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Analysis of lipid metabolism-related candidate genes in swine

Karina Meidtner

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Contents

1 Introduction

Domestication and breeding of pigs for different purposes have created a diversity of phenotypes among different breeds [1]. Over the last 50 years objectives of pig breeding based on leanness, growth rate and feed efficiency, which are determined by energy- and lipid metabolism. Although, the goals of genetic studies in pigs are agronomic, homologous loci in humans are likely to play a role in the genetic predisposition to obesity [2]. Therefore, the variety of pig breeds provides a valuable model for research into genetics of human obesity, especially because of the high anatomical and physiological similarity between pigs and humans [3].

According to the World Health Organization [4], there are more than 1 billion adults overweight worldwide with at least 300 million of them clinically obese. Obesity and overweight causes a high risk to develop chronic diseases, including type 2 diabetes, cardiovascular disease, hypertension, stroke, and certain forms of cancer [4]. The high incidence of obesity nowadays may be an evolutionary legacy. Over centuries, famine was a periodic problem in many countries. This resulted consequently in the survival of people able to store sufficient energy in their adipose tissue [5]. The rise in obesity prevalence started when people became surrounded by unlimited, energy-rich food. Knowing the genetic reason for susceptibility to obesity will help to prevent obesity and find new treatment strategies.

There are many different approaches to identify genes responsible for a complex trait like obesity, or in respect to the pig, for fatness. In general, all these approaches fall into two categories: candidate-gene studies and genome- wide studies, like linkage mapping and genome-wide association studies [6]. Genome-wide studies require genotyping of many genetic markers over all chromosomes and result in the identification of chromosomal regions linked to the trait under investigation. The detection of the actual functional variant requires further studies in the identified region. Candidate-gene studies focus on the analysis of a single or a few genes. The analysed genes are chosen according to their location in a region of linkage and (or) according to functional evidence for the gene involved in development of the trait under investigation.

In various pig populations genome wide studies have been carried out to map quantitative trait loci (QTL) and develop genetic markers for breeding. This resulted in more than 400

QTL for fatness [7]. The numerous QTL studies revealed chromosomal regions repeatedly linked to fatness traits. Some of the most significant QTL for backfat were identified on porcine chromosome 7 [8-11] . In this QTL region the peroxisome proliferative activated receptor delta (PPAR-δ) gene was mapped [12, 13]. PPAR-δ is involved in the regulation of lipid metabolism, energy balance and insulin sensitivity [14]. Based on these facts, *PPARD* is considered a functional as well as positional candidate gene for fatness related traits in pigs. The purpose of this thesis is to test this candidate gene hypothesis.

Melanocortin 4 Receptor (*MC4R*) is another candidate gene for fatness, because MC4R is implicated in mediating the effect of leptin on food intake. Furthermore, *MC4R* was mapped at a region of chromosome 1, where QTL for backfat thickness were detected in some studies [15-17]. Sequence variants in the human *MC4R* leading to the most common monogenic form of early-onset severe obesity [18-20]. In swine a non-synonymous Asp298Asn polymorphism in *MC4R* has been described [21], but reports about association with backfat thickness are controversial [21-23]. The aim of this thesis was to study the effect of the Asp298Asn polymorphism in previously uninvestigated breed in order to evaluate the importance of the described *MC4R* variant for pig breeding.

2 Literature review

2.1 MC4R

The first known and eponymous member of melanocortin receptor family, melacortin 1 receptor (MC1R) is responsible for controlling skin and hair pigmentation. MC4R was identified after it had been demonstrated that widespread ectopic expression of the MC1R antagonist agouti resulted in a yellow, surprisingly hyperphagic and obese mouse. The antagonistic effect of agouti on the presumably in hypothalamus expressed MC4R was revealed as reason for the observed metabolic changes. MC4R knock out mice exhibit a similar hyperphagic phenotype that leads to a maturity onset obesity syndrome and helped to uncover the role of the MC4R in the regulation of energy balance [24].

MC4R is a seven transmembrane spanning G-protein coupled receptor and transduces signal by activating adenylyl cyclase [25]. The endogenous ligand of the MC4R is α melanocyte-stimulating hormone (α-MSH) that is cleaved from its precursor proopiomelanocortin (POMC) [26]. The production of POMC in the nucleus arcuatus of the hypothalamus is up-regulated by leptin (Figure 1) [27]. High leptin levels in obese individuals enhance α-MSH production and induce a reduction in food intake through activation of MC4R. Agouti related protein (AgRP) antagonises the effect of α-MSH on MC4R. In contrast to α-MSH, AgRP synthesis is down-regulated by leptin [28]. Hence, high leptin levels lead to activation of MC4R through changes in α-MSH and AgRP abundance and, consequently, reduce food intake.

Furthermore, neuropeptide Y (NPY) regulates feed intake in a leptin dependent manner. Like AgRP, production of NPY is reduced by leptin. Activation of the NPY pathway leads to an increase in feed intake. In healthy subjects, an increase in body fat stores is counterbalanced by a leptin-dependent reduction in food intake mediated through activation of MC4R and inhibition of NPY pathway.

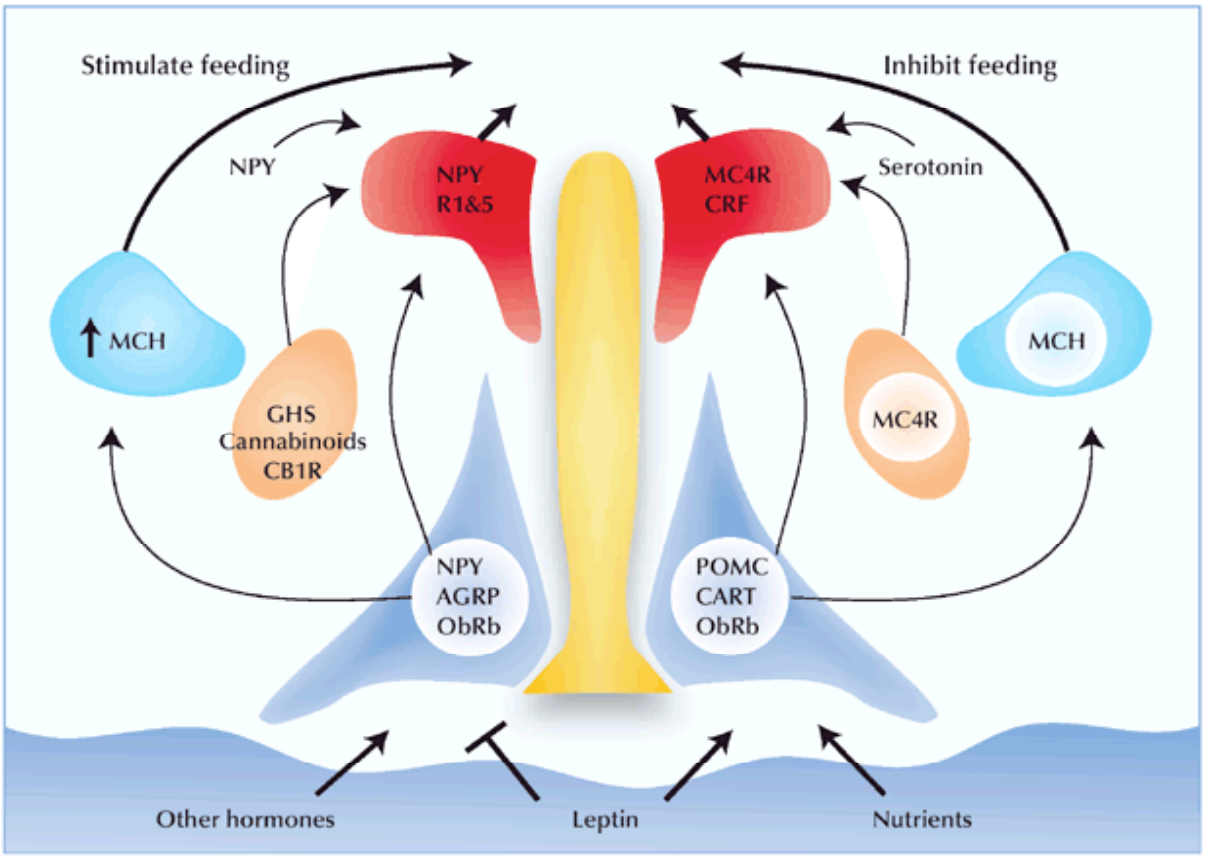


Figure 1: The neural circuit regulating food intake and body weight. [29]

Several sequence variants in the human *MC4R* gene are known and lead to the most common monogenic form of early-onset severe obesity [18-20]. In swine *MC4R* has been mapped about 50 cM distal to most backfat QTL on chromosome 1 [8, 23, 30, 31]. Nonetheless, there are individual reports about backfat QTL at the chromosomal location of *MC4R* [15, 17]. A non-synonymous Asp298Asn polymorphism in *MC4R* has been described [21]. The allele encoding for an asparagine at position 298 leads to a MC4R protein that is defective in agonist stimulated signalling, whereas cell surface expression and agonist binding are not impaired [32]. Association between the Asp298Asn polymorphism and feed intake, backfat and growth have been described, with the asparagine variant causing an increased feed intake, faster growth and greater backfat [21, 22]. However, Park [23] found no influence of the Asp298Asn polymorphism on backfat, growth and abdominal fat content and concluded that further studies are required to evaluate the significance of the *MC4R* Asp298Asn variant.

2.2 PPAR-δ

Peroxisome proliferator-activated receptors (PPARs) belong to the superfamily of nuclear receptors. Nuclear receptors are ligand-activated transcription factors that regulate expression of target genes. The first member of the PPAR family was identified in 1990 and termed

"peroxisome proliferator-activated receptor α" because of its ability to bind chemicals known to induce peroxisome proliferation [33]. Until now, three isoforms of PPAR, namely PPAR-α (NR1C1), PPAR-γ (NR1C3) and PPAR-δ/β (NR1C2), have been identified, each of them encoded by a separate gene.

PPARs are activated by fatty acids, eicosanoids and various synthetic ligands. They bind to their lipid-ligands with a low affinity. For that reason, the formation of a complex between PPAR and its ligand depends on ligand concentration that can be affected by dietary intake of fatty acids. Suggested PPAR-δ ligands are several 14- to 18-carbon saturated fatty acids and 16- to 20-carbon polyunsaturated fatty acids as well as prostaglandin A_1 and synthetics like iloprost, carbaprostacyclin and GW501516 [34, 35]. However, the physiological importance of these PPAR-δ ligands remains to be established. Very low-density lipoprotein- derived fatty acids cause PPAR-δ dependent expression of target genes suggesting an important role of dietary triglycerides for PPAR-δ activation [36].

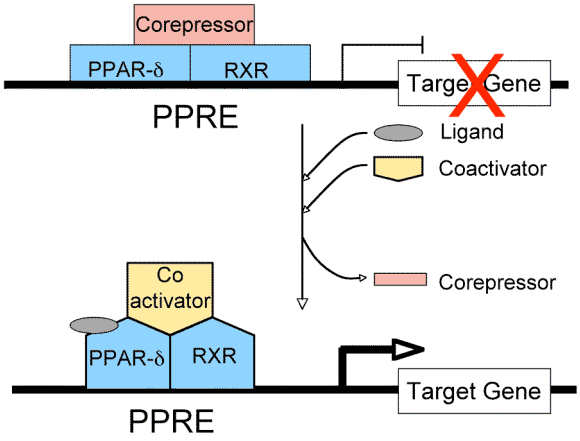


Figure 2: Gene transcription mechanism of PPAR-δ

PPARs form heterodimers with the retinoid X receptor (RXR). These heterodimers bind to the peroxisome proliferator responsive element (PPRE) in the promoter region of target genes (
Figure 2). PPRE consists of direct repeats of two hexanucleotides with the consensus sequence AGGTCA separated by one basepair [37]. In the inactive state, the PPAR-δ/RXR heterodimer is associated with corepressors like nuclear receptor corepressors (NCoRs) or silencing mediator for retinoid and thyroid hormone receptor (SMRT) [38]. Binding of a ligand results in a conformation change of the PPAR that is accompanied by the release of the

corepressors. Subsequently, transcriptional activation of target genes is mediated by the recruitment of cofactors like steroid receptor coactivator 1 (SRC1) [39].

PPAR-δ is expressed ubiquitously in contrast to PPAR-α and PPAR-γ that are both predominantly expressed in liver and adipose tissue [40-42]. PPARs do not seem to function independently from each other. Shi *et al.* [43] showed that PPAR-δ is able to modulate the activity of PPAR-α and PPAR-γ. PPAR-α is predominantly involved in fatty acid catabolism and PPAR-γ is known as a central regulator of adipogenesis [44]. PPAR-δ serves as a widespread regulator of lipid catabolism in muscle [14, 45]. It regulates target genes required for fatty acid transport, fatty acid oxidation, energy dissipation and mitochondrial respiration [14, 45]. PPAR-δ activation in mice prevents insulin resistance and obesity - both high-fat diet induced and genetically predisposed [14, 45]. Overexpression of PPAR-δ in skeletal muscle results in an increase of muscle fibres with oxidative metabolic capabilities, reduction of body fat and enhanced running endurance [46, 47]. Furthermore, it was demonstrated that exercise up-regulates PPAR-δ in wild-type mice [46]. Beside the effect on lipid metabolism, PPAR-δ agonists are suggested to influence glucose metabolism [48-50]. Lee *et al.* [50] revealed the liver as most responsive tissue to PPAR-δ agonists. In contrast to PPAR-δ action in muscle, PPAR-δ activation in the liver up-regulates genes involved in fatty acid synthesis, elongation and modification. Additionally, an increase in glucose catabolism through the pentose phosphate pathway was described [50]. Based on these findings the authors suggest that PPAR-δ regulates metabolic homeostasis and insulin sensitivity by enhancing fat-production in the liver to consume glucose, counterbalanced by fat burning in the muscle [50]. This hypothesis is supported by the fact that PPAR-δ activation results in reduced glucose utilization of the skeletal muscle caused by a switch in mitochondrial substrate preference from glucose to fatty acids [49]. Therefore, PPAR-δ is discussed to be both a target to treat insulin resistance in type 2 diabetes and a candidate gene for predisposition to this disease.

Association studies revealed an effect of *PPARD* polymorphisms on fasting plasma glucose, body mass index, high-density lipoprotein cholesterol and plasma triglyceride levels in humans [51-53]. Furthermore, PPAR-δ has been discussed to be involved in atherosclerosis [54-56], cancer [57], wound healing [39] and fertility [58]. The porcine peroxisome proliferator-activated receptor delta gene (*PPARD*) has been assigned between SW1856 (61.5 cM) and S0102 (70.1 cM) on chromosome 7 [12, 13]. This region has been repeatedly

reported to contain a QTL for backfat thickness (Table 1). Most studies report a paradox of lower backfat caused by the allele originating from the breed, usually Meishan, with the higher backfat mean [8, 11, 59, 60].

Table 1: Backfat QTL on chromosome 7

Author [Reference]	Animals	QTL span in cM (QTL center in cM)	Trait
Bidanel [30]	Meishan x Large White	48.2 - 70.1 (57.1)	Backfat (13 weeks), Backfat (40 kg), Backfat (17 weeks), Backfat (22 weeks), Backfat (60 kg)
de Koning [11]	Meishan x Dutch	30.2 - 70.1 (50) 30.2 - 79.3 (61.5)	Average Backfat Backfat
Malek [10]	Berkshire x Yorkshire	57 - 134.9 (79.3) 32.9 - 134.9 (78.2)	Average Backfat Last Lumbar Backfat
Milan [9]	Meishan x Large White	57 - 73 (65)	Backfat Weight, Backfat (3-4 Ribs)
Rattink [61]	Meishan x Dutch	22.3 - 82.8 (57)	Average Backfat
Rohrer [8]	Meishan x White	30.2 - 73.4 (58.9) 32.9 - 58.9 (40)	Average Backfat Last Lumbar Backfat
Rohrer [31]	Meishan x White	58 58 57 121	Last Lumbar Backfat (26 weeks) Last Rib Backfat (26 weeks) Last Lumbar Backfat (14 weeks) Last Rib Backfat (14 weeks)
Su [62]	Dabai x Meishan	65 60 60	Rear Backfat Backfat (6-7 Ribs) Backfat
Wada [63]	Meishan x Göttingen miniture pig	32.9 - 48.2 (39) 57 - 75.3 (66)	Backfat Backfat

A metabolic and histochemical characterisation of fat and muscle tissue from pigs with the SSC7 QTL alleles from Meishan and Large White showed smaller adipocyte size and higher number of adipocytes in backfat together with a low basal rate of glucose incorporation into lipids and low activity of lipogenic enzymes [64]. No change in oxidative and glycolytic metabolism was observed in muscle. Therefore, the authors conclude further studies aiming at the understanding of the QTL effect on chromosme 7 should focus on candidate genes involved in adipogenesis rather than catabolic oxidative pathways. In the eyes of the authors,

PPARD is one of most promising candidate genes [64]. A microarray-based experiment aiming at the identification of differentially expressed genes between lean Piétrain and 'obese' German Landrace pigs revealed an up-regulation of *PPARD* in Piétrain [65]. This suggests a possible effect on lipid deposition and strengthens the hypothesis of *PPARD* being a candidate gene for fatness. Nonetheless, no study investigating genetic variation within porcine *PPARD* was carried out until now.

2.3 Pig Breeds

2.3.1 Piétrain

The Piétrain breed is one of the most import pig breeds in South Germany and accounted for 21 % of herd book registered pigs in Germany in 2004 [66].The name originates from a Belgian village with the name Piétrain, where this breed was established from the French breed Bayeux. Bayeux itself stems from the English Berkshire breed [67]. The Piétrain swine is of medium size, has shorter legs than most swine breeds and is quite broad along the back. It is white with black spots and ears are carried erected. The Piétrain breed is known to exhibit an enormously high proportion of lean to fat that is caused by extremely bulging and muscular hams [68]. However, breeding for high meatiness resulted in relatively low feed intake and daily gain. Furthermore, Piétrain pigs are very stress susceptible. Stress or exposure to the anaesthetic halothane triggers malignant hyperthermia, a disorder in which uncontrolled muscle contraction can cause lethal overheating and reduce meat quality [1]. The malignant hyperthermia syndrome is accompanied by a high lean-muscle content. Breeding for high lean-muscle content resulted in a high proportion of Piétrain pigs exhibiting the malignant hyperthermia syndrome. The phenotype is caused by a missense mutation (Arg614Cys) in the ryanodine receptor 1 gene (*RYR1*) [69]. *RYR1* encodes for an ion channel that regulates the release of calcium ions in skeletal muscle [70]. Selection against stress - susceptibility is applied successfully since several years with the help of a DNA test [67].

2.3.2 Mangalitsa

The Mangalitsa breed originates from Hungary, where it was bred by crossing Serbian Sumadia Pigs with ancient Hungarian pig breeds (Bakyoner, Szalontaer) [67]. There are three lines, namely the Blond, the Red and the Swallow Belly Mangalitsa [68]. The Mangalitsa breed is characterised by a thick, bristly coat and excellent meat quality but accompanied by high fatness [71]. Mangalitsas were selected for high backfat thickness till the demand for bacon and lard reduced. Mangalitsa lost its importance as pork producer in the 1950th due to

weak reproductive characteristics, low daily gain and the demand for lean meat [67]. Nowadays, Mangalitsas are kept and bred for conservation of the breed.

2.3.3 German Landrace (Deutsche Landrasse)

The German Landrace breed is the most common pig breed in Germany and represented 59.5 % of herd book registered pigs in Germany in 2004 [66]. The German Landrace breed is characterised by hanging ears, a long and massive body, fast growth and high fertility. Breeding German Landrace started at the end of the 19th century by crossing many different local German Landrace breeds with the Yorkshire breed. Since the 1950th breeding focuses on high carcass length and meatiness.

2.3.4 German Large White (Deutsches Edelschwein)

The German Large White breed is accounts for 12.3% of herd book registered pigs in Germany in 2004 [66]. The German Large White was bred from the Old German Marschschwein by replacement breeding with English Large White. The German Large White pig has medium length, erected ears, fast growth and good fertility.

2.3.5 Duroc

The Duroc breed has its origin in the United States. It is proposed that the Duroc breed descends from pigs coming from Guinea and Spain [67]. The colour of Duroc pigs varies from very light golden to a very dark red [68]. Durocs have a medium length, a slight dish of the face, drooping ears and exhibit a good feed efficiency. In 2004, the Duroc breed accounted for only 0.4 % of German herd book registered pig population [66] and is predominantly used in hybrid lines

Animals, Material and Methods

2.4 Animals

2.4.1 Mangalitsa x Piétrain intercross

Most analyses presented in the following chapters were performed within a Mangalitsa x Piétrain resource population. Mangalitsa and Pietrain are highly divergent with respect to lipid deposition characteristics. An intercross Mangalitsa x Pietrain is therefore well suitable to identify genes responsible for lipid deposition traits.

Initially, the parental generation of the crossbreed consisted of two Swallow Bellied Mangalitsa boars and 22 Piétrain sows. All 22 Piétrain sows were homozygous for the mutant Cys614 allele at the *RYR1* locus. They were mated to produce an F1 generation. Selected F1 animals were used to produce an F2 generation. Within the F2 generation the feeding regime was changed from restricted feeding to *ad libitum* feeding and the feed composition was altered. Parallel to these changes in feeding, the technical equipment to record feed intake per individual became available. To minimise the environmental influences on the production traits, only F2 pigs raised under *ad libitum* feeding were used for analysis (Table 2). Therefore, actually progeny of 13 Piétrain sows was used to produce approximately 600 F2 animals that were available for association studies. Unfortunately, from one of the 13 Piétrain founder dams neither blood nor tissue samples were taken. Therefore, the term 'parental generation' in the following chapters refers to the 12 Piétrain sows and 2 Mangalitsa boars that produced the analysed F2 progeny.

The pigs were raised in the experimental station in Thalhausen (Germany). After birth, every piglet was registered and weighed. After 28 days, the piglets were weaned and separated from their mothers. At a weight of about 30 kg, a transponder was fixed at the ear of every F2 pig and pigs were kept in a feeding station to record feed intake per individual. Once every 14 days animals were weighed. When the pigs reached the weight of 40 to 50 kg a blood sample was taken. By reaching a weight of 90 to 95 kg pigs were slaughtered at the research slaughterhouse in Grub (Germany). At this time various carcass and meat quality traits were measured according to German Pig Breeders Standards [72, 73]. This included body length, weight at slaughtering, several backfat values, muscle depth and pH values. Moreover, a longissimus muscle sample was taken from the 12^{th} and 13^{th} rib from every pig. In these

samples, intramuscular fat content was determined by fat extraction according to the method proposed by Hallermayer [74, 75]. Fatty acid composition was determined by gas chromatography of esterified fatty acids according to a standard protocol [76].

Table 2: Pedigree information of the Mangalitsa x Piétrain resource population

For the parental (P) and F1 generation animal ids of mated animals are given. The number of F2 progeny of a particular F1 sire or dam is indicated in the last column.

P (animal id)		**F1 (animal id)**	**Number of F2 progeny under *ad libitum* feeding**
Mangalitsa sire	Piétrain dam		
		sire	
2	3	24	227
1	4	36	63
1	7	55	257
1	9	63	205
2	10	67	239
		dam	
1	12	173	61
1	12	175	61
1	19	182	70
1	19	184	24
2	20	193	58
1	21	201	40
1	21	205	68
1	21	206	48
2	22	213	54
2	22	217	49
2	15	237	78
2	15	238	44
2	266	243	29
2	266	244	57
2	266	246	77
1	267	251	66
1	267	252	55
1	10	265	52

Association analyses were carried out in the F2 generation. F3 sows were backcrossed with Piétrain boars to introduce a desired *PPARD* gene variant (haplotypes 4 and 5) that was lost in F3. The genotypes of Piétrain boars were determined in order to assure the presence of the *PPARD* haplotypes 4 and 5 in the employed Piétrain boars. Seven F4 litters were used for gene expression studies. These seven litters correspond to a total number of 56 animals, 31 of them male and 25 female. They were slaughtered in proximity to the experimental station Thalhausen at 80 ± 2 days of age. At that time, these pigs had an average weight of 25.6 kg.

Tissue samples (approximately 0.5 g) for RNA isolation were collected from liver, longissimus muscle, backfat, heart, spleen, brain, kidney, ham and lung within 30 min after exsanguination and stored in 5 ml of RNAlater™ (Qiagen, Hilden, Germany).

2.4.2 Commercial Pig Breeds

2.4.2.1 Piétrain

Samples were collected for DNA isolation from different Piétrain groups. The first group consisted of 41 boars and was collected from the artificial insemination centres in Bergheim (Germany), Landshut (Germany) and Neustadt/Aisch (Germany) from 2000 to 2002. This group was used to estimate allele frequencies. Samples from a second group were provided from the artificial insemination centre in Bergheim (Germany) in 2005 in form of hairs from all 197 Piétrain boars that were available at this time. Selected boars of this population were crossed with the F3 generation of the Mangalitsa x Piétrain resource population.

2.4.2.2 German Landrace (Deutsche Landrasse)

A total of 41 boars were used for estimating allele frequencies in German Landrace. These samples were collected from the artificial insemination centres in Bergheim (Germany), Landshut (Germany) and Neustadt/Aisch (Germany) from 2000 to 2002. DNA from a second German Landrace group was provided by the Landesanstalt für Landwirtschaft Bayern, Grub (Germany) and contained 722 animals from performance testing that were slaughtered within 2002 - 2005 in Grub (Germany) and Schwarzenau (Germany).

2.4.2.3 German Large White (Deutsches Edelschwein)

Sperm from twelve boars were collected from artificial insemination centres in Bergheim (Germany), Landshut (Germany) and Neustadt/Aisch (Germany) from 2000 to 2002.

2.4.2.4 Duroc

Ear tissue samples of 39 Duroc animals were provided by the SUISAG (Sempach, Switzerland).

2.4.2.5 Piétrain x German Landrace (Deutsche Landrasse)

F1 offspring of Piétrain and German Landrace are widely used as fattened pigs in Germany. A total of 101 animals were chosen from a population of 13016 F1 Piétrain x German Landrace animals according to their feed intake. The pigs were used for performance testing in Grub (Germany) and Schwarzenau (Germany) between 2002 and 2005. About 50 animals

with the highest (2.05 kg/day ≤ feed intake ≤ 2.27 kg/day) and lowest (1.42 kg/day ≤ feed intake ≤ 1.68 kg/day) feed intake were selected for analysis. DNA from these animals was provided by the Landesanstalt für Landwirtschaft Bayern, Grub (Germany).

2.5 Materials and Methods

2.5.1 DNA isolation

2.5.1.1 DNA isolation from blood (standard protocol)

About 40 ml blood was collected for DNA isolation. Coagulation was prevented by addition of 3 ml 0.5 M EDTA resulting in a final EDTA concentration of about 50 µM. The haemolysis of 10 ml whole blood (containing EDTA) was performed by dilution with 40 ml water and addition of 5 ml 1.8 % NaCl solution. Cell constituents were separated from the liquid fraction by centrifugation at 4000 g for 20 min at 8°C. The pellet was resuspended in 40 ml of 0.1 % NP40 (Roche, Penzberg, Germany). After centrifugation at 5000 g for 20 min at 8°C the supernatant was discarded and proteinase K digestion was carried out. For that purpose, 2.5 ml of buffer (20 mM Tris/HCl, 4 mM EDTA, 0.1 M NaCl), 200 µl of 10 % SDS and 50 µl of 10 g/l proteinase K (Roche, Penzberg, Germany) were mixed with the pellet and incubated overnight at 55°C. After incubation, proteins were precipitated by 2 ml of a saturated NaCl solution. After centrifugation at 7000 g for 20 min at 8°C the DNA containing supernatant was transferred to a new tube and DNA was precipitated by isopropanol and washed 3 times with 700 µl of 70 % ethanol. DNA was air dried until all ethanol had been vaporised. Next, 200 µl TE buffer (10 mM Tris, 1 mM EDTA) were added and DNA was dissolved overnight at 55°C. This DNA stock solution was diluted 1:5, concentration was determined by measuring the absorption at 260 nm using an Ultraspec® III UV/Visible Photometer (Pharmacia LKB Biotechnology, Uppsala, Sweden), and a working solution with a DNA concentration of 25 ng/µl was prepared.

2.5.1.2 DNA isolation from blood (quick method)

The method was applied when fast genotyping results were required.

Haemolysis was performed with 60 µl water and 10 µl NaCl (1.8 %) that were added to 20 µl whole blood containing EDTA. After centrifugation at 16000 g for 5 min the supernatant was discarded and the pellet was resuspended in 80 µl NP40 (0.1%) for core lysis. Another centrifugation at 16000 g for 5 min followed. After the supernatant was discarded, the pellet was incubated in 5 µl buffer (20 mM Tris/HCL. 4 mM EDTA. 0.1 M NaCl), 4 µl SDS (1%)

and 1 µl proteinase K (1 mg/ml) for 30 min at 55°C. A 1:20 dilution of the resulting solution was used for PCR.

2.5.1.3 DNA isolation from hair

Approximately 10 hair roots of a length of about 0.5 cm were cut and incubated for 3 hours at 58°C in a lysis buffer (pH 8.3) containing 10 mM Tris, 50 mM KCl and 0.5 % Tween 20 as well as proteinase K (Roche, Penzberg, Germany) at a final concentration of 3.3 µg/ml. This solution was used for PCR after denaturation for 10 min at 96°C.

2.5.1.4 DNA isolation from Ear Tissue

About 0.5 g ear tissue was cut in small pieces and incubated in a lysis buffer (0.05 M Tris, 0.1 M NaCl, 0.1 M EDTA, 0.5 % SDS) containing 2 g/l proteinase K (Roche, Penzberg, Germany) at 58°C overnight. In the morning, samples were checked for complete lysis. If tissue parts were still visible, 0.8 mg proteinase K were added to the lysis buffer and the sample was incubated until the tissue was completely lysed. Subsequently, the sample was transferred to a Vacutainer SST™ tube (BD, Franklin Lakes, NJ, USA) and unwanted cell components were extracted by phenol/chloroform extraction. In the Vacutainer SST™ tube, the organic phase is collected on the bottom of the tube separated from the upper aqueous phase by a wax layer. The aqueous, DNA containing phase was transferred into a new tube and DNA was precipitated by ice-cold, absolute ethanol. Next, DNA was transferred into a new tube by pipetting the visible precipitate with a truncated pipette tip and washed with 70 % ethanol. After washing and after all ethanol was evaporated, DNA was dissolved in 0.1 M TE (10 mM Tris, 1 mM EDTA) buffer and handled like described in the protocol for DNA isolation from blood.

2.5.1.5 DNA isolation from semen

DNA preparation from semen was carried out in context of another thesis and is described elsewhere [77].

2.5.2 PCR

Primers for PCR were designed with Primer 3 Software [78] and ordered from MWG (MWG, Ebersbach, Germany). Primers sequences can be accessed from appendix 11.1 by the given primer number.

A standard PCR reaction contained 0.25 µM of each primer, 200 µM of each dNTP (Fermentas, St. Leon-Rot, Germany), 0.025 units/µl Taq-Polymerase (Qiagen, Hilden, Germany), 2.5 ng/µl template DNA and the PCR buffer supplied by Qiagen (Tris/HCl buffer (pH = 8,7) that contain 15 mM $MgCl_2$, KCl, $(NH_4)_2SO_4$). The total volume of PCR reaction was 20 µl, when PCR was followed by sequencing reaction and 10 µl, when PCR was followed by restriction fragment length polymorphism analysis. After preincubation at 94 °C for 3 min, the PCR mixture underwent 30 cycles of denaturation at 94 °C for 30 s, annealing for 60 s and extension at 72°C for 60 s. A final elongation step at 72°C for 3 min followed. If PCR failed under standard conditions, the reaction was optimized by adding Q-Solution (Qiagen, Hilden, Germany) or 5 % DMSO or by adjusting the annealing temperature to the requirements of the primers. In order to check the success of the PCR, 2 µl of the PCR products were mixed with 4 µl of a 1:4 diluted Orange Loading Dye (Fermentas, St. Leon-Rot, Germany) and loaded onto an 1.5 % agarose gel with 0.5 TBE buffer and ethidium bromide staining. The electrophoresis of PCR products was carried out at 100 V for 30 min.

2.5.3 Sequencing

PCR product must be cleaned from remaining primers and dNTPs before sequencing. Cleaning of PCR products was undertaken in a MultiScreen® $PCR_{\mu 96}$ Plate (Millipore, Schwalbach, Germany). 50 µl water were pipetted to the MultiScreen® $PCR_{\mu 96}$ Plate (Millipore, Schwalbach, Germany) and 18 µl of PCR product mixture were added. The MultiScreen® $PCR_{\mu 96}$ Plate (Millipore, Schwalbach, Germany) was placed on a vacuum manifold and vacuum was applied until the wells were completely empty. Afterwards the plate was removed from the manifold and the samples were dissolved in 40 µl of 10 mM Tris buffer (pH = 8,0) by shaking on a plate mixer for 5 minutes. After shaking, the samples were transferred to a new 96-well PCR plate. The amount of cleaned PCR product used for the following sequencing reaction varied from 2 to 5.5 µl depending on fragment size and PCR product yield. The sequencing reaction consisted of a 1 to 2 µl reaction mix of the BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and 0.5 µM primer in addition to the PCR products. The total volume of the sequencing reaction mix was adjusted to 10 µl using water. Thermal cycling for each reaction was initiated by denaturation at 95°C for 10 s, followed by 35 cycles of 51°C for 5 s and 60°C for 4 min. Cleaning of sequencing reaction to remove unincorporated dye terminators was carried out via gel filtration. In order to perform cleaning of the sequencing products, 45 µg Sephadex G-50 (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) were incubated with 300 µl water in a MultiScreen 96 well filtration plate (Millipore, Schwalbach, Germany) at room temperature

for three hours. After thermal cycling the sequencing reaction mixtures were transferred onto Sephadex columns and centrifuged (Centrifuge 5804, eppendorf, Hamburg, Germany) at 950 g for 5 min. The plate with the filtrate was placed in a vacuum centrifuge (Savant Speed Vac Plus SC110A, Thermo Savant, Holbrook, NY, USA) and vacuum was applied until all liquid of the filtrate had been vaporised. Subsequently, 2 µl of formamide mixed with dextranblue (Fluka, Buchs, Switzerland) were transferred into each well to dissolve the sequencing products. The mixture was shaken on a plate mixer for 5 min and sequencing products were denatured for 2 minutes at 96°C, before they were loaded onto a polyacrylamide gel.

DNA sequencing was performed according to Sanger [79] on an ABI PRISM® 377 DNA Sequencer (Applied Biosystems, Foster City, CA, USA). A sequencing gel contained 21 g urea (Roth, Karlsruhe, Germany), 20 ml water (HPLC Grade water, Roth, Karlsruhe, Germany), 6 ml 10x TBE buffer, 8.4 ml of 30 % NF-acrylamid / bisacrylamid (29:1) solution (Roth, Karlsruhe, Germany), 300 µl 10 % APS and 20 µl TEMED. Gels were prepared for 36, 48, 64 or 96 samples, respectively. The amount of sample applied to the gel varied between 0.5 µl (for 96 samples) and 1.2 µl (for 36 samples). The gel was run under standard conditions for 3 - 9 hours.

Obtained sequences were analysed using the Phred/Phrap/Polyphred/Consed software suite [80-83].

2.5.4 BAC clones

Bacterial artificial chromosome (BAC) clones containing the gene of interest were identified *in-silico* with the help of the genomic location of the human gene from the Porcine BAC End Sequencing Project [84] and ordered from the distributors of the BAC libraries [85, 86]. Colony PCRs with specific primers for each gene (*MC4R*: 3744/3745, *PPARD*: 3709/3710 + 3383/3384) were carried out to ensure that the clones contain the gene of interest. A positive clone for each gene was sequenced in a shotgun approach at the Helmholtz Centre for Infection Research, Braunschweig, Germany.

2.5.5 Restriction Fragment Length Polymorphism (RFLP) analyses

Enzymes for RFLP analyses were chosen with the help of the NEBcutter [87] and bought from Fermentas (St. Leon-Rot, Germany). For RFLP analysis, 3 - 7 µl of the appropriate PCR product were mixed with the enzyme and the supplied buffer. The volume was adjusted to

10 µl using water. An overview of enzymes used and reaction conditions employed is given in Table 3. After incubation at 37°C the resulting fragments were separated on a 2 % agarose (Sigma-Aldrich, Steinheim, Germany) gel. For the separation, 2 µl Orange Loading Dye (Fermentas, St. Leon-Rot, Germany) were added to the reaction mixture and the resulting solution was loaded onto the gel. Electrophoresis was performed at 100 V for at least 45 min.

Table 3: Restriction enzymes

Gene	SNP	Enzyme	Specificity* 5' → 3'	Primer pair	Units per reaction	Incubation time
PPARD	-159G>A	*Eco72*I	CAC**G**TG	3392 3393	5	3 h
PPARD	+1137G>T	*EcoO109*I	PuG**G**NCCPy	3383 3384	0.5	14 h
PPARD	-281-244A>C	*Bsp143*II	PuG**C**GCPy	3598 3626	0.5	14 h
PPARD	+768A>G	*Esp3*I	**C**GTCTC	3981 3982	0.5	14 h
PPARD	-180C>T	*Bsh1236*I	CG**C**G	3392 3393	0.5	14 h
MC4R	+707A>G	*HpyF10*VI	GCNNNNNNN**G**C	4133 4134	0.5	14 h
MC4R	+892A>G	*Bsu15*I	ATC**G**AT	3744 3745	5	4 h

*polymorphic nucleotide marked in bold

2.5.6 RNA isolation

2.5.6.1 RNA isolation from liver

Total RNA from 20 mg of RNAlater™ (Qiagen, Hilden, Germany) stabilised liver tissue was isolated using the RNeasy® Plus Mini Kit (Qiagen). Homogenisation of the tissue was achieved by processing the sample in the FastPrep® Instrument (Qbiogene, Inc, CA, USA) for 40 seconds at a speed setting of 6.5 m/s using Lysing Matrix D (Qbiogene).

2.5.6.2 RNA isolation from backfat

Total RNA from backfat was isolated from 60 mg of RNAlater™ (Qiagen) stabilised adipose tissue using the RNeasy® Lipid Tissue Mini Kit (Qiagen) with a modified protocol. Adipose tissue was homogenised in 1.3 ml RLT buffer (Qiagen) using Lysing Matrix D (Qbiogene) and the FastPrep® Instrument (Qbiogene), for 30 seconds at a speed setting of 5.0 m/s. Homogenised samples were incubated for 5 min at room temperature to promote dissociation of nucleoprotein complexes. The sample was transferred to a new reaction tube, mixed with 300 µl chloroform and incubated for 5 min at room temperature. After centrifugation at

3300 g for 15 min at 4°C the upper aqueous phase was transferred to a new reaction tube and mixed with 650 µl 96% 2-propanol. The reaction mix was incubated 2 h on ice and transferred to an RNeasy Mini Spin Coloumn. Further steps were carried out according to the manufacturer's protocol (RNeasy® Lipid Tissue Mini Handbook Step 10).

2.5.6.3 RNA isolation from skeletal muscle

Total RNA from muscle was isolated from 30 mg of RNAlater™ (Qiagen) stabilised skeletal muscle tissue using the RNeasy® Fibrous Tissue Mini Kit (Qiagen). The recommended on-column DNase digestion was omitted, since it affected negatively both RNA quality and quantity. Homogenisation of the tissue was achieved by processing the sample in the FastPrep® Instrument (Qbiogene), for 40 seconds at a speed setting of 6.0 m/s using Lysing Matrix D (Qbiogene).

2.5.6.4 RNA isolation from other tissues

Approximately 50 mg of RNAlater™ (Qiagen) stabilised spleen, kidney, brain, lung, heart and ham tissue was homogenised in 0.5 ml TRIZOL (Invitrogen, Carlsbad CA, USA) by the FastPrep® Instrument (Qbiogene, Inc, CA) using Lysing Matrix D (Qbiogene, Inc, CA). Speed setting was optimised for every tissue. The homogenate was mixed with 100 µl chloroform, incubated 10 min at room temperature and centrifuged at 12000 rpm at 4°C for 15 min. The upper RNA containing phase was transferred to a new tube. RNA was precipitated with 500 µl 2-propanol. The RNA pellet was washed three times with 75 % ethanol and dried at 37°C after the last washing step. RNA was dissolved in 30 µl RNase free water.

2.5.7 RNA quality and quantity

The integrity of total RNA was analysed on a 1.5 % denaturing, ethidium bromide stained agarose gel containing 1% formaldehyde. A total of 3 µl RNA from muscle and adipose tissue and 2 µl RNA from liver were mixed with the corresponding volume of 2X Loading Dye Solution (Fermentas, St.-Leon Roth, Germany) and denatured at 60°C for 10 min.

RNA was quantified and checked for purity by UV spectroscopy. Absorbance of a diluted RNA sample (dilution 1:100 with 10 mM Tris buffer pH 7.5) was read at 260, 280 and 320 nm by an Ultraspec® III UV/Visible Photometer (Pharmacia LKB Biotechnology, Uppsala, Sweden). The RNA concentration was calculated using the Beer-Lambert law (Equation 1), where A is the absorbance, e is the extinction coefficient of the absorbing component, c the

concentration of the absorbing component and l the path length of the spectrophotometer cuvette.

Equation 1: Beer-Lambert law

$$A = \varepsilon \cdot c \cdot l$$

The extinction coefficient e of RNA at 260 nm is 0.025 ml · µg-1 · cm-1. As a consequence one absorbance unit corresponds to a concentration of 40 mg/l. The ratio of the readings at 260 nm and 280 nm was used to estimate RNA purity. A ratio of 1.85 - 2.1 indicates highly purified RNA.

2.5.8 Synthesis of complementary DNA (cDNA)

Reverse transcription polymerase chain reaction (RT-PCR) of RNA was carried out using First Strand cDNA Synthesis Kit (Fermentas, St.-Leon Roth, Germany). Test approaches were carried out with 200 ng RNA and 200 ng random hexamer primers according to the Kit protocol (Table 4). As opposed to the Kit protocol, a mastermix was prepared that contained reaction buffer, dNTPs, Ribonuclease Inhibitor as well as M-MuLV reverse transcriptase. This mastermix was added to RNA and primers after 5 min incubation at 70°C. All cDNA reaction mixtures were incubated 10 min at 25°C, 1 h at 37°C and 10 min at 70°C. In order to improve cDNA representativeness, cDNA preparations were carried out from 200 ng RNA with random pentadecamer primers and a gene-specific primer for *PPARD* according to the protocol described by Nolan et al [88], Table 4. Finally, cDNA synthesis from liver and muscle was carried out for all samples at the same time with 1 µg RNA and 500 ng random pentadecamer primers (Table 4, optimised reaction) and used for all following analyses.

Table 4: Reverse transcription reactions

	Random hexamer (test reaction)	**Random pentadecamer** (test reaction)	**Gene- specific** (test reaction)	**Random pentadecamer** (optimised reaction)
RNA	10 mg/l	10 mg/l	10 mg/l	50 mg/l
Primer	10 mg/l	2.5 mg/l	100 nM	25 mg/l
Reaction buffer	1 x	1 x	1 x	1 x
Ribonuclease Inhibitor	1 U/µl	1 U/µl	1 U/µl	1 U/µl
dNTPs	1mM	1mM	1mM	1mM
M-MuLV	2 U/µl	2 U/µl	2 U/µl	2 U/µl

2.5.9 5' Rapid Amplification of cDNA ends (5'RACE)

The First Choice® RLM-RACE kit (Ambion Inc, Austin, TX, USA) was used to carry out 5'RACE of *PPARD* with primer 4931 as outer primer and primer 5045 for PPAR-δ_v1 and primer 5166 for PPAR-δ_v2 as inner primers for each splice variant. The resulting PCR products were separated on a 3 % agarose gel and two bands isolated by gel extraction with NucleoSpin® Extract II kit (MACHEREY-NAGEL, Düren, Germany). The two extracted PCR products were sequenced according to the standard protocol.

2.5.10 Allelic imbalance study

Standard PCRs were carried out with primer pair 3392 / 3393 from genomic DNA and with primer pair 4999 / 5045 from 1:5 diluted cDNA. The PCR products were cleaned with the help of ExoSAP-IT® (usb, Cleveland, Ohio, USA) according to the manufacturer's instructions. Sequencing was carried out using the standard protocol using with primer 5199 for both genomic DNA and cDNA. Peak heights and peak areas were either extracted by a python script from the .poly output file of Phred [81, 83, 89] or measured using the Edit View Software [90].

The allele1 / allele2 or allele1 / (allele1 + allele2) ratio was calculated for each DNA type (cDNA and genomic DNA) of heterozygous animals. Repeatability (r) was estimated using a linear mixed-effects model where the repeated measurement of peak height or peak area per animal was fitted as fixed effect within one experiment and one DNA type. P-values were obtained empirically by a permutation technique (number of permutations = 10000) via shuffling the fixed effect "DNA type" randomly across the observed ratios. For the permutation test a linear "mixed-effects" model was applied that contained the repeated

measurement of peak height or peak area per animal as fixed effect. The DNA type was included as a fixed effect as well. These computations were performed in R and required the R package "lme4" [91, 92].

2.5.11 Quantitative Real-Time PCR

Quantitative Real-Time PCR was carried out on an ABI PRISM® 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Real-Time PCR reaction consisted of Power SYBR® Green PCR Master Mix (Applied Biosystems), primers in an optimised concentration and diluted cDNA in a reaction volume of 20 µl. Optimisation of primer and cDNA template concentration according to Nolan [88] resulted in primer concentrations listed in Table 5.

Table 5: Primers used for Real-Time PCR

Gene	Forward primer (concentration in PCR)	Reverse primer (concentration in PCR)
PPARD	5278 (100 nM)	5279 (300 nM)
FASN	5066 (200 nM)	5067 (200 nM)
SCD	5353 (300 nM)	5354 (200 nM)
ACTB	5089 (300 nM)	5090 (300 nM)
GAPDH	4128 (200 nM)	4129 (200 nM)
HPRT1	5070 (300 nM)	5071 (200 nM)
TBP	5091 (200 nM)	5092 (300 nM)
TOP2B	5191 (200 nM)	5094 (500 nM)

After activation of Hot Start Polymerase by 10 min incubation at 95°C a 2-step PCR program was used consisting of 45 cycles of 15 s at 95°C and 1 min at 60°C. In case of *PPARD* annealing temperature had to be increased to 66°C to avoid primer dimers.

Crossing point (CP) and efficiency were calculated for each individual PCR reaction using ABI PRISM® 7000 SDS Software [93] and the MoBPA package in R [92, 94], respectively. For statistical analysis a modified version of the REST© (Relative Expression Software Tool) method was applied [95]. The algorithm of REST© allows group-wise comparison of relative expression data in Real-Time PCR. However, this method assumes equal amplification efficiencies in all samples. The method used here was adapted to account for differences in PCR efficiency.

Mean expression differences between different *PPARD* genotypes for all genes of interest were calculated as follows. In a first step, the expression E for each animal and each gene was determined (Equation 2).

Equation 2: $E = Efficiency^{CP}$

The geometric mean $\overline{E}_g$ of these expression values was calculated within a group of N_{gt} animals with the same genotype *gt* (Equation 3).

Equation 3: $\overline{E}_g = (\prod_{i=1}^{N_{gt}} E_i)^{1/N_{gt}}$

Finally, the mean expression of one genotype (*gt2*) was divided by the mean expression of the other genotype (*gt1*). For SNPs where 3 genotypes were present in the analysed animals the expression of one genotype (*gt1*) was compared to a pool of animals with the two other genotypes (*gt2*). The mean expression difference *R* was calculated by dividing the ratio for the gene of interest by the geometric mean of the ratios for *M* reference genes (Equation 4).

Equation 4: $R = \frac{\frac{\overline{E}_g\,(\text{target})_{gt2}}{\overline{E}_g(\text{target})_{gt1}}}{(\prod_{j=1}^{M} \frac{\overline{E}_g(\text{reference}_j)_{gt2}}{\overline{E}_g(\text{reference}_j)_{gt1}})^{1/M}}$

A mean expression difference $R = 1$ characterises no effect of the genotype on expression of the analysed gene. The significance of a derivation from $R = 1$ was estimated by a permutation technique (number of permutations = 5000). The natural logarithm of *R* was used to obtain valid p-values for a two-sided significance test, because the untransformed values of *R* are left-skewed.

2.5.12 Statistical analyses of association studies

Haplotypes were inferred using PHASE software version 2.1.1 [96, 97] with default parameters (number of iterations = 100, thinning interval = 1, burn-in = 100).
All other statistical analyses were carried out using the R language and environment for statistical computing [92]. Allele frequencies and tests for Hardy-Weinberg Equilibrium were calculated using the R package "genetics" [98].

Traits for association analyses were chosen according to the biological function of the respective gene. As a result, association of *MC4R* with feed intake, daily gain and backfat

thickness was studied. *PPARD* was tested for association with (a) backfat thickness, (b) fat thickness at the side, (c) intramuscular fat content, (d) indices and ratios characterising the fatty acid composition of meat and (e) enzyme activities [99]. The following values were calculated (Fatty acids in the equations are abbreviated by a "C" followed by the number of C atoms and the number of double bonds of the particular fatty acid separated by a colon):

Equation 5: saturated fatty acid content (SFA) [%]

$$SFA = C14:0 + C16:0 + C18:0$$

Equation 6: monounsaturated fatty acid content (MUFA) [%]

$$MUFA = C16:1 + C18:1$$

Equation 7: polyounsaturated fatty acid content (PUFA) [%]

$$PUFA = C18:2 + C18:3 + C20:4 + C20:5 + C22:4 + C22:6$$

Equation 8: elongation index (EI)

$$EI = \frac{C18:0 + C18:1}{C16:0 + C16:1 + C18:0 + C18:1}$$

Equation 9: desaturation index C16 (DI16)

$$DI16 = \frac{C16:1}{C16:0 + C16:1}$$

Equation 10: desaturation index C18 (DI18)

$$DI18 = \frac{C18:1}{C18:0 + C18:1}$$

Equation 11: polyunsaturated/saturated fatty acids ratio(PS)

$$PS = \frac{C18:2 + C18:3}{C14:0 + C16:0 + C18:0}$$

Association analyses between *MC4R* or *PPARD* genotypes on the one and 'candidate' traits on the other hand required the R package "car" for analysis of variance (ANOVA) type II [100]. A linear model was applied that besides genotype contained additional covariates. The genotype was included as a factor. For *PPARD*, the genotype was expressed as number of alleles of the tested haplotype. Covariates were gender, sire, dam and weight for all models analysed in the Mangalitsa x Piétrain population. Intramuscular fat content was added as covariable to the models for analysing fatty acid pattern. The feeding station was additionally

included as cofactor in the models for feed intake and daily gain. The model for estimating the effect of *PPARD* variants in the German Landrace population contained the performance testing station and weight as the only covariates because unrelated castrated animals were chosen. For model selection, non-significant covariates were removed from the model. At the end, p-values were estimated with the model containing only significant effects. If needed, normal distribution of residuals was established by transformation of phenotypic data. Least square means and their standard errors were calculated with untransformed data based on the respective model using the R package "effects" [101]. Outliers were detected by the Grubbs Test and removed if they were still present after transformation of the data [102, 103].

Correction for multiple testing was achieved by Bonferroni correction for association studies of *MC4R*. A multiple testing corrected significance threshold was calculated for association studies of *PPARD*. The significance thresholds of 1 % and 5 % were divided by the product of effective traits and number of tested haplotypes. The number of effective traits was calculated through correlation between the analysed traits [104].

2.5.13 Bioinformatics

The BLAST algorithm was used to screen the genomic survey sequences (GSS) division of GenBank with the porcine PPAR-δ RefSeq mRNA (NM_214152) [105, 106]. The *PPARD* gene was annotated using GenomeThreader to predict gene structure [107]. Subsequently, Apollo was used to view and edit the predicted gene model [108]. Sequences of each melanocortin receptor and peroxisome proliferator-activated receptor from different species were obtained from GenBank or Ensembl [106, 109]. Alignments were produced using the MUSCLE algorithm [110, 111] to some extend within CLC Free Workbench 3 [112]. The protein domain structure of *MC4R* and *PPARD* was annotated according to UniProtKB/Swiss-Prot database [113, 114].

Transcription factor binding sites were predicted by Cister [115], P-Match [116] and MatInspector [117]. Transcription starting site of bovine *PPARD* was predicted by Neural Network Promoter Prediction version 2.2 [118] and by Eponine Transcription Start Site Finder [119].

Prediction of mRNA secondary structure was carried out using the Mfold web server [120].

3 Results

3.1 MC4R

3.1.1 Polymorphism analysis of porcine *MC4R*

Association of the *MC4R* Asp298Asn missense mutation with fatness, growth and feed intake has been reported previously [21, 22, 32, 121]. However, none of the authors of these association studies screened the entire *MC4R* gene for variations. The genomic sequence of *MC4R* was obtained by sequencing a BAC (PigI-486E6) and the resulting sequence was submitted to gene bank (EU169096). Porcine *MC4R* (RefSeq: NM_214173) consists of one exon of 2113 bp and starts at position 5559 in the obtained genomic sequence (EU169096) and ends at position 7670. The derived cDNA sequence is to 99 % (2111 bp / 2113 bp) identical to the RefSeq. In addition to the *MC4R*, approximately 1000 bp of the 5' and 3' flanking region were screened for variations by re-sequencing the parental animals of the Mangalitsa x Piétrain resource population. Detected variants are summarised in Table 6.

Table 6: Sequence variants within *MC4R*. Nucleotide numbering is according to the nomenclature proposed by Dunnen *et al.* [122]. A description of nucleotide numbering is given in appendix 11.2.

Variant	Localisation	Function
-533-605C>T	promoter	-
-533-169Cdel	promoter	-
-135C>T	5' UTR	-
+175C>T	coding sequence	synonymous
+707A>G	coding sequence	non-synonymous His236Arg (R236H)
+892A>G	coding sequence	non-synonymous Asp298Asn (D298N)
*427A>T	3' UTR	-
*580+646A>T	3', not transcribed	-

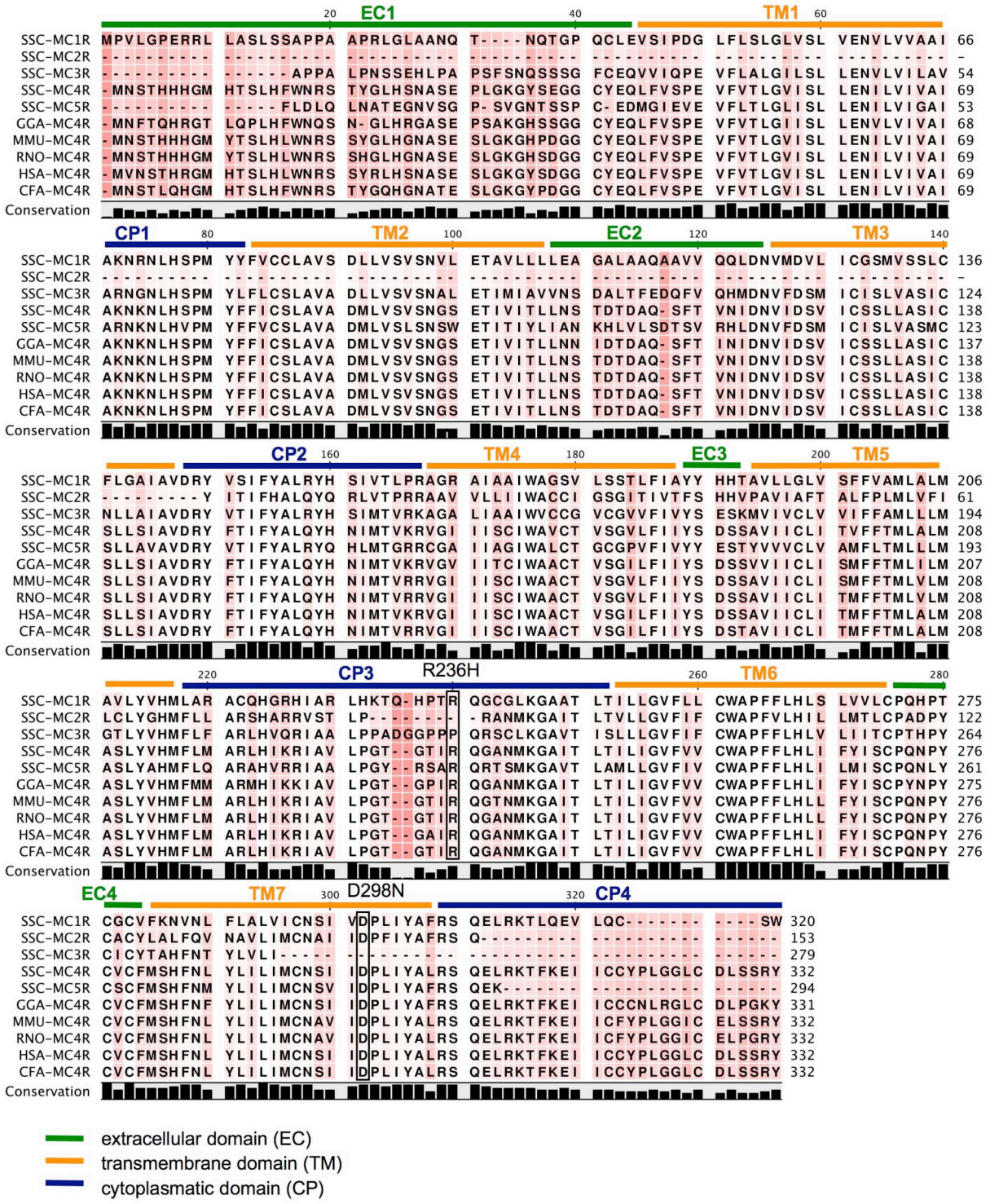


Figure 3: Multiple alignment of MCRs.

Protein sequence of porcine (SSC) MCRs 1, 2, 3, 4 and 5 were aligned with chicken (GGA), mouse (MMU), rat (RNO), human (HSA) and canine (CFA) melanocortin 4 receptor.

Two non-synonymous SNPs were detected. The +892G>A polymorphism has been described previously and was intensively studied [32, 123]. The resulting amino acid exchange is located in the seventh transmembrane domain of the heptahelical melanocortin 4 receptor that

is highly conserved over all porcine types of melanocortin receptors and many species (Figure 3). The exchange from aspartic acid to asparagine at position 298 in the amino acid sequence represents an exchange from a negatively charged, acidic residue to an uncharged (neutral) residue. The chemical structure of both amino acids is similar, since asparigine is formed by amidizing aspartic acid. The second non-synonymous SNP (+707G>A) causes an amino acid exchange from arginine to histidine at codon 236 and has been reported to occur in Vietnamese pigs [32]. Both amino acids are positively charged and for that reason basic, but differ in chemical structure. The side chain of arginine is formed by three C atoms terminated by a guanidinium group, whereas the side chain of histidine consists of an imidazole group. The Arg236His polymorphism is located in an area of the third cytoplasmatic loop where the MC4R is conserved over a variety of different species (Figure 3). The remaining sequence variants are synonymous or occurring in untranslated or untranscribed regions.

Table 7: Haplotypes of the porcine *MC4R*

Haplotype	-533-605C>T	-533-169Cdel	-135T>C	+175C>T	+707G>A	+892G>A	*427A>T	*580+646A>T
1	C	I	T	C	G	G	A	T
2	C	I	T	C	G	A	A	T
3	T	D	C	T	A	G	T	A

Out of the eight polymorphisms three haplotypes could be derived. Haplotype 1 represents the wild type. Haplotype 2 differs only in SNP +892G>A (Asp298Asn) from haplotype 1. The third haplotype comprise the second amino acid exchanging variant +707G>A, despite several other polymorphism in the untranslated and untranscribed region. No haplotype containing both amino acid exchanges was detected. All three haplotypes were present in Piétrains. The two Mangalitsas possess the wild type haplotype 1 allele.

3.1.2 Association studies

The two non-synonymous SNPs were used to tag the three *MC4R* haplotypes. Genotyping was carried out only for offspring from those Piétrain sows that possessed haplotypes 2 or 3 and that were used for breeding F2. Two sows of the F1 generation exhibited diplotype 1/2. These two sows produced 103 F2 offspring that were analysed for association of the Asp298Asn polymorphism with daily gain and feed intake. Only 97 animals were involved in the study with backfat (Table 8) because of missing backfat data for some animals. Haplotype 3 was not passed to the F1 generation of the Mangalitsa x Piétrain cross and consequently absent in the F2 generation.

Table 8: Association of the *MC4R* Asp298Asn polymorphism with feed intake, daily gain and backfat in F2 Mangalitsa x Piétrain pigs

Trait	Number of animals		LSM ± SE		*p*	p_{cor}^{*}
	Asp/Asp	Asp/Asn	Asp/Asp	Asp/Asn		
Average feed intake [g/day]	55	48	1989 ± 21	2070 ± 22	0.009	0.028
Daily gain [g/day]	55	48	539 ± 6	563 ± 6	0.008	0.025
backfat [mm]	54	43	28.6 ± 0.7	29.4 ± 0.8	0.505	1

* P-values were corrected for multiple testing by Bonferroni correction.

Average feed intake and daily gain of heterozygous Asp/Asn animals differed significantly ($p < 0.05$) from the corresponding values in homozygous Asp/Asp pigs (Table 1). Heterozygous animals ingested 81 g more feed and gained 24 g more per day than homozygous animals for the wild type allele. These results agree with the majority of association studies on *MC4R* Asp298Asn polymorphism [21, 22, 121, 123-125]. In contrast to some of those studies, however, no significant association between backfat thickness and the *MC4R* Asp298Asn polymorphism was detected, perhaps because our study population was relatively small and contained only heterozygous pigs for the Asn298 allele. However, several authors [23, 126] reported lacking evidence for an association with backfat thickness, too.

Table 9: Frequency of the *MC4R* Asp298Asn polymorphism in a Piétrain x German Landrace cross

	Number of animals				Allele frequency	
	Total	Asp/Asp	Asp/Asn	Asn/Asn	Asp	Asn
Low feed intake	47	42	5	0	0.95	0.05
High feed intake	52	42	10	0	0.90	0.10

Additionally, the *MC4R* Asp298Asn polymorphism was examined in animals of a commercial Piétrain x German Landrace cross. About 50 animals with the highest (2.05 kg/day ≤ feed intake ≤ 2.27 kg/day) and lowest (1.42 kg/day ≤ feed intake ≤ 1.68 kg/day) feed intake were chosen from a population of 13016 Piétrain x German Landrace F1 offspring, respectively. Allele frequency of the *MC4R* Asp298Asn polymorphism was determined in each group. From the previously described results the frequency of the mutant Asn298 allele is expected to be higher in animals with high feed intake. Indeed, a frequency of 0.10 in animals with high feed intake and 0.05 in animals with low feed intake was observed (Table 9). However, a Chi-Square-Test revealed no significant differences ($\chi^2 = 0.83$, $p = 0.36$) between the two groups.

Table 10: Allele frequency of two *MC4R* variants in different pig breeds

Arg236His						
	Number of animals			Allele frequency		HWE
	Arg/Arg	Arg/His	His/His	Arg	His	p
Duroc	34	0	0	1	0	-
Pietrain	39	2	0	0.98	0.02	1
Landrace	32	8	0	0.90	0.10	1
Large White	9	0	0	1	0	-
Asp298Asn						
	Number of animals			Allele frequency		HWE
	Asp/Asp	Asp/Asn	Asn/Asn	Asp	Asn	p
Duroc	0	21	16	0.28	0.72	0.07
Pietrain	38	5	0	0.94	0.06	1
Landrace	35	5	0	0.94	0.06	1
Large White	2	4	3	0.44	0.56	1

The allele frequencies of two *MC4R* variants in four pig breeds are shown in Table 10. The data suggests that the Arg236 allele is fixed in Duroc and Large White pigs. Furthermore, the mutant His236 allele is quite infrequent in the Piétrain and the German Landrace breeds. Summarising these results, variant His236 is very uncommon in German pig breeds. For that reason, it was impossible to estimate the impact of this variant on fatness, growth and meat quality traits in the available animals. The frequency of mutant Asn298 varies widely across the different pig breeds. It is relatively low in Piétrain and German Landrace. In Large White a frequency of about 56 % was estimated and in Duroc an even higher frequency of 72 % was observed. Interestingly, Piétrain exhibit the lowest and Duroc the highest daily gain within the investigated pig breeds, nearly parallel to the lowest and highest frequency of the Asn298 allele.

3.2 PPARD

3.2.1 Structure of the porcine *PPARD* gene

The genomic structure of the porcine *PPARD* was previously unknown. Therefore, it had to be determined from the porcine RefSeq mRNA sequence (NM_214152). A BLAST [105] search of the genomic survey sequences (GSS) division of GenBank with the porcine RefSeq mRNA revealed porcine genomic sequence. Additional genomic sequence data was obtained by direct sequencing of a porcine BAC (PigE-255B24). The assembly of the BAC shot gun sequences resulted in two genomic contigs containing *PPARD* that were submitted to gene bank (EU169095).

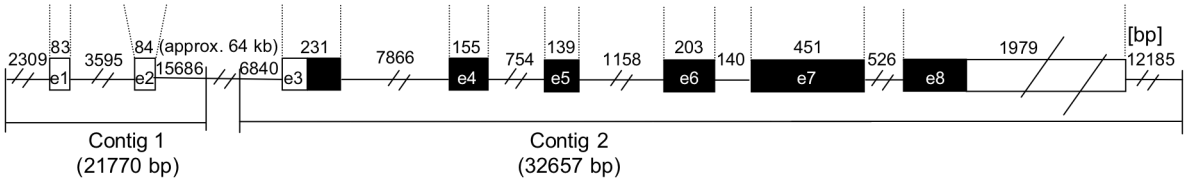


Figure 4: Exon-intron structure of *PPARD*

The resulting mRNA sequence (appendix 11.3.1) contains the full 3' UTR, and therewith sequence information that is missing in the mRNA RefSeq (NM_214152). All introns follow the GT-AG rule. The first contig comprises the putative promoter, exon 1, the complete intron 1, exon 2 and approximately 15.7 kb of intron 2. The second contig covers the region from exon 3 to exon 8 and 12.2 kb of the region 3' of the last exon. The protein-coding sequence starts in exon 3 and ends in exon 8. The derived amino acid sequence is to 94.6 % identical with the sequence of the human PPAR-δ (Figure 6).

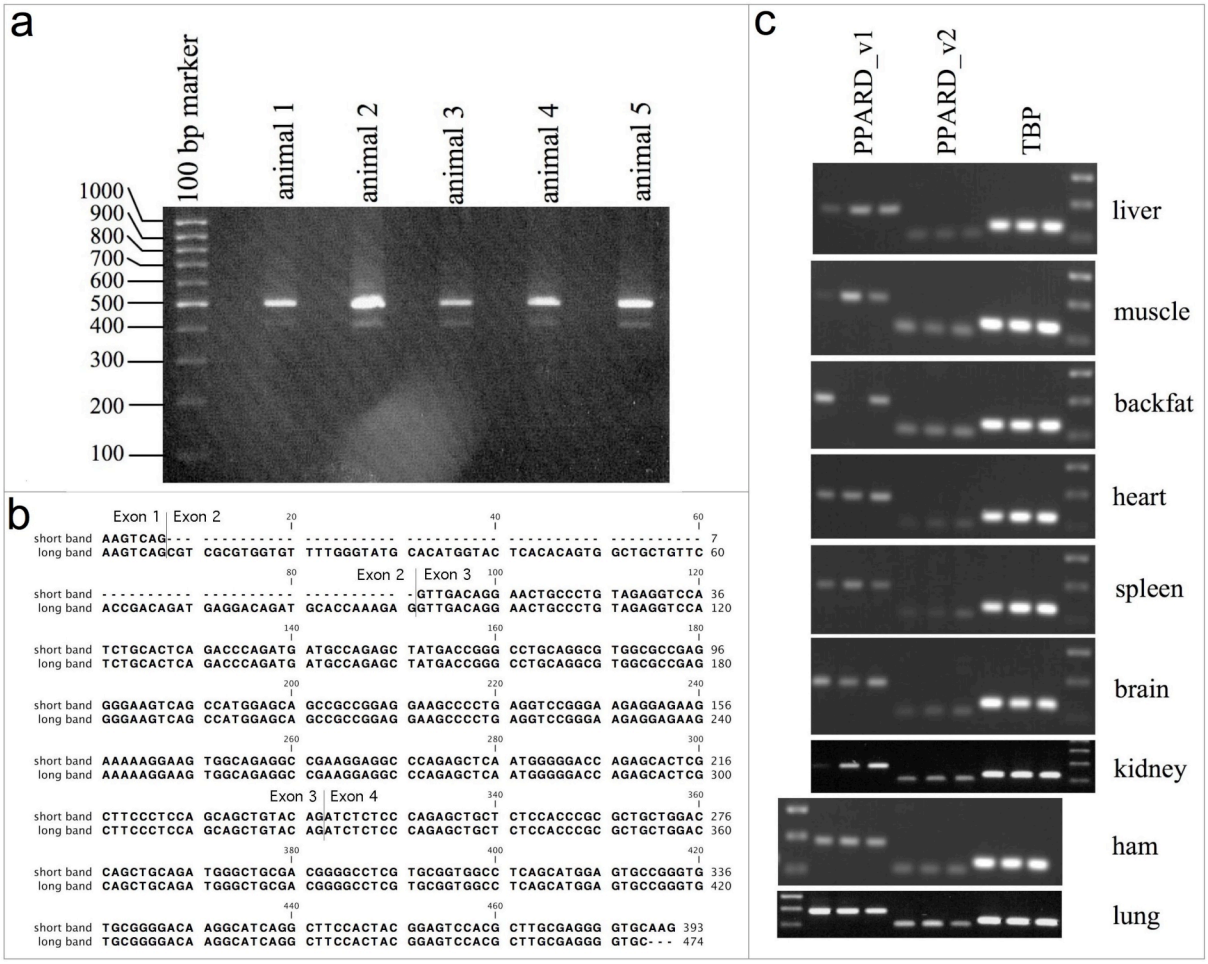


Figure 5: PPAR-δ splice variants and their expression (a) PCR product of primers 5011 and 4931, (b) Alignment of sequenced PCR products of primers 5011 and 4931, (c) Expression of PPAR-δ splice variants in various porcine tissues. Expression of PPAR-δ_v1 (primer 4999/5045), PPAR-δ_v2 (primer 4999/5166) and Tata box binding protein (*TBP*, primer 5091/5092) was analysed in three different animals. The bands lying upon each other on the right (and left side for ham and lung) of PCR products, respectively, are the 100 bp, 200 bp and 300 bp band of the 100 bp marker.

The common PPAR-δ_v1 transcript includes all eight exons. In addition, a splice variant without exon 2 was detected. PCR with the forward primer located in exon 1 (primer 5011) and the reverse primer located on the border of exons 4 and 5 (primer 4931) resulted in two PCR products (Figure 5a). Beside the expected 502-bp long PCR product, a shorter fragment was detected. The two bands were isolated by gel extraction from an agarose gel and sequenced. Resulting sequences were aligned (Figure 5b). The alignment illustrates the absence of exon 2 in the shorter PCR product. Expression of the PPAR-δ splice variants PPAR-δ_v1 and PPAR-δ_v2 was analysed with primer spanning the exon 1 / exon 2

boundary (PPAR-δ_v1) and exon 1 / exon 3 (PPAR-δ_v2) boundary, respectively. Both *PPARD* splice variants are ubiquitously expressed (Figure 5c). One animal appears to express only PPAR-δ_v2 and not PPAR-δ_v1 in backfat.

The protein of the porcine PPAR-δ is structured like a typical member of the nuclear hormone receptor superfamily [127]. It consists of a highly conserved DNA-binding domain and a C-terminal ligand-binding domain that is also conserved but less so than the DNA-binding domain (Figure 6). The core DNA-binding domain contains two highly conserved zinc finger motives that promote the binding of PPARs to the peroxisome proliferator response element (PPRE) [128]. A hinge region is located between the DNA-binding and ligand-binding domains. This hinge region permits protein flexibility and thereby enables binding of cofactors that rely on ligand-induced conformation changes.

The C-terminal domain encompasses the ligand-binding domain, a second dimerisation interface that is dependent on DNA-binding and the ligand-dependent activation function (AF-2). The third domain is the less well-defined N-terminal domain that is not conserved. Especially the three porcine peroxisome proliferator-activated receptors (PPAR) show large differences in the latter domain (Figure 6). The N-terminal domain of some nuclear receptors is responsible for ligand-independent activation of transcription. It has been shown that PPAR-γ isoforms exhibit a so-called ligand-independent activation function (AF-1), whereas PPAR-δ is lacking AF-1 [129].

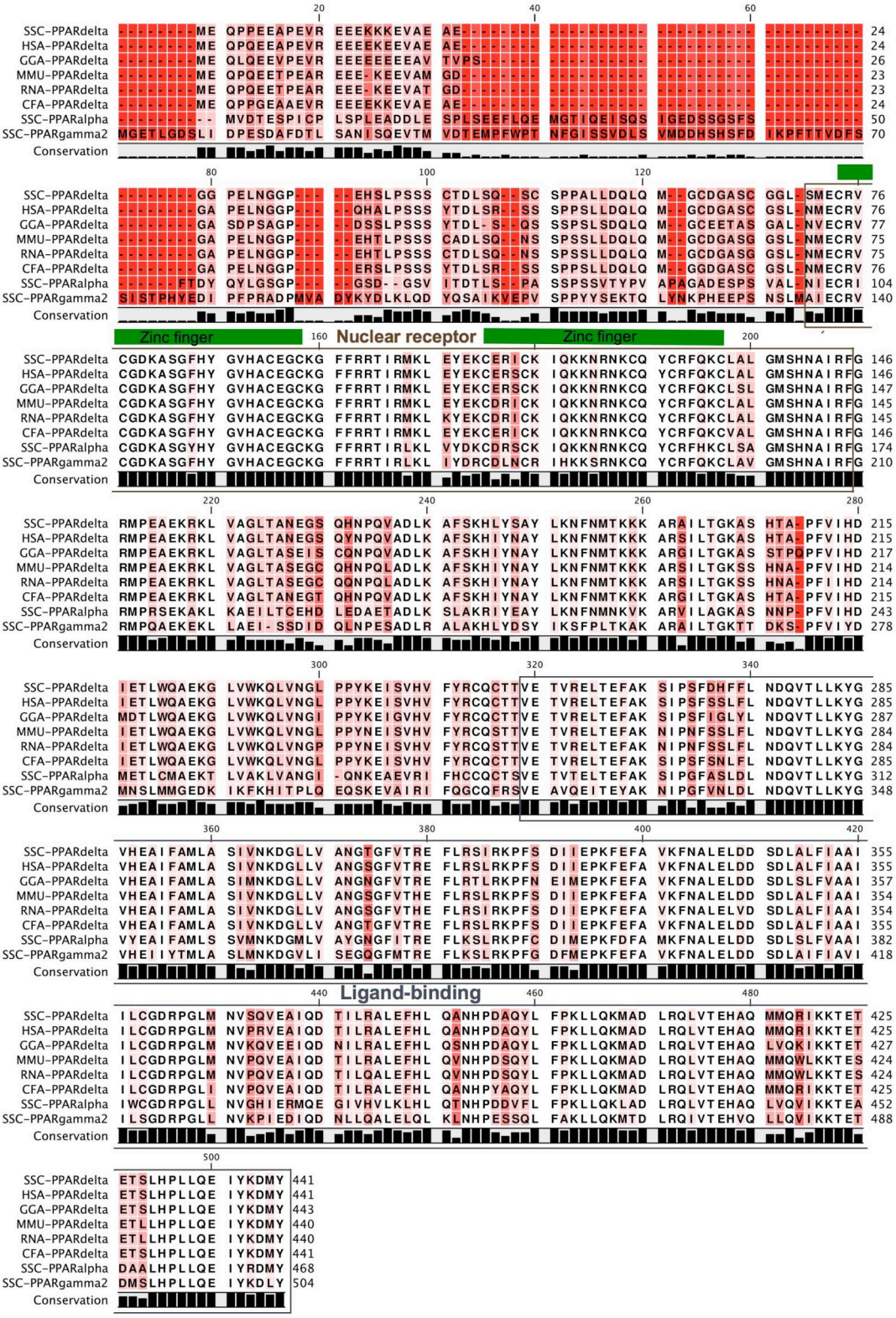


Figure 6: Multiple alignment of PPARs. Porcine (SSC) PPAR-δ, PPAR-γ2 and PPAR-α were aligned with chicken (GGA), murine (MMU), rat (RNO), human (HSA) and canine (CFA) PPAR-δ.

3.2.2 Polymorphism analysis of porcine *PPARD*

All eight exons of porcine *PPARD*, exon flanking intronic regions and 2000 bp of the putative promoter region have been screened for genetic variation by resequencing the parental generation of the Mangalitsa x Piétrain intercross and three unrelated animals of each of the German Landrace, German Large White and Duroc breeds. A total of 25 variants were identified, comprising 22 SNPs, two insertion / deletion polymorphisms and one stretch of a variable number of Cytosins (Table 11). Two out of 22 SNPs are located in the protein-coding region, but they do not affect the amino acid sequence. The number of Cytosins in the polyC stretch varied between 11 and 14 Cs in the analysed animals. However, it was impossible to determine the exact number of Cs of the polyC stretch in the 3' UTR in some heterozygous animals. For that reason, only 24 polymorphisms were used to infer haplotypes.

A total of five haplotypes were detected with haplotypes 4 and 5 being identical at 23 of 24 polymorphisms (Table 12). Both founding Mangalitsa boars of the resource population were homozygous for haplotype 1. The haplotype frequencies were 17 %, 46 %, 17 %, 8 % and 12 % for haplotype 1, 2, 3, 4 and 5, respectively, in the twelve Piétrain parental animals. Four SNPs (polymorphisms -281—244A>C, -159G>A, +768G>A and +1137G>T) are sufficient to tag the five haplotypes.

Table 11: Sequence variants within *PPARD*

Nucleotide numbering is according to the nomenclature proposed by Dunnen *et al.* [122]. A description of nucleotide numbering is given in appendix 11.2.

Variant number	Variant	Localisation	Predicted effect
1	-281-1377A>G	Promoter	-
2	-281-889A>G	Promoter	-
3	-281-689A>G	Promoter	-
4	-281-686C>T	Promoter	-
5	-281-244A>C	Promoter	affects putative ETS-domain TFBS
6	-185-118A>G	Intron 1	-
7	-180C>T	Exon 2 (5' UTR)	minor effect on mRNA structure
8	-159G>A	Exon 2 (5' UTR)	minor effect on mRNA structure
9	+130+25A>G	Intron 3	-
10	+285+107C>T	Intron 4	-
11	+285+516A>G	Intron 4	-
12	+424+367C>T	Intron 5	-
13	+425-181delA	Intron 5	-
14	+425-47C>T	Intron 5	-
15	+425-19A>G	Intron 5	-
16	+425-6A>G	Intron 5	-
17	+627+28C>T	Intron 6	-
18	+627+42_47delCCCT	Intron 6	-
19	+768G>A	Exon 7 (coding)	synonymous, minor effect on mRNA structure
20	+1137G>T	Exon 8 (coding)	synonymous, minor effect on mRNA structure
21	*1305G>T	Exon 8 (3' UTR)	minor effect on mRNA structure
22	*1478C>T	Exon 8 (3' UTR)	no effect on mRNA structure
23	*1515C>T	Exon 8 (3' UTR)	no effect on mRNA structure
24	*1584C>T	Exon 8 (3' UTR)	minor effect on mRNA structure
25	*1575_1588delCCCC	Exon 8 (3' UTR)	minor effect on mRNA structure

Table 12: *PPARD* haplotypes

Haplotye	-281-1377A>G	-281-889A>G	-281-689A>G	-281-686C>T	-281-244A>C	-185-118A>G	-180C>T	-159G>A	+130+25A>G	+285+107C>T	+285+516A>G	+424+367C>T	+425-181delA	+425-47C>T	+425-19A>G	+425-6A>G	+627+28C>T	+627+42_47delCCCT	+768G>A	+1137G>T	*1305G>T	*1478C>T	*1515C>T	*1584C>T
1	A	G	A	C	A	A	C	G	G	T	G	C	I	T	A	A	C	D	G	G	G	C	T	T
2	A	G	A	C	A	A	C	G	G	T	G	C	I	C	G	A	C	D	G	T	G	T	C	C
3	A	A	G	C	A	G	C	A	A	T	G	T	I	C	G	A	C	D	G	T	T	C	T	C
4	G	G	G	T	C	G	T	A	G	C	A	C	D	C	G	G	T	I	G	T	G	C	T	C
5	G	G	G	T	C	G	T	A	G	C	A	C	D	C	G	G	T	I	A	T	G	C	T	C

Tag SNPs were used to estimate allele frequencies of the observed haplotypes in different economically import breeds. Frequencies of *PPARD* haplotypes vary greatly among the German Large White, Piétrain, German Landrace and Duroc breed (Figure 7). Haplotype 1 (43 %) is the most important haplotype in Piétrain. Haplotype 2 (44 %) and 4 (37 %) are the most frequent haplotypes in German Landrace pigs. The Large White (55 %) and Duroc (62 %) breeds exhibit haplotype 4 as most common haplotype. Haplotype 5 is very rare or absent in all investigated breeds. Haplotype 3 occurs most often in Piétrain (20 %) and does not occur in Duroc. Haplotype 4 and 5 are absent in Meishan (data not shown).

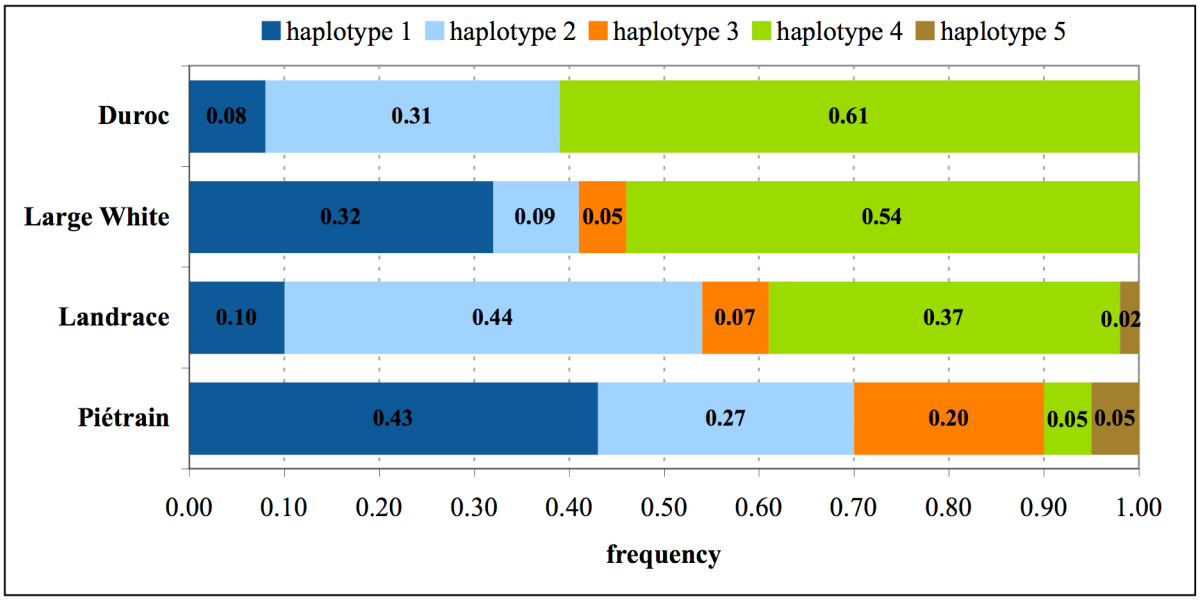


Figure 7: Frequency of *PPARD* haplotypes in different pig breeds

3.2.3 Association studies

Haplotype 4 was not passed on to the F1 generation of the Mangalitsa x Piétrain resource population and was consequently absent in the F2 generation. Therefore, SNPs -281-244A>C, -159G>A and +1137G>T were sufficient to distinguish the remaining four haplotypes and were used to genotype 599 F2 animals by diagnostic restriction enzyme assays.

Table 13: *PPARD* haplotype and diplotype distribution of analysed F2 Mangalitsa x Piétrain animals. The '-' and '+' signs mark absence and presence of the particular haplotype, respectively.

Haplotype	Number of animals		
	-/-	+/-	+/+
1	97	280	222
2	300	247	52
3	522	77	-
5	553	46	-

Diplotype	Number of animals
1/1	222
1/2	202
1/3	51
1/5	27
2/2	52
2/3	26
2/5	19

PPARD was chosen as a candidate gene for this thesis because it is located in a QTL region for backfat on chromosome 7. Therefore, association analyses of *PPARD* variants and backfat were carried out and revealed a significant association between *PPARD* haplotype 5 and backfat thickness ($p = 0.022$, Table 14) in the Mangalitsa x Piétrain cross. Furthermore, a suggestive association with the fat depth at the side and haplotype 3 was detected ($p = 0.079$, Table 14). Both associations are not significant after correction for multiple testing. Nevertheless, heterozygous animals carrying one haplotype 5 allele showed a backfat thickness increase by 2.43 mm. However, the effect of haplotype 5 allele cannot be reliably estimated, because just 46 animals carried haplotype 5 while 553 pigs did not carry haplotype 5 (Table 14).

Table 14: Association of *PPARD* haplotypes with lipid deposition and fatty acid metabolism Multiple testing corrected significance thresholds: 5 % - 0.006, 1% - 0.0003
The '-' and '+' signs mark absence and presence of the particular haplotype, respectively.

Trait	Trans formation	Covariates	Out liers	Haplo type	Haplotype LS means (SE)			P-value
					-/-	+/-	+/+	
backfat thickness middle [mm]	^0.75	gender, mother, weight	-	1	29.50 (0.56)	29.29 (0.32)	29.16 (0.37)	0.902
				2	29.30 (0.33)	29.14 (0.35)	29.80 (0.81)	0.744
				3	29.38 (0.24)	28.57 (0.72)	-	0.311
				5	29.09 (0.23)	31.52 (1.00)	-	0.022
fat depth at the side [mm]	-	gender, mother, father, weight	-	1	42.36 (0.85)	42.84 (0.48)	42.92 (0.57)	0.861
				2	42.70 (0.50)	42.75 (0.53)	43.54 (1.22)	0.814
				3	43.06 (0.36)	41.01 (1.07)	-	0.079
				5	42.82 (0.34)	42.51 (1.48)	-	0.842
intramuscular fat content [%]	log	gender, father, mother	-	1	2.06 (0.06)	2.06 (0.04)	2.07 (0.04)	0.802
				2	2.08 (0.04)	2.05 (0.04)	2.04 (0.09)	0.493
				3	2.06 (0.03)	2.08 (0.08)	-	0.656
				5	2.06 (0.03)	2.12 (0.11)	-	0.623
saturated fatty acid content [%]	-	mother, father, gender, imf, imf^2, imf^3	< 30 > 60	1	36.98 (0.14)	36.90 (0.09)	36.89 (0.10)	0.836
				2	36.89 (0.09)	36.87 (0.10)	37.16 (0.20)	0.364
				3	36.93 (0.07)	36.74 (0.17)	-	0.286
				5	36.89 (0.07)	37.09 (0.23)	-	0.414
mono unsaturated fatty acid content [%]	-	imf, imf^2, imf^3	< 30	1	48.11 (0.22)	48.22 (0.15)	48.35 (0.15)	0.597
				2	48.39 (0.14)	48.06 (0.16)	48.24 (0.30)	0.186
				3	48.22 (0.12)	48.50 (0.25)	-	0.269
				5	48.27 (0.11)	48.03 (0.31)	-	0.458
poly unsaturated fatty acid content [%]	square root	mother, father, imf, imf^2, imf^3	-	1	13.68 (0.26)	13.53 (0.16)	13.40 (0.17)	0.669
				2	13.37 (0.15)	13.70 (0.17)	13.55 (0.36)	0.274
				3	13.48 (0.13)	13.59 (0.30)	-	0.727
				5	13.51 (0.12)	13.32 (0.42)	-	0.659
elongation index [%]	-	gender, mother, father, imf, imf^2,	< 45	1	66.48 (0.11)	66.41 (0.07)	66.32 (0.08)	0.484
				2	66.32 (0.07)	66.45 (0.07)	66.53 (0.16)	0.335
				3	66.40 (0.05)	66.33 (0.14)	-	0.667
				5	66.38 (0.05)	66.45 (0.19)	-	0.731
desaturation index C16	-	mother, father, imf, imf^2, imf^3	-	1	14.25 (0.14)	14.53 (0.09)	14.91 (0.10)	0.0001
				2	14.84 (0.09)	14.46 (0.09)	14.13 (0.19)	0.0004
				3	14.65 (0.07)	14.59 (0.17)	-	0.746
				5	14.67 (0.07)	14.25 (0.23)	-	0.080
desaturation index C18	log	mother, father, imf, imf^2, imf^3	> 50	1	79.18 (0.17)	79.36 (0.11)	79.41 (0.12)	0.508
				2	79.44 (0.11)	79.27 (0.12)	79.15 (0.24)	0.373
				3	79.32 (0.09)	79.54 (0.21)	-	0.348
				5	79.37 (0.09)	79.15 (0.29)	-	0.462
ps ratio	-	mother, father, gender, imf, imf^2, imf^3	> 0.5 > 2.2	1	1.67 (0.01)	1.68 (0.01)	1.68 (0.01)	0.751
				2	1.68 (0.01)	1.68 (0.01)	1.67 (0.02)	0.752
				3	1.68 (0.01)	1.69 (0.01)	-	0.439
				5	1.68 (0.01)	1.65 (0.02)	-	0.095

Intramuscular fat content was not found to be associated with any of the *PPARD* haplotypes. Association of *PPARD* variants with fatty acid compositions in muscle was analysed because PPAR-δ modulates fatty acid synthesis in the liver as well as fatty acid oxidation in muscle. Haplotypes 1 and 2 are significantly associated with desaturation index C16 even after correcting for multiple testing (Table 14). While haplotype 1 increases desaturation index C16, haplotype 2 decrease this index. The presence of haplotypes 1 and 2 are inversely correlated (Table 13). This could explain the association of both haplotypes. Desaturation index C16 refers to the proportion of palmitoleic acid compared to the total amount of C16 fatty acids in muscle. Desaturation index C16 reflects the activity of delta-9 desaturase that catalyse the reaction from palmitic acid to palmitoleic acid. The same enzyme is required for transformation of stearic acid to oleic acid. The desaturation index for this reaction (desaturation index C18) is not significantly influenced by any *PPARD* variant (Table 14). However, a small increase in desaturation index C18 can be observed in presence of haplotype 1 as well as a decrease in presence of haplotype 2 in accordance with the trend in desaturation index C16.

A second association study was carried out in order to confirm or refute the hypothesis of association between backfat thickness and haplotype 5. The selection of a suitable study population was problematical because haplotype 5 is infrequent in all analysed pig populations (Figure 7). The highest frequency is observed in Piétrain and is estimated at 5% (Figure 7). Nevertheless, haplotype 4 is identical to haplotype 5 at all but SNP +768G>A (Table 12) and haplotype 4 is frequent in German Landrace, German Large White and Duroc (Figure 7). German Landrace was chosen because it was expected to exhibit both a relative high frequency of haplotype 4 and possibly a few animals carrying haplotype 5. A total of 722 animals were genotyped. However, the number of animals for which haplotypes could be derived was reduced to 681 due to weak PCR products. The allele frequency were 10 %, 44 %, 7%, 37 % and 2% for haplotypes 1, 2, 3, 4 and 5, respectively. None of the tag SNPs deviates from Hardy-Weinberg equilibrium.

A significant association of *PPARD* haplotype 4 and backfat thickness was observed ($p = 0.034$). However, haplotype 4 decreases backfat thickness while haplotype 5 in the Mangalitsa x Piétrain population increases backfat thickness (Figure 8). Haplotype 5 has no significant effect on backfat thickness ($p = 0.401$). However, the very low allele frequency of haplotype 5 reduces markedly the power to detect an association. Nonetheless, the mean

backfat thickness of pigs carrying haplotype 5 is higher (mean backfat thickness = 2.12 ± 0.10 cm) than in pigs carrying no haplotype 5 (mean backfat thickness = 2.06 ± 0.01 cm).

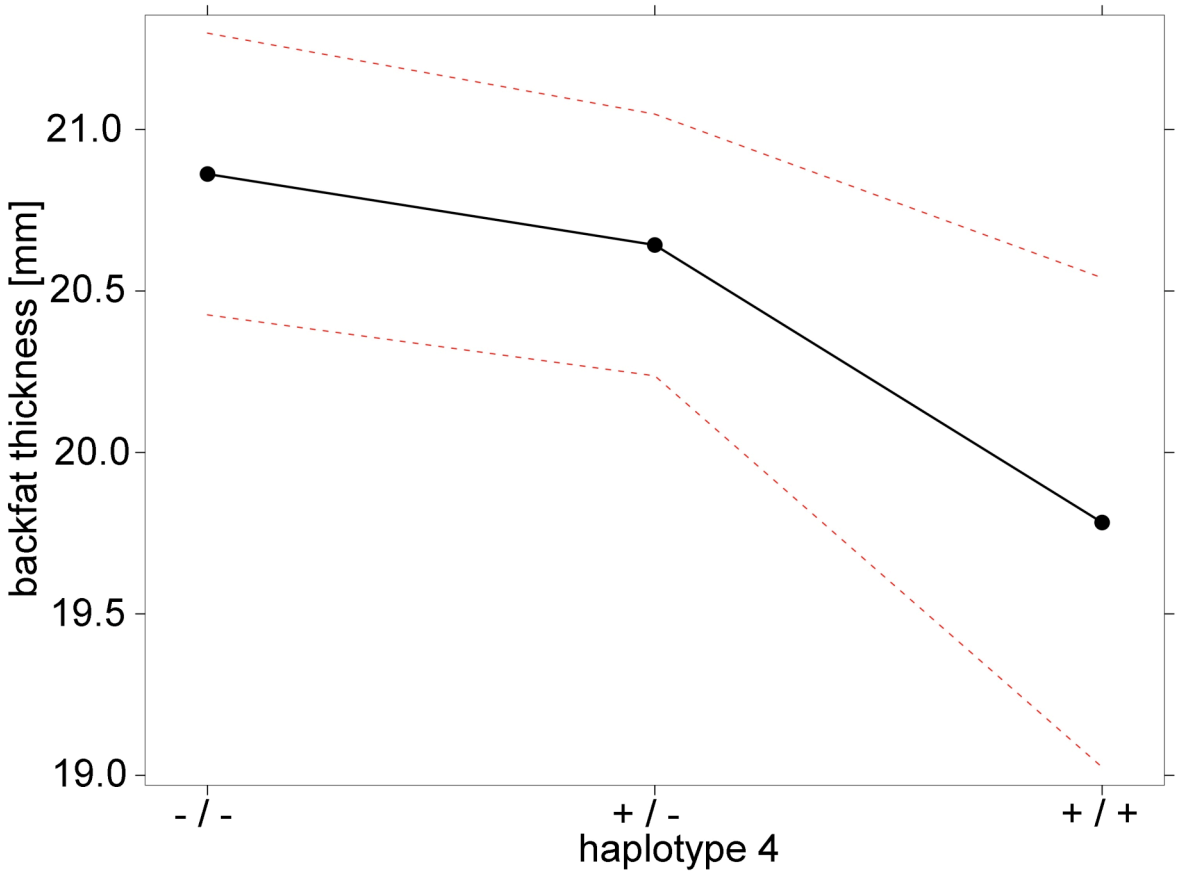


Figure 8: Effect of haplotype 4 on backfat thickness in a German Landrace population
Dashed lines indicated 95 % confidence interval. Solid line marks least square means.

The two association studies provide evidence for association between *PPARD* variants with backfat thickness and desaturation index C16. However, it is unclear if the observed associations are caused by a variation of *PPARD* or a causal mutation in linkage disequilibrium to *PPARD*. None of the SNPs located in the coding region cause an amino acid exchange. For this reason, there is no obvious functional candidate for the observed association. However, numerous studies have identified cis-regulatory and synonymous mutations with functionally significant consequences for morphology, physiology and behaviour [130-133]. Consequently, the function of SNPs located in the putative promoter region, the effect of exonic SNP on mRNA structure and changes in codon usage due to coding SNPs were analysed.

3.2.4 mRNA secondary structure of *PPARD* variants

A total of eight SNPs and one variable polyC stretch were identified in exons. The effect of these polymorphisms on the mRNA secondary structure was predicted by means of the RNA MFold software through a web interface [120]. Secondary structures of mRNAs corresponding to the five porcine *PPARD* haplotypes were compared with each other on the basis of the predicted structure with the least energy because this structure is the most stable and therefore hypothesised to be the naturally occurring structure.

Table 15: Gibbs free energy (ΔG) for full length mRNA corresponding to the five porcine *PPARD* haplotypes

Haplotype	ΔG [kcal/mol]
1	-1360.99
2	-1360.66
3	-1356.43
4	-1356.91
5	-1354.49

Gibbs free energy for full length mRNA of haplotype 1 is only 6.5 kcal/mol less than for haplotype 5 (Table 15). Differences in Gibbs free energy between haplotype 5 and haplotypes 2, 3, 4 are lower than 6.5 kcal/mol (Table 15). Therefore, the differences in Gibbs free energy for full length mRNA are small. However, these numbers are not informative with respect to the effect single SNPs to local secondary structures. In oversimplified picture differences in Gibbs free energy of haplotypes could be seen as adding up the effect of single SNPs on local structures. However, SNPs, which lie in close proximity interact with each other and cannot be approximated as being independent. Therefore, the effect of SNPs was estimated within local structures which were identified from prediction for full length mRNA and exhibit one or more SNP (Figure 9).

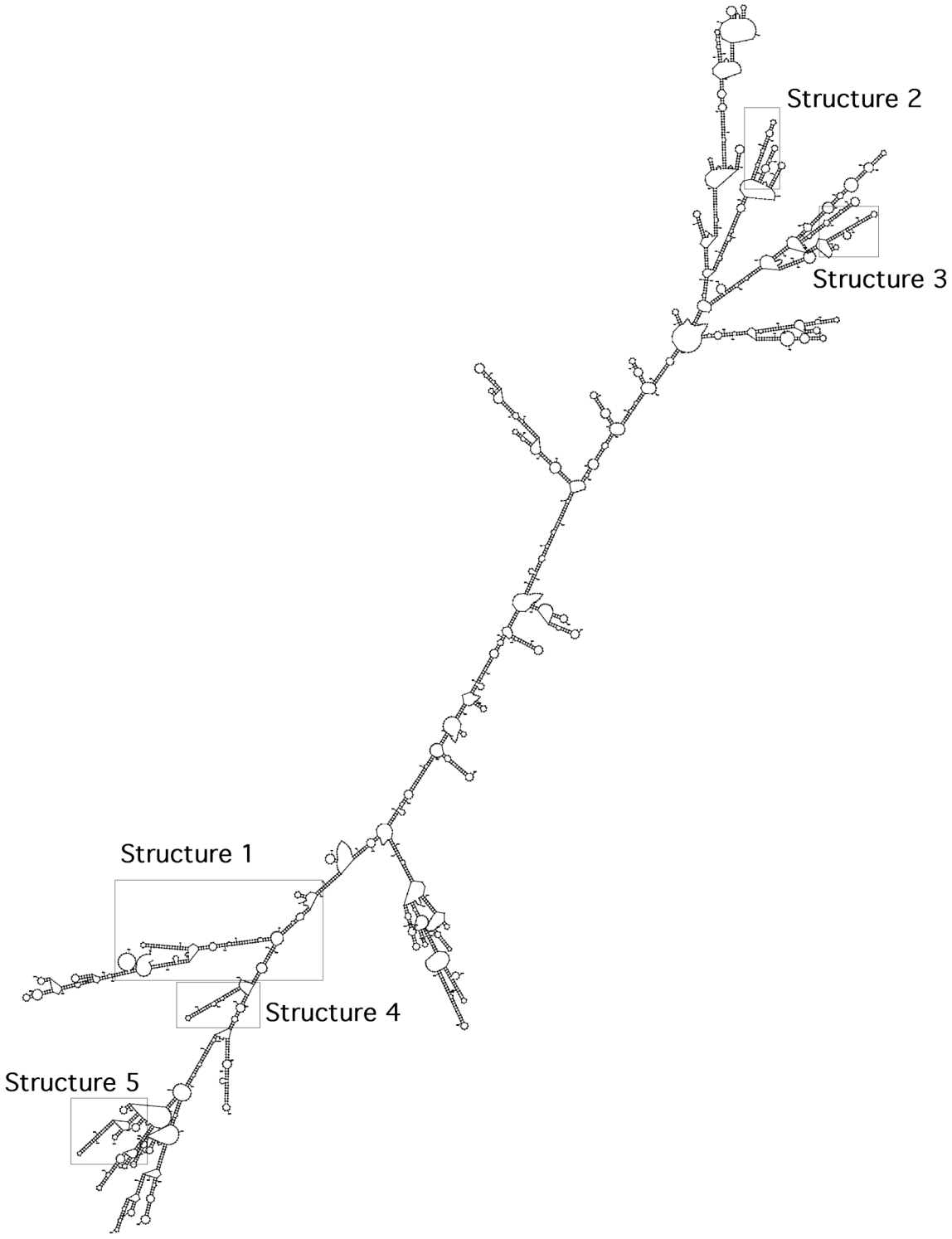
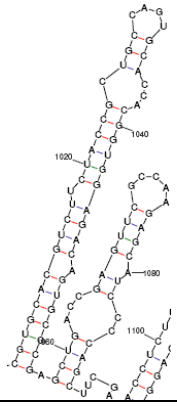
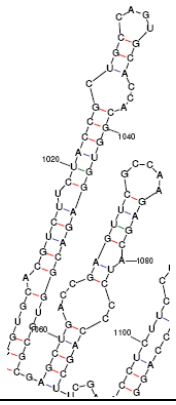
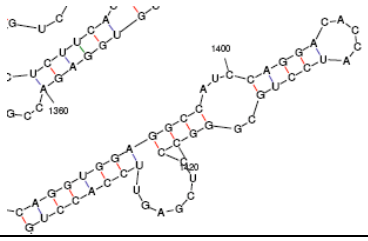
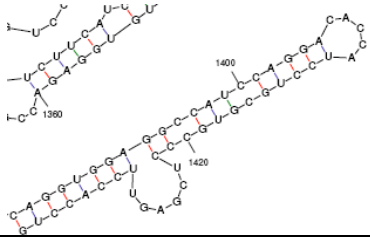
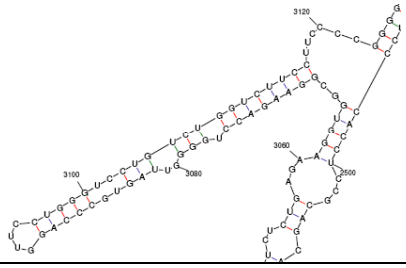
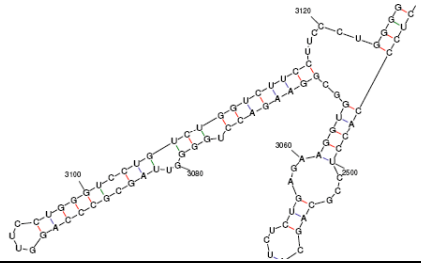
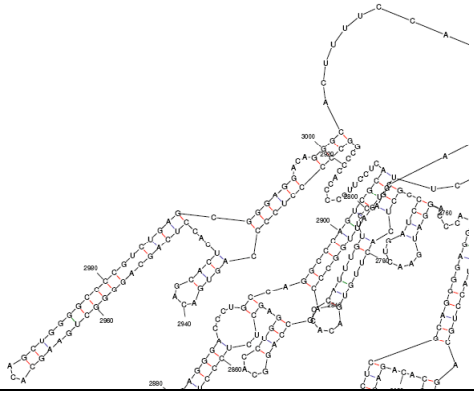
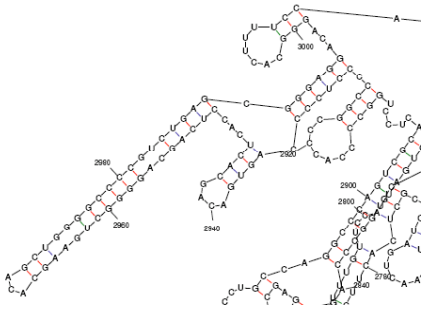


Figure 9: Full length mRNA secondary structure

Local secondary structures that are affected by SNPs are marked and numbered. Structure 1 is affected by SNPs -180C>T, -159G>A, *1584C>T and *1575_1588delCCCC, structure 2 by SNPs +768G>A, structure 3 by SNP +1137G>T, structure 4 by SNPs *1478C>T and *1515C>T and structure 5 by SNP *1305G>T.

Table 16: Local mRNA secondary structures affected by SNPs

Structure (SNPs)	Base pairs	ΔG [kcal/mol]					dΔG [kcal/mol]
1 (-180C>T, -159G>A, *1584C>T)	1-147, 2449-2490, 3128-3338	HT 1	HT 2	HT 3	HT 4	HT 5	
		-156.5	-158.9	-158.3	-155.7	-155.7	
2 (+768G>A)	974-1157		HT 5		HT 1, 2, 3, 4		2.4
			-74.9		-77.3		
							
3 (+1137G>T)	1203-1464		HT 1		HT 2, 3, 4, 5		-3.3
			-91.8		-88.5		
							
4 (*1478C>T *1515C>T)	3068-3123		HT2		HT 1, 3, 4, 5		-0.5
			-28.3		-27.8		
							
5 (*1305G>T)	2815-3017		HT 3		HT 1, 2, 4, 5		3.1
			-85.8		-88.9		
							

Local mRNA structures are slightly affected or not affected by individual SNPs and neighboring SNPs corresponding to haplotypes, respectively (Table 16). The thermal energy at body temperature is approximately 0.6 kcal/mol. Energy differences in the order of the thermal energy cannot be distinguished. Hence, SNPs *1478C>T and *1515C>T have no effect on mRNA structure. The SNPs responsible for structure 1, 2, 3 and 5 have minor effects on secondary structure. The biggest impact on mRNA structure has SNP +1137G>T (3.3 kcal/mol) and SNP *1305G>T (3.1 kcal/mol). SNP +1137G>T is tag SNP of haplotype 1 that is associated with desaturation index C16. SNP +1137G>T is tag SNP of haplotype 3. No significant association between haplotype 3 and any phenotypic trait was observed. Compared to results from literature a difference in Gibbs free energy of approximately 3 kcal/mol is very small. Nackley *et al.* demonstrated significant differences in a local stem-loop structure of 17 kcal/mol leading to an alteration in protein expression of Catechol-O-Methyltransferase [133]. It is, therefore, highly unlikely that the differences observed in porcine PPAR-δ mRNA structure affect mRNA stability.

3.2.5 Codon Usage

Nearly all amino acids are encoded by more than one base triplet. Most species show preference for one of the codons that encode a specific amino acid. Particularly, differences in the third codon position result in large differences in codon usage [134]. Synonymous polymorphisms inserting a rare codon can alter translation efficiency and protein folding [132].

Table 17: Changes in codon usage caused by two synomynous SNPs of *PPARD*
Values for codon usage were obtained from the codon usage database [135, 136]. Codon usage is stated as fraction of the observed codon within all codons for the particular amino acid and as relative synonymous codon usage (RSCU). RSCU values are the number of times a particular codon is observed, relative to the number of times that the codon would be observed in the absence of any codon usage bias. The RSCU value would be 1.00 in case of uniform usage of synonymous codons.

SNP	Codon 1	Codon 2	Amino acid	Codon 1 usage (RSCU)	Codon 2 usage (RSCU)
+768G>A	ACG	ACA	Thr	0.14 (0.56)	0.23 (0.92)
+1137G>T	CGG	CGT	Arg	0.21 (1.21)	0.08 (0.48)

SNPs +768G>A and +1137G>T could potentially affect codon usage due to their location in the coding sequence. The codon ACA which is produced by the rare A allele of SNPs +768G>A is used more frequently than the ACG in pigs. The T allele of SNP +1137G>T is inserting a rare codon for arginine. The value for relative synonymous codon usage (RSCU) of 0.48 indicates a frequency of less than 50 % as expected in absence of any codon usage bias [136].

SNP +768G>A

codon	TTC	TAC	CGC	TGC	CAG	TGC	ACC
freq / 1000	24.5	19.3	12.0	14.6	34.9	14.6	22.6

+768G>A

codon	ACG	GTG	GAG	ACG	GTG	CGC	GAG
freq / 1000	7.7	33.0	41.0	7.7	33.0	12.0	41.0
				ACA			
				12.4			

codon	CTG	ACC	GAG	TTC	GCC	AAG	AGC
freq / 1000	46.0	22.6	40.9	24.5	31.6	33.5	19.9

SNP +1137G>T

codon	CAG	GTG	GAG	GCC	ATC	CAG	GAC
freq / 1000	34.9	33.0	41.0	31.6	25.0	34.9	28.3

+1137G>T

codon	ACC	ATC	CTG	CGG	GCC	CTC	GAG
freq / 1000	22.6	25.0	46.0	11.6	31.6	23.2	41.0
				CGT			
				4.1			

codon	TTC	CAC	CTG	CAG	GCC	AAC	CAC
freq / 1000	24.5	15.7	46.0	34.9	31.6	22.2	15.7

Figure 10: Codon usage in regions surrounding SNPs +768G>A and +1137G>T. Ten codons upstream and downstream of the particular SNP and their frequency per 1000 codons are displayed.

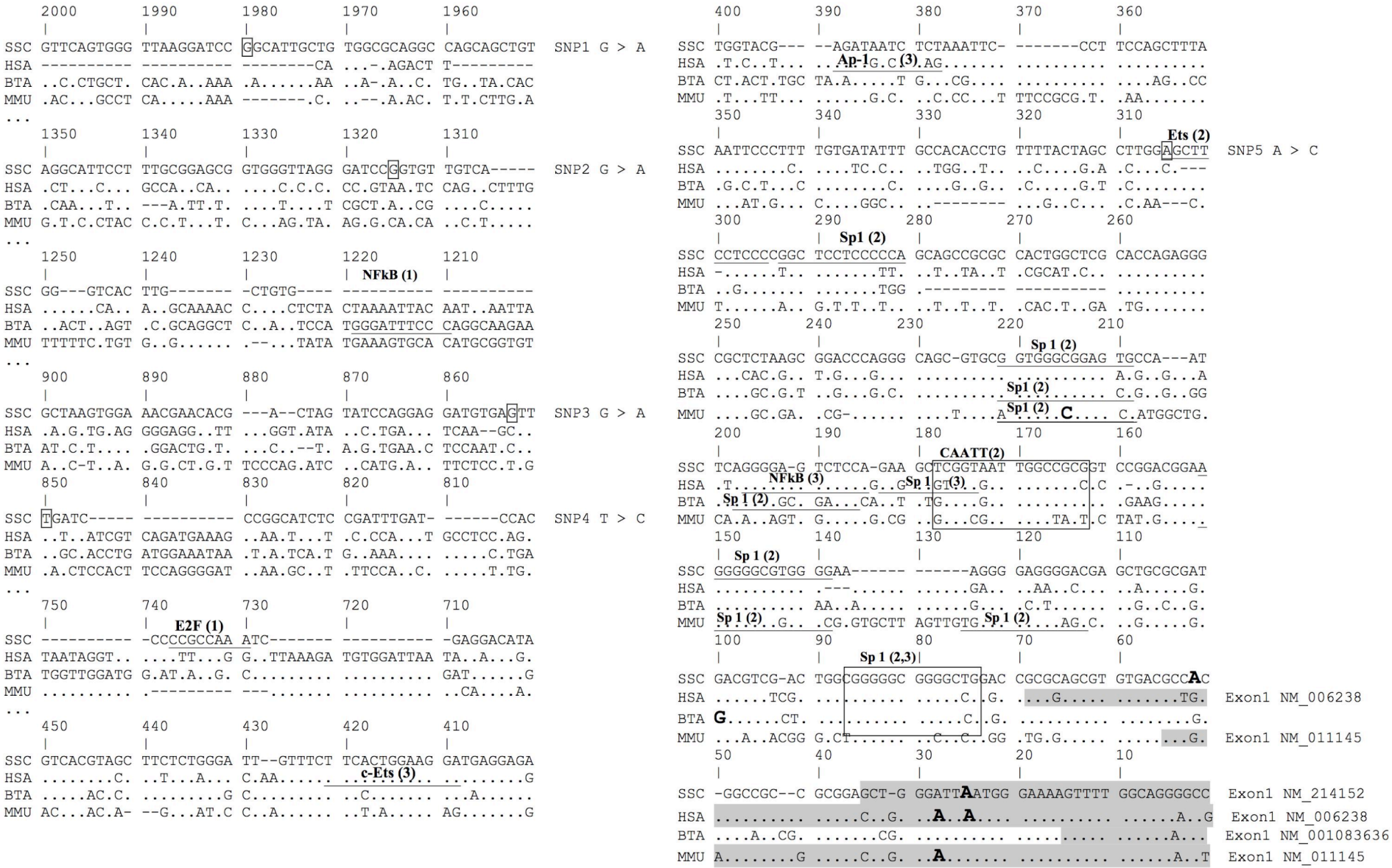
It was hypothesised that when frequent codons are changed to rare codons in a cluster of infrequently used codons, the timing of cotranslational folding is affected and may result in altered function [132]. Both SNPs are not located in a cluster of infrequent codons and both

codons of each of the two SNPs are infrequent compared to the surrounding codons. This makes a marked effect of codon usage on PPAR-δ cotranslational folding highly unlikely. An effect on translation efficiency cannot be excluded.

3.2.6 Analysis of the *PPARD* promoter region

Identification of potential transcription factor binding sites (TFBSs) was attempted by finding conserved sequences in the corresponding porcine, human, bovine and murine *PPARD* promoter and applying bioinformatic tools for TFBS prediction. The transcription start site (TSS) for both porcine *PPARD* splice variants was determined in liver by 5' RACE. Nested PCR with the inner primers for each splice variant resulted in two PCR products with difference in length of approximately 30 bp in each case. The two bands were isolated by gel extraction and sequenced with gene-specific primers. Both *PPARD* splice variants exhibit two TSSs at the same position (Figure 11). The TSSs are 27 bp away from each other. However, the sequence had a strong background, indicating that there are more, probably minor TSSs. More than 10 human TSS can be found in the database of transcriptional start sites (DBTSS) [137]. One of the two main TSS is located at orthologous position to one of the porcine TSS. The second main TSS is only 3 bp away from this site.

Figure 11: Partial alignment of *PPARD* promoter (see next page). About 2000 bp upstream of the porcine (SSC), human (HSA), bovine (BTA) and murine (MMU) *PPARD* RefSeq were aligned. Only conserved regions or regions containing porcine SNPs are displayed. In the alignment dots represent similarity, minus represents gaps and letters represent sequence differences compared to the porcine sequence. Transcription factor binding sites (TFBSs) are underlined; a box marks TFBSs at orthologous positions in all four species. TFBSs are shown as detected by P-Match (1) and Cister (2), or published by Skogsberg (3) [40]. A bold letter indicates the transcription start site (TSS). Porcine and murine TSS were identified by RACE [41]. Human TTS are displayed according to DBTSS [137]. The TSS in BTA is shown as predicted by the Neural Network Promoter Prediction [118]. SNPs are numbered by variant number (Table 11).


2000 1990 1980 1970 1960
SSC GTTCAGTGGG TTAAGGATCC GGCATTGCTG TGGCGCAGGC CAGCAGCTGT SNP1 G > A
HSA ---------- ---------- --------CA ...-.AGACT T---------
BTA ..C.CTGCT. CAC.A..AAA .A......AA ..A-.A..C. TG..TA.CAC
MMU .AC...GCCT CA.....AAA -------.C. ..--.A.AC. T.T.CTTG.A
1350 1340 1330 1320 1310
SSC AGGCATTCCT TTGCGGAGCG GTGGGTTAGG GATCCGGTGT TGTCA----- SNP2 G > A
HSA .CT...C... GCCA..CA.. ....C.C.C. CC.GTAA.TC CAG..CTTTG
BTA .CAA...T.. ---A.TT.T. ....T....T CGCT.A..CG ....C.....
MMU G.T.C.CTAC C.C.T...T. C...AG.TA. AG.G.CA.CA ..C.T.....
1250 1240 1230 1220 1210
NFkB (1)
SSC GG---GTCAC TTG------- -CTGTG---- ---------- ----------
HSA ......CA.. A..GCAAAAC C....CTCTA CTAAAATTAC AAT..AATTA
BTA ..ACT..AGT .C.GCAGGCT C..A..TCCA TGGGATTTCC CAGGCAAGAA
MMU TTTTTC.TGT G..G..... .--...TATA TGAAAGTGCA CATGCGGTGT
900 890 880 870 860
SSC GCTAAGTGGA AACGAACACG ---A--CTAG TATCCAGGAG GATGTGAGTT SNP3 G > A
HSA .A.G.TG.AG GGGAGG..TT ...GGT.ATA ..C.TGA... TCAA--GC..
BTA AT.C.T.... .GGACTG.T. ...C..--T. A.G.TGAA.C TCCAAT.C..
MMU A..C-T..A. G.G.CT.G.T TCCCAG.ATC ..CATG.A.. TTCTCC.T.G
850 840 830 820 810
SSC TGATC----- ---------- CCGGCATCTC CGATTTGAT- ------CCAC SNP4 T > C
HSA ..T..ATCGT CAGATGAAAG ..AA.T...T .C.CCA...T GCCTCC.AG.
BTA ..GC.ACCTG ATGGAAATAA .T.A.TCA.T G..AAA.... .....C.TGA
MMU .A.CTCCACT TCCAGGGGAT ..AA.GC..T .TTCCA..C. .....T.TG.
750 740 730 720 710
E2F (1)
SSC ---------- -CCCCGCCAA ATC------- ---------- -GAGGACATA
HSA TAATAGGT.. ....TT...G G..TTAAAGA TGTGGATTAA TA..A...G.
BTA TGGTTGGATG G.AT.A..G. C......... .... GAT......G
MMU ....--------- ---..... ....CA....A.
450 440 430 420 410
SSC GTCACGTAGC TTCTCTGGGA TT--GTTTCT TCACTGGAAG GATGAGGAGA
HSA ....C. ..T...A... C.AA..... ..c-Ets (3).. ....G
BTA .....AC.C. ....G C......... ..C..... ...A......
MMU AC...AC.A- --G...AT.C C..A..... ..T.A..... AG.......G
400 390 380 370 360
SSC TGGTACG--- --AGATAATC TCTAAATTC- -------CCT TCCAGCTTTA
HSA .T.C..T... ..Ap-1G.C(3).AG..... ....
BTA CT.ACT.TGC TA.A.....T G...CG.... ....AG..CC
MMU .T...TT... ......G.C. ..C.CC...T TTCCGCG.T. .AA.....
350 340 330 320 310
Ets (2)
SSC AATTCCCTTT TGTGATATTT GCCACACCTG TTTTACTAGC CTTGGAGCTT SNP5 A > C
HSA ....C. ....TC.C.. ..TGG..T.. ..C....G.A .C...C.---
BTA .G.C.T...C ....C. ....G..G.. .C.....G.T .C.......
MMU ...AT.G... C....GGC.. ..-------- ...G..C... .C.AA---C.
300 290 280 270 260
Sp1 (2)
SSC CCTCCCCGGC TCCTCCCCCA GCAGCCGCGC CACTGGCTCG CACCAGAGGG
HSA -......T.. ....TT. ..T..TA..T .CGCAT.C.. ....
BTA ..G..... ....TGG .--------- ---------- ....
MMU T......A.. G.T.T..T.. ..T..T..T. .CAC.T..GA .TG.....
250 240 230 220 210
Sp 1 (2)
SSC CGCTCTAAGC GGACCCAGGG CAGC-GTGCG GTGGGCGGAG TGCCA---AT
HSA ...CAC.G.. T.G...G... .... A.G..G...A
BTA ....GC.G.T ..G...G.C. .....Sp1 (2)..... C.G..G..GG
MMU ....GC.GA. .CG-..... ....T....A.....Sp1 (2).C.... C.ATGGCTG.
200 190 180 170 160
CAATT(2)
SSC TCAGGGGA-G TCTCCA-GAA GCTCGGTAAT TGGCCGCGGT CCGGACGGAA
HSA .T..... .....G..G NFkB (3) Sp 1 GT(3).G.. .....C.C .-..G.....
BTA .Sp 1 (2).GC. GA...CA..T .TG....G.. ..... .GAAG.....
MMU CA.A..AGT. G.....G.CG ..G...CG.. ....TA.T.C TAT.G.....
150 140 130 120 110
Sp 1 (2)
SSC GGGGGCGTGG GGAA------ ------AGGG GAGGGGACGA GCTGCGCGAT
HSA .....Sp 1 (2)..... .---..... .....GA.. ..AA..C... ..A.....G.
BTA ..... AA..A..... .....G... .C.T..... ..G..C..G.
MMU Sp 1 (2)..G.. .CG.GTGCTT AGTTGTG.Sp 1 (2)...AG.C. ..G.....G.
100 90 80 70 60
Sp 1 (2,3)
SSC GACGTCG-AC TGGCGGGGGC GGGGCTGGAC CGCGCAGCGT GTGACGCCAC
HSA ......TCG. ....C..G. ...G..... .....TG. Exon1 NM_006238
BTA G......CT. ....C..G. ....G.
MMU ...A..ACGG G.CT..... ..C..C..GG .TG.G..... .....G. Exon1 NM_011145
50 40 30 20 10
SSC -GGCCGC--C GCGGAGCT-G GGATTAATGG GAAAAGTTTT GGCAGGGGCC Exon1 NM_214152
HSA ....C..G. ..A..A.... ....A..G Exon1 NM_006238
BTA ....A..CG. .....CG. .... Exon1 NM_001083636
MMU A.....G .....C..G. ..A..... ....A..T Exon1 NM_011145

PPARD expression in mouse is controlled by four alternative promoters [41]. The main promoter is orthologous to the human and porcine promoter and has multiple TSS. The preferred TSS in mouse is located about 190 bp away from the first TSS in pigs (at position 215 in Figure 11). A minor TSS is located at orthologous positions to the human and porcine TSS. There are no reports about experimental determined TSS in cattle. Hence, the TSS of the bovine *PPARD* was estimated by the Neural Network Promoter Prediction (NNPP) version 2.2 [118] and by Eponine Transcription Start Site Finder [119] based on the preliminarily annotated draft sequence encompassing the *PPARD* gene. Eponine predicted a TSS at 104 bp and NNPP at 100 bp (Figure 11). Alignment of orthologous *PPARD* promoter and comparison of identified TSS shows the existence of multiple TSS in human, mouse and pig. The TSSs are distributed over a region of approximately 200 bp upstream of exon 1 of the particular RefSeq. Nevertheless, a 4 bp section located downstream of the 5' end of mRNA reference sequence contains TSSs of human, mouse and pig.

Previous reports of the human *PPARD* promoter lack evidence for the existence of a TATA box [40]. Analyses using Cister [115] and P-Match [116] also failed to detect a TATA box in all four species. TATA-less promoters are typically not highly conserved [138], hence it is not unexpected that TSSs are not present at orthologous positions in different species. A pyrimidine-rich initiator sequence (INR) that is usually situated at the TSS and that defines a specific transcription start even in GC-rich promoters is absent in the *PPARD* promoter [139]. A 373-bp-long CpG island that extends from the promoter to intron1 was detected by EMBOSS CpGPlot [140]. Promoters within a CpG island typically lack a TATA box and other core promoter elements and are often characterised by the presence of multiple TSS [139]. CpG-rich promoters are frequently found in ubiquitously expressed genes.

A common feature of CpG-rich promoters is the presence of multiple binding sites for transcription factor Sp1 located 40-80 bp upstream of the TSS [139]. Four Sp1 binding sites were identified in the porcine *PPARD* promoter. One of the SP1 binding sites and the CAAT box are both conserved. However, both are located downstream from the preferred murine TSS. In addition, the preferred murine TSS is located in a predicted Sp1 binding site. Nonetheless, this site shows high homology between the different species suggesting an important function due to either a TSS or a TFBS. Additionally, one binding site for an ETS-domain transcription factor and one for an E2F transcription factor were predicted in the porcine *PPARD* promoter (Figure 11). Of the five SNPs detected in the promoter region, only

SNP -281-244A>C (SNP 5 in Figure 11) is located in a predicted TFBS, namely an ETS-domain binding site (Figure 11). Prediction by Cister showed allele C to have a higher matching score than allele A and suggests a possible effect of the polymorphism on the transcription of *PPARD*.

3.2.7 Allelic Imbalance Studies

3.2.7.1 Background

As shown, SNP -281-244A>C of haplotype 4 and 5 possibly affects the binding of an ETS-domain transcription factor in the *PPARD* promoter. This could lead to allelic differences in PPAR-δ mRNA expression. SNP -180C>T lies in complete linkage disequilibrium with SNP -281-244A>C and is located in exon 2 of the PPAR-δ_v1 mRNA. Thus, SNP -180C>T can be employed to investigate the effect of the promoter SNP -281-244A>C on PPAR-δ mRNA expression. For that reason, sequencing of genomic DNA and cDNA of heterozygous carrier of haplotype 4 or 5 was carried out. Heterozygous carriers of haplotype 4 or 5 are consequently heterozygous carrier of both above-mentioned SNPs. In case of no allelic differences in gene expression the ratios of allele peak areas or heights are equal for both alleles of SNP -180C>T in genomic DNA and cDNA. A deviation of the C/T ratio of cDNA from that of genomic DNA would characterise allelic imbalance.

3.2.7.2 Optimisation of quantitative sequencing

Preliminary analysis showed a deviation of the C/T ratio of cDNA from that of DNA, however, there was no consistent trend. In some cases the C/T ratio of cDNA was higher than that of DNA, while in a few cases was observed. To test if these observations reflect a heterogenic expression of the two *PPARD* alleles or are due to experimental errors, further experiments were designed. Duplicates in cDNA synthesis, PCR and sequencing were carried out and revealed highly fluctuating C/T ratios from cDNA (Figure 12). In contrast, the C/T ratios of replicates from genomic DNA are very stable indicating that the cDNA synthesis is particularly error prone (Figure 13). Therefore, an optimisation of cDNA sythesis was carried out.

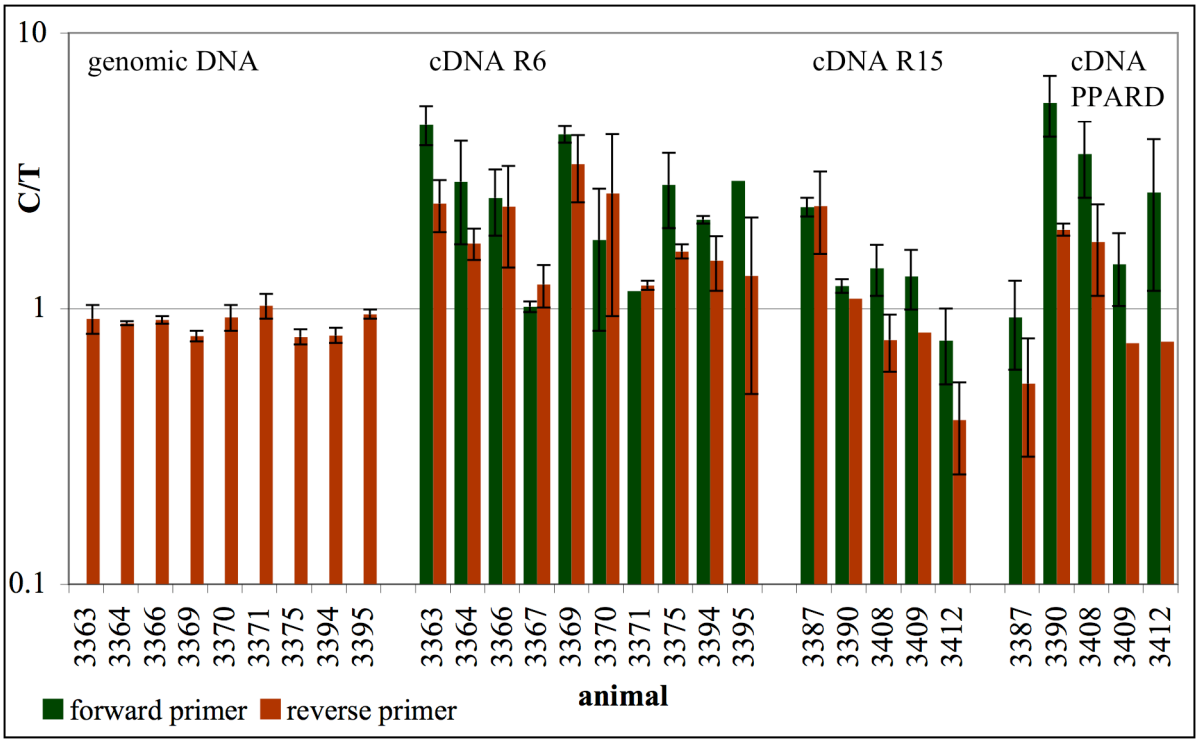


Figure 12: Repeatability of quantitative sequencing of SNP -180C>T in genomic and cDNA. PCR products from genomic and cDNA were sequenced with forward and reverse primers in duplicate. The cDNA was synthesised applying random hexamer (R6), random pentadecamer (R15) and gene-specific primer for *PPARD* (*PPARD*). C/T ratios of SNP -180C>T were calculated on the basis of allele peak area. Height of bars indicate mean value of C/T ratio, error bars show difference between the two measurements. The y-axis is log scaled.

In the preliminary studies, the cDNA synthesis was carried out with random hexamer primers. Technical replicates of cDNA synthesis with random pentadecamer primers as well as gene-specific primers for *PPARD* were carried out. PCR and sequencing of the cDNA was carried out in parallel to DNA replicates (Figure 12). Results indicate a higher repeatability of cDNA synthesis initiated with random pentadecamer ($r = 0.50$) or gene-specific primers ($r = 0.54$) than with random hexamer primers ($r = 0.31$). Studies from Stangegaard et al [141] showed cDNA priming with random pentadecamer primers increases cDNA yield as well as quality and suggested a better coverage of the transcriptome by random pentadecamer primers. The experiments presented here agree with this publication and in addition suggest no important advantage of gene-specific cDNA priming for studying allelic imbalance in PPAR-δ expression. Thus, cDNA priming for the final allelic imbalance study was carried out using random pentadecamer primers. Furthermore, the RNA input amount was increased with the

aim to enhance repeatability.

In addition, the accuracy to measure peak heights was improved for the final analysis by using the software package EditView [90], which allows measurement of peak heights at the peak of each individual allele and not on the same location for both peaks as carried out by Phred [81, 83]. Measuring the height of both allele peaks at the highest point of the highest peak is a major disadvantage if the peaks are shifted (Figure 13, SNP2). For that reason, the peak area was measured in the preliminary studies. Analysis of the peak area of several samples indicated that in some cases the lower peak is wider (Figure 13, SNP1). This may lead to nearly equal peak areas, even if significant differences in peak heights are visible.

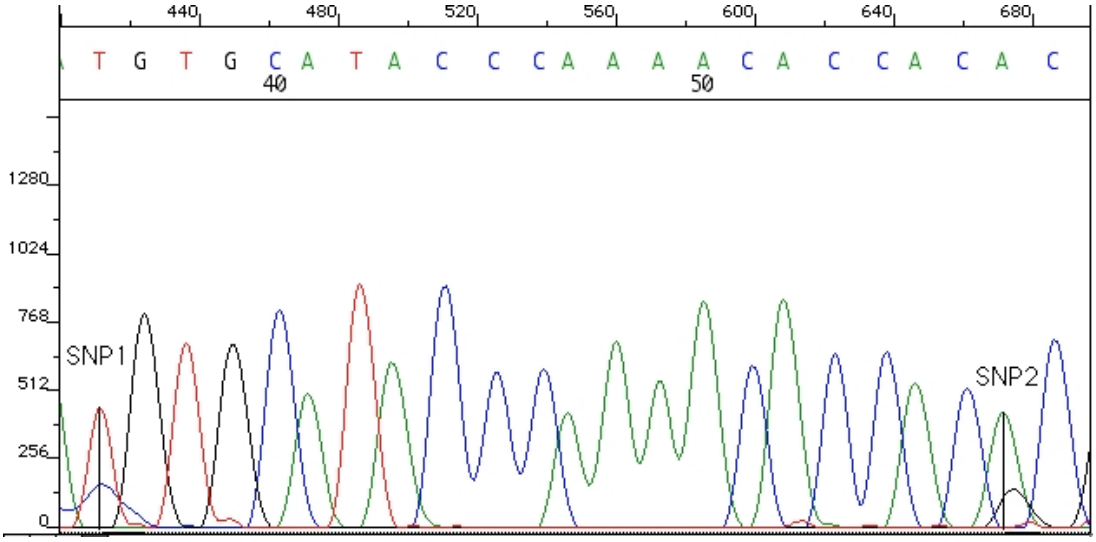


Figure 13: Measurement of peak heights. Allele peaks of SNP1 are at the same position whereas allele peaks of SNP2 are shifted.

The analysis was improved with the calculation of the C/(C+T) ratio so that the ratio is normalised between 0 and 1. Furthermore, a second SNP was included in the calculation. The SNP -159G>A is located 21 bp downstream of SNP -180C>T and linked to SNP -281-244A>C, too. Haplotype 4 and 5 possess the T allele at SNP -180C>T and the A allele at SNP -159G>A (Figure 14). Haplotype 3 exhibits also the A allele at SNP -159G>A. This results in less animals that can be analysed for allelic imbalance of SNP -159G>A than for SNP -180C>T, because animals carrying diplotypes 3/4 and 3/5 are not heterozygous for SNP -159G>A in contrast to SNP -180C>T (Figure 14). For SNP -159G>A the G/(A+G) ratio was calculated because it equals the C/(C+T) ratio.

haplotypes

haplotye	-281-244A>C	-180C>T	-159A>G
1	A	C	G
2	A	C	G
3	A	C	A
4	C	T	A
5	C	T	A

diplotypes 1/4, 1/5, 2/4, 2/5

-281-244A>C	-180C>T	-159A>G
A	C	G
C	T	A

diplotypes 3/4, 3/5

-281-244A>C	-180C>T	-159A>G
A	C	A
C	T	A

Figure 14: Linkage between SNPs involved in allelic imbalance study

SNP -281-244A>C is located in the *PPARD* promoter and potentially affects an ETS-domain transcription factor binding site. SNPs -180C>T and -159G>A are located in exon 2 and were used to undermine the effect of SNP -281-244A>C on allelic expression of PPAR-δ.

3.2.7.3 Analysis of allelic imbalance

Reactions were carried out twice for cDNA and once for genomic DNA (Figure 15). The repeatability of the calculated ratios for cDNA is 0.49 for SNP -159G>A and 0.42 for SNP -180C>T. Combining the two SNPs and defining them as repetition yielded in a repeatability of r = 0.43. In case of SNP -180C>T a significant alteration in C/(C+T) ratio between genomic DNA and cDNA is observed, whereas no significant alteration in G/(G+A) ratio is observed for SNP -159G>A. This result is unexpected, because ratios of both SNP should be completely linked because they are sharing the same haplotype. The discrepancy could be due to the high variability of G/(G+A) ratios within genomic DNA in case of SNP -159G>A that is reflected by a high standard error of the mean (Table 18).

Table 18: Statistics of allelic imbalance study

		SNP -159G>A	SNP -180C>T	Joint analysis
cDNA	Min / max ratio	0.289 / 0.605	0.314 / 0.638	0.289 / 0.638
	Mean ratio (SE)	0.422 (0.009)	0.463 (0.008)	0.444 (0.006)
	Repeatability	0.49	0.42	0.43
Genomic DNA	Min / max ratio	0.351 / 0.533	0.411 / 0.562	0.351 / 0.562
	Mean ratio (SE)	0.412 (0.079)	0.506 (0.005)	0.464 (0.057)
Permutated p-value		0.630	0.0004	0.038

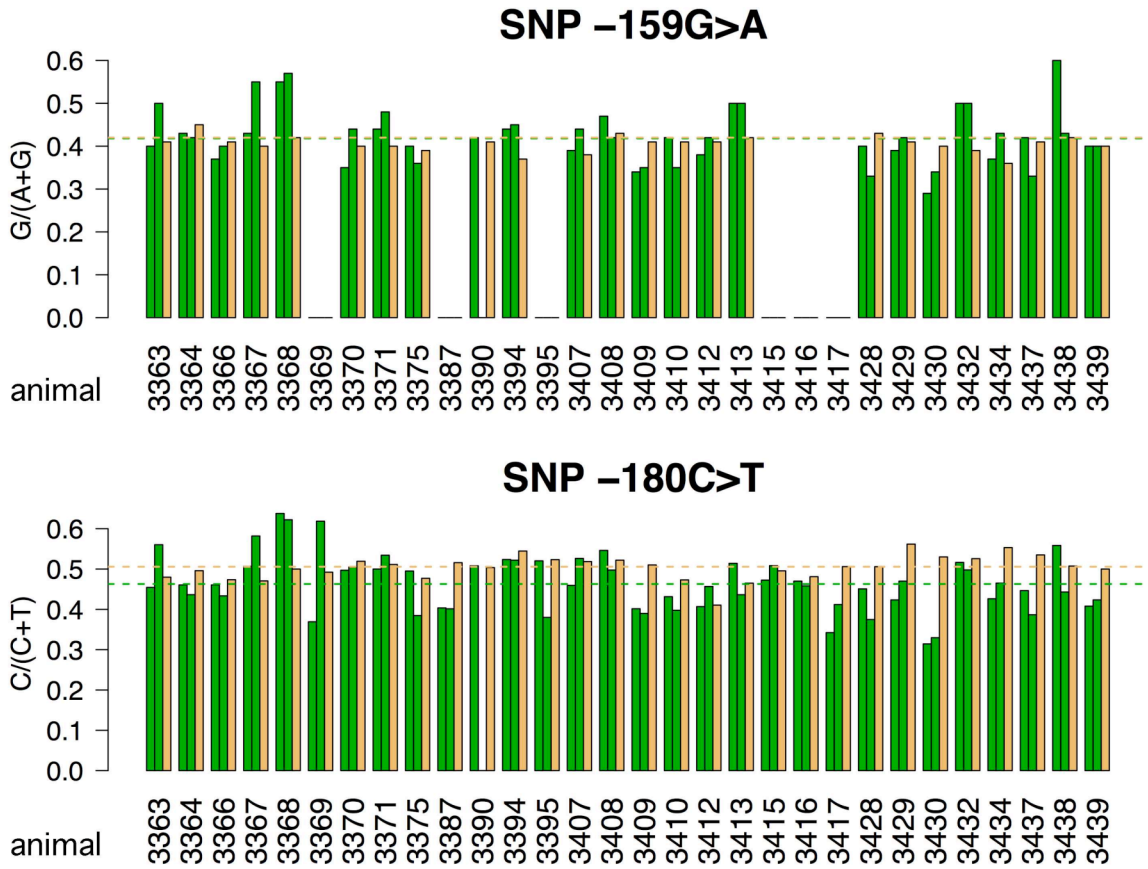


Figure 15: Allelic Imbalance of the -159G>A and -180C>T polymorphisms of *PPARD*. Ratios were calculated on the basis of peak heights measured with EditView. Green and yellow bars represent cDNA and genomic DNA, respectively. Haplotypes 4 and 5 exhibit the A allele at SNP -159G>A and the T allele at SNP -180C>T.

A combined analysis of both SNPs was carried out to circumvent the problem of the disagreement and resulted in a significant p-value ($p = 0.038$). This supports the assumption of an allelic imbalance within the *PPARD* haplotypes. However, the observed effect is very small. Pastinen suggests a ratio difference of 0.1 as limit for allelic imbalance [142]. This limit is reached only by a small numbers of animals suggesting incomplete allelic imbalance. However, since *PPARD* promoter is TATA-less no strong expression differences were expected. A ratio difference of 0.05 was defined as evidence for allelic imbalance in this study and the number of animals exceeding the threshold was counted for all diplotypes (Table 19). The majority of animals showing allelic imbalance at SNP -180C>T exhibit diplotype 1/5 (Table 19). In contrast, only one animal with diplotype 1/4 possesses allelic imbalance. If SNP -281-244A>C would affect *PPARD* expression, animals with both diplotypes should exhibit allelic imbalance to the same extend. Since this is not true, it is

highly unlikely that -281-244A>C changes expression of PPAR-δ. However, the study provides evidence for allelic differences in expression of PPAR-δ haplotype 1 and 5 possibly through a different mechanism than alteration of transcription factor binding sites. The C/(C+T) ratio of cDNA was decreased in 8 of 12 animals with diplotype 5, suggesting either enhanced expression of haplotype 5 or decreased expression of haplotype 1.

Table 19: Number of animals showing allelic imbalance at SNP -180C>T
Animals with a difference of 0.05 in C/C+T ratio between genomic and cDNA were defined to exhibit allelic imbalance, if both cDNA duplicates exceeded the threshold. The C allele corresponds to haplotype 1, 2 and 3, respectively. The T allele corresponds to haplotype 4 and 5, respectively.

	Number of animals per diplotype			
	1/4	1/5	2/4	3/4
Total	10	12	6	6
Genomic DNA(C/(C+T)) > cDNA(C/(C+T))	1	8	0	2
Genomic DNA(C/(C+T)) < cDNA(C/(C+T))	0	0	1	0

3.2.8 Expression of PPAR-δ and two potential target genes

The expression of PPAR-δ variants was investigated in liver. In order to test the function of the *PPARD* variants existent, a gene expression experiment was carried out. Since PPAR-δ acts as transcription factor, expression of PPAR-δ target genes should be linked to the presence of a particular *PPARD* variant, if this variant is functional.

Association studies with *PPARD* haplotypes provide evidence for association with backfat thickness and desaturation index C16. PPAR-δ has been shown to up-regulate several enzymes responsible for fatty acid and triglyceride synthesis in the murine liver [50]. One of these genes is the fatty acid synthase (*FASN*). A porcine BAC clone (CH242-192P8) of this gene was sequenced and provided the necessary sequence information for promoter analysis and primer design. MatInspector [117] identified two potential peroxisome proliferative response elements for PPAR-δ in the *FASN* promoter. For that reason, FASN expression was investigated for association with *PPARD* variants. Gene expression analysis of *FASN* was carried out in liver tissue because it was shown that liver is the most responsive tissue to

PPAR-δ agonists [50]. A total of 56 animals were included in this analysis.

Desaturation index C16 depends on the delta-9 desaturase activity in muscle. Stearoyl - CoA desature 1 (SCD1) exhibits delta-9 desaturase activity and has been shown to be regulated by PPARs [143]. MatInspector [117] identified three potential peroxisome proliferative response elements predominantly for PPAR-α. Ligand-induced transcriptional activity of PPAR-α can be inhibited by PPAR-δ [43]. PPAR-δ could control *SCD1* expression in another way through sterol-responsive element binding protein 1 (SREBP1) that binds to sterol-responsive element (SRE) in the promoter region of *SCD1* [144, 145]. *SREBP1* expression is diminished by PPAR-δ agonists [48]. These facts show that *SCD1* is good candidate to generate a potential effect of PPAR-δ on desaturation index. For that reason, SCD expression of 29 animals with different *PPARD* genotypes was investigated in muscle tissue by quantitative Real-Time PCR.

A relative quantification approach was chosen to determine gene expression of the chosen genes. In relative quantification, the expression of the gene of interest is normalized by the expression of a not regulated housekeeping gene. Reliability of relative quantification relies on stably expressed housekeeping genes. For that reason, expression of five housekeeping genes, namely beta-actin (*ACTB*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), hypoxanthine phosphoribosyltransferase 1 (*HPRT1*), TATA box binding protein (*TBP*) and topoisomerase (DNA) II beta (*TOP2B*) was measured in 10 animals in liver and muscle and the most stably expressed genes were chosen as reference genes for the relative quantification. The expression stability of one gene was evaluated relative to the other genes by the geNorm algorithm [146]. Only the two most stably expressed genes and not a single most stably expressed gene can be identified with this procedure. A decrease in average expression stability M indicates a more stable gene expression. Figure 16 shows a large difference in gene expression of the analysed housekeeping genes between muscle and liver. For example, *ACTB* is one of the two most stably expressed genes in muscle, but the least stably expressed gene in liver. ACTB and TBP were chosen as reference genes in muscle and TBP and TOP2B in liver, because they show the most stable expression in the relevant tissue.

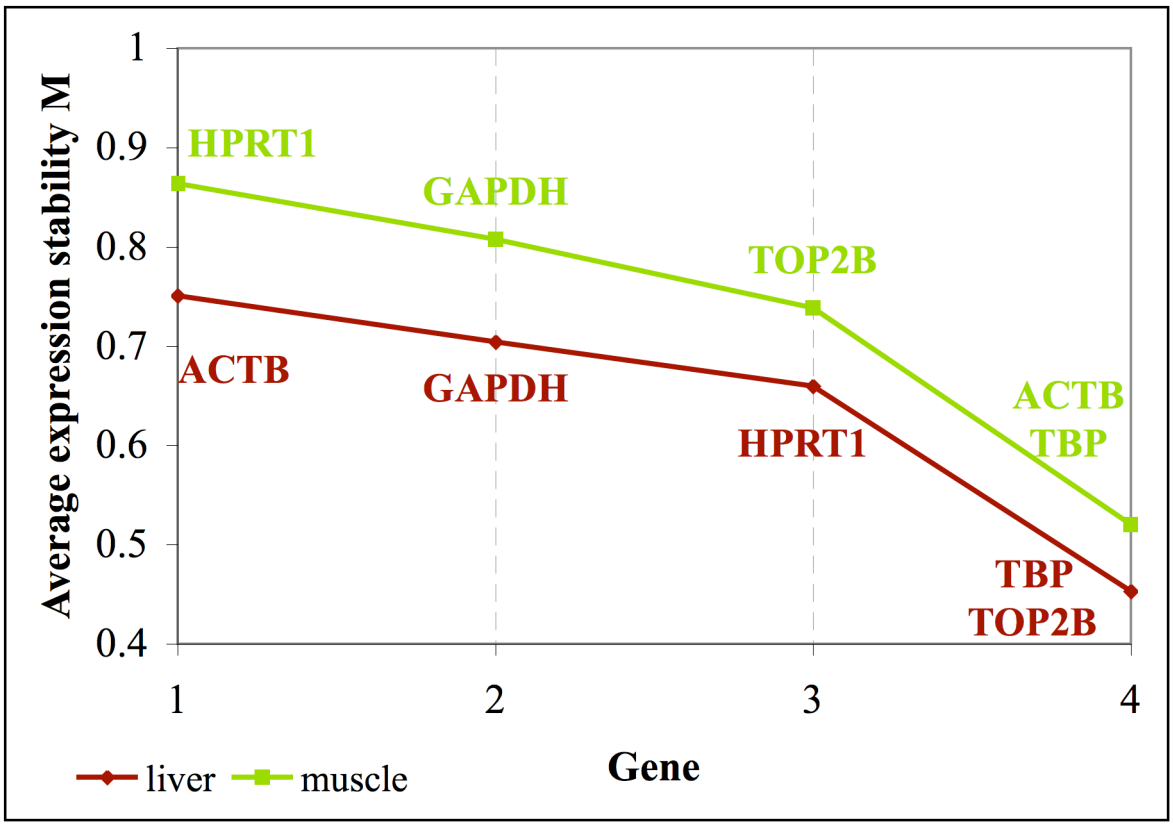


Figure 16: Expression stability of housekeeping genes in muscle and liver according to geNorm [146]. Stably expressed genes are characterised by a low expression stability M. Expression stability of one gene is estimated relative to the other genes. The analysis is carried out in consecutive steps. In each step (x-axis) the least stably expressed gene is removed from the analysis. For this reason, two stably expressed genes and not a single most stably expressed gene can be identified with this procedure.

The relative quantification method enables the investigation of variations in gene expression between two groups. In this study, differences in gene expression between one group of animals carrying a particular haplotype heterozygously and a second group without this haplotype were analysed. Within the analysed pigs, three animals carried haplotyope 1 homozygously. Data from these animals was pooled with data from animals carrying haplotype 1 heterozygously. No animal was found to be homozygous for haplotypes 2, 3, 4 and 5, respectively.

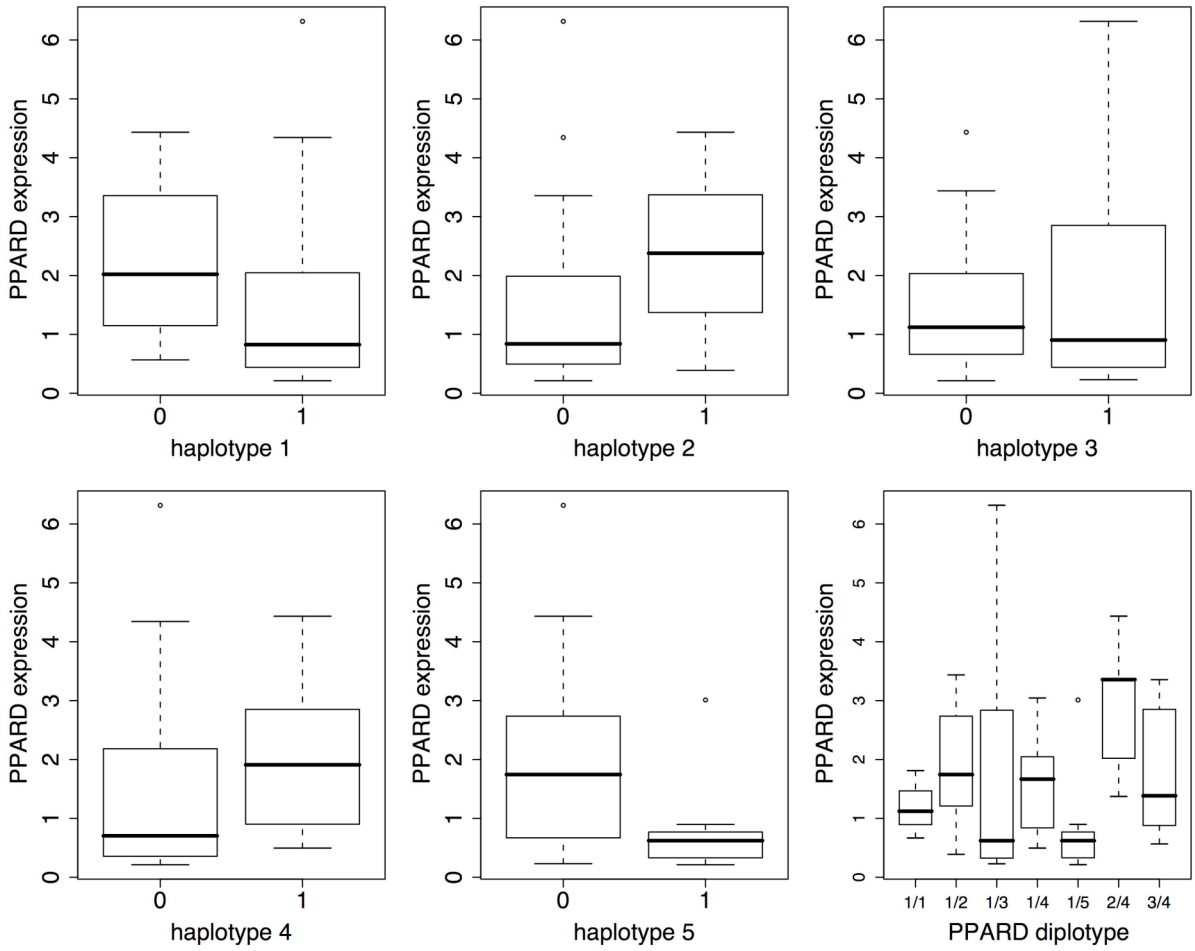


Figure 17: mRNA expression of PPAR-δ variants in liver

Expression values *E* were normalised by *E* of two reference genes. A value of 0 for the haplotype indicates absence of the particular haplotype, 1 indicates presence of one allele of the particular haplotype.

Figure 17 illustrates differentially expression of certain *PPARD* haplotypes. PPAR-δ expression is significantly reduced by haplotypes 1 and 5, respectively and increased by haplotype 4 (Table 20). Interestingly, haplotypes 4 and 5 showed an effect on backfat thickness. The presence of haplotype 5 increased backfat thickness in a Mangalitsa x Piétrain cross and the presence of haplotype 4 decreased backfat thickness in a German Landrace population. In accordance with these findings, PPAR-δ expression is altered in opposite directions by these two haplotypes. Haplotype 1 was found to be associated with desaturation index C16 and animals carrying haplotype 1show decreased mRNA expression of PPAR-δ.

Table 20: mRNA expression of PPAR-δ and FASN in porcine liver. Ratios indicate the influences of a *PPARD* haplotype on mRNA levels. Ratio R = 1 indicates no influence, R > 1 indicates mRNA levels are increase by the haplotype and R < 1 indicates a decrease. P-values were estimated by a permuation method.

Haplo type	Number of animals (without litter 7)		PPARD		FASN		FASN without litter 7	
	- / -	+ / -	R	P-value	R	P-value	R	P-value
1	11 (8)	42 (40)	0.47	0.044*	0.67	0.399	1.63	0.228
2	43 (38)	10 (10)	1.69	0.182	0.55	0.212	0.80	0.525
3	35 (35)	18 (13)	1.14	0.704	3.37	0.001***	1.52	0.209
4	32 (30)	21 (18)	2.13	0.014*	0.96	0.917	0.69	0.219
5	41 (36)	12 (12)	0.35	0.004**	0.58	0.240	0.86	0.651

Table 21: mRNA expression of SCD in porcine muscle. Ratios indicate if presence of particular haplotype influences mRNA expression. Ratio R = 1 indicates no influence, R > 1 increase and R < 1 decrease. P-values were estimated by a permuation method.

Haplo type	Number of animals (without litter 7)		SCD		SCD without litter 7	
	- / -	+ / -	R	p-value	R	p-value
1	12 (9)	17 (15)	0.50	0.191	0.76	0.507
2	18 (13)	11 (11)	0.25	0.005**	0.47	0.051.
3	18 (18)	11 (6)	5.36	0.0004***	2.04	0.118
4	17 (15)	12 (9)	1.99	0.193	1.31	0.521
5	24 (19)	5 (5)	0.71	0.628	1.24	0.681

However, no significant effect of haplotypes 4 and 5 on FASN expression and no significant effect of haplotype 1 on SCD expression are observed. Nevertheless, FASN expression is slightly decreased by haplotype 5 (Table 20) and SCD expression is moderately decreased by haplotype 1. Surprisingly, pigs carrying haplotype 3 have a 3.37-fold increase in FASN expression in liver and a 5.36-fold increase in SCD expression in muscle (Table 20, Table 21). Genetic effects or environmental influences may cause the enormous increases in FASN and SCD expression. All animals of litter 7 carry haplotype 3 and exhibit a much higher FASN and SCD expression than all other animals (Figure 18). If litter 7 is excluded from analysis, the ratio for FASN and SCD expression of haplotype 3 drops and significance is

lost. These changes are paralleled by an increase of SCD expression ratio of haplotype 2. In case of haplotype 2, significance remains at a suggestive level. The effect of removing litter 7 on ratios of all other haplotypes is moderate.

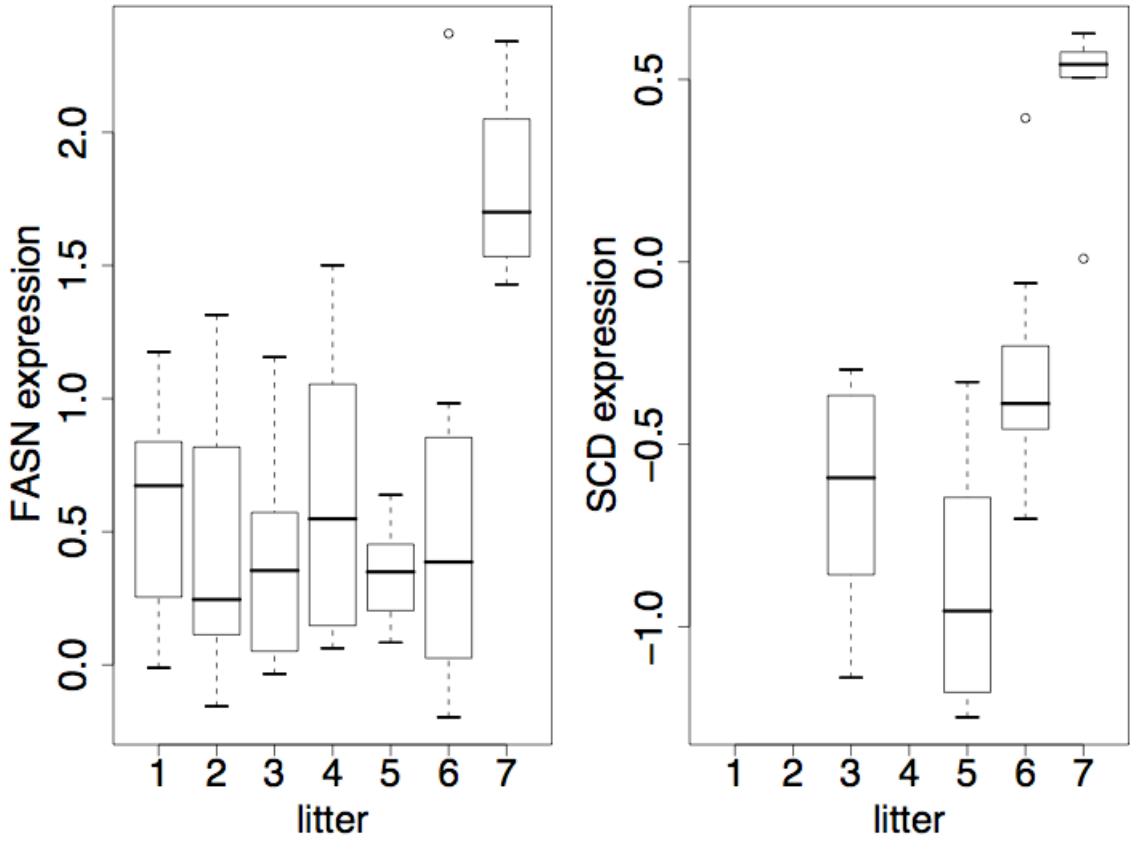


Figure 18: mRNA expression of FASN and SCD within litters
The natural logarithm of expression values *E* normalised by *E* of two reference genes is displayed.

Gene expression studies were carried out to provide functional evidence for association of *PPARD* haplotypes with backfat thickness and desaturation index C16. A significant alteration of PPAR-δ expression by all haplotypes that were found to be associated with phenotypes was observed. However, none of these haplotypes causes a significant alteration in expression of the two analysed target genes. Haplotype 3 causes significant increase of both target genes, however, only if litter 7 is included in the analysis. It is questionable, if the effect on FASN and SCD expression is due to *PPARD* haplotype 3 or environmental influences.

4 Discussion

4.1 Main findings

The aim of this thesis was to evaluate the association between the candidate genes *MC4R* and *PPARD* and lipid deposition in swine. With respect to *MC4R*, results from the association study carried out in this thesis are discussed within the context of existing literature in the following chapter. The most important findings regarding *MC4R* are:

- The Asp298Asn missense mutation of *MC4R* is associated with feed intake and growth, but not with backfat thickness in F2 Mangalitsa x Piétrain pigs.
- No Polymorphisms in linkage disequilibrium with the Asp298Asn missense mutation were detected in *MC4R* and in 5' and 3' adjacent genome regions.
- The Asp298Asn polymorphism is the most common genetic alteration in porcine *MC4R*.

The initial situation for candidate gene analysis of *PPARD* was completely different from that of *MC4R*, as no prior reports about porcine *PPARD* were available. Therefore, the main findings for association, gene expression and bioinformatic analyses are discussed. The following is a summary of the most important results:

- Only silent mutations were identified in the porcine *PPARD*.
- *PPARD* haplotype 5 is associated with increased backfat thickness in F2 Mangalitsa x Piétrain pigs, whereas *PPARD* haplotype 4 is associated with decreased backfat thickness in German Landrace.
- PPAR-δ mRNA levels are elevated in pigs with *PPARD* haplotype 4 and reduced in pigs with *PPARD* haplotype 5.

4.2 MC4R

4.2.1 The Asp298Asn polymorphism of *MC4R* as causal mutation

MC4R plays an important role in the neural circuit regulating food intake. Previous reports [21, 22] of association between *MC4R* Asp298Asn polymorphism and feed intake as well as daily gain in pigs could be confirmed in the Mangalitsa x Piétrain cross. Furthermore, the systematic search for polymorphism in the *MC4R*, the *MC4R* promoter and the region 3' adjacent to the *MC4R* locus revealed no polymorphisms in linkage disequilibrium with the Asp298Asn missense mutation. The Asn298 variant of the porcine *MC4R* is defective in agonist-stimulated signalling [32]. This suggests an impaired regulation of food intake trough MC4R by the Asn298 allele. Taken together, these findings suggest that the Asp298Asn polymorphism of *MC4R* rather than another linked variant of *MC4R* or a neighbouring gene is responsible for the observed associations with feed intake.

Acceleration of growth probably results from increased feed intake. The effect of elevated feed consumption on lipid deposition varies among different studies. Despite the lack of significant association [126], the Asn298 allele of *MC4R* has been reported to be associated with higher as well as lower backfat thickness [21, 22, 123, 124]. Therefore, it is discussed controversially whether or not *MC4R* is the causative mutation for the fat / lean meat QTL on chromosome 1. In a Landrace x Hampshire cross a QTL for fatness traits was observed on chromosome 1 [16, 125]. In this study, the statistical significance of the QTL is lost after inclusion of *MC4R* genotype in the model for QTL analysis. This supports the hypothesis that *MC4R* is a positional candidate gene for this fat / lean meat QTL [125]. QTL analysis in a Large White x Wild Boar intercross did not reveal any significance for fatness traits on chromosome 1 [23]. In accordance with the latter study [23], neither a QTL for fatness traits on chromosome 1 (A. Pertek (2007), personal communication) nor an association with backfat thickness was observed in the analysed F2 offspring of the Mangalitsa x Piétrain cross. In the Mangalitsa x Piétrain cross investigated in this thesis, the lack of significance in the association as well as the QTL study may be explained by reduced statistical power to detect a small effect of about 1 mm on backfat thickness [21, 125] under the observed low frequency of Asn298 allele. Furthermore, only a small number of F2 offspring was analysed in the QTL study. This limits the power to detect a QTL of a small to moderate effect.

Feed composition as well as feeding regime play a pivotal role in lipid deposition, and are likely to interact with the *MC4R* genotype. In studies under *ad libitum* feeding, an association between the Asn298 allele of *MC4R* and increased backfat thickness was observed [21, 22], whereas the Asn298 allele was associated with decreased backfat thickness under restricted feeding [124]. The importance of feeding was evaluated in another study that demonstrated a significantly higher frequency of the Asp298 allele after selection for lean feed conversion and lean growth under *ad libitum*, but not under restricted feeding [22]. The influence of the feeding regime and the apparently conflicting outcomes of various investigations might be explained by the "general linear / plateau theory for lean and fatty tissue growth" [147]. This general theory used in pig growth models says that as feed intake increases, a linear response in lean growth is observed leading to a plateau phase at the maximum lean growth rate. Since muscle has a higher priority in nutrient partitioning [148], adipose tissue growth is restrained to a minimum level in the linear response phase for lean growth [147]. If feed intake exceeds the requirements for maximum lean growth, feed is partitioned to fat deposition [149]. Therefore, enhancing feed intake will initially enhance growth instead of fatness until the lean potential is reached. Further increase in feed intake will result in fat deposition [147]. The maximum lean gain potential varies between genders and genetic strains of pigs, and may be determined by genetic influences [149]. For that reason, it is not unexpected to observe different outcomes in different studies. It is safe to assume that feed intake exceeded requirements for maximum lean gain in studies showing an increased feed intake, growth and fatness due to the Asn298 allele [21, 22]. Moreover, this theory is able to explain an enhanced lean gain in some form of restricted feeding [124], where the feed intake is below the threshold needed for maximum lean gain.

4.2.2 Relevance of the Asp298Asn mutation for pig breeding

Several authors [123-126, 150] reported allele frequencies of the Asp298Asn polymorphism in different breeds (Table 22). The allele frequencies of the German and Swiss pig breeds analysed here are similar to those estimated by other authors in the respective Polish [126], US-American [150], Lithuanian [124], Danish [125] and Korean [123] breeds. Interestingly, Bruun *et al.* [125] showed a significant increase in the frequency of the Asn298 allele in Landrace and Duroc (Table 22) over a 12 year period. Bruun *et al.* [125] conclude from their findings, that the increased occurrence of Asn298 could be explained by selection for daily gain in these breeds. Furthermore, they assume that selection for daily gain in the Yorkshire breed affects other loci, because the allele frequency of the same variant is not influenced by selection for daily gain in Yorkshire pigs. The allele frequency of the Asn298 allele in

German Landrace is similar to that of the Danish Landrace in 1990. An increase in Asn298 frequency of 26 % was observed in the Danish Landrace within 12 years.

Faster growth would be desirable especially in slow growing Piétrain pigs. Breeding for higher feed intake and daily gain with the help of gene-assisted selection for the Asn298 variant could help to accelerate growth. However, pork production costs are determined by the amount of feed and the time required to produce quality lean meat [149]. An enhanced feed intake is in itself not desirable, but can be accepted if a shorter fattening period is achieved and if the lean meat yield is increased. Selection for Asn298 allele under *ad libitum* feeding may result in a small to moderate increase of fatness, which might be a reason not to consider the Asp298Asn polymorphism as selection criterion. Since the effect of the Asn298 allele on fatness seems to depend on the feeding regime, a well-designed restricted feeding regime could possibly avoid an increase in fatness and potentially even enhance lean meat content.

Table 22: Frequency of the mutant Asn298 allele in various pig breeds

The frequency of the analysed German (Pietrain, Landrace, Large White) and Swiss (Duroc) breeds were compared with figures reported in American [150], Polish [126], Danish [125] in 1990 [a] and 2002 [b], Lithuanian [124] and Korean [123] breeds.

breed	German / Swiss	US-American	Polish	Danish	Lithuanian	Korean
Pietrain	0.06					
Landrace	0.06	0.28	0.29	0.06 [a] 0.32 [b]		
Large White	0.56	0.44	0.76		0.41	
Yorkshire				0.74 [a] 0.55 [b]		
Duroc	0.72	0.95		0.59 [a] 0.96 [b]		0.73
Hampshire				1.0 [a,b]		

4.3 PPARD

4.3.1 *PPARD* as a functional candidate gene for backfat thickness

PPARD was chosen as a candidate for backfat thickness because of the vital role of PPAR-δ in the regulation of lipid metabolism and of the localisation of *PPARD* in a major QTL region for backfat thickness on chromosome 7. The candidate gene analysis of the porcine *PPARD* presented here reveals an association of *PPARD* haplotypes 4 and 5 with backfat thickness. Haplotype 5 is associated with increased backfat thickness in F2 Mangalitsa x Piétrain pigs and haplotype 4 with decreased backfat thickness in the German Landrace population. It was not possible to carry out an association study with haplotypes 4 and 5 in the same pig population, as haplotype 4 is absent in the F2 Mangalitsa x Piétrain generation and haplotype 5 is extremely rare in German Landrace. Since the only SNP (+768G>A) differing between haplotypes 4 and 5 is synonymous, it seems unlikely that this SNP causes the opposing effects on backfat thickness. Therefore, it is more reasonable to assume that the observed association is due to a polymorphism in linkage disequilibrium with the *PPARD* variant rather than the *PPARD* itself. Especially in the F2 generation of the Mangalitsa x Piétrain cross, linkage disequilibrium could be a plausible explanation for the observed association, because in F2 crosses long chromosomal segments with high linkage disequilibrium are generated. Due to the inability to separate linkage from association, genetic studies in intercrosses are not suitable to detect associations with a single gene. Nevertheless, the use of experimental animals from the Mangalitsa x Piétrain cross provided a possibility to study the effect of *PPARD* haplotypes on mRNA levels of PPAR-δ and PPAR-δ - regulated genes. Interestingly, the opposite effect of *PPARD* haplotypes 4 and 5 on backfat thickness is reflected by an opposite effect of these two haplotypes on PPAR-δ mRNA levels. Haplotype 4 is associated with reduced backfat thickness, and it significantly increases mRNA expression of PPAR-d in liver. Haplotype 5 is associated with higher backfat thickness, and it significantly decreases PPAR-δ expression in liver. These findings are in line with studies demonstrating a decrease of body fat in mice caused by PPAR-δ overexpression [46]. In conclusion, findings from the association study, when considered together with results from the PPAR-δ expression study suggest a causative effect of *PPARD* on backfat thickness. However, no influence of haplotype 4 and 5 on expression of FASN, a potential PPAR-δ target gene could be detected. The failure to detect changes in FASN expression might hint at a lack of functionality of the *PPARD* variants, but this might as well be explained by other facts. Firstly, *FASN* may not be

regulated by PPAR-δ in the porcine liver. It was shown that PPAR-δ regulates FASN expression in murine liver, but the primary site for fatty acid synthesis in pigs is adipose tissue, whereas in mice it is the liver [151, 152]. Secondly, differences in FASN expression could occur before or after the time of sampling. It is difficult to reveal the reason for the missing effect of *PPARD* haplotypes on FASN expression. For that reason, a characterisation of *PPARD* target genes in swine is required prior to further evaluation of how *PPARD* haplotypes affect expression of PPAR-δ target genes in various tissues. Such analyses could also elucidate the basis for the observed association of *PPARD* haplotype 1 with desaturation index C16:0 in the Mangalitsa x Piétrain cross. Similar to haplotype 4 and 5, a significant influence of haplotype 1 on PPAR-δ mRNA levels was noted, but no changes in mRNA expression of the potentially PPAR-δ regulated gene *SCD1* in muscle was detected.

In summary, it is still unclear whether the detected association of *PPARD* haplotypes with backfat thickness is caused by PPARD or a variant in linkage disequilibrium. If it is argued that *PPARD* causes the observed association, the question then arises which genetic variant could be the potential cause. None of the SNPs located in coding sequence results in an amino acid exchange. Studies of allelic imbalance that could have been caused by one SNP located in a potential transcription factor binding site led to the exclusion of this variant as functional candidate. None of the other SNPs in the analysed 2000 bp region of the *PPARD* promoter is located in a conserved region or a region that is predicted to be a transcription factor binding site. Since functional SNPs are rarely found in introns, SNPs located in the coding region, 5' or 3' UTR are more likely to produce functional effects. 'Silent' SNPs in exons might affect translational efficiency and mRNA stability as well as by create or disrupt microRNA target sites, splicing control elements and UTR functional elements [131, 153, 154].

Two synonymous SNPs are located in the coding region, each of them being a tag SNP for a haplotype associated with the phenotypic traits. SNP +1137G>T is a tag SNP of haplotype 1 with the G allele being unique to haplotype 1. Another tag SNP for haplotype 1 is SNP *1584C>T. The second synonymous polymorphism is SNP +768G>A that tags haplotype 5. Furthermore, this SNP is the only SNP with different alleles for haplotypes 4 and 5. SNP -180C>T differentiates haplotypes 4 and 5 from all other haplotypes. Due to their absence or presence in particular haplotypes, these four SNPs are candidates for causing functional effects. However, only small differences in codon usage of the synonymous SNPs were detected. Furthermore, none of the SNPs located in mRNA exhibits a large influence on the

mRNA secondary structure.
Differences in mRNA levels between *PPARD* haplotypes suggest that functionality is caused either by influences on mRNA stability or by differences in mRNA expression. However, control elements located in introns or far away from the gene can enhance or inhibit mRNA expression. This makes it difficult to identify the functional variant, especially as the observed effect might be due to not only one, but several genetic variants interacting with each other. In conclusion, this study was not able to detect a genetic variant in *PPARD* that is likely to cause the observed association.

4.3.2 *PPARD* as a positional candidate gene for backfat thickness

PPARD is also a positional candidate gene for backfat thickness of pigs. The genetic study of the PPAR-δ gene locus in pigs carried out in this thesis demonstrated an effect on backfat thickness. Analogous to numerous QTL studies [8, 11, 59, 60], the same paradox of lower backfat caused by the allele originating from the breed with more backfat was revealed. In the Mangalitsa x Piétrain cross, haplotype 5 originates from the lean Piétrain breed and causes higher backfat. This may indicate that *PPARD* represents the QTL on chromosome 7. However, in the present study, it remains unclear whether or not *PPARD* causes the observed association. Furthermore, no backfat QTL at the position of *PPARD* was discovered in the Mangalitsa x Piétrain cross ([155]; A. Pertek (2007), personal communication). Additionally, haplotype 5 is very rare or even absent in all analysed pig breeds making it highly unlikely that it was fixed in one of the pig breeds used for QTL studies by different authors [8, 11, 59, 60]. In summary, this study does not support the assumption that *PPARD* may represent the backfat QTL on chromosome 7.

Furthermore, the metabolic and histochemical characterisation of fat and muscle tissue from pigs with the SSC7 QTL alleles from Meishan and Large White showed differences in adipogenesis, but no change in oxidative and glycolytic metabolism in muscle [64]. PPAR-δ has been shown to regulated target genes presumably located in liver and muscle, but not in adipose tissue [50]. Therefore, other candidate genes located in the QTL region on chromosome 7 and involved in adipogenesis, such as mitogen-activated protein kinase 14 (*MAPK14*), high mobility group AT-hook 1 (*HMGA1*) or cyclin-dependent kinase-inhibitor 1 (*CDKNA1*) [64] deserve further study.

4.4 Relevance of findings for human obesity

PPARD has been shown to be associated with body mass index in humans [51, 156]. Likewise, this study of the porcine *PPARD* revealed an association with fatness. However, whether or not the observed association in swine is caused by the *PPARD* gene remains unclear. Nevertheless, the association of *PPARD* with fatness in two different species strengthens the argument that *PPARD* could be the cause for the observed influence on lipid deposition in swine as well as in humans.

Genetic alterations in human *MC4R* are associated with severe forms of obesity [19, 157]. An effect of a porcine *MC4R* variant on pig fatness could be shown in several studies, but the effect seems to be small [21, 124]. Different functional characteristics of the studied mutations in humans and pigs can explain the observed differences in effect size. Associations with feed intake and daily gain in pigs provide information about the mode of action of genetic alterations in *MC4R* and contribute valuable information about traits that are difficult to measure precisely in humans. According to the Genetic Association Database [158], association studies between food consumption and *MC4R* mutations are inexistent in humans. Only association studies of eating disorders like Bulimia nervosa [159] and Binge-Eating [160-162] were carried out and revealed no clear association with *MC4R* variants.

For both analysed genes first reports about association with human obesity occurred before the gene was mapped in pigs [163, 164]. This demonstrates the problems scientists working with the animal model pig are facing. Since the porcine genome is not fully sequenced yet, it is very labour-intensive and time-consuming to identify new genes involved in the development of fatness. Database resources for annotated genes, SNPs and haplotypes are almost inexistent or not comparable to resources available for humans or other animal models like mouse or cattle [109]. Although mapping of numerous QTL for fatness in divergent pig breeds revealed several interesting genomic regions, the identification of genes and mutations that underlie this QTL was rarely successful [165]. Consequently, the pig provides a valuable genetic resource for research into development of obesity, but the tools to use this resource are not yet available. Nevertheless, sequencing of the porcine genome is underway [166] and the fast progress achieved in publishing genomes and related information of other species in recent years raises optimism that tools for genetic studies in pigs will improve markedly within the next few years.

5 Summary and Conclusions

The aim of this thesis was to evaluate the effect of the melanocortin 4 receptor gene (*MC4R*) and peroxisome proliferator-activated receptor delta gene (*PPARD*) on backfat thickness in pigs. Backfat thickness is used as a selection criterion for meatiness, thereby being of economic importance to pork production. Furthermore, since backfat thickness is correlated with body fatness, it is a well-suited trait to study factors associated with lipid deposition. Both analysed genes can be viewed as functional and positional candidate genes. *PPARD* is a key regulator of lipid metabolism and has been mapped in a major QTL region for backfat thickness on chromosome 7. MC4R is involved in the neural circuit regulating food intake. The *MC4R* gene is located on chromosome 1 in a region where backfat QTL were mapped in some studies.

For both genes, a BAC clone was sequenced to obtain the genomic sequence. *MC4R* and *PPARD* were screened for genetic variation by re-sequencing animals of the 'fat' Mangalitsa breed and the 'lean' Piétrain breed. These animals had been mated to produce a Mangalitsa x Piétrain resource population. Haplotypes of each gene were inferred from genotype data. Tag SNPs comprising the observed haplotypes were genotyped in the F2 generation of the Mangalitsa x Piétrain intercross and analysed for association with backfat thickness and other traits.

The Asp298Asn missense mutation of *MC4R* was found to be significantly associated with feed intake and daily gain in the F2 generation of the Mangalitsa x Piétrain cross. These findings agree with previous reports. However, in contrast to some, but not all prior studies, no significant association with backfat thickness was observed. For the first time, it could be demonstrated that no additional polymorphisms of the *MC4R* and adjacent genome regions are in linkage disequilibrium with the Asp298Asn missense mutation. The SNP underlying this missense mutation is likely to be the causative mutation for the observed association with feed intake. Enhanced feed intake due to the Asn298 allele results in accelerated growth and may affect carcass composition and fatness as shown in other studies.

Screening for genetic variation in porcine *PPARD* revealed only silent mutations. Nevertheless, significant associations between haplotype 1 and fatty acid pattern in muscle as well as haplotype 5 and backfat thickness were observed in the F2 generation of the Mangalitsa x Piétrain cross. Additional association and gene expression studies were carried

out to determine whether these results are due to *PPARD* or a gene in linkage disequilibrium with *PPARD*. Association between *PPARD* and backfat thickness was also detected in German Landrace. Haplotype 5 is associated with increased backfat in the F2 Mangalitsa x Piétrain population, whereas haplotype 4 is associated with lower backfat thickness in the German Landrace population. Haplotype 4 and 5 carry the same alleles at all but one of the discovered SNPs. Interestingly, the opposite effects of *PPARD* haplotypes 4 and 5 on backfat thickness are reflected by opposite effects of these two haplotypes on PPAR-δ mRNA levels. Haplotype 4 significantly increases PPAR-δ mRNA levels, whereas haplotype 5 decreases mRNA levels of PPAR-δ. In line with these findings it has been reported that overexpression of PPAR-δ can prevent obesity. Alteration of PPAR-δ mRNA levels by haplotypes 4 and 5 does not result in altered expression of two potential PPAR-δ target genes.

In conclusion, this work adds evidence for association between the Asp298Asn missense mutation of *MC4R* and feed intake as well as daily gain. The observed association is in all probability due to a direct effect on feed consumption and a subsequent acceleration of growth. The resulting effect on meatiness is likely to be strongly influenced by feed availability. Therefore, the Asp298Asn polymorphism can be applied to study gene - environment interactions and might be used in selection for accelerated growth without a negative effect on carcass composition with an optimised feeding regime.

Studies regarding *PPARD* revealed association between *PPARD* variants and backfat thickness. However, it is unclear whether or not the association is caused by *PPARD*, especially because no obvious functional variant was identified. Further studies are required to determine whether the observed associations are present in other pig populations. If they are present, it would be important to first identify PPAR-δ target genes in pigs and then test them for *PPARD* variant-dependent expression in order to proof a functional effect. Finally, functional studies that pinpoint the effect of the detected variants would be required to clarify the importance of *PPARD* variants as genetic determinants of fatness.

6 Zusammenfassung

Das Ziel dieser Arbeit war es den Einfluss der Gene des Melanocortin 4 Rezeptors (*MC4R*) und des Peroxisomen Proliferator aktivierenden Rezeptors delta (*PPARD*) auf die Rückenspeckdicke des Schweins zu untersuchen. Die Rückenspeckdicke ist ein Hilfsmerkmal für die Fleischigkeit und ist damit von ökonomischer Bedeutung für die Produktion von Schweinefleisch. Ferner ist die Rückenspeckdicke mit dem Fettanteil des Körpers korreliert und kann daher verwendet werden, um Einflussfaktoren auf die Fetteinlagerung zu untersuchen. Die beiden analysierten Gene können sowohl als funktionelle als auch als positionelle Kandidatengene angesehen werden. PPAR-δ reguliert Schlüsselstellen im Fettstoffwechsel, und wurde in einem der wichtigsten QTL für Rückenspeckdicke kartiert. MC4R ist Bestandteil eines neuronalen Netzes, welches die Nahrungsaufnahme reguliert. Das *MC4R* Gen ist auf dem Chromosom 1 in einer Genomregion lokalisiert in der in einzelnen Schweinepopulationen ein QTL für Rückenspeckdicke identifiziert wurde.

Ein BAC Klon für jedes der beiden Gene wurde sequenziert, um die genomische Sequenz zu erhalten. Zur Identifizierung von genetischen Veränderungen in den Genen wurden diese in alle Tieren der Parentalgeneration einer Mangalitza x Pietrain Kreuzung sequenziert. Mit Hilfe der identifizierten SNPs wurden Haplotypen konstruiert. Tag SNPs, welche die gefundenen Haplotypen repräsentieren wurden in der F2 Generation der Mangalitsa x Pietrain Kreuzung genotypisiert und auf Assoziation mit Rückenspeckdicke und weiteren Merkmalen untersucht.

Es konnte gezeigt werden, dass die Asp298Asn Missense Mutation des *MC4R* mit der Futteraufnahme und der täglicher Zunahme assoziiert ist. Diese Ergebnisse stimmen mit denen der Literatur überein. Im Gegensatz zu einigen, aber nicht allen vorangegangenen Studien konnte jedoch keine Assoziation zwischen der Asp298Asn Mutation und der Rückenspeckdicke festgestellt werden. Im *MC4R* und in angrenzenden Genomregionen wurde keine weitere Mutation gefunden, die sich im Kopplungsungleichgewicht mit dem Asp298Asn Polymorphismus befindet. Die Asp298Asn Mutation ist wahrscheinlich die kausale Variante für den Effekt auf die Futteraufnahme. Eine Erhöhung der Futteraufnahme durch das Asn298 Allel resultiert in einem beschleunigten Wachstum und könnte auch für die in anderen Studien beobachteten Effekte auf die Schlachtkörperzusammensetzung verantwortlich sein.

Im *PPARD* Gen wurden ausschließlich stille Mutationen identifiziert. Trotzdem konnte eine signifikante Assoziation des *PPARD* Haplotyps 1 mit dem Fettsäuremuster im Muskel beziehungsweise des *PPARD* Haplotyps 5 mit der Rückenspeckdicke in der F2 Generation einer Mangalitza x Pietrain Kreuzung nachgewiesen werden. Weitere Assoziations- und Genexpressionsstudien wurden durchgeführt um zu untersuchen, ob die beobachtete Assoziation durch *PPARD* oder ein mit den *PPARD* Varianten gekoppeltes Gen verursacht wird. Eine Assoziationsstudie in Schweinen der Deutschen Landrasse ergab eine signifikant verringerte Rückenspeckdicke in Schweinen mit dem Haplotyp 4. Im Gegensatz dazu ist die Rückenspeckdicke in F2 Mangalitza x Pietrain Tieren mit Haplotyp 5 erhöht. Die Allele der einzelnen SNPs für Haplotyp 4 und 5 unterscheiden sich nur an einer der 24 identifizierten Mutationen. Interessanterweise spiegelt sich der gegensätzliche Effekt der *PPARD* Haplotypen auf die Rückenspeckdicke auch in einem entgegengesetzten Effekt auf die PPAR-δ mRNA Level wieder. PPAR-δ mRNA Level sind signifikant erhöht in Tieren mit Haplotyp 4 und vermindert in Tieren mit Haplotyp 5. Dieses Ergebnis stimmt mit Berichten aus der Literatur überein, die zeigten, dass die Überexpression von PPAR-δ vor Übergewicht schützen kann. Allerdings konnte in den analysierten Tieren kein Einfluss der *PPARD* Haplotypen auf die Expression von potentiell durch PPAR-δ regulierten Genen festgestellt werden.

Abschließend kann festgestellt werden, dass die Untersuchung der Asp298Asn Missense Mutation in der F2 Mangalitza x Pietrain Kreuzung weitere Belege für die Assoziation mit der Futteraufnahme und der täglicher Zunahme liefert. Höchstwahrscheinlich werden die beobachteten Assoziationen durch einen direkten Effekt auf die Futteraufnahme und einen daraus resultierenden Effekt auf das Wachstum und die Fetteinlagerung hervorgerufen. Ein wichtiger Faktor für die Ausprägung des Einflusses auf die Fetteinlagerung scheint die Verfügbarkeit von Futter zu sein. Die Asp298Asn Mutation im Schwein kann daher zur Untersuchung von Gen - Umwelt - Interaktionen herangezogen werden.

Die Untersuchungen des *PPARD* Gens ergaben eine Assoziation der Rückenspeckdicke mit einzelnen Haplotypen. Es bleibt jedoch unklar, ob diese Assoziation durch *PPARD* oder ein gekoppeltes Gen verursacht wird. Weitere Studien sind notwenig, um dies abklären zu können.

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8 Bibliography

1. Andersson, L, Georges, M: **Domestic-Animal Genomics: Deciphering the Genetics of Complex Traits**. *Nature Reviews Genetics* 2004, **5**(3): 202-212.
2. Pomp, D: **Genetic Dissection of Obesity in Polygenic Animal Models**. *Behav Genet* 1997, **27**(4): 285-306.
3. **Comparative Anatomy and Physiology of the Pig** http://www.nal.usda.gov/awic/pubs/swine/swine.htm#art
4. **Obesity and Overweight Information Sheet** http://www.who.int/dietphysicalactivity/publications/facts/obesity/en/index.html
5. Bjorntorp, P: **Thrifty Genes and Human Obesity. Are We Chasing Ghosts?** *The Lancet* 2001, **358**(9286): 1006.
6. Hirschhorn, JN, Daly, MJ: **Genome-Wide Association Studies for Common Diseases and Complex Traits**. *Nature Reviews Genetics* 2005, **6**(2): 95.
7. Hu, Z-L, Dracheva, S, Jang, W, Maglott, D, Bastiaansen, J, Rothschild, M, Reecy, J: **A QTL Resource and Comparison Tool for Pigs: PigQTLdb**. *Mammalian Genome* 2005, **16**(10): 792.
8. Rohrer, GA, Keele, JW: **Identification of Quantitative Trait Loci Affecting Carcass Composition in Swine: I. Fat Deposition Traits**. *J Anim Sci* 1998, **76**(9): 2247-2254.
9. Milan, D, Bidanel, JP, Iannuccelli, N, Riquet, J, Amigues, Y, Gruand, J, Le Roy, P, Renard, C, Chevalet, C: **Detection of Quantitative Trait Loci for Carcass Composition Traits in Pigs**. *Genet Sel Evol* 2002, **34**(6): 705-728.
10. Malek, M, Dekkers, JC, Lee, HK, Baas, TJ, Rothschild, MF: **A Molecular Genome Scan Analysis to Identify Chromosomal Regions Influencing Economic Traits in the Pig. I. Growth and Body Composition**. *Mamm Genome* 2001, **12**(8): 630-636.
11. de Koning, DJ, Janss, LLG, Rattink, AP, van Oers, PAM, de Vries, BJ, Groenen, MAM, van der Poel, JJ, de Groot, PN, Brascamp, EW, van Arendonk, JAM: **Detection of Quantitative Trait Loci for Backfat Thickness and Intramuscular Fat Content in Pigs (Sus Scrofa)**. *Genetics* 1999, **152**(4): 1679-1690.
12. Tanaka, M, Suzuki, K, Morozumi, T, Kobayashi, E, Matsumoto, T, Domukai, M, Eguchi-Ogawa, T, Shinkai, H, Awata, T, Uenishi, H: **Genomic Structure and Gene Order of Swine Chromosome 7q1.1-->Q1.2**. *Anim Genet* 2006, **37**(1): 10-16.
13. Barbosa, A, Demeure, O, Urien, Cl, Milan, D, Chardon, P, Renard, C: **A Physical Map of Large Segments of Pig Chromosome 7q11-Q14: Comparative Analysis with Human Chromosome 6p21**. *Mammalian Genome* 2004, **15**(12): 982-995.
14. Tanaka, T, Yamamoto, J, Iwasaki, S, Asaba, H, Hamura, H, Ikeda, Y, Watanabe, M, Magoori, K, Ioka, RX, Tachibana, K, Watanabe, Y, Uchiyama, Y, Sumi, K, Iguchi, H, Ito, S, Doi, T, Hamakubo, T, Naito, M, Auwerx, J, Yanagisawa, M, Kodama, T, Sakai, J: **Activation of Peroxisome Proliferator-Activated Receptor {delta} Induces Fatty Acid {beta}-Oxidation in Skeletal Muscle and Attenuates Metabolic Syndrome**. *PNAS* 2003, **100**(26): 15924-15929.
15. Nezer, C, Moreau, L, Wagenaar, D, Georges, M: **Results of a Whole Genome Scan Targeting QTL for Growth and Carcass Traits in a Pietrain X Large White Intercross**. *Genet Sel Evol* 2002, **34**(3): 371-387.
16. Karlskov-Mortensen, P, Bruun, CS, Braunschweig, MH, Sawera, M, Markljung, E, Enfalt, AC, Hedebro-Velander, I, Josell, A, Lindahl, G, Lundstrom, K, Seth, G, Jorgensen, CB, Andersson, L, Fredholm, M: **Genome-Wide Identification of Quantitative Trait Loci in a Cross between Hampshire and Landrace I: Carcass Traits**. *Animal Genetics* 2006, **37**(2): 156-162.

17. Geldermann, H, Muller, E, Moser, G, Reiner, G, Bartenschlager, H, Cepica, S, Stratil, A, Kuryl, J, Moran, C, Davoli, R, Brunsch, C: **Genome-Wide Linkage and QTL Mapping in Porcine F2 Families Generated from Pietrain, Meishan and Wild Boar Crosses**. *Journal of Animal Breeding and Genetics* 2003, **120**(6): 363-393.
18. Farooqi, IS, Keogh, JM, Yeo, GSH, Lank, EJ, Cheetham, T, O'Rahilly, S: **Clinical Spectrum of Obesity and Mutations in the Melanocortin 4 Receptor Gene**. *N Engl J Med* 2003, **348**(12): 1085-1095.
19. Hinney, A, Hohmann, S, Geller, F, Vogel, C, Hess, C, Wermter, A-K, Brokamp, B, Goldschmidt, H, Siegfried, W, Remschmidt, H, Schafer, H, Gudermann, T, Hebebrand, J: **Melanocortin-4 Receptor Gene: Case-Control Study and Transmission Disequilibrium Test Confirm That Functionally Relevant Mutations Are Compatible with a Major Gene Effect for Extreme Obesity**. *J Clin Endocrinol Metab* 2003, **88**(9): 4258-4267.
20. Tao, Y-X: **Molecular Mechanisms of the Neural Melanocortin Receptor Dysfunction in Severe Early Onset Obesity**. *Molecular and Cellular Endocrinology* 2005, **239**(1-2): 1-14.
21. Kim, KS, Larsen, N, Short, T, Plastow, G, Rothschild, MF: **A Missense Variant of the Porcine Melanocortin-4 Receptor (MC4R) Gene Is Associated with Fatness, Growth, and Feed Intake Traits**. *Mammalian Genome* 2000, **11**(2): 131-135.
22. Houston, RD, Cameron, ND, Rance, KA: **A Melanocortin-4 Receptor (MC4R) Polymorphism Is Associated with Performance Traits in Divergently Selected Large White Pig Populations**. *Animal Genetics* 2004, **35**(5): 386-390.
23. Park, HB, Carlborg, O, Marklund, S, Andersson, L: **Melanocortin-4 Receptor (MC4R) Genotypes Have No Major Effect on Fatness in a Large White X Wild Boar Intercross**. *Animal Genetics* 2002, **33**(2): 155-157.
24. Huszar, D, Lynch, CA, Fairchild-Huntress, V, Dunmore, JH, Fang, Q, Berkemeier, LR, Gu, W, Kesterson, RA, Boston, BA, Cone, RD: **Targeted Disruption of the Melanocortin-4 Receptor Results in Obesity in Mice**. *Cell* 1997, **88**(1): 131-141.
25. MacKenzie, RG: **Obesity-Associated Mutations in the Human Melanocortin-4 Receptor Gene**. *Peptides* 2006, **27**(2): 395.
26. Marsh, DJ, Hollopeter, G, Huszar, D, Laufer, R, Yagaloff, KA, Fisher, SL, Burn, P, Palmiter, RD: **Response of Melanocortin-4 Receptor-Deficient Mice to Anorectic and Orexigenic Peptides**. *Nat Genet* 1999, **21**(1): 119-122.
27. Elmquist, JK: **Hypothalamic Pathways Underlying the Endocrine, Autonomic, and Behavioral Effects of Leptin**. *Int J Obes Relat Metab Disord* 2001, **25 Suppl 5**: S78-82.
28. Haskell-Luevano, C, Monck, EK: **Agouti-Related Protein Functions as an Inverse Agonist at a Constitutively Active Brain Melanocortin-4 Receptor**. *Regul Pept* 2001, **99**(1): 1-7.
29. Friedman, JM: **Modern Science Versus the Stigma of Obesity**. *Nat Med* 2004, **10**(6): 563-569.
30. Bidanel, JP, Milan, D, Iannuccelli, N, Amigues, Y, Boscher, MY, Bourgeois, F, Caritez, JC, Gruand, J, Le Roy, P, Lagant, H, Quintanilla, R, Renard, C, Gellin, J, Ollivier, L, Chevalet, C: **Detection of Quantitative Trait Loci for Growth and Fatness in Pigs**. *Genet Sel Evol* 2001, **33**(3): 289-309.
31. Rohrer, GA: **Identification of Quantitative Trait Loci Affecting Birth Characters and Accumulation of Backfat and Weight in a Meishan-White Composite Resource Population**. *J Anim Sci* 2000, **78**(10): 2547-2553.
32. Kim, KS, Reecy, JM, Hsu, WH, Anderson, LL, Rothschild, MF: **Functional and Phylogenetic Analyses of a Melanocortin-4 Receptor Mutation in Domestic Pigs**. *Domestic Animal Endocrinology* 2004, **26**(1): 75-86.

33. Issemann, I, Green, S: **Activation of a Member of the Steroid Hormone Receptor Superfamily by Peroxisome Proliferators**. *Nature* 1990, **347**(6294): 645.
34. Barish, GD, Narkar, VA, Evans, RM: **PPARdelta: A Dagger in the Heart of the Metabolic Syndrome**. *J Clin Invest* 2006, **116**(3): 590-597.
35. Xu, HE, Lambert, MH, Montana, VG, Parks, DJ, Blanchard, SG, Brown, PJ, Sternbach, DD, Lehmann, JM, Wisely, GB, Willson, TM: **Molecular Recognition of Fatty Acids by Peroxisome Proliferator-Activated Receptors**. *Molecular Cell* 1999, **3**(3): 397.
36. Chawla, A, Lee, CH, Barak, Y, He, W, Rosenfeld, J, Liao, D, Han, J, Kang, H, Evans, RM: **PPARdelta Is a Very Low-Density Lipoprotein Sensor in Macrophages**. *Proc Natl Acad Sci U S A* 2003, **100**(3): 1268-1273.
37. Kota, BP, Huang, TH-W, Roufogalis, BD: **An Overview on Biological Mechanisms of PPARs**. *Pharmacological Research* 2005, **51**(2): 85-94.
38. Krogsdam, AM, Nielsen, CA, Neve, S, Holst, D, Helledie, T, Thomsen, B, Bendixen, C, Mandrup, S, Kristiansen, K: **Nuclear Receptor Corepressor-Dependent Repression of Peroxisome-Proliferator-Activated Receptor delta-Mediated Transactivation**. *Biochem J* 2002, **363**(Pt 1): 157-165.
39. Tan, NS, Michalik, L, Desvergne, B, Wahli, W: **Multiple Expression Control Mechanisms of Peroxisome Proliferator-Activated Receptors and Their Target Genes**. *The Journal of Steroid Biochemistry and Molecular Biology* 2005, **93**(2-5): 99-105.
40. Skogsberg, J, Kannisto, K, Roshani, L, Gagne, E, Hamsten, A, Larsson, C, Ehrenborg, E: **Characterization of the Human Peroxisome Proliferator Activated Receptor delta Gene and Its Expression**. *Int J Mol Med* 2000, **6**(1): 73-81.
41. Larsen, LK, Amri, EZ, Mandrup, S, Pacot, C, Kristiansen, K: **Genomic Organization of the Mouse Peroxisome Proliferator-Activated Receptor beta/delta Gene: Alternative Promoter Usage and Splicing Yield Transcripts Exhibiting Differential Translational Efficiency**. *Biochem J* 2002, **366**(Pt 3): 767-775.
42. Braissant, O, Foufelle, F, Scotto, C, Dauca, M, Wahli, W: **Differential Expression of Peroxisome Proliferator-Activated Receptors (PPARs): Tissue Distribution of PPAR-alpha, -beta, and -gamma in the Adult Rat**. *Endocrinology* 1996, **137**(1): 354-366.
43. Shi, Y, Hon, M, Evans, RM: **The Peroxisome Proliferator-Activated Receptor delta, an Integrator of Transcriptional Repression and Nuclear Receptor Signaling**. *PNAS* 2002, **99**(5): 2613-2618.
44. Evans, RM, Barish, GD, Wang, YX: **PPARs and the Complex Journey to Obesity**. *Nat Med* 2004, **10**(4): 355-361.
45. Wang, Y-X, Lee, C-H, Tiep, S, Yu, RT, Ham, J, Kang, H, Evans, RM: **Peroxisome-Proliferator-Activated Receptor [delta] Activates Fat Metabolism to Prevent Obesity**. *Cell* 2003, **113**(2): 159-170.
46. Luquet, S, Lopez-Soriano, J, Holst, D, Fredenrich, A, Melki, J, Rassoulzadegan, M, Grimaldi, PA: **Peroxisome Proliferator-Activated Receptor {delta} Controls Muscle Development and Oxidative Capability**. *FASEB J* 2003, **17**(15): 2299-2301.
47. Wang, Y-X, Zhang, C-L, Yu, RT, Cho, HK, Nelson, MC, Bayuga-Ocampo, CR, Ham, J, Kang, H, Evans, RM: **Regulation of Muscle Fiber Type and Running Endurance by PPAR delta**. *PLoS Biology* 2004, **2**(10): e294.
48. Kramer, DK, Al-Khalili, L, Perrini, S, Skogsberg, J, Wretenberg, P, Kannisto, K, Wallberg-Henriksson, H, Ehrenborg, E, Zierath, JR, Krook, A: **Direct Activation of Glucose Transport in Primary Human Myotubes after Activation of Peroxisome Proliferator-Activated Receptor {delta}**. *Diabetes* 2005, **54**(4): 1157-1163.

49. Brunmair, B, Staniek, K, Dorig, J, Szocs, Z, Stadlbauer, K, Marian, V, Gras, F, Anderwald, C, Nohl, H, Waldhausl, W, Furnsinn, C: **Activation of PPAR-delta in Isolated Rat Skeletal Muscle Switches Fuel Preference from Glucose to Fatty Acids**. *Diabetologia* 2006.
50. Lee, C-H, Olson, P, Hevener, A, Mehl, I, Chong, L-W, Olefsky, JM, Gonzalez, FJ, Ham, J, Kang, H, Peters, JM, Evans, RM: **PPAR{delta} Regulates Glucose Metabolism and Insulin Sensitivity**. *PNAS* 2006, **103**(9): 3444-3449.
51. Shin, HD, Park, BL, Kim, LH, Jung, HS, Cho, YM, Moon, MK, Park, YJ, Lee, HK, Park, KS: **Genetic Polymorphisms in Peroxisome Proliferator-Activated Receptor {delta} Associated with Obesity**. *Diabetes* 2004, **53**(3): 847-851.
52. Skogsberg, J, McMahon, AD, Karpe, F, Hamsten, A, Packard, CJ, Ehrenborg, E: **Peroxisome Proliferator Activated Receptor delta Genotype in Relation to Cardiovascular Risk Factors and Risk of Coronary Heart Disease in Hypercholesterolaemic Men**. *J Intern Med* 2003, **254**(6): 597-604.
53. Chen, S, Tsybouleva, N, Ballantyne, CM, Gotto, AM, Jr., Marian, AJ: **Effects of PPARalpha, gamma and delta Haplotypes on Plasma Levels of Lipids, Severity and Progression of Coronary Atherosclerosis and Response to Statin Therapy in the Lipoprotein Coronary Atherosclerosis Study**. *Pharmacogenetics* 2004, **14**(1): 61-71.
54. Muscat, GE, Dressel, U: **Cardiovascular Disease and PPARdelta: Targeting the Risk Factors**. *Curr Opin Investig Drugs* 2005, **6**(9): 887-894.
55. Oliver, WR, Jr., Shenk, JL, Snaith, MR, Russell, CS, Plunket, KD, Bodkin, NL, Lewis, MC, Winegar, DA, Sznaidman, ML, Lambert, MH, Xu, HE, Sternbach, DD, Kliewer, SA, Hansen, BC, Willson, TM: **A Selective Peroxisome Proliferator-Activated Receptor delta Agonist Promotes Reverse Cholesterol Transport**. *PNAS* 2001, **98**(9): 5306-5311.
56. Vosper, H, Patel, L, Graham, TL, Khoudoli, GA, Hill, A, Macphee, CH, Pinto, I, Smith, SA, Suckling, KE, Wolf, CR, Palmer, CNA: **The Peroxisome Proliferator-Activated Receptor delta Promotes Lipid Accumulation in Human Macrophages**. *J Biol Chem* 2001, **276**(47): 44258-44265.
57. Burdick, AD, Kim, DJ, Peraza, MA, Gonzalez, FJ, Peters, JM: **The Role of Peroxisome Proliferator-Activated Receptor-[beta]/[delta] in Epithelial Cell Growth and Differentiation**. *Cellular Signalling* 2006, **18**(1): 9.
58. Lim, H, Gupta, RA, Ma, W-g, Paria, BC, Moller, DE, Morrow, JD, DuBois, RN, Trzaskos, JM, Dey, SK: **Cyclo-Oxygenase-2-Derived Prostacyclin Mediates Embryo Implantation in the Mouse Via PPARdelta**. *GENES & DEVELOPMENT* 1999, **13**(12): 1561-1574.
59. Yue, G, Stratil, A, Cepica, S, Schroffel, J, Schroffelova, D, Fontanesi, L, Cagnazzo, M, Moser, G, Bartenschlager, H, Reiner, G, Geldermann, H: **Linkage and QTL Mapping for Sus Scrofa Chromosome 7**. *Journal of Animal Breeding and Genetics* 2003, **120**(s1): 56-65.
60. de Koning, DJ, Rattink, AP, Harlizius, B, Groenen, MAM, Brascamp, EW, van Arendonk, JAM: **Detection and Characterization of Quantitative Trait Loci for Growth and Reproduction Traits in Pigs**. *Livestock Production Science* 2001, **72**(3): 185-198.
61. Rattink, AP, De Koning, DJ, Faivre, M, Harlizius, B, van Arendonk, JAM, Groenen, MAM: **Fine Mapping and Imprinting Analysis for Fatness Trait QTLs in Pigs**. *Mammalian Genome* 2000, **11**(8): 656-661.
62. Su, YH, Xiong, YZ, Zhang, Q, Jiang, SW, Lei, MG, Yu, L, Zheng, R, Deng, CY: **[Mapping Quantitative Trait Loci for Fat Deposition in Carcass in Pigs]**. *Yi Chuan Xue Bao* 2002, **29**(8): 681-684.

63. Wada, Y, Akita, T, Awata, T, Furukawa, T, Sugai, N, Ishii, K, Ito, Y, Kobayashi, E, Mikawa, S, Yasue, H, Inage, Y, Kusumoto, H, Matsumoto, T, Miyake, M, Murase, A, Shimanuki, S, Sugiyama, T, Uchida, Y, Yanai, S: **Quantitative Trait Loci (QTL) Analysis in a Meishan X Göttingen Cross Population**. *Animal Genetics* 2000, **31**(6): 376-384.
64. Demars, J, Riquet, J, Sanchez, MP, Billon, Y, Hocquette, JF, Lebret, B, Iannuccelli, N, Bidanel, JP, Milan, D, Gondret, F: **Metabolic and Histochemical Characteristics of Fat and Muscle Tissues in Homozygous or Heterozygous Pigs for the Body Composition QTL Located on Chromosome 7**. *Physiol Genomics* 2007, **30**(3): 232-241.
65. Ponsuksili, S, Murani, E, Walz, C, Schwerin, M, Wimmers, K: **Pre- and Postnatal Hepatic Gene Expression Profiles of Two Pig Breeds Differing in Body Composition: Insight into Pathways of Metabolic Regulation**. *Physiol Genomics* 2007, **29**(3): 267-279.
66. **Schweineproduktion 2004 in Deutschland**. Bonn; 2004.
67. Sambraus, HH: **Atlas der Nutztierrassen**, 4. edn. Stuttgart: Ulmer; 1994.
68. **Breeds of Livestock** [http://www.ansi.okstate.edu/breeds/swine/]
69. Fujii, J, Otsu, K, Zorzato, F, de Leon, S, Khanna, VK, Weiler, JE, O'Brien, PJ, MacLennan, DH: **Identification of a Mutation in Porcine Ryanodine Receptor Associated with Malignant Hyperthermia**. *Science* 1991, **253**(5018): 448-451.
70. Andersson, L: **Genetic Dissection of Phenotypic Diversity in Farm Animals**. *Nat Rev Genet* 2001, **2**(2): 130-138.
71. Ratky, J, Brussow, KP, Solti, L, Torner, H, Sarlos, P: **Ovarian Response, Embryo Recovery and Results of Embryo Transfer in a Hungarian Native Pig Breed**. *Theriogenology* 2001, **56**(5): 969.
72. **Richtlinie für die Stationsprüfung auf Mastleistung, Schlachtkörperwert und Fleischbeschaffenheit beim Schwein** http://www.zds-bonn.de/download.php/790/rlnkp04.pdf
73. Littmann, E, Götz, K-U, Dodenhoff, J: **Schweinezucht Und Schweineproduktion**, vol. 7. Freising: Bayrische Landesanstalt für Landwirtschaft; 2006.
74. Hallermayer, R: **Eine Schnellmethode zur Bestimmung des Fettgehaltes in Lebensmitteln**. *Deutsche Lebensmittelrundschau* 1976, **10**: 356-359.
75. Bligh, EG, Dyer, WJ: **A Rapid Method of Total Lipid Extraction and Purification**. *Can J Biochem Physiol* 1959, **37**(8): 911-917.
76. Arens, M, Schulte, E, Weber, K: **Fettsaeuremethylester, Umesterung Mit Trimethylsulfoniumhydroxid(Schnellverfahren) - Gemeinschaftsarbeiten Der Dgf, 138.Mitteilung: Deutsche Einheitsmethoden Zur Untersuchung Von Fetten, Fettprodukten, Tensiden Und Verwandetenstoffen, 105.Mitt.:Analyse Von Fetten Xxxi**. *Fat Science Technology* 1994, **96**(2): 67-68.
77. Binder, S: **Einfluss von Varianten des alpha (1,2)Fucosyltransferase (FUT1) - Gens auf den Escherichia Coli F18 - Adhäsionsphänotyp und auf Merkmale der Mastleistung Und Der Schlachtkörperqualität in Bayerischen Schweinepopulationen**. Freising: Technische Universität München; 2004.
78. **Primer3** http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi
79. Sanger, F, Nicklen, S, Coulson, AR: **DNA Sequencing with Chain-Terminating Inhibitors**. *Proc Natl Acad Sci U S A* 1977, **74**(12): 5463-5467.
80. Nickerson, DA, Tobe, VO, Taylor, SL: **Polyphred: Automating the Detection and Genotyping of Single Nucleotide Substitutions Using Fluorescence-Based Resequencing**. *Nucleic Acids Res* 1997, **25**(14): 2745-2751.
81. Ewing, B, Green, P: **Base-Calling of Automated Sequencer Traces Using Phred. II Error Probabilities**. *Genome Res* 1998, **8**(3): 186-194.

82. Gordon, D, Abajian, C, Green, P: **Consed: A Graphical Tool for Sequence Finishing**. *Genome Res* 1998, **8**(3): 195-202.
83. Ewing, B, Hillier, L, Wendl, MC, Green, P: **Base-Calling of Automated Sequencer Traces Using Phred. I. Accuracy Assessment**. *Genome Res* 1998, **8**(3): 175-185.
84. **Porcine Bac End Sequencing Project** http://www.sanger.ac.uk/Projects/S_scrofa/BES.shtml
85. **BAC PAC Resources** http://bacpac.chori.org/
86. **INRA BAC-YAC Resource Center** http://dga.jouy.inra.fr/grafra/
87. **Nebcutter V2.0** http://tools.neb.com/NEBcutter2/index.php
88. Nolan, T, Hands, RE, Bustin, SA: **Quantification of Mrna Using Real-Time RT-PCR**. *Nat Protoc* 2006, **1**(3): 1559-1582.
89. Python Software Foundation: **Python**. version 2.4.4, 2007 www.python.org
90. **Edit View**. version 1.0.1, *Applied Biosystems* http://www.appliedbiosystems.com/support/software/dnaseq/installs.cfm
91. Bates, D: **Lme4: Linear Mixed-Effects Models Using S4 Classes**. version 0.99875-6, 2007 www.r-project.org
92. R Development Core Team: **R: A Language and Environment for Statistical Computing**. version 2.4.1, *R Foundation for Statistical Computing* 2004 http://www.R-project.org
93. Applied Biosystems: **ABI Prism® 7000 SDS Software**. version 1.1,
94. Alvarez, MJ, Vila-Ortiz, GJ, Salibe, MC, Podhajcer, OL, Pitossi, FJ: **Model Based Analysis of Real-Time PCR Data from DNA Binding Dye Protocols**. *BMC Bioinformatics* 2007, **8**: 85.
95. Pfaffl, MW, Horgan, GW, Dempfle, L: **Relative Expression Software Tool (REST) for Group-Wise Comparison and Statistical Analysis of Relative Expression Results in Real-Time Pcr**. *Nucleic Acids Res* 2002, **30**(9): e36.
96. Stephens, M, Donnelly, P: **A Comparison of Bayesian Methods for Haplotype Reconstruction from Population Genotype Data**. *Am J Hum Genet* 2003, **73**(5): 1162-1169.
97. Stephens, M, Smith, NJ, Donnelly, P: **A New Statistical Method for Haplotype Reconstruction from Population Data**. *Am J Hum Genet* 2001, **68**(4): 978-989.
98. Warnes, G, Leisch, F: **Genetics: Population Genetics**. version 1.2.1, 2006 www.r-project.org
99. De Smet, S, Raes, K, Demeyer, D: **Meat Fatty Acid Composition as Affected by Fatness and Genetic Factors: A Review**. *Animal Research* 2004, **53**: 81-98.
100. Fox, J: **Car: Companion to Applied Regression**. version 1.2-1, 2006 www.r-project.org
101. **Effects: Effect Displays for Linear and Generalized Linear Models.** http://www.r-project.org
102. Komsta, L: **Test for Outliers**. version 0.12, 2005 http://www.r-project.org
103. Grubbs, FE: **Sample Criteria for Testing Outlying Observations.** *Ann Math Stat* 1950, **21**(1): 27-58.
104. Cheverud, JM: **A Simple Correction for Multiple Comparisons in Interval Mapping Genome Scans**. *Heredity* 2001, **87**(Pt 1): 52-58.
105. Altschul, SF, Madden, TL, Schaffer, AA, Zhang, J, Zhang, Z, Miller, W, Lipman, DJ: **Gapped Blast and Psi-Blast: A New Generation of Protein Database Search Programs**. *Nucl Acids Res* 1997, **25**(17): 3389-3402.
106. Benson, DA, Karsch-Mizrachi, I, Lipman, DJ, Ostell, J, Wheeler, DL: **Genbank**. *Nucl Acids Res* 2007, **35**(suppl_1): D21-25.

107. Gremme, G, Brendel, V, Sparks, ME, Kurtz, S: **Engineering a Software Tool for Gene Structure Prediction in Higher Organisms**. *Information and Software Technology* 2005, **47**(15): 965.
108. Lewis, SE, Searle, SMJ, Harris, N, Gibson, M, Iyer, V, Richter, J, Wiel, C, Bayraktaroglu, L, Birney, E, Crosby, MA, Kaminker, JS, Matthews, BB, Prochnik, SE, Smith, CD, Tupy, JL, Rubin, GM, Misra, S, Mungall, CJ, Clamp, ME: **Apollo: A Sequence Annotation Editor**. *Genome Biology* 2002, **3**(12): research0082.0081 - 0082.0014.
109. Hubbard, TJP, Aken, BL, Beal, K, Ballester, B, Caccamo, M, Chen, Y, Clarke, L, Coates, G, Cunningham, F, Cutts, T, Down, T, Dyer, SC, Fitzgerald, S, Fernandez-Banet, J, Graf, S, Haider, S, Hammond, M, Herrero, J, Holland, R, Howe, K, Howe, K, Johnson, N, Kahari, A, Keefe, D, Kokocinski, F, Kulesha, E, Lawson, D, Longden, I, Melsopp, C, Megy, K, Meidl, P, Ouverdin, B, Parker, A, Prlic, A, Rice, S, Rios, D, Schuster, M, Sealy, I, Severin, J, Slater, G, Smedley, D, Spudich, G, Trevanion, S, Vilella, A, Vogel, J, White, S, Wood, M, Cox, T, Curwen, V, Durbin, R, Fernandez-Suarez, XM, Flicek, P, Kasprzyk, A, Proctor, G, Searle, S, Smith, J, Ureta-Vidal, A, Birney, E: **Ensembl 2007**. *Nucl Acids Res* 2006: gkl996.
110. Edgar, RC: **Muscle: A Multiple Sequence Alignment Method with Reduced Time and Space Complexity**. *BMC Bioinformatics* 2004, **5**: 113.
111. Edgar, RC: **Muscle: Multiple Sequence Alignment with High Accuracy and High Throughput**. *Nucleic Acids Res* 2004, **32**(5): 1792-1797.
112. Knudsen, B, Knudsen, T, Flensborg, M, Sandmann, H, Heltzen, M, Andersen, A, Dickenson, M, Bardram, J, Steffensen, PJ, Mønsted, S, Lauritzen, T, Forsberg, R, Thanbichler, A, Bendtsen, JD, Görlitz, L, Rasmussen, J, Tordrup, D, Værum, M, Ravn, MN: **CLC Free Workbench**. version 3.2.1, *CLC bio A/S* 2005 www.clcbio.com
113. Bairoch, A, Apweiler, R, Wu, CH, Barker, WC, Boeckmann, B, Ferro, S, Gasteiger, E, Huang, H, Lopez, R, Magrane, M, Martin, MJ, Natale, DA, O'Donovan, C, Redaschi, N, Yeh, L-SL: **The Universal Protein Resource (Uniprot)**. *Nucl Acids Res* 2005, **33**(suppl_1): D154-159.
114. Boeckmann, B, Blatter, M-C, Famiglietti, L, Hinz, U, Lane, L, Roechert, B, Bairoch, A: **Protein Variety and Functional Diversity: Swiss-Prot Annotation in Its Biological Context**. *Comptes Rendus Biologies* 2005, **328**(10-11): 882.
115. Frith, MC, Hansen, U, Weng, Z: **Detection of Cis -Element Clusters in Higher Eukaryotic DNA**. *Bioinformatics* 2001, **17**(10): 878-889.
116. **Gene Regulation** [http://www.gene-regulation.com]
117. Cartharius, K, Frech, K, Grote, K, Klocke, B, Haltmeier, M, Klingenhoff, A, Frisch, M, Bayerlein, M, Werner, T: **Matinspector and Beyond: Promoter Analysis Based on Transcription Factor Binding Sites**. *Bioinformatics* 2005, **21**(13): 2933-2942.
118. Reese, MG: **Application of a Time-Delay Neural Network to Promoter Annotation in the Drosophila Melanogaster Genome**. *Comput Chem* 2001, **26**(1): 51-56.
119. Down, TA, Hubbard, TJ: **Computational Detection and Location of Transcription Start Sites in Mammalian Genomic DNA**. *Genome Res* 2002, **12**(3): 458-461.
120. Zuker, M: **Mfold Web Server for Nucleic Acid Folding and Hybridization Prediction**. *Nucl Acids Res* 2003, **31**(13): 3406-3415.
121. Hernandez-Sanchez, J, Visscher, P, Plastow, G, Haley, C: **Candidate Gene Analysis for Quantitative Traits Using the Transmission Disequilibrium Test: The Example of the Melanocortin 4-Receptor in Pigs**. *Genetics* 2003, **164**(2): 637-644.
122. Dunnen, Jd, Antonarakis, S: **Nomenclature for the Description of Human Sequence Variations**. *Human Genetics* 2001, **V109**(1): 121.

123. Kim, KS, Lee, JJ, Shin, HY, Choi, BH, Lee, CK, Kim, JJ, Cho, BW, Kim, TH: **Association of Melanocortin 4 Receptor (MC4R) and High Mobility Group at-Hook 1 (HMGA1) Polymorphisms with Pig Growth and Fat Deposition Traits**. *Animal Genetics* 2006, **37**(4): 419-421.
124. Jokubka, R, Maak, S, Kerziene, S, Swalve, HH: **Association of a Melanocortin 4 Receptor (MC4R) Polymorphism with Performance Traits in Lithuanian White Pigs**. *Journal of Animal Breeding and Genetics* 2006, **123**(1): 17-22.
125. Bruun, CS, Jorgensen, CB, Nielsen, VH, Andersson, L, Fredholm, M: **Evaluation of the Porcine Melanocortin 4 Receptor (MC4R) Gene as a Positional Candidate for a Fatness QTL in a Cross between Landrace and Hampshire**. *Animal Genetics* 2006, **37**(4): 359-362.
126. Stachowiak, M, Szydlowski, M, Obarzanek-Fojt, M, Switonski, M: **An Effect of a Missense Mutation in the Porcine Melanocortin-4 Receptor (MC4R) Gene on Production Traits in Polish Pig Breeds Is Doubtful**. *Anim Genet* 2006, **37**(1): 55-57.
127. Edwards, DP: **The Role of Coactivators and Corepressors in the Biology and Mechanism of Action of Steroid Hormone Receptors**. *J Mammary Gland Biol Neoplasia* 2000, **5**(3): 307-324.
128. Chawla, A, Repa, JJ, Evans, RM, Mangelsdorf, DJ: **Nuclear Receptors and Lipid Physiology: Opening the X-Files**. *Science* 2001, **294**(5548): 1866-1870.
129. Werman, A, Hollenberg, A, Solanes, G, Bjorbak, C, Vidal-Puig, AJ, Flier, JS: **Ligand-Independent Activation Domain in the N Terminus of Peroxisome Proliferator-Activated Receptor gamma (PPARgamma). Differential Activity of PPARgamma 1 and -2 Isoforms and Influence of Insulin**. *J Biol Chem* 1997, **272**(32): 20230-20235.
130. Wray, GA: **The Evolutionary Significance of Cis-Regulatory Mutations**. *Nat Rev Genet* 2007, **8**(3): 206.
131. Chamary, JV, Parmley, JL, Hurst, LD: **Hearing Silence: Non-Neutral Evolution at Synonymous Sites in Mammals**. *Nat Rev Genet* 2006, **7**(2): 98.
132. Kimchi-Sarfaty, C, Oh, JM, Kim, I-W, Sauna, ZE, Calcagno, AM, Ambudkar, SV, Gottesman, MM: **A "Silent" Polymorphism in the MDR1 Gene Changes Substrate Specificity**. *Science* 2007, **315**(5811): 525-528.
133. Nackley, AG, Shabalina, SA, Tchivileva, IE, Satterfield, K, Korchynskyi, O, Makarov, SS, Maixner, W, Diatchenko, L: **Human Catechol-O-Methyltransferase Haplotypes Modulate Protein Expression by Altering Mrna Secondary Structure**. *Science* 2006, **314**(5807): 1930-1933.
134. D'Onofrio, G, Mouchiroud, D, Aissani, B, Gautier, C, Bernardi, G: **Correlations between the Compositional Properties of Human Genes, Codon Usage, and Amino Acid Composition of Proteins**. *J Mol Evol* 1991, **32**(6): 504-510.
135. **Codon Usage Database** http://www.kazusa.or.jp/codon/
136. Nakamura, Y, Gojobori, T, Ikemura, T: **Codon Usage Tabulated from International DNA Sequence Databases: Status for the Year 2000**. *Nucl Acids Res* 2000, **28**(1): 292-.
137. Suzuki, Y, Yamashita, R, Sugano, S, Nakai, K: **DBTSS, Database of Transcriptional Start Sites: Progress Report 2004**. *Nucl Acids Res* 2004, **32**(suppl_1): D78-81.
138. Carninci, P, Sandelin, A, Lenhard, B, Katayama, S, Shimokawa, K, Ponjavic, J, Semple, CAM, Taylor, MS, Engstrom, PG, Frith, MC, Forrest, ARR, Alkema, WB, Tan, SL, Plessy, C, Kodzius, R, Ravasi, T, Kasukawa, T, Fukuda, S, Kanamori-Katayama, M, Kitazume, Y, Kawaji, H, Kai, C, Nakamura, M, Konno, H, Nakano, K, Mottagui-Tabar, S, Arner, P, Chesi, A, Gustincich, S, Persichetti, F, Suzuki, H,

Grimmond, SM, Wells, CA, Orlando, V, Wahlestedt, C, Liu, ET, Harbers, M, Kawai, J, Bajic, VB, Hume, DA, Hayashizaki, Y: **Genome-Wide Analysis of Mammalian Promoter Architecture and Evolution**. *Nat Genet* 2006, **38**(6): 626.

139. Smale, ST, Kadonaga, JT: **The Rna Polymerase II Core Promoter**. *Annual Review of Biochemistry* 2003, **72**(1): 449-479.
140. Rice, P, Longden, I, Bleasby, A: **Emboss: The European Molecular Biology Open Software Suite**. *Trends Genet* 2000, **16**(6): 276-277.
141. Stangegaard, M, Dufva, IH, Dufva, M: **Reverse Transcription Using Random Pentadecamer Primers Increases Yield and Quality of Resulting Cdna**. *Biotechniques* 2006, **40**(5): 649-657.
142. Pastinen, T, Sladek, R, Gurd, S, Sammak, Aa, Ge, B, Lepage, P, Lavergne, K, Villeneuve, A, Gaudin, T, Brandstrom, H, Beck, A, Verner, A, Kingsley, J, Harmsen, E, Labuda, D, Morgan, K, Vohl, M-C, Naumova, AK, Sinnett, D, Hudson, TJ: **A Survey of Genetic and Epigenetic Variation Affecting Human Gene Expression**. *Physiol Genomics* 2004, **16**(2): 184-193.
143. Miller, CW, Ntambi, JM: **Peroxisome Proliferators Induce Mouse Liver Stearoyl-CoA Desaturase 1 Gene Expression**. *Proc Natl Acad Sci U S A* 1996, **93**(18): 9443-9448.
144. Shimomura, I, Shimano, H, Korn, BS, Bashmakov, Y, Horton, JD: **Nuclear Sterol Regulatory Element-Binding Proteins Activate Genes Responsible for the Entire Program of Unsaturated Fatty Acid Biosynthesis in Transgenic Mouse Liver**. *J Biol Chem* 1998, **273**(52): 35299-35306.
145. Tabor, DE, Kim, JB, Spiegelman, BM, Edwards, PA: **Identification of Conserved Cis-Elements and Transcription Factors Required for Sterol-Regulated Transcription of Stearoyl-Coa Desaturase 1 and 2**. *J Biol Chem* 1999, **274**(29): 20603-20610.
146. Vandesompele, J, De Preter, K, Pattyn, F, Poppe, B, Van Roy, N, De Paepe, A, Speleman, F: **Accurate Normalization of Real-Time Quantitative RT-PCR Data by Geometric Averaging of Multiple Internal Control Genes**. *Genome Biology* 2002, **3**(7): research0034.0031 - research0034.0011.
147. Whittemore, CT: **An Approach to Pig Growth Modeling**. *J Anim Sci* 1986, **63**(2): 615-621.
148. Mitchell, AD: **Impact of Research with Cattle, Pigs, and Sheep on Nutritional Concepts: Body Composition and Growth**. *J Nutr* 2007, **137**(3): 711-714.
149. Clutter, AC, Brascamp, EW: **Genetics of Performance Traits**. In: *The Genetics of the Pig.* Edited by Rothschild MF, Ruvinsky A. Oxon, UK: CABI; 1998.
150. Kim, KS, Thomsen, H, Bastiaansen, J, Nguyen, NT, Dekkers, JC, Plastow, GS, Rothschild, MF: **Investigation of Obesity Candidate Genes on Porcine Fat Deposition Quantitative Trait Loci Regions**. *Obes Res* 2004, **12**(12): 1981-1994.
151. O'Hea, EK, Leveille, GA: **Significance of Adipose Tissue and Liver as Sites of Fatty Acid Synthesis in the Pig and the Efficiency of Utilization of Various Substrates for Lipogenesis**. *J Nutr* 1969, **99**(3): 338-344.
152. Hems, DA, Rath, EA, Verrinder, TR: **Fatty Acid Synthesis in Liver and Adipose Tissue of Normal and Genetically Obese (Ob/Ob) Mice During the 24-Hour Cycle**. *Biochem J* 1975, **150**(2): 167-173.
153. Pesole, G, Liuni, S: **Internet Resources for the Functional Analysis of 5' and 3' Untranslated Regions of Eukaryotic Mrnas**. *Trends Genet* 1999, **15**(9): 378.
154. Clop, A, Marcq, F, Takeda, H, Pirottin, D, Tordoir, X, Bibe, B, Bouix, J, Caiment, F, Elsen, J-M, Eychenne, F, Larzul, C, Laville, E, Meish, F, Milenkovic, D, Tobin, J, Charlier, C, Georges, M: **A Mutation Creating a Potential Illegitimate Microrna**

Target Site in the Myostatin Gene Affects Muscularity in Sheep. *Nat Genet* 2006, **38**(7): 813-818

155. Goßner, H: **Kartierung von QTL in einer F2-Kreuzungspopulation des Schweines unter Verwendung der QTL-Express-Applikation**. Freising-Weihenstephan: Technische Universität München; 2002.
156. Aberle, J, Hopfer, I, Beil, FU, Seedorf, U: **Association of Peroxisome Proliferator-Activated Receptor delta +294T/C with Body Mass Index and Interaction with Peroxisome Proliferator-Activated Receptor alpha L162v**. *Int J Obes* 2006, **30**(12): 1709.
157. Lubrano-Berthelier, C, Dubern, B, Lacorte, J-M, Picard, F, Shapiro, A, Zhang, S, Bertrais, S, Hercberg, S, Basdevant, A, Clement, K, Vaisse, C: **Melanocortin 4 Receptor Mutations in a Large Cohort of Severely Obese Adults: Prevalence, Functional Classification, Genotype-Phenotype Relationship, and Lack of Association with Binge Eating**. *J Clin Endocrinol Metab* 2006, **91**(5): 1811-1818.
158. Becker, KG, Barnes, KC, Bright, TJ, Wang, SA: **The Genetic Association Database**. *Nat Genet* 2004, **36**(5): 431-432.
159. Hebebrand, J, Fichter, M, Gerber, G, Gorg, T, Hermann, H, Geller, F, Schafer, H, Remschmidt, H, Hinney, A: **Genetic Predisposition to Obesity in Bulimia Nervosa: A Mutation Screen of the Melanocortin-4 Receptor Gene**. *Mol Psychiatry* 2002, **7**(6): 647-651.
160. Hebebrand, J, Geller, F, Dempfle, A, Heinzel-Gutenbrunner, M, Raab, M, Gerber, G, Wermter, AK, Horro, FF, Blundell, J, Schafer, H, Remschmidt, H, Herpertz, S, Hinney, A: **Binge-Eating Episodes Are Not Characteristic of Carriers of Melanocortin-4 Receptor Gene Mutations**. *Mol Psychiatry* 2004, **9**(8): 796-800.
161. Tao, YX, Segaloff, DL: **Functional Analyses of Melanocortin-4 Receptor Mutations Identified from Patients with Binge Eating Disorder and Nonobese or Obese Subjects**. *J Clin Endocrinol Metab* 2005, **90**(10): 5632-5638.
162. Potoczna, N, Branson, R, Kral, JG, Piec, G, Steffen, R, Ricklin, T, Hoehe, MR, Lentes, KU, Horber, FF: **Gene Variants and Binge Eating as Predictors of Comorbidity and Outcome of Treatment in Severe Obesity**. *J Gastrointest Surg* 2004, **8**(8): 971-981; discussion 981-972.
163. Chagnon, YC, Chen, WJ, Perusse, L, Chagnon, M, Nadeau, A, Wilkison, WO, Bouchard, C: **Linkage and Association Studies between the Melanocortin Receptors 4 and 5 Genes and Obesity-Related Phenotypes in the Quebec Family Study**. *Mol Med* 1997, **3**(10): 663-673.
164. Kim, KS, Larsen, NJ, Rothschild, MF: **Rapid Communication: Linkage and Physical Mapping of the Porcine Melanocortin-4 Receptor (MC4R) Gene**. *J Anim Sci* 2000, **78**(3): 791-792.
165. Rothschild, MF, Hu, ZL, Jiang, Z: **Advances in QTL Mapping in Pigs**. *Int J Biol Sci* 2007, **3**(3): 192-197.
166. **Porcine Genome Sequencing Project** [http://www.sanger.ac.uk/Projects/S_scrofa/]

9 Lists of abbreviations, tables and figures

9.1 Abbreviations

A	adenine
ABI	Applied Biosystems
ACTB	actin, beta
BAC	bacterial artificial chromosome
BLAST	basic local alignment search tool
bp	base pair
C	cytosine
cDNA	complementary/copy deoxyribonucleic acid
CFA	Canis familiaris
cM	centi Morgan
DNA	deoxyribonucleic acid
dNTP	nucleotides
DTT	dithiothreitol
EDTA	ethylendiamintetraacetat
FASN	fatty acid synthase
G	guanine
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GGA	Gallus gallus
HPRT1	hypoxanthine phosphoribosyltransferase 1
HSA	Homo sapiens
HT	haplotype
kb	kilo base pairs
MC1R	melanocortin 1 receptor
MC2R	melanocortin 2 receptor
MC3R	melanocortin 3 receptor
MC4R	melanocortin 4 receptor
MC5R	melanocortin 5 receptor
MCR	melanocortin receptor
MMU	Mus musculus
M-MuLV	Moloney Murine Leukemia Virus reverse transcriptase
mRNA	messenger ribonucleic acid
N	A, C, G, T, U
NaCl	sodium chloride
NCBI	National Center for Biotechnology Information
PCR	polymerase chain reaction
PPAR	peroxisome proliferator-activated receptor
PPARA	peroxisome proliferator-activated receptor alpha gene
PPAR-α	peroxisome proliferator-activated receptor alpha
PPARD	peroxisome proliferator-activated receptor delta gene
PPAR-δ	peroxisome proliferator-activated receptor delta
PPARG	peroxisome proliferator-activated receptor gamma gene
PPAR-γ	peroxisome proliferator-activated receptor gamma

QTL	quantitative trait locus / loci
RACE	Rapid Amplification of cDNA ends
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RNO	Rattus norvegicus
rpm	rounds per minute
RT-PCR	reverse transcription PCR
SCD1	stearoyl-CoA desaturase 1
SDS	sodium dodecylsulfat
SNP	single nucleotide polymorphism
SSC	Sus Scrofa
SSC7	porcine chromosome 7
T	thymine
TBE	tris-borate-EDTA buffer
TBP	TATA box binding protein
TE	tris EDTA buffer
TEMED	N', N', N', N', tetramethylethylendiamin
TFBS	transcription factor binding site
TOP2B	topoisomerase (DNA) II beta
Tris	tris (hydroxymethyl) aminomethane
TSS	transcription start site
UTR	untranslated region

9.2 Tables

9.3 Figures

10 Appendix

10.1 Primer sequences

10.1.1 Primers used for sequencing and genotyping of *PPARD*

primer number	direction	pair number	sequence	localisation	$T_{annealing}$ [°C]	product length [bp]
3269	forward	3270	CTACAGCGCCTACCTGAAAAAC	exon 6	60	644
3270	reverse	3269	GAGAGCCAGGTCACTATCATCG	exon 7	60	644
3327	forward	3391	GGATTAATGGGAAAAGTTTTGG	promoter	59	281
3381	forward	3382	TCACCCTCTCATCCTCTACACC	intron 2	60	410
3382	reverse	3381	GCTGATTAGCGATAGAGTGACC	intron 3	60	410
3383	forward	3384	CCAAGGTCCCCTGTCCTC	intron 7	60	489
3384	reverse	3383	GAGAGGAGGCAGGGCTATAAG	exon 8	60	489
3385	forward	3386	TCTCTGTCTTTGCTCGTGTACC	intron 5	67	921
3386	reverse	3385	CCAGGAGGGCTGAGTGTG	intron 7	67	921
3391	reverse	3327	AGCAACTAACGACCGTGGAC	intron 1	59	281
3392	forward	3393	TCCAGGATTGAGAAAAATCTGC	intron 1	60	324
3393	reverse	3392	CAAGAATCCTAAACCTGGGATG	intron 2	60	324
3396	forward	3397	AACCATCTTTCTCCCTTCTTCG	intron 4	60	280
3397	reverse	3396	GCACTCCCTTCTGTCTCTGG	intron 5	60	280
3414	reverse	3445	AGGAAGAACCTACAAGCACCAC	intron 4	65	565
3417	forward	3418	GCTTCCACTACGGAGTCCAC	intron 4	59	778
3418	reverse	3417	GATGAGGGAGGGTGAGAAAAG	intron 4	59	778
3421	forward	3422	GCTGGGCATGTCTCACAAC	intron 5	59	1192
3422	reverse	3421	CAAAGCGAATGGCTGCATAG	intron 5	59	1192
3423	forward	3424	TAGTGACCTGGCTCTCTTCATC	intron 7	59	609
3424	reverse	3423	ATGGCCTCCACCTGTGAC	intron 7	59	609
3445	forward	3414	CTGCCCCTGCTGTGTCTG	intron 4	65	565
3598	reverse	3626	GCTGCTTGCCTATCCACTTC	promoter	60	587
3626	forward	3598	GCAGCACAGTTTCCTCCAG	promoter	60	587
3709	forward	3710	GGCAAGGAGGTTAACATCTGA	promoter	60	845
3710	reverse	3709	GAGACTCCCCTGAATCACCA	promoter	60	845
3735	reverse	3776	CTGTTGCAGTCACACGGTTC	promoter	60	988
3776	forward	3735	TGAGGATCCCCTCTGTGGTA	promoter	60	988
3805	forward	3806	GAATGCCTCTTCCTGAATGG	promoter	60	697
3806	reverse	3805	CCTCCTTGCCTTTGATATTGA	promoter	60	697
3807	forward	3808	CTGCAGTCGACTCTGAGGAT	promoter	60	597
3808	reverse	3807	TCTGTACCCTCCTTTGAGGTTT	promoter	60	597
3831	forward	3832	TTCAGTCCTTGGCCTCACTC	intron 3	60	626
3832	reverse	3831	TAGGCGCTGTAGAGGTGCTT	intron 3	60	626
3981	forward	3982	GCATCTCTGGGGAGTTCCTA	intron 5	60	846
3982	reverse	3981	TCGTTGAGGAAGAGGTGGTC	exon 7	60	846
3983	forward	3984	GGGCTGGGAAGCTAAACTCT	intron 1	60	379
3984	reverse	3983	CACTGCCTTTGGTGTTTGTG	intron 1	60	379
4870	forward	4871	GGACGTTTCCGTTGACCAG	exon 8	56	951
4871	reverse	4870	GTGATTTCAGTGCTCGTGCT	exon 8	56	951
4912	forward	4913	AGCTGAAGCGCACACTCC	exon 8	60	821
4913	reverse	4912	CCGAGGACAAACACTGTGAA	exon 8	60	821
5199	reverse	3392	CTTTGGTGCATCTGTCCTCA	exon 2	60	280

10.1.2 Primers used for sequencing and genotyping of *MC4R*

primer number	direction	pair number	sequence	localisation	$T_{annealing}$ [°C]	product length [bp]
3712	reverse	4212	GCCTCGCAGAGAGAATCAGT	exon	60	997
3744	forward	3745	ATTGGGGTCTTTGTGGTCTG	exon	60	548
3745	reverse	3744	GCAATGTGTCAAATCCTTTCG	exon	60	548
4133	forward	4134	TCGATTGCAGTGGACAGGTA	exon	60	662
4134	reverse	4133	GAAAATGCTGTTGTTGAAGCA	exon	60	662
4212	reverse	3711	CCAACCCGCTTAACTGTCAT	exon	60	997
4213	forward	5014	AGCTCCTTACTCGCCTCAA	exon	60	1000
4214	reverse	5013	ACCTCTACACTTCAGCGTTGTAT	exon	60	1000
4215	forward	5016	CGAAAGGATTTGACACATTGC	exon	60	1105
4216	reverse	5015	CAGACCATGAGGATTTGAGGA	3' not transcribed	60	1105
4217	forward	5018	CAAATTAGCCTTCCACAAGCA	3' not transcribed	60	849
4218	reverse	5017	TTGGTTTTACCAGAGAAAGAGGA	3' not transcribed	60	849
4219	forward	5020	TCTGGTAAAACCAAGATTCTGC	3' not transcribed	60	809
4220	reverse	5019	GCAGAAATGCAAAATGCAAA	3' not transcribed	60	809
4221	forward	5022	CAGTTGCCAAGGTATGTGTGA	promoter	60	959
4222	reverse	5021	GGATCAATAACTAGGCGATACTCA	promoter	60	959

10.1.3 Primers used for various PCR protocols from cDNA

gene	primer number	direction	pair number	sequence	localisation	$T_{annealing}$ [°C]	product length [bp]
ACTB	5089	forward	5090	TCTGGCACCACACCTTCT	exon 2	60	114
ACTB	5090	reverse	5089	TGATCTGGGTCATCTTCTCAC	exon 2/3	60	114
FASN	5066	forward	5051	TGATCGGATCCACCAAGTCG	exon 8	60	78
FASN	5067	reverse	5050	ACAGCAGCACCTTGATCAGC	exon 8/9	60	78
GAPDH	4128	forward	4129	TCCCACGGCACAGTCAA		60	536
GAPDH	4129	reverse	4128	GCAGGTCAGGTCCACAA		60	536
HPRT1	5070	forward	5071	TTATGGACAGGACTGAACGGC	exon 2/3	60	115
HPRT1	5071	forward	5070	TCCAGCAGGTCAGCAAAGAAT	exon 3	60	115
PPARD	4126	forward	4127	GTCTCACAACGCCATTCGCT	exon 5/6	60	642
PPARD	4127	reverse	4126	CACCAGCAGCCCATCCTTAT	exon 7	60	642
PPARD	4646	forward	4647	CACAGCGCCCTTTGTGAT	exon 6/7	60	657
PPARD	4647	reverse	4646	TCGGTCTCGGTCTTCTTGAT	exon7	60	657
PPARD	4931	reverse	4286	CGGAAGAAGCCCTTGCAC	exon 4/5	60	490
PPARD	4999	forward	5000	GAGCTGGGATTAATGGGAAA	exon 1	60	407
PPARD	5000	reverse	4999	GTACAGCTGCTGGAGGGAAG	exon 3	60	407
PPARD	5011	forward	4931	CGG GGG GCA GCC AAG TCA	exon1	60	537
PPARD	5012	forward	4931	AGG ACC CTG CGG AGC CTG CC	exon1	60	501
PPARD	5045	reverse	4999	CAACCTCTTTGGTGCATCTG	exon 2/3	60	181
PPARD	5166	reverse	4999	TGTCAACCTGACTTGGCTGC	exon 1/3	60	100
PPARD	5199	reverse	4999	CTTTGGTGCATCTGTCCTCA	exon 2	60	176
PPARD	5278	forward	5279	CATGTCTCACAACGCCATTCG	exon 5/6	66	237
PPARD	5279	reverse	5278	ATGTCGTGGATCACAAAGGGC	exon 6/7	66	237
SCD	5353	forward	5354	GATGGCATTCCAGAATGACG	exon 3/4	60	132
SCD	5354	reverse	5353	ACAAGCAGCCAACCCACG	exon 4	60	132
TBP	5091	forward	5092	GATGGACGTTCGGTTTAGG	exon 3	60	124
TBP	5092	reverse	5091	AGCAGCACAGTACGAGCAA	exon 3/4	60	124
TOP2B	5094	reverse	5191	TGGAAAAACTCCGTATCTGTCTC	exon 12	60	117
TOP2B	5191	forward	5094	GCTGGTGGCAAACACTCACTGG	exon 11/12	60	117

10.2 Nucleotide numbering for the description of sequence variations

10.2.1 Coding regions

Nucleotide +1 is the A of the ATG-translation initiation codon.

10.2.2 Non-coding regions

The nucleotide 5' of the ATG-translation initiation codon is –1, the nucleotide 3' of the translation termination codon is *1.

10.2.3 Intronic regions

10.2.3.1 Beginning of the intron

The position of the mutant base is described by the number of the last nucleotide of the preceeding exon, a plus sign, and the position in the intron

10.2.3.2 End of the intron

The position of the mutant base is described by the number of the first nucleotide of the following exon, a minus sign, and the position upstream in the intron.

10.2.4 Promoter region

The position of the mutant base is described by the number of the first nucleotide of the mRNA RefSeq, a minus sign, and the position upstream in the promoter.

10.3 Nucleotide Sequences

10.3.1 Predicted mRNA sequence of *PPARD*

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>SSC PPARD mRNA predicted 3325 bases
00001 AATGGGAAAA GTTTTGGCAG GGGCCGGAGG ACCCTGCGGA GCCTGCCGGA
00051 CGGTGGCGGT GGCGCGGGGG GCAGCCAAGT CAGCGTCGCG TGGTGTTTTG
00101 GGTATGCACG TGGTACTCAC ACAGTGGCTG CTGTTCACCG ACAGATGAGG
00151 ACAGATGCAC CAAAGAGGTT GACAGGAACT GCCCTGTAGA GGTCCATCTG
00201 CACTCAGACC CAGATGATGC CAGAGCTATG ACCGGGCCTG CAGGCGTGGC
00251 GCCGAGGGGA AGTCAGCCAT GGAGCAGCCG CCGGAGGAAG CCCCTGAGGT
00301 CCGGGAAGAG GAGAAGAAAA AGGAAGTGGC AGAGGCCGAA GGAGGCCCAG
00351 AGCTCAATGG GGGACCAGAG CACTCGCTTC CCTCCAGCAG CTGTACAGAT
00401 CTCTCCCAGA GCTGCTCTCC ACCCGCGCTG CTGGACCAGC TGCAGATGGG
00451 CTGCGACGGG GCCTCGTGCG GTGGCCTCAG CATGGAGTGC CGGGTGTGCG
00501 GGGACAAGGC ATCAGGCTTC CACTACGGAG TCCACGCTTG CGAGGGGTGC
00551 AAGGGCTTCT TCCGCCGGAC AATCCGCATG AAGCTGGAGT ACGAGAAGTG
00601 TGAGCGGATC TGCAAGATCC AGAAGAAGAA CCGCAACAAG TGCCAGTACT
00651 GCCGCTTCCA GAAATGCCTG GCGCTGGGCA TGTCTCACAA CGCCATTCGC
00701 TTTGGCCGGA TGCCCGAGGC AGAGAAAAGG AAGCTGGTGG CTGGGCTGAC
00751 GGCAAACGAG GGGAGTCAGC ACAACCCGCA GGTGGCTGAC CTGAAGGCCT
00801 TCTCCAAGCA CCTCTACAGC GCCTACCTGA AAAACTTCAA CATGACCAAA
00851 AAGAAGGCCC GCGCCATCCT CACCGGCAAG GCCAGCCACA CAGCGCCCTT
00901 TGTGATCCAC GACATCGAGA CGTTGTGGCA GGCCGAGAAG GGCCTGGTGT
00951 GGAAGCAGCT GGTGAATGGC CTGCCGCCCT ACAAGGAGAT CAGCGTGCAC
01001 GTCTTCTACC GCTGCCAGTG CACCACGGTG GAGACGGTGC GCGAGCTGAC
01051 CGAGTTCGCC AAGAGCATCC CCAGCTTCGA CCACCTCTTC CTCAACGACC
01101 AGGTGACCCT TCTCAAGTAC GGCGTGCACG AGGCCATCTT CGCCATGCTG
01151 GCCTCCATCG TCAATAAGGA TGGGCTGCTG GTGGCCAACG GCACTGGTTT
01201 TGTCACCCGC GAGTTCCTGC GCAGCATCCG AAAGCCCTTC AGTGACATCA
01251 TTGAGCCCAA GTTTGAGTTC GCTGTCAAGT TCAATGCCCT GGAACTCGAT
01301 GATAGTGACC TGGCTCTCTT CATCGCAGCC ATCATTCTGT GTGGAGACCG
01351 GCCAGGCCTC ATGAACGTGT CACAGGTGGA GGCCATCCAG GACACCATCC
01401 TGCGTGCCCT CGAGTTCCAC CTGCAGGCCA ACCACCCCGA CGCCCAGTAC
01451 CTCTTCCCCA AGCTGCTGCA GAAGATGGCA GACCTGCGGC AGCTGGTCAC
01501 CGAGCACGCC CAGATGATGC AGCGGATCAA GAAGACCGAG ACCGAGACCT
01551 CGCTGCACCC CCTGCTCCAG GAGATCTACA AGGACATGTA CTGAGGGGTG
01601 CGCCTTGGGC CTCCCAACAG GCCTCCCGGA GCAGGTGGAC GGCGCGGGGA
01651 CAGACACTGC CTGCGGGACG TTTCCGTTGA CCAGCCCGAG CCCTCAGCCG
01701 AGCAGCAGGT CACAGGCTCA GCCAGACGCA CGGCCTCCCA CTCCTTATAG
01751 CCCTGCCTCC TCTCCCTCCT CAGCTCCCCT CTCTCTCATC TCTTTGCTCT
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01801 TTCTTTTCCT TCCTCTCTCA GCCTCGCTTT CTCTCTCCCC ATCCTGTCTG
01851 TCCATCTTTC TCTTCCTGTG AGACAGTTTG TGTTATTTCA CCAGCACCAA
01901 AACAAGACCG CTGCTTTGTC CCCTGCTCCC CGGCCCCGGA GCAGGAGGGG
01951 GCAGGGCCTG CCCTCTGCAC CAACCATCGC CTTCTCCAGT CTTCAAAGGA
02001 CACGCAGGCC ATCCAAAGAA ACACTAAGCT CTCCGGGCCT GGCTTACTGG
02051 GGAAGCCACG CAGGGCCTGG GCTGAGTGCC GAGCAGCCCT AGCCACAGGG
02101 TCCCTGGGGG AGGCCGCCCA CCCGAGGCTG AGGCTGGCAC CCCATGGCTG
02151 AGCGGACCCC GCTCCTGCAG CATGCCTCAG CCCCACAGAC GCCCACCCCT
02201 CTTTTGTTTT TCTTTGCACC AGTCTTCCAG GCCAGTGCCA CTGTGCTGGC
02251 TGCTGGCGGA TGCCCCCAGC CTGGATGGAG GTGGGATTCC CTCCAGGTGG
02301 GGGCGCCCAC ACCCCCATTG AAGAGGAGCA TGCCTCAAGG GAGCAGTTGG
02351 TAGGGAAGGC AGTGGGCAGC AGACTTGATT CTGACCCCAG GCCTTGGGTG
02401 GGTCCTCCCT CAGCACCCCA CTCTCTCCAG CCTCTGCAGC AGCCACTGAG
02451 CCCTGCCCAC GCTGTGTCAG CATCGCACCT CCCACCTCCG CAGCACCCCG
02501 GCTTGGCCTC AGCCACGCCC TTCTTTCTCC AGCCGGGCGA CACTGGCTCC
02551 AGCCCAGCTG AAGCGCACAC TCCCTGGAGC GCCTCCAGCA CACGCAGCAC
02601 GAGCACTGAA ATCACTTTAC CTGCAGGTTC CACAACCTCG GCCTCCCTCC
02651 TGAGGCAGGT GGACCACAGA GCTGTGCCCC TGACTCCCCG GGCGGGCGGG
02701 GAGCCCTGCT GCCCCAGCCC AGCACTGCTC GCAGGGGAGG TACCCAGGAT
02751 GAACTGATCC CGCTCACTTG TGACACCCAT TTGTTCCAGC AGCTCTGCTG
02801 CCCTCCCCTT TCCTTGTGAT TGGCCCAGCC AGGCACCTGG AGCTCTCCCT
02851 GCACCGCTTC TGGTGACCAG GGACCCTGCC AGGCCCAGTC GCACTCCTGG
02901 CCCCACCCCG GCCCCCTCCC CAGTGACAGC ACTCACCTCA GCAGGGGCTG
02951 AAGCACAGCT GGGGCCCCGT CTGAGCGGGA GGACAGGGCA CTTTTCCAGG
03001 GAGGAAGCTG ACCTGGAGCG CCCAGGGGCT GGCTAGGGTC TCTGAGAAGG
03051 TGGCGGAAGA CCTGGGGTTA GCGCCCAGGT TCCTGGGTCC TGTCTGGTCT
03101 TCCTTCCCCG GGGGTACTCT GTGCTGGCAG GATTGTCCCC TCCCGGCCAC
03151 ACACCACTTC CCATCCTTGC CCCCCCCCCC CGGTCTGGTA CTGAGGCTAC
03201 AGCTCTTCTC AGTGTCTGAA CAATCTCCAA AACTGAAATG TATATTTTTG
03251 CTAGGAGCAG CTTCCTGTTT TTAATATAAA TAGTGTACAC AGACTGACAG
03301 AACTTTAAAT AAATGGGAAT TAAAT
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10.4 Descriptive statistics of pigs used for association analyses

10.4.1 Mangalitsa x Piétrain

	Weight [kg]	Test period [d]	Test daily gain [g / day]	Test average food intake [kg / day]	Meat content	Backfat depth (middle of the back) [cm]	Average backfat thickness [cm]	Fat depth at side [cm]
Minimum	70.00	57.00	340.30	1489.42	44.51	1.05	1.98	1.11
Maximum	118.00	183.00	852.11	2903.28	72.40	5.10	4.91	6.69
1. Quartile	94.50	99.00	500.00	1894.46	51.07	2.55	3.04	3.74
3. Quartile	100.00	120.00	604.20	2165.17	55.68	3.29	3.73	4.88
Mean	97.24	110.04	556.08	2029.33	53.67	2.93	3.37	4.28
Median	97.00	107.00	557.55	2010.00	53.27	2.92	3.38	4.30
SE Mean	0.16	0.74	3.23	8.60	0.15	0.02	0.02	0.36
Variance	16.22	331.20	6268.62	44298.90	14.02	3.10	2.63	76.75
SD	4.03	18.20	79.17	210.47	3.74	0.56	0.51	8.76

	Intramuscular fat content	Saturated fatty acid content [%]	Monounsaturated fatty acid content [%]	Polyunsaturated fatty acid content [%]	Ps ratio	Desaturation index C16 [%]	Desaturation index C18 [%]	Elongase index [%]
Minimum	0.98	26.68	22.93	3.54	0.55	9.49	44.99	43.99
Maximum	4.59	62.90	63.12	32.40	2.71	19.82	87.45	77.37
1. Quartile	1.61	35.64	45.41	12.04	1.62	13.30	77.82	65.63
3. Quartile	2.37	37.74	49.38	16.89	1.77	15.28	79.96	67.07
Mean	2.06	36.67	47.28	14.69	1.70	14.32	78.92	66.34
Median	1.95	36.72	47.39	14.44	1.69	14.27	79.02	66.37
SE Mean	0.03	0.08	0.14	0.17	0.01	0.06	0.09	0.07
Variance	0.40	4.06	11.67	15.67	0.02	0.02	0.05	0.03
SD	0.63	2.02	3.42	3.96	0.14	1.40	2.23	1.65

10.4.2 German Landrace

	Weight [kg]	Test period [d]	Test daily gain [g / day]	Test average food intake [kg / day]	Meat content	Backfat depth (middle of the back) [cm]	Average backfat thickness [cm]	Fat depth at side [cm]
Minimum	99.00	64	562.0	0.06	46.17	1.21	0.40	1.20
Maximum	116.00	134	1127.0	3.15	66.16	3.68	3.30	4.90
1. Quartile	106.00	85	781.0	2.16	54.34	1.79	1.40	2.80
3. Quartile	108.50	99	905.0	2.46	57.51	2.28	1.90	3.50
Mean	107.09	93	844.1	2.31	55.96	2.06	1.64	3.17
Median	107.00	92	837.0	2.31	55.94	2.02	1.60	3.20
SE Mean	0.08	0.4	3.4	0.01	0.09	0.01	0.02	0.02
Variance	4.75	113	8073.3	0.06	5.98	0.14	0.17	0.33
SD	2.18	11	89.9	0.25	2.44	0.38	0.41	0.57