in feces) or on log-transformed data (all other parameters). No further data pre-treatment was carried out. The PCA was performed after a Varimax rotation with Kaiser-normalization. Only principal components with initial eigenvalues above 1 were further analyzed.

4.5 RESULTS

4.5.1 EFFECTS OF ABATTOIR, FARM AND PRE-SLAUGHTER CONDITIONS ON SKATOLE, INDOLE AND ANDROSTENONE IN ADIPOSE TISSUE, AND ON ENDOCRINE PARAMETERS

The results of the ANOVA and LS-means for boar taint compounds and endocrine measurements are summarized in Table 4.2 and Table 4.3.

Table 4.2: LS-means ± SEM for the physiological parameters and estimated increase per hour of transport and pre unloading time (ANOVA Model 1)

compound	substrate	N	LS Mean ± SEM	increase/hour transport	increase/hour pre-unloading
androstenone	**fat (µg/g)**	**169**	**0.9 ± 0.1**	**+0.09**	
skatole	**fat (ng/g)**	**169**	**73.8 ± 5.0**	**+3.6**	**+21.5**
indole	**fat (ng/g)**	**169**	**34.1 ± 1.5**	**+6.8**	**+10.6**
testosterone	**plasma (ng/ml)**	**165**	**9.7 ± 0.8**	**+2.2**	
	urine (ng/mg creatinine)	**153**	**10.1 ± 0.6**	**+1.6**	
	feces (ng/g)	**124**	**22.6 ± 0.7**	**+1.4**	
cortisol	**urine (ng/mg creatinine)**	**153**	**71.7 ± 2.2**		
	feces (ng/g)	**124**	**49.7 ± 1.65**	**+4.3**	

Abattoir had a significant effect on skatole and indole concentrations in adipose tissue, but not on androstenone concentrations. The factor farm had a significant effect on androstenone and indole in adipose tissue, but not on skatole. Testosterone concentrations in plasma were significantly affected by farm and abattoir, but neither in feces nor in urine. The factor farm did influence the cortisol concentrations in the long-term parameter feces, but not in urine. Androstenone concentrations were furthermore affected by the duration of transport, whereas skatole and indole levels were influenced by both pre-unloading time and

transport time. Testosterone concentrations were affected by transport time in all measured substrates, plasma, urine, and feces. Cortisol concentrations in urine were not affected by transport time or pre-unloading time, whereas cortisol concentrations in feces were significantly influenced by transport time. Coefficients of regression indicate a positive relationship between these compounds and duration of transport, pre-unloading time and several physiological parameters (Table 4.2). A surprising result is the effect of transport time on androstenone concentrations with an increase of 0.09 µg/h transport time. Skatole concentrations in fat increase by about 21.5 ng with every hour of pre-unloading time and by 10.6 ng/h in case of indole. The effect of transport time on testosterone was distinct in case of plasma, less pronounced in urine, and least obvious in feces as reflected by the coefficients of regression. Plasma testosterone concentrations increased by 2.2 ng/h transport. In urine, the corresponding increase was 1.6 ng/h, and in feces 1.4 ng/h. Cortisol concentrations in feces increased by 4.3 ng/h transport.

Table 4.3: Effects of farm, abattoir and pre-slaughter conditions (transport and pre-unloding time) on the investigated parameters given by ANOVA model 1

			ANOVA			
compound	**substrate**	**n**	**Farm**	**abattoir**	**pre-unloading time**	**transportation time**
androstenone	**fat (µg/g)**	**169**	***	n.s.	n.s.	**
skatole	**fat (ng/g)**	**169**	n.s.	*	***	***
indole	**fat (ng/g)**	**169**	*	***	***	***
testosterone	**urine (ng/mg creatinine)**	**149**	n.s.	n.s.	n.s.	**
	feces (ng/g)	**124**	n.s.	n.s.	n.s.	**
	plasma (ng/ml)	**165**	***	*	n.s.	***
cortisol	**urine (ng/mg creatinine)**	**153**	n.s.	n.s.	n.s.	n.s.
	feces (ng/g)	**119**	***	n.s.	n.s.	***

*:p<0.05; **:p<0.01; ***:p<0.001;n.s.:p>0.05

Fig.4.1 illustrates the sustained impact of transport time on testicular function as reflected by androstenone concentrations in adipose tissue and testosterone values in urine. Even though the individual levels of androstenone in boars from different farms (A,B,C) differ due to age and genotype effects, an increase in transport time leads to a higher number of animals with androstenone concentrations above the threshold of 0.5 µg/g in adipose tissue. In case of farm A, 6.9% of the animals delivered to abattoir 1 had androstenone concentrations above the threshold, whereas this amount increased to 19.0% with the longer transport time to abattoir 2 (60 vs 240 min). Similarly, the amount of carcasses from farm B above this threshold was 64.5% at abattoir 1, and 90.9% at abattoir 2 (150 vs 270 min), and 96.4% and 88.9% for farm C (300 vs 90 min), respectively. Testosterone concentrations in urine also vary in accordance with transport time.

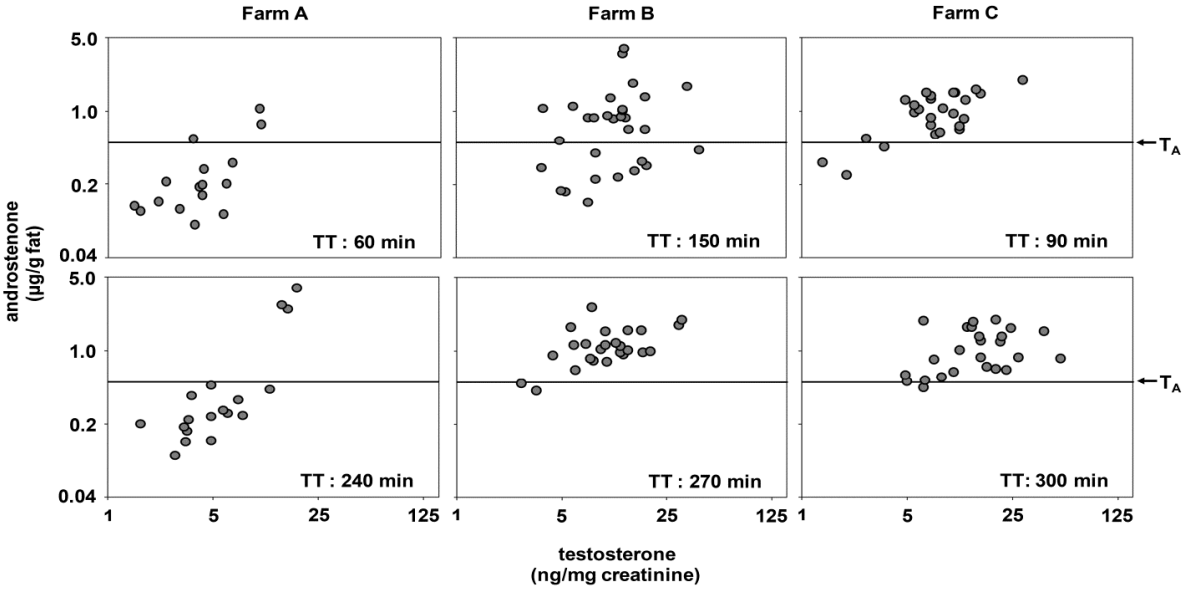

Fig.4.1: Androstenone in adipose tissue and testosterone concentrations in urine as influenced by transport time (TT) in the animals delivered from three farms (A, B, C) to two different abattoirs. The threshold for androstenone tainted carcasses used in this study (TA = 0.5 µg/g fat) is indicated by the solid black line. (Fig. basing on log-transformed data, axis were back transformed for illustration)

The effect of pre-unloading time on skatole and indole in adipose tissue for animals from all three farms is given in Fig.4.2 Skatole and indole concentrations in adipose tissue of animals from the same farm tend to increase with a prolonged pre-unloading time. 24.1% of the animals delivered to abattoir 1 from farm A (pre-unloading time: 480 min) were above the

threshold of 150 ng skatole/g fat, whereas only 14.3% of the animals from the same farm delivered to abattoir 2 (pre-unloading time: 202 min) exceeded the threshold. No animals from farm B did exceed the threshold, irrespective of the abattoir they were delivered to. About 10.7% of the animals from farm C delivered to abattoir 1 (pre-unloading time: 260 min) were above the threshold, but none of the animals delivered to abattoir 2 (pre-unloading time: 17 min).

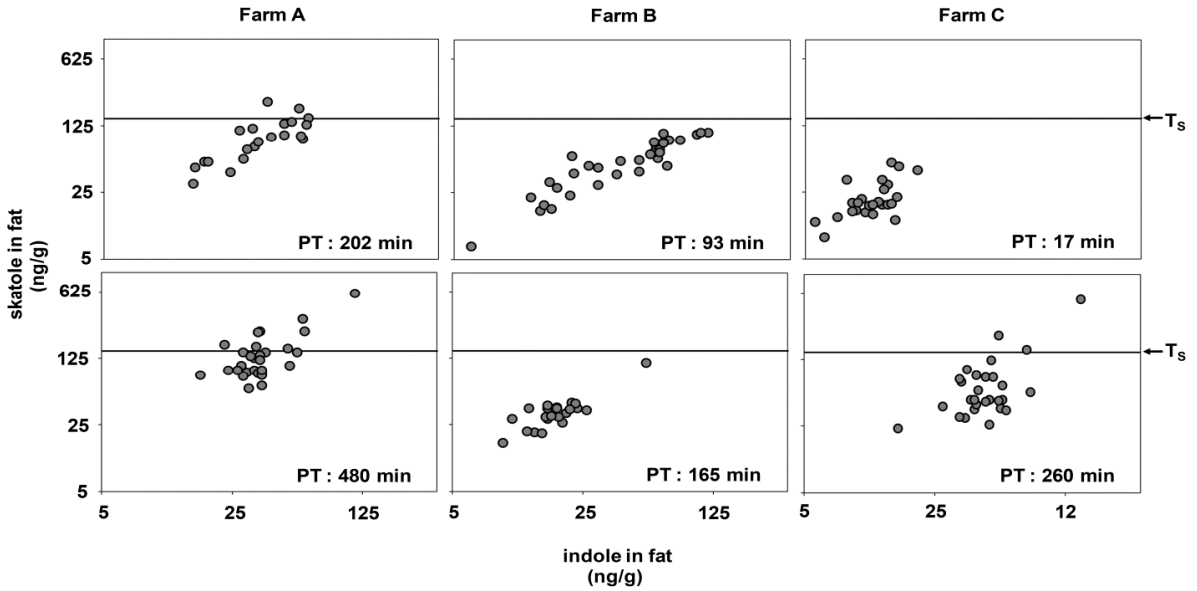

Fig.4.2: Skatole and indole in adipose tissue as influenced by pre unloading time (PT) in the animals delivered from three farms (A, B, C) to two different abattoirs. The threshold for skatole tainted carcasses used in this study (T_S = 150 ng/g fat) is indicated by the solid black line. (Fig. basing on log-transformed data, axis were backtransformed for illustration)

4.5.2 RESULTS OF THE PCA

The use of a PCA for the data was suggested by a Kaiser-Meyer-Olkin measure of sampling adequacy of 0.62, and by a Bartlett's Test of Sphericity result below 0.001. The analysis of principal components (PCs) after Varimax rotation with an eigenwert value above 1.0 revealed 3 factors, as given in Table 4.4, which together explained 61.6% of total variance. PC1, which explained about 23.3% of variance, mainly correlates with transport time and the long-term parameters adrenal (cortisol in feces), testicular function (androstenone in fat, testosterone in feces), as well as testosterone in urine which indicates testicular functions some hours earlier. PC1 is inversely related to pre-unloading time. PC2, which explains about 21.6% of variance, is positively related to skatole and indole in fat, to pre-unloading

time and to lesion score. PC3 explains 16.7% of total variance and is related to overall testicular function, especially testosterone in plasma, urine, and feces, and cortisol in urine. Moreover, PC3 is inversely related to lesion score.

Table 4.4: Results of the PCA after rotation

	PC1	PC2	PC3
total variance %	**23.3**	**21.6**	**16.7**
androstenone in fat	**0.79**		
cortisol in feces	**0.72**		
pre-unloading time	**-0.65**	**0.60**	
transportation time	**0.63**		
testosterone in feces	**0.52**		**0.52**
indole in fat		**0.93**	
skatole in fat		**0.89**	
testosterone in urine	**0.42**		**0.70**
testosterone in plasma			**0.67**
cortisol in urine			**0.49**
lesion score		**0.41**	**-0.48**

4.5.3 RELATIONSHIP BETWEEN LESION SCORE AND SKATOLE OR INDOLE CONCENTRATIONS

A lesion score could be obtained from 153 of the 169 sampled carcasses. 49.0% scored in lesion score class 0, 39.9% in lesion score class 1, and 11.1% lesion score class 2. As the PCA pointed to a concomitant influence of pre-unloading time and lesion score on skatole and indole concentrations, differences in the parameters between the three lesion score classes were further analyzed (LS-means see Fig.4.3). The influence of lesion score class on both parameters was significant in the Kruskal-Wallis test. Carcasses with the highest score (class 2) had significantly higher skatole concentrations in fat than in class 0 ($p<0.01$) and class 1 ($p<0.05$). The differences between lesion score classes in indole concentrations in fat

showed similar influences. Indole concentrations in lesion score class 2 were significantly higher than in class 0 and class 1 (both $p<0.05$).

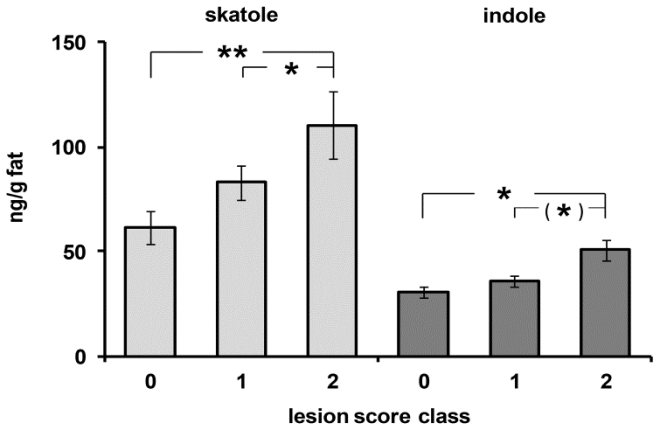

Fig.4.3: LS-means for skatole and indole concentrations in fat according the lesions score classes. Class 0: no lesions/side (n=72); class 1: 1 - 8 lesions/side (n=61); class 2: > 8 lesions/side (n=17) (*:$p<0.05$; **: $p < 0.01$; (*): $p = 0.054$)

4.5.4 CORRELATIONS

Androstenone in fat was positively correlated with testosterone concentrations in long- and mid-term parameters of testicular function, but not in the short-term parameter plasma, as illustrated in Table 4.5 Skatole and indole concentrations were positively correlated with testosterone in feces. In addition, indole tended to be positively correlated with cortisol in feces (r = 0.17; n = 124; p = 0.055). Testosterone and cortisol concentrations did correlate positively in various substrates as shown in Table 4.5 below.

Table 4.5: Pearson correlations

	indole in fat	**testosterone in urine**	**testosterone in feces**	**cortisol in urine**
androstenone in fat		**0.49****	**0.22****	**0.17***
N		**142**	**124**	**142**
skatole in fat	**0.76****		**0.26****	
N	**169**		**124**	
indole in fat			**0.23****	
N			**124**	
testosterone in plasma	**-0.22****	**0.33*****		
N	**165**	**138**		
testosterone in urine			**0.41*****	
N			**124**	
testosterone in feces				
N				
cortisol in urine		**0.16***	**0.23****	
N		**124**	**124**	
cortisol in feces	**0.17(*)**		**0.28****	
n	**124**		**124**	

4.6 DISCUSSION

To our knowledge, this is the first study analyzing the effect of pre-slaughter conditions on concentrations of boar taint compounds in fat, and on endocrine parameters in different substrates which allows the monitoring of changes in those parameters over the preceding hours. To exclude an experimental bias effect of genotype, age and housing, animals from the same farm were transferred to two different abattoirs on two consecutive days. Such a brief delay between transports is not likely to affect boar taint compounds, if groups are not mixed between pens (Fàbrega et al., 2011).

The effects of farm on androstenone can mainly be explained by the different genotypes used in this study, as it is known that androstenone concentrations are higher in Duroc than in landrace crossbreeds (Xue et al., 1996). In contrast, skatole and indole concentrations in fat are mainly influenced by nutrition and environmental factors (Wesoly & Weiler, 2012). Even if feeding and housing of the animals in this study were not standardized, the experimental bias of such effects was excluded as animals from the same farms were delivered to the two abattoirs.

To ascribe differences in boar taint compounds to environmentally-induced endocrine changes, measurements of testicular steroids were carried out in different substrates. Testosterone in blood plasma is known to react rapidly, within minutes, either to sexual stimuli such as the presence of a sow in estrous, or to pharmacological challenges such as hCG (human chorionic gonadotropin) (Bonneau et al., 1982; Claus and Alsing, 1976). The increase of testosterone metabolites in urine starts in parallel to the endocrine changes in blood. A urine sample, however, represents a pool sample over several hours (2 - 3 h) (Hay et al., 2000). The excretion of fecal testosterone metabolites is further delayed by the transit time of digesta along the colon. Thus elevated testosterone concentrations may be expected with a delay of 6 to 30 h, depending on the transit time and fecal deposition frequency, which may vary considerably under stressful conditions (Palme et al., 1996; Désautés et al., 1999). Androstenone in fat is assumed to be another long-term parameter of testicular function, which is also reflected by the close relationships with testosterone in feces and urine in the PCA (PC1). It was therefore quite surprising to see that transport time affected androstenone concentrations in fat so rapidly. However, similar differences in the proportion of androstenone-tainted carcasses have been mentioned in another study comparing short (less than 2 h) and long (6 h) transport times (Moerlein et al., 2012). Most of the studies which show an increase of androstenone in fat resulting from a distinct stimulus have been carried out with a larger time span between application of the stimulus and fat collection (Chen et al., 2006 and Oskam et al., 2010). A similar rapid increase of androstenone

concentrations in fat as shown in our study has been described in an individual boar after hCG application and frequent fat biopsies (Claus & Karg, 1976). The course of androstenone in fat induced by transport should be further clarified by intensive studies with frequent biopsies. Irrespective of the magnitude of reaction, the impact of transport on increased testicular function is further supported by the PCA results for our data. Transport time and long-term adrenal and testicular parameters are related to PC1, which may represent a sustained stimulation of the endocrine system by the novel environment. Such an increase in testicular steroid concentrations in blood and fat of a boar has been identified as the result of a transfer from a familiar pen to an unfamiliar environment (Claus & Alsing, 1976). A novel environment has similar stimulatory effects on adrenal activity (Désautés et al., 1999). After experimental stimulation of the adrenals by ACTH injection, an increase in both cortisol and testosterone concentrations in plasma have been reported (Bilandžić et al., 2012, Liptrap and Raeside, 1975; Pitzel et al., 1984).

PC3 confirmed the relationship between all testicular parameters and fecal cortisol levels, but pointed to an inverse relationship with regard to lesion score. Thus we assume that PC3 reflects the overall testicular development, which is highly variable within a group of boars during puberty. Testicular function also depends on the social rank of the individual within a group (Giersing et al., 2000). The relationship between rank, sexual activity and puberty with mounting behavior has been subject to controversy (Fredriksen and Hexeberg, 2009; Hintze et al., 2013; Rydhmer et al., 2010; Rydhmer et al., 2006). The assumption that high ranking boars are more active in mounting and fighting, whereas low ranking males are mounted more frequently and reveal more skin lesions (Rydhmer et al., 2006) is supported by PC3, which revealed an inverse relationship between parameters of testicular function and lesion score. In another study, mounting behavior was linked to androstenone levels in fat (Prunier, Brillouët, Merlot, Meunier-Salaün, & Tallet, 2013). PC3 in our study revealed a much closer relationship to other parameters of testicular function.

In contrast to androstenone, skatole and indole were mainly increased by pre-unloading time, even though the duration of transport had a significant but less pronounced effect. Thus, differences in skatole levels between boars from the same farm were explained by accordant differences in pre-unloading time in the case of farms A and C, but not in the case of farm B. Farm B had the smallest difference in pre-unloading time (72 min), whereas this difference was about 4 h for the two other farms (A: 278 min; C: 243 min). Such a rapid accumulation of skatole in the adipose tissue of pigs has not been described before. The infusion of ^{14}C-skatole in goats, however, resulted in a rapid storage of radioactive skatole in adipose tissue (Bradley & Carlson, 1982).

The results of the PCA (PC2) also show that both indolic compounds increased in accordance with the lesion scores of the carcasses. Similarly, more lesions due to fighting and higher boar taint scores have been observed for the second lot of carcasses after the split marketing of boars (Fàbrega et al., 2011), whereas in other studies such a significant relationship between skin lesions and skatole concentrations in fat were not found (Bekaert et al., 2012; Prunier et al., 2013).

Our findings of higher skatole/indole concentrations in fat after increased pre-unloading time and higher numbers of skin lesions support the hypothesis of Claus et al. (1994), who considered stress as a reason for the formation and accumulation of both indoles. Fighting, and as a result higher lesion scores, point to stressful conditions during the pre-slaughter period. Although transport time was related to cortisol levels in feces, the pre-unloading time did not further affect cortisol in feces or urine. Only a tendency toward a significant correlation between cortisol in feces and indole in fat was found. As described above, stress leads to both elevated adrenal and testicular activities. This is reflected by the correlation between cortisol and testosterone in feces in our study, and might be ascribed to the loading procedure at the farm. This effect however, might mask that of stressful conditions before slaughter on corticoids in urine and feces. Other possible mechanisms, such as specific effects of stress on colonic mucosa as described for other species, or a shift in microbial composition in favor of skatole-producing bacteria (Bailey et al., 2011; Cook et al., 2007; Gareau et al., 2007; Jensen and Jensen, 1998; Savignac et al., 2011; Whithehead et al., 2008), require further investigation. An influence of stress on the enzymes involved in skatole degradation has been shown for another species (Maksymchuk & Chashchyn, 2012). In barrows, intestinal infections may increase skatole concentrations in fat due to higher tryptophan availability and decreased skatole degradation (Škrlep et al., 2012).

In the present study, weak positive correlations were observed between skatole in fat and testosterone concentrations in feces, but not in urine or plasma. The effect of testosterone on skatole metabolism has been discussed controversially. Claus et al. (1994) reported lower skatole concentrations in adipose tissue after testosterone infusion in barrows. In the in vitro study of Rasmussen, Zamaratskaia, and Ekstrand (2011) testosterone was not effective to inhibit skatole metabolizing enzymes such as CYP2E1 in hepatocytes from boars. In contrast, the prolonged application of testosterone propionate (ten days) led to an androgen-dependent down-regulation of CYP2A19 and CYP2E1 genes in Meishan and Landrace, a condition which is assumed to favor high skatole levels in fat (Kojima & Degawa, 2013). In our study testicular function and hormone secretion were stimulated by transport and by the novel environment, but had a less pronounced effect on skatole levels. We assume that the effect of the transport-related increase in testicular steroids on skatole may be masked by a

fighting activity between boars during the pre-unloading time. The physiological background is the effect of stress on skatole levels, which had been suggested already by Claus et al. (1994). An even closer relationship between testosterone and indole concentrations in fat was found, but a striking explanation for the difference between skatole and indole could not be deduced from our data.

From our results, we conclude that stress during transport and pre-unloading times leads to higher skatole accumulation in fat through several mechanisms: stress favors skatole-producing bacteria through chances in the intestinal milieu, increases tryptophan availability through mucosal damage, and leads to a higher uptake of skatole from colon and lower hepatic degradation.

4.7 CONCLUSIONS

We therefore conclude that androstenone and skatole respond differently to pre-slaughter conditions. Androstenone is influenced by the duration of transport. Skatole, on the other hand, is affected by both transport and pre-unloading times, possibly reflecting stressful conditions and fighting encounters before slaughter. This study points to a new challenge for pig production with regard to pre-slaughter conditions for entire male pigs. We recommend a reduction in both transport and pre-unloading times to minimize the risk for boar taint.

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CHAPTER 5

INFLUENCE OF SAMPLING PROCEDURE, SAMPLING LOCATION AND SKIN CONTAMINATION ON SKATOLE AND INDOLE CONCENTRATIONS IN ADIPOSE TISSUE OF PIGS

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5 INFLUENCE OF SAMPLING PROCEDURE, SAMPLING LOCATION AND SKIN CONTAMINATION ON SKATOLE CONCENTRATIONS IN ADIPOSE TISSUE OF PIGS

5.1 ABSTRACT

Skatole leads to off-odor in pork and is influenced by several factors such as sex and management conditions of pigs, but the causal relationships have not yet been clarified. In the present study, physiological skatole concentrations along the carcass were monitored and the transdermal diffusion of skatole was experimentally studied with skatole-spiked feces. Additionally, the impact of different biopsy techniques on skatole in fat and blood was studied. Monitoring of skatole along the carcass revealed higher skatole concentrations in the belly than in dorsal cuts. Topical application of spiked feces increased skatole in fat strictly at the application site. In contrast to punch biopsies, surgical biopsies significantly affected skatole and cortisol levels in blood, but not in fat. We conclude that biopsies for skatole measurements should be taken without anesthesia from the dorsal side of the animals. Fecal contaminations on the ventral side are not likely to influence overall concentrations.

5.2 INTRODUCTION

The European pig production industry faces the challenge to end pig castration by 2018, even if the occurrence of boar taint, the main reason why piglet castration is undertaken, has not yet been resolved. One approach to reduce the amount of tainted carcasses is the selection for low-tainted lines (Baes et al., 2013; Strathe et al., 2013). Measurements of androstenone and skatole in adipose tissue, the two main compounds leading to off-odors in boars, are therefore included in an increasing number of breeding programs (Frieden et al., 2012). Whereas in earlier studies such measurements were limited to samples from offspring after slaughter, more recent sampling is also carried out in order to assess the individual breeding value for boar taint for the breeding candidates (Baes et al., 2013). While detailed information about the variability of androstenone along the carcass of boars is available (Claus, 1978), similar data for skatole is scarce. Comparative measurements in adipose tissue from different anatomical locations of carcasses are limited to a small number of sampling sites such as neck, flare (visceral fat) and belly (Hawe, Moss, Walker, & Porter, 1989; Lösel, Lacorn, Buttner, & Claus, 2006). These studies revealed significantly higher skatole concentrations in flare and belly compared to dorsal fat samples. In contrast to measurements in carcasses, samples obtained by biopsies may be influenced by different levels of stress during the sampling procedure, which in turn may affect various physiological parameters. In previous studies, an influence of stress on skatole formation and accumulation in fat has been discussed, but the mechanisms have not been investigated in detail for pigs (Claus, Weiler, & Herzog, 1994; Wesoly, Jungbluth, Stefanski, & Weiler, 2015). A possible effect of stress on skatole levels and physiological parameters due to the choice of biopsy technique should therefore be quantified before routine application in breeding programs. So far, such an impact of biopsy technique and the effect of the sampling site has not been investigated in detail. Moreover, the contribution of fecal contamination of the skin to skatole levels in adipose tissue is not clear and has been subject to controversial debate for decades. Transdermal diffusion of ^{3}H-skatole has been described (Hansen, Larsen, Jensen, Hansen-Møller, & Barton-Gade, 1994), but the relevance of this route of absorption on overall concentrations in adipose tissue has not yet been established. In some studies, this transdermal diffusion after soiling was used to explain elevated skatole concentrations in adipose tissue (Hansen et al., 1994; Hansen, 1998). In other studies such an effect could not be proven, even if daily cleaning of pigs was included as a control mechanism while the experimental group was anointed with its own feces twice daily (Aluwé et al., 2011, Bekaert et al., 2012). In this study, we therefore aimed at analyzing the effect of sampling locations and sampling techniques as well as fecal contamination of the skin on skatole concentrations in fat.

5.3 MATERIAL & METHODS

5.3.1 EXPERIMENTAL STRATEGY

Three different experiments were carried out to analyze the effect of sampling site, sampling technique, and fecal contamination of the skin on skatole concentrations in fat. In experiment 1, variations of skatole and indole concentrations were monitored along the carcasses of boars. In experiment 2, the influence of transdermal skatole diffusion was investigated, whereas experiment 3 compared the effects of two different biopsy techniques on endocrine stress parameters and skatole concentrations in blood and fat. For experiments 2 and 3, barrows were used to ensure low initial concentrations of skatole. All samples from each animal were measured either within the same assay or two consecutive assays within one week to avoid artefacts due to storage time or inter-assay variations.

5.3.1.1 EXPERIMENT 1: MONITORING OF SKATOLE AND INDOLE CONCENTRATIONS ALONG THE CARCASS

Carcasses from 8 adult boars (German Landrace; 12 - 14 months; 200 kg live weight on average, range: 194 – 215 kg) were used for this study. In total, 36 samples per animal were collected from 18 anatomical locations on both left and right sides of the carcass as given in Fig.5.1. The samples from the dorsal region were assigned to three cuts: neck (N1 - N3; distance between samples: 6.5 cm), loin (L1 - L6; distance between samples: 7.0 cm) and ham (H1 - H4; distance between samples: 10.0 cm). Samples from the ventral region represent the belly (B1 - B5; distance between samples: 7.0 cm). From each sampling site, subcutaneous adipose tissue (approximately 2 cm x 2 cm x 4 cm) was collected and stored at - 20°C until assayed.

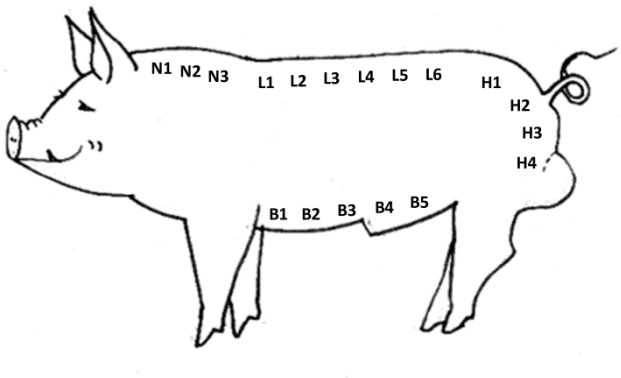

Fig.5.1: Sampling procedure in different cuts along the carcass (N: neck; L: loin; H: ham; B: belly).

5.3.1.2 EXPERIMENT 2: TRANSDERMAL SKATOLE DIFFUSION

Six crossbred barrows (German Landrace x Pietrain) were individually housed in pens (2 m x 3 m) with concrete floors and fed 3 kg/day of a standard diet (13.4 MJ/kg; 17% crude protein; 1% total lysine). The average live weight at the beginning of the experiment was about 200 ± 10 kg. Feces with high skatole concentrations (HSF, High Skatole Feces) were prepared, as follows. Two kg of fresh pooled normal feces (dry matter: 19.5%) with 1.0 g of skatole (Sigma Aldrich, M51458) which had been dissolved in 20 ml MeOH. After the addition of the dissolved skatole the feces were mixed thoroughly for 15 min with a commercial immersion blender. The even distribution was confirmed by measuring concentrations skatole and indole (not added) in four randomly collected samples from this mixture (coefficients of variation: skatole 19 %, indole 9%). Analysis of skatole in HSF revealed 455 µg skatole/g fresh feces and 20 µg indole/g, respectively. High skatole feces were stored in portions of 25 g each at 4°C until the start of the treatment.

In order to characterize the initial skatole and indole pretreatment concentrations in adipose tissue, a punch-biopsy of adipose tissue was taken as a control sample (CON1) from the neck region of each barrow before the first topical HSF application. In order to ensure standardized application of HSF, a 5 x 5 cm square was marked in the shoulder region on both sides of each animal as shown in Fig.5.2. For seven days, the marked area on the left side was covered twice daily at 8:00 a.m. and 3:00 p.m. with 25 g of HSF (HSF-area). The same region on the right side was rinsed twice daily with 50% EtOH and served as post-treatment control (CON2).

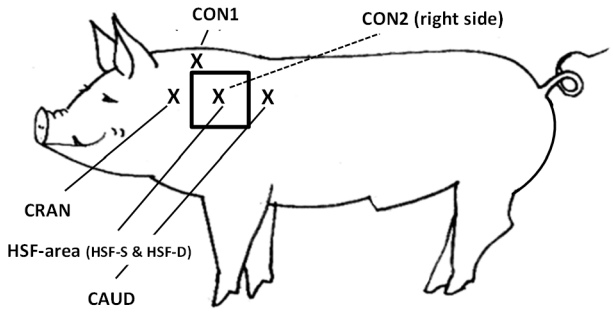

Fig.5.2: Treatment site, site of punch biopsy on day 0 (CON1) and sampling procedure on day 7 (CON2, CRAN, CAUD, HSF-S, HSF-D).

On day 7 all animals were euthanized and adipose tissue samples were collected. Samples of subcutaneous adipose tissue were taken from four locations as shown in Fig.5.2. Two

samples were collected from the HSF-area, one sample from the control area (CON2) and one sample each from cranial (CRAN) and caudal (CAUD), 5 cm from the HSF-area respectively. The post-treatment CON2 was taken from the marked area on the contra-lateral side, which had been cleaned twice daily during the treatment period. The samples from the HSF-area were split into a superficial (HSF-S) and a deep layer (HSF-D). The HSF-S samples represented the first cm of subcutaneous fat below the skin, while HSF-D comprised tissue from 1 - 2 cm below the skin.

To avoid contamination of subcutaneous adipose tissue with the topically applied HSF or other superficial skin contaminations during sampling, all areas were cleaned carefully with water and ethanol before the incision was made. The skin was carefully removed from the subcutaneous fat with a scalpel blade parallel to the surface. The adipose tissue was then divided into two layers (HSF-S and HSF-D), and samples were collected from each layer. All samples were immediately frozen and stored at -20°C until assayed.

5.3.1.3 EXPERIMENT 3: INFLUENCE OF BIOPSY TECHNIQUE

A total of ten barrows (German Landrace x Pietrain) of 110 ± 6 kg live weight were included in this experiment. All animals had been fitted surgically with indwelling jugular vein catheters 6 days before the start of the experiment (Kraetzl & Weiler, 1998) and had been acquainted with the blood sampling procedure and the experimentators. Catheters were rinsed twice daily at 8:00 a.m. and 3:00 p.m. and plasma samples were collected at 3:00 p.m. throughout the experiment. Four animals were subject to repeated surgical fat biopsies (surgical biopsy group, SBG). A total of 5 surgical biopsies (SB) in weekly intervals (day 0, 7, 14, 21, and 28) were carried out in the neck area under total anesthesia (15 mg ketamine [Ursotamin®]/kg LW and 2 mg azaperon [Stresnil®] /kg LW i.v. via catheter). To ensure an undisturbed wound healing only each second biopsy was carried out on the same side.

In the SBG, an additional plasma sample was collected before the SB at 8:00 a.m. The surgical fat biopsies were then carried out between 09:00 a.m. and 10:00 a.m. Before the incision, the skin was cleaned thoroughly and rinsed with iodine. A 3 cm long incision was made and a 2 cm x 2 cm x 1 cm slice of adipose tissue excised and stored deep frozen (-20°C) for further analysis. The wound was closed with a suture, and covered with iodine (Mundipharma, Limburg, Germany) and a layer of aluminum spray. Blood samples were centrifuged directly after collection and stored deep frozen (-20°C) until further analyzed.

The other six animals were subject to a punch biopsy (punch biopsy group, PBG) to monitor the effect of a punch biopsy (PB) taken from the neck region without anesthesia. The PB-tool was a modified captive bolt gun for rabbits with a fixed biopsy needle (diameter 6 mm) as described by Baes et al. (2013). For sampling, the spring was tensioned and the PB-tool was placed in the neck region, approximately 5 cm lateral from the spine. The PB-tool was placed slightly aslope to minimize the trauma to muscle tissue and maximize adipose tissue gain. With this procedure, a fat sample of approximately 300 mg per sampling could be extracted. Directly after sampling, the wound was covered with iodine and aluminum spray. Blood samples were taken 5 min prior as well as 5, 15, 30, 60, 120, and 480 min after punch biopsy. The daily samples (8:00 a.m. and 3:00 p.m.) collected in the SBG correspond to the samples -5 min and +480 min.

5.3.2 ANALYTICAL METHODS

5.3.2.1 SKATOLE AND INDOLE DETERMINATION IN FECES

Skatole measurements in feces were carried out after solid phase extraction as described by Dehnhard, Bernal-Barragan, & Claus (1991), with modifications for fluorescence detection. A Dionex UPLC system (Ultimate RS 3000) with a fluorescence detector (Ultimate 3000 RS FLD-3400RS) was used for skatole and indole determination. Separation of the analytes was performed on an Acclaim 120 C18 2.1x100 mm 3 µm (Thermo Scientific, Karlsruhe, Germany) column. The mobile phase was 0.011 M acetic acid in H_2O:Acetonitrile:2-propanol (55:30:15). A volume of 2.5 µl was injected with a flowrate of 0.4 ml/min and a runtime of 5.5 min. Peak recording was performed with Chromeleon 7.1 (Dionex, Sunnyvale, USA) at emission wavelength 275 nm and extinction wavelength 352 nm. Fluorescence detection revealed good linearity with UV-detection (skatole: $y=1.0453x - 0.499$; $R^2= 0.99$; indole: $y= 1.063x - 2.0495$; $R^2= 0.99$).

Precision was determined by measuring skatole- and indole-spiked fecal samples. The mean recovery rate for skatole and indole was 107.4% and 106.3%, respectively. Intra-assay and inter-assay variability were determined with biological samples for skatole and indole, and were below 10% each.

5.3.2.2 SKATOLE AND INDOLE DETERMINATION IN ADIPOSE TISSUE

The determination of skatole and indole concentrations in adipose tissue were carried out as described by Dehnhard, Claus, Hillenbrand, & Herzog (1993) and by Wesoly et al. (2015).

Precision and reproducibility were comparable to the results described by Wesoly et al. (2015). All concentrations are given in liquid fat further on (ng/g fat).

5.3.2.3 SKATOLE AND INDOLE DETERMINATION IN BLOOD

Skatole and indole concentrations in plasma were determined according to Claus, Dehnhard, Herzog, Bernal-Barragan, & Giménez (1993) with modifications for the UPLC-system. In brief, 2.0 ml diethyl ether were added to 500 µl of plasma sample spiked with internal standard (2.4 ng IS per test), mixed vigorously for 30 seconds, and then centrifuged (15 min, 800g, 4°C). Afterwards, the aqueous phase was frozen for 1 hour and the supernatant was transferred into a vial with 500 µl of eluent A (0.011M acetic acid in H_2O:acetonitrile:2-propanol; 55:30:15). The ether was evaporated at 60°C, and the remaining skatole-containing phase was transferred into vials for UPLC analysis as described above. The injection volume was 50 µl and the run time 9 minutes. The mobile phase consisted of eluent A (0 - 4 min 100%) and B (100% acetonitrile and 0.011 M acetic acid; 4 - 9 min 100%). Precision was determined by measuring skatole- and indole-spiked plasma samples. The mean recovery rate for skatole and indole was 96% and 97%, respectively. Intra-assay variability and inter-assay variability were determined for skatole and indole with biological samples and were below 5% each.

5.3.2.4 CORTISOL DETERMINATION IN BLOOD PLASMA

Cortisol concentrations in plasma were determined with a radioimmunological analysis after ethyl acetat extraction according to Grün et al. (2013). In order to establish procedural losses, the recovery rate was determined with ^{3}H-cortisol and revealed a mean recovery rate of 91.2%. Cortisol-spiked plasma samples revealed a mean recovery rate of 105.5%. The intra-assay and inter-assay variability of biological samples were below 3% and 12%, respectively.

5.4 STATISTICAL ANALYSIS

Statistical analyses were carried out with JMP 11.2.0 (SAS Institute Inc.) statistic software. Cortisol concentrations in plasma and skatole and indole concentrations in all substrates were analyzed after logarithm transformation to ensure normal distribution of the data, which was then verified with the Kolmogorov-Smirnov test. All results are given as LS-means of untransformed data.

The following models were used for the statistical analysis of the experiments. In experiment 1, the side of sampling (left/right), the cut (neck, loin, ham, and belly), the location within a cut (N1 - N3, L1 - L6, H1 - H4, B1 - B5) and the individual animal were included into the analysis. The model used for ANOVA considered the cut, the side of sampling, location nested within cut, side x cut, side x location nested within cut and the individual animal as random effects. Differences between sides, cuts and locations were tested for significance with Tukey HSD.

In experiment 2, an ANOVA model was established with animal and sampling location (CON1, CON2, CRAN, CAUD, HSF-S, HSF-D) as random effects. Differences between sampling locations were tested for significance with Tukey HSD.

For experiment 3, the first 5 days after jugular vein cannulation were not further considered for statistical analysis to exclude artefacts during convalescence. In case of surgical biopsies, the results one day before SB until five days after SB were renumbered for each of the five biopsies per animal as follows: times of sampling were related to the day of SB (day before SB: SB_{-1} to SB_{+5}). On the day of SB two samples were analyzed ($SB0_{before}$ and $SB0_{after}$) which correspond to the morning and afternoon sample on the day of SB. The ANOVA included individual animal and renumbered time of sampling (SB_{-1} to SB_{+5}) as random effects. In case of the PBG, the model included animal and time (-5 min before PB up to 480 min after PB) as random effects. Time was considered as samples gained prior and after PB. The level of significance was set at p-values lower than 0.05.

5.5 RESULTS

5.5.1 EXPERIMENT 1

The average skatole and indole concentrations for all animals and all samples (n=288) were 52.5 ± 44.0 ng/g and 63.4 ± 45.7 ng/g liquid fat (mean ± SD), respectively. Individual pigs differed considerably in their skatole and indole levels (skatole: 17.6 ng/g fat to 154.5 ng/g fat; indole: 19.4 ng/g to 169.3 ng/g fat). As shown in Table 5.1, the ANOVA revealed a significant effect of the individual but also of the cut for both skatole and indole concentrations in adipose tissue (each $p<0.001$). In case of skatole, the sampling side also had a significant impact ($p < 0.01$). Concentrations were 5.9% higher in samples collected from the right side than in those from the left side of the carcasses. This effect, however, was small compared to the differences between individuals as described above, or the more pronounced effect of the cut. Differences between cuts were limited to significantly higher concentrations in samples from the belly ($p< 0.001$) which exceeded those from the dorsal cuts by 25.5% in case of skatole and by 20.0% in case of indole (Table 5.2). Concentrations of skatole and indole between the dorsal cuts did not differ significantly and were uniform within each cut.

Table 5.1: Results from ANOVA in Exp.1

	indole		**skatole**	
effect	F-value	p-value	F-value	p-value
Side	0.84	p=0.3601	93.59	**p=0.0025**
Cut	1223.71	**p<0.0001**	1672.84	**p<0.0001**
location (cut)	0.22	p=0.9987	0.56	p=0.8965
side*cut	0.24	p=0.8684	12.90	p=0.2786
location*side (cut)	0.26	p=0.9970	0.26	p=0.9968
animal	1017.58	**p<0.0001**	1421.96	**p<0.0001**

Table 5.2: Mean skatole and indole concentrations in different cuts of 8 boars (each cut represents 3 [N1-N3] to 6 [L1-L6] samples per boar)

Cut	total samples	skatole (ng/g fat)	Indole (ng/g fat)
neck (N1-N3)	24	48.0 ± 0.88 [a]	59.1 ± 0.69 [a]
loin (L1-L6)	48	48.5 ± 0.63 [a]	59.5 ± 0.49 [a]
ham (H1-H4)	32	47.3 ± 0.77 [a]	59.3 ± 0.59 [a]
belly (B1-B5)	40	64.2 ± 0.69 [b]	74.8 ± 0.53 [b]

Different superscripts within a column indicate significant differences.

5.5.2 EXPERIMENT 2

Skatole concentrations were low at the beginning of the study and remained at that level in control samples as indicated in Fig.5.3. (CON1 vs CON2 : 27.2 ± 0.23 ng/g vs 28.3 ± 0.23 ng/g; LS-means ± SE; n.s.). Topical treatment with HSF for one week was effective to increase skatole concentrations in both layers of adipose tissue in the HSF-area. Skatole concentrations in HSF-S were 10 times higher (262.0 ± 0.23 ng/g) than in CON1 or in adipose tissue from CON2 (each $p < 0.001$). Concentrations in HSF-D were 66% lower than in HSF-S ($p<0.05$) but still higher than in both control samples (HSF-D vs CON1/ CON2, both $p<0.05$).

CAUD and CRAN samples revealed a high variability in skatole concentrations ranging from 27.2 ng/g up to 75.6 ng/g and from 32.1 up to 220.4 ng/g, respectively. Mean skatole concentrations in CAUD and CRAN adipose tissue were significantly lower than HSF-S (CRAN: $p<0.0001$ and CAUD: $p<0.01$). Concentrations in HSF-D, however, did not differ significantly from levels in CAUD and CRAN.

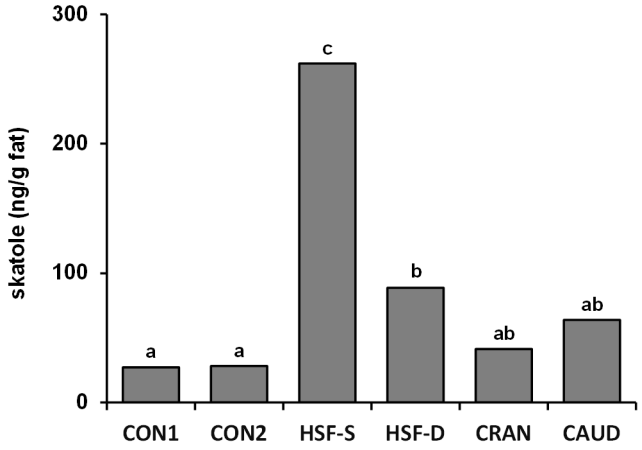

Fig.5.3: Skatole concentrations according to treatment and sampling site. Different superscripts mark significant differences.

Concentrations of indole in adipose tissue of all samples measured were low (<30 ng/g) as the topically applied feces were not spiked with indole. Irrespective of the low concentrations, significant differences between the sampling sites were obvious. Indole concentrations in all samples were significantly higher than in CON1 and further increased significantly in HSF-S as shown in Fig.5.4. Indole concentrations in HSF-S were significantly higher than in all other samples, except HSF-D (Fig.5.4).

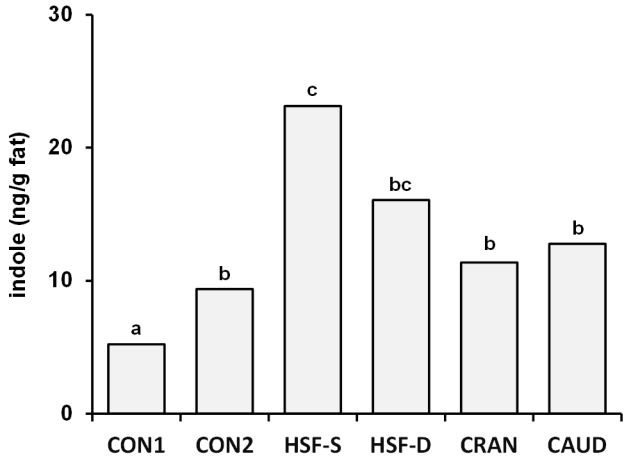

Fig.5.4: Indole concentrations according to treatment and sampling site. Different superscripts mark significant differences.

5.5.3 EXPERIMENT 3

The course of cortisol, skatole, and indole in blood plasma is given for all animals of the SBG in Fig.5.5 over the whole experimental period. It is obvious that each biopsy under total anesthesia is followed by a transient increase in cortisol concentrations, as well as in skatole and indole in plasma. Cortisol concentrations after surgical biopsy reach maximum concentrations of up to 117.2 ng/ml compared to an average of 28.5 ng/ml before the biopsy. This increase occurred regularly after each biopsy under anesthesia throughout the study. Skatole and indole concentrations resembled the cortisol pattern and increased transiently in blood after each surgical biopsy.

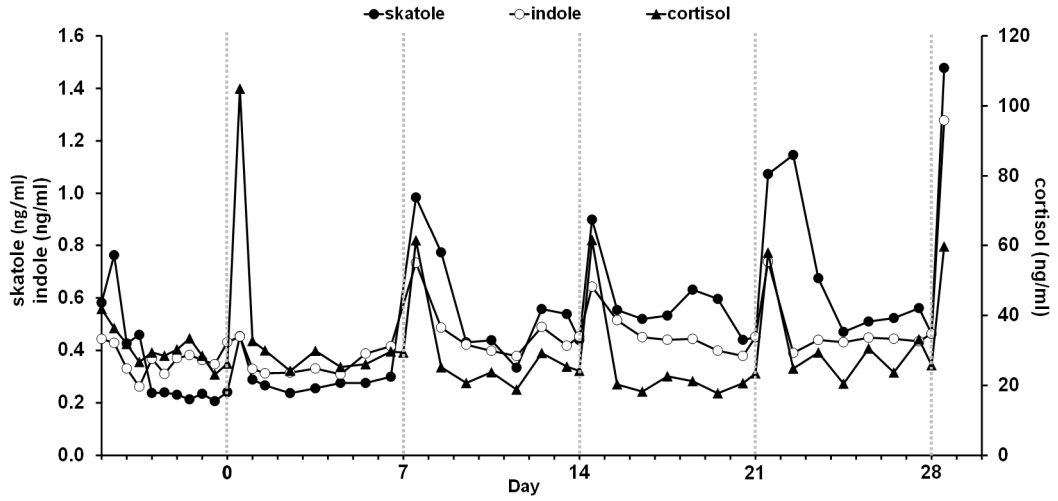

Fig.5.5: Skatole, indole and cortisol concentrations in the course experimental period in SBG. Grey lines marks days of surgical biopsy under total anesthesia.

As illustrated in Table 5.3, the ANOVA revealed a significant impact of the time of sampling in relation to SB for all the blood parameters measured. Cortisol, skatole, and indole concentrations were each $p<0.001$. The concentrations of all parameters at SB_{-1} and $SB0_{before}$ are in a similar order as from SB_{+1} until SB_{+5} and did not differ significantly from each other. The concentrations of skatole and indole show a twofold increase in SB0 after samples ($p<0.001$ each) when compared to all other sampling times (exception: skatole at SB_{+1}: n.s.).

Table 5.3: LS-Means ± SEM for cortisol, skatole and indole in blood on different days prior and post surgical biopsy (SB).

	SB_{-1}	SB_{before}	SB_{after}	SB_{+1}	SB_{+2}	SB_{+3}	SB_{+4}	SB_{+5}
cortisol (ng/ml)	24.62 [a] ± 0.09	28.48 [a] ± 0.10	62.5 [b] ± 0.09	21.16 [a] ± 0.09	21.67 [a] ± 0.10	23.96 [a] ± 0.10	21.3 [a] ± 0.10	22.83 [a] ± 0.10
skatole (ng/ml)	0.37 [a] ± 0.07	0.36 [a] ± 0.07	0.86 [b] ± 0.07	0.55 [ab] ± 0.07	0.42 [a] ± 0.07	0.40 [a] ± 0.07	0.39 [a] ± 0.07	0.44 [a] ± 0.07
indole (ng/ml)	0.39 [a] ± 0.12	0.43 [a] ± 0.12	0.64 [b] ± 0.11	0.40 [a] ± 0.12	0.39 [a] ± 0.12	0.38 [a] ± 0.12	0.38 [a] ± 0.12	0.41 [a] ± 0.11

Different superscripts within a row indicate significant differences

Skatole and indole concentrations in fat samples collected from SBG animals had generally low concentrations (7.80 ± 1.10 ng skatole/g and 8.37 ± 1.09 ng indole/g). Skatole and indole concentrations in adipose tissue remained at this low level during the investigation period and were not affected by the experimental procedure.

In contrast to SBG animals, cortisol concentrations in the PBG were not influenced by PB fat sampling in conscious animals, as shown in Fig.5.6. Similarly, skatole and indole concentrations after PB remained at the same level measured prior to PB. As a consequence, the ANOVA confirmed significant differences only between animals, but not between sampling times for all parameters.

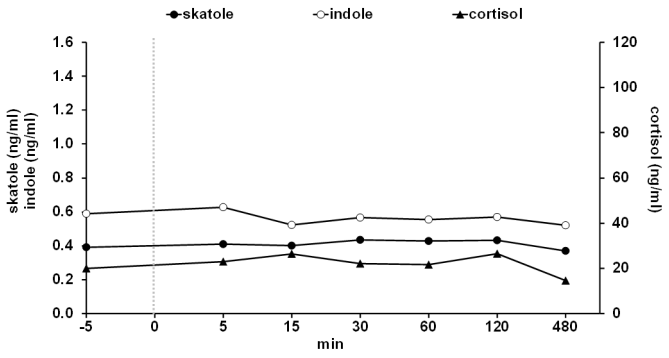

Fig.5.6: Skatole, indole and cortisol concentrations before and after PB. Grey line marks time of PB.

5.6 DISCUSSION

Our study provides new data in the controversially discussed field of skatole physiology. For the first time, a low contribution of transdermal diffusion to overall skatole levels was experimentally proven. Moreover, we could show a close relationship between skatole and cortisol concentrations under the stressful conditions of a SB.

The present study reveals that in mature boars skatole is evenly distributed within the different regions of adipose tissue, except for a 20% higher skatole accumulation in adipose tissue from bellies. Thus our data confirms the high predictive value of even a single sample from the dorsal region to characterize the level of skatole accumulation within a carcass. A more or less pronounced tendency towards elevated skatole and indole concentrations in the belly has been reported in earlier studies, irrespectively of the genotype, even if the sampling frequency was much lower than in the present research (Hawe et al., 1989; Lösel et al., 2006; Weiler, Dehnhard, Herbert, & Claus, 1995). Further increased skatole and indole concentrations have been described for flare fat, which even exceed those in adipose tissue from the belly (Lösel et al., 2006; Weiler et al., 1995). Even if we did not determine fatty acid composition in our samples, our data supports the hypothesis that skatole accumulates in relation to the amount of saturated fatty acids along the body of pigs. Higher amounts of saturated fatty acids were reported for belly and flare fat compared to dorsal fat (Franco, Escamilla, García, Garcia Fontan, & Carballo, 2006; Jeremiah, 1982; Monziols, Bonneau, Davenel, & Kouba, 2007). In a recent study of Mörlein & Tholen (2014), boars were divided into two groups with low (37.5 ng/g in the average) and high skatole (464.5 ng/g in the average) concentrations. Boars with high skatole concentrations also showed higher amounts of saturated fatty acids. Boars from the low skatole groups had leaner carcasses and more unsaturated fatty acids in adipose tissue. Similarly, the higher skatole concentrations in the belly are in accordance with previously reported higher amounts of saturated fatty acids in ventral adipose tissue (Franco et al., 2006; Jeremiah, 1982; Monziols et al., 2007).

Even if differences between the left and right side of the body were small, the reasons for this discrepancy are unknown and further research is required to clarify this surprising result.

To our knowledge, this is the first study describing skatole diffusion through skin in detail. Hansen et al. (1994) reported higher skatole concentrations in pigs with severe fecal skin contaminations. In his study, however, skatole concentrations were higher in the inner layer (near the muscle) than in the outer layer (closer to the skin) of subcutaneous fat. These findings contradict the results of our study, where a decreasing gradient was found from the outer (HSF-S) to the inner (HSF-D) fat layer. The tendency towards higher skatole

concentrations in the samples close to the treatment area (CRAN, CAUD), but without a corresponding increase in concentrations on the contra-lateral side points to a close restriction of transdermal diffusion to the contaminated area, a limited diffusion within the subcutaneous fat and a negligible systemic effect. It was surprising, however, that indole concentrations did differ in the treated area, although HSF was not spiked with indole. The changes in concentrations were statistically significant but small and not in an order relevant to cause boar taint.

More recent studies could not confirm different levels of skatole in pigs raised under differing hygienic conditions or after experimental fecal skin contamination (Aluwé et al., 2011; Bekaert et al., 2012; Van Wagenberg et al., 2013). The small increase of indole after soiling with HSF in our study permits the assumption that skatole and indole from native feces may diffuse through the skin in small amounts, but are not responsible for concentrations leading to off-odor. This could explain the lack of differences in the very systematic approach by Aluwé et al. (2011). In order to explain elevated skatole levels in farms with heavily soiled pigs and a poor level of hygiene, we favor the hypothesis of an endogenous origin of skatole, as in experiment 3 skatole and cortisol concentrations in blood were similarly affected by stressful treatment. Increasing concentrations of skatole in adipose tissue of about 20 ng//h were also found after prolonged lairage times on the transportation vehicle with increased intermale aggression (Wesoly et al., 2015).

In the study of Hansen et al. (1994), heavily soiling of pigs was caused experimentally by reducing pen sizes and omitting the cleaning of the pen during the investigation period. Such a treatment has to be regarded as stressful to pigs, as maintenance of functional areas within a pen is no longer possible for the animals. Endocrine parameters, however, were not included in this study.

Based on the results of our experiment 3, where skatole was affected similarly to cortisol by treatment, we therefore support the theory of a possibly endogenous origin of skatole in stressed pigs. A previous study showed that the skatole metabolism, especially the activity of the metabolizing enzymes (CYP2E1), can be influenced by various conditions like nutrition or hormones (Rasmussen, Zamaratskaia, Andersen, & Ekstrand, 2012; Rasmussen, Zamaratskaia, & Ekstrand, 2011). From others species influences of stress on the P450 enzymes, especially CYP2E1, are described recently (Maksymchuk & Chashchyn, 2012). Continuous stress reduced the activity of CYP2E1 in mice. A similar effect on the CYP2E1 enzymes in pigs may be assumed, but has not been proven yet. Dirty conditions may act as a stressor *per se* and influence the skatole metabolism, but it may also reflect poor overall management with suboptimal conditions for pigs. An earlier study showed lower hepatic

activity, especially of P450 enzymes, in rats kept in dirty conditions (Vesell, Lang, White, Passananti, & Tripp, 1973). Similar studies for pigs are missing, although Claus et al. (1994) showed that the application of prednisolone to pigs increased the production of indolic compounds in the colon.

The results of our experiment 3 point to a strong relationship between the stress-associated parameter cortisol and skatole in blood. The elevated cortisol levels were most likely caused by the ketamine anesthesia, which is known to influence cortisol concentrations in blood (Clapper, 2008). Skatole concentrations followed the course of cortisol after biopsy in our study. However, it is not clear if skatole concentrations increased due to high cortisol levels, or due to depressed liver metabolism as a result of anesthesia (Meneguz, Fortuna, Lorenzini, & Volpe, 1999). Such an increase in cortisol was missing in our PBG, which reflects similar results shown for cortisol in an earlier study (Geverink et al., 1999). The missing effects on skatole concentrations in adipose tissue in the SBG could be explained by the transient increase on treatment days only, followed by low concentrations in plasma from day SB+2 until the next SB. Skatole is accumulated rapidly in adipose tissue, but is also released more rapidly than androstenone as shown by Claus et al., 1994. Moreover, hepatic skatole metabolism in barrows, as used in our study, is not hindered by testicular steroids as has been shown for boars (Brunius et al., 2012; Kojima & Dewaga, 2013). However, the influence of acute and chronic stress on skatole and indole formation and metabolism needs to be further investigated in detail.

Sampling techniques of adipose tissue used on living animals by several breeding companies seem to favor this method. Skatole concentrations may be less influenced by the punch biopsy procedure due to lower invasiveness as described earlier by other authors (Baes et al., 2013), especially when repeated measurements in living animals are required.

In conclusion, skatole diffusion through skin has been confirmed by this study. However, the strong local limitation of skin diffusion and the low variability of skatole concentrations along the carcass demonstrate that the impact of soiling on skatole concentrations in pigs is low. An effect of skin contamination would reveal a stronger variability along the animal body, especially in the cuts exposed to feces like ham and belly. The low variability of skatole concentrations along the carcass does not force sampling of adipose tissue on a narrowed location. Samples obtained in the dorsal region can give representative information for the whole carcass, although reasons for elevated skatole concentrations in the belly region could not be clarified in detail. For boar taint detection at slaughter plants with the human nose technique, these findings could be helpful. Such human nose tests regularly reveal low number of tainted carcasses when compared to analytical studies (Mathur et al., 2012).

However, based on our study, it could be at least recommended to detect smell in the belly region to increase the probability of successfully detecting carcasses with high skatole concentrations. This method, however, would not improve androstenone detection.

We can conclude that the environmental factor stress and its influence on off-odors in entire male pig production requires further investigation.

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CHAPTER 6

GENERAL DISCUSSION

6 GENERAL DISCUSSION

In the European Union about 125 million male pigs are raised each year and castration has been performed traditionally in about 80% of these animals surgically without anesthesia (PIGCAS, 2009). During the last decade, this topic has gained increasing interest in the public discussion, as castration is regarded as infringement in the welfare of male piglets. The European declaration on alternatives for pig castration has been signed in 2010 to end voluntary castration by January 1st, 2018. In Germany castration without anesthesia will be illegal after January, 1st 2019. Most of the pig castrations are performed with analgesia since April 2009 in Germany, but also the percentage of entire male pigs in German pork production has increased in parallel from about 0% in 2006 up to 5 - 10% in 2014. However, most of the European countries still adhere to pig castration mainly due to the problem of boar taint (Backus et al., 2015). Surgical castration of male piglets is actually the most reliable method to prevent boar taint in pork at the consumer level, as detection of boar taint in entire male pigs at the slaughter lines does not provide convincing results. Thus, pork production with entire males suffers from a high risk of tainted boar meat for consumers.

Therefore measures to reduce boar taint formation in entire males are needed as a basic requirement before the given stipulated period of 2019 to end castration in Germany. Thus, to eliminate the boar taint problem in pork production with entire males, basic knowledge about exogenous factors influencing formation, metabolism and accumulation is crucial to optimize management accordingly. As described earlier, boar taint is caused by two main compounds, androstenone and skatole. Recent studies underline the high importance of skatole for adverse consumer reactions, as boar meat samples were scored less negative when skatole levels were low, even if androstenone concentrations were high, due to a considerable frequency of androstenone anosmia in the population of European consumers (Meier-Dinkel et al., 2015; Bonneau & Chevillion, 2012).

As skatole formation occurs in the gut as a result of microbial activity, one aim of this thesis was to evaluate and summarize nutritional factors influencing skatole concentrations in pigs. Several feeding strategies and especially feed additives were identified in the past to reduce skatole concentrations in fat of pigs as discussed in Chapter 3. Successful feeding strategies and additives to reduce skatole concentrations under practical conditions need to fulfill the following requirements: they have to reduce skatole concentrations in fat reliably, they have to be effective in low dosage, and they should not have a negative influence on fattening traits or the overall economic performance. Moreover, from a physiological point of view, successful skatole-reducing feeding strategies have to affect more than one of the steps of cascade of skatole formation. It has been shown that feeding of pigs with at least 20% of raw potato starch resulted in a significant reduction of skatole concentrations in adipose tissue

but also in the colon, suggesting a reduced formation as the key mechanism for the reduction in fat (Lösel et al., 2006). The feeding of raw potato starch for 100 days even up to 30% of the ration did not influence growth performance traits in pigs (Fang et al., 2014; Øverland et al., 2011; Pauly et al., 2008). However, it slightly affected the concentrations of androstenone (Rasmussen et al., 2013; Chen et al., 2007).

Tannins have been shown to be effective in reducing ruminal skatole formation *in vitro* or *in vivo* in ewes as further discussed in 3.3.2. Recently, it was proven that adding 1 to 3% of tannins from chestnut wood extract in diets of pigs for 70 days before slaughter led to reduced skatole concentrations in the colon content and to an elevated activity of skatole metabolizing enzymes in the liver (Čandek-Potokar et al., 2015). These changes were reflected in a tendency to lower skatole concentrations in fat which, however, was not statistically significant. Thus tannins show positive potential for practical implementation, since they are active on more than on level of the formation cascade of skatole. However, there are no published data on specific requirements for tannin usage like, the accurate dosage, duration of application and no data are published for the effect of tannin diets on androstenone concentration.

The addition of inulin to diets has been proven sufficiently to be effective in reducing skatole (Table 3.1). Zammerini et al. (2012) reported an increase of androstenone concentrations in fat of boars during the last two weeks prior to slaughter. This increase was not accompanied by the expected increase of skatole concentrations in fat if inulin via chicory supplementation (9%) was included into the diet. The addition of inulin resulted in 56% of animals with very low skatole concentrations in fat (< 0.05 µg/g) whereas only 7% of the boars in the control group revealed such low concentrations (Zammerini et al., 2012). These results confirm the reducing effect of inulin on skatole concentrations. Inulin is known to increase hepatic skatole metabolism and decrease the rate of microbial TRP degradation by favoring microbial protein synthesis due to its function as microbial energy source (see Fig 3.2). In contrast, androstenone is known to decrease hepatic skatole metabolism (see 3.2.4). Recently, it has been described, that feeding inulin reduces androstenone concentrations possibly via enhanced degradation in the liver (Rasmussen & Ekstrand, 2014; Rasmussen et al., 2013). Regarding the resulting skatole concentrations in fat, the effect of androstenone on skatole degradation seems to be of minor importance when compared to the effect of decreased skatole production from TRP, as described above. The increase of androstenone along the study of Zammerini et al. (2012) was discussed by the authors as the effect of establishing a new social hierarchy, as boars were removed from the group in weekly intervals. The effect of continuous social stress from this formed hierarchy may neutralize the effect of inulin on hepatic androstenone degradation.

Furthermore, elevated androstenone concentrations were also reported in blood collected at slaughter plant when compared to blood samples obtained one week earlier (Tuomola et al., 2002). Both studies point to a rapid increase of androstenone in blood or adipose tissue after exogenous stimuli which affect testicular function such as e.g. inter-male aggression or a novel environment but an effect of age cannot be excluded.

In the present study such an age effect could be excluded and the increase in androstenone and skatole, respectively, could be ascribed either to transport or pre-unloading time. Stressful conditions while transport to slaughter were associated with inter-male aggression and quality drawbacks in pig meat when the driven distance extended 170 km in a recent study (Arduini et al., 2014). Our findings also support earlier results showing an increasing effect of the transport to a novel environment on androstenone concentrations; however, this was shown for an individual animal (Claus & Alsing, 1976). Our results also added a more systematic approach, including a high number of animals, where effects of farm, and genotype could be excluded. As discussed in 4.6 stress seems to be one major trigger of the cascades leading to increased boar taint. The impact of stress on skatole concentrations is further shown and supported from our results of Exp.3 (see 5.5.3) as discussed later.

In the study of Zammerini et al. (2012) skatole concentrations were low in the inulin fed group, although social stress for the pigs was assumed. The reduction of skatole formation in the gut by inulin in this study predominated the effect of stress. Thus, results of the present study as well as those of Zammerini et al. (2012) may have an important impact for the management of pigs before slaughter. Reducing transport as well as pre-unloading time are effective measures to decrease the risk of a pre-slaughter increase of the two boar taint compounds. The suggested measures to reduce boar taint compounds before slaughter are already in state of implementation in national slaughter plants (Bubeck, 2014). Additional inulin feeding may further reduce the risk for skatole accumulation in case of stressful conditions but this suggestion has to be further investigated in detail.

The effect of housing conditions on skatole concentrations has been discussed extensively in literature (for review see: Frieden et al., 2014; Prunier et al., 2013; Van Wagenberg et al., 2013; Bekaert et al., 2012; Aluwé et al., 2011; Hansen et al., 1994). However, the mechanisms leading to elevated skatole concentrations in fat under different housing conditions have not been finally clarified and are still subject to controversial discussions. Therefore this thesis aimed to clarify a suggested contribution of soiling to overall skatole levels. Hansen et al. (1994) discussed exogenous sources of skatole for elevated concentrations in fat and suggested a skatole uptake via inhalation if animals were kept in heavily fouled pens. Maximum concentrations up to 335µg skatole /m^3 air were measured in facilities with low hygienic conditions, which are about tenfold higher than the average

skatole concentrations of 40 µg/m^3 measured in regularly cleaned pens (for review see: Ni et al., 2012). Using such concentrations for a theoretical calculation and a total volume of 31m^3 air/pig and day (tidal volume of 9 ml/kg BW and 15 - 20 breaths/min) (Klein & Reinold, 2001), a total amount of 10.4 mg skatole per day and pig may result. Assuming a resorption rate of the 100% from the inhaled air this amount is only about 10% of daily upper skatole resorption from the gut of entire male pigs (Laue et al., 1998; Hawe et al., 1993; Jensen & Jensen, 1993). Studies with multi-catheterized pigs and peroral and intracecal skatole challenge experiments showed, that skatole uptake rates from the gut into portal vein may vary between individuals but can reach almost 100%. Most of this amount is metabolized immediately by the liver and liver extraction rates of skatole may reach up to 96%. Liver extraction rates out of plasma were proven to be a rapid mechanism since skatole clearance in peripheral plasma was determined to be 0.045 mg skatole/min compared to total hepatic clearance of peripheral and portal vein blood with 0.081 mg skatole/min, respectively (Agergaard & Laue, 1998). Thus, a considerable contribution of skatole from inhaled air is only possible if 100% would be resorbed in the lungs and no further metabolism would occur, which is an unlikely scenario. In addition, this route cannot explain sufficiently differences between individual animals within a pen (Walstra et al., 1999). As a further source of skatole in carcasses coprophragy was discussed. Adding supraphysiological concentrations of skatole (450 mg/day) to the diet of pigs led to elevated skatole concentrations in fat (Pederson et al., 1986). Coprophagy in general, however is not frequently observed in pigs (Saucier et al., 2007). To resemble the amount of skatole applied per pig and day in the study of Pederson et al. (1986) the amounts of feces taken up by coprophagy have to be in an order of 25 kg feces per day (Hansen et al., 1994; Hawe & Walker, 1991).

More convincing was the discussion about a possible transdermal diffusion of skatole into adipose tissue, since ^{3}H-Skatole has been reported to pass through the epidermis of pigs (Hansen et al., 1994). In support of this hypothesis, Aluwé et al. (2011) tested the effect of transdermal skatole diffusion but did not find a clear and significant effect of soiling pigs with feces on skatole concentrations in adipose tissue. Therefore this thesis aimed to evaluate the influence of transdermal diffusion basically. Our study was designed in such a way that every animal represents a control and inter-comparison of parts of the same animal was possible in this way. Whit this approach the transdermal diffusion was proven. It is known from the results from one part of the studies (5.5.1) that within an individual skatole concentrations do to not vary considerably along the carcass, except homogenous higher concentrations in the belly. The locally restricted diffusion of skatole without any effect on skatole concentrations of the contralateral side in Exp.2 shows that a relevant contribution of soiling to the overall skatole concentrations in a carcass is unlikely (5.5.2). This result confirms to the observation

from previous studies, where soiling of entire male pigs was not correlated to skatole concentrations in adipose tissue (Van Wagenberg et al., 2013; Bekaert et al., 2012).

The close relationship between cortisol and skatole found in Exp.3 points to a physiological link between stress and the accumulation of skatole in adipose tissue of pigs. An extended pre-unloading period with inter-male aggressions before slaughter was shown to increase skatole concentrations in fat (Table 4.3). In our study (Chapter 4) cortisol concentrations in urine and feces were correlated to testicular steroids, however only small correlations were observed for indole but not for skatole. Even if the particular mechanism is not known for the pig, the increased skatole deposition into fat could be explained through a decreased hepatic degradation rate, as similarly described for mice under stressful conditions, where a reduced CYP2E1 activity was found (Maksymchuk, & Chashchyn, 2012). Such a mechanism would also explain elevated skatole levels in pigs from pens with bad hygienic conditions due to heavy fouling, as this may be regarded as stressful conditions.

Also, the reported increase in skatole concentrations in plasma after weaning (Lanthier et al. 2006) coincide with elevated cortisol concentrations, as these are increased after weaning for almost two weeks in piglets (Meunier-Salaün et al., 1991). Lanthier et al. (2006) explained that the increase of skatole concentrations is a result of formation a new microbial flora in the intestine and can be less likely explained by elevated apoptosis after weaning. The multiple hints to a link between cortisol and skatole suggest, that stress may further contribute to the increase in skatole concentrations in blood after weaning. Thus, considering stress as an important factor may also offer a new approach to discuss environmental effects of housing or at slaughter on skatole levels. However, the mechanisms, how stress leads to elevated skatole levels in pigs need to be clarified in detail.

The ban of pig castration within this decade all over Europe is an ambitious goal, which has been mainly driven by the motivation to improve animal welfare. On the first glance, this goal contradicts the aim to preserve high meat quality free of boar taint and thus a good consumer protection. The results presented in our two experimental studies point to the importance of a careful treatment of pigs on farm as well as during the transport to slaughter both, due to animal welfare aspects and to maintain a high meat quality low in skatole concentrations. A reduced transport time and a prevention of social conflicts between boars further contribute to a good meat quality low in androstenone concentrations. Our studies thus provide some of the information needed to find a solution in the field of boar production and point to a perspective in this conflict of aims.

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CHAPTER 7

SUMMARY

7 SUMMARY

Castration of male piglets without anesthesia and analgesia has been an ancient and traditional practice in several European countries to prevent problems with boar taint.

The commencement of the National Protection of Animals Act on 13th July 2013 intends to end castration without anesthesia in Germany by 2019. In Europe, the goal is to end castration of piglets by raising entire male pigs only from 2018 onwards. This, however, is a challenge for the entire supply chain of the pig production industry, since sufficient consumer protection from tainted boar meat cannot be guaranteed so far. Off-odors in boar meat are caused by the testicular steroid androstenone and the tryptophane metabolite skatole. Exogenous and endogenous factors which favor the formation of both boar taint substances, but especially of skatole, have so far been only partially identified. Thus the aim of the present thesis was to determine exogenous influences on skatole formation and deduce measures to reduce the amount of tainted carcasses.

In the first part of the thesis (Chapter 3), the current state of research on the impact of feeding strategies on skatole physiology was summarized. The mechanisms of different feeding strategies and feed additives were described in their effect on formation, metabolism and fat deposition of skatole. Based on a deduced formation cascade of skatole, different feeding strategies aiming to reduce skatole can be evaluated in a simplified manner. It could be shown that promising feeding strategies to reduce skatole have to be effective on more than one level of the formation cascade at the same time.

In the second part of this thesis (Chapter 4), the reasons for varying androstenone and skatole concentrations found at slaughter plants in animals from the same origin were investigated. In order to identify the reasons leading to said differences, a study with 169 boars from three different farms was conducted. Each farm delivered animals, split into two groups, to two different slaughter plants with a time interval of one day. The duration of transport as well as the duration of pre-unloading and the time spent on the vehicle before unloading were recorded. During the slaughter process, samples of blood, feces and urine were collected for hormone analysis. Carcasses were scored visually for lesions in cold storage after slaughter, and adipose tissue was removed for boar taint analysis. Even in animals of the same origin, the same genotype as well as the same feeding regimen, significant differences in androstenone and skatole concentrations in fat could be measured, which could be traced back to the different transport and pre-unloading times. In contrast to androstenone, skatole in the fat of the animals was influenced predominately by the

pre-unloading time and increased by more than 20 ng/g with every hour of pre-unloading time. In addition, animals with higher lesion scores had higher skatole concentrations in fat. Transport time, on the other hand, had an effect on androstenone concentrations in fat, which increased by about 0.1 µg/g per hour transport. Skatole concentrations, however, were only slightly affected by transport time. Positive correlations could be found between cortisol and testosterone in various substrates with deposition of boar taint substances in fat. However, further research is required to clarify the mechanisms of these effects in detail. Minimizing transport and pre-unloading times before the slaughter process, however, seems mandatory to reduce the amount of tainted carcasses.

The third part of this thesis (Chapter 5) investigated the impact of management factors on variability of skatole concentrations in blood and fat. Modern breeding companies take fat biopsy samples to estimate the breeding value for the trait boar taint in AI boars. However, it is not yet known to which extent repeated biopsies or different sampling locations may themselves affect skatole levels. Furthermore, the influence skin contamination of animals and of transdermal skatole diffusion have been matters of heated discussion for decades. The published results, however, are in part contradictory and it is not clear to which extent skatole can diffuse through the skin and to which degree this source may contribute to the concentrations in the carcass. As a consequence, the clarification of these complex relationships was the subject of the third study. The results show that skatole concentrations reveal a low variability in samples from the dorsal part of the carcass, although 20% higher concentrations were measured in the ventral area. The transdermal diffusion of skatole was confirmed in this study, but it was also shown that this diffusion is local and that skatole levels of the carcass in general were not increased. Repeated biopsies under total anesthesia temporarily increased skatole levels in blood and were accompanied by a similar course of cortisol concentrations in blood. Punch biopsies in conscious animals had no effect on the course of skatole or cortisol in blood.

The present thesis illustrates that, besides nutrition, stress is an important factor for off-odors in boar carcasses. The results from the experimental studies suggest that an improvement of animal welfare can reduce the risk of off-odor in entire male pig production and thus contribute to consumer protection. However, the results also show that any success of the farmer in the reduction of boar taint can be partially undone on the way to slaughter.

CHAPTER 8

ZUSAMMENFASSUNG

8 ZUSAMMENFASSUNG

Die betäubungslose Kastration männlicher Ferkel ist seit Jahrhunderten in vielen europäischen Ländern übliche Praxis um Schlachtkörper mit Ebergeruch zu verhindern. Mit Inkrafttreten der Novellierung des Tierschutzgesetzes am 13. Juli 2013, wurde beschlossen bis zum Jahr 2019 in Deutschland aus der betäubungslosen Ferkelkastration auszusteigen. Europaweit ist man bestrebt bereits 2018 auf die Ferkelkastration gänzlich zu verzichten und Jungeber zu mästen. Dies stellt allerdings eine Herausforderung für die gesamte Kette der Schweinefleischerzeugung dar, da bisher ein ausreichender Schutz der Verbraucher vor geruchsbelastetem Fleisch nicht sichergestellt ist. Die Geruchsbelastungen von Eberfleisch werden durch das Hodensteroid Androstenon und den Tryptophanmetaboliten Skatol verursacht. Exogene und endogene Faktoren die die Bildung der beiden Ebergeruchstoffe - insbesondere von Skatol - begünstigen, sind allerdings nur zum Teil identifiziert. Das Hauptziel dieser Arbeit war es daher exogene Einflussgrößen auf die Bildung von Skatol zu untersuchen und hieraus Maßnahmen zur Verminderung des Anteils geruchsbelasteter Schlachtkörper abzuleiten.

Im ersten Teil dieser Arbeit (Chapter 3) wurde der aktuelle Kenntnisstand zur Auswirkung von Fütterungsmaßnahmen auf die Skatolphysiologie zusammengefasst. Dabei wurden die Mechanismen beschrieben, über die verschiedene Fütterungsstrategien oder Futterzusätze in die Bildung, Metabolisierung und Einlagerung von Skatol ins Fett eingreifen. Anhand einer daraus abgeleiteten Bildungskaskade können skatolreduzierende Fütterungsmaßnahmen hinsichtlich ihrer Wirksamkeit vereinfacht beurteilt werden. Dabei konnte gezeigt werden, dass erfolgversprechende Fütterungsmaßnahmen zur Reduzierung von Skatol möglichst gleichzeitig auf mehreren Stufen der Bildungskaskade ihre Wirkung entfalten müssen.

Im zweiten Teil dieser Arbeit (Chapter 4) wurde untersucht, warum Androstenon- und Skatolkonzentrationen bei Tieren gleicher Herkunft zwischen Schlachthöfen variieren können. Zur Identifikation der Ursachen wurde eine Studie an zwei verschiedenen Schlachthöfen durchgeführt, an denen 169 Eber aus drei verschieden Betrieben geschlachtet wurden Jeder Betrieb lieferte seine Tiere in zwei Gruppen an die beiden Schlachthöfe im Anstand von einem Tag an. Dabei wurden die Transportzeit und die Wartezeit im LKW vor dem Abladen am Schlachthof erfasst. Während des Schlachtvorgangs wurden Blut, Kot und Urin für die Hormonanalytik gesammelt. Im Kühlhaus nach dem Schlachten wurden die Schlachtkörperhälften nach Verletzungsspuren bonitiert und Fettgewebe für die Ebergeruchsbestimmung entnommen. Bei Tieren gleicher Herkunft, gleicher Genetik sowie gleicher Fütterung konnten Unterschiede in Androstenon- und Skatolwerten aufgezeigt werden, welche durch die unterschiedlichen Transport- und

Wartezeiten erklärt werden konnten. Dabei wurde im Gegensatz zu Androstenon Skatol im Fett der Tiere maßgeblich von der Dauer der Wartezeit beeinflusst, je Stunde Wartezeit stiegen die Skatolkonzentrationen um mehr als 20 ng/g Fett an. Zudem wiesen Tiere mit höherem Verletzungsgrad auch höhere Skatolkonzentrationen auf. Die Transportzeit hatte hingegen Einfluss auf die Androstenonkonzentrationen, diese stiegen je Stunde Fahrt um etwa 0,1 µg/g Fett an, während die Skatoleinlagerung nur geringfügig beeinflusst wurde. Dabei konnten positive Beziehungen zwischen Cortisol und Testosteron in den verschiedenen Substraten und der Einlagerung geruchsaktiver Substanzen ins Fett nachgewiesen werden, allerdings sind weitere Untersuchungen notwendig um die detaillierten Mechanismen darstellen zu können. Eine Minimierung von Transport- und Wartezeit bei der Schlachtung von Jungebern scheint zwingend, um den Anteil geruchsbelasteter Schlachtkörper zu minimieren.

Im dritten Teil dieser Arbeit (Chapter 5) wurde der Einfluss von Managementfaktoren auf die Variabilität von Skatolkonzentration in Blut und Fett untersucht. Moderne Zuchtunternehmen entnehmen Fettbiopsien am lebenden Schwein um die Eigenleistung für den Zuchtwert Ebergeruch potentieller Besamungseber bestimmen zu können. Es ist jedoch unklar inwieweit wiederholte Biopsien oder unterschiedliche Entnahmestellen die gemessenen Skatolkonzentrationen beeinflussen können. Zudem wird der Einfluss des Verschmutzungsgrades der Tiere und einer Diffusion von Skatol durch die Haut bereits seit langem diskutiert. Allerdings sind die publizierten Ergebnisse hierzu teilweise widersprüchlich und lassen die Frage offen, in welchem Maß Skatol es durch die Haut diffundieren kann und welchen Beitrag diese Quelle zu den Konzentrationen im Schlachtkörper leistet. Die Klärung dieser komplexen Zusammenhänge war Gegenstand der dritten Untersuchung. Die Ergebnisse zeigen, dass die Skatolkonzentration in Proben aus dem dorsalen Bereich eines Schlachtkörpers nur eine geringe Variation aufweisen, allerdings im ventralen Bereich um 20% höher sind. Die transdermale Diffusion von Skatol konnte prinzipiell bestätigt werden. Dabei konnte auch gezeigt werden, dass die Diffusion lokal begrenzt ist und nicht systemisch die Skatolkonzentrationen eines Schlachtkörpers erhöht. Wiederholte Biopsien unter Narkose erhöhen vorübergehend die Skatolkonzentrationen im Blut und sind von einem gleichsinnigen Verlauf der Cortisolkonzentrationen begleitet. Eine Schussbiopsie ohne Narkose zur Gewebenentnahme hatte keinen Effekt auf den Verlauf der Skatol- und Cortisolkonzentrationen im Blut.

Die vorgelegte Arbeit zeigt, dass neben der Ernährung der Faktor Stress eine wichtige Einflußgröße auf die Geruchsbelastung von Eberschlachtkörpern ist. Die Ergebnisse des experimentellen Teils legen nahe, dass eine Verbesserung des Tierschutzes im Produktionsverfahren Ebermast das Risiko von Geruchsabweichungen bei Ebern vermindern

kann und somit auch zum Verbraucherschutz beiträgt. Die Ergebnisse zeigen aber auch, dass auf dem Weg zur Schlachtung die Erfolge des Landwirts bei der Reduzierung des Ebergeruchs partiell wieder zunichte gemacht werden können.

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Ort und Datum Unterschrift

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