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Transcriptomics and proteomics analysis to identify molecular mechanisms associated with meat quality traits

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Widmung

Dedicated to my family

Meiner Familie

In Gedenken an meine Mutter

Transcriptomics and proteomics analysis to identify molecular mechanisms associated with meat quality traits

Boar taint and water holding capacity (WHC) are important quality criteria in pig production and affect the financial output, the nutritional composition as well as the consumer appeal of pork. Both, boar taint and WHC are measured by several traits which can be characterized by complex genetic architecture and molecular mechanisms. Thus, the analysis of the transcriptome and proteome using high-throughput technologies are necessary to elucidate the molecular mechanisms and to identify biomarkers with the potential to be developed as markers that can be monitored in such traits. The aim of this study was therefore to provide a transcriptome and proteome analysis in liver and muscle samples from crossbred animals with as well different androstenone and skatole levels and high and low drip loss.

In the first study, microarray analysis using the porcine Affymetrix gene chip in liver tissues from 10 boars of a Pietrain F_2 crossbred with high and low androstenone, high and low skatole levels and grouping of combined phenotypes revealed 264 differentially expressed genes (DEGs). Only two genes could be identified in liver between high and low androstenone group, whereas 92 DEGs ($p \leq 0.05$) between high and low skatole group were identified. Out of these genes, 49 were up - and 43 down-regulated in samples with high skatole level. In addition, when a combined phenotype of androstenone and skatole was analyzed, 170 DEGs were identified of which 86 showed an increased and 84 a decreased level of expression. The differentially expressed genes were mainly assigned in metabolic processes, oxidative reductase activity and lipid metabolism. In summary, this study could be obtained an insight into the biology of complex characteristic. The high number of genes identified by comparing groups of combined phenotypes suggests a strong relationship between androstenone and skatole and should be considered in future investigation.

In the second study samples of *musculus longissimus dorsi* with high and low drip loss from a Duroc × Pietrain (DuPi) F_2 resource population (n = 42) was used. The relative protein quantification was done using isotope-coded protein labeling techniques (ICPL) and electrospray ionization liquid-chromatography-tandem mass spectrometry (LC-MS/MS). In total, 763 different proteins were identified. Among these different proteins, PYGL, PYGM, HSPA8, EE1A1, ACTA1, CASQ1, FLN-C, MYOM1, TNNT3, and HSP27 were up-regulated and TNNI1, MYL3, MYL2, MB, MYBPC1, FHL1C, TPM1, TPM2, AK1, TNNC2, MYL11, CK, PGK1 and MYH7 down-regulated in animals with low drip loss compared to animals with high drip loss. Results revealed that with high drip loss meat was characterized by a higher level of glycolytic enzymes than in low drip loss meat. Additionally, we could observe that higher levels of chaperone proteins were associated with a low drip loss level. In conclusion, proteomics studies contribute to understand the underlying metabolisms of different meat quality traits. In further steps combining genomics, proteomics and metabolomics data should enable a holistic view of the relevant biological systems.

Transcriptomics und Proteomics-Analyse zur Identifizierung molekularer Mechanismen von Fleischqualitätsmerkmalen

Ebergeruch und Wasserbindungsvermögen sind wichtige Qualitätskriterien des Schweinefleisches und beeinflussen sowohl den finanziellen Output, die Nährwertzusammensetzung als auch die Attraktivität für den Verbraucher von Schweinefleisch. Beide Merkmale werden von einer Vielzahl an Genen beeinflusst weshalb man von einer komplexen Vererbung mit komplexen molekularen Mechanismen ausgehen kann. Die Analysen des Transkriptoms und Proteoms mittels High-Throughput-Technologien sind daher von Nutzen für die Aufklärung dieser Mechanismen, um im Anschluss geeignete Biomarker entwickeln zu können. Das Ziel dieser Studie war es, eine Transkriptom und Proteom Untersuchung in Leber- und Muskelproben von Kreuzungstieren mit sowohl unterschiedlichen Androstenon- und Skatolgehalten sowie mit hohem und niedrigem Tropfsaftverlust durchzuführen.

Im ersten Experiment konnten mittels des porcinen Affymetrix Genchip in Leberproben von jeweils 10 Ebern mit hohem/niedrigem Androstenon/Skatol Gehalt einer Pietrain F_2 Kreuzung 264 unterschiedlich exprimierte Gene aufgedeckt werden. Nur zwei Gene konnten im Vergleich der Gruppe hohem mit niedrigem Androstenongehalt identifiziert werden, während 92 Gene beim Vergleich zwischen hohem und niedrigem Skatolgehalt ein unterschiedliches ($p \leq 0.05$) Expressionsniveau aufwiesen. Von diesen waren 49 Gene hoch- und 43 runter reguliert in Proben mit hohem Skatolgehalt. Darüber hinaus wurden bei der Untersuchung eines kombinierten Androstenon/Skatolphänotyps 170 unterschiedlich regulierte Gene identifiziert, wovon 86 ein erhöhtes und 84 ein niedrigeres Expressionsniveau zeigten. Die unterschiedlich exprimierten Gene konnten überwiegend Stoffwechselprozessen, oxidativer und reduktiver Aktivitäten und dem Lipidmetabolismus zugewiesen werden. Zusammenfassend konnte mit dieser Studie ein Einblick in die Biologie des komplexen Merkmales gewonnen werden. Die hohe Anzahl der identifizierten Gene beim Vergleich des kombinierten Phänotypes lässt auf eine starke Beziehung zwischen Androstenon und Skatol schließen und sollte in zukünftigen Untersuchen berücksichtigt werden.

In der zweiten Studie wurden Proben vom *musculus longissimus dorsi* einer Duroc × Pietrain (DuPi) F_2 Kreuzungspopulation (n = 42) mit hohem und niedrigem Tropfsaftverlust verwendet. Die relative Quantifizierung von Proteinen erfolgte mittels Isotope coded proteine labeling (ICPL) und Elektrospray Ionisation Flüssigchromatographie/Tandem-Massenspektrometrie (LC-MS/MS). Insgesamt wurden 763 Proteine identifiziert. Von diesen Proteinen, waren PYGL, PYGM, HSPA8, EE1A1, ACTA1, CASQ1, FLN -C, MYOM1, TNNT3 und HSP27 hoch und TNNI1, MYL3, MYL2, MB, MYBPC1, FHL1C, TPM1, TPM2, AK1, TNNC2, MYL11, CK, PGK1 und MYH7 herunter reguliert bei Tieren mit niedrigem Tropfsaftverlust im Vergleich zu Tieren mit hoher Tropfsaftverlust. Die Ergebnisse zeigten, dass ein erhöhter Tropfsaftverlust im Fleisch durch eine höhere Regulation von glykolytischen Enzymen gekennzeichnet war. Zusätzlich konnte festgestellt werden, dass eine höhere Regulation von Chaperon-Proteinen mit einem niedrigen Tropfverlust verbunden war. Proteomics Untersuchungen sind bei komplexen Merkmalen notwendig um die zugrunde liegenden Stoffwechselvorgänge besser verstehen zu können. In weiteren Schritten sollte die Kombination von genomischen, proteomischen und metabolomischen Daten eine ganzheitliche Sicht auf die relevanten biologischen Systeme ermöglichen.

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List of abbreviations

2AAP	2 aminoacetophenone
2-DE	2D gele electrophoresis
2D-LC	Two-demensional liquid chromatography
3MOI	3-metyloxyindole
ACN	Acetonitrile
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
AUC	Area under curve
BP	Biological processes
C2C12	Mouse myoblast cell line
Ca^{2+}	Calcium ion
CAST	Calpastatin
CBB	Coomassie brilliant blue
CC	Cell components
cDNA	Copy deoxyribonucleic acid
cM	Centi morgan
COUP	Chicken ovalbumin upstream promoter
CP	Creatine phosphate
CPS	Campus syndrome
DAVID	Database for annotation, visualization and integrated discovery
DEGs	Different expressed genes
DFD	Dark, firm, dry
DIGE	Differnce gel electrophoresis
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuklease
DuPi	Duroc × Pietrain resource population
e.g.	For example
ELISA	Enzyme linked immunosorbent assay
eQTL	Expression quantitative trait loci
ESI	Electrospray ionization
F	Forward
F_1	Filial generation 1

F_2	Filial generation 2
FC	Folde change
FD	Fluorescence detector
FDR	False discovery rate
FTICR	Fourier transform ion cyclotron resonance
GC-MS	Gas chromatography–mass spectrometry
gcRMA	GeneChip robust multiarray averaging
GeXP	Genetic analysis system
Glm	General linear model
GO	Gene ontology
GWAS	Genome-wide association study
H	Hour
h^2	Heritability
I3C	Indole-3-carbinol
ICAT	Isotope coded affinity tag
ICPL	Isotope coded protein labeling
IEC	Ion-exchange chromatography
IEF	Isoelectric focusing
IMF	Intramuscular fat content
IPG	Immobilized pH gradien
iTRAQ	Isobaric tags for relative and absolute quantitation
Kb	Kilo bases
kDa	Kilo dalton
KEGG	Kyoto encyclopedia of genes and genomes database
LC-MS	Liquid chromatography–mass spectrometry
LD	Landrace
LDM	Laing distal myopathy
LH	Lutenizing hormone
LIMMA	Linear models for microarray data
LM	Musculus longissimus dorsi
LW	Large wight
MALDI	Matrix-assisted laser desorption ionization
MF	Molecular functions
Min	Minute

MRM	Multiple reaction monitoring
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
MSM	Body myopathy
MUFA	Mono unsaturated fatty acids
MW	Molecular weight
MyoD	Myogenic differentiation
Nano-LC-ESI	Nano electrospray liquid chromatography tandem mass spectrometry
NCBI	National center for biotechnology information
OGE	Off-gel electrophoresis
PCA	Prinicipal component analysis
PCR	Polymerase chain reaction
pH_{24}	pH 24 h post mortem in m. long. dorsi
pH_1	pH 45 min post mortem in m. long. dorsi
pH_{45min}	pH 45 min post mortem in m. long. dorsi
pHu	pH ultimate
pI	Isoelectric point
PMF	Peptide mass fingerprinting
PPAR	Peroxisome proliferator-activated receptors
pQTL	Phenotypic quantitative trait loci
PSE	Pale soft and exudative
PSN	Pale soft and non exudative
PUFA	Poly unsaturated fatty acids
Q	Quadrupole
QTL	Quantitative trait loci
QTLdb	Quantitative trait loci database
R	Correlation
R	Reverse
RFN	Reddish, firm and nonexudative
Rg	Genetic correlation
RNA	Ribonucleic acid
RNase	Ribonuclease
RNA-seq.	RNA sequencing
Rp	Phenotypic correlation

RP-HPLC	Reverse-phase high performance liquid chromatography
RSE	Reddish soft and exudative
RT	Reverse transcriptase
SCD	Stearoyl-CoA desaturase
SDS	Sodium dodecyl sulfate
SILAC	Stable isotope labeling by amino acids
SLS	Sodium lauryl sulfate
SM	Semimembranosus muscle
SNP	Single nucleotide polymorphism
SRM	Selected reacted monitoring
SSC	Sus scrofa chromosome
T_le	Testis length
T_wei	Testis weight
T_wi	Testis wide
TFA	Trifluoroacetic acid
TOF	Time of flight
tQTL	Transcription/translation quantitative trait loci
WBSF	Warner bratzler shear force
WHC	Water holding capacity

A list of abbreviations for gene and protein names are shown in appendix table 10

Chapter 1: General introduction

1.1. Meat quality in pigs

The pig (*Sus scofa domesticus*) is one of the most important animals for meat production. The main objective of pig production is to produce high quality and healthy pork that provides a positive eating experience for the consumer. Commercially processed meat products, like cooked and dry cured ham, smoked pork sausages are worldwide known and consumed. Europe is with 152 million pigs and a yearly production of about 23 million t carcass weight the worlds second biggest producer of pig meat after China and also the biggest exporter. In Germany 2011 the consumption per head of pig meat was 54 kg in comparison to beef and veal with 13.1 kg or poultry meat with 18.9 kg (BVDF).

Meat quality can be defined in various ways from palatability to technological aspects to safety. A common definition of quality is that it is a 'measure of traits that are sought and valued by the consumer'. Hoffman (1990) described meat quality as the 'sum of all quality factors of meat in terms of the sensoric, nutritive, hygienic and toxicological and technological properties'. Consumers want a safe, healthy, tasty and inexpensive product which is easy to prepare, whereas tradesman and producer want to produce attractive products, and want to deliver this efficiently at peak cost. Sensory properties of fresh meat and cooked meat include tenderness, boar taint, flavor and color while nutritive factors include fat, proteins and connective tissue content. Technological quality refers to the utility characteristics of meat in the current production processes, which constitute a set of technological and physicochemical properties such as: water holding capacity (WHC), pH, intensity and homogeneity of color, firmness and processing yield. For the consumer, sensory characteristics including appearance, color, and palatability (tenderness, texture, juiciness, and flavor) are key factors (McIlveen and Buchanan 2001, Verbeke et al. 2009). Till now the measuring of meat quality traits based on laboratory methods require an expenditure of time, personnel and cost. Most procedures are generally not quick enough or adaptable enough to an on-line or at-line situation. Ideally, the ultimate eating quality of meat needs to be predicted in the early post-mortem (24-48 h post slaughter) period (Mullen et al. 1998, Troy et al. 1998).

Meat quality is influenced by a lot of different factors such as: environmental factors, animal handling, post slaughter handling, nutrition and genetic aspects. In the next sections the genetically aspects of two important meat quality traits (boar taint and WHC) are displayed.

1.2. The meat quality traits boar taint and water holding capacity

Boar taint and WHC are important meat quality traits in pig production. Both traits influence financial output, nutritional value, consumer appeal and/or technological properties of porcine meat (Malmfors and Lundström 1983, Offer and Knight 1988). The causes for boar taint and WHC are totally different and will be explained in detail in the next sections.

1.2.1. Boar taint

Boar taint is a well-known, but due to the current discussion in the media at present, problem in pig production. Boar taint is an unpleasant odour and flavour of the meat and occurs in high proportion of uncastrated male pigs (Grindflek et al. 2011b) and is mainly caused by high levels of androstenone (Patterson 1968) and/or skatole (Vold 1970) in adipose tissue. Till now, intact boars are rarely used for fattening, because consumers would object to the boar taint, which tends to develop with sexual maturity and renders meat inedible. Consumer thresholds for acceptance levels of androstenone and skatole are approximately 500 - 1000 ng/g fat and 200 – 250 ng/g fat, respectively (Lundström et al. 2009, Walstra et al. 1999). These authors commonly showed a strong aversion to tainted meat (Grindflek et al. 2011b). Diverse studies showed that the acceptability varied depending on the sex of the animal and/or the levels of boar taint (Diestre et al. 1990, Font i Furnols et al. 2008, Matthews et al. 2000). Besides, people react very differently to boar taint, depending on the country of origin, gender, age and their sensitivity (Font i Furnols et al. 2000, Font i Furnols et al. 2003, Matthews et al. 2000, Weiler et al. 2000). Almost all the consumers are sensitive to skatole, whereas some people are anosmic for androstenone. The perception of androstenone is determined genetically (Keller et al. 2007) and, generally, women are more sensitive than men. To eliminate this problem, boars are usually castrated at a young age, a practice which is painful and has been criticized repeatedly as not in line with animal welfare. In 2008, representatives of the German pig farming community, the processing industry and the trade drafted a resolution („Düsseldorfer Erklärung“) to stop castration of piglets without anesthetization. European pig farmers and their union (COPA-COGECA) agreed in December 2010 to terminate surgical castration by 2018 (DBV 2008). This means that castration of piglets with anesthesia will only be accepted as a transitional step until castration will be completely banned in Europe. However, if intact boars are fattened, negative consumer response to boar taint in pork has to be prevented: by testing carcasses routinely with sufficient speed and accuracy and by reducing the incidence of boar taint at slaughter

age. This may be approached in different ways: by genetic selection, nutrition and/or management (Frieden et al. 2011).

1.2.1.1. Causes for boar taint

Androstenone (5α-androst-16-en-3-one) is basically a pheromone and is produced in the Leydig cells of the testis, among other anabolic hormones in a stepwise conversion involving numerous enzymes (Doran et al. 2004, Gower 1972, Kwan et al. 1985, Patterson 1968). The androstenone was described as urine-like odour and flavour in meat (Wysocki et al. 1989). The production of androstenone and other testicular steroids is controlled by the neuroendocrine system, particularly, by lutenizing hormone (LH). Androstenone is subsequently released into the spermatic vein for circulation around the body where, due to its hydrophobicity, it accumulates well in fat (Brooks and Pearson 1986). From there androstenone is transported to the submaxillary glands, where it is released into the saliva to act as a pheromone that influences sow reproduction (Mattioli et al. 1986, Sinclair et al. 2006). In the salivary gland and hepatocytes, androstenone is converted to two other 16-androstene steroids, 3β-androstenol and 3α-androstenol (Mason et al. 1997, Penning 1997a), with 3β-androstenol as the major product produced by the 3-hydroxysteroids dehydrogenase enzymes present in the liver (Sinclair et al. 2005b). Androstenone is metabolised in two phases: phase I consists of metabolism by hydrogenation and phase II consists of metabolism by sulfoconjugation in testis or in liver (Doran et al. 2004, Robic et al. 2008, Sinclair et al. 2005a, Sinclair and Squires 2005). After metabolisation, androstenone will be storage with a poor loss in adipose tissue and produced taint (Bonneau and Terqui 1983, Doran et al. 2004). In figure 1 an overview of the metabolic pathways of androstenone are shown. Therefore, in theory, high levels of androstenone in fat can be dedicated to a high intensity of testicular synthesis and/or a low intensity of liver degradation (Robic et al. 2008).

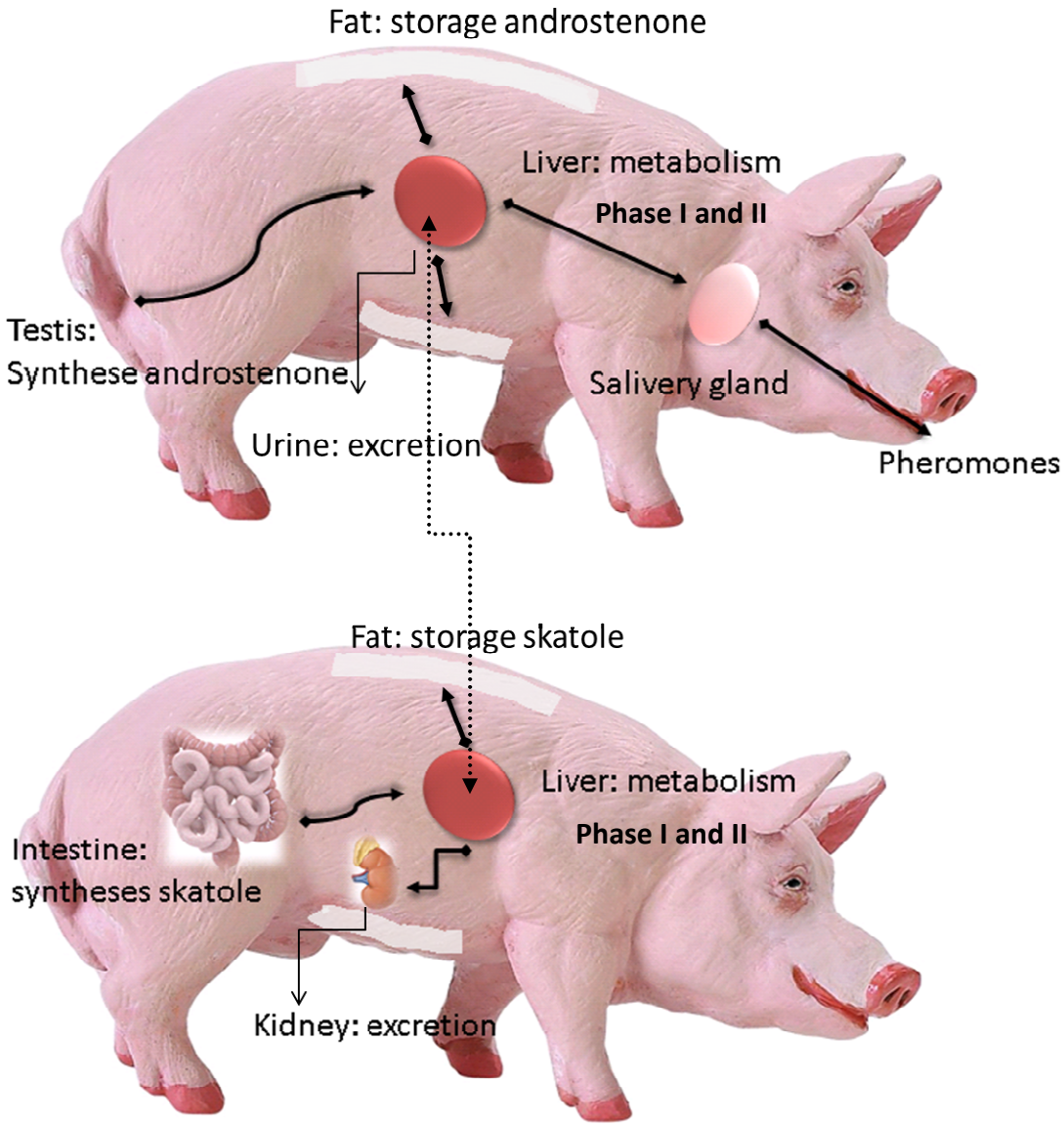

Figure 1: Summary of the metabolic pathways of androstenone and skatole showing the role of enzymes in liver

In comparison to androstenone, skatole is a fermentation product of L-tryptophan and is produced by specific bacteria (E. coli, Lactobacillus and Clostridia) in the large intestine (Lösel et al. 2006, Lundström et al. 2009, Vold 1970). Therefore, the amount of skatole is thus primarily dependent on the availability of tryptophan and the composition and activity of the intestinal bacteria. A significant source of tryptophan represents the turnover rate of the intestinal mucosa cells (Claus et al. 1994). It has a fecal-like odor. Unlike the smell of androstenone the vast majority of people are able to detect the smell of skatole (Andresen 2006). Skatole is synthesized in the intestine of all genders of pigs but only some entire males accumulate skatole in the adipose tissue in high concentrations. A part of skatole is excreted with faces and the remaining part is absorbed through the gut wall and released into the blood

(Agergaard et al. 1998). Skatole is metabolized in the liver to a variety of metabolites (Bæk et al. 1998, Diaz et al. 1999). There are two phases of metabolism. Phase I is a chemical modification to add a hydroxyl group that can be used to attach a conjugate in Phase II. The conjugate makes the modified compound more hydrophilic so that it can be excreted in the urine or bile (Zamaratskaia 2004) (figure 1).

A relationship between androstenone and skatole has been already described in several studies, but till now the mechanisms behind are unclear (see also Chapter 2). There are two hypotheses: (1) male sex hormones may increase the skatole production (Claus et al. 1994) and (2) androgens inhibit the catabolism of skatole in the liver (Doran et al. 2002b). Regulation mechanisms acting on the promoter of one cytochrom P450 family gene (*CYP2E1*) are very important to explain the variability of skatole in fat (Tambyrajah et al. 2004). They have shown that androstenone can inhibit the binding of COUP trancription factor 1 to the promoter the *CYP2E1* gene, which is a member of the steroid hormone receptor family, therefore it is not surprising that androstenone associates with it.

1.2.2. Water holding capacity

Water is the major constituent of meat accounting for approximately 75 % of its weight (Borisova and Oreshkin 1992, Offer and Knight 1988). The other main components include protein (nearly 20 %), lipids (approximately 5 %), carbohydrates (nearly 1 %) and vitamins as well as minerals (round about 1 %). In fact, approximately 85 % of the water in muscle is held within the myofibrils and the cell membrane (sarcolemma) and between the muscle cells and muscle bundles (Huff-Lonergan and Lonergan 2005, Offer and Knight 1988, Offer and Cousins 1992). The amount and distribution of water inside the meat has a considerable influence on its properties. High losses of water in meat affect financial output, nutritional value, consumer appeal and/or technological properties of porcine meat (Offer and Knight 1988).

During the conversion from muscle to meat many changes occur, first of all, a gradual depletion of available energy. The metabolism shifts from aerobic to anaerobic modes favouring the production of lactic acid, resulting in pH declining. Once pH has reached the isoelectric point of the major proteins (e.g. myosin), positive and negative electrical charge on the proteins are equal. These positive and negative groups within the protein attract each other and cause a reduction of amount of water that can be attracted and held by this protein (Huff-Lonergan and Lonergan 2005). Additionally, since like charges repel, as the net charge of the proteins that make up the myofibril approaches zero repulsion of structures within the myofibril is reduced allowing those structures to pack more closely together. The end result of this is a reduction of space within the myofibril. Partial denaturation of the myosin head at low pH (especially if the temperature is still high) is also thought to be responsible for a large part of the shrinkage in myofibrillar lattice spacing (Offer 1991).

The main factor influencing the meat quality in technological and sensory context is muscle pH. It influences characteristics such as color, WHC, cooking losses, processing yield etc. Muscle pH is measured 45 min. and 24 h after slaughtering and shows dynamics of biochemical changes in muscle post-mortem. If the pH decline is rapid and reaches 5.5 to 5.8 while the muscle temperature is still high (more than 36°C), the meat may become pale, soft exudative (PSE). Conversely, if the muscle pH will be abnormally high (6.0 to 7.0) making the meat appear dark, firm and dry (DFD) (Sellier and Monin 1994). Additionally, there are two intermediate forms as reddisch soft and exudative (RSE) and pale soft and non-exsudative (PSN) (Fischer 2007). A rapid pH decline and muscle with high temperature results in denaturation of many proteins including those involved in binding of cellular water

(Huff-Lonergan and Lonergan 2005). This it is accompanied by leakage of muscle cells and loss of water, ions and proteins (Greaser 2001, Offer and Knight 1988). There is also a variation of fluid released from the muscle during conversion of muscle to meat, which is caused by various environmental effects and different genotypes (Greaser 2001).

WHC is defined as the capacity of meat to retain its water during application of external forces (Hamm 1985). This characteristic can be measured by drip loss, but other traits can be used as well (Honikel and Hamm 1994, Honikel 1998). In the past the assessment of drip loss was done by several methods (Borchers et al. 2007, Otto et al. 2004). All methods measure the inherent ability of the cellular and subcellular structures of meat to hold on to part of its own and/or added water. A ccomparison of methods used to measure WHC in pork was given by Kauffman et al. (1986a, 1986b), and a general overview of WHC methods by Honikel and Hamm (1994).

The ability of WHC develops as a result of early post-mortem biochemical and biophysical processes such as rate and extent of pH decline, proteolysis and even protein oxidation that occur in muscle (Huff-Lonergan and Lonergan 2005).

Drip loss is defined as a fluid consisting of water and protein expelled from the meat surface without any mechanical force other than gravity (Offer and Knight 1988). The highest drip loss is often found in PSE meat from pigs that have inherited a mutation on the ryanodine receptor/calcium release channel (*RYR1*) gene in the sarcoplasmic reticulum (Fujii et al. 1991). Because of the existence of a commercial test for this mutation, the German pig production has mostly eliminated this mutation in the pig populations. However there are other factors that cause PSE meat for example the short term stress before slaughter, which determine a rapid pH decline, protein denaturation and therefore higher drip loss (Rosenvold and Andersen 2003).

1.3. Understanding the genetics behind meat quality traits

Genetic effects play a crucial role in meat quality. The heritability (h^2) of such traits varies from low to high (see section 1.3.1.). The reason for these phenomena could be that most of the traits are complex (multifactorial) traits (Andersson and Georges 2004, Andersson 2007). The term "complex trait" described any phenotype which does not exhibit classical Mendelian inheritance attributable to a single gene locus. Several phenotypes may be controlled by several genes, which could influence the trait in varying levels (Lander and Schork 1994). This phenotypic variation could be influenced by different factors such as specific modifier genes, the genetic background, epigenetic mechanisms, stochastic effects in morphogenesis and influences of the environment. It is often impossible to find a genetic marker that shows perfect co-segregation with a complex trait. The reasons for this can be ascribed to a few basic problems: incomplete penetrance and phenocopy, genetic heterogeneity, high frequency of causing allele and other transmission factors (Wolf 1997).

To understand the whole mechanisms behind different meat quality traits in the last years new technologies have been applied to livestock production, including genomics, transcriptomics, proteomics and metabolomics. Together, these technologies offer a holistic view on the relevant biological systems behind traits. Figure 3 shows a schematic representation of these technologies currently available for quantification of different phenotypes. The combination of these technologies with bioinformatics should provide a real time picture of the cell's biology and might allow capture of all the possible effects on the cell/tissue (Wheeler et al. 2010). In the next sections an overview about the state of affairs of the levels of genomics, transcriptomics and proteomics in the meat quality traits boar taint and WHC are given. In the chapters 2 and 3 results of the transcriptomic study with divergent boar taint components and the proteomics study in different drip loss samples are presented.

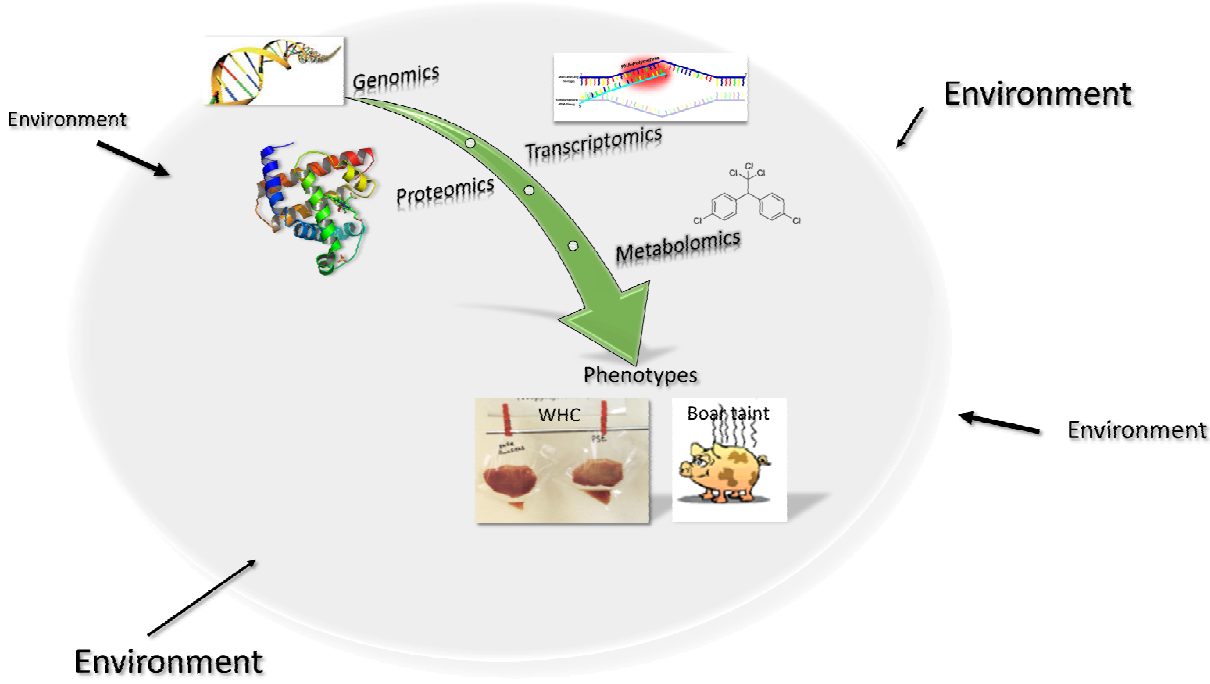

Figure 2: Schematic representation of the major technologies currently available for quantification of different phenotypes

Livestock production started ~8,000 – 10,000 years ago. At this time classical breeding approach where parents of the next generation were selected on the basis of their phenotype for livestock improvement was used. To understand the genetic components of phenotypic variation was an important step in the productivity enhancement in the livestock species. In the 1980s the molecular genetics revolution led to the emergence of a new scientific discipline, genomics, resulting from the convergence of genetics, molecular biology and bioinformatics (Sodhi and Schook 2011). Because the major goal of genomics is to gain an exhaustive understanding of the structure and the functions of the genomes, genomics is classically divided into two basic areas: structural genomics (section 1.3.2.), the characterization of the physical nature of whole genomes and the functional genomics (section 1.3.3.), the characterization of overall patterns of gene expression (Eggen 2003).

1.3.1. Quantitative genetics of meat quality traits

1.3.1.1. Boar taint

The estimated h^2 of the key components for boar taint are at a high level. For androstenone h^2 estimates are found to range from 0.5 to 0.87 and for skatole from 0.3 to 0.5 (Baes et al. 2011, Bergsma et al. 2007, Frieden et al. 2011, Grindflek et al. 2001, Karacaören et al. 2010, Knol et al. 2010, Sellier et al. 2000, Tajet et al. 2006a, Varona et al. 2005, Windig et al. 2012). The lower h^2 for skatole may reflect the greater susceptibility of skatole to environmental factors such as feeding, husbandry and hygiene management, with effects on the bacterial metabolism of L-tryptophan in the intestine of boars.

In a series of studies a positive relationship between androstenone and skatole were published. This relationship can be described from a physiological point of view through the inhibitory action from androstenone of skatole degradation (Doran et al. 2002a). The phenotypic and genetic correlations vary between close to 0 (Zamaratskaia et al. 2005) and 0.3 - 0.4 (Bergsma et al. 2007, Merks et al. 2009, Tajet et al. 2006a, Windig et al. 2012). Skatole and indole were found to be strongly correlated (0.71 to 0.78) (e.g. Baes et al. 2011, Windig et al. 2012). This was expected, since both components are interconnected by a common biological precursor L- tryptophan (Claus et al. 1994).

Relationship between androstenone and fertility

Androstenone is produced in the testis of boars and shows a close relationship with other sex steroids such as androgens and estrogens. For this reason, antagonistic relationships between androstenone and features of reproduction are probably (Claus et al. 1994). This expectation is confirmed by the phenotypic correlations (rp) between androstenone and testicular weight (rp = 0.19 to 0.50), epididymis weight (rp = 0.13 to 0.50), salivary gland (rp = 0.34 to 0.46), seminal vesicle (rp = 0.44 to 0.53) and weight of bulbourethral gland (rp = 0.28 to 0.72). For details see Bracher-Jakob (2000). A relationship between androstenone and paternal fertility shows a moderate undesirable genetic relationship (rg) between androstenone and lifespan of sperm (rg = 0.11), ejaculate volume (rg = 0.18) and sperm motility (rg = 0.32), while sperm concentration shows a desired genetic correlation with rg = -0.22 (Bergsma et al. 2007). In a similar study by Engelsma et al. (2007) they identified an unwanted relationship between the androstenone in backfat and maternal reproductive traits such as age of first insemination (rg –0.24), weaning-conception-interval (rg = 0.44) and stillborn piglets (rg = 0.59).

1.3.1.2. Water holding capacity

The heritability's for traits related to WHC are low. Van Wijk et al. (2006) estimated in 1855 commercial crossbred pigs the h^2, the phenotypic and genetic correlation of 14 meat quality trait. For drip losses h^2 of 0.08 for EZ – drip loss and of 0.11 for the bag method was estimated. Furthermore, the h^2 for ultimate pH was found at 0.11. The genetic correlation between WHC and drip loss is naturally very high (rg = -0.9). Both traits are correlated with pH value 45 min after slaughtering (rg = -0.6/-0.3, respectively), ultimate pH (rg = 0.4/-0.7), reflectance (rg = -0.4/0.5) and cooking loss (rg = -0.2/0.6) (Bidanel et al. 1994, De Vries 1989, Hermesch et al. 2000, Knapp et al. 1997). In a study by Tholen et al. (2005) was found that the presence or absence of the mutation in the *RYR1* locus influences the estimate of heritability for these traits. Three genotypes with a corresponding phenotype on *RYR1* were observed: NN - without mutation, stress stable, normal meat quality; PP - with mutation, susceptible to stress, poor meat quality, often PSE meat and NP - carriers of P allele, heterozygous, phenotype between NN and PP. The heritability for meat quality in homozygous PP populations was higher than in in P- free populations, such as Landrace, Large White or Pietrain PP. In a similar study by Borchers et al. (2007) genetic parameters were estimated directly for drip losses for purebred Pietrain, both in animals with genotype NN and PP genotype. The h^2 of animals with the mutation in the *RYR1* locus was 0.34, whereas h^2 at NN genotypes reduced to 0.14. Genetic correlations between drip loss and early pH were -0.91 for the genotype NN and -0.66 for the genotype PP and complied the study by Tholen et al. (2005). The defects in meat quality traits resulting from the mutation in the *RYR1* locus, such as PSE meat could be processed by breeding. However, the problem is the genetically antagonistic relations between the lean meat content and meat quality. The presented estimations are an indication that different genes are possibly involved in the expression of the examined traits. Drip loss and the other WHC-related meat quality traits are complex quantitative traits, which are influenced by many genes. Therefore, it is difficult to identify single genes with a significant effect on meat quality (Jennen et al. 2007). However, the low h^2 of traits related to WHC implicated that many environmental factors play a significant role to control the traits.

1.3.2. Structural genomics

The search for genetic variation relevant to meat quality has proceeded via quantitative trait loci (QTL) mapping, candidate gene analysis, and the application of genome-wide association study (GWAS) approaches. A large database of QTL for farm animal species is located at http://www.genome.iastate.edu/cgi-bin/QTLdb/index (Hu et al. 2013). Until now, there are 8,919 QTL from 370 publications curated into the database (update: October 2013). Those QTL represent 644 different traits. From this, 973 QTL for drip loss, 68 QTL for androstenone and 26 QTL for skatole were identified. The application of GWAS is a relatively new–omics approach and is facilitated by massively parallel single-nucleotide polymorphisms (SNP) arrays (Pant et al. 2010, Snelling et al. 2010). Once QTL regions have been identified, candidate genes in the interval can be examined by sequencing phenotypically divergent individuals at candidate loci. This may lead to the identification of individual SNP or haplotypes that are causative for traits of interest. Haplotypes may also be used in genome-assisted selection (Hayes et al. 2009). Mainly due to the high h^2 it can be assumed that some important genes with large effect are influencing boar taint (reviewed by Robic et al. 2008). Quantitative traits such as boar taint may be influenced by the same locus (pleiotropic effects) and each single trait may be affected by several loci (polygenic effects). In recent works, the aim was to understand the underlying pathways responsible for the expression of boar taint and to identify the genes involved (Wimmers et al. 2010). On the basis of genetic markers such as microsatellites or SNP, which are distributed as possible over the entire porcine genome, the studies were performed.

1.3.2.1. Quantitative trait loci and single nucleotide polymorphisms identified for boar taint components

Basic work on this was performed by Lee et al. (2005), Quintanilla et al. (2003) and Varona et al. (2005) using microsatellites as marker. Quintanilla et al. (2003) studied in 485 F_2 boars, a cross between Large White and Meishan, the androstenone in fat at different ages of the animals. Various statistical approaches have made it possible to identify QTL on porcine chromosomes (SSC) 3, 4, 6, 7, 9 and 14, which were significantly associated with androstenone level in the backfat of boars. The identified QTL on SSC7 explained up to 14.5 % of the phenotypic variance of androstenone. Lee et al. (2005) used 178 F_2 animals to identify QTL for androstenone, skatole and subjectively evaluated the taste of boar meat. Overall, five QTL were detected on SSC 2, 4, 6, 7 and 9 for androstenone. Varona et al.

(2005) identified in their study in a Landrace population (N = 217) no QTL for androstenone and only one QTL for skatole on SSC6.

The establishment of high-throughput SNP typing techniques like array technology allowed, with a significantly larger number of markers, to genotype the pig genome comprehensively and efficiently. In a GWAS by Duijvesteijn et al. (2010) of 987 commercial endproduct Duroc pigs significant QTL regions on SSC1 and SSC6 for androstenone were identified. The analysis of the skatole in the same population showed 16 significant SNP on SSC6 which did not correspond with the identified QTL for androstenone (Ramos et al. 2011). Grindflek et al. (2011a) conducted a GWAS and linkage analysis for androstenone and skatole in a Norwegian Landrace (n = 1251) and Duroc population (n = 918). A total of 25 QTL for androstenone in the backfat was identified, of which seven could be detected in both breeds. For skatole 12 QTL were detected, of which only four in both populations have been identified. A common QTL for androstenone, skatole and indole was identified on SSC7 in which the candidate gene CYP1A1 is located. This gene is responsible for the synthesis of an enzyme that is involved in both, the metabolism of skatole and in the steroidogenesis (Diaz and Squires 2003). A similar approach using haplotype analysis was applied by Gregersen et al. (2012). The authors examined Duroc pigs (n = 265), Landrace pigs (n = 393) and Yorksire pigs (n = 265) in which 46 significant (suggestive) QTL and 25 haplotypes were found. Only one QTL on SSC12 was in all three breeds detected. The remaining detected QTLs were specific to only one breed or in, two breeds exceptional.

1.3.2.2. Genetic variation related with water holding capacity

Until now 973 QTL (update: October 2013) have been detected for drip loss (http://www.genome.iastate.edu/cgi-bin/QTLdb/index) in several pig breeds and crosses. In a study by Liu et al. (2007) 24 significant and 47 suggestive QTL for meat quality traits and carcass composition traits in a F_2 Duroc × Pietrain resource population were identified. For drip loss several QTL on SSC2, 3, 5 and 18 were detected. Edwards et al. (2008) found 94 QTL regions for meat quality in another F_2 Duroc × Pietrain resource population, but only one QTL on SSC9 was detected for drip loss. The QTL on SSC2 and SSC5 (Liu et al. 2007) as well as the QTL on SSC9 (Edwards et al. 2008) were identified in the study of Thomsen et al. (2004), where a Berkshire × Yorkshire F_2 population was used. Consequently, by using low density of microsatellite markers, QTL are often mapped to a large interval of more than 20 centimorgans (cM). Jennen et al. (2007) reviewed a list of potential candidate genes presently

mapped in or near to QTL regions, which were selected based on their function in muscle development and metabolism and/or their association with meat quality traits.

Much research has focused on the search for genetic variation underpinning drip loss, pale color, and poor texture related to poor WHC. Up to now, several genes influencing body composition and meat quality have been identified using both candidate gene and genome scan approaches. Two SNPs have been identified that affecting strongly WHC and having effects on the muscle proteome: (1) halothane gene or ryanodine receptor (*RYR1* gene) that regulates Ca^{++} transport across muscle cell membranes (Fujii et al. 1991) and (2) the rendement napole (*RN*) gene that affects glycogen content of muscle (Milan et al. 2000a). The recessive mutation (R614C missense mutation) at the *RYR1* results in susceptibility to stress-induced death in pigs or porcine stress syndrome (Fujii et al. 1991). Individuals carrying the halothane gene are highly susceptible to stress accompanying pre-slaughter treatment, even with careful handling. This results in a 90 – 95 % incidence of PSE, production of carcasses that are 3 – 4 % leaner with less backfat (Klont et al. 1994). The *RYR1*-locus was mapped to SSC6 (Sellier 1998) and a DNA test for the defective allele is patented and widely used throughout the world (Fujii et al. 1991). The *RN* gene, identified in the Hampshire breed, has two alleles, a dominant mutant allele RN- and a recessive normal RN+ allele. A dominant mutation in the AMP-activated protein kinase, y-3 subunit (*PRKAG3*), also known as RN gene, is associated with reduced Napole yield and leaner carcasses, and it results in poor meat quality having a lower pH because of post-mortem degradation of glycogen (often called as 'acid meat') (Milan et al. 2000b, Škrlep et al. 2010, van der Steen et al. 2005). Several SNP alleles in the *PRKAG3* gene revealed to have positive effects on pork quality traits including ultimate pH, meat color, WHC, drip loss, tenderness, and cooking loss (Ciobanu et al. 2001). Recent studies in beef indicated that a mutation in bovine *PRKAG3* is also associated with WHC and pH-related traits (Reardon et al. 2010). While coding mutations in these key signalling genes negatively impact WHC, significant variability remains when these are controlled for (Borchers et al. 2007).

It is likely that a number of other genes contribute to quantitative variation in this trait. In pork, calpastatin haplotypes have been found to be positively correlated with several WHC traits (Ciobanu et al. 2004, Škrlep et al. 2010). These associations have been patented (Rothschild and Ciobanu 2003) and commercialized. Casas et al. (2006) identified an association between an SNP in the 3' untranslated region of Calpastatin (*CAST*) and juiciness in a U.S. Bos Taurus population, whereas an SNP in the *CAST* gene (Schenkel et al. 2006) was found to be significantly associated with ultimate pH color in loin and ham muscle in

bovine M. longissimus and M. semimembranosus (Reardon et al. 2010). In a later study, although the ultimate pH values were within the acceptable range, GG genotype animals were more likely to produce dark-cutting beef, compared with other genotypes. Other genes for which associations with pH have been reported include myogenin (Kim et al. 2009), pyruvate kinase muscle isozyme 2 (*PKM2*) (Sieczkowska et al. 2010), and troponin I (Yang et al. 2010). In pig, *PKM2* and *CYP21* are associated with drip loss and 2,4-dienoyl CoA reductase 1 (*DECR1*) has been associated with glycolytic potential (Kamiński et al. 2010, Sieczkowska et al. 2010).

The establishment of high-throughput SNP typing techniques like array technology allowed, with a significantly larger number of markers, to genotype the pig genome comprehensively and efficiently. In a genome-wide association study by Luo et al. (2012) in a 455 Large White x Minzhu intercross population 45 SNP showed significant association with one or multiple meat quality traits. Ma et al. (2013) used for they GWA analysis two different pig populations 434 Sutai pigs and 933 F_2 White Duroc x Erhulian intercross prigs. Meat quality traits, including pH, color, drip loss, moisture content, protein content and intramuscular fat content, marbling and firmness scores in *musculus longissimus dorsi* (LM) and semimembranosus muscle (SM) were verified in the two populations. The authors identified in total 127 chromosome-wide significant SNP for these traits. Among them, 11 SNP reached genome-wise significance level, including 1 on SSC3 for pH (pH drop between 45 min and 24 h post-mortem), 1 on SSC3 and 3 on SSC15 for drip loss. The 3 SNPs on SSC15 were in a haplotype block spanning 178 kb.

1.3.3. Functional genomics

Beside genomics, functional genomics focuses on the dynamic aspects such as gene transcription, translation, and protein–protein interactions, as contrary to the static aspects of the genomic information such as DNA sequence or structures. The aim of functional genomics is to answer questions about the function of DNA at the levels of genes, RNA transcripts, and protein products and to understand the relationship between an organism's genome and its phenotype (Hieter and Boguski 1997). Gene “function” can be considered from several points of view: it can mean biochemical function (e.g. protein kinase), cellular function (e.g. a role in a signal transduction pathway), developmental function (e.g. a role in pattern formation), or adaptive function (the contribution of the gene product to the fitness of the organism) (Rahman et al. 2013). With these approaches the focus of the analysis is shifted from individual components to biological systems. Functional genomics involves the use of

high-throughput methods for the study of large numbers of genes (ideally the entire set) in parallel. Having identified a new sequence, the comparison with sequence databases is the simplest way to obtain (essentially biochemical) functional information (Bouchez and Höfte 1998).

1.3.3.1. Tools for transcriptome analysis: a short overview

Functional genomics as described in section 1.3.3. are characterize as a overall patterns of gene expression (Eggen 2003). For the identification of a range of genes that differ in expression between experimental samples, in the last 20 years techniques for evaluating gene expression have progressed from methods for single specific genes (northern blot, semi-quantitative polymerase chain reaction, PCR). The simultaneous measurement of thousands of transcripts is possible today by microarray platform technologies (Bilitewski 2009) or next generation sequencing (Shendure and Ji 2008).

DNA microarray technology has been advancing rapidly. Gene expression microarray results have produced much important information about how trancriptome is deployed in different cell types (Chtanova et al. 2001), and tissues (Kononen et al. 1998). DNA microarrays are typically composed of DNA "probes" that are bound to a solid substrate such as glass. Each spot (50 to 150 μm) in the array lattice is composed of many identical probes that are complementary to the gene of interest. During hybridization DNA "targets" diffuse passively across the glass surface, when sequences complementary to a probe will anneal and form a DNA duplex. Hybridized targets can then be detected using one of many reporter molecule systems (Call 2001). In essence, a microarray is a reverse dot-blot that employs the same principles of hybridization and detection used for many years with membrane-bound nucleic acids (e.g. Southern and Northern blots) (Schulze and Downward 2001).

The completion of genome sequences in livestock species will almost certainly allow for measurement of the whole transcriptome. Whole transcriptome sequencing using the next-generation sequencing technologies is rapidly developing, which will provide another means to measure the abundance of all transcribed RNA including non-coding RNA and splice variants (Forrest and Carninci 2009). The advent of the next-generation sequencing appears a more appealing technology for the whole transcriptome analysis compared to the microarray technology (Ansorge 2009).

Principle, RNA sequencing (RNA seq) analyzes complementary DNA (cDNA) by means of highly efficient, next-generation methods and subsequent mapping of short sequence fragments (read) onto the reference genome (Twine et al. 2011). A population of RNA (total

or fractionated, such as poly (A)+) is converted to a library of cDNA fragments with adaptors attached to one or both ends. Each molecule, with or without amplification, is then sequenced in a high-throughput manner to obtain short sequences from one end (single-end sequencing) or both ends (pair-end sequencing). The reads are typically 30 – 400 bp, depending on the DNA sequencing technology used. The resulting sequence reads are aligned with the reference genome or transcriptome, and classified as three types: exonic reads, junction reads and poly(A) end-reads. These three types are used to generate a base-resolution expression profile for each gene (Pascual et al. 2010, Wang et al. 2009).

However, until now the acquisition and implementing cost of potential companies still relatively high, because of this for transcriptome analysis in this study a microarray analysis was selected for the identification of differentially expressed genes.

1.3.3.2. Whole transcriptome analysis to identified candidate genes related with meat quality traits

QTL regions are generally large and contain several putative causal genes. Combining microarray data with QTL linkage studies offers new options of understanding the biology at a global level and the genetic factors affecting the trait of interest. To elucidate the biological mechanisms through fine-mapping functional analysis of potential candidate genes are necessary. Up to now, using microarray techniques a number of candidate genes and their key enzymes involved in the metabolism of androstenone and skatole were identified (Leung et al. 2010, Moe et al. 2007b, Moe et al. 2008). The detected gene transcripts were involved in protein synthesis with key functions on the expression of boar taint components. Peacock et al. (2007) examined the expression profile of genes with the identified SNP by Lin et al. (2005) using real-time PCR. In studies of Moe et al. (2007b, 2008) gene expression profiles using microarrays of liver tissue and testicular tissue from each of 29 boars with very high and very low androstenone level was created. Large scales of genes were differentially expressed between the two groups. A lot of these genes were previously found to affect androstenone levels in boars as well as genes from pathways not formerly described in this aspect. These authors confirm the involvement of *CYP17* and *CYB5* and detected a number of other genes involved in the steroid hormone pathway that seem to be essential for androstenone levels. Besides *SULT2A1* other conjugation enzyme genes that might be important, including *SULT2B1, AKR1C4, GSTO1, MGST1* and *HSD17B4* were identified. Morever, Moe et al. (2008) revealed differential expressed genes encoding 17beta-hydroxysteroid dehydrogenases *(HSD17B2, HSD17B4, HSD17B11 and HSD17B13*) and

plasma proteins alpha-1-acid glycoprotein (*AGP*) and orosomucoid (*ORM1*). Based on the results of Moe et al. (2008), Grindflek et al. (2010) selected 15 candidate genes and validated the associated gene expression patterns. Comparable to the study of Peacock et al. (2007) only for the gene *CYB5A* an association between gene expression and SNP was observed. Stewart et al. (2005), Moe et al. (2007b, 2008) and Leung et al. (2010) identified genes that affect metabolic pathways of steroidogenesis, of steroid metabolism and the fat and energy metabolism. Therefore a relationship between fatty acid metabolism and the concentration of skatole and androstenone in adipose tissue could be derived.

Information on the use of whole transcriptome sequencing using the next generation sequencing technologies in boar taint are rare. Gunawan et al. (2013a, 2013b) used RNA-sequencing (RNA-Seq) technology to identified differentially regulated genes which are involved in the metabolism of androstenone and skatole. For skatole, approximately 448 genes were differentially regulated. Among them, 383 genes were up-regulated in higher skatole group and 65 were down-regulated. Differentially regulated genes in the high skatole liver samples were enriched in metabolic processes such as small molecule biochemistry, protein synthesis, lipid and amino acid metabolism (Gunawan et al. 2013a). The RNA-Seq analysis to identify polymorphism revealed that mutations in *ATP5B, KRT8, PGM1, SLC22A7* and *IDH1* genes could be potential markers for skatole levels in boars. In a second study by Gunawan et al. (2013b) the expression of testis and liver samples with divergent androstenone levels revealed 46 differentially regulated genes. Among them, 25 genes showed differential expression in the liver. The DEGs in high androstenone testis and liver samples were enriched in metabolic processes such as lipid metabolism, small molecule biochemistry and molecular transport. Gunawan et al. (2013b) identified also mutations in *IRG6, MX1, IFIT2, CYP7A1, FMO5* and *KRT18* genes which could be potential candidate markers for androstenone levels in boars.

For other meat quality traits in pig, whole transcriptome analysis were developed in the last years. To identify biological processes as well as molecular markers for drip loss, a parameter for WHC of meat, Ponsuksili et al. (2008b) used Affymetrix Porcine Genome Array to analyze the LM transcriptome of six divergent sib pairs. Genes, which were up-regulated in high drip loss samples belong to groups of genes functionally categorized as genes of membrane proteins, signal transduction, cell communication, response to stimulus, and cytoskeleton. Whereas, down-regulated genes in high drip loss samples belongs to functional groups of oxidoreductase activity, lipid metabolism and electron transport. Also a second study by Ponsuksili et al. (2008a) in samples of LM of 74 F_2 animals of a resource population

revealed 1,279 transcrips with trait correlated expression to WHC. Furthermore, using a linkage analysis 104 expression QTL (eQTL) coinciding with QTL regions for WHC were obtained.

Canovas et al. (2012) performed an mRNA expression study in obtained samples from LM of 59 Duroc × Landrace/Large White (LD/LW) pigs to search for gene sequences related to meat quality traits (pH_{24min}, pH_{45min}, "Lab" color coordinates, curing yield, and exudation at 3 different times) or meat composition traits (intramuscular fat, content of several fatty acids (C16:0, C18:0, C18:1, and C18:2), ratio of saturated, mono unsaturated, and poly unsaturated fatty acids, and protein and humidity contents) to find target genes for selection. The result exposed that more differentially expressed genes are related to meat quality (506) than to meat composition traits (279). A large number of the overexpressed genes are related to muscle development and functionality and repair mechanisms. Therefore, they could be good candidates for breeding programs in which the main goal is to enhance meat quality.

In 2013, Yu et al. compared meat quality traits between Lantang (a Chinese indigenous breed) and Landrace (a typical lean breed). The Lantang pigs indicated higher lightness values and intramuscular fat content, lower pH_{45min}, pH_{24h} and shear force in LM than Landrace. A fatty acid analysis demonstrated a lower mono unsaturated fatty acids (MUFA) and higher poly unsaturated fatty acids (PUFA) percentage in Lantang LM compared to Landrace LM ($p < 0.05$). Using microarray technology in LM samples Yu et al. (2013) observed 586 transcripts as differentially expressed, of which 267 transcripts were highly expressed in Lantang pigs. Validation by real-time quantitative PCR, revealed 13 genes as potential candidate genes for fatty acid composition of muscle, including stearoyl-CoA desaturase (SCD). Additionally, in vitro a SCD over-expression plasmid was transfected into a mouse myoblast cell line (C2C12) to reveal the effect of SCD on the fatty acid composition in vitro.

For aspects of palatability such as tenderness, flavour and juiciness the Warner-Bratzler shear force (WBSF) and % intramuscular fat content (IMF) are objective meat quality measurements which are significantly correlated. Because of this Hamill et al. (2012b) performed transcriptomic profiles of LM with lower or higher IMF (n=8) and WBSF values on day 1 post mortem (n = 8). The authors identified 101 DEGs in relation to WBSF, whereas 160 genes were associated with differences in IMF.

Muscle tenderness is an important complex trait for meat quality. In the transcriptome analysis of Lobjois et al. (2008), they found a relationship between gene expression variability and tenderness. He authors used LM samples from 30 F_2 pigs, which were

characterized by WBSF on cooked meat as a characterization of tenderness. The identified genes are involved in three functional networks: cell cycle, energy metabolism and muscle development. Among them, 12 genes were found to be located in regions previously reported to contain QTL affecting pig meat tenderness (SSC 2, 6 and 13). Some genes seem to be therefore as positional candidate genes.

1.4. Proteomics: the study of the proteome

The proteome is the entire complement of proteins. The word proteome was first coined by Marc Wikins 1994 in a symposium on "2D Electrophoresis (2-DE): from protein maps to genomes" held in Siena in Italy and was a blend of protein and genome (Wilkins et al. 1996b). The proteome is larger than the genome, especially in eukaryotes. It is well known that there are more proteins than genes according to alternative splicing and post-translational modifications like glycosylation or phosphorylation. Moreover the proteome has at least two levels of complexity absent in the genome. While the genome is defined by the sequence of nucleotides, the proteome cannot be limited to the sum of the sequences of the proteins present. Knowledge of the proteome requires knowledge of (1) the structure of the proteins in the proteome and (2) the functional interaction between the proteins. In contrast to the genome the proteome is continuously changing. This is due to factors influencing on either protein synthesis or degradation (Bendixen 2005, Hollung et al. 2007b).

Proteomics is the large-scale study of proteins in a cell, tissue or whole organism at a given time point and under defined conditions. Typically it gives us a better understanding of an organism than genomics (Anderson and Anderson 1998, Blackstock and Weir 1999). Peter James (1997) used firstly the term proteomics to make an analogy with genomics or transcriptomics, the study of the genes. The field of proteomics has grown at an surprising rate, mainly through to remarkable enhancements in the accuracy, sensitivity, speed and throughput off mass spectrometry (MS) and development of powerful analytical software (Abdallah et al. 2012). Most of the early developments in quantitative proteomics were driven by research on yeast and mammalian cell lines (Schulze and Usadel 2010). Following genomic and transcriptomic approaches, proteomics are the next step in the study of biological systems. It is more complicated than genomics because an organism's genome is more or less constant, whereas the proteome differs from cell to cell and from time to time. Distinct genes are expressed in different cell types, which mean that even the basic set of proteins that are produced in a cell needs to be identified. In the past this phenomenon was done by mRNA analysis, but it was not found to correlate with protein contents (Dhingra et al. 2005, Rogers et al. 2008). It is now known that mRNA is not always translated into protein (Buckingham 2003) and the amount of protein produced for a given amount of mRNA depends on the gene it is transcribed from and on the current physiological state of the cell. Proteomics confirms the presence of the protein and provides a direct measure of the quantity present. Typical steps of a proteome analysis using 2-DE and MS are shown in figure 4.

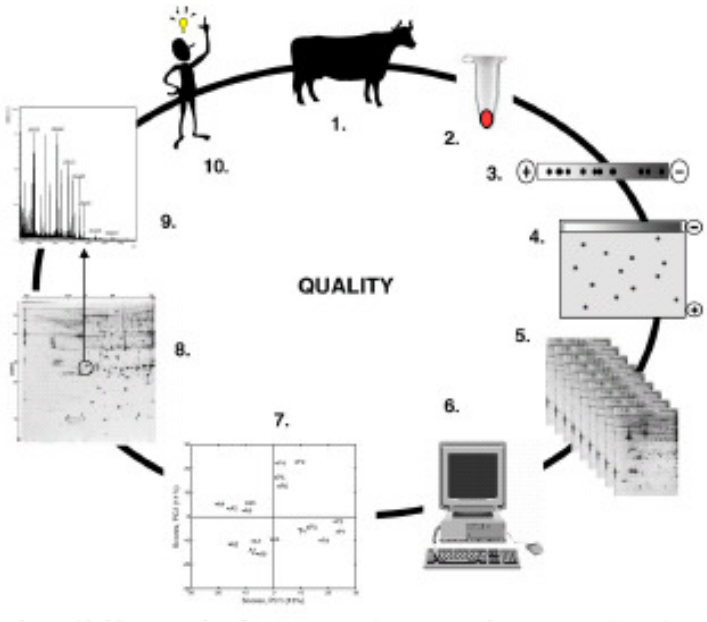

Figure 3: Schematic representation of proteome analysis using 2-D gel electrophoresis and mass spectrometry. (1) Animal or sample chose for analysis, (2) sample extraction, (3) isoelectric focusing, (4) SDS-PAGE, 2 DE, (5) alignments and comparisons of 2-DE images, (6) data analysis, (7) data interpretation and selection of significantly changed proteins, (8) extraction of these protein spots, (9) identification of protein spots by MALDI-TOF MS, (10) interpretation of the results (Hollung et al. 2007b)

1.4.1. Tools for the investigation of the proteome

For the investigation of the proteome different approaches and methods are available. In the following section three main methods for separation of proteins are described: (A) 2-DE gel electrophoresis, (B) gel-free method and (C) labeled method.

(A) Tow-dimensional gel electrophoresis

In meat science, the most common method for proteome analyses is based on separation of protein using 2-DE followed by identification of specific proteins with MS. Separation of proteins by 2-DE is an old method (O'Farrell 1975). However, major technical improvements such as the introduction of immobilized pH gradients have been important for the reproducibility of the method, for a review see Görg et al. (2000, 2004). In addition to semiquantitative information on the proteins that are present in the sample, 2-DE also provides information on modifications, as the same protein may occur in several spots in the gels. Following extraction and sample preparation, the proteins are first separated by charge using isoelectric focusing (IEF), and then the focused proteins are separated by mass using

sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-page). The protein composition and complexity of the sample determines which gradient will provide most information. Proteomes of low complexity can usually be separated on wide gradients, while a narrow gradient is needed to separate more complex samples. A narrow gradient will reduce the number of overlapping protein spots. Very hydrophobic proteins, membrane proteins, and high MW proteins are often difficult to solubilize and to analyze by 2-DE (Fey and Larsen 2001, Görg et al. 2004). These are among the major limitation with 2-DE analyses compared to gel-free systems, which will be described in the next section

There are two different principles to visualize the proteins on a 2-DE gel. Either the proteins are labeled with radioactivity or fluorescent tags prior to separation or the proteins are stained in the gel after separation using either fluorescent or nonfluorescent dyes. The other principle is to label the proteins prior to electrophoresis of which the most common method is the difference gel electrophoresis (DIGE) (Ünlü et al. 1997). A major advantage with DIGE is the simultaneous separation of sample in a single gel using different labels for each sample. Up tow three samples are commonly separated on the same gel. An advantage of this is that comigration of the same proteins from different samples are secured, thus simplifying the analysis between the samples.

Several statistical approaches have been used to analyze proteomics data. Multivariate analyses such as principal component analysis (PCA) have proven to be a very useful tool for validation of data from 2-DE experiments and to identify and remove outliers from the analyses. Multivariate approaches have also been used for selection of significant changes in the 2-DE data according to the design parameters (Jessen et al. 2002, Jia et al. 2006, Kjærsgård et al. 2006). Assessment of hierarchical clustering methodologies, commonly used in transcriptomics studies, has also been discussed in several papers (Jacobsen et al. 2007, Maurer et al. 2004).

(B) Gel-free methods

Gel-free analyses of complex samples are based on different combinations of LC and MS. The proteins are usually digested with trypsin prior to LC for a prefractionation of the peptides. There are numerous methodologies of separation: the ion-exchange chromatography (IEC), reversed-phase chromatography (RP), two-dimensional liquid chromatography (2D-LC) and off-gel electrophoresis (OGE) (descried in detail in Chapter 3) (Abdallah et al. 2012). The different fractions then are analyzed by MS. In some LC-MS approaches a first separation is performed by SDS-page. The lane containing the sample is then divided in areas

that are excised and digested to release the peptides that are then used in LC for the next separation step. The mass analyzer separates the ions according to their mass-quadrupole ion trap, quadrupole (Q), and Fourier transform ion cyclotron resonance (FT-ICR) mass analyzers (Shen et al. 2001).

(C) Labeled method

Several methods have been developed to assist in quantitative comparisons between samples using MS-based approaches (review by Elliott et al. 2009). The label based methods for relative quantification can be classified into two main groups, the chemical isotope tags and the metabolic labeling. In chemical labeling, proteins or peptides are tagged through a chemical reaction, in metabolic labeling the label is introduced to the whole cell organism through the growth medium. The principle of these methods were that both labeled and unlabeled peptides exhibit the same chromatographic and ionization properties but can be distinguished from each other by a mass-shipft signature (Schulze and Usadel 2010). Metabolic labeling methods include stable isotope labeling by amino acids (SILAC) (Ong et al. 2002) and 15N labeling mostly used for autotrophic organisms such as plant and bacteria (Wang et al. 2002). The chemical labeling include the isotope coded protein label (ICPL) technique (in detail described in Chapter 3) based on the isotopic labeling of all free amino groups on the proteins (Schmidt et al. 2005), the isotope coded affinity tag (ICAT), the proteins are labeled using a thiol-specific reagent containing a biotin moiety (Gygi et al. 1999). Also included in this group are the isobaric tags for relative and absolute quantification (iTRAQ) (Ross et al. 2004) and the mass differential tags for relative and absolute quantification (mTRAQ) an amine-reactive isotope-coded reagent (DeSouza et al. 2010).

Other methods are based on inclusion of internal standards such as multiple reactions monitoring (MRM) (Lange et al. 2008a) and selected reacted monitoring (SRM) (Lange et al. 2008b, Picotti et al. 2010). However, these techniques require that the target proteins selected for quantification are identified and have been applied on a selected number of proteins in each analysis. High-throughput SRM methods based on crude synthetic peptide libraries are being developed and will greatly improve this strategy for quantitative comparisons between samples (Picotti et al. 2010). Label-free approaches are also being used. These are either based on comparisons of signal intensities of most intense peptides from specific proteins in different samples or by counting and comparisons of the number of MS/MS spectra obtained from a single protein (Wang et al. 2008).

1.4.2. Identification of the protein sequence: Mass spectrometry

To obtain the amino acid sequence of the peptides, the peptides need to be fragmented and analyzed by a separate analyzer. Mass spectrometers are used either to measure simply the molecular mass of a polypeptide or to determine additional structural features including the amino acid sequence or the site of attachment and type of posttranslational modifications (Domon and Aebersold 2006). The two primary methods for ionization of whole proteins are electrospray ionization (ESI) (Fenn et al. 1989) and matrix-assisted laser desorption/ionization (MALDI) (Karas and Hillenkamp 1988). In keeping with the performance and mass range of available MS, two approaches are used for characterizing proteins. In the first, intact proteins are ionized by either of the two techniques and then introduced to a mass analyzer called as "top-down" strategy of protein analysis. In the second, proteins are enzymatically digested into smaller peptides using a protease such as trypsin and subsequently introduced into the mass spectrometer and identified by peptide mass fingerprinting or tandem mass spectrometry. Hence, this latter approach (also called "bottom-up" proteomics) uses identification at the peptide level to infer the existence of proteins (Aebersold and Mann 2003).

Whole protein mass analysis is primarily conducted using either time-of-flight (TOF) MS, or FT-ICR. These two types of instruments are preferable here because of their wide mass range, and in the case of FT-ICR, its high mass accuracy. Mass analysis of proteolytic peptides is a much more popular method of protein characterization, as cheaper instrument designs can be used for characterization. Additionally, sample preparation is easier once whole proteins have been digested into smaller peptide fragments. The most widely used instrument for peptide mass analysis are the MALDI time-of-flight instruments as they permit the acquisition of peptide mass fingerprints (PMFs) at high pace (1 PMF can be analyzed in approx. 10 sec). Multiple stage quadrupole-time-of-flight and the quadrupole ion trap also find use in this application (Viant and Sommer 2013).

In meat science, PMF analysis has been successfully applied to identify proteins from muscles of different species (Hamelin et al. 2006, Hamelin et al. 2007, Kwasiborski et al. 2008b, Kwasiborski et al. 2008a, Lametsch et al. 2002, Lametsch et al. 2006, Lamont et al. 2006, Laville et al. 2007, Morzel et al. 2004, Morzel et al. 2008, Sayd et al. 2006).

In contrast to the gel free methods, only the proteins that are changed according to the experimental design are identified. 2-DE followed by MS based techniques is still the most popular approach to relatively quantify and identify proteins within complex mixtures (Klose et al. 2002). Because of the limitation factors of these methods: the difficult automation of the

workflow and the rare detection of low-abundant or membrane proteins, for our experiment the ICPL method was used.

1.5. Proteomics and meat quality

Over the last decades proteomics investigation in livestock, such as pig and cattle developed rapidly. The emphases of these studies include muscle growth, breed differences, meat quality traits, such as tenderness, juiciness and flavour, color and WHC. In the next section a general overview about relevant proteomic studies in pig meat science in the last few years are presented.

1.5.1. Proteomics in pig meat science

In pig most of the proteome studies were investigating skeletal muscles in different breeds or specific proteins which are related to meat quality traits. In table 1 an overview about the most important proteins related to meat quality traits is given. In 2010, Kim et al. investigated the pig muscle proteome by means of 2-DE of white and red skeletal muscles (longissimus dorsi and soleus, respectively) in Large White, Landrace and Duroc crossed animals. Significant breed-related differences were highlighted, including differential expression of heat shock proteins (HSPs) and metabolism-related proteins. Mach et al. (2010) studied proteome breed differences on 2 muscles from 5 different pure pig breeds and identified proteins which may be used as potential biomarkers for breed classification. Kwasiborski et al. (2008b, 2008a) identified, by using 2-DE, in an original sire breed potential gender specific markers including an actin isoform, a myosin light chain 2 isoform and CYPBc1. Also Hollung et al. (2008) suggested that protein-wide signatures also exist in relation to pig age, including structural (actin) and metabolic proteins (enolase or triosephosphate isomerise).

In the study by Liu et al. (2009) on the LM from several species, it emerged that interindividual variability in intramuscular fat content might arise essentially from differences in early adipogenesis. Indeed, Cagnazzo et al. (2006) showed that these differences arise already at the 14 days in embryo development.

During the meat processing a number of biochemical changes occur; such alterations are accompanied by changes in the proteomic profiling. Lametsch and Bendixen (2001) used 2-DE technology to characterize the changes that occur in pig muscle proteins during post mortem storage of the carcass. For the comparison of the different used time points the authors identified 15 proteins which changed significantly their profiles. The same team

identified the differentially expressed proteins using MALDI TOF. They found major proteins such as actin, myosin heavy chain, troponin T, as well as glycogen phosphorylase, creatin kinase and dihydrolipoamide succinyltransferase. These proteins were likely related to proteolytic activities involved in the post mortem conversion of muscle to meat (Lametsch et al. 2002). In a third study by Lametsch et al. (2003) the relation between changes in post mortem proteome of porcine muscle and tenderness development was studied and identified 103 protein spots which changed significantly. Because of the identified proteins it could be suggested that post mortem degradation of actin and myosin heavy chain is strongly related to meat tenderness. Laville et al. (2007) compared in their study longissimus sacroplasmic protein abundance between two groups: tough and tender meat of cooked pork. The authors identified 14 differentially expressed proteins which included adipocyte fatty acid binding protein and acyl-CoA binding protein, enoyl-CoA hydratase, aldose reductase and triophosphate isomerize with a higher expression level in the tender group. Laville et al. (2007) finally suggest that the lower post-cooking shear force could be partly related to muscle adipogenetic and/or myogenetic status.

Also from pre-slaughter handling procedures it's well known that these influence pork meat quality. Morzel et al. (2004) studied the influence of post mortem storage time and pre-slaughter conditions (transport the day before slaughter or immediately before slaughter) on proteome changes over a 72 h ageing period and found 37 spots varied significantly as a consequence of aging. The pre-slaughter condition affected significantly 8 spots, including: mitochondrial ATPase and myosin light chain.

Formation of drip is generally considered as a result of denaturation on contractile proteins and shrinkage of myofibrils during rigor development (Bertram et al. 2004a, Offer et al. 1989). In pig longissimus dorsi proteom analysis by 2-DE has identified candidate protein markers highly relevant to the process of drip loss (van de Wiel and Zhang 2007). The most clearly identifed proteins were creatine phosphokinase M-Type, desmin and a transcription activator (SWI/SNF related matrix-associated actin-dependent regulator of chromatin subfamily A member1, SNF2L1). Di Luca et al. (2011) subjected centrifugal drip sample to 1-DE following MS to identify proteins of different time points post mortem and in meat with different levels of WHC, including PSE and DFD meat. In total, the authors identified 44 proteins in the drip proteome and 5 bands showed different volumes with aging, most of them related with the glycolytic pathway. Also, one HSP 70 was associated with changes in the WHC. The second study of the same group (Di Luca et al. 2013) revealed the importance of stress response for improved WHC. By using 2-DE DIGE followed by MS analyses and

Western blot di Luca et al. (2013) identified proteins including metabolic enzymes, stress response proteins and structural proteins. Triosephosphate isomerase and transfer showed a major difference between the two phenotypes and may have potential as biological markers for WHC. In another study, Hwang et al. (2005) identified three main proteins, adenylate kinase, substrate protein of mitochondrial ATP-dependent proteinase SP-22 and troponin T slow-type isoform, correlated with changes in meat drip loss. In a recent study by Yu et al (2009), the correlation between meat quality and HSP expression in LM of pigs was investigated with a proteomic approach. The four heat shock proteins tested (L-ß crystallin, HSP27, HSP70 and HSP90) by ELISA in the LM tissue of pigs tended to decrease after transportation. The authors found a relationship between the decline in HSP expression and increased drip loss in LM, pointing at this mechanism as a possible cause resulting in poor meat quality in the LM. It is well known that the HAL gene is considered to be a genetic cause of PSE. Laville et al. (2009b) compared sacroplasmic protein profiles of early post mortem pig semimembranosus muscle using 2-DE, sampled from pigs of the different HAL genotype. The group identified in total 55 proteins. In NN pigs, glycolytic enzymes and few proteins of oxidative metabolism pathway had a higher abundance, whereas Nn and NN animals had a higher expression of small HSP and myofibrillar proteins.

Table 1: Proteins involved in the variability of meat quality traits modified by Hamill et al. (2012a)

Quality traits	Species/muscle	Proteins involved in quality changes	References
Texture (WBSF)	Pig (LM)	Actin fragments, MHC fragments, MLC II glycolytic enzyme triose phosphate isomerase I	Lametsch et al. (2003)
Texture (WBSF)	Pig (LM)	Adipocyte fatty acid-binding protein, acyl CoA-binding protein, enoyl CoA hydratase, aldose reductase, triosephophate isomerise, initiation factor elf-3ß, chaperonin subunit 2, profiling II	Laville et al. (2007)
Texture (WBSF) Color WHC (drip loss)	Pig (LM)	MLC I, desmin, troponin T, cofilin 2, F-actin capping protein ß subunit, ATP syntase, carbonate dehydratase, triosephosphate isomerise, actin and its relevant peptides, peroxiredoxin 2, α-ß crystalline and HSP 27 kDa	
Texture (WBSF) Color WHC (drip loss)	Pig (Semimenbranosus)	Triosephosphate isomerase, phosphoglucomutase, pyruvate dehydrogenase, succinyl CoA ligase and dihydropilamide succinyltransferase,	Laville et al. (2009b)

		ubiquinol cytochrome c reductase, ubiquinone oxidoreductase, mitochondrial ATP synthase, HSP27, Rß-C, HSP71, HSP70/HSP90	
Color	Pig (Semimembranosus)	ATP sythase ß subunit, NADH dehydrogenase, succinate dehydrogenase, enolase 1, enolase 3, glycerol-3-phosphate dehydrogenase, hemoglobin α- and ß-chain, HSP27, RB-crystallin, glucose-regulated protein 58 kDa, low-molecular-weight protein tyrosine phosphatase, S-transferase ω, cyclophilin D	Sayd et al. (2006)
Color WHC (drip loss)	Pig (LM)	Cofilin 2, troponin T, α-ß crystalline, HSP27 kDa, group chain A aldehyde dehydrogenase, glycerol-3 phosphate, dehydrogenase, haemoglobin α-chain, DJ-1 protein	Hwang (2004)
Color WHC (drip loss, EM)	Pig (LM)	A-ß crystalline, HSP 27, HSP 70, HSP 90	Yu et al. (2009)
WHC (drip loss)	Pig (LM) (Biceps femoris)	Creatine phosphokinase M-type, desmin,	van de Wiel and Zhang (2007)

		SWI/SNF-related matrix-associated actin-dependent regulator of chromatin a 1 isoform b	
Color WHC (drip loss)	Pig (L lumborum)	Myoglobin isoform, HSP 72, CK, calcium-binding protein, cytosolic glycerol-3-phosphate dehydrogenase, dimeric dihydrodiol dehydrogenase, isoform M1 of pyruvate kinase	Kwasiborski et al. (2008b)

1.6. Metabolomics: what does it mean?

Metabolomics is an interdisciplinary study that involves the exhaustive quantitative profiling of metabolites in a target organism using sophisticated analytical technologies (Putri et al. 2013). The metabolome represents the collection of all metabolites in a biological cell, tissue, organ or organism, which are the end products of cellular processes (Jordan et al. 2009). Thus, while mRNA gene expression data and proteomic analyses do not tell the whole story of what might be happening in a cell, metabolic profiling can give an instantaneous snapshot of the physiology of that cell. One of the challenges of systems biology and functional genomics is to integrate proteomic, transcriptomic, and metabolomic information to give a more complete picture of living organisms. Two metabolomics approaches exist. The metabolic fingerprinting and the metabolic profiling (reviewed by Hocquette et al. 2009). Till now studies of whole metabolomics has been rarely used to livestock. Some example: metabolomics has been used to find anabolic hormones in bovine urine (Rijk et al. 2009), to evaluate the effect of L-arginine supplementation in pig (Ansorge 2009) and to investigate the effect of high-fiber rye buns in hypercholesterolemic pigs used for medical research (Bertram et al. 2009). To investigate metabolic disease or nutritional status in blood of livestock metabolic profiles has been used frequently (Herdt 2000, Ingraham and Kappel 1988).

1.7. Scope of the thesis

In general complex traits such as boar taint and WHC are influenced by many genes. Thus, the analysis of the transcriptome and proteome using high-throughput technologies for elucidating the molecular mechanisms and to identify biomarkers with the potential to be developed as markers that can be monitored is necessary. To identify important genes/proteins which are related to meat quality traits, two different experiments were conducted on transcriptional as well as on proteomic level.

Experiment I:

Analysis of the transcriptome of boar liver sample with divergent androstenone and skatole level in their backfat to identify relevant genes for (a) single trait (androstenone/skatole) but also for (b) the relationship between androstenone and skatole

Experiment II:

Identification of relevant proteins in muscle samples with high and low drip loss to get an clearer overview in the mechanism behind WHC of meat

1.8. Experiment I: Transcriptome analysis in liver from boars with divergent androstenone and skatole level in their backfat

To achieve the analysis of the transcriptome of boar liver sample with divergent androstenone and skatole level the porcine microarray Affymetrix gene chip were used (figure 5). The materials and methods are described in details in the chapters 2 of this thesis. The importance of some methods and their descriptions are briefly summarized here. The goal of the study was to identify DEGs and pathways related by using whole transcriptome analysis. Three comparisons were taken into account: the comparison of high vs. low androstenone (A), high vs. low skatole (B) and the comparison of a combined phenotype (high skatole/low androstenone vs. low skatole/high androstenone) (C).

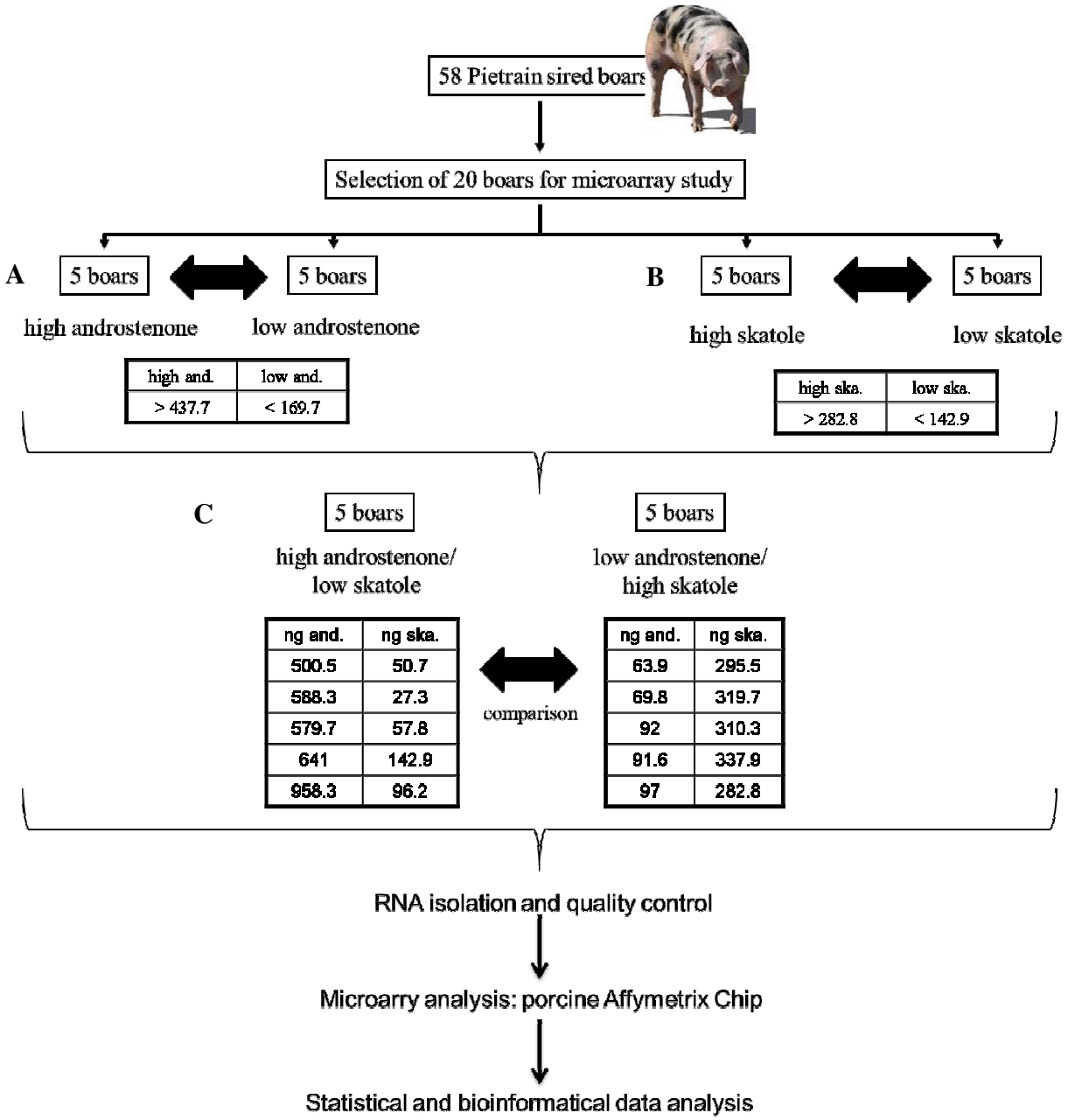

Figure 4: Experimental design of the transcriptome study in boars with divergent androstenone and skatole levels. Three comparisons were taken into account: the comparison of high vs. low androstenone (A), high vs. low skatole (B) and the comparison of a combined phenotype (high skatole/low androstenone vs. low skatole/high androstenone) (C)

1.8.1. Sample collection

For the microarray study, 20 animals of 58 crossing boars (Pietrain × F_2) with high and low androstenone and skatole levels were selected. Average levels of androstenone were at > 470 ng/g fat and of skatole at > 250 ng/g fat. All boars were kept and tested at the Boxberg pig testing station (Landesanstalt für Schweinezucht, Germany). Animals were kept according to the guidelines of the German performance test regulations (ZDS 2003). Liver samples were obtained at slaughterhouse, snaps frozen in liquid nitrogen and stored at -80°C until RNA isolation. Fat samples were collected from the neck and stored at -20°C until used for androstenone and skatole measurements. The quantitative determination of the three main components of boar taint was conducted by Fraunhofer Institute for Molecular Biology and Applied Ecology (IME) Schmallenberg. For the measurement of androstenone and skatole a total of about 4 g of back fat sample was needed, which were taken at five different locations of the input sample. These samples were heated in a microwave oven, followed by separation, freeze out and centrifugation steps. For the quantification of androstenone an adapted gas chromatography / mass spectrometry (GC / MS) (Garcìa-Regueiro and Diaz 1989) was used. The quantitative determination was carried out with this method based on the mass / charge ratio of the ionized components. The detection limit was at 4 ng per sample (equivalent to 10 ng / g melted fat). The content of skatole and indole was purified by reverse-phase high performance liquid chromatography (RP-HPLC) (Dehnhard et al. 1993) using a fluorescence detector (FD) is determined. The skatole level was due to the wavelength of the emitted fluorescence of the components determined (Haugen et al. 2012, Skoog and Leary 1996). The detection limit for both substances, skatole and indole, was 2 ng per sample (equivalent to 5 ng / g melted fat).

1.8.2. RNA isolation, amplification and microarray hybridization

Total RNA was extracted from 20 liver samples using RNeasy Mini Kit according to manufacturer's recommendations (Qiagen). The RNA was cleaned up using the RNeasy Kit (Quiagen, Hilden, Germany). RNA concentration was measured using NanoDrop ND-8000 (Thermo-Scientific). The integrity and the absence of contamination was checked using the Agilent RNA 6000 Nano Kit (Agilent Technologies, Waghäusel - Wiesental, Germany). In addition, absence of DNA contamination was checked using the RNA as a template in a PCR amplifying with GAPDH (Accession number: XP_003126582). All RNA was stored at -80°C until use.

The expression patterns of the RNAs were assessed using the GeneChip Porcine Genome Array (Affymetrix). According to Affymetrix protocols, 100 ng of total RNA were reversely transcribed into cDNA, and labelled using the Affymetrix One cycle synthesis and labelling kit to prepare antisense biotinylated RNA targets. The quality of hybridization was assessed in all samples following the manufacturer's recommendations. Data were analysed with Affymetrix GCOS 1.1.1 software using global scaling to a target signal of 500. The microarrays were scanned using a ScanArray Express scanner (Perkin Elmer Inc., MA, USA). Signal intensities were quantified using the ScanArray Express software.

1.8.3. GenomeLab expression analysis

Although qRT-PCR is the most commonly practiced methods for gene quantification, it is not cost-effective for the quantification of a set of genes in a large numbers of tissues when compared to the GenomeLab Genetic Analysis System (GeXP). The qRT-PCR results are reported to vary according to the system (Lu et al. 2010). The Beckman Coulter GeXP genetic analysis system allows for multiplexed detection and quantitation of up to 35 genes in 192 samples in a single analysis (Rai et al. 2009). The results of GeXP are comparable to quantitative real-time PCR (Raghunathan et al. 2009). Therefore, GeXP is a faster and cheaper method in this regard (Rai et al. 2009). The analytical procedure includes modified reverse transcription and PCR amplification, followed by capillary electrophoretic separation (Rai et al. 2009). All the forward primers are mixed together (forward-plex) while ‘reverse-plex’ is prepared by mixing all the reverse primers. Each of these primers is chimeric, having a 3′gene-specific end and a 5′end containing a quasi-T7 universal sequence, which serves as a template in subsequent amplification steps (Rai et al. 2009). The GeXP software matches each fragment peak with the appropriate gene, and reports peak height and area-under-the-curve (AUC) for all peaks in the electropherogram. Electrophoretic separation is needed to be done by GenomeLab™ GeXP Genetic Analysis System (Beckman Coulter, Fullerton, CA, USA). Kanamycin RNA internal positive control is to be included and produces a peak at 326 bp when samples are separated via electrophoresis. All experiments usually included “no template” (i.e. without RNA) and “no enzyme” (i.e. no reverse transcriptase) as negative controls to confirm the absence of peaks at the expected target sizes. The “no template” sample produces a single peak at 326 bp, corresponding to the externally spiked-in kanamycin RNA.The data set is exported from the GeXP software after normalization to kanamycin, with AUC set to 1 which minimizes inter-capillary variation (Rai et al. 2009). This data were used for subsequent analyses after normalization against reference genes.

1.9. Results

Some of the important results are briefly described here. The detailed results can be found in chapters 2 in this thesis.

1. The analysis of the microarrays revealed in total 264 differentially expressed genes in the liver sample with divergent androstenone and skatole level.
2. Only two genes were identified in liver between high and low androstenone group.
3. 92 genes were differentially expressed ($p \leq 0.05$) in the comparison of high vs. low skatole. Of which 49 were up and 43 down regulated in high level of skatole compared to the low level of skatole.
4. Using linear contrast to compare the combined phenotypes of androstenone and skatole (high skatole/low androstenone vs. low skatole/high androstenone) it was possible to identified 170 differentially expressed genes, of which, 84 genes were down regulated and 86 genes were up regulated in high skatole/low androstenone compared to low skatole/high androstenone.
5. A total of 84 genes were found to be common for high vs. low skatole and the combined phenotypes group. On the other hand, both identified genes for high vs. low androstenone were found to be common in the group of combined phenotypes.

1.10. Experiment II: Proteomic study in *musculus longissimus dorsi* sample with high and low drip loss

To achieve the identification of relevant proteins in muscle samples with high and low drip loss several materials and methods were used (figure 6). The materials and methods are described in details in the chapter 3 of this thesis. The importance of some methods and their descriptions are briefly summarized here. The goal of the study was to identify relevant proteins and pathways, which are related with WHC, using a whole proteome analysis.

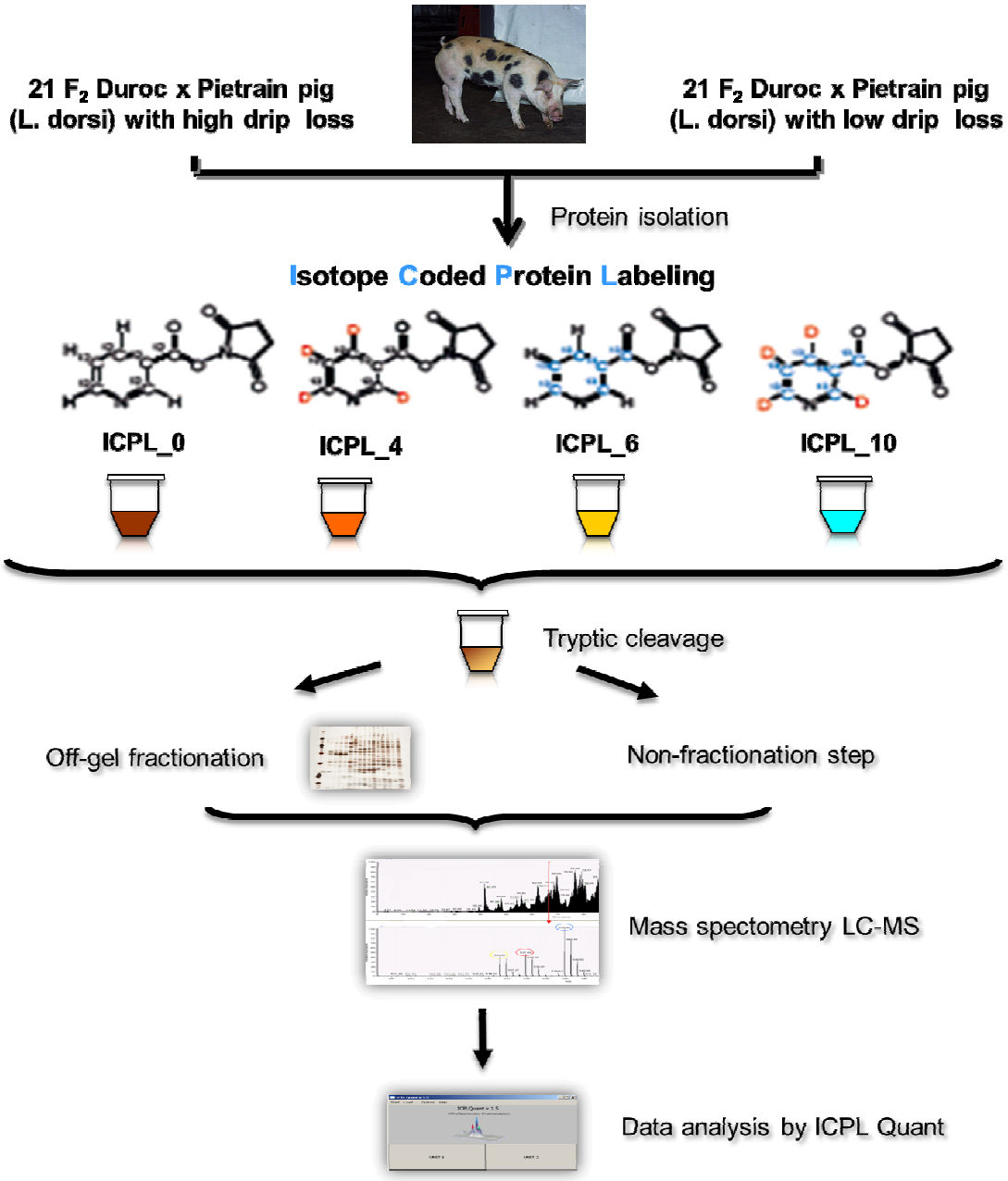

Figure 5: Experimental design of the proteome study in muscle samples with high and low drip loss

1.10.1. Sample collection

In this study, 42 F_2 animals of a reciprocal cross of the Duroc (Du) and Pietrain (Pi) breeds were used. All F_2 animals were kept and performance tested at the Frankenforst experimental farm of the University of Bonn. The phenotypes were recorded in a commercial slaughterhouse according to the rules of German performance stations (ZDS 2003). Tissue samples from musculus longissimus dorsi (LM) were frozen in liquid nitrogen immediately after slaughter and stored at -80°C until used for protein extraction. The 42 F_2 animals were selected for their extreme phenotype in meat quality characteristic drip loss. Pigs having drip loss level from 0.5 % to 1.1 % as well as and from to 3.2 % to 5.0 % were defined as low and high drip loss samples, respectively. In total, 4 meat quality traits (drip loss, pH_1, pH_{24} and shear force) were analyzed.

1.10.2. Protein extraction and isotope coded protein labeling

After complete cell lysis 200 µl to 300 µl ICPL lysis buffer (kit component plus protease inhibitors complete mini, Roche and phosphatase inhibitor cocktails I and II, Sigma) was added to each sample. Then the sample was homogenized by grinding. The lysis was done using the grinding kit (GE Healthcare, No. 80-6483-37) according to the manufacturer's instructions. Following a centrifugation step to remove cellular debris the supernatant was transferred into a low bind tube for subsequent ICPL labeling. An aliquot was used for determine the protein concentration by a Bradford assay.

In total 100 µg proteins were used for each ICPL labeling reaction. Labeling was performed according to the manufacturer's instructions. The sample labeling with ICPL_4 and ICPL_10 to ICPL_6 was switched in order to prevent data bias. The pool of all samples served as material for the normalization between the 14 MS runs. The pool was aliquoted and subsequently labeled with the ICPL_0 label. The enzymatic cleavage was performed using trypsin (ratio 50:1, sequencing grade, porcine, SERVA Electrophoresis GmbH, Germany) and Glu-C (ratio 75:1, MS grade, Protea Biosciences, Inc.). Then the peptides were acidified to 1 % formic acid for subsequent MS analysis.

1.10.3. Pre-fractionation

For detection and relative quantification of lower abundant protein species the proteome was sub fractionated into 15 fractions using Off-gel fractionation. First two ICPL labeling were prepared for the comparison of 2 x 4 animals in total. The animals were selected because of their drip loss value and grouped in low (n = 4) and high (n = 4) drip loss. Subsequently, the

ICPL labeled protein pools were separated according to their isoelectric point (pI). Fifteen fractions covering the whole pH separation range were analyzed by electrospray ionization liquid-chromatography-tandem mass spectrometry (nano-LC-ESI-MS/MS) for each labeling. The individual proteins were quantified relatively using the ICPL Quant software (Brunner et al. 2010).

1.10.4. LC-ESI-MS/MS after tryptic cleavage

For nano-LC-ESI-MS/MS the peptides were separated with an analytical column (C18, 25cm length, 35°C) with a 140 min linear gradient (A: 0.1 % formic acid, B: 80 % acetonitrile (ACN) and 0.1 % formic acid) at a flow rate of 300 nl/min. The gradient used was: 5-50 % of B. Mass spectrometry was performed on a linear ion trap MS (LTQ Orbitrap Velos, Thermo Scientific) operating in positive polarity mode online coupled to the nano-LC system. The MS method consisted of a cycle combining one full MS scan (Mass range: 300 - 1500 m/z) with ten data dependent MS/MS events of the highest signals with a dynamic exclusion (CID; 35 % collision energy).

Statistical analysis was done using SAS v. 9.2. To map protein-protein interactions the STRING 9.0 software (Franceschini et al. 2013) was used.
In order to link protein and gene information, the obtained data were additionally annotated using the biomaRt package (version 2.14.0) in R (www.r-project.org). A hyper geometric gene set enrichment test (GOstats package version 2.24.0) was performed.

1.11. Results

Some of the important results are briefly described here. The detailed results can be found in chapters 3 in this thesis.

1. In the present study, ICPL and off-gel fractionation analysis resulted in the identification of 763 proteins.
2. Among them, 60 show a different abundance due to their ratio. Proteins with a ratio < 0.7 (20 proteins) were under-expressed and with a ratio > 1.3 (60 proteins) over-expressed.
3. By off-gel fractionation a total of 134 proteins were identified. 33 of these proteins were additionally new to the ICPL method, 131 are identified in both, ICPL and off-gel fractionation.
4. In the comparison of all 42 animals three proteins shows a different regulation between high and low drip loss samples: TPM1, AK1 and HSPA8 ($p \leq 0.05$).
5. In the comparison of the 2 x 5 extreme animals we observed 10 significantly differentially expressed ($p \leq 0.05$) proteins: PYGL, PYGM, TNNI1, MYL3, MYL2, MB, MYBPC1, FHL1C, AK1 and MYH7.
6. Protein-protein interaction of all annotated proteins resulted in three main functional areas: proteins of glycolysis, proteins of cytoskeleton part and proteins of mitochondrial.
7. The down regulated proteins are mainly assigned in their interaction in the fields of muscle contraction and metabolic processes, while the up-regulated proteins were in involved in glycogen breakdown.
8. To investigate potential gene set enrichments, the proteins were additionally annotated with corresponding gene information. Based on the identified 763 proteins 663 were assigned to Entrez gene ID. Among these, 474 were able to the group biological processes (BP), 447 to cellular components (CC) and 577 to molecular function (MF) of the GO database.
9. BP were identified amongst others as glycolysis, response to stress, actin cytoskeleton organization and actin filament-based process. CC was identified as actin filament, myofibril, troponin complex, cytoskeletal part, stress fiber and myosin complex. MF

was identified as ATPase activity, actin filament binding, structural constituent of cytoskeleton and actin binding.

Chapter 2: Gene expression profiling in liver of boars revealed a divergent relationship between androstenone and skatole levels in backfat

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2.1. Abstract

Boar taint is an unpleasant smell and taste of porcine meat derived from uncastrated male pigs. The aim of the present study was to investigate the transcriptome differences in liver tissues from boars with divergent androstenone and skatole levels using Affymetrix porcine microarray. Therefore, equal number of boars (n = 10) were selected for each of high and low androstenone or skatole. Only two genes were identified in liver between high and low androstenone group, whereas 92 genes were differentially expressed ($p \leq 0.05$) between high and low skatole group. The analysis of combined androstenone and skatole phenotypes revealed 170 differently expressed genes. The most common pathways of the differentially regulated genes in liver samples were fatty acid metabolism, retinol metabolism and PPAR signalling pathway.

2.2. Highlights

- Liver microarray analysis reveals numerous transcripts associated with boar taint
- DEGs in the group of combined phenotypes indicates a high relationship in the regulation of the two boar taint components in liver
- Identification of several genes which have potential influence on fatty acid metabolism, PPAR signalling pathway, retinol metabolism

Keywords: boar taint, microarray, liver, pigs, androstenone, skatole

2.3. Introduction

Male piglets are usually castrated to prevent the development of an unpleasant smell of their meat called boar taint (Windig et al. 2012). Castration is undesirable both for ethical as well as for economical reasons and declared without anaesthesia to be banned in the European Community until 2018 (Morlein et al. 2012). While, the fattening of uncastrated male pigs is profitable for the swine production, because it improves feed efficiency and leads to a greater lean yield of the carcass (Moe et al. 2007a). The use of intact male pigs for pork production is limited because of the possible occurrence of boar taint, an off-odor and off-flavour in meat from some uncastrated male pigs (Babol and Squires 1995). Therefore, alternative methods are needed to prevent tainted meat (Moe et al. 2007a). Selected breeding of intact pigs would be a more preferable method to reduce tainted meat from entries male pigs, therefore, a better knowledge of the genes involved in boar taint is warranted in order to use genetic markers to select against boar taint (Leung et al. 2010).

Boar taint is caused predominantly by increased levels of two compounds, androstenone and skatole, in the fat (Bonneau et al. 2000). Androstenone (5α-androst-16-en-3-one) is a 16-androstene steroid produced from pregnenolone in the steroid hormone pathway in boar testis near sexual maturity (Gower 1972, Kwan et al. 1985, Robic et al. 2008). It is metabolized in liver and partly deposited in adipose tissue because of its lipophilic properties (Doran et al. 2004). Metabolism of androstenone is presented in two phases: phase I consists of metabolism by hydrogenation and phase II consists of metabolism by sulfoconjugation in testis or in liver (Doran et al. 2004, Robic et al. 2008, Sinclair et al. 2005a, Sinclair and Squires 2005). Therefore, in theory, high levels of androstenone in fat can be dedicated to a high intensity of testicular synthesis and/or a low intensity of liver degradation (Robic et al. 2008). Skatole (3-methyl-indole) is produced from tryptophan by bacteria in the hindgut of pigs (Yokoyama and Carlson 1979). *Lactobacillus sp.* strain 11201 is considered as the main organism producing skatole causing boar taint (Deslandes et al. 2001). Tryptophan both from the diet and from cell debris degradation of intestinal mucosa can be metabolised to skatole. In pigs skatole is absorbed by the intestinal mucosa into the portal vein and passes through the liver where it is efficiently metabolised and similar to androstenone, skatole does also seem to be easily transferred from plasma to adipose tissue. Beside this, in some boars a proportion of skatole, nevertheless passes the liver without being metabolised and accumulates in adipose tissue. The reason could be related to metabolic process of boar taint in the liver (Andresen 2006).

A close relationship between the key components and the subjective perception of boar taint was found in a variety of studies with trained expert panels. Experiments of Bonneau and

Chevillon (2012) showed that the acceptance limit of consumers for androstenone were in a much higher range (2.000 - 3.000 ng/g fat) if skatole is very low (< 50 ng/g fat) as with a high skatole level (> 200 ng/g fat). Thus, at very low skatole levels, the limits for androstenone could be less sharply drawn.

The genetic background of boar taint traits have been investigated by numerous of researches (reviewed by Robic et al. 2008). Moderate to high heritabilities (h^2) and differences between sire lines have been reported for boar taint compounds (Merks et al. 2009, Tajet et al. 2006b). A number of quantitative trait loci (QTL) and genome-wide association analysis (GWA) have been conducted for androstenone in purebred and crossbred pig populations (Duijvesteijn et al. 2010, Gregersen et al. 2012, Grindflek et al. 2011a, Quintanilla et al. 2003). Gene expression analysis has been used to identify candidate genes related to the trait of interest. Several candidate genes have been proposed for divergent androstenone levels in different pig populations by global transcriptome analysis in boar testis and liver samples (Leung et al. 2010, Moe et al. 2007b, Moe et al. 2008). Till now, most of the studies are performed in purebreds. It is therefore of interest to investigate gene expression patterns in individuals with high or low levels of boar taint compounds in crossbreed pigs. Functional genomics provides an insight into the molecular processes underlying phenotypic differences for androstenone and skatole levels. Also a molecular genetic relation could be observed between androstenone and skatole metabolism in liver by Doran et al. (2002a). They identified that skatole induced *CYP2E1* protein expression while androstenone antagonised this effect in isolated hepatocytes.

Therefore, the objective of this study was to identify differentially expressed genes (DEGs) that could point towards pathways associated with extreme levels of androstenone and skatole in pigs. Because of the relationship between these two components, a comparison of combined phenotypes was performed additionally. For this purpose, boars with extreme levels of androstenone and skatole were selected from the Pietrain sired commercial breeding populations for gene expression analysis in liver using microarray technology.

2.4. Material and methods

2.4.1. Animals and phenotype

Tissue samples were collected from Pietrain × F_2 cross animals (n = 58). F_2 was created by crossing F_1 animals (Leicoma × German Landrace) with Large White pig breed. Pietrain × F_2 boars were on average 116 days old and had on average 90 kg live weight when slaughtered. All the pigs were slaughtered in a commercial abattoir. Carcass and meat quality data were collected according to guidelines of the German performance test (ZDS 2003). Tissue samples from liver were frozen in liquid nitrogen immediately after slaughter and stored at -80°C until used for RNA extraction. Fat samples were collected from the neck and stored at -20°C until used for androstenone and skatole measurements. The quantitative determination of the two main components of boar taint was conducted by Fraunhofer Institute for Molecular Biology and Applied Ecology (IME) Schmallenberg. For the quantification of androstenone an adapted gas chromatography/mass spectrometry (GC/MS) (Garcìa-Regueiro and Diaz 1989) was used. The content of skatole was purified by reverse-phase high performance liquid chromatography (RP-HPLC) (Dehnhard et al. 1993) using a fluorescence detector (FD). The skatole level was due to the wavelength of the emitted fluorescence of the components determined (Haugen et al. 2012, Skoog and Leary 1996). In order to determine the significance of testicular dimensions regarding to boar taint the testes were removed from the carcass. The length, wide and weight of testis without epididymis and scrotum were held.

For the microarray study, 20 boars of the Pietrain x F_2 pigs were selected on the basis of phenotype of androstenone and skatole levels. The effect of slaughter season was considered during selection in order to control confoundings. Equal number of boars (n = 10) were selected with extreme high and low androstenone or skatole, respectively. A list of the used phenotypes for the microarray study is shown in table 2. For a association study and validation of selected genes all 58 animals are used.

Table 2: List of the used phenotypes for the microarray study

Animal number	Skatole ng/g fat	Androstenone ng/g fat	Slaughter day
2	145.1	56.1	24.08.2010
5	295.5	63.9	10.08.2010
6	319.7	69.8	17.08.2010
1	55.1	85.5	17.08.2010
7	310.3	92.0	17.08.2010
9	282.8	97.0	03.08.2010
8	337.9	91.6	17.08.2010
20	76.7	92.5	03.08.2010
3	103.9	102.6	31.08.2010
4	76.6	169.7	31.08.2010
15	352.1	437.7	03.08.2010
17	365.2	451.9	03.08.2010
16	339.9	470.1	27.07.2010
10	50.7	500.5	24.08.2010
18	524.4	543.4	31.08.2010
12	57.8	579.7	10.08.2010
11	27.3	588.3	24.08.2010
13	142.9	641.0	24.08.2010
19	357.5	759.2	24.08.2010
14	96.2	958.3	17.08.2010

2.4.2. RNA isolation and microarray process

Total RNA was extracted from the 58 liver samples using RNeasy Mini Kit according to manufacturer's recommendations (Qiagen). Briefly, 15 mg of the samples were extracted using 800 µl of RLT + ß-Mercaptoethanol buffer and homogenised. Centrifugation was then performed 3 min by max. speed. For further steps the supernatant was used. To elute the RNA 50 µl of RNase-DNase free water (Gibco®) was used. Contamination with DNA was removed by DNase treatment (Promega). The extracted RNA was treated with 5 µl RQ1 DNase buffer (Promega), 5 units DNase and 40 units RNase inhibitor (Promega) in a 40 µl reaction volume. The mixture was incubated at 37°C for 1 h followed by purification using chemicals and protocols of the RNeasy Mini Kit (Qiagen). The RNA samples were visualized on 1.5 % agarose gels to check the integrity, and the concentration was measured by NanoDrop ND8000 spectrophotometer (Thermo-Scientific). RNA quality was assessed using

an Agilent 2100 Bioanalyser and RNA Nano 6000 Labchip kit (Agilent Technologies). In addition, absence of DNA contamination was checked using the RNA as a template in a PCR amplifying with GAPDH (Accession number: XP_003126582). All RNA was stored at -80°C until use. The expression patterns of the RNAs were assessed using the GeneChip Porcine Genome Array (Affymetrix). This array contains 23,937 probe sets that interrogate approximately 23,256 transcripts from 20,201 *Sus scrofa* genes. According to Affymetrix protocols, 100 ng of total RNA were reversely transcribed into cDNA, and labelled using the Affymetrix One cycle synthesis and labelling kit to prepare antisense biotinylated RNA targets. The quality of hybridization was assessed in all samples following the manufacturer's recommendations.

2.4.3. Image capturing, quantification and data analysis

Data were initially analysed with Affymetrix GCOS 1.1.1 software using global scaling to a target signal of 500. The microarrays were scanned using a ScanArray Express scanner (Perkin Elmer Inc., MA, USA). Signal intensities were quantified using the ScanArray Express software. The analysis of microarray raw data was performed with the R software (http://www.r-project.org). For normalization and background correction of the data, the algorithm gcRMA (GeneChip Robust Multichip Average) was used. The analysis of expression differences was performed with a linear model for microarray analysis (LIMMA) (Smyth 2004). Three comparisons were taken into account by means of linear contrasts: the comparison of (1) high vs. low androstenone and (2) high vs. low skatole. A third comparison (3) was performed using groups with divergent levels of androstenone and skatole (high skatole/low androstenone vs. low skatole/high androstenone, further termed as combined phenotype group, Figure 6). Differentially regulated genes were identified on the basis of a $p \leq 0.05$, a fold changes ≥ 1 and a false discovery rate (FDR) ≤ 0.3 as already used for microarray analysis by (Ponsuksili et al. 2008b, Wimmers et al. 2010).

The functional annotation and KEGG pathway analysis of DEGs was performed by the DAVID (The Database for Annotation, Visualization and Integrated Discovery) gene annotation tool (http://david.abcc.ncifcrf.gov/) (Huang et al. 2008) in order to assign DEGs to categories of biological functions and to canonical pathways. A Gene ontology (GO) analysis was divided in three parts: biological process (BP), cell components (CC) and molecular functions (MF).

2.4.4. Selection of candidate genes for GenomeLab GeXP verification

The microarray expressions of some selected genes were verified using a GenomeLab GeXP (GeXP) analysis (Rai et al. 2009). Total RNA was isolated from liver. The validation was done in the 58 animals. Twelve interested DEGs and three housekeeping genes (*YWHAZ, RPL4* and *PPIA*) were selected from the results of the microarray study (table 3) for a GeXP multiplex analysis. Two gene, *CYP2E1* and *MGSTI* was selected from literature.

Table 3: List of genes, primer sequence (F: forward, R: reverse) used for GeXP

Gene	Accession number	Sequence w/Universal	Product size
Cell death activator (CIDEBL)	NM_001112688.1	AGGTGACACTATAGAATACGGCCCAAAGAAAATACTCA	208
Cell death activator (CIDEBR)		GTACGACTCACTATAGGGAAAGGTCAGAGCTCCTGCTCA	
Cytochrome P450 2D6 (CYP2D6L)	NM_214394.1	AGGTGACACTATAGAATAGCATGGCCTGGTCTTAGGTA	278
Cytochrome P450 2D6 (CYP2D6R)		GTACGACTCACTATAGGGAAAGCTGAGACGTGGGTCTTG	
Cytochrome P450 39A1 (CYP39A1L)	NM_001101027.1	AGGTGACACTATAGAATAGAACTAGCCGTGCAAAATCC	144
Cytochrome P450 39A1 (CYP39A1R)		GTACGACTCACTATAGGGAGTCCCCATTTTCCCTTTCAT	
Cytochrome P450 3A7 (CYP3A7L)	NM_214422.1	AGGTGACACTATAGAATAATGTTCCCCATCATTAGCCA	222
Cytochrome P450 3A7 (CYP3A7R)		GTACGACTCACTATAGGGATTTTCCACAAAGGGGTCTTG	
Cytochrome P450, family 2, subfamily E (CYP2E1L)	NM_214421.1	AGGTGACACTATAGAATAACAGAATCCCTGCCATCAAG	201
Cytochrome P450, family 2, subfamily E (CYP2E1R)		GTACGACTCACTATAGGGAAATTACCACTGTGCCCTTGG	
Dehydrogenase/reductase SDR family member 7 (DHRS7L)	NM_001244160.1	AGGTGACACTATAGAATATTAGGGACGGTGTCCTTGAC	339
Dehydrogenase/reductase SDR family member 7 (DHRS7R)		GTACGACTCACTATAGGGAATCTTGTGGGATTGGTCTGC	
Flavin-containing monooxygenases (FMO1L)	NM_214064.1	AGGTGACACTATAGAATATGGCCAGGCATCACTATACA	137
Flavin-containing monooxygenases (FMO1R)		GTACGACTCACTATAGGGAGGGTAGTAAGGAGCCCAAGG	
Glutathione S-transferase theta 1 (GSTT1L)	XM_001929370.1	AGGTGACACTATAGAATAGCCATCCTGCTCTACCTGAC	264
Glutathione S-transferase theta 1 (GSTT1R)		GTACGACTCACTATAGGGAAGGGTCACATCCAACTCTGC	
Microsomal glutathione S-transferase 1 (MGST1L)	NM_214300.1	AGGTGACACTATAGAATAGCATTGGCCTTCTGTATTCC	352
Microsomal glutathione S-transferase 1 (MGST1R)		GTACGACTCACTATAGGGATCTTAATTCCTCGGCTCCCT	
NAD(P)-dependent steroid dehydrogenase (NSDHLL)	NM_001167636.1	AGGTGACACTATAGAATAGGCTATGCCGTCAACGTATT	299
NAD(P)-dependent steroid dehydrogenase (NSDHLR)		GTACGACTCACTATAGGGACACTGGCACTGCTGGTTAAA	
Peptidylprolyl isomerase A (PPIAL*)	NM_214353.1	AGGTGACACTATAGAATAACTGGGGAGAAAGGATTTGG	271
Peptidylprolyl isomerase A (PPIAR)		GTACGACTCACTATAGGGACTTGGCAGTGCAAATGAAAA	
Retinol dehydrogenase 11 (RDH11L)	XM_001928767.1	AGGTGACACTATAGAATAAAGAGAAGCCCAATCCCCTA	179
Retinol dehydrogenase 11 (RDH11R)		GTACGACTCACTATAGGGACTGATTTGGGGTACAGCGAT	
Ribosomal protein L4 (RPL4L*)	DQ845176.1	AGGTGACACTATAGAATATCAGCGAATGAGAGCTGGTA	158
Ribosomal protein L4 (RPL4R)		GTACGACTCACTATAGGGATCCAGGGATGTTTCTGAAGG	
Trifunctional enzyme alpha subunit (HADHAL)	NM_213962.1	AGGTGACACTATAGAATACTTTGCTGACCAGAACCCAT	306
Trifunctional enzyme alpha subunit (HADHAR)		GTACGACTCACTATAGGGATGCTTCCTGTGATATTTGCG	
Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWHAZL*)	XM_001927228.4	AGGTGACACTATAGAATAATGATGGGAGGCAGTGAGTC	151
Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWHAZR)		GTACGACTCACTATAGGGACAAAGGGCTGTTATTTCCCA	
UDP-glucuronosyltransferase 2B17 (UGT2B17L)	NM_001244124.1	AGGTGACACTATAGAATACTAAAGCCTTGGCAAACCAG	229
UDP-glucuronosyltransferase 2B17 (UGT2B17R)		GTACGACTCACTATAGGGAGGCTGAATATGGGAGTGGAA	

Total RNA, measured by using the NanoDrop ND-8000 spectrophotometer (Thermo Scientific) was diluted to 50 ng/µl. The mRNA expression of the 13 selected genes and three housekeeping genes were detected in a GeXP (GenomeLab Expression Analysis) multiplex system as described earlier (Gandolfi et al. 2011). Briefly, an amount of 250 ng RNA was used as a template for reverse transcriptase (RT) reaction, performed using GenomeLab GeXP Start Kit (Beckman Coulter, Fullerton, CA, USA), in a total volume of 20 µl. In the RT reaction, a pool of all reverse primers (table 3) at a final concentration of 50 nM was used. Primers were designed using proprietary software provided by Beckman-Coulter. Each of these primers is chimeric, having a 3′ gene-specific end and a 5′ end containing a quasi-T7 universal sequence, which serves as a template in subsequent amplification steps. The RT reaction was performed following the conditions: 1 min at 48°C, 60 min at 42°C, 5 min at 95°C, hold at 4°C, in a thermal cycler (Bio-Rad). After RT reaction, 9.3 µl of the products were used as template for a PCR with 20 nM of each forward primer and 1 U Beckman Coulter Thermo-StartR DNA polymerase (Beckman Coulter). Each of the forward primers contains an SP6 universal sequence at the 5′ end and a gene-specific sequence at the 3′ end (table 2). The PCR reaction was performed in a thermal cycler following the conditions: 10 min at 95°C, followed by 35 cycles of 30 s at 94°C, 30 s at 55°C and 1 min at 70°C; with a hold at 4°C. PCR products were electrophoretically separated by the fragment analysis method on the GenomeLab GeXP (Beckman Coulter), by diluting 1 µl PCR reaction with 28.5 µl SLS buffer and 0.50 µl size standard-400. Kanamycin RNA internal positive control was included and produced a peak at 326 bp when samples were separated via electrophoresis. All experiments include “no template” (i.e. without RNA) and “no enzyme” (i.e. no reverse transcriptase) as negative controls to confirm the absence of peaks at the expected target sizes. The “no template” sample produces a single peak at 326 bp, corresponding to the externally spiked-in kanamycin RNA. Electrophoretic separation was done by the GenomeLab™ GeXP Genetic Analysis System (Beckman Coulter, Fullerton, USA). The GenomeLab GeXP software matches each fragment peak with the appropriate gene, and reports peak height and area under curve (AUC) for all peaks in the electropherogram.

2.4.5. Image capturing, quantification and data analysis

The data were exported from the expression analysis module of the GenomeLab GeXP software as expression data for subsequent analyses. The expression of the genes was normalized by dividing for the geometric mean of the expression of three house keeping

genes (*YWHAZ, RPL4* and *PPIA*) which are reported as comparatively stably expressed (Uddin et al. 2011). These normalized expression values were used for further statistical analysis using SAS Version 9.2 (SAS Institute Inc., Cary, NC, USA). Differences in gene expression levels between groups were determined using proc *t*-test. P-value ≤ 0.05 was considered statistically significant. To investigate the relationship between the gene expression levels and the boar taint traits a correlation analysis was performed for all 58 animals. To avoid a bias towards environmental effects the data were precorrected using a generalized linear model. The proc GLM procedure with following model was applied.

$exp_i = \mu + season_i + \beta_1 age_i + \beta_2 weight_i + e_i$

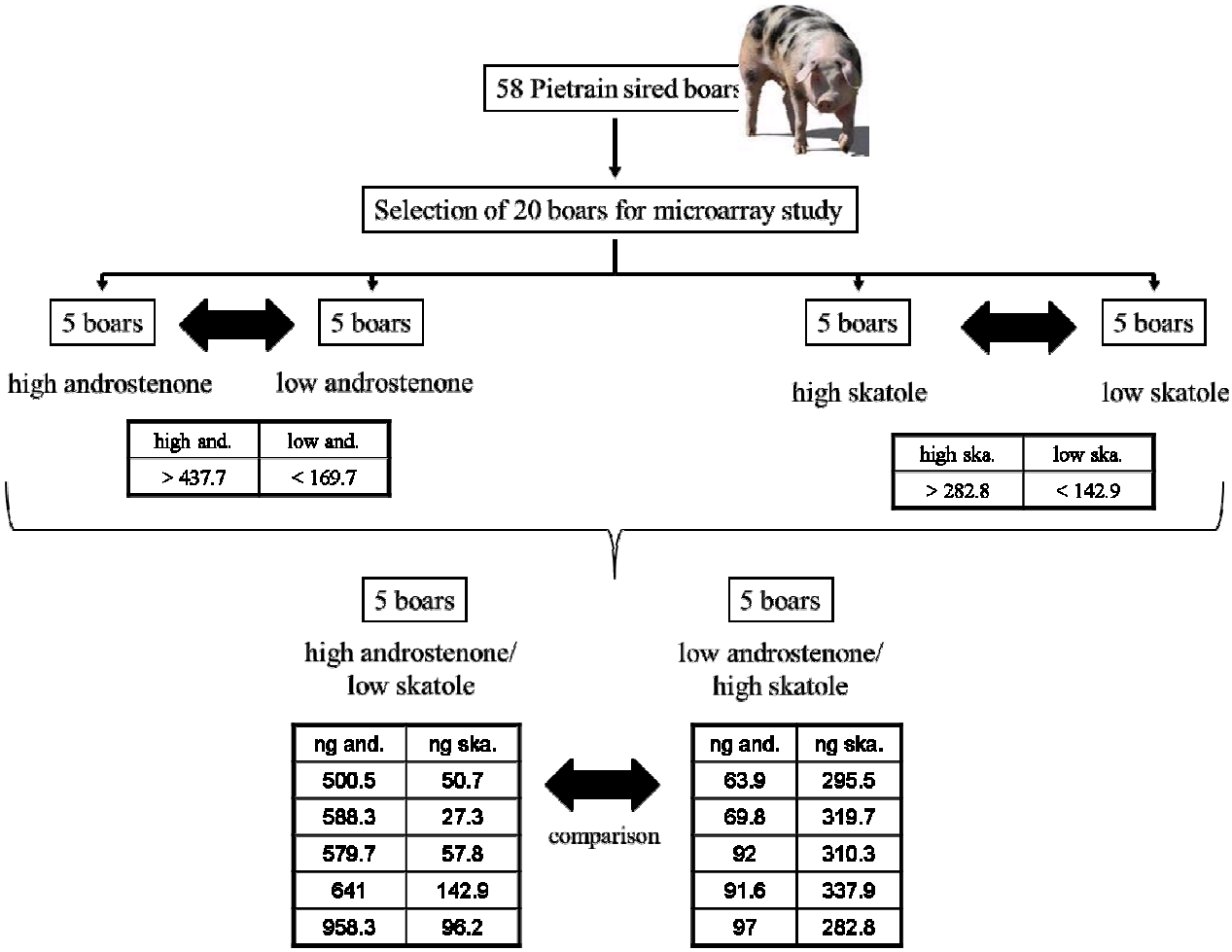
where exp is the gene expression for the ith animals; μ is the overall mean; season is the fixed effect; slaughter age and weight are covariables with their regression coefficient β_1 and β_2, respectively, and e is the random residual error.

Further more, the boar taint traits were log transformed (base e) and in order to meet the assumption of normal distributed data precorrected for the same influencing factors as described in model (1). The relationship between boar taint traits and gene expression abundance was determined using the Pearson's correlation coefficient as implemented in proc corr. A significant correlation was considered for P-value ≤ 0.05.

2.5. Results

2.5.1. Phenotypes

All 58 pigs having a fat androstenone and skatole level between 0.04 µg/g and 0.9 µg/g fat and 0.02 µg/g and 0.8 µg/g fat, respectively. In literature a fat androstenone and skatole level less than 0.5 µg/g and 1 µg/g fat and greater than 0.5 µg/g and 0.25 µg/g fat were defined as low and high androstenone and skatole samples, respectively (Andersen 2006, Andresen 2006, Claus et al. 1994, Fischer and Weiler 1995, Lundström and Zamaratskaia 2006, Lundström et al. 2009, Rhodes 1971, Walstra et al. 1999). Therefore, the average androstenone and skatole values of the 20 selected animals in this study were 0.47 ± 0.05 µg/g and 0.25 ± 0.02 µg/g, respectively. Figure 7 gives an overview of the average levels of androstenone and skatole and the comparison which were used. Among the 20 boars it was possible to divide the animals according a divergent phenotype (figure 6, comparison 3)



ng and.	ng ska.
500.5	50.7
588.3	27.3
579.7	57.8
641	142.9
958.3	96.2

ng and.	ng ska.
63.9	295.5
69.8	319.7
92	310.3
91.6	337.9
97	282.8

Figure 6: Experimental design with the average levels of androstenone and skatole and the comparison which are used for microarray analysis

2.5.2. Overview of differential gene expression

The Affymetrix porcine microarrays containing 26,877 clones were used to examine the Pietrain × F_2 pigs with divergent levels of androstenone and skatole. The analysis of the microarrays revealed only two genes in the high/low levels androstenone comparison with FDR ≥ 0.8, no different expressed genes was identified. 92 genes were differentially expressed in the comparison of high vs. low skatole, of which 49 were up and 43 down regulated. Moreover, statistical analysis identified that 170 genes differentially expressed in the comparison of the combined phenotype of androstenone and skatole, of which 84 genes were down regulated and 86 genes were up regulated in high skatole/low androstenone compared to low skatole/high androstenone. Venn diagram was created to explore any overlap between the three comparisons (figure 7). The top 20 differently expressed genes are shown in table 4. A total of 84 genes were found to be common for high vs. low skatole and the

combined phenotype group. On the other hand, both identified genes were found to be common for high vs. low androstenone and the group of combined phenotypes.

Table 4: Top 20 differently expressed gens of the three groups: comparison of high and low androstenone (A), high and low skatole (B) and the combined phenotypes (C)

A

Gene name		Accession number	logFC[1]	P-Value	FDR[2]
CYP3A7	Cytochrome P450 3A7	NM_214422.1	1.34	0.0017	0.84
INHBA	Inhibin beta A chain precursor	AJ746628	-1.07	0.0019	0.84

B

Gene name		Accession number	logFC	P-Value	FDR
Q5TCH4	Cytochrome P450, family 4, subfamily A, polypeptide 22	NM_214424.1	-3.93	0.000214	0.07
SCD	Acyl-CoA desaturase	NM_213781.1	-3.54	0.000695	0.12
FASN	Fatty acid synthase	CN166778	-3.25	0.000028	0.03
CYP4A11	Cytochrome P450 4A11 precursor	NM_214425.1	-3.11	0.000108	0.05
PSPHL	L-3-phosphoserine phosphatase	CF364971	-2.59	0.000039	0.03
PPAP2A	Lipid phosphate phosphohydrolase 1	CF789276	-2.55	0.002424	0.21
ACAS2	Acetyl-coenzyme A synthetase, cytoplasmic	AW483183	-2.39	0.000789	0.13
DOCK1	Dedicator of cytokinesis protein 1	BF710297	-2.14	0.000020	0.03
PTPRD	Protein-tyrosine phosphatase delta precursor	BI341817	-2.06	0.000369	0.09
FDPS	Farnesyl pyrophosphate synthetase	CK463813	-2.05	0.000132	0.05
C10orf45	---	BI119156	1.65	0.003455	0.24
Q68D10	---	CN159402	1.81	0.003599	0.24
PLA2G7	Platelet-activating factor acetylhydrolase precursor	BQ603958	1.86	0.000112	0.05
DHRS4	Dehydrogenase/reductase SDR family member 4	NM_214019.1	2.08	0.003558	0.24
LDHB	L-lactate dehydrogenase B chain	U07180.1	2.54	0.000004	0.01
ABCD3	ATP-binding cassette, sub-family D, member 3 (70 kDa peroxisomal membrane protein)	CK466741	2.84	0.000012	0.02
G0S2_HUMAN	Putative lymphocyte G0/G1 switch protein 2.	CF361311	2.98	0.001957	0.19
PMM1	Phosphomannomutase 1	CN159771	3.33	0.000028	0.03
ADFP	Adipophilin	AY550037.1	3.67	0.001301	0.15
HMGCS2	Hydroxymethylglutaryl-CoA synthase, mitochondrial precursor	NM_214380.1	4.53	0.000114	0.05

C

Gene name		Accession number	logFC	P-Value	FDR
Q5TCH4	Cytochrome P450, family 4, subfamily A, polypeptide 22	NM_214424.1	-3.42	0.0005545	0.09
SCD	Acyl-CoA desaturase	NM_213781.1	-3.22	0.0012922	0.12
FASN	Fatty acid synthase	CN166778	-3.21	0.0000299	0.02
ALDRL6	Inositol oxygenase	NM_214102.1	-3.04	0.0026559	0.18
CYP4A11	Cytochrome P450 4A11 precursor	NM_214425.1	-2.90	0.0001794	0.06
PSPHL	L-3-phosphoserine phosphatase	CF364971	-2.72	0.0000272	0.02
Q5VT40	PREDICTED: similar to gene	BX676064	-2.59	0.0044821	0.21
NP_054778	Response gene to complement 32; RGC32 protein	BI183661	-2.08	0.0057780	0.24
ACACA	Acetyl-CoA carboxylase 1	BF441762	-2.05	0.0008440	0.10
CYP3A7	Cytochrome P450 3A7	NM_214422.1	-2.04	0.0000987	0.04
DHRS4	Dehydrogenase/reductase SDR family member 4	NM_214019.1	2.03	0.0040706	0.20
Q96L03	---	BF712467	2.28	0.0072945	0.25
CPT1A	Carnitine O-palmitoyltransferase I, mitochondrial liver isoform	AF288789.1	2.62	0.0019500	0.15
LDHB	L-lactate dehydrogenase B chain	U07180.1	2.90	0.0000015	0.00
G0S2_HUMAN	Putative lymphocyte G0/G1 switch protein 2	CF789113	3.57	0.0004899	0.09
ABCD3	ATP-binding cassette, sub-family D, member 3	CK466741	3.77	0.0000013	0.00
PMM1	Phosphomannomutase 1	CN159771	4.07	0.0000059	0.01
APOA4	Apolipoprotein A-IV precursor (Apo-AIV)	NM_214388.1	4.65	0.0068373	0.25
ADFP	Adipophilin (Adipose differentiation-related protein) (ADRP)	AY550037.1	4.71	0.0002461	0.07
HMGCS2	Hydroxymethylglutaryl-CoA synthase, mitochondrial precursor	NM_214380.1	6.02	0.0000135	0.02

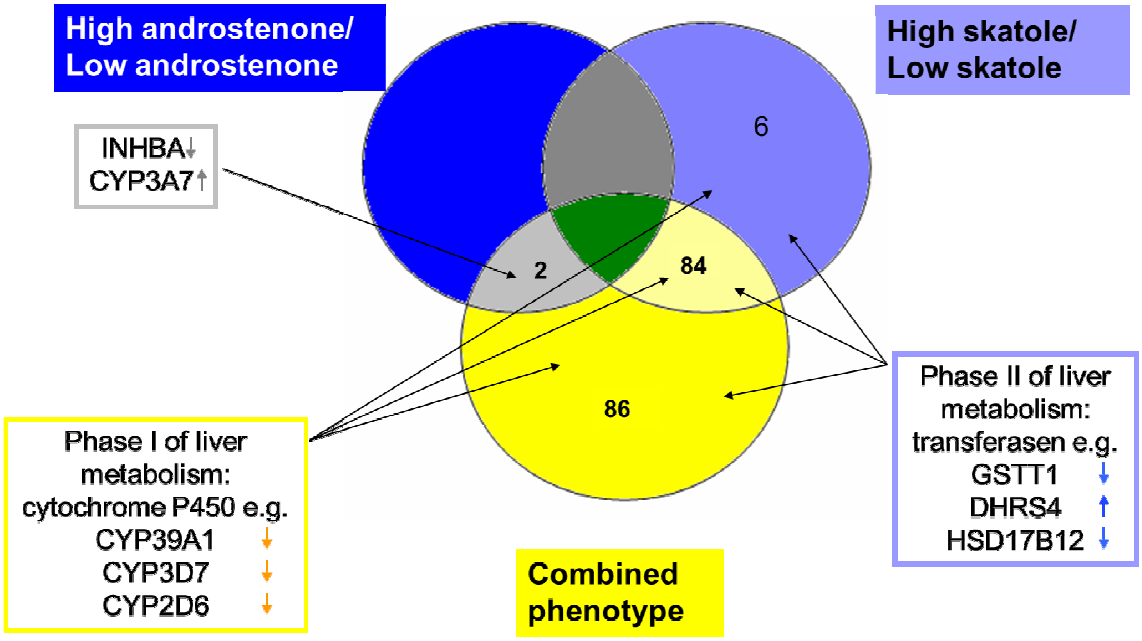


Figure 7: Venn diagram of the DEGs showing differentially expressed genes between high and low androstenone, high and low skatole and between the combined phenotype

2.5.3. Biological function analysis for DEGs

The classification into the 3 groups, BP, CC and MF of all DEGs, identified in high vs. low skatole and of the combined phenotypes was performed by the GO database DAVID. When all the DEGs were analysed in DAVID, metabolic process, cellular metabolic process, oxidative reduction and lipid metabolism process are found to be the most dominant biological processes in boar's liver (figure 8A). The CC analysis showed cytoplasmic part, membrane-bound organelle and cell fraction as the important component in this study (figure 8B). Additionally, oxidoreductase activity, cofactor binding, identical protein and coenzyme binding were found to be the pivotal molecular functions active in divergent boar's liver tissues (figure 8C).

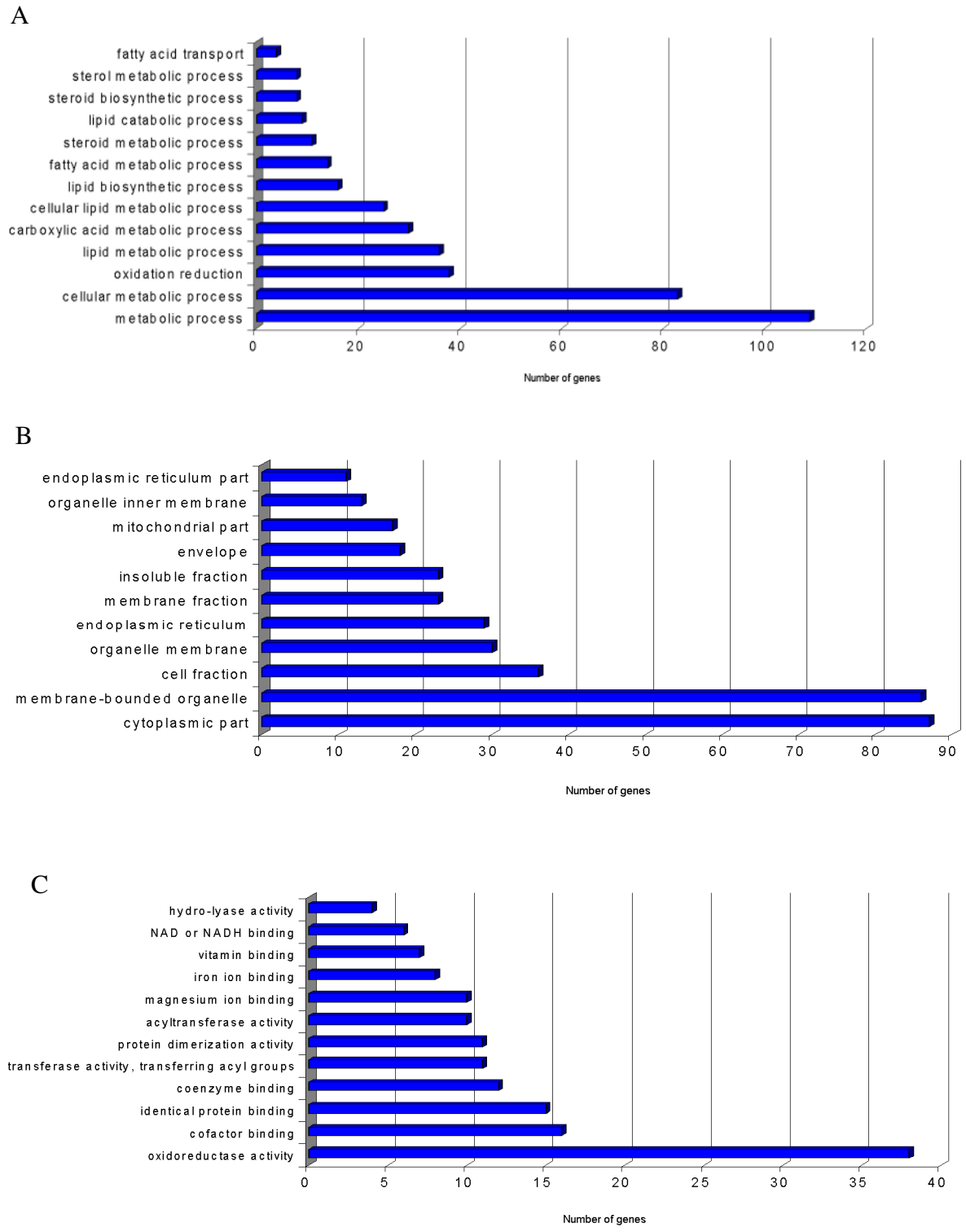
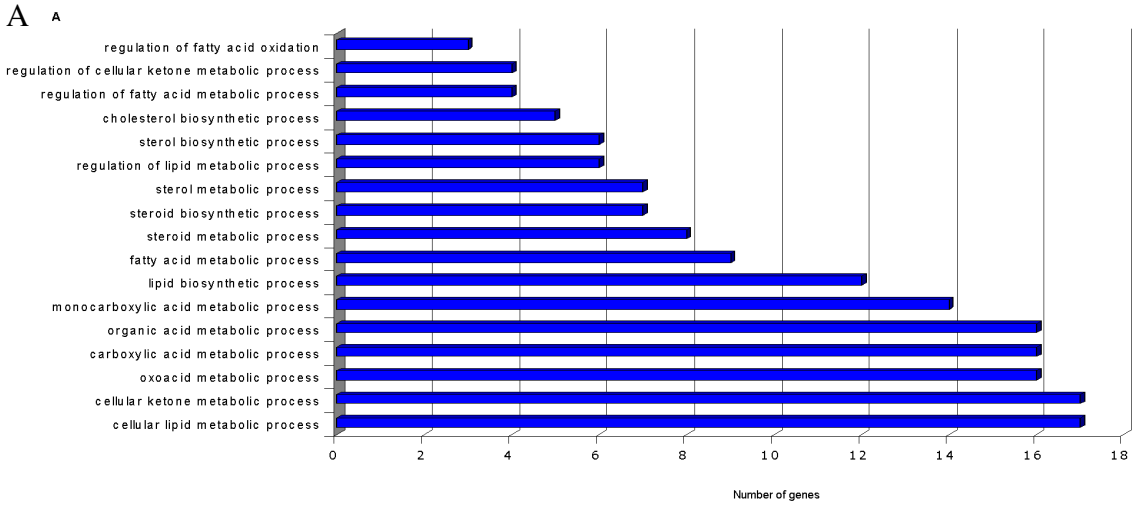


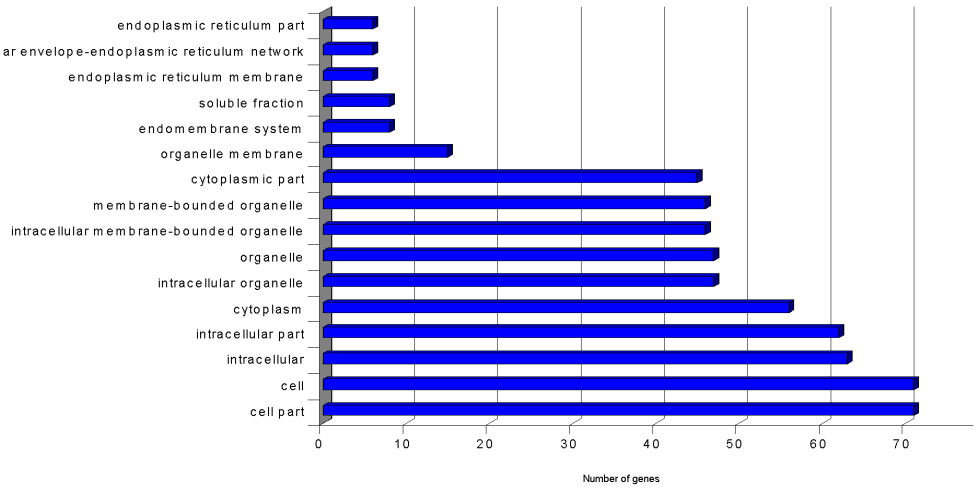
Figure 8: Ontological classification of all DEG. The genes were classified according to cell components (A), biological processes (B) and molecular function (C)

In the comparison between high skatole and low skatole cellular lipid metabolic process, cellular ketone metabolic process and oxacid metabolic process, carboxylic acid metabolic process and organic acid metabolic process were the most abundant biological process (Figure 9A). CC analysis identified cell part, cell, and intracellular component as the abundant in liver tissues (figure 9B). The observed GO term enrichment for molecular function for the DEGs between divergent skatole levels were catalytic activity and oxidoreductase activity (figure 9C).

A A



B



C

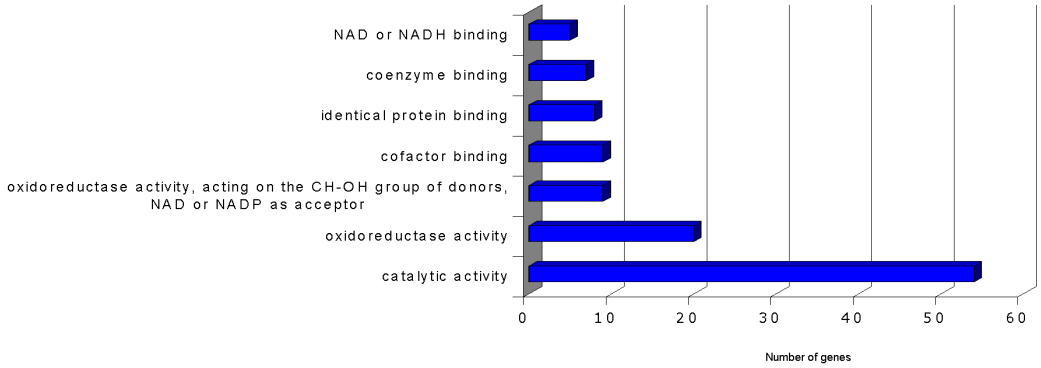
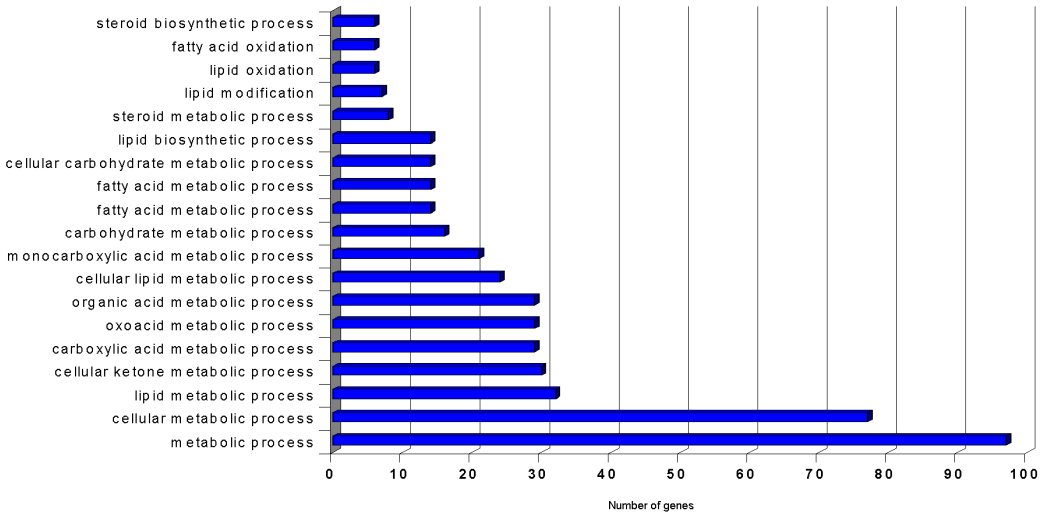


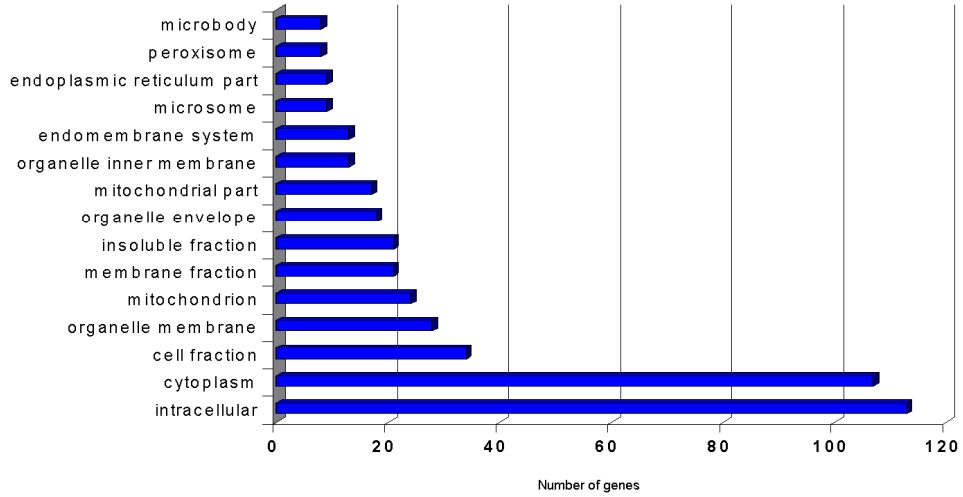
Figure 9: Ontological classification of DEG in the comparison of high skatole and low skatole. The genes were classified according to cell components (A), biological processes (B) and molecular function (C)

The DEGs of the combined phenotypes showed important features when analysed in DAVID (figure 10). The most important biological processes were metabolic process, cellular metabolic process, lipid metabolic process and cellular ketone metabolic process (figure 10A). Intracellular, cytoplasmic and cell fraction were the abundant cellular component in interaction DEGs (figure 10B). The molecular function for the interaction DEGs identified catalytic activity, cofactor binding, and oxidoreductase activity as the important features of molecular functions in this study (figure 10C).

A



B



C

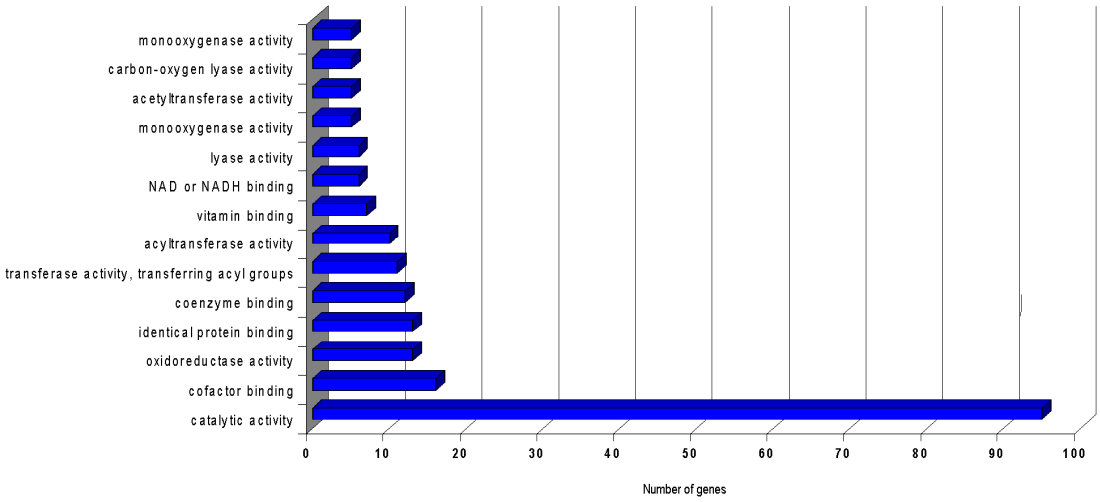


Figure 10: Ontological classification of DEG in the comparison of the combined phenotypes. The genes were classified according to cell components (A), biological processes (B) and molecular function (C)

2.5.4. Identified KEGG pathways for DEGs

The KEGG pathway analysis using DAVID for all DEGs highlighted several pathways such as fatty acid metabolism, retinol metabolism, PPAR signaling, pathway, beta-alanine metabolism, glutathione metabolism, propanoate metabolism, steroid biosynthesis, biosynthesis of unsaturated fatty acids, metabolism of xenobiotics by cytochrome P450, ether lipid metabolism, adipocytokine signaling pathway and glycerophospholipid metabolism.

DAVID assigned 27 DEGs between high and low skatole liver samples to ten different pathways. The pathway analysis highlighted pathways involving retinol metabolism, propanoate metabolism, PPAR signaling pathway, fatty acid metabolism, steroid biosynthesis, glycerophospholipid metabolism, ether lipid metabolism, pyruvate metabolism, fatty acid biosynthesis, amino sugar and nucleotide sugar metabolism.

The DEGs between the combined phenotypes of liver samples were analysed into different pathways by DAVID. Fatty acid metabolism, PPAR signaling pathway, retinol metabolism, beta-alanine metabolism, glutathione metabolism, propanoate metabolism, pyruvate metabolism, biosynthesis of unsaturated fatty acids, steroid biosynthesis, valine, leucine and isoleucine degradation, arginine as well as proline metabolism were detected as the dominant pathways involved in boar's liver with divergent skatole and androstenone levels (table 5).

Table 5: List of the identified KEGG pathways

	Pathways	Differentially Expressed Genes
All DEGs of the microarray study	Fatty acid metabolism	Acaa1, ACSL1, ACSLS, ACADVL, ADH4, Cpt1a, CYP4A11, HADHA
	Retinol metabolism	UGT2B12, ADH4, aldh1a1, CYP3A1, CYP4A11, Dhrs3, Dhrs4, rdh11
	PPAR signaling pathway	HMGCS2, Acaa1, ACSL1, ACSL5, Cpt1a, CYP4A11, PPARA, scd
	Beta-alanine metabolism	ABAT, DPYS, HADHA, Sms, UPB1
	Glutathione metabolism	ANPEP, Ggct, GSTT1, gpx3, IDH1, Sms
	Propanoate metabolism	ABAT, ACACA, ACSS2, HADHA, Idhb
	Steroid biosynthesis	DHCR7, nsdhl, lipA, tm7sf2
	Drug metabolism	UGT2B12, ADH4, CYP2d6, CYP3A7, FMO1, GSTT1
	Pyruvate metabolism	ACACA, ACSS2, Idhb, Idhd, Pc
	Drug metabolism	UGT2B17, CYP3A7, DPYS, UPB1, UPP2
	Biosynthesis of unsaturated fatty acids	Acaa1, HADH4, HSD17B12, scd
	Metabolism of xenobiotics by cytochrome P450	UGT2B17, ADH4, CYP3A7, dhdh, GSTT1
	Ether lipid metabolism	AaPAT3, PPAP2A, PLA2G7, Pla2q12a
	Adipocytokine signaling pathway	ACSL1, ACSL5, adipor2, cpt1a, PPARA
	Glycerophospholipid metabolism	AGPAT3, GPD1, GPD1L, PPAP2A, Pla2q12a
	Valine, leucine and isoleucine degradation	HMGCS2, ABAT, Acaa1, HADHA
DEGs of high vs. low skatole	Retinol metabolism	Q5TCH4, RDH11, UGT2B17, DHRS3, ALDH1A1, DHRS4
	Propanoate metabolism	HADHA, ACAS2, LDHB, ACACA
	PPAR signaling pathway	SCD, Q5TCH4, HMGCS2, LCF1, PPARA
	Fatty acid metabolism	Q5TCH4, HADHA, LCF1, ACADVL
	Steroid biosynthesis	TM7SF2, DHCR7, NSDHL
	Glycerophospholipid metabolism	PLA2G12A, GPD1, GPD1L, PPAP2A
	Ether lipid metabolism	PLA2G7, PLA2G12A, PPAP2A
	Pyruvate metabolism	ACAS2, LDHB, ACACA
	Fatty acid biosynthesis	FASN, ACACA
	Amino sugar and nucleotide sugar metabolism	PGM3, PMM1, GALT
DEGs of combined phenotypes	Fatty acid metabolism	ACAA1, Q5TCH4, HADHA, LCF1, CPT1A, ACADVL, ADH4, ACSL5
	PPAR signaling pathway	ACAA1, SCD, Q5TCH4, HMGCS2, LCF1, CPT1A, ACSL5, PPARA
	Retinol metabolism	Q5TCH4, RDH11, ADH4, CYP3A7, DHRS3, ALDH1A1, DHRS4
	beta-Alanine metabolism	ABAT, UPB1, HADHA, DPYS, SMS
	Glutathione metabolism	GPX3, GSTT1, C7orf24, IDH1, ANPEP, SMS
	Propanoate metabolism	ABAT, HADHA, ACAS2, LDHB, ACACA
	Pyruvate metabolism	ACAS2, LDHB, ACACA, LDHD, PC
	Biosynthesis of unsaturated fatty acids	ACAA1, SCD, HADHA, HSD17B12
	Drug metabolism	GSTT1, ADH4, CYP3A7, CYP2D6, FMO1
	Adipocytokine signaling pathway	ADR2, LCF1, CPT1A, ACSL5, PPARA
	Steroid biosynthesis	TM7SF2, DHCR7, LIPA
	Valine, leucine and isoleucine degradation	ACAA1, ABAT, HADHA, HMGCS2
	Arginine and proline metabolism	ALDH4A1, DAO, AGMAT, SMS

2.5.5. Validation of expressions of selected genes

In order to validate the expression patterns of microarray data, *FMO1, CYP2E1, CYP2D6, CYP39A1, DHRS7, HADHA, MGST1, CYP3A7, RDH11, GSTT1, UGT2B12, NSDHL* and *CIDEB* and three housekeeping genes *YWHAZ, RPL4* and *PPIA* were selected for GeXP analysis (table 3). The expressions of patterns of the selected genes were found to be similar in microarray and GeXP (figure 11). The genes were selected from the microarray comparison of high skatole with low skatole and between the combined phenotype. The genes *RDH11* and *GSTT1* were significantly ($p < 0.05$) different expressed in the comparison of high skatole with low skatole and show the same regulation to the common microarray data, while *HADHA* show an ambivalence trend in their regulation (figure 11A). The analysis of the combined phenotype data show no significant different in the expression (figure 11B) but the fold change show the same trend of regulation in common to the microarray data.

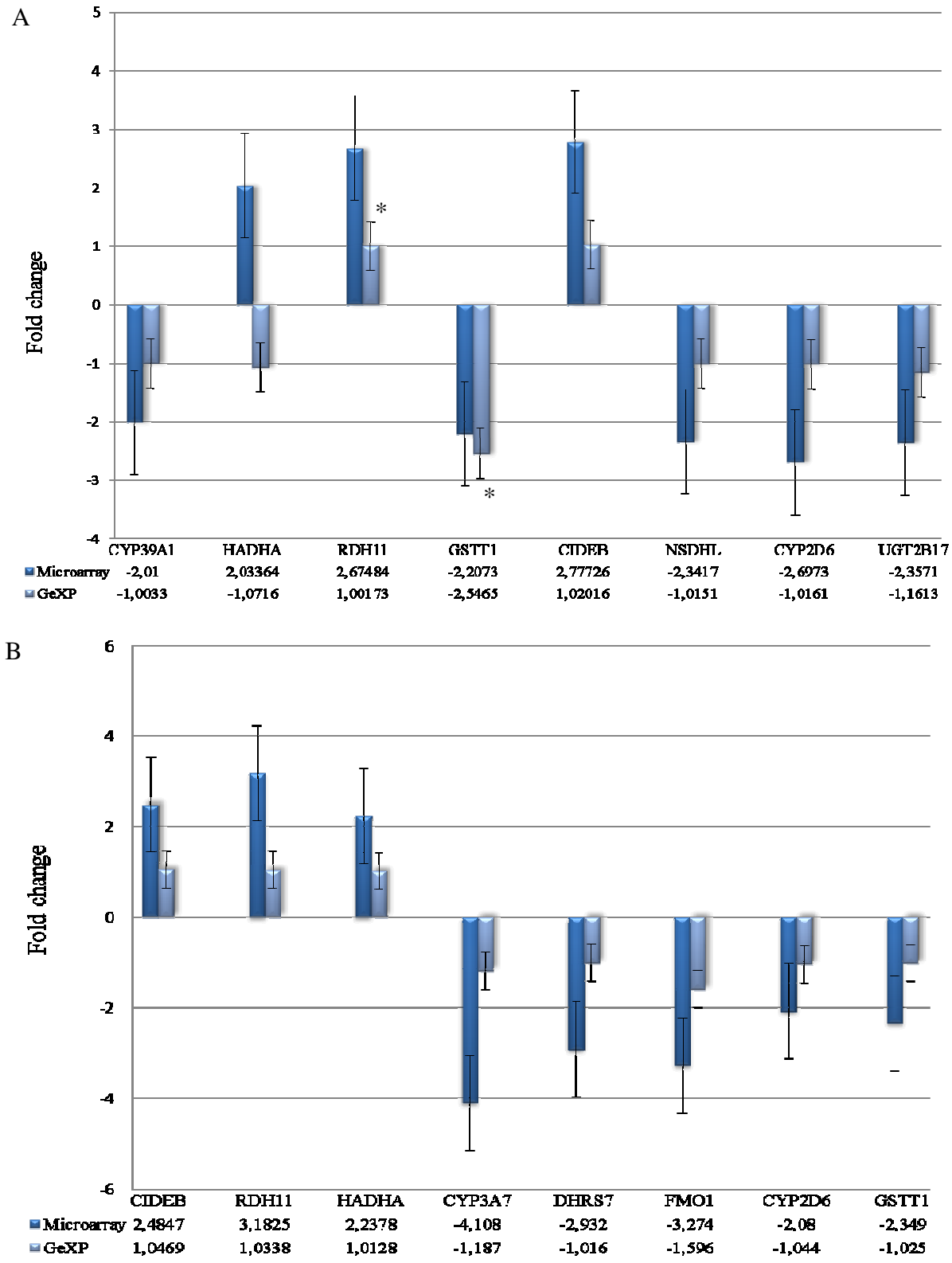


Figure 11: Fold change from the microarray and GeXP analysis of the validated genes: comparison high vs. low skatole (A) and comparison of the combined phenotype (B). * $p < 0.05$ in GeXP

2.5.6. Association between genes expressions and phenotypes

A linear relationship between the expression of selected genes and boar taint components and testicular size was performed using a correlation analysis (n = 58). The results are shown in table 6. Androstenone found to be associated with all measurements of testicular sizes (table 6). *DHRS3, ADFP, CYP3A7 and CYP2E1* were associated with androstenone levels, while *CYP2E1* and *HSD17B12* were associated with skatole levels. The two genes *FMO1* and *CYP2E1* were associated with the testis weight.

Table 6: Association of expression of selected gene with boar taint components and testicular size

	Andro.	Skatol	T_wi 1	T_wi 2	T_le 1	T_le 2	T_wei 1	T_wei 2
Andro.	n.s.	n.s.	***	***	***	***	***	****
Skatol	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
UGT2B31	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
DHRS3	***	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
DHRS4	*	n.s.	*	n.s.	n.s.	n.s.	n.s.	n.s.
ADFP	**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
CYP3A7	***	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
CYP2E1	**	***	**	n.s.	n.s.	n.s.	n.s.	n.s.
HSD17B12	n.s.	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
RDH11	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
NSDHL	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
CYP4A21	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
FMO1	n.s.	n.s.	**	**	n.s.	n.s.	*	n.s.
CYP2D25	n.s.	n.s.	n.s.	n.s.	n.s.	*	n.s.	n.s.
ACADVL	n.s.	n.s.	n.s.	*	n.s.	n.s.	n.s.	n.s.

Andro: Androstenon; T_wi 1: testis wide 1; T_wi 2: testis wide 2; T_le 1: testis length 1; T_le 2 : testis length 2 ; T_wei 1: testis weight 1; T_wei 2: testis weight 2; n.s.: non significant. ***** $p \leq 0.0001$, **** $p \leq 0.001$, *** $p \leq 0.01$, ** $p \leq 0.05$, * $p \leq 0.1$

2.6. Discussion

Boar taint is very important from the consumer point of view which is highly related to the acceptance value of the pork. Before starting selection it is important to understand the complex genetic system controlling boar taint and to take into account possible correlated effects on other traits in the breeding goal. The relationship between boar taint and the fertility but also between skatole and androstenone is well known (Claus et al. 1994) and therefore the selection against boar taint is difficult. Holistic transcriptome analyses is one important approach to understand the genes and pathways involved in the metabolism of boar taint compounds in the porcine liver.

This study identified several genes and pathways that might be involved in the metabolism of androstenone and skatole in the liver of boars. In total, 264 genes were identified to be differentially expressed with a FDR < 0.3 and $p < 0.05$. Two genes were observed comparing the levels of androstenone. There are two explanations why we only observed two genes related to androstenone although the FDR threshold was ignored. Firstly the number of used sample was small and secondly, the androstenone levels of the Pietrain F_2 crossbred animals used in this study was at the button edge of threshold for boar taint and therefore the differences between low and high maybe not clear enough.

Among all differentially expressed genes, CYP 450 family genes such as *CYP39A1, CYP4A11, CYP4A22, CYP2D6* and *CYP3A7* were found to be differently expressed in boars liver tissues with divergent boar taint compounds. *CYP4A22, CYP4A11* and *CYP2D6* were found to be down regulated in the group of high skatole compared to low skatole as well as in the comparison of the combined phenotypes. *CYP39A1* was down regulated in high skatole compared to low skatole and *CYP3A7* was down regulated in the group of the combined phenotypes and up regulated in the comparison of high androstenone compare to low androstenone. Several gene expression studies identified genes of the Cytochrome P450 family as important for the regulation of boar taint (Grindflek et al. 2011a, Gunawan et al. 2013b, Leung et al. 2010, Moe et al. 2007b, Moe et al. 2008). Cytochrome P450 isoenzymes are the main enzymes playing roles in phase 1 skatole metabolism, where skatole is degraded to several intermediate products such as indole-3-carbinol (I3C), 2-aminoacetophenone (2AAP) and 3-metyloxyindole (3MOI), details (reviewed by Deslandes et al. 2001, Robic et al. 2008, Wesoly and Weiler 2012). High levels of the metabolite 6-sulphatoxyskatole in plasma were suggested as indicators of entire male pigs with the ability to rapidly metabolise and excrete skatole (reviewed by Andresen 2006). Skatole is synthesised in the intestine of all genders of pigs but only entire males accumulate skatole in the adipose tissue in high concentrations. A part of skatole is excreted with faeces and the remaining part is absorbed through the gut wall and released into the blood. Skatole is metabolised in the liver to a variety of metabolites (reviewed by Andresen 2006). Several of the identified genes belong to the group of CYP P450 family, which are mainly involved in the skatole and androstenone metabolisms in pig (Babol et al. 1999). The CYP2E1 gene, from which is well known that skatole induced the protein expression while androstenone antagonised this effect in isolated hepatocytes (Doran et al. 2002a) was not found in our microarray study and also showed no significant differently regulation in the GeXP analysis but is associated with the three boar taint components androstenone, skatole, indole and one testis size in this study. For further studies it could be very interesting to analyse the different genes of the CYP 450 family and the corresponding pathway (drug metabolism) in a single way.

Different groups of transferases including UDP-glucuronosyltransferase 2B17 (*UGT2B17*) (down regulated in high skatole compare to low skatole) and Glutathione S-transferase theta 1 (*GSTT1*) (down regulated in high skatole compared to low skatol and in the combined phenotypes) were found to be differentially regulated in this study. Glutahione S-transferases (GSTs) are functionally diverse enzymes mostly known to catalyse conjugation reactions of endogenous substances, haem, fatty acids, xenobiotics and products of oxidative processes

(Litowsky et al. 1988). UGT is a family of conjugation enzymes and members of the UGT enzyme family such as *UGT1A5* and *UGT2A1* are reported to be differentially regulated in porcine transcriptome analysis for androstenone (Moe et al. 2008). Beside, *UGT2B* genes, Glutathione S-transferase theta 1 (*GSTT1*) genes were found to be involved in high vs. low skatole group. During phase 2 metabolism, the water solubility of the skatole metabolism is increased to facilitate excretion via urine and the GSTs, reported to transport different molecules (Litowsky et al. 1988).

Members of the dehydrogenase/reductase (SDR) family are enzymes involved in the process of oxidation of 3b-hydroxysteroid precursors into ketosteroids. Several family members have previously been shown to be important in catalyzing an essential step in the biosynthesis of all classes of active steroid hormones (Penning 1997b). *DHRS4* and *DHRS7* are differentially expressed in this study. *DHRS4* was significantly up regulated with a fold change of 2.0 in both groups and *DHRS7* was down regulated (FC -1.5) in the comparison of combined phenotypes. Moe et al. (2007b) and Grindflek et al. (2010) identified *DHRS4* to be highly up-regulated in high androstenone boars in testis sample in both Landrace and Duroc. Interestingly, the *DHRS4* was recently shown to have a role in 3b-hydroxysteroid synthesis, and *DHRS4* was shown to be induced via *PPARA* activation (Matsunaga et al. 2008). *PPARA* has previously been shown to regulate various genes controlling gluconeogenesis, ketone body synthesis, heme synthesis and cholesterol metabolism (Michalik et al. 2006). Furthermore, in our study the PPAR signalling pathway was identified as one of the important pathway in (a) all DEGs (b) in the comparison of high and low skatole and (c) in the combined phenotypes (table 5).

The PPARA gene is identified as DEGs in this study in the comparison between high and low skatole as well in the comparison of combined phenotypes up regulated. The *PPARA* gene affects the expression of target genes involved in cell proliferation and differentiation process and regulates the peroxisomal beta-oxidation pathway of fatty acids. Importantly, it functions as transcription activator for the *ACOX1* and P450 genes (Gulick et al. 1994). A connection to the metabolism of skatole is till now unclear, but different regulation in liver with combined skatole levels and in the comparison of the combined phenotypes of androstenone and skatole is maybe a evidence for a relationship between androstenone and skatole.

The pathway analysis show two more interesting metabolism which are identified in all DEGs, in the comparison of high and low skatole and in the combined phenotype: retinol metabolism and fatty acid metabolism.

For the retinol metabolism two genes are also members of the SDR family *RDH11* and *DHRS3* (also called *RDH17*) and found to be differently regulated in both group of this study. DHRS3 is significantly down regulated with a fold change of -1.0 in high skatole compared to low skatole and in the combined phenotype group while *RDH11* are significantly up regulated (FC 1.6) in both groups. The identification of these genes in both groups and the fact that DHRS3 show a relationship to androstenone (table 6) indicates a relationship for the regulation of androstenone and skatole in liver. The retinol dehydrogenase (RoDH) gene family catalyze the conversion of retinol to retinal as well as efficient oxidative 3alpha-HSD activity with 3alpha-Adiol and androsterone as substrates (Duester 2000, Napoli 1999). Grindflek et al. (2011a) identified a QTL for androstenone on SSC5 within this region the *RDH5* gene, which is found to be involved in hydroxysteroid dehydrogenase activity by recognizing 5alpha-androstan-3alpha, 17beta-diol and androsterone as substrates (Wang et al. 1999). Leung et al. (2010) showed in their study one RDH gene which to be expressed at a higher level in high androstenone boars in three different breeds. This result suggested that the genes of the retinol metabolism maybe involved in the regulation of boar taint. The identification of several genes which are involved in steroid biosynthesis suggested the hypothesis of Claus et al. (1994) that male sex hormones increase skatole production.

Another important part for the regulation of boar taint could be the process of the fat metabolism in liver and their gene expression. In this study we identified several genes of the fat metabolism, like *ACSL1, ACSL2, PLA2G, HADHA, FASN* and *LCF1*. *ACSL1* and *ACSL5* are found to be important DEGs that might be involved in boar taint compound metabolism in liver. ACSL, a large family of isozymes convert free long-chain fatty acids into fatty acyl-CoA esters, and thereby play a key role in lipid biosynthesis and fatty acid degradation (Stanczak et al. 1992). *PLA2G* (Phospholipase A2) family members hydrolyze the sn-2 fatty acid ester bond of glycerophospholipids to produce lysophospholipids and free fatty acid. These genes have diverse biological functions including roles in inflammation, cell growth, signaling and death and maintenance of membrane phospholipids (Dennis 1994). *HADHA* was found to be differentially expressed in the liver tissues from divergent boar taint compounds. This gene encodes the alpha subunit of the mitochondrial trifunctional protein, which catalyzes the mitochondrial beta-oxidation of long chain fatty acids (Das et al. 2006). The identification of these DEGs not only in the group of divergent skatole levels but also in the group with combined phenotypes could be indicate that this gens are important for both the regulation of skatole and androstenone.

An important gene family found to be differentially expressed in our transcriptome analysis is the flavin-containing monooxygenases (FMOs) gene family. The FMO family of enzymes converts lipophilic compounds into more polar metabolites and decreases activity of the compounds (Cashman 2005), a similar activity to that of the cytochrome P450s (Cashman 2005). Moe et al. (2008) found that flavin-containing monooxygenase 1 (*FMO1)* is up-regulated in pigs with higher androstenone level. In contrast, *FMO5* was found to be down-regulated in high androstenone liver samples in our previous study (Gunawan et al. 2013b), whereas *FMO1* is down regulated in this study comparing the group of combined phenotypes. The *FMO1* is a gene of phase I metabolism which could be involved in the metabolism of skatole and androstenone and thus for boar taint. Because of the monooxygenase activity and its regulation by steroid hormones makes it an interesting candidate gene for boar taint (Moe et al. 2008). A mutations in flavin containing mono-oxygenase 3 (*FMO3*) are known to be associated with a fishy off flavour in both chicken eggs (Honkatukia et al. 2005) and cow's milk (Lundén et al. 2002). A quantitative trait locus (QTL) for off flavour was identified previously on SSC9 (Thomsen et al. 2004). The *FMO1* and *FMO3* genes were both linkage mapped to SSC9 all these data suggested that *FMO1* could be an important candidate genes for further association and functional study to validate their involvement in boar taint (Glenn et al. 2007, Moe et al. 2008).

A relationship between androstenone and skatole has been already described in several studies, but till now the mechanisms behind are unclear. There are two hypotheses: at first male sex hormons may increase the skatole production (Claus et al. 1994). A second hypothesis considering that androgens inhibit the catabolism of skatole in the liver (Doran et al. 2002b). In the comparison of the combined phenotypes 84 genes were not in common with the results of the analysis of the androstenone and skatole levels and could be therefore of interest as candidate genes for selection against both traits. Different expression of two of these genes (*FMO1* and *HSP17B12*) (already described above) are already known to play an important role in the regulation of androstenone in liver (Moe et al. 2008). The pathway analysis suggested that there is a high relationship between the two components. The most common pathways in all DEGs (1), the comparison of high and low skatole (2) and the combined phenotypes (3) are fatty acid metabolism, retinol metabolism and PPAR signalling pathway (table 4). These results may serve as a further evidence for the second hypothesis. Therefore, a pleiotropic effect of these genes can not be ruled out. Till now only few studies published about pleiotropic effects of genes that influenced both androstenone and skatole. *CYP2E1* is well know to influence as well as androstenone and skatole (Doran et al. 2002a,

Doran et al. 2002b). Only a few groups studied the pleiotropic effect of QTL regions for androstenone and other important traits such as birth weight, backfat thickness, growth rate, total number born, litter birth weight, teat number, sperm motility, and number of spermatozoa per ejaculation and identified a favorable effect of the low-androstenone haplotype on number of spermatozoa per ejaculation on SSC6 (Hidalgo et al. 2013).

2.7. Conclusion

It is crucial to understand the transcriptome of boar taint to select pigs for lower androstenone and skatole levels and thus reduce boar taint. Our study identified several genes which have potential influence on fatty acid metabolism, steroid biosynthesis, retinol metabolism and PPAR signalling pathway which might be involved in the metabolism of androstenone and skatole in porcine liver. The identification of a lot of DEGs in group of combined phenotypes indicates a high relationship in the regulation of androstenone and skatole in liver. Best of our knowledge, only one study (Gunawan et al. 2013a) was devoted to perform a global transcriptome analysis for divergent skatole levels in boar fat of Duroc x F_2 cross animals as well as to identify the pathways that might be involved in skatole metabolism in liver. Also, till now no study has examined the context of androstenone and skatole on the transcriptome level. Further studies covering polymorphism identification and association study of these genes in pig populations may help to validate them as important candidate genes in order to utilize them in the pig breeding with an aim to reduce boar taint.

2.8. Acknowledgement

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2.9. Author’s contributions

CN performed the experiments and wrote the manuscript; CGB analyzed the microarray data, discussed the experiment and revised the manuscript; LF, MP contributed in sampling and phenotyping; DT was responsible for kits and reagents; ET were responsible for the statistical

analysis; CL revised the manuscript; AZ and AB performed microarray preparation and scanning; MUC discussed the experiment; MJU revised the manuscript. All authors read and approved the final manuscript; KS edited manuscript and designed the experiment. The authors declared of no competing interest.

Chapter 3: The effect of protein abundance in porcine skeletal muscle of different water holding capacity

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3.1. Highlights

- Proteome analysis of the *Musculus longissimus dorsi* in pig with divergent drip loss
- By using the techniques ICPL and Off-gel fractionation 763 proteins were identified
- Twenty-four proteins showed altered expression between high and low drip loss
- Glycolytic enzymes, chaperone proteins and myofibrillar regulatory proteins were highly abundant

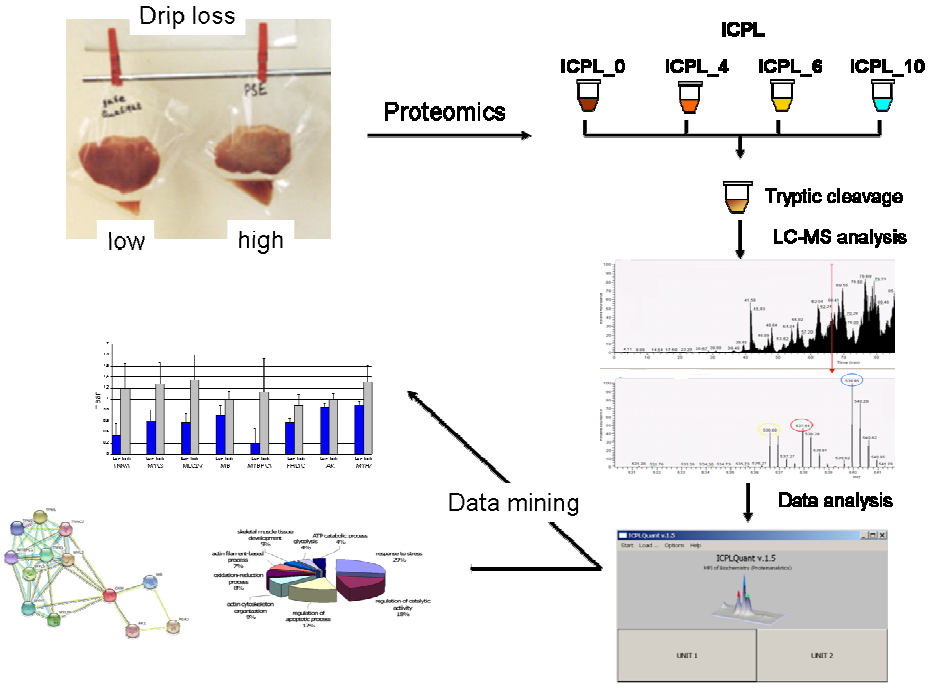

Figure 12: Graphical Abstract

3.2. Abstract

Water holding capacity (WHC) measured as drip loss is the ability of the skeletal muscle to retain water post-mortem. Proteolysis and protein oxidation are key factors influencing the loss of water in meat and degradation of cytoskeletal proteins may result in increased shrinking of muscle cells and drip loss. The aim of this work was a global proteome analysis in order to identify the relevant biological mechanism of porcine muscle proteins, with potential functional relevance for drip loss. For this experiment a Duroc × Pietrain (DuPi) F_2 resource population (n = 42) was used. Proteins were isolated from the *Musculus longissimus dorsi* of the F_2 animals. The relative protein quantification was performed using isotope-coded protein labeling techniques (ICPL) and electrospray ionization liquid-chromatography-tandem mass spectrometry (nano-LC-MS/MS). In total, 763 different proteins were identified. Of all these different proteins, PYGL, PYGM, HSPA8, EE1A1, ACTA1, CASQ1, FLN-C, MYOM1, TNNT3, and HSP27 were up regulated and TNNI1, MYL3, MYL2, MB, MYBPC1, FHL1C, TPM1, TPM2, AK1, TNNC2, MYL11, CK, PGK1 and MYH7 down-regulated in animals with low drip loss compared to animals with high drip loss. Results revealed that the high drip loss meat was characterized by higher level of glycolytic enzymes than in low drip loss meat. Additionally, we could observe that higher levels of chaperone proteins were associated with a low drip loss level.

3.3. Biological significance

Proteome analysis of porcine meat by isotope-coded protein labeling of protein peptide fractionation revealed 763 proteins in total. The differentially regulated proteins may facilitate the understanding of biochemical, physiological, and post-mortem structural changes that modulate the amount and distribution of water within muscle/meat. This protein dataset might be a useful resource for development of biomarkers for drip loss in pork.

Keywords: Proteomics; Drip loss; Water-holding capacity; Isotope-coded protein labeling; Pig

3.4. Introduction

Meat quality is a complex trait that is influenced by a number of different factors such as genetic components, environmental factors like animal handling and slaughtering processes (Gao et al. 2007, Janss et al. 1997). Meat quality can be assessed by various criteria, including palatability, color, water-holding capacity and nutritional value (McIlveen and Buchanan 2001, Verbeke et al. 2009). The importance of these characteristics can vary both between muscles within a carcass as well as between different breeds and species.

A key attribute of fresh pork for both, producers and consumers, is the ability of meat to retain water, known as water-holding capacity (WHC) during processing and can be measured as drip loss (Hamm 1960, Huff-Lonergan and Lonergan 2005). Pork with low WHC is characterised to be less tender, flavorsome, and juicy and results in not be tolerated cooking losses (Mullen and Troy 2005). For producers, pork with low WHC show often pale color, soft consistency and is not suitable for several processing lines. Losses up to 10 % and 12 % of carcass weight are observed (Melody et al. 2004, van de Wiel and Zhang 2007). In processed meat, variation in WHC leads to a variation of product quality, e.g., binding ability in comminuted products, and slicing properties in hams. However, as well the biochemical, physiological, and post-mortem structural changes that modulate the amount and distribution of water within muscle/meat have not yet been study in whole (Schellander 2007). Also, the underlying molecular mechanisms determining these quality traits are complex and far from understood (Hollung et al. 2007b). Together with genomics and transcriptomics, proteomics is a crucial step for understanding the biological systems. While transcriptomic studies on meat quality have revealed a number of candidate genes it is now known that mRNA is not always translated into protein, and the amount of protein produced for a given amount of mRNA depends on the gene which is transcribed from and in the current physiological state of the cell (Nie et al. 2007).

The proteome is the protein complements of the genome and consists of the total amount of proteins expressed at a certain time point (Wilkins et al. 1996a) and contains information on genes that are actually being expressed and translated into proteins. The continuous change of the proteome is influenced either by protein synthesis or degradation. In this regard, the proteome can be seen as the molecular link between the genome and the functional quality of the muscle or meat (Hollung et al. 2007a). Thus, understanding the variation and different components of the proteome in regard to certain quality or processing parameters will lead to

knowledge that can be used in optimizing the conversion of muscle to meat (Bendixen 2005, Hollung et al. 2007b, Hollung et al. 2007a, Mullen et al. 2006).

It is generally accepted that the source of drip loss from pork is intracellular water which is lost from the muscle fibre post-mortem, driven by a pH and calcium-induced shrinkage of myofibrils during rigor development (Honikel et al. 1986). The rate and quality of drip formation in fresh meat is believed to be influenced by the extent of protein denaturation (Bertram et al. 2004b, Offer et al. 1989). Proteome analysis of porcine muscle by 2D gel electrophoresis (2-DE) has identified candidate proteins like adenylate kinase, substrate protein of mitochondrial ATP-dependent proteinase SP22, troponin T slow-type isoform and heat shock proteins highly relevant to the process of drip loss (Hwang et al. 2005, van de Wiel and Zhang 2007, Yu et al. 2009).

For meat quality, quantitative proteomic analyses of complex protein mixtures in animals have been typically accomplished by 2-DE e.g. van de Wiel et al. (2007) in combination with Mass Spectrometry (MS)-based techniques (Klose et al. 2002, Link et al. 1997). In addition to this technology, quantitative novel techniques based on differential labeling of peptides with stable isotopes, like the well-known isotope-coded affinity tags (ICAT) method (Gygi et al. 1999) or the stable isotopic labeling with amino acids in cell culture (SILAC) (Ong et al. 2002), have been established in the last few years. The proteomic analysis of the present study was performed using the isotope-coded protein label (ICPL) method, a novel approach for the accurate quantitative comparative analysis of proteomes (Schmidt et al. 2005).

In the present study, we performed a proteomics analysis, along with functional annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. This data will help us characterizing and comparing expression profiles in the porcine *Musculus longissimus dorsi* (LM) muscle with different drip loss levels. Regarding the current literature the 2-DE was used for quantitative proteomic analysis in meat quality. To get a clearer picture of the different expressed proteins in LM the high sensitive high-throughput method ICPL occurs as the best choice.

The aim of the current study was first to gather the proteome with the use of proteomic approach of porcine LM. Furthermore, to evaluate the influence of different drip loss levels on the muscle proteome post-mortem to contribute to the understanding of the biochemical changes and molecular mechanisms modulating the amount and distribution of water within muscle/meat.

3.5. Materials and methods

3.5.1. Sample collection

In this study, 42 F_2 animals of a reciprocal cross of the Duroc (Du) and Pietrain (Pi) breeds were used (Liu et al. 2007). All F_2 animals were kept and performance tested at the Frankenforst experimental farm of the University of Bonn. The phenotypes were recorded in a commercial slaughterhouse according to the rules of German performance stations (2003). Tissue samples from LM were frozen in liquid nitrogen immediately after slaughter and stored at -80°C until used for protein extraction.

Meat samples of cross breed pigs (F_2-cross) which have extreme divergent drip loss values were used for protein analysis. In order to avoid a confounding of extreme phenotypes with families and litters, two discordant full sibs of each family were selected. A list of the used samples is reported in appendix table 1.

In total, 4 meat quality traits (drip loss, pH_1, pH_{24} and shear force) were analyzed. Drip loss was determined according to the bag method of Honikel (1987). Samples of about 2.5 cm thickness and weighing approximately 80 g were removed from the LM of the 13.-14. thoracic vertebra of each animal. They were suspended by a string in an expanded bag, so that the meat did not come in contact with the bag. The samples were subsequently suspended at 4°C for 48 h, at which time the surface was lightly blotted with a paper tissue and reweighted. Drip loss was expressed as a percentage of the original weight of the steak.

Pigs having drip loss level from 0.5 % to 1.1 % as well as and from to 3.2 % to 5.0 % were defined as low and high drip loss samples, respectively. Muscle pH was measured at two different time points after slaughtering using star-series equipment (Rudolf Mathaeus Company, Germany). pH_1 was measured between spinous processes of the 13.-14. thoracic vertebra after 45 min post mortem and pH_{24} was measured at the same position 24 h later.

For Warner Bratzler shear force (WBS) samples were weighted and then cooked for 50 min into a plastic bag in a water bath at 75°C until an internal temperature of 71°C was achieved. After cooling in cold water bath for 40 min, 4 muscle cores (1×1×4 cm) were cut parallel to the long axis of the muscle fibers and WBS value were taken on the cylindrical cores using an Instron apparatus (Instron LTd., UK) equipped with a WBS device. The texture analyzer was set with an extension between 0 and 40.0 and crosshead speed of 200 mm min^{-1}.

For the proteomics study approximately 40 mg tissue of each sample was crushed and stored at -80°C for further analysis. A list of the used samples is reported in appendix table 11.

3.5.2. Proteomics

3.5.2.1. Lysis and Bradford assay

The complete cell lysis was performed on ice. First 200 µl to 300 µl ICPL lysis buffer [kit component plus protease inhibitors complete mini, (Roche) and phosphatase inhibitor cocktails I and II (Sigma)] was added to each sample. Then the sample was homogenized by grinding. The lysis was done using the grinding kit (GE Healthcare, No. 80-6483-37) according to the manufacturer's instructions. Subsequently, the samples were centrifuged at 12,000 × g for 10 min at 4°C to remove cellular debris. The supernatant was transferred into a low bind tube for subsequent ICPL labeling. An aliquot was used for the determination of the protein concentration by a Bradford assay (see appendix table 11). Samples were stored at -80°C of the original samples and processed samples before different analysis steps.

3.5.2.2. Pre-fractionation

For detection and relative quantification of lower abundant protein species the proteome was sub fractionated into 15 fractions using Off-gel fractionation. First two ICPL labeling were prepared for the comparison of 2 x 4 animals in total. The animals were selected because of their drip loss value and grouped in low and high drip loss. Subsequently, the ICPL labeled protein pools were separated according to their isoelectric point (pI). Fifteen fractions covering the whole pH separation range were analyzed by electrospray ionization liquid-chromatography-tandem mass spectrometry (nano-LC-ESI-MS/MS) for each labeling. The individual proteins were quantified relatively using the ICPL Quant software (Brunner et al. 2010).

3.5.2.3. Off-gel fractionation

The lysed samples prepared in the ICPL analysis for part I were used. The 3100 off-gel fractionator (Agilent Technologies) with a 24-well setup was used for the pH-based protein separation. Thirty min prior to sample loading, 24 cm long IPG gel strips with a linear pH gradient ranging from 3.5 to 4.5 were rehydrated in the 24-well assembled device with 30 µl of off-gel buffer (8.4 M urea, 2.4 M thiourea, 6 % glycerin, 1.2 % servalyte 3-5) per well at room temperature. Sixty µl off-gel buffer was applied in the anodic and the cathodic well. The two off-gel runs were performed in parallel. For each off-gel run, 400 µg of the ICPL labeled proteins were diluted in the same buffer to a final volume of 3.6 ml. One hundred fifty µl of this sample solution was loaded in each well. The proteins were focused with a maximum of 6000 V, 100 µA, 400 mW until 100 kVh. After 76 kVh the isoelectric focusing (IEF) was

paused. The cathodic well was emptied by transferring the solution into a fresh LoBind tube. Additionally, the electrode wicks were exchanged and each well was filled up to 150 µl with off-gel buffer. Afterwards focusing was restarted and continued to a total of 100 kVh. The proteins in each well were transferred into fresh LoBind tubes. Moreover, each well was filled again with 100 µl off-gel buffer and focusing was continued for further 30 min in order to enhance the recovery of proteins out of the IPG strip gel matrix into the liquid phase. The recovered fractions were pooled respectively and stored at -20°C until enzymatic cleavage.

3.5.2.4. ICPL labeling and cleavage

The protein concentration was adjusted to the recommended concentration of 5 mg/ml for ICPL labeling. 100 µg total proteins were used for each ICPL labeling reaction. Labeling was performed according to the manufacturer's instructions. The sample labeling with ICPL_4 and ICPL_6 to ICPL_10 was switched in order to prevent data bias. For the normalization between the 14 mass spectrometry (MS) runs a pool of all 42 samples served as material. This pool was aliquoted and subsequently labeled with the ICPL_0 label and used for all MS runs (see Appendix table 11). For the off-gel fractionated samples 50-100 µl of the neighboring fractions were pooled and used for enzymatic cleavage. Prior to digestion, the samples were diluted 4-fold with 25 mM Tris pH 8.0. The enzymatic cleavage was performed using trypsin (ratio 50:1, sequencing grade, porcine, SERVA Electrophoresis GmbH, Germany) and Glu-C (ratio 75:1, MS grade, Protea Biosciences, Inc.). First the proteins were cleaved with trypsin at 37°C overnight. Then Glu-C endoproteinase was added and the cleavage was performed at room temperature for approx. 8 h. Then the peptides were acidified to 1 % formic acid for subsequent mass spectrometry analysis. One hundred µl of the acidified peptide mixture per fraction pool were desalted using C18 Omix Tips according to the manufacturer's instructions. The elutes were dried down in a speed vac and redissolved in 20 µl 0.1 % TFA. Six µl per sample were analyzed by nano-LC-ESI-MS/MS.

3.5.2.5. ICPL labeling for off-gel fractionation

The protein concentration was adjusted to the recommended concentration of 5 mg/ml for ICPL labeling. One hundred µg of total protein were used for each ICPL labeling reaction. Labeling was performed according to the manufacturer's instructions. The labeling with ICPL_0, ICPL_4, ICPL_6 and ICPL_10 was switched in the second labeling according to the sample type (low or high drip loss) in order to prevent data bias. The labeled proteins were acetone precipitated and re-dissolved in 160 µl 2 M urea, 25 mM Tris pH 8.0.

3.5.2.6. LC-ESI-MS/MS after tryptic cleavage

For nano-LC-ESI-MS/MS the peptides were separated with an analytical column (C18, 25 cm length, 35°C) with a 140 min linear gradient (A: 0.1 % formic acid, B: 80 % ACN and 0.1 % formic acid) at a flow rate of 300 nl/min. The gradient used was: 5-50 % B. Mass spectrometry was performed on a linear ion trap mass spectrometer (LTQ Orbitrap Velos, Thermo Scientific) operating in positive polarity mode online coupled to the nano-LC system. The MS method consisted of a cycle combining one full MS scan (Mass range: 300-1500 m/z) with ten data dependent MS/MS events of the highest signals with a dynamic exclusion (CID; 35% collision energy).

3.5.2.7. ICPL quantification

The raw data were converted to mzXML format using the software msconvert from the Trans-Proteomic Pipeline. The MS2 spectra were deisotoped using the Markey method (MS2Denoise 1 500 true). Then the peak detection, deconvolution, deisotoping, quadruplet detection and quantification of the peaks were performed using ICPL-ESIQuant (Brunner et al. 2013).

3.5.2.8. Database queries and ICPL identification

To identify proteins from primary sequence databases the MASCOT software were used. The MASCOT identification data were imported with a threshold ion score of 30. Individual ions scores > 30 indicate identity or extensive homology ($p < 0.05$). The database contained the sequences of *sus scrofa* (pig); NCBI Build Number: 4, Version: 1, Release date: 21 December 2011. For each nano-LC-ESI-MS/MS run four separate database queries were done using always one of the ICPL labels as second fixed modification (ICPL_* (K)). The -.mgf files were created from the -.raw file format using the software msconvert from the Trans-Proteomic Pipeline with the MS filter setting: threshold count 200 most-intense.

The identified peptides were combined to the respective proteins and the quantitative ratio to ICPL_0 for all peptides was calculated for each protein. This was done using the ICPL *Quant* software. The protein ratios to ICPL_0 represent the normalized values because the ICPL_0 labeled sample was equal and enabling a comparison of all samples independent of the MS run. The different animals were grouped in low and high drip loss. 21 animals for each drip loss group were compared. The average and median for each group and the ratio for these values between these groups were calculated. The recommended filter excludes outliers with less than 2 quadruplets and/or a coefficient of variation above 40 %.

3.5.3. Statistical analysis and bioinformatic approaches

For further statistical analysis Pearson's correlation coefficients between protein expression values and meat quality traits (drip loss, pH_1, pH_{24} and shear force) were calculated by SAS v. 9.2. Correlation were significant different from zero using a p-value < 0.05.

Because of the high variation in the drip loss phenotype we compared also the 10 most extreme animals (5 samples of low drip loss, between 0.4 % and 0.6 % and 5 samples with high drip loss from 4.6 % to 5.1 %) in order to identify proteins those have comparable expression pattern than the phenotype. The comparison based on a student t-test.

3.5.3.1. Protein-protein interaction analysis

To map protein-protein interactions the STRING 9.0 software (Franceschini et al. 2013) was used. Proteins identified experimentally for high and low drip loss meat have been updated in the software along with indications of the proteomic analysis and the species under investigation (*Sus scrofa*).

3.5.3.2. Functional enrichment of GO terms and pathway analysis

In order to link protein and gene information, the obtained data were additionally annotated using the biomaRt package (version 2.14.0) in R (www.r-project.org). A hyper geometric gene set enrichment test (GOstats package version 2.24.0) was performed. Overrepresentation of gene sets defined by particular groups of Biological Processes (BP), Cellular Components (CC) and Molecular Function (MF) in the Gene Ontology (GO; http://www.geneontology.org/) database or the Kyoto Encyclopedia of Genes and Genomes database (KEGG; http://www.genome.jp/kegg/) was tested using Fisher's exact test. A gene-set was considered significant if p-value < 0.05.

3.6. Results and discussion

3.6.1. Principle of the method

In the present study, we used the ICPL technology for the characterization of the porcine meat proteome with different levels of the meat quality trait drip loss. ICPL is a gel-free differential proteomic technique. In contrast to the ICAT methodology, which employs isotopic labeling of cysteine residues at the peptide level, ICPL is based on the labelling of the free amino groups of proteins (lysine and N-terminus) with stable isotopes. With this technology it is possible to identify and quantify thousands of proteins in complex protein samples in an accurate and reproducible mode (Schmidt et al. 2005). The method is compatible with all known separation techniques on the protein and the peptide level and provides a highly accurate quantification of differentially expressed proteins (Sarioglu et al. 2006). Moreover, because of the simultaneous quantification and identification of ICPL analysis this method provides information about non-differential proteins as opposed to 2D gel-based methods in which protein identifications are focused on differential proteins. In contrast to other MS-based differential proteomic approaches (e.g. SILAC, iTRAQ), this technique can be applied both to cell cultures and to tissue samples.

A schematic representation of the experimental approach is shown in figure 13. After protein isolation, proteins of high and low drip loss sample were isotopically labeled with the four forms of the ICPL: ICPL_0, ICPL_4, ICPL_6 and ICPL_10. Corresponding peptides from different proteomic states show identical chemical and physical characteristics, only differing in mass, introduced with the label. With this method it is possible to quantify four different proteome samples in one experiment.

For the normalization between the 14 MS runs a pool of all 42 samples was used. This pool was aliquoted and subsequently labeled with the ICPL_0 label and used for all MS runs. After ICPL labeling the samples were merged and digested with Glu-C and trypsin. Subsequent to the combining of the four samples, a separation method can be adopted to reduce the complexity of the sample on the protein level. For the present study an off-gel fractionation step with 8 (4 high drip loss sample and 4 low drip loss samples) samples were performed.

During a nano-LC-ESI run (after digestion) corresponding peptides co-elute and show the typical mass pattern, introduced during ICPL labeling (mass differences 0, +4, +6, +10). For relative quantification peak the intensities of the isotopologues were used. Identification of differentially regulated peptide (and corresponding proteins) was done by MS/MS fragmentation and identification in sequence databases.

The quantification of multiplexed ICPL experiments was greatly facilitated by the recently published ICPL*Quant* software (Brunner et al. 2010, Brunner et al. 2013). In figure 14 we show an example of an typical MS spectrum (A) and an elution profile (B) detected by the ICPL*Quant* software for the protein desmin along with the different regulation.

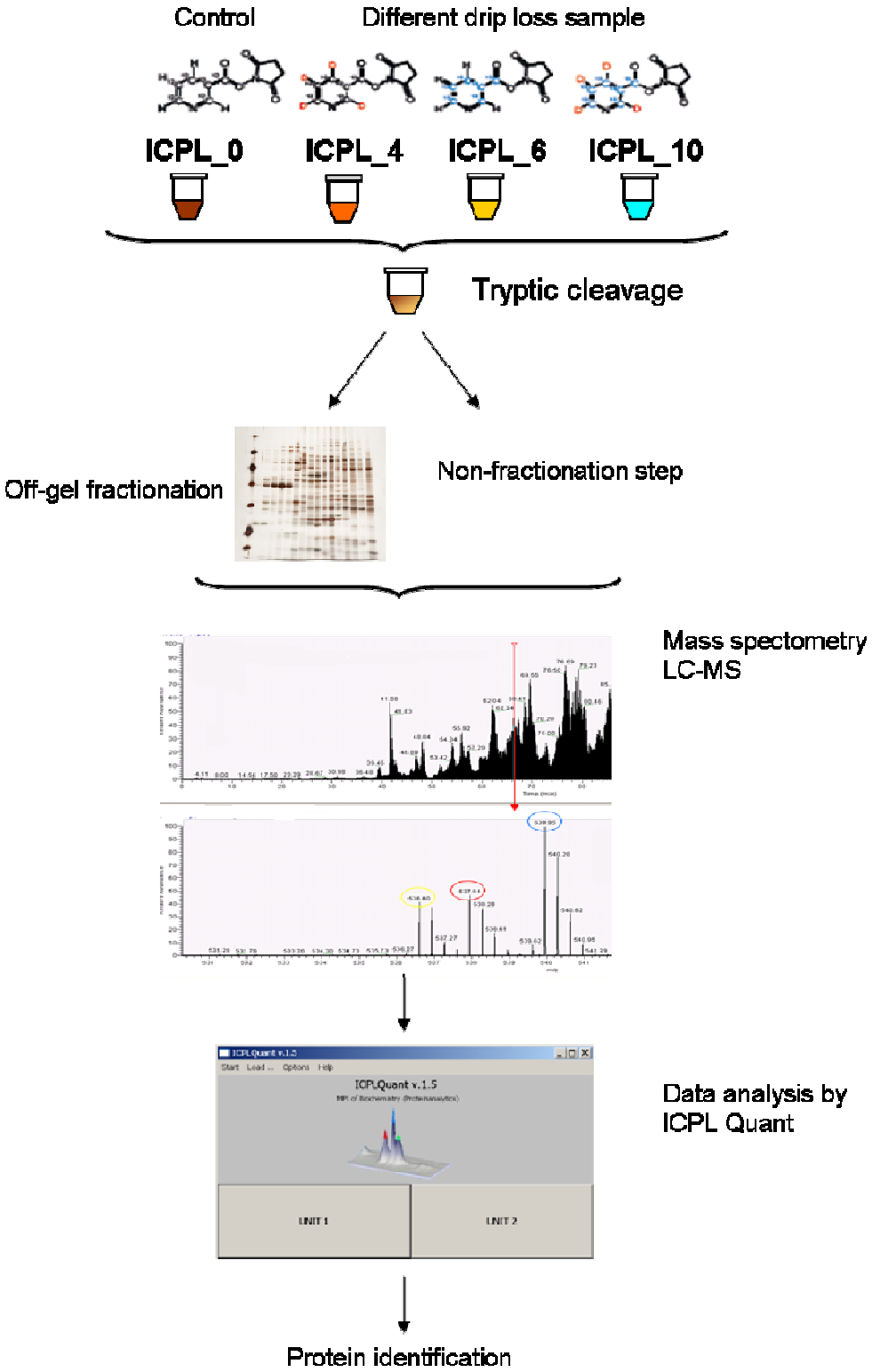

Figure 13: Scheme of the ICPL workflow employed in the present study. For experimental details see material and method

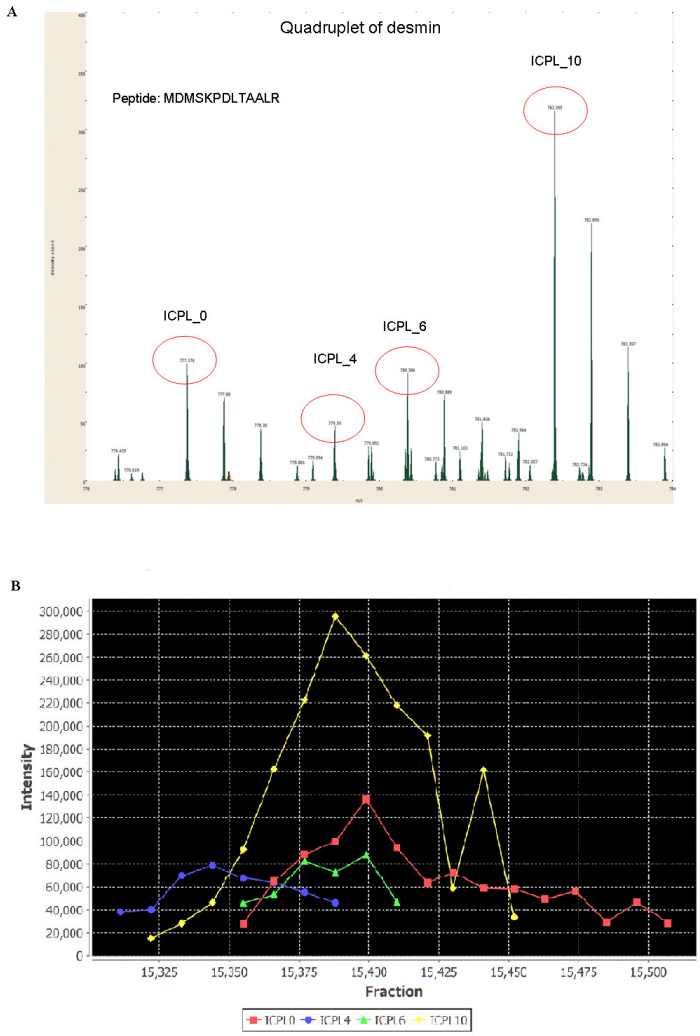

Figure 14: Spectra of MS/MS for the identification of desmin. Quadruplet of desmin peptide MDMSKPDLTAALR; Regulation of the protein: ICPL4/0 = 0.6, ICPL6/0 = 0.7, ICPL10/0 = 3. ICPL0 777.376, ICPL4 779.39, ICPL6 780.386, ICPL10 782.395 (A). Elution profile of the same protein detected by the ICPL*Quant* software (B)

3.6.2. Protein identification

For analyzing differences in growth, breeds etc. in pigs proteomics analyses on muscle samples have been extensively performed over the last decade (Di Luca et al. 2011, Lametsch and Bendixen 2001, Lametsch et al. 2006, Morzel et al. 2004, Xu et al. 2009, Yu et al. 2009) and have been recently reviewed (Bendixen et al. 2011, D'Alessandro and Zolla 2012, de Almeida and Bendixen 2012). However proteomic analyses in specific meat quality traits are rare in pigs (Di Luca et al. 2013, van de Wiel and Zhang 2007). In our study identified peptides were combined to the respective proteins and the quantitative ratio to ICPL_0 for all peptides was calculated for each protein (ICPL_4/ICPL_0; ICPL_6/ICPL_0 and

ICPL_10/ICPL_0). The protein ratios to ICPL_0 were analyzed in Microsoft Excel. These ratios represent the normalized values because the ICPL_0 labeled sample was equal and enabling a comparison of all samples independent of the MS run. The different animals were grouped according low and high drip loss and 21 animals for each drip loss group were compared. Based on the average and median for each group and the ratio for these values 763 proteins could be identified with the ICPL and off-gel fractionation analysis. Out of these proteins, 60 proteins showed different abundance due to their ratio. Proteins with a ratio < 0.7 (20 proteins) were under-expressed and with ratio > 1.3 (60 proteins) over-expressed. By off-gel fractionation, a total of 134 proteins were identified. 33 of these proteins were additionally new to the ICPL method, 131 are identified in both the ICPL and off-gel method. The number of identified proteins using particular methods can be found in figure 15. For further analyzes, proteins from the two methods were jointed together.

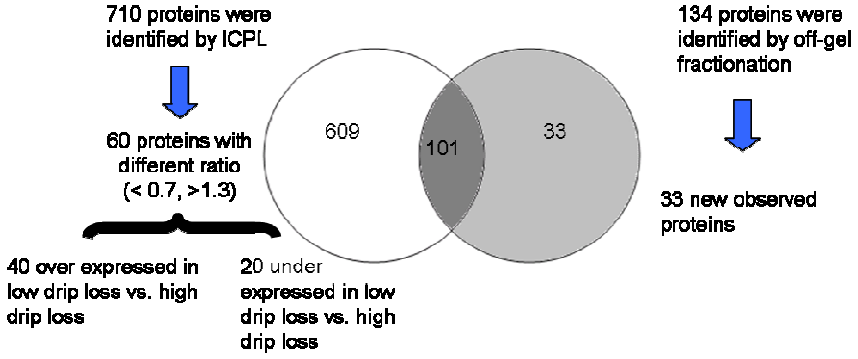

Figure 15: Venn-Diagram of the identified proteins from the two methods, ICPL and off-gel fractionation and their different ratio

3.6.3. Differentially regulated proteins in high vs. low drip loss sample

For the identification of differentially regulated proteins we performed a student t-test for comparing of two groups. At first we compare 21 high drip loss samples with 21 low drip loss samples to get an overview of all 42 animals in this study.

Statistical analysis for all 42 animals revealed three proteins to differ ($p < 0.05$) between high and low drip loss samples: tropomyosin 1 (alpha) (TPM1), adenylate kinase 1 (AK1) and heat shock 70kDa protein 8 (HSPA8). The remaining proteins troponin C Type 2 (TNNC2), heat shock 27kDa protein 1 (HSPA27), creatine kinase (CKM), tropomyosin 2 (beta) (TPM2), myosin light chain (MYL11), phosphorylase, glycogen, muscle (PYGM), eukaryotic translation elongation factor 1 alpha 1 (EE1A1), actin, alpha 1, skeletal muscle (ACTA1) and

phosphoglycerate kinase 1 (PGK1), were not found to be significant at the criteria of $p < 0.05$ but found to be different at $p < 0.1$. The relative intensities of the different expressed proteins are shown in figure 16.

In the comparison of the 2 x 5 extreme animals we observed 10 significantly differentially expressed ($p < 0.05$) proteins phosphorylase, glycogen, liver (PYGL), phosphorylase, glycogen, muscle (PYGM), troponin I slow-twitch iso form (TNNI1), myosin light chain 3 (MYL3), myosin light chain-2 (MYL2), myoglobin (MB), myosin-binding protein C, slow-type (MYBPC1), four and a half LIM domain protein 1 (FHL1C), adenylate kinase 1 (AK1) and myosin heavy chain beta isoform (slow twitch) (MYH7). The remaining proteins calsequestrin 1 (fast-twitch, skeletal muscle) (CASQ1), filamin-C (FLN-C), myomesin 2-like (MYOM1), troponin T type 3 (skeletal, fast) (TNNT3), eukaryotic translation elongation factor 1 alpha 1 (EEF1A1), actin, alpha 1, skeletal muscle (ACTA1), only revealed a tendency to be different ($p < 0.1$). The relative intensities of the different expressed proteins are shown in figure 17. Only the protein AK1 was significantly differentially expressed ($p < 0.05$) in both groups, for the 42 animals and for the 10 animals, respectively.

Among of all these different proteins (including $p < 0.1$), PYGL, PYGM, HSPA8, EE1A1, ACTA1, CASQ1, FLN-C, MYOM1, TNNT3, and HSP27 were up regulated and TNNI1, MYL3, MYL2, MB, MYBPC1, FHL1C, TPM1, TPM2, AK1, TNNC2, MYL11, CKM, PGK1 and MYH7 down-regulated in animals with low drip loss compared to animals with high drip loss.

The different regulated proteins are reported in table 7, along with protein name, Accession number, regulation, *p*-value, multiplet count, unique peptides and MASCOT peptide significant score sum.

Table 7: Differentially regulated proteins between drip loss sample

Accession Number	Protein name	Regulation factor[a]	*p*-value[b]	Multiplet count[c]	Unique peptides[d]	Mascot peptides significance score sum[e]
gi|148222268|ref|NP_001090952.1|	Tropomyosin alpha-1 chain [Sus scrofa]	0.9	0.047	21	8	1333
gi|350579686|ref|XP_003122225.3|	PREDICTED: adenylate kinase isoenzyme 1 isoform 1 [Sus scrofa]	0.9	0.002*	8	6	555
gi|345441750|ref|NP_001230836.1|	Heat shock 70kDa protein 8 [Sus scrofa]	1.2	0.038	4	3	395
gi|49274641|ref|NP_001001862.1|	Troponin C, skeletal muscle [Sus scrofa]	0.9	0.07	5	4	629
gi|55926209|ref|NP_001007519.1|	Heat shock protein beta-1 [Sus scrofa]	1.1	0.097	3	6	340
gi|194018722|ref|NP_001123421.1|	Creatine kinase M-type [Sus scrofa]	0.9	0.099	17	15	1682
gi|194018702|ref|NP_001123419.1|	Tropomyosin beta chain [Sus scrofa]	0.9	0.11	14	6	802
gi|335306231|ref|XP_003135255.2|	PREDICTED: phosphoglycerate kinase 1-like, partial [Sus scrofa]	0.9	0.1	3	1	191
gi|268607671|ref|NP_001161267.1|	Actin, alpha skeletal muscle [Sus scrofa]	1	0.085	31	13	5772
gi|147899784|ref|NP_001090887.1|	Elongation factor 1-alpha 1 [Sus scrofa]	1.0	0.098	1	1	65
gi|178056753|ref|NP_001166644.1|	Glycogen phosphorylase, liver form [Sus scrofa]	1.0	0.0037*	1	1	629
gi|47523262|ref|NP_998956.1|	Myosin light chain 2V [Sus scrofa]	0.9	0.018*	5	8	491
gi|311268794|ref|XP_003132211.1|	PREDICTED: myosin light chain 3-like [Sus scrofa]	0.7	0.016*	1	3	383
gi|335281566|ref|XP_003122636.2|	PREDICTED: glycogen phosphorylase, muscle form isoform 1 [Sus scrofa]	1.1	0.037*	21	34	3273
gi|47522664|ref|NP_999077.1|	Troponin I, slow skeletal muscle [Sus scrofa]	0.7	0.0083*	1	4	225
gi|47523546|ref|NP_999401.1|	Myoglobin [Sus scrofa]	1.0	0.018*	3	2	166
gi|47523806|ref|NP_999540.1|	Four and a half LIM domains 1 protein, isoform C [Sus scrofa]	0.9	0.027*	4	5	208
gi|55741486|ref|NP_999020.1|	Myosin-7 [Sus scrofa]	0.9	0.04*	46	34	4677
gi|343098472|ref|NP_001230198.1|	Calsequestrin-1 [Sus scrofa]	1.0	0.057*	5	7	399
gi|311275457|ref|XP_003134747.1|	PREDICTED: filamin-C isoform 1 [Sus scrofa]	1.4	0.038*	1	14	1206
gi|350593312|ref|XP_003483654.1|	PREDICTED: myomesin-2-like, partial [Sus scrofa]	1	0.064*	5	10	568
gi|55741811|ref|NP_001001863.1|	Troponin T, fast skeletal muscle [Sus scrofa]	1	0.06*	8	10	585

[a] high drip loss vs. low drip loss. [b] significant from zero using a p-value < 0.05 * significant different in 10 animals (5 high drip loss vs. 5 low drip loss). [c] Multiplet count: amount of peptides, which are ICPL labeled and therefore are quantified. [d] Unique peptides: amount of sequence specific peptides. [e] MASCOT peptide significance score sum: sum of all ion scores of the individual peptides to this protein. The more significant peptides and the higher the protein is identified.

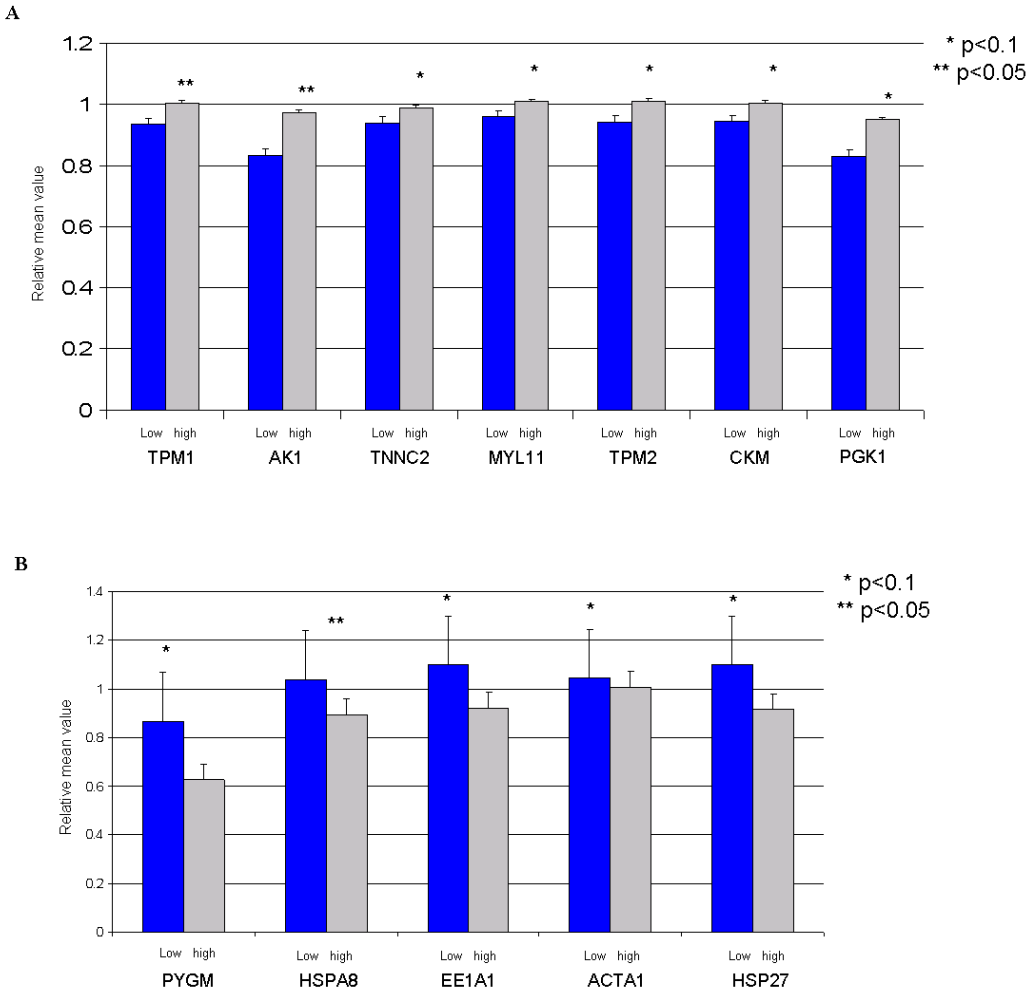

Figure 16: Differentially expressed proteins between high and low drip loss of all 42 samples (* $p < 0.1$, ** $p < 0.05$). Down regulated proteins in low drip loss sample compared to high drip loss sample (A). Up regulated proteins in low drip loss sample compared to high drip loss (B)

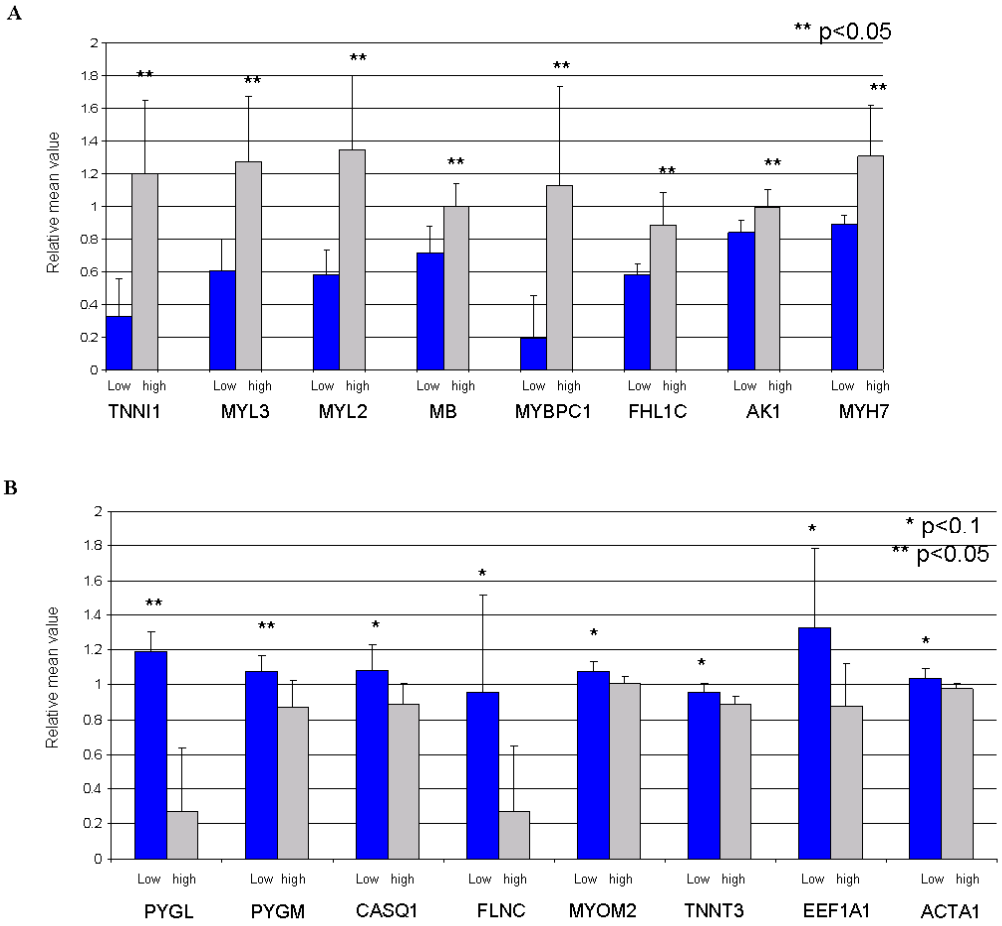

Figure 17: Differentially expressed proteins between high and low drip loss of the selected 10 samples (* $p < 0.1$, ** $p < 0.05$). Down regulated proteins in low drip loss sample compared to high drip loss sample (A). Up regulated proteins in low drip loss sample compared to high drip loss (B)

3.6.4. Bioinformatics analysis

Further information could be aggregated due to protein-protein interaction analysis of differential regulated proteins with bioinformatic web tool STRING 9.0 (Franceschini et al. 2013) based upon experimental evidence on *Homo sapiens* orthologous (figure 18A-18C). The protein-protein interaction of all annotated proteins resulted in 3 main functional areas summarized as follow: 1. proteins of glycolysis, 2. proteins of cytoskeleton part and 3. proteins of mitochondrial (figure 18A). The down-regulated proteins are mainly assigned in their interaction in the fields of muscle contraction (MYL2, TPM1, MYL3, MYL11, MYH7, TNNI1, TPM1, TPM2, MYBPC1, TNNC2) and metabolic processes (CKM, AK1, PGK1)

(figure 18B). While the protein-protein interactions of the up-regulated proteins were involved in glycogen breakdown (PYGM and PYGL) (figure 18C).

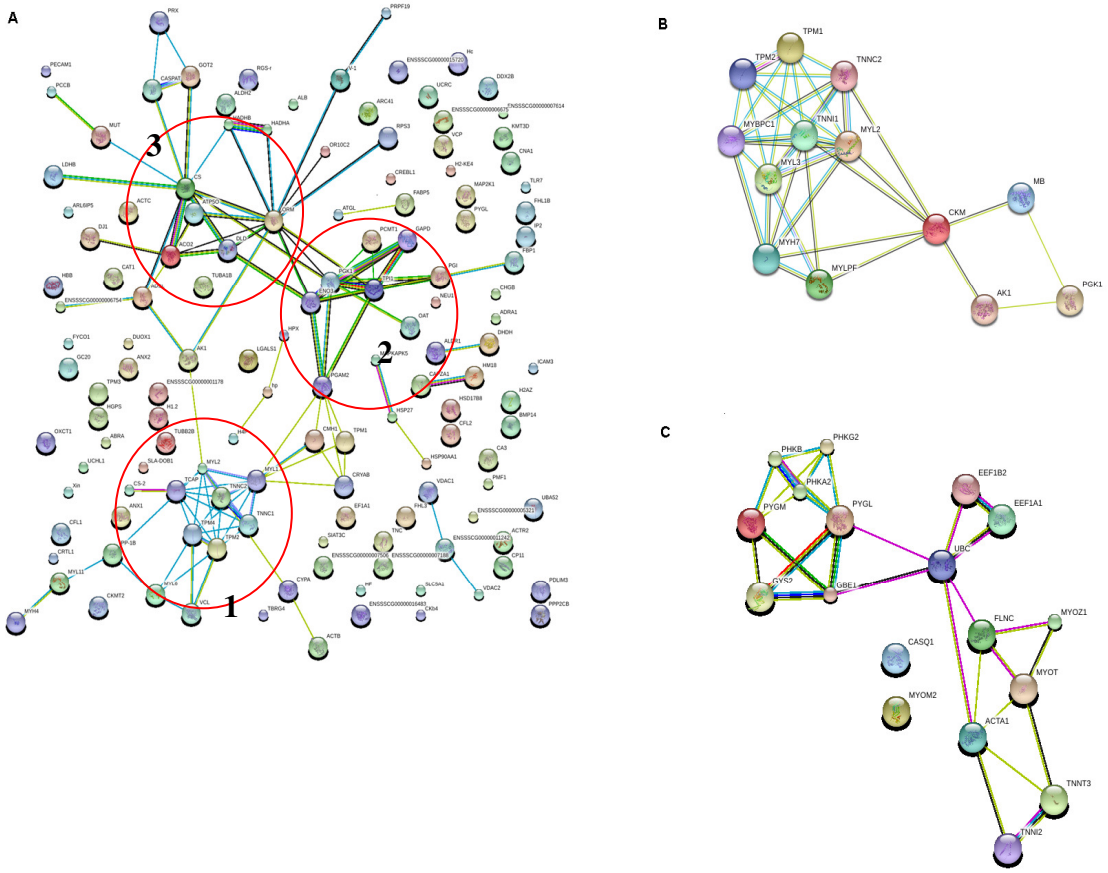

Figure 18: Protein-protein interaction analysis of all identified proteins in the LD muscle could be summarized as follow 3 main functional areas: 1. proteins of glycolysis, 2. proteins of cytoskeleton part and 3. proteins of mitochondria (A). Protein-protein interaction analysis of the down-regulated proteins in low drip loss compared to high drip loss (B). Protein-protein interaction analysis of the up-regulated proteins in low drip loss compared to high drip loss (C)

In order to investigate potential gene set enrichments, the proteins were additionally annotated with corresponding gene information. This annotation was performed using the biomaRt package in R (version 2.14.0). Further information was obtained from the Ensemble database. The classification of proteins into the 3 groups, Biological Processes (BP), Cellular Components (CC) and Molecular Function (MF) was performed by the GO database. Based on the identified 763 proteins 663 were assigned to Entrez gene ID. Among these, 474 proteins could be assigned to the group BP, 447 to CC and 577 to MF of the GO database. The review of the identified clusters and pathways through the KEGG database was

performed using the hyper-geometric genes set's enrichment test (GoStats package version 2.24.0). The GO term enrichment and pathway analysis was performed on the overall differential dataset of both ICPL and off-gel fractionation experiment. The CC (figure 19A) was identified as actin filament, myofibril, troponin complex, cytoskeletal part, stress fiber, and myosin complex. GO term enrichment for biological processes confirmed these results. The biological compartments (figure 19B) were identified amongst others as glycolysis, response to stress, actin cytoskeleton organization, actin filament-based process. The observed GO term enrichment for molecular function (figure 19C) was identified as ATPase activity, actin filament binding, structural constituent of cytoskeleton and actin binding and assigned the results of the different regulated proteins. In the table 8 the identified pathways are listed for all identified proteins and confirmed the results of the protein-protein analysis.

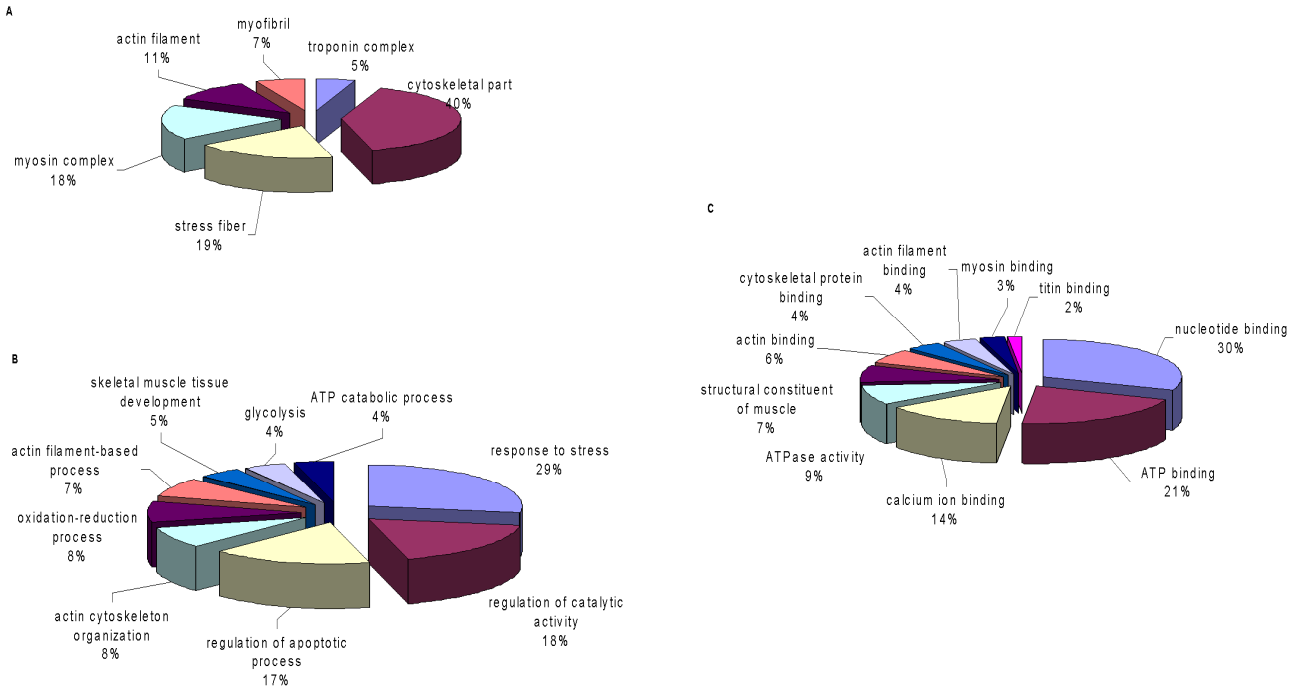

Figure 19: Ontological classification of all identified proteins. The proteins were classified according to cell components (A), biological processes (B) and molecular function (C)

Table 8: KEGG pathways of the identified proteins

Pathways	Genes
Metabolic pathways	SIAT7B, AKR1B1, BCAT2, MDH1, ACY1, GOT1, GOT2, ACO2, HADHA, HADHB, FBP1, UGP2, DLD, CKM, CS, PGM1, GPI, MUT, PRDX6, LDHA, LDHB, PGK1, ODC1, SDHB, ENO3, PFKM, CKMT2, ATP5O, ALDH2, AK5, ATP5J2, COX6B, AMPD1, FBP2, PCK1, PCYT1B, LDHAL6B, DUROC-HSD17B8, COMT, UQCR10, OAT, UQCRC1, ATP5B, MMAB, POLG, TPI1, ATP5A1, PCCB, PKM, ADSL, PGAM2, ACSM2B, NAT1, ALDOC, NDUFS5, AHCYL2, PDHB, POLR3C, POLR1B, HYI, SPR, AK1, PGM2, UQCRFS1, UQCRC2, UQCRH, APIP
Glycolysis / Gluconeogenesis	FBP1, DLD, PGM1, GPI, LDHA, LDHB, PGK1, ENO3, PFKM, ALDH2, FBP2, PCK1, LDHAL6B, TPI1, PKM, PGAM2, ALDOC, PDHB, PGM2
Cardiac muscle contraction	TPM2, RYR2, TPM4, TPM3, TPM1, COX6B, ACTC1, UQCR10, TNNC1, UQCRC1, UQCRFS1, UQCRC2, UQCRH
Tight junction	MLC2V, MYH3, MYH7, MYH2, PPP2CA, ACTB, MYLPF, MYH2, MAGI3, ACTN2, EPB41, ACTN3, CSDA
Purine metabolism	PGM1, AK5, AMPD1, GMPR, PKM, ADSL, NPR2, POLR3C, POLR1B, AK1, PGM2, PDE8B
Regulation of actin cytoskeleton	MLC2V, VCL, PPP1CB, ACTB, MYLPF, CFL2B, ACTN2, ARPC1B, PFN1, ACTN3, APC, MYLK2, TIAM1
Hypertrophic cardiomyopathy (HCM)	MLC2V, TPM2, DES, RYR2, TPM4, TPM3, ACTB, TPM1, LMNA, ACTC1, TNNC1, MYL3
Dilated cardiomyopathy	MLC2V, TPM2, DES, RYR2, TPM4, TPM3, ACTB, TPM1, LMNA, ACTC1, TNNC1, MYL3
Oxidative phosphorylation	SDHB, ATP5O, ATP5J2, COX6B, UQCR10, UQCRC1, ATP5B, ATP5A1, NDUFS5, UQCRFS1, UQCRH,
Calcium signaling pathway	PPP3CB, RYR2, VDAC1, VDAC2, TNNC2, ADRA1D, CALM2, TNNC1, ATP2A1, SLC25A4, MYLK2, PHKA1
Pyruvate metabolism	AKR1B1, MDH1, DLD, LDHA, LDHB, ALDH2, PCK1, LDHAL6B, GLO1, PKM, PDHB
Vascular smooth muscle contraction	MYL6, PPP1CB, ADRA1D, ACTA2, CALM2, MYL6B, ARHGEF11, NPR2, MYLK2
Valine, leucine and isoleucine degradation	BCAT2, OXCT1, HADHA, HADHB, DLD, MUT, ALDH2, PCCB
Arginine and proline metabolism	ACY1, GOT1, GOT2, CKM, ODC1, CKMT2, ALDH2, OAT
Pentose phosphate pathway	FBP1, PGM1, GPI, PFKM, FBP2, ALDOC, PGM2
Fructose and mannose metabolism	AKR1B1, FBP1, PFKM, FBP2, TPI1, ALDOC, PFKFB2
Citrate cycle (TCA cycle)	MDH1, ACO2, DLD, CS, SDHB, PCK1, PDHB
Propanoate metabolism	HADHA, MUT, LDHA, LDHB, ALDH2, LDHAL6B, PCCB
Cysteine and methionine metabolism	GOT1, GOT2, LDHA, LDHB, LDHAL6B, AHCYL2, APIP
Glyoxylate and dicarboxylate metabolism	MDH1, ACO2, CS, MUT, PCCB, HYI
Starch and sucrose metabolism	UGP2, PGM1, GPI, GYS1, PYGL, PGM2
Galactose metabolism	AKR1B1, UGP2, PGM1, PFKM, PGM2
Amino sugar and nucleotide sugar metabolism	UGP2, PGM1, GPI, PGM3, PGM2
Pentose and glucuronate interconversions	AKR1B1, UGP2, DHDH, ALDH2
Butanoate metabolism	OXCT1, HADHA, ACSM2B, PDHB
Phenylalanine metabolism	GOT1, GOT2, PRDX6
Phenylalanine, tyrosine and tryptophan biosynthesis	GOT1, GOT2

3.6.5. Correlation between proteins and selected meat quality traits

The correlation between proteins and selected meat quality traits are shown in table 9. The down regulated proteins in low drip loss compared to high drip loss (TPM1, TPM2, AK1, MYHC and MYL3) were positively correlated with drip loss, while the up regulated proteins in low drip loss compared to high drip loss (HSPA8, ACTA1 and EEF1A1) were negatively correlated with drip loss.

The glycolytic enzymes, TPM1, TPM2 and AK1 were observed to increase in high drip loss sample, showing a positive correlation with drip loss ($r = 0.284$, $r = 0.255$ and $r = 0.430$). These results approved the hypothesis that higher post-mortem glycolytic potential turns in extended pH decline and therefore reducing net charge decreasing myofilament spacing and protein solubility, and contributes to decrease WHC. We found significant correlation between the glycolytic enzymes and pH or shear force. In particular, heat shock 70kDa protein 8 (HSPA8) was significantly negative correlated with drip loss and pH_1 ($r = -0.310$ and $r = -0.359$), respectively and negatively correlated with pH_{24} ($r = 0.393$).

One myosin heavy chain (MYH3) were positively correlated with drip loss ($r = 0.406$) and negatively correlated with shear force ($r = -0.416$), respectively. This MYH3 is a fast isoform of myosin heavy chain and processes an fast ATPase and high anaerobic capacity in single muscle fibers (Choi and Kim 2009). These result were in agreement with other studies (Ryu and Kim 2005) and (Kang et al. 2011) were a relationship between drip loss and the fast-glycolytic fiber type IIB ($r = 0.36$ and $r = 0.32$) was observed already.

An unexplained exception found in this experiment is that MYL3 was positively correlated with drip loss ($r = 0.291$) and negatively with share force and pH_{24} ($r = -0.416$ and $r = -0.462$), respectively. MYL3 is an isoform of the myosin light chain group and expressed in slow twitch fiber typs. In general, glycolytic (fast) fiber types have a higher glycogen concentration and are metabolically better provided for anaerobic glycoglyses and therefore positively correlated with drip loss (Lee et al. 2010). To our best knowledge this is the first study investigating the expression level of proteins of different fiber types and their correlation to meat quality parameters.

Table 9: Pearson's correlation coefficient between meat quality traits and identified proteins

Drip loss	Coefficient	*p*-value
HSPA8 (Heat shock 70kDa protein 8)	-0.310	0.046
AK1 (Adenylate kinase isoenzyme 1-like)	0.430	0.004
TNNT1 (Troponin T type 1)	-0.260	0.096
TPM1 (Tropomyosin 1 (alpha))	0.284	0.103
TPM2 (Tropomyosin 2 (beta))	0.255	0.068
PYGL (Glycogen phosphorylase, liver form)	-0.363	0.018
MYH3 (Myosin heavy chain 3)	0.406	0.026
ACTA1 (Actin, alpha 1, skeletal muscle)	-0.311	0.045
MYL3 (Myosin light chain 3-like)	0.291	0.061
MAP (Ensconsin-like)	0.481	0.069
EEF1A1 (Elongation factor 1-alpha 1)	-0.269	0.085
AAC1 (ADP/ATP translocase 1-like)	-0.273	0.093
TMOD4 (Tropomodulin 4 (muscle))	0.263	0.106
pH_1 in loin	Coefficient	*p*-value
HSPA8 (Heat shock 70kDa protein 8)	-0.359	0.019
HSP70-2 (Heat shock-related 70 kDa protein 2-like)	-0.350	0.023
GAPDH (Glyceraldehyde-3-phosphate dehydrogenase)	0.326	0.035
MYH1 (Myosin-1)	-0.295	0.058
pH_{24} in loin	Coefficient	*p*-value
MYL3 (Myosin light chain 3-like)	-0.462	0.002
HSPA8 (Heat shock 70kDa protein 8)	0.393	0.010
DES (Desmin)	0.371	0.016
KBTBD10 (Kelch repeat and BTB domain-containing protein 10)	0.353	0.022
MYBPH (Myosin-binding protein H)	0.314	0.043
ACTN2 (Alpha-actinin-2)	0.312	0.044
PFKM (6-Phosphofructokinase, muscle type)	-0.310	0.046
PYGL (Glycogen phosphorylase, liver form)	0.285	0.067
EEF1A1 (Elongation factor 1-alpha 1)	0.281	0.071
Shear force	Coefficient	*p*-value
MYH3 (Myosin-3)	-0.416	0.022
KBTBD10 (Kelch repeat and BTB domain-containing protein 10)	0.298	0.055
PVALB (Parvalbumin)	0.382	0.066

pH_1= pH 45min post mortem LD muscle, pH_{24}= pH 24 h post mortem LD muscle

3.6.6. High drip loss displays higher levels of glycolytic enzymes

Among the differentially regulated proteins, in high drip loss meat we observed an increase in glycolytic enzymes (TPM1 and TPM2, PGK1, TNNC2). In most of the published proteomics paper a positive correlation between glycolytic enzyme levels and meat quality traits in bovine and pig were described (D'Alessandro et al. 2012, Laville et al. 2009a, Xu et al. 2012). The development of pig meat quality traits are largely influenced by the rate and extent of post-mortem pH decline during the conversion of muscle to meat. Classically, the rate and extent of pH decline are a consequence of post-mortem glycolysis, with the conversion of glycogen to lactate and H^+ (Kastenschmidt et al. 1966). In general, pH of LD muscle decrease gradually from 7.4 in living muscle to an ultimate pH (pH_u) of about 5.5 post-mortem. In contrast, in dark, firm, and dry (DFD) meat the ultimate pH is higher than normal and results post-mortem in 6.2 to 6.5 (Bendall and Swatland 1988). Having relatively low muscle glycogen content therefore results in limitation in the post-mortem glycolysis and thus the pH drop.

On the contrary, pale, soft, and exudative (PSE) meat is the result of a rapid post-mortem pH decline. Muscle with an extended pH decrease demonstrates a normal rate of pH decline early post-mortem, but continues to a low pH_u around 5.3. Low pH_u is near the isoelectric point of major muscle proteins. This causes decreasing in myofilament spacing and protein solubility, and contributes to decreased WHC and poor processing yield (Enfält et al. 1997, Irving et al. 1989, Joo et al. 1999). Pork with low pH_u is often described as “acid meat” to differentiate it from PSE meat caused by rapid glycolysis. Monin et al. (1985) suggested that greater initial muscle glycogen content confers an increased capacity for post-mortem glycolysis, or high “glycolytic potential,” that in turn, extends pH decline.

The troponin complex is an important factor in the regulation of muscle contraction and relaxation and is located on the thin filament of muscle (Li et al. 2008). The subunit troponin C (calcium binding subunit) is involved in the attachment of the complex to tropomyosin (TPM) and plays a key role in initiating muscle contraction in fast-twitch muscle fibers by binding Ca^{2+} (Farah and Reinach 1995). The troponin C consists of two isoforms: slow skeletal troponin C and fast skeletal troponin C (TNNC2) (Parmacek et al. 1990). TNNC2 plays a critical role in skeletal muscle contraction (Farah and Reinach 1995). TNNC2 gene is expressed during the myoblast differentiation and skeletal muscle development (Bucher et al. 1988). The T allele has positive effects on tenderness and marbling score, moreover, on the intracellular Ca^{2+} concentration, an important factor affecting the meat tenderness in pig

(Küchenmeister et al. 1999) and as a primary functional factor for TNNC2 activity (Luo and Rall 2006). Thus, the gene involved in skeletal muscle growth, myoblast differentiation and skeletal muscle contraction and was considered usually as potential candidate gene for meat quality (Pas and Soumillon 2001), and identified to be an excellent model system for studies of developmentally regulated gene expression in skeletal muscle (Parmacek et al. 1990). Tropomyosin 1 (TPM1) is expressed in fast-twitch muscle fibers, while TPM2 is mainly expressed in slow-switch muscle fibers. Tropomyosin functions with the troponin complex to regulate muscle contraction by restricting myosin from binding to actin (Weymouth et al. 2011). Therefore, the three proteins (TNNC2, TPM1 and TPM2) together could be of interest as molecular marker for meat quality traits like drip loss.

The conversion of muscle to meat is an energy-demanding process. After death, the energy in the muscle is provided by reducing ATP to ADP and inorganic phosphorus. Creatine kinase (CKM) is known for enzymatic conversation of creatine phosphate (CP) into creatine and ATP (Henckel et al. 2002). CKM is located in the myofibrils particularly in the M-line, in the membrane of mitochondria, and in the cytoplasm (Mejsnar et al. 2002). The M-line is the place in the sarcomere where thick or myosin filaments are kept in lateral register both transversely and longitudinally. Myosin together with actin belongs to the so called contractile proteins, which are responsible for the mechanical contraction of the muscle (van de Wiel and Zhang 2007). Therefore, CKM may have an effect on the rate of muscle contraction post-mortem, by its effect on CP degradation, rate of decline of glycogen and concomitant change of pH. Our observation that CKM show a higher level in muscle with high drip loss were the same like van de Wiel et al. (2007) and fit their hypothesis, that high CKM levels cause shortening of the delay phase by rapidly degrading creatine phosphate. This may in turn cause more rapid pH decline and muscle contraction, and therefore result in higher drip loss.

An important unresolved issue is the relationship between muscle design and metabolic pathways maintaining cellular energy homeostasis. Adenylate kinases (AK) are evolutionary strongly conserved enzymes that catalyze the reaction ATP + AMP $\rightleftarrows$ 2ADP (Schulz et al. 1986). This reaction is one of principal steps in adenine nucleotide metabolism and high energy phosphoryl transfer in the cellular bioenergetic network. Among several AK isoenzymes found in mammals, skeletal muscle is particularly rich in AK1, the major cytosolic isoform (Janssen et al. 2003). AK1 is localized in the cytosol, clustered within myofibrils or bound to membranes. In fact, the AK system has been found to facilitate high-

energy phosphoryl transfer from mitochondria to myofibrils. Moreover, evidence is accumulating in support of an integrated intracellular high-energy phosphoryl transfer network comprised of AK, CKM and glycolytic phosphortransfer enzymes (Janssen et al. 2000).

3.6.7. Higher levels of chaperone proteins were related to low drip loss level

Heat shock proteins (HSPs), also called stress proteins, play an important physiological role in both normal and stressed cells. In the present study we identified 2 heat shock proteins both up regulated in low drip loss compared to high drip loss sample. Two essential functions of HSPs are to protect against stress and to act as molecular chaperones. In general, HSPs respond to heat or other stresses and are induced for maintaining cellular homeostasis (Kim et al. 2010).

HSP27 (up-regulated in low drip loss) is believed to act in stabilizing cytoskeletal structures and plays a central role in the structural and functional organization of the 3-D intermediate filament and actin microfilament system, which were larger in oxidative fibers and increased in response to an increased oxidative metabolism (Liu and Steinacker 2001, Neufer and Benjamin 1996). Which were in agreement with the higher level of slow fiber type proteins in paragraph 3.5. Several studies have revealed that HSP27 show abundant constitutive expression in skeletal muscle (Kato et al. 1992, Sugiyama et al. 2000). Morzel et al. (2008) reported that beside structural proteins chaperone proteins like HSP27 to be main substrates of post-mortem proteolysis and samples with a increased level of HSP27 in the early post-mortem stage displayed limited actin proteolysis immediately after slaughter, although actin fragmentation accelerated with storage time and resulted in meat with good tenderness characteristics. HSP27 is localized on specific sarcomeric structure such as Z-or I-band and plays therefore an important role in organizing and protecting the myofibril (Sugiyama et al. 2000). The HSP27 also showed regulated expression in pig muscle during development and in a mouse myoblast cell line (C2C12) during differentiation (Ito et al. 2001, Sugiyama et al. 2000, Tallot et al. 2003). In agreement with our results, Yu et al. (2009) found a relationship between the decline in heat shock protein expression and increased drip loss in LD muscle, pointing at this mechanism as a possible cause resulting in poor meat quality in the LD.

HSPA8 is a constitutively expressed member of the 70 kDa heat-shock protein (HSP70) family. The HSPA8 has been related to muscle growth, and it was found that the expression of HSPA8 increased postnatal both in pigs and bovine skeletal muscle (Guerriero et al. 1989, McComb and Spurlock 1997). Hasselgren et al. (2002) reported that the HSPA8 is required

to regulate the fate of aberrantly folded damaged proteins and thus has a critical role in regulating the proteasome pathway. Protein degradation by the proteasome pathway is a dominant mechanism involved in myofibrillar protein degradation in vivo and may also be involved in the control of myoblast proliferation through the selective degradation of MyoD and regulatory proteins of the cell cycle such as cyclins. Also Di Luca et al. (2011) identified a higher abundance of HSP70 protein in low drip loss muscle as well as Yu et al. (2009) who found a correlation between increased drip loss in LD muscle caused by transport stress and a decline in HSP abundance.

The different functions that have been described for the 2 members of the heat shock family HSP70 and HSP27 indicate that the 2 proteins may be important for the biochemical mechanism behind drip loss.

3.6.8. Muscle fiber composition and drip loss

In the present study, we could observe both fast and slow twitch isoforms of proteins showing differential regulation in high and low drip loss sample. Five of our identified proteins (TNNI1, MYL3, MYH7, and MYBPC1, MYL2) belonged to type I fiber (slow-oxidative) and were down regulated ($p < 0.05$) in low drip los compared to high drip loss. Whereas 2 identified proteins (CASQ1 and TNNT3) belonged to type II fiber (fast-glycolytic) and were up regulated ($p < 0.1$) in low drip loss sample compared to high drip loss sample.

One of the main factors determining muscle biochemical pathways is the fiber type composition. Skeletal muscle is composed of different types of fibers, which are the results of coordinated expression of distinct sets of structural proteins and metabolic enzymes (Chang et al. 2003, Schiaffino and Reggiani 1996). The porcine LD muscle was previously analyzed with classical histochemical techniques (Beecher et al. 1965, Karlsson et al. 1993) and found to contain 10 % of fibers are type I, 7 % type IIA and 83 % type IIB (Choi and Kim 2009). The type I and IIB fibers, also known as slow-oxidative and fast-glycolytic twitch muscle fibre, respectively, are characterized by two extreme metabolic profiles. The type IIA fiber are intermediate fast oxidative-glycolytic fibers (Klont et al. 1998).

Several research groups reported that fiber type composition may be associated with post-mortem changes in the conversion of muscle to meat and subsequently meat quality (Karlsson et al. 1999). Therefore, the variation in fiber type characteristics can explain proportionally the variation of some meat quality traits in beef (Maltin et al. 1998, Ozawa et al. 2000) and in pork (Eggert et al. 2002, Karlsson et al. 1999). Recent work revealed that meat quality parameters showed different correlation to the fiber types (Schellander 2007, Wimmers et al.

2008). Ryu et al. (2005) reported that drip loss was negatively correlated with fiber I and IIA and positively to fiber type IIB. These results suggested that increasing of fiber type IIB and decreasing of fiber type I and IIA is related to drip loss. However, in this study we can not make disclosures of fiber types composition in the different muscle samples. It could be observed the identified fiber proteins belonged to slow fiber type proteins and were up regulated in high drip loss.

In general, glycolytic type IIA and IIB fiber has a higher glycogen concentration and are metabolically better provided for anaerobic glycogen utilization than type I fibers. If there are mainly glycolytic fibers in individual muscle, rapid post-mortem glycolysis would be induced, and accumulation of lactate results in a rapid muscle pH decline and therefore lead to a poorer WHC (Lee et al. 2010).

Nevertheless, till now there are no analysis with the previous methods which show a correlation of the expression of fiber type proteins and meat quality traits. To the best of our knowledge, this is one of the first studies which found a difference in the expression of genes related to fiber type.

3.6.9. Myofibrillar regulatory proteins

Myofibrillar filaments are responsible for generating the physical movement of skeletal muscles. Our analysis specified that many proteins were related to the myofibrillar network. All significant identified myofibrillar proteins (Two myosin light chain: myosin light chain 3-like (MYL3) and myosin light chain 2 (MYL2), one myosin binding protein C, slow type (MYBPC1) and one myosin heavy chain 7, cardiac muscle, beta (MHC7) were down regulated in low drip loss samples.

The MYL is the part of myosin that plays an important role in the structure of the muscle fiber. It has previously been reported that the post-mortem level of MYL2 may be related to tenderness (Lametsch et al. 2003). However, the implication of MYL on tenderness is unclear. Recently, it was reported that MYL is dephosphorylated post-mortem, and that the dephosphorylation is related to the post-mortem metabolism (Morzel et al. 2004). Furthermore, it has been reported that myosin is cleaved in the neck region of the globular myosin head domain (Lametsch et al. 2002), which also contains the binding site for MYL 2 and 3 (Rayment et al. 1993) and it can be speculated that this cleavage would release MYL 2 and 3 from the actomyosin complex into the sarcoplasmic fraction. If this is the case, then the greater intensity of the MYL 2 and 3 in the samples from the pig showing higher drip loss will indicate a greater proteolytic activity post-mortem in these pigs.

Myosin heavy chain (MYH) is the primary component of the thick filament and is the predominant protein in skeletal muscle. MYH is encoded by a multigene family consisting of several members (Talmadge and Roy 1993). One is the myosin heavy chain 7 (MYH7), which is a gene encoding a myosin heavy chain beta (MHC-β) isoform (slow twitch). MYH7 is expressed in cardiomyocytes and typ I striated muscle fibers and has been identified as responsible of a few cardiac and skeletal muscle diseases in human (Tasca et al. 2012). Diseases in muscle have been classified into two subgroups, according to clinical and pathological findings: laing distal myopathy (LDM) and body myopathy (MSM) (Oldfors and Lamont 2008). Clinical signs of MPD1 partially resemble to the campus syndrome (CPS) in pig, which occurs in a muscle tremor starting in the hind limbs followed by a tremors in the fore limbs briefly after (Tammen et al. 1999). Murgiano et al. (2012) and Meredith et al. (2004) reported already that a mutation in MYH7 gene to be associated with MPD1 in human and also with CPS in pig, respectively.

3.7. Conclusion

Using the ICPL method, we have identified a large number of proteins whose expression is different between high and low drip loss sample. This method is a useful and reliable alternative to 2-DE gel electrophoreses because of the large number of identified proteins.

The proteome analysis by use of ICPL allows the identification of a large number of proteins belonging to different functional classes to be studied and provides fine details of quantitative changes in skeletal muscle protein expression.

The alterations in the meat quality depend on the different drip loss levels and therefore the alterations in protein abundance indicate wide variability in metabolism, myofibrillar filaments, cytoskeleton, contractile activity and stress response.

We could observe that the glycolytic metabolism enzymes were increased in high drip loss sample in agreement with the correlation proposed by several groups e.g. (D'Alessandro et al. 2012, van de Wiel and Zhang 2007). Other important modifications in the muscle samples were the large number of identified myofibrillar proteins with the same trend of their regulation. Nevertheless, the implication of myofibrillar proteins on meat quality traits is unclear. A higher abundance of these proteins indicates a greater proteolytic activity post-mortem, which is in accordance with a higher proteolytic activity resulting in a lower ultimate pH and therefore in drip loss (Enfält et al. 1997, Irving et al. 1989, Joo et al. 1999).

This proteomic approach was recognized as the next step to understand the underlying metabolisms of different meat quality traits. Therefore, further steps should be to combine data of gene expression profiles and metabolomic to get a holistic view on the relevant biological systems.

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Appendix

Table 11: List of used sample for the ICPL experiment

Chapter 4: General discussion and conclusion

Meat quality traits such as boar taint and WHC are important in meat production and affected financial outputs, nutritional values, consumer appeals and/or technological properties of porcine products (Malmfors and Lundström 1983, Offer and Knight 1988). The causes of boar taint and WHC are totally different and are described in detail in chapter 1. In general, the genetic regulation of the relevant measured traits of boar taint (androstenone and skatole) and WHC (e.g. drip loss) are complex and poorly understood. Because of the complexity of the traits it is important to understand the whole mechanisms of biological processes. The results of the study show us that the mechanisms behind these traits are very multifaceted and the analyses of the different levels (Transcriptomic/Proteomic) are necessary for the identification of potential biomarkers.

Boar taint and WHC are characterized by many different traits. These traits can be measured in a sensorial (Bonneau et al. 2000) but also in a technical way (Haugen 2010). However, till now there are no official international analysis methods approved for androstenone, skatole (Haugen et al. 2012, Thompson and Wood 1995, Thompson et al. 2002) or drip loss (Honikel and Hamm 1994). In general, most procedures are not quick or adaptable enough to an on-line or at–line situation during slaughtering processing. Ideally, the ultimate eating quality of meat should be predicted in the early post-mortem period (Mullen et al. 1998, Troy et al. 1998). There is also a need for a method of assessing meat quality at the point of sale. Presently, routine methods of measuring meat quality, within a typical European meat company, revolve around a few measurements. These include measurements of pH and temperature. Some more complex measurements (e.g. ultra-sound to measure back fat or muscle depth) are undertaken by some poducers (Mullen 2002). However, in general these measurements are not systematically recorded in a centralized way that would allow the data to be used effectively in selection programs (Williams 2008).

The analysis of boar taint can be performed in two different ways: (1) by a trained sensory panel related to the levels of androstenone and skatole in boar adipose tissue (Lundström et al. 2009) and/or (2) chemical methods developed for the measurement of concentrations of androstenone and/or skatole in adipose tissue (Haugen 2010, Haugen et al. 2012). However, sensory characterization of boar taint is very complex, even with an extensive training of the panel and statistical correction of discrepancies among panelist (Bonneau et al. 2000, Dijksterhuis et al. 2000, Font-i-Furnols 2012, Morlein et al. 2012). This is probably due to the fact that there are no clear references during the training of the assessors to approve by consensus the quantitative and qualitative use of the attributes (Font-i-Furnols et al. 2009). People react very differently to boar taint, depending on their sensitivity. Approximately 99 %

of consumers are sensitive to skatole (Meier-Dinkel et al. 2013), whereas the proportions of individuals that are insensitive or anosmic to androstenone vary in different studies (Bonneau et al. 2000). The chemical methods are not applicable on the slaughter line since they involve complicated sample preparation steps and are usually labor-intensive, but they permit quantification of boar taint compounds which is essential for research purposes. However, regardless of the plethora of analytical protocols, there still exists no harmonized approved method for detecting boar taint in the EU (Haugen et al. 2012). Most of the laboratory-based methods require an expenditure of time, personnel and cost. In last few years, a research group from Switzerland performed a method to select small adipose tissue sample from live boars via biopsy and measured androstenone, skatole and indole. The results show that data on boar taint compounds from small adipose samples obtained by biopsy provide similar genetic parameters as that described in the literature for larger samples and are therefore a reliable performance test for boar taint in live breeding candidates (Baes et al. 2013).

In comparison to boar taint the measurements of WHC of meat is carried out in many different ways reviewed by several authors (Honikel and Hamm 1994, Kauffman et al. 1986a, Kauffman et al. 1986b, Offer and Knight 1988, Offer et al. 1989) and can be only conducted after slaughtering. All measure the inherent ability of the cellular and subcellular structures of meat to hold on to part of its own and/or added water. Traditional methods of measuring WHC are the filter paper press method (Grau and Hamm 1953), the filter paper method (Hamm 1986, Kauffman et al. 1986a, Kauffman et al. 1986b) the bag method (Honikel 1987), and the tray method (Lundström and Malmfors 1985). Recent studies showed that there is need to find more suitable methods for determination of drip loss. Rasmussen and Anderson (1996) recommend a method working with EZ-Drip loss containers. Another group of researchers (Walukonis et al. 2002) used absorptive material in the early post-mortem stage. However, there is high diversity in procedures such as in sample size, or the force applied to the meat during measurement. For instance, bag method is carried out with approximately 100 g samples, whereas the EZ-Drip loss method uses approximately 10 g and for centrifugation almost 3 - 4 g. However, because of the variation in the methods used, the results for drip loss in the literature are difficult to compare (Honikel 1998).

Beside the different measurement techniques and other environmental factors affecting the phenotype the genetic background of boar taint and WHC has been widely investigated.

The genetic determinism of androstenone and skatole levels has been investigated primarily through the estimation of genetic parameters. The heritability's of androstenone and skatole

are at a moderate to high level (see Chapter 1.3.1.) and suggested a high genetical contribution which indicates a good basis for genetic selection (Baes et al. 2011, Bergsma et al. 2007, Frieden et al. 2011, Grindflek et al. 2001, Karacaören et al. 2010, Knol et al. 2010, Sellier et al. 2000, Tajet et al. 2006a, Varona et al. 2005, Windig et al. 2012). Androstenone is produced in the testis among other anabolic hormones. Therfore, the environmental factor is less. In comparison to androstenone, the lower heritability for skatole may reflect the greater suggestibility of skatole by environmental influence such as feeding, husbandry and hygiene management, with affects the bacterial metabolism of L-tryptophan in the intestine of boars. Mapping data of QTL involved in boar taint demonstrate its multifactorial nature. A number of QTL analyses have been conducted for androstenone and skatole mostly in intercross or backross pig populations (Lee et al. 2005, Quintanilla et al. 2003, Varona et al. 2005). While GWA analyses are performed in purebred pig population (Duijvesteijn et al. 2010, Gregersen et al. 2012, Grindflek et al. 2011a) (see Chapter 1.3.2.1.). The influence of different breeds on boar taint are already examined (Fredriksen et al. 2006, Squires 2006). Gene expression analysis has been used to identify candidate genes related to the trait of interest. Several candidate genes have been proposed for divergent androstenone levels in different pig populations by global transcriptome analysis in boar testis and liver samples (Gunawan et al. 2013b, Leung et al. 2010, Moe et al. 2007b, Moe et al. 2008). Functional genomics provides an insight into the molecular processes underlying phenotypic differences such as androstenone and skatole levels. Several studies are devoted to identify the genes and pathways involved in the androstenone metabolism in liver (Gunawan et al. 2013b, Leung et al. 2010, Moe et al. 2008) but to best of our knowledge, only one study (Gunawan et al. 2013a) was devoted to perform a global transcriptome analysis for divergent skatole levels in boar fat of a Duroc x F_2 cross animals as well as to identify the pathways that might be involved in skatole metabolism in liver. Both components are metabolized in the liver. This suggested a positive genetic correlation between skatole and androstenone levels (0.36 – 0.62) (Tajet et al. 2006a). Quantitative traits such as boar taint may be influenced by the same locus (pleiotropic effects) and each single trait may be affected by several loci (polygenic effects). A relationship between androstenone and skatole has been already described in several studies, but till now the mechanisms behind are unclear (see Chapter 1.3.1.). The comparison of the combined phenotype in our study revealed several genes which are differently expressed. This genes have may be a pleiotropic effect on androstenone and skatole and could be therefore of interest as candidate genes for selection programs.

In comparison to boar taint, the genetically influence on WHC are less (see Chapter 1.3.1.). The low heritability of traits related to WHC implicate that many environmental factors play a significant role to influence the traits. However, the different measurements techniques and the high environmental factor suggested that the heritability may be are underestimated. Because of that, it is surprising that until now many studies revealed numerous of single QTL to identify promising regions influencing complex traits. Until now 973 QTL (update: October 2013) have been detected for drip loss (http://www.genome.iastate.edu/cgi-bin/QTLdb/index) (Hu et al. 2013) in several pig breeds and crosses. The high number of identified QTL could be evidence that there are a lot of interesting regions with non additive genetic effects such as dominance effect or epistasis (Große-Brinkhaus et al. 2010). Our Bonner Duroc-Pietrain (DUPI) resource population was used in last years for different studies to get an explanation of the genetically influence of WHC. QTL were identified (Liu et al. 2007) and also expression profiles were performed (Heidt et al. 2013, Ponsuksili et al. 2008b). By combining QTL mapping and microarray analyses, it is possible to identify regulatory networks underlying the quantitative trait of interest and localize genomic variation, the so-called genetical genomics approach (Jansen and Nap 2001, Jansen 2003). For this approach Ponsuksili et al.(2010) and Heidt et al. (2013) identified several expression QTL (eQTL) to connect the variation at the level of RNA expression to the variation at the level of the DNA.

Together with genomics and transcriptomics, proteomics is a crucial step for understanding the biological systems. Thus, understanding the variation and different components of the proteome in regard to certain quality or processing parameters will lead to knowledge that can be used in optimizing the conversion of muscle to meat (Bendixen 2005, Hollung et al. 2007b, Hollung et al. 2007a, Mullen et al. 2006). Briefly, proteomics has been successfully applied for the analysis of the porcine skeletal muscle. Till now, most of the quantitative proteomic analyses of complex protein mixtures in animals have been typically accomplished by 2-DE (van de Wiel and Zhang 2007) in combination with MS-based techniques (Klose et al. 2002, Link et al. 1997). The proteomic analysis of the present study was performed using the ICPL method, a novel approach for the accurate quantitative comparative analysis of proteomes (Schmidt et al. 2005). With this technology it is possible to identify and quantify thousands of proteins in complex protein samples in an accurate and reproducible mode (Schmidt et al. 2005). The method is compatible with all known separation techniques on the protein and the peptide level and provides a highly accurate quantification of differentially expressed proteins (Sarioglu et al. 2006). Moreover, because of the simultaneous quantification and identification of ICPL analysis this method provides information about non-differential proteins as opposed

to 2D gel-based methods in which protein identifications are focused on differential proteins. However, the number of articles published to date using ICPL is noticeably low (Nakatsuji et al. 2008, Paradela et al. 2010, Sarioglu et al. 2006, Shi et al. 2007) when compared with other methods and no clear consensus exists on its feasibility as a quantitative tool.

However, proteomic studies by itself provide an insight in the expression of proteins which are involved in the phenotypes, but only in connection with the other "omics" (structural genomics, functional genomics and metabolomics) conclusion as the potential candidate marker can be drawn.

Therefore, one important further step for WHC should be combining the proteomic data with gene expression profiles and metabolomics profiles in order to get a holistic view on the relevant biological systems. Up to now studies combining transcriptional, proteomic and metabolomics analysis of skeletal muscle are limited (D'Alessandro et al. 2011, D'Alessandro et al. 2012, Hornshoj et al. 2009, Kim et al. 2010, Xu et al. 2012). The proteomic and transcriptional analysis only measure the changes at the protein and mRNA level respectively. While the expression on many genes is controlled at the transcriptional level (epistasis, gene x gene interaction) other genes are also employed to posttranscriptional regulation processes involving mRNA stability, translation initiation, and protein stability (Chen et al. 2002, Tian et al. 2004). Epistasis and gene by gene interaction are important effects and should be not neglected when studying complex traits such as WHC (Cheverud and Routman 1995, de Visser et al. 2011, Roff and Emerson 2006). The use of bioinformatics approaches to identify QTL on different levels such as transcriptional level (eQTL) but also on translational level (tQTL) and to combine these data with phenotypic QTL (pQTL) (Cheng et al. 2013) could be useful tool to understand the mechanisms that control complex traits.

For boar taint, further works might be to analyze the holistic transcriptome in a bigger population and in different breed or crosses to confirm the results and to study potential candidate genes related to their function in the metabolism of androstenone but also in skatole. It could be of much interest to clarify in detail the relationship of androstenone and skatole and to examine genes which may be influence both androstenone and skatole. On the other hand, the study of gene regulation at the transcript level may be misleading as the amount of RNA coding for a specific protein may not correlate very accurately with the concentration of that protein in the cell. There are many mechanisms in the cell which influence the rate of translation of an RNA molecule into a protein (Alberts et al. 2002). For the future it could be necessary to investigate the proteome and metabolome to see whether there is any difference between the mRNA expression and the translation into the protein. For

meat quality traits related to WHC such as drip loss, pH in pig but also for traits such tenderness, color or texture in bovine a lot of studies are already published (see Chapter 1.4.2.). However, till now proteome studies related to boar taint are scarce. In 2006 van de Wiel et al. indicated that proteomics could be a highly effective tool for the purpose of identifying candidate marker genes for boar taint. In their study in Duroc pigs with a range of androstenone levels in back fat they identified four candidate marker proteins. The specific proteins affected are not reported.

In general, different factors on the transcriptional level and posttranscriptional level influence the expression of many genes. Therefore, the analysis and the connection of the different "omics" are necessary to understand the whole mechanisms of biological processes of complex traits such as boar taint and WHC.

Chapter 5: Summary

In complex traits such as boar taint and WHC it is often not feasible to determine the number of genes affecting a particular trait and the individual effects of the genes on the phenotype. Transcriptomics and proteomics analysis could help to get a holistic view on the relevant biological systems. Therefore, two studies were performed to investigate the transctiptome and proteome in liver/muscle tissue from animals with divergent androstenone and skatole level and drip loss, respectively.

Boar taint is the nasty odour or taste that can be evident during the cooking or eating of pork or pork products derived from non-castrated male pigs once they reach puberty (Moe et al. 2008). Previous studies showed that about 75% of consumers are sensitive to boar taint, therefore it is necessary for pig producers to control it (Bonneau et al. 1992). Normally pigs are castrated very early in life to prevent boar taint in the meat. However, the castration also removes the source of natural anabolic androgens that stimulates lean growth. Moreover, non-castrated males have improved feed efficiency and greater lean yield of the carcass compared to barrows (Babol and Squires 1995). Boar taint is caused by the accumulation of two main substances androstenone and skatole (Babol et al. 1999).

Water-holding capacity (WHC) is defined as the capacity of meat to retain its water during application of external forces (Hamm 1985) and can be measured by drip loss (Honikel 1987, Honikel 1998). It is generally accepted that the source of drip loss from pork is intracellular water which is lost from the muscle fibre post-mortem, driven by a pH and calcium-induced shrinkage of myofibrils during rigor development (Honikel et al. 1986). The rate and quality of drip formation in fresh meat is believed to be influenced by the extent of protein denaturation (Bertram et al. 2004b, Offer et al. 1989).

The aim of the first study was to investigate the transcriptome of liver samples from boars with high and low androstenone and skatole levels in their backfat. For this experiment, 20 animals of 58 cross bred boars (Pietran × F_2) with high and low androstenone and skatole levels were selected. Average levels of androstenone were at > 470 ng/g fat and of skatole at > 250 ng/g fat. Liver samples were obtained at slaughterhouse and RNA was isolated. Liver gene expressios patterns were produced using 20 GeneChip Porcine Array (Affymetrix). This array contains 23,937 probe sets that interrogate approximately 23,256 transcripts from 20,201 *Sus scrofa* genes. The analysis of the microarrays revealed that 92 genes differentially expressed in the comparison of high vs. low skatole. Of which 49 were up and 43 down regulated in high level of skatole compared to the low level of skatole. Only two genes were identifed in liver between high and low androstenone group. Moreover, statistical analysis

identified that 170 genes differentially expressed in the comparison of the combined phenotypes in which 84 genes were down regulated and 86 genes were up regulated in skatole high/androstenone low compared to skatole low/androstenone high. A total of 84 genes were found to be common for high vs. low skatole and the combined phenotype group. On the other hand, both identified genes were found to be common for high vs. low androstenone and the group of combined phenotypes. The DAVID ontology classification was performed in order to assign differentially expressed genes to categories of Biological Processes (BP), Cellular Components (CC) and Molecular Function (MF) and to canonical pathways. Differentially expressed genes between the group of combined phenotypes and between high and low skatole levels, showed the most significant features in catalytic activity, in metabolic processes, in fatty acid metabolism and lipid metabolic processes. The most relevant pathways of these two parameters were retinol-, fatty acid- and steroid metabolism pathways. In conclusion, the results of this study identified candidate genes which have potential influence on pathways (steroid pathway, retinol pathway) and in connection with the metabolism of androstenone and skatole production. Further studies covering polymorphism identification and validation in other pig populations may be needed for the candidate genes in order to prove their candidacy for pig breeding.

The aim of the second study was a global proteome analysis and to identify the relevant biological mechanism of porcine muscle proteins, with potential functional relevance for drip loss. For this experiment a Duroc × Pietrain (DuPi) F_2 resource population (n = 42) was used. Proteins of the m*usculus longissimus dorsi* from the F_2 animals were isolated. The relative protein quantification was done using isotope-coded protein labeling techniques (ICPL) and electrospray ionization liquid-chromatography-tandem mass spectrometry (LC-MS/MS). In total, 763 proteins could be identified with the ICPL and off-gel fractionation analysis. Out of these proteins, 60 proteins showed different abundance due to their ratio. Proteins with a ratio < 0.7 (20 proteins) were considered under-expressed and with ratio > 1.3 (60 proteins) were considered over-expressed. Applying a t-test revealed that 10 proteins PYGL, PYGM, HSPA8, EE1A1, ACTA1, CASQ1, FLN-C, MYOM1, TNNT3, and HSP27 were up-regulated and 14 proteins TNNI1, MYL3, MYL2, MB, MYBPC1, FHL1C, TPM1, TPM2, AK1, TNNC2, MYL11, CK, PGK1 and MYH7 down-regulated in animals with low drip loss compared to animals with high drip loss ($p < 0.1$). The protein-protein interaction of all annotated proteins resulted in 3 main functional areas summarized as follow: 1. proteins of glycolysis, 2. proteins of cytoskeleton part and 3. proteins of mitochondria. The down-regulated proteins are mainly assigned in their interaction in the fields of muscle contraction

and metabolic processes. While the protein-protein interactions of the up-regulated proteins were involved in glycogen breakdown. The classification of the corresponding genes of the proteins using gene set enrichments analysis was identified among others as actin filament, myofibril, troponin complex, cytoskeletal part, stress fiber, and myosin complex. In conclusion, this proteomic approach was recognized as the next step to understand the underlying metabolisms of different meat quality traits. Therefore, further steps should be to combine data of gene expression profiles and metabolomics to get a holistic view on the relevant biological systems.

In general, this study revealed the importance of transcriptomic and proteomic analysis for understanding the biological mechanisms of complex traits.

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Chapter 7: Appendix

Table 10: Abbreviations of genes and proteins

Protein or gene name	
AAC1	ADP/ATP translocase 1-like
ACOX1	Acyl-Coenzyme A oxidase 1
ACSL1	Long-chain-fatty-acid—CoA ligase 1 i
ACSL5	Long-chain-fatty-acid—CoA ligase 5
ACTA1	Actin, alpha 1, skeletal muscle
ACTN2	Alpha-actinin-2
ADFP	Adipophilin
AGP	Alpha-1 acid glycoprotein
AK1	AdenylaAdenylate kinase isoenzyme 1-like
AKR1C4	Aldo-keto reductase family 1, member C4.
ATP5B	ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide.
CASQ1	Calsequestrin 1
CIDEB	Cell death-inducing DFFA-like effector b
CK	Creatine kinase
CYB5	Cytochrome b5
CYBc1	cytochrome b562 1
CYP11A1	Cytochrome P450, family 11, subfamily A, polypeptide 1
CYP17	Cytochrome 17
CYP1A1	Cytochrome 1 type A 1
CYP1A1	Cytochrom P450-1A1
CYP21	Cytochrome 21
CYP2D6	Cytochrome 2 type D6
CYP2E1	Cytochrome P450 2E1
CYP39A1	Cytochrome 39 A1
CYP3A43	Cytochrome P450, family 3, subfamily A, polypeptide 43
CYP3A7	Cytochrome P450, family 3, subfamily A, polypeptide 7
CYP4A21	Cytochrome 4 A21
CYP4A24	Cytochrome P450 4A24
CYP4B25	Cytochrome 4 B25
CYP7A1	Cytochrome P450, family 7, subfamily A, polypeptide 1
CYP7A1	Cytochrome P450 7A1
DECR1	2,4-dienoyl CoA reductase 1, mitochondrial.

DES	Desmin
DHRS3	Dehydrogenase/reductase (SDR family) member 3.
DHRS7	Dehydrogenase/reductase (SDR family) member 7
EEF1A1	Elongation factor 1-alpha 1
FASN	Fatty acid synthase.
FHL1C	Four-and-a-half LIM domain protein 1 isoform C
FLNC	Folliculin
FMO1	Flavin containing monooxygenase 1
FMO3	Flavin containing monooxygenase 3
FMO5	Flavin containing monooxygenase 5
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GSTO1	Glutathione S-transferase omega-1
GSTT1	Glutathione S-transferase theta 1
HADHA	Hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase
HSD17B11	Hydroxysteroid (17-beta) dehydrogenase 11.
HSD17B12	Hydroxysteroid (17-beta) dehydrogenase 12.
HSD17B13	Hydroxysteroid (17-beta) dehydrogenase 13
HSD17B2	Hydroxysteroid (17-beta) dehydrogenase 2.
HSD17B4	Hydroxysteroid (17-beta) dehydrogenase 4.
HSP27	Heat shock 27kDa protein 1
HSP70-2	Heat shock-related 70 kDa protein 2-like
HSP90	90-kDa heat shock protein
HSPA8	Heat shock 70kDa protein 8
IDH1	Isocitrate dehydrogenase 1 (NADP+), soluble
IFIT2	Interferon-induced protein with tetratricopeptide repeats 2
INHBA	Inhibin, beta A
IRG6	Inflammatory response protein 6
KBTBD10	Kelch repeat and BTB domain-containing protein 10
KRT8	Keratin 8
LCF1	Long-chain-fatty-acid-CoA ligase
MAP	Ensconsin-like
MB	Myoglobin
MGST1	Microsomal glutathione S-transferase 1
MGST1	Microsomal glutathione S-transferase 1 i

MPD1	Protein disulfide-isomerase
MX1	Myxovirus (influenza virus) resistance 1, interferon-inducible protein p78
MYBPC1	Myosin binding protein C
MYBPH	Myosin-binding protein H
MYH1	Myosin-1
MYH3	Myosin heavy chain 3
MYH7	Myosin, heavy chain 7
MYL11	Myosin light chain 11
MYL2	Myosin, light chain 2,
MYL3	Myosin light chain 3-like
MYOM1	Myomesin 1
NSDHL	Sterol-4-alpha-carboxylate 3-dehydrogenase, decarboxylating
ORM	Orosomucoid
PFKM	6-Phosphofructokinase, muscle type
PGK1	Phosphoglycerate kinase 1
PGM1	Phosphoglucomutase 1
PKM2	Pyruvate kinase, muscle
PLA2G	Phospholipases A2
PPARA	Peroxisome proliferator-activated receptor alpha
PPIA	Peptidylprolyl Isomerase A
PRKAG3	Protein kinase, AMP-activated, gamma 3 non-catalytic subunit.
PVALB	Parvalbumin
PYGL	Glycogen phosphorylase, liver form
PYGM	Phosphorylase, glycogen, muscle
RDH11	Retinol dehydrogenase 11
RDH5	Retinol dehydrogenase 5
RN	Rendement Napole
RPL4	Ribosomal protein L4
RYR 1	Ryanodine receptor 1
SLC22A7	Solute carrier family 22 (organic anion transporter), member 7
SNF2L1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 1
SULT2A1	Sulfotransferase family, cytosolic, 2A, dehydroepiandrosterone (DHEA)-preferring, member 1

SULT2B1	Sulfotransferase family, cytosolic, 2B, member 1.
TMOD4	Tropomodulin 4 (muscle)
TNNC2	Troponin C type 2
TNNI1	Troponin I type 1
TNNT1	Troponin T type 1
TNNT3	Troponin T type 3
TPM1	Tropomyosin 1 (alpha)
TPM2	Tropomyosin 2 (beta)
UGT1A5	Glucuronosyltransferase 1 family, polypeptide A5
UGT2A1	Lucuronosyltransferase 2 family, polypeptide A1
UGT2B12	UDP glucuronosyltransferase 2 family, polypeptide B12
UGT2B17	UDP glucuronosyltransferase 2 family, polypeptide B17
YWHA7	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide

Appendix: Chapter 3

Table 11: List of used sample for the ICPL experiment

Animal nr.	Class drip loss	Sex	Litter nr.*	Slaughter date	Drip loss %	Tissue mg	Bradford.1:10 µg/µl	ICPL labels	ICPL MS run
28	Low drip loss	1	17	07.11.2005	0.4	35	4.821	ICPL_4	1
36	Low drip loss	1	24	28.11.2005	0.4	34	4.544	ICPL_10	1
38	Low drip loss	1	25	28.11.2005	0.4	31	3.954	ICPL_4	2
11	Low drip loss	1	8	26.06.2002	0.5	60	1.006	ICPL_10	2
26	Low drip loss	2	16	05.04.2006	0.5	30	4.034	ICPL_4	3
27	Low drip loss	1	16	30.08.2006	0.5	28	4.848	ICPL_10	3
34	Low drip loss	2	22	10.01.2007	0.5	42	2.603	ICPL_10	4
39	Low drip loss	1	26	07.11.2005	0.5	31	4.821	ICPL_4	4
2	Low drip loss	1	1b	20.09.2004	0.6	25	4.696	ICPL_4	5
19	Low drip loss	2	13b	31.01.2005	0.6	44	5.086	ICPL_10	5
20	Low drip loss	2	13c	16.02.2005	0.6	35	3.739	ICPL_6	8
21	Low drip loss	1	13d	19.12.2005	0.6	30	3.846	ICPL_4	6
23	Low drip loss	2	14b	16.02.2005	0.6	37	3.505	ICPL_6	9
25	Low drip loss	1	15	27.05.2003	0.6	40	3.529	ICPL_10	6
40	Low drip loss	1	27a	13.03.2006	0.6	52	2.888	ICPL_10	7
16	Low drip loss	1	11b	07.03.2005	0.7	38	6.751	ICPL_6	10
1	Low drip loss	1	1a	19.02.2003	0.8	39	4.777	ICPL_6	11
10	Low drip loss	1	7b	24.11.2004	0.8	50	5.294	ICPL_6	13
22	Low drip loss	2	14a	18.10.2004	0.8	30	2.869	ICPL_6	12
15	Low drip loss	1	11a	31.03.2004	0.9	40	3.06	ICPL_6	14
31	Low drip loss	1	19	13.06.2007	0.9	35	5.309	ICPL_4	7
3	High drip loss	1	2	08.10.2002	3.1	36	5.95	ICPL_6	7
37	High drip loss	1	24	26.04.2006	3.1	43	3.857	ICPL_6	1
41	High drip loss	2	27b	04.09.2006	3.1	41	2.839	ICPL_6	2
13	High drip loss	2	10	26.06.2002	3.3	70	6.619	ICPL_6	3
14	High drip loss	2	11a	06.04.2004	3.3	46	5.186	ICPL_6	4
4	High drip loss	1	3	15.05.2002	3.4	10	1.168	ICPL_6	5
29	High drip loss	2	18	04.09.2006	3.5	30	4.069	ICPL_6	6
42	High drip loss	2	28	27.07.2005	3.6	35	4.135	ICPL_4	8
18	High drip loss	1	13a	29.04.2003	3.7	60	4.71	ICPL_10	8
33	High drip loss	1	21	17.07.2006	3.7	39	4.4	ICPL_4	9
9	High drip loss	2	7a	08.10.2002	4.1	70	6.531	ICPL_10	9
5	High drip loss	1	4	14.08.2002	4.3	35	3.319	ICPL_4	11
30	High drip loss	2	19	30.08.2006	4.3	31	4.712	ICPL_10	10
32	High drip loss	1	20	17.07.2006	4.3	63	6.994	ICPL_4	10
24	High drip loss	1	14b	27.07.2005	4.6	30	4.615	ICPL_10	11
7	High drip loss	2	6a	27.05.2003	4.8	46	4.306	ICPL_4	12
17	High drip loss	1	12	11.02.2004	4.8	40	4.534	ICPL_10	12
6	High drip loss	1	5	24.04.2002	4.9	27	2.493	ICPL_4	13
12	High drip loss	2	9	14.08.2002	4.9	70	5.019	ICPL_10	13
8	High drip loss	1	6b	16.02.2005	5	29	3.522	ICPL_4	14
35	High drip loss	2	23	29.11.2006	5.3	38	4.508	ICPL_10	14

*The animals that were chosen for protein analysis were selected on a high degree of relationship with the F_2 crosses and their extreme value of drip loss. Therefore in order to avoid a confounding of extreme phenotypes with families and litters, two discordant full sibs of each family were selected.

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