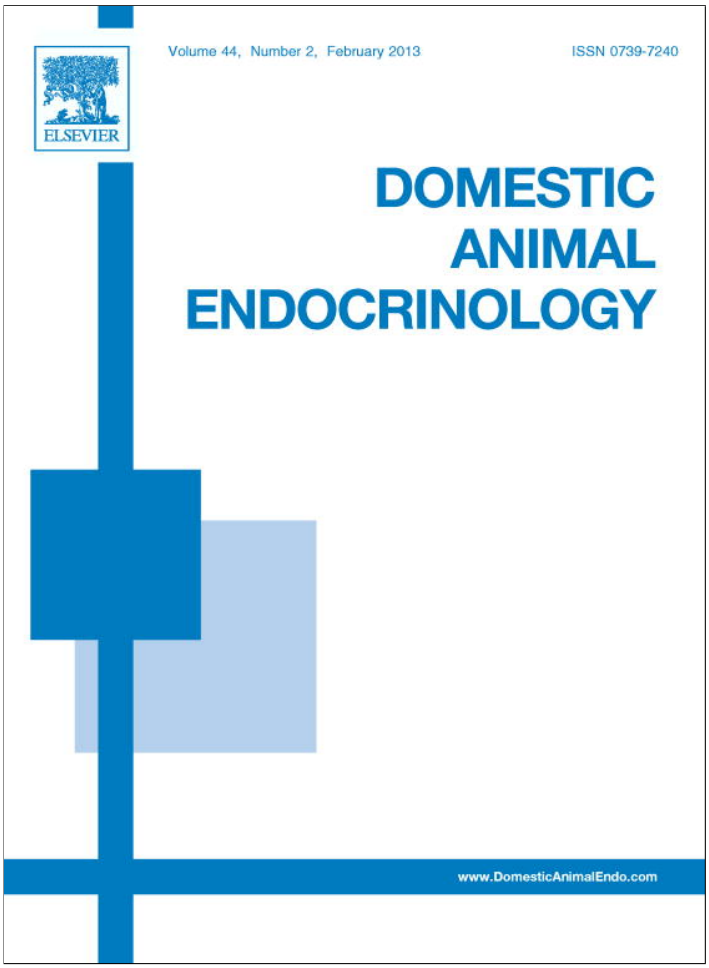

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Association study of molecular polymorphisms in candidate genes related to stress responses with production and meat quality traits in pigs

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Abstract

The hypothalamic-pituitary-adrenal (HPA) axis exerts a large range of effects on metabolism, the immune system, inflammatory processes, and brain functions. Together with the sympathetic nervous system, it is also the most important stress-responsive neuroendocrine system. Both systems influence production traits, carcass composition, and meat quality. The HPA axis may be a critical target for genetic selection of more robust animals. Indeed, numerous studies in various species have demonstrated the importance of genetic factors in shaping the individual HPA axis phenotype, and genetic polymorphism can be found at each level of the axis, including hormone production by the adrenal cortices under stimulation by adrenocorticotropic hormone (ACTH), hormone bioavailability, or receptor and postreceptor mechanisms. The aim of the present experiment was to extend these findings to the brain neurochemical systems involved in stress responses. To this end, a number of candidate genes were sequenced for molecular polymorphisms and their association was studied with stress neuroendocrine and production traits in a genetically diverse population consisting of 100 female pigs from an advanced intercross (F10-F12) between 2 highly divergent breeds, Large White (LW) and Meishan (MS). The LW breed has a high production potential for lean meat and a low HPA axis activity, and the MS breed has low growth rate, fat carcasses—but large litters of highly viable piglets—and a high HPA axis activity. Candidate genes were chosen in the catecholaminergic and serotonergic pathways, in the pituitary control of cortisol production, among genes previously demonstrated to be differentially expressed in ACTH-stimulated adrenal glands from LW and MS pigs, and in cortisol receptors. Sixty new polymorphisms were found. The association study with carcass and meat quality traits and with endocrine traits showed a number of significant results, such as monoamine oxidase (*MAOA*) polymorphisms with growth rate ($P = 0.01$); lean content and intramuscular fat ($P < 0.01$), which are the most important traits for carcass value; dopamine receptor D3 (*DRD3*) with carcass composition ($P < 0.05$); and vasopressin receptor 1B (*AVPR1B*) with meat quality traits ($P \leq 0.05$). The effect of these polymorphisms on neuroendocrine parameters (eg *DRD3* and HPA axis or *AVPR1B* and catecholamines) indicates information regarding their biological mechanism of action.

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1. Introduction

The hypothalamic-pituitary-adrenal (HPA) axis exerts a large range of effects on metabolism, the immune system, inflammatory processes, and brain functions. It is also the most important stress-responsive neuroendocrine pathway, together with the autonomic nervous system. In farm animals, the HPA axis plays a critical role in the balance between production and robustness traits [1,2]. Glucocorticoid hormones (cortisol in pigs) act via the activation of 2 receptors, the glucocorticoid receptor (GR) encoded by the *NR3C1* gene and the mineralocorticoid receptor, encoded by the *NR3C2* gene. These receptors are transcription factors regulating the expression of hundreds of genes [3]. Glucocorticoid hormones favor the deposit of fat at the expense of proteins [4] but there is some evidence for a positive influence on so-called functional traits, such as newborn survival [5] or resistance to heat stress [6]. Continuous selection for high feed efficiency, fast growth rate, and leaner carcasses has been paralleled by decreased HPA axis activity [7], which may be one of the physiological mechanisms involved in the reduction of robustness when selection focuses on production traits only [8,9]. The current evolution of animal production systems (increase of economic pressure, diversification of production environments, reduction of individual animal management, increase of parasitic load with outdoor production) combined with global warming reinforces the importance of adaptation and robustness traits in sustainable breeding goals [10].

Several breeding strategies can be implemented to increase robustness [1]. Genetic improvement in functional traits, such as leg soundness, mortality rates in various stages of the animal's life, and functional longevity, is possible when these traits are properly included in breeding goals and selection criteria and is being realized in existing breeding programs [9]. Although valuable, this approach requires substantial phenotyping of the animals for multiple and frequently difficult-to-measure traits. Alternatively, the global sensitivity to the environment can be measured by techniques such as the reaction norm analysis by comparing animals with identical genotypes in different environments. This is a difficult endeavor and heritability of the trait is low [11]. Sensitivity to the environment may also contribute to the variance of a trait, which has been shown to be under genetic control. The reduction of trait variance by genetic selection is also known as canalizing selection or canalization [12]. Up to now, this approach has not opened practical solutions for genetic selection. The third strategy, which we are currently exploring more thoroughly, focuses on the molecular genetics of neuroendocrine stress responses, more specifically the HPA axis, with the objective of improving robustness via a genetically selected increase in HPA axis activity [1,2].

Indeed, the activity of the HPA axis is highly heritable and many molecular polymorphisms have been described in the components of the axis, with consequences on the functioning of the system and its numerous physiological targets, leading to large individual variation with wide physiopathological implications [13]. The most important sources of genetic variation can be found in glucocorticoid hormone production, which is mainly related to the sensitivity of the adrenal cortex to adrenocorticotropic hormone (ACTH) [14], in hormone bioavailability with a special influence of the synthesis and binding properties of transcortin [15], and in hormone receptor function via complex transduction mechanisms that are still largely unexplored in farm animals [16]. These different genetic polymorphisms and their functional consequences have been explored mainly in 2 highly divergent breeds of pigs, the Large White (LW), with a high production potential for lean meat and a low HPA axis activity, and the Meishan (MS), with low growth rate, fat carcasses—but large litters of highly viable piglets—and a high HPA axis activity. Compared with LW, MS pigs have higher cortisol concentrations in blood and urine [17,18], an increased sensitivity of the adrenal gland to ACTH [19], and higher levels of circulating transcortin [20,21]. The molecular mechanisms of the genetic variation in CBG levels [22] and adrenal sensitivity to ACTH [14] were previously explored in these breeds.

The highest level of regulation of stress responses in the brain [23] is difficult to explore directly for genetic effects in farm animals. For this purpose, genes of the catecholamine and serotonin pathways, as well as of the pituitary regulation of ACTH release (corticotropin-releasing hormone [CRH] and vasopressin pathways), were sequenced to find polymorphisms to be used in an association study with endocrine and production traits in an advanced intercross between LW and MS that was extensively phenotyped previously [18]. This study was

extended to other candidate genes, such as the cortisol receptors or candidate genes differentially expressed in the adrenal gland [14].

2. Materials and methods

2.1. Animals

We studied a population of 100 female pigs from an advanced intercross between LW and MS breeds (F10-F12), which were extensively phenotyped for stress responses, production, and meat quality traits (see [18] for experimental details). Briefly, animals were reared in a breeding nucleus farm in Southwestern France. Data were collected on the farm for growth and ultrasonic back fat and urine samples were collected to measure stress hormone levels (cortisol and catecholamines) when spontaneously voided at the farm in the morning (between 8:00 AM and 10:00 AM) during the week preceding slaughter. At approximately 193 d of age the animals were transported to overnight lairage at a research abattoir in Spain (IRTA, Monells). The next morning (between 7:00 AM and 1:00 PM), animals were weighed and harvested after CO_2 stunning (85% concentration). Blood was collected during exsanguination in a dry tube, left at room temperature for 20 min for coagulation, and centrifuged. Serum aliquots were frozen until analysis. Measurements of fat (G34FOM) and muscle (M34FOM) depths at 45 min postmortem (pm) were made using the Fat-O-Meat'er equipment (SFKTech, Herlev, Denmark) at 60 mm from the midline between the third and fourth last rib level. Estimated carcass lean content (plean) was calculated using the Spanish official equation [24], plean = 61.56 – 0.878 * G34FOM + 0.157 * M34FOM.

Approximately 1 h after slaughter, carcasses were chilled in a cold room at 2°C. At 24 h pm, muscle pH was measured using a Crison portable meter equipped with a xerolyt electrode (Crison, Barcelona, Spain) in the *longissimus lumborum* muscle at the level of the caudal end of the last rib and in the *semimembranosus* muscle in the middle of the muscle in the exposed visible part. The surface area of the eye of the *longissimus lumborum* muscle was measured between the third and fourth last rib level by making a transversal cut at this point and taking a digital image. This image was used to calculate the surface using a specific program [25]. Each left half carcass was cut and dissected following the method of Walstra and Merkus [26]. To have more commercial cuts, some parts were jointed as hind hand plus leg (ham), loin minus subcutaneous fat of the loin (loin), and belly. A sample of *semimembranosus* and *longissimus lumborum* muscles was taken for the determination of intramuscular fat content by near infrared transmittance (Infratec 1265, FOSS Tecator, Sweden). Mean values (and SD) of these production phenotypes in the population are given in Table 1.

2.2. Hormonal and biochemical assays

Urinary cortisol and cortisone were assayed using a solid-phase extraction procedure on C18 cartridges followed by high-performance liquid chromatography with ultraviolet absorbance detection (254 nm), as previously described [27]. The intra- and interassay coefficients of variation were 7.4% and 10.6%, respectively. Urinary catecholamines (adrenaline and noradrenaline) were assayed using an ion exchange purification procedure followed by high-performance liquid chromatography with electrochemical detection, as previously described [28]. The intra- and interassay coefficients of variation were 7.0% and 7.1% for adrenaline and 6.5% and 11.6% for noradrenaline, respectively. Concentrations of hormones in urine were expressed as the ratio to creatinine content (nanograms per milligram creatinine) to correct for the variable dilution of urine related to water intake. Creatinine levels were determined using a colorimetric quantitative reaction (Creatinine, BIOLABO, Fismes, France). Plasma cortisol was measured by radioimmunoassay as described [19]. The intra- and interassay coefficients of variation were 7.3% and 12.3%, respectively. Glucose, lactate, and creatine kinase activity were measured with a clinical biochemistry automate (Hitachi 911) and assay kits from Roche Diagnostics (Meylan, France). Mean values (and SD) of these endocrine phenotypes in the population are given in Table 1.

2.3. Candidate genes

Candidate genes were chosen in the components of the HPA axis [13], at the level of the pituitary gland (*CRH* and vasopressin receptors that regulate the release of ACTH: *CRHR1, AVPR1A, AVPR1B*), among genes previously demonstrated to be differentially expressed in ACTH-stimulated adrenal glands from LW and MS pigs [14] such as ACTH receptor (*MCR2*), cholesterol suppliers (*LDLR, SCARB1, STAR*), regulatory factors (*CREM*), metabolic enzymes (*CYP11A*), and several genes with a currently unknown function in the adrenal glands (*EIF1B, RNF2, PPAP2B*), and finally the 2 receptors for cortisol (*NR3C1* and *NR3C2*). Brain neurochemical systems were also explored because of the importance of brain neurotransmitters in the regulation of

Table 1
Phenotypic value (mean ± SD) of the traits measured in this experiment.

Phenotypes		Mean	Standard deviation	Nb animals
Production phenotypes:				
testdg	Daily weight gain (g)	762.95	87.99	100
arealt	Loin area (cm^2)	39.88	4.93	94
rflareft	Relative flare fat (% carcass weight)	10.83	3.36	100
lr34fom	Fat depth/muscle depth (fat-o-meter)	24.09	4.94	94
plean	Percentage lean meat	48.66	4.48	94
pham	Ham percentage	22.95	0.81	100
ploin	Loin percentage	16.72	0.88	82
imfsm	Ham intramuscular fat (%)	2.19	1.00	89
imfll	Loin intramuscular fat (%)	1.98	0.96	92
phusm	Final pH ham	5.51	0.08	100
phull	Final pH loin	5.59	0.09	100
lminolta	Loin cut surface color	47.22	2.23	100
driploss	Drip loss	2.82	1.25	96
Endocrine/biological phenotypes:				
lucort1	Urinary cortisol/creatinine basal (ng/mg log)	1.31	0.27	91
leftot1	Urinary cortisol + cortisone/creatinine basal (ng/mg, log)	1.69	0.26	89
leftrat1	Ratio urinary cortisone/cortisol (log)	0.13	0.17	98
luad1	Urinary adrenaline/creatinine basal (ng/mg, log)	0.41	0.24	94
luna1	Urinary noradrenaline/creatinine basal (ng/mg, log)	0.91	0.19	100
lucarat1	Urinary adrenaline/noradrenaline basal (log)	−0.50	0.25	94
lbcort	Plasma cortisol at slaughter (ng/mL, log)	1.44	0.27	100
lbck	Plasma creatine kinase at slaughter (IU/mL, log)	3.16	0.18	94
bglucose	Plasma glucose at slaughter (mmol/L)	6.46	1.03	94
blactate	Plasma lactate at slaughter (mmol/L)	3.13	1.33	100
radrenl	Relative weight left adrenal gland (g/100 kg live weight)	2.39	0.48	100
radrenr	Relative weight right adrenal gland (g/100 kg live weight)	3.07	0.50	96

the neuroendocrine stress responses [23]. Candidate genes are involved in the regulation of monoaminergic, serotonergic (*HTR1A, HTR1B, HTR2A, HTR2C, SLC6A4, COMT, MAOA*). and dopaminergic (*DRD1, DRD2, DRD3, SLC6A3*) systems.

2.4. Sequencing and genotyping

A tissue sample was taken from the adrenal cortex. Genomic DNA was extracted using the Wizard genomic purification kit (Promega, Madison, WI, USA). Extracts had a DNA concentration of about 5×10^2 ng/μL. DNA was diluted to a working concentration of 10 ng/μL. All exons were sequenced within a gene, with sequencing primers being designed for every exon and approximately 1,000 bp before the first exon was sequenced as an important part of the gene regulatory region. Nucleotide sequences were obtained from Ensembl (http://www.ensembl.org) and PubMed (http://www.ncbi.nlm.nih.gov/pubmed) databases. All primers were designed using the Primer Express software (Applied Biosystems, Villebon Sur Yvette, France) and Primer 3 (http://frodo.wi.mit.edu). PCR primers were supplied by Eurogentec (Liege, Belgium; Table 2).

DNA samples of 24 animals with the most extreme (low, n = 12, and high, n = 12) basal urinary cortisol concentrations (urinary cortisol/creatinine ratio) were selected for sequencing. Prior to the polymerase chain reaction (PCR), the samples were analyzed to verify the recommended DNA concentration of 10 ng/μL using NanoDrop. The PCR reactions were done using Taq polymerase (Promega) in a final volume of 20 μL (5 μL DNA, 5 μL forward and reverse primers [1 ng/μL], 10 μL PCR mix containing dNTP). PCR conditions were 95°C for 6 min (extended initial denaturation step); 95°C for 20 sec (denaturation), 58°C for 20 sec (dimer annealing), 72°C for 45 sec (extension), 35 cycles; and 72°C for 10 min (final extension step). The annealing temperature was modified to 56°C in some cases. The presence and size (base pairs) of PCR products were confirmed by electrophoresis on 2% agarose gel.

Sequencing was done on the ABI 3730 DNA sequencer (Applied Biosystems, Villebon Sur Yvette, France) according to standard protocols. The sequencing reaction conditions using Big Dye Terminator version 3.1 were 94°C for 1 min, 50°C for 15 sec, and 60°C for 4 min. Sequencing products were purified by

Table 2
Gene-specific primers used for sequencing.

SNP	Forward primer	Reverse primer
HTR2C(−101T>C)	TGGTACACAGGGTGATTGCT	ACTTAGGCAGATTGAGGCAAA
HTR2C(−752G>A)	TGGTACACAGGGTGATTGCT	ACTTAGGCAGATTGAGGCAAA
TPH2(−853A>C)	CCCAGGACTCAGGCTCAATA	GCTACCCTAGTAAACTCTCCTCTGG
SLC6A4(−960G>A)	ACCCAGAAAGTTGAGGCACA	GAATGGACGCTGACACACAT
SLC6A4(4653C>G)	GGGCCTTTCTTTCTCATTCAC	CCAGAGTCCTCCTCACCTTG
MAOA(−1072A>C)	GCGTAACAATGAAAGCACCA	CTATGGAGGAGTCGGAGAGC
MAOA(−598T>C)	GCGTAACAATGAAAGCACCA	CTATGGAGGAGTCGGAGAGC
MAOA(−430A>T)	GCGTAACAATGAAAGCACCA	CTATGGAGGAGTCGGAGAGC
DRD3(−680C>T)	CAGGAAGCCAAGAGAAGCAG	TGCAGTAGGACAGGGCATAG
CRHR1(24590C>T)	GATGAGACCCGTGTAGGATG	CTCAGATACCTCCAACAGTGC
CRHR1(24605A>G)	GATGAGACCCGTGTAGGATG	CTCAGATACCTCCAACAGTGC
CRHR1(51301C>T)	CAAATGGACCCAGATGACC	TGTACGTGATGCCCAGGAG
AVPR1A(−601G>T)	GCTGTGGGAATCAGGCTTT	CCGACTGGTATTGGCATCTC
AVPR1A(−454C>G)	GCTGTGGGAATCAGGCTTT	CCGACTGGTATTGGCATCTC
AVPR1A(609C>T)	CGGAGATGCCAATACCAGTCGG	CAGATTCCAGGGCTCCATT
AVPR1B(−938T>C)	TGCTGGGCTAACAAGGAAC	CAAACAGGCAGGTAACCTCA
AVPR1B(99G>A)	TCCTCACCCTCCTCCTTTCT	AGCTGCGATGAGTGGGTAG
NR3C1(12905C>T)	ATTCAGCAGGCCACTACA	GTATTGCCTTTGCCCATT
NR3C1(29517G>A)	AATTCCCAGAGATGTTAGCTG	AAATGAGCAAGCGTAGTTCAC
NR3C2(36328T>C)	CACCCAGTTCACACATTTCT	CAGGTCTCACCAAGTCCAC
STAR(m.1662G>T)	TCCACTCTAGTAAATCGCAAC	CCATTCAGTCACTCCCTTT
CREM(9160C>T)	CTATGCTGGTGGGGCTTT	CATCCTTACACATGGTCTCCTT

SNP, single nucleotide polymorphism.

ethanol precipitation. The software packages CodonCode Aligner and BioEdit were used for sequence assembly and single nucleotide polymorphism (SNP) detection. All animals (n = 100) were genotyped for these SNP using the Sequenom mass spectrometry–based genotyping assay technology (Sequenom, Hamburg, Germany).

2.5. Statistical analysis

Linkage disequilibrium between SNPs in the same gene was quantified as r^2 using Haploview (v4.2) [29]. The association strategy was designed to account for the small effective population size and the high degree of relatedness that exist in the design because of the founder effect. The pigs were also genotyped with the iSelect Custom 7 K porcine SNP Chip [25,26] (data not shown) for calculating kinship among animals. The association analysis between candidate gene genotypes and animal phenotypes was performed in R software using the GenABEL package that allows considering kinship [30]. A pedigree-based association analysis was performed with the genome-wide rapid association using mixed model and regression approach and the Genomic Control (GC) method, which were shown to be powerful for association analysis in related populations [31,32].

Corrected phenotypes were used as the dependent trait in a simple linear regression for each SNP, $\hat{e}_i = \mu + kg_i + e_i$, where μ is the mean, g_i is the genotype at the marker under study, k is the marker genotype effect, and e_i is the random residual. *P* values were corrected by the GC method using an estimate of the inflation factor (λ_{GC}). Significant association between candidate gene genotypes and phenotypes was set at $P < 0.05$. SNP or animals were excluded from analyses if they met one of the following criteria: low (<5%) minor allele frequency; out of Hardy-Weinberg equilibrium; too high autosomal heterozygosity (FDR < 1%); or too high identity by state (≥0.95).

3. Results

3.1. Molecular polymorphisms in candidate genes

Fifty-two new SNPs were found in the regulatory and coding regions of the genes studied (Tables 3 and 4; only regions with identified SNPs are shown in Table 4). All SNPs in the same gene were in linkage disequilibrium (data not shown), and only 1 representative SNP was considered.

3.2. Association with carcass composition and meat quality traits

Significant associations ($P < 0.05$) of SNPs in *HTR2C, SLC6A4, MAOA, DRD3, CRHR1, AVPR1A, AVPR1B, CREM,* and *NR3C1* genes with several car-

Table 3
Number and localization of new single nucleotide polymorphisms (SNPs).

Gene	Symbol	Number of new SNPs	
		Promotor region	coding region
5-Hydroxytryptamine (serotonin) receptor 2A	*HTR2A*	1	1
5-Hydroxytryptamine (serotonin) receptor 2C	*HTR2C*	4	0
Tryptophane hydroxylase 2	*TPH2*	2	1
Serotonin transporter	*SLC6A4*	2	2
Monoamine oxidase A	*MAOA*	3	0
Dopamine receptor D2	*DRD2*	4	0
Dopamine receptor D3	*DRD3*	2	0
Dopamine β-hydroxylase	*DBH*	2	0
Corticotropin-releasing hormone receptor 1	*CRHR1*	0	4
Vasopressin receptor 1A	*AVPR1A*	5	7
Vasopressin receptor 1B	*AVPR1B*	1	2
Scavenger receptor class B, member 1	*SCARB1*	0	2
Steroidogenic acute regulatory protein	*STAR*	0	2
cAMP responsive element modulator	*CREM*	0	1
Nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)	*NR3C1*	0	3
Nuclear receptor subfamily 3, group C, member 2 (mineralocorticoid receptor)	*NR3C2*	0	1
Total number of new SNPs		26	26

cass composition and meat quality traits were found (Table 5). Significant results were obtained for an SNP in the regulatory region of the monoamine oxidase A gene, *MAOA*(−430A>T), strongly associated with loin area ($P < 0.024$), fat/muscle depth ratio ($P < 0.004$), percentage of lean meat ($P < 0.005$), and intramuscular fat content of loin ($P < 0.009$) and ham ($P < 0.013$), which are the most important traits for carcass value. For all traits, T/T-homozygous animals were significantly different from other genotypes ($P < 0.05$); however, differences between A/T and A/A animals did not achieve statistical significance (Fig. 1).

The *MAOA*(−598T>C) SNP was also associated ($P < 0.05$) with loin area, ham percentage, loin pH, and daily weight gain. The *DRD3*(−680C>T) SNP was significantly associated with the percentage of loin and intramuscular fat level in loin (Fig. 2). The *HTR2C*(−101T>C), *SLC6A4*(4653C>G), and *SLC6A4*(−960G>A) SNPs related to the serotonergic system showed significant associations ($P < 0.05$) with loin and ham percentage and daily weight gain, respectively.

3.3. Association with endocrine traits

The *DRD3*(−680C>T) SNP in the regulatory region of the dopamine receptor D3 gene showed an association with several neuroendocrine parameters measured in urine collected under basal conditions (Table 6): cortisol ($P < 0.028$), total glucocorticoid (cortisol + cortisone) ($P < 0.035$), and adrenaline ($P < 0.008$) levels and adrenaline/noradrenaline ratio ($P < 0.003$) (Fig. 2). *MAOA* SNP was associated with the urinary cortisone/cortisol ratio ($P < 0.05$) and slaughter plasma glucose level ($P < 0.002$; Fig. 1). SNPs in serotonin-related genes *HTR2C* and *TPH2* were significantly associated with basal urinary adrenaline level and plasma cortisol concentrations at slaughter, respectively ($P < 0.025$).

CRHR1 gene SNPs were associated with the urinary cortisone/cortisol ratio ($P < 0.001$) and the relative weight of adrenal glands ($P < 0.05$). Two SNPs in the glucocorticoid receptor (*NR3C1*) gene (12905C>T and 29517G>A) were associated with slaughter plasma lactate level ($P > 0.01$) and relative weight of the left adrenal gland ($P > 0.02$). The *NR3C2*(36328T>C) SNP of the mineralocorticoid receptor gene was associated with the basal level of total urinary glucocorticoids (cortisol + cortisone; $P > 0.03$). SNPs in the *AVPR1B* gene were associated with plasma cortisol level at slaughter ($P < 0.05$).

4. Discussion

Numerous new SNPs were detected in candidate genes, with some showing an association with phenotypes related to carcass composition, meat quality, and stress hormones. Indeed, strong relationships were shown previously between HPA axis hormones (cortisol/cortisone) and carcass composition, with levels of glucocorticoid hormones in urine positively correlated with carcass fat content, and between catecholamine (adrenaline/noradrenaline) levels and meat pH [33]. These relationships reflect the metabolic activity of stress hormones. Indeed, cortisol favors the accretion of lipids in fat at the expense of proteins from muscle and other tissues [4]. Postmortem acidification of meat (and therefore meat pH and drip loss, major components of meat quality [34]) depends on the anaerobic catabolism of muscle glycogen [33]. Cortisol is a gluconeogenic hormone that increases glycogen stores in liver and muscles and could therefore influence meat pH. Cat-

Table 4
Candidate genes and single nucleotide polymorphism (SNP) location.

Gene	Name	SNP	NCBI ss No.	Position	Amino acid change	Sequence
HTR2C	5-Hydroxytryptamine (serotonin) receptor 2C	*HTR2C*(−101T>C)	538291348	Promoter		AGTTTAATATTGATTTTTATCACTTAA TAAGTATATTTACATTTATTTAATT GAATATCATGCAAGTTATTTAAACT TTTAACTAATTACCTTATTCTC**[T/C]** CTTTTTTTTTCTCTTCCCAGA AAGGATGGCATAATGAATCCAGTC TATTAAATTTCCTCTTCTCAATTT TAAACTTTGGTTGCTTAAGACTAA AGCAATC
HTR2C	5-Hydroxytryptamine (serotonin) receptor 2C	*HTR2C*(−752G>A)	538291349	Promoter		GTTGTTGTTGTTTGGCTGGGTCCATG AAAGTTCCCAGGCCAGGGATTGAA CCCACACCACAGCAGCAACCCAAG CCACT**[G/A]**CAGAGACAATGCCA GATCCTTAACCCACTGCACCACAAGA GAACTCCACTTTTCATGTTTCTATAA GATGAAAGTTTTTCACTTTTTAAT GCTACCTAA
TPH2	Tryptophan hydroxylase 2	*TPH2*(−853A>C)	538291350	Promoter		CTGCCTGAGCTTAAATCCTGCCTCAG CCACTCTTTAATTGTGTAAACCTTT GGCAAGTTGTCTAAATTCTTTGTGC CTCCGTTTCTT**[A/C]**ATATGTTAAGT GAAAATAATTGTTACTGCAGTCTTT TCTTTCCTGCTTAGTAAGTGTTGTTT GANTTTTTTNACTTATTCTAGTTTC AAGTTATCAAAGGAATGACTGATA TATAGGTATTTAGTTTC
SLC6A4	Solute carrier family 6 (neurotransmitter transporter, serotonin), member 4	*SLC6A4*(−960G>A)	538291351	Promoter		GGTGGCCTGGACACAGCTGGGAACA GCTAGGTTGCTAAGAGCTGGGCTCCT GGAAGAGTGAAAAAGTCTGCAGCAA AGGAGCTAAGGGTGCCTC**[A/G]**CAG AGGGTGGCCTTTTCACTTCTCTC CTATGCTCTTTGCCTTTTAAAGTC ACACCTATGGTTTATATAAAATAAG TTTTGAGAGAGTTCACGTTGTGGC
		SLC6A4 (4653C>G)	538291352	Exon5	I40M	GTCCTCTCAGAGGTGTCGCACCGTCA TGTGCAGGGGACCGTTCAGACCAGG TCATGGGCCTTTCTTTCTCATTCAC TCAGACGCCACGTCCTGCAGAT**[C/G]** CACCGGTCTAAGGGACTCCAGGACCT GGGGGGCATCAGCTGGCAGCTTGCC

(continued on next page)

Table 4
(continued)

Gene	Name	SNP	NCBI ss No.	Position	Amino acid change	Sequence
MAOA	Monoamine oxidase A	*MAOA*(−1072A>C)	538291353	Promoter		GCGTAACAATGAAAGCACCAGGTTGA AAGCCCCACGTGGCCACGCCCACGCT CTCTGGCCACACCCCCCCACTTTAGA GGCGTTGCCGGGCCACACCAGG**[A/C]** GGGAGGGCTTCCCGCGCGCGCGCGC GCCCGGTACAGACGGGGAAGGCGGA GCC
		MAOA(−598T>C)	538291354	Promoter		GTGGCGGACTGCCTCCCGTCCGTTTT CCAGCTCCCCGTCGAACGCGTCCTCA CCAGACGTAACACACTCCCGAAGCTT CAGCGCGCCCTCGCGACT**[T/C]**GG CTCCAGTCCTGGCCCGATAACTGACA AGGACATCAAGATTGGCCTCTGGGGC TTTGGCGCCCGCGGCCTGAGCAACCG GATCCCCATGTCTGAC
		MAOA(−430A>T)	538291355	Promoter		GCAACCGGATCCCCATGTCTGACCC CCGCTCGACCACCTCAGATGGGGCCG CCCCAGCCTCTAACCTGAACACCAGC AACCCCCCAGAATCTGT**[A/T]**CAGGG ATCTGTCCCGCCCTCAGCCTCCCCC CAGTTGTCCCCCCAAGAGCCCAC CCCACCCTCCGTGTCCGCCTCCCCT GGGGAATCAGTCCCAGAAA
DRD3	Dopamine receptor D3	*DRD3*(−680C>T)	538291356	Promoter		CACGCTGCCCCTGCCTCACCTGACTC CATCCAGACGGGCCTCCCTGTTGTC CCTTCCACCTACCAGCTGCCCTGCCT TTGTA**[C/T]**CTGTTCCTGCCATTCC TCCTGCCTGGATATCTGCATGGTT GGCTTCCTCAGGTCCCCCTGGTCATG
CRHR1	Corticotropin-releasing hormone receptor 1	*CRHR1*(24590C>T)	538291357	Intron		TCTCAGCTGTCACCACCTCTCTGAGG CCTCCACCACCCCAGGGCTCTCAGA GCCTGCACACAGAGCCGTGCCCTGGG GCAGGAAGAGCGGGGAGATTGCCTCT CTAGGGCTCCAAGCTGTCTAC**[C/T]** GAGGGCCAAGTGCCNTGTTGAGGAG AATCTTGAAGTTGGGCGCAAGTGTGG TGGGAAGGAGCTATGATTAGGTGACA GCATCCGCCCGGGGCACAGAGGGGCT TGTGGCAAGTGA

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Table 4
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Gene	Name	SNP	NCBI ss No.	Position	Amino acid change	Sequence
		CRHR1(24605A>G)	538291358	Intron		CCTCCACCACCCCAGGGCTCTCAGAG CCTGCACACAGAGCCGTGCCCTGGGG CAGGAAGAGCGGGGAGATTGCCTCT CTAGGGCTCCAAGCTGTCTACNGAGG GCCAAGTGCC**[A/G]**TGTTGAGGAGA ATCTTGAAGTTGGGCGCAAGTGTGGT GGGAAGGAGCTATGATTAGGTGACAG CATCCGCCCGGGGCACAGAGGGGCTT GTGGCAAGTGA
		CRHR1(51301C>T)	538291359	Intron		GGTTTGGCAAAAGGCCTGGGGTATAC ACTGACTACATCTACCAGGGCCCCA TGATATTGGTCCTGCTGGTAAGAGCC TGGGTAGGGGGGCAGGAGAGAGGNN GCTCAGTGGGGA**[C/T]**GGGCAAGCGG TGCTTTCTGTGGACAGAAAGGATCCC TCTCCCAGAGGTGAAGGTTGTGGGG CCAGGAATGTGCATGGGCTCAC GAAGGCAGTGGGGAGCA
AVPR1A	Vasopressin receptor 1A	*AVPR1A*(−601G>T)	538291360	Promoter		GCTGGAGTAGGTAGAGTCACACGCTA AGTTAAGGTGCAAGAGCACACGCGC CTGCAGGTGGATTCGGGATTTCCAG GTACCAAATTGACTACATCTGTT**[G/T]** AAGTCAAATGTAGGGTTAAGGTGAC TCTTGAGGCTTAAGGAAATGGTGG TAA
		AVPR1A(−454C>G)	538291361	Promoter		ATGGCATGTCCAGTTCTCCAAGTTT TTTAGAGCAGCGGGAAAACTCCGGGA TCAACCCTCTTTTATGCCCAGTAA TCCTTGA**[G/C]**TCTCCCAGNCCCA CCCCTGCATTGGNAAGCAAAGACA AATCACCGACGTAGNGGGGAGGGGGA TAAAACCCCGGNGGAATAGGGAAGG GAGGTGGCGAGAGTAGGCACCGAGCA GTCTCCAACCACTGGGGTGAGAAGC AGCGCGATCTGGGGAAAGAACTCCCC ATAAATAGA

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Table 4
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Gene	Name	SNP	NCBI ss No.	Position	Amino acid change	Sequence
		AVPR1A(609C>T)	538291362	Exon1	C204C	ATCGCCGCCGCCTGGGTGCTGAGCTT CGTGCTGAGCATTCCGCAGTACTTC GTCTTCTCCATGGTCGAGGTGAGCA ATGTCACCAAGGCCTACGACTG**[C/T]** TGGGCCAACTTCATCCAGCCCTGGGG TCTCCCCGCCTACGTGACCTGGATGA CTGGCGGCATCTTCGTGGTGCCCGTG GTCATCCTGGGCACCT
AVPR1B	Vasopressin receptor 1B	*AVPR1B*(−938T>C)	538291363	Promoter		CCTGCCGTTCTTGGACCCTTGCTGGGC TAACAAGGAACCCAACAGCGCTGCT AGCCTTAGGGTCCCCACATGGTGAGT GGCCACCCCCCATTCCC**[C/T]** GGG TATGCATCTGTCTTGGGAGACAGTC CGATGGTTCCAAGCGCGAGCCAAGA TGAGGTTACCTGCCTGTTTGCGGAT CCATGG
		AVPR1B(99G>A)	538291364	Exon1	L34L	TGGCCAACCCCACCCCTGGAGGC ACTCTCTCTGTCCCCAATGCCACCAC ACCCTGGCTGGGCCGGGATGAGGAAC T**[G/A]**GCCAAGGTGGAGATTGGAGT GTTGGCCACTGTCCTGGTGCTGGCGA CAGGGGGCAACCTGACTGTGCTGCTG ACCTTGGGACAGCCAGGCCGCAAGCG
NR3C1	Glucocorticoid receptor, nuclear receptor subfamily 3, group C, member 1	*NR3C1*(12905C>T)	538291365	Exon5	P546P	CACAAGAAACTTCTGAAAATTCTGCT AATAAAACAATAGTTCCTGCAACG CTACCACAGCTCACCCCCACCCTGG TGTCACTGCTGGAAGTCATTGAAC C**[C/T]**GAGGTGTTGTATGCAGGAT ATGACAGCTCGATTCCAGATTCCA CCTGGCGGATCATGACCGCACTCAA CATGTTAGGCGGGCGGCAGGTGATT GCGGCAG
		NR3C1(29517G>A)	538291366	3′		AAGGAGAAAGTGATAGGTGGTGAAT CCCAAAAATGAGAAATAATAACTTG GGGGCAAGCTCCACTGTCAGAGAAG GATGGCACCTAAACCACCAGTGCCC **[G/A]**AAGTTTGTGTGACAGACTT TCTGCTCATACTTTTCACGGTTGGA CAAAATTTTCTAGACTTTCGTTGGT GTATTTTCCCCCATATAGTTAGG ACAGCATT

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Table 4
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Gene	Name	SNP	NCBI ss No.	Position	Amino acid change	Sequence
NR3C2	Mineralocorticoid receptor, nuclear receptor subfamily 3, group C, member 2	*NR3C2*(36328T>C)	538291367	3′		TTTTGCAGGAAAGGCACGTTCCATG CAGCAGACACCAGCATCCTTCTTCC TTCCACAAAGCAGGTGACTTAGCC ACATCACTTAGGCAGCTTCCTAGA CA**[T/C]**GGTTGAATCTGGGAAGGTT GTTGTAAGCTTATAGCAGTGTTA AGATGCAGAGGGGCCCAGGGACAG GGTGAGAATCAGGCCCCATGGTCAT GGCCCCTGT
STAR	Steroidogenic acute regulatory protein	*STAR*(m.1662G>T)	538291368	3′		TCTAGTAAATCGCAACTTCATAC TAAAGGAGTCGTGGACGAAGATTTTA ATTAGTTAAACTAAATCCCCACCTT GTTCAAAGGAAAAACTGCTGGGGACT **[G/T]**TGGTCATTTAATTACATAGA ATCTGTTATCAGAATCTTCCGTAAA ATCTAATTTGCACAAGCCCAAAT CTGTTCTTAAAAGAACTCTATGGC TGGTAGCT
CREM	cAMP responsive element modulator	*CREM*(9160C>T)	538291369	Intron		GACCAAAAAAAAAAAAAAAGCAAA GTTGTTTTCAGCTTTCTATGCTG GTGGGGCTTTTTTTGTTTGTTTTTT AGTTTTCTGGAAATTGGGGCATTCT GAT**[C/T]**TAGTTTTGAGAGATT TTAATTTTTTTAATAAAGTCTATT ATGGTCTTCCA

Location and coding sequence of SNP significantly associated with studied phenotypes. SNP location is indicated by the number of nucleotides starting at the beginning of the first exon. SNPs are located in the coding region except when indicated by a minus sign (promotor region) or an "m" (change in the mRNA, genomic sequence not available for comparison).

Table 5
Phenotype-genotype association ($P < 0.05$), carcass composition, and meat quality traits.

Phenotype	testdg	arealt	rflareft	lr34fom	plean	pham	ploin	imfsm	imfll	phusm	phull	lminolta	driploss
SNP													
HTR2C(−101T>C)							0.023						
HTR2C(−752G>A)													
TPH2(−853A>C)													
SLC6A4(4653C>G)						0.031							
SLC6A4(−960G>A)	0.021												
MAOA(−1072A>C)													
MAOA(−430A>T)		0.024		0.004	0.005			0.013	0.009				
MAOA(−598T>C)	0.011	0.049				0.02					0.01		
DRD3(−680C>T)							0.039		0.049				
CRHR1(24590C>T)											0.025		
CRHR1(24605A>G)													
CRHR1(51301C>T)		0.023											
AVPR1A(609C>T)						0.031	0.035	0.047					
AVPR1A(−454C>G)													
AVPR1A(−601G>T)							0.043					0.019	
AVPR1B(99G>A)									0.054	0.005			0.043
AVPR1B(−938T>C)													
STAR(m.1662G>T)													0.049
CREM(9160C>T)										0.027			
NR3C1(12905C>T)													0.032
NR3C1(29517G>A)			0.017										
NR3C2(36328T>C)													

SNP, single nucleotide polymorphism.
Only SNPs with significant associations ($P < 0.05$) are listed. See Table 1 for the meaning of trait abbreviations and Table 2 for gene full names and SNP annotation.

echolamines are glycolytic hormones; high levels of catecholamines in urine as measured at slaughter are correlated with a low meat pH [33]. Catecholamine release is triggered by preslaughter physical activity, emotional activation, and aggressive encounters among animals, which also cause glycogen depression directly because of muscular activity [35]. Furthermore, activation of β-adrenergic receptors increases mobilization of fat and reduces protein catabolism, mostly via β2-type receptors [36]. A direct action of adrenaline, a potent β2 agonist, in these processes should therefore reduce the fat content of the carcass and increase the yield of muscle in the case of a sustained high adrenergic tone. Therefore, genetic variation in HPA axis and sympathetic nervous system activity, as well as in behavioral reactivity to preslaughter stress, is expected to influence carcass composition and meat quality [18,37].

CRH is the primary hypothalamic neuropeptide promoting the release of ACTH by the pituitary gland. The influence of pituitary receptor gene (*CRHR1*) SNP on HPA axis function is shown by an association with the urinary cortisone/cortisol ratio and the relative weight of adrenal glands, reflecting the trophic effect of pituitary peptides released by CRH on adrenal glands [38]. The association between 2 SNPs and loin area also reflects the catabolic effect of cortisol on muscle proteins. Their association with final pH of ham reflects the influence of cortisol on glycogen stores. In rodent studies, the *CRHR1* gene was identified previously as a candidate gene for stress-related quantitative trait loci [39–41]. The porcine *CRH* gene was proposed as a functional-positional candidate for growth and body composition in quantitative trait loci mapping [42,43] and Murani and collaborators described the association between an SNP in the *CRH* gene with glucose concentration and creatine kinase level in plasma collected at slaughter, suggesting that this *CRH* SNP may also be involved in preslaughter behavioral interaction between animals [37]. Finally, an SNP in the *CRH* gene was associated with end-of-test rib-eye area and hot carcass weight in steers [44].

Vasopressin potentiates CRH to release ACTH from the anterior pituitary gland via 1b-type receptors (*AVPR1B*) [45]. However, vasopressin is also implicated in interneuronal communication within various areas of the brain to modulate HPA axis function, as well as emotional and social behavioral and physiological responding, via both *AVPR1A* (most abundant) and *AVPR1B* receptor subtypes [46]. In pigs, D'Eath and collaborators showed that aggressive animals have a higher number of vasopressin-labeled cells in the medial amygdale and Murani and collaborators showed

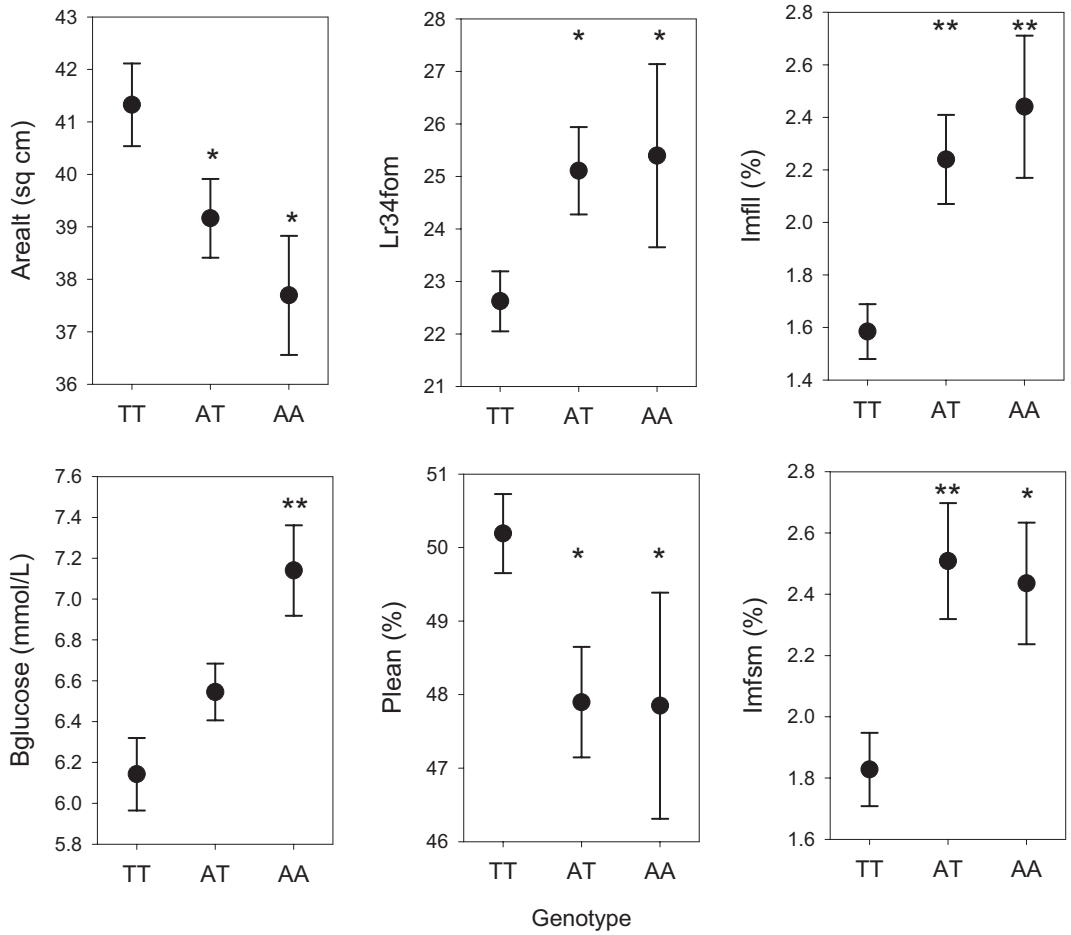

Fig. 1. Phenotypic effect of the *MAOA*(−430A>T) single nucleotide polymorphism in the promoter region of monoamine oxidase A gene. Phenotypic means (± SEM) for TT (n = 40) and AA-homozygous (n = 11 to 13 because of missing phenotypes) and AT-heterozygous (n = 41) animals (*$P < 0.05$, **$P < 0.01$ vs TT). Variables: loin area (arealt); plasma glucose slaughter (bglucose); fat depth/muscle depth, fat-o-meter (lr34fom); percentage lean meat (plean); loin intramuscular fat (imfll); ham intramuscular fat (imfsm).

that SNPs in *AVPR1B* had consistent effects on middle body lesion score as a result of different levels of aggressive behavior [37,47]. This pleiotropic action of vasopressin receptors may explain the large range of effects of their SNPs on neuroendocrine traits and, consequently, on carcass composition and meat quality. More work will be necessary to sort out the relative contribution of direct neuroendocrine effects and the consequences and different levels of aggressive behaviors.

Sensitivity of adrenal cortices to ACTH is an important source of genetic variation of HPA axis activity [13]. We analyzed here several genes important for adrenal cortex function that we previously showed to be differentially expressed in MS and LW adrenals [14]. The only significant effects were found for *STAR* and *CREM* SNPs on meat quality measures. The low level of preslaughter stress in the present experiment may have impaired the full expression of potential differences linked to variation in the adrenal response to ACTH.

Several associations were found between meat quality and SNPs in the coding region of cortisol receptor genes *NR3C1* (encoding GR) and *NR3C2* (*encoding mineralocorticoid receptor*). These receptors play different roles. GR is the main receptor mediating the metabolic effects of cortisol, but both receptor types are involved in the feedback regulation of HPA axis activity and in the regulatory effects of cortisol on the autonomic nervous system, brain function, and behavior [38,48]. Although we obtained some indications that molecular SNPs in these genes are associated with both neuroendocrine parameters and carcass composition or meat quality traits, the effects were rather modest.

Major monoaminergic systems are involved in the regulation of stress neuroendocrine and behavioral reactivity, including aggressive tendencies [49,50]. Two SNPs in the promoter region of the *MAOA* gene are

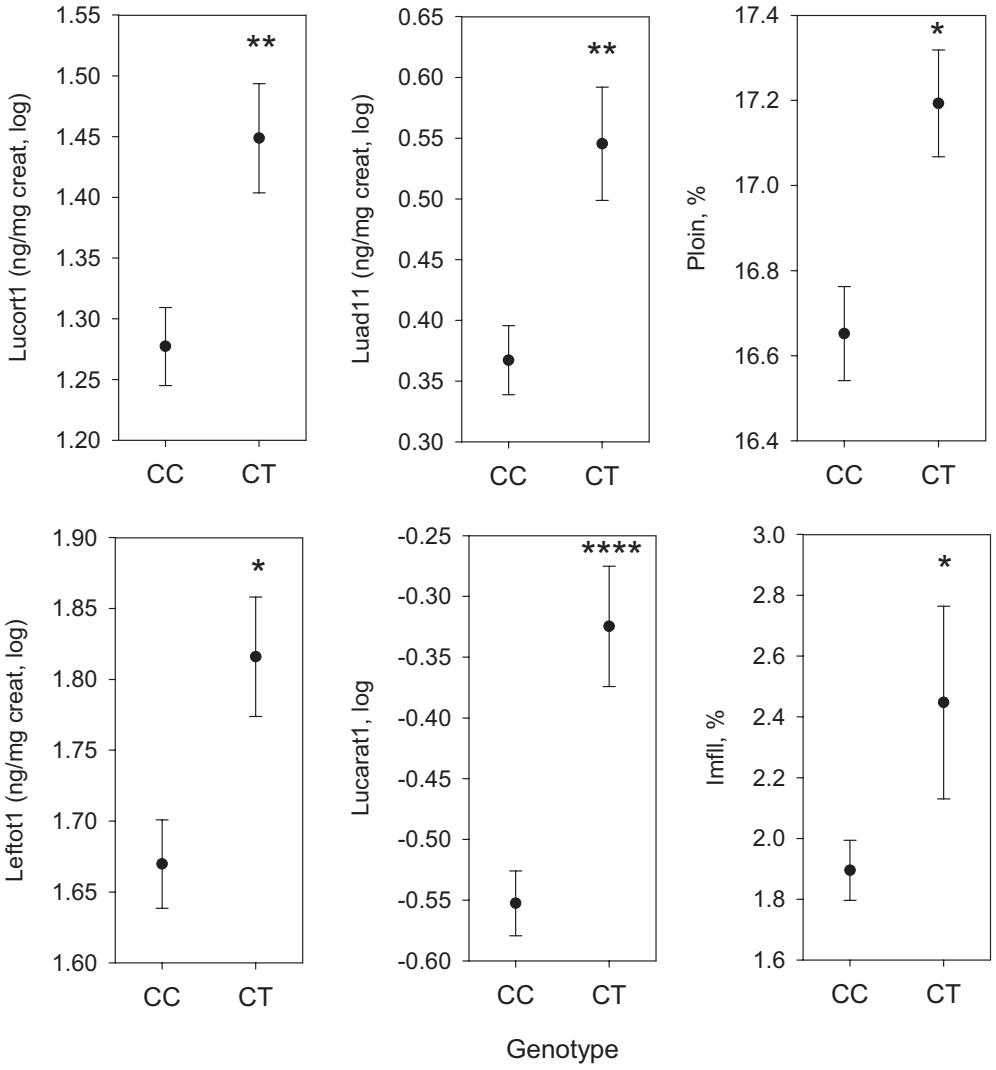

Fig. 2. Phenotypic effect of the *DRD3*(−680C>T) single nucleotide polymorphism in the promoter region of dopamine receptor D3 gene. Phenotypic means (± SEM) for CC-homozygous (n = 66 to 68 because missing phenotypes) and CT-heterozygous (n = 13 to 20) animals (*$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$). The small number of TT-homozygous animals (n = 3) was excluded from the analysis. Variables: urinary cortisol/creatinine basal, log (lucort1); urinary cortisol + cortisone/creatinine basal, log (leftot1); urinary adrenaline/creatinine basal, log (luad1); urinary adrenaline/noradrenaline basal, log (lucarat1); loin percentage (ploin); loin intramuscular fat (imfll).

strongly associated with most traits of carcass composition and meat quality, such as loin area, fat depth/muscle depth ratio, ham and loin intramuscular fat for *MAOA*(−430A>T), and daily weight gain, loin area, and ham percentage for *MAOA*(−598T>C) SNP. These SNPs were also associated with biological traits: the *MAOA*(−1072A>C) SNP with urinary cortisone/cortisol ratio and the *MAOA*(−430A>T) SNP with slaughter plasma glucose. Loin area is larger and adiposity (both subcutaneous and intramuscular) is lower in T/T animals and plasma glucose level at slaughter is higher in A/A animals than in homozygous T/T animals (Fig. 1). MAOA is a ubiquitous enzyme that inactivates monoaminergic neurotransmitters (serotonin and catecholamines) both in the periphery (sympathetic nervous system) and in the central nervous system, so that variations in the molecular form of the enzyme can have wide effects. The limited effect of *MAOA* SNPs on neuroendocrine parameters, compared with their large metabolic consequences, suggests that they modulate the effect of catecholamines at the level of target organs, rather than at higher levels of control.

The association between the dopamine receptor D3 *DRD3*(−680C>T) SNP with urinary cortisol and cortisone levels reflects the influence of this receptor on cortisol production. The change in adrenaline levels (independently from noradrenaline) may result from the influence of cortisol on adrenaline production [51] and the differences in carcass composition reflect the metabolic effects of cortisol and adrenaline (cf ante). Little

Table 6
Phenotype-genotype association ($P < 0.05$), endocrine traits.

Phenotype	lucort1	leftot1	leftrat1	luad1	luna1	lucarat1	lbcort	lbck	bglucose	blactate	radrenl	radrenr
SNP												
HTR2C(−101T>C)												
HTR2C(−752G>A)				0.021								
TPH2(−853A>C)							0.025					
SLC6A4(4653C>G)												
SLC6A4(−960G>A)												
MAOA(−1072A>C)			0.038									
MAOA(−430A>T)									0.002			
MAOA(−598T>C)												
DRD3(−680C>T)	0.028	0.035		0.008		0.003						
CRHR1(24590C>T)												
CRHR1(24605A>G)											0.034	0.047
CRHR1(51301C>T)			0.008									
*AVPR1*A(609C>T)					0.019	0.016						
AVPR1A(−454C>G)								0.047				
AVPR1A(−601G>T)	0.033	0.048										
AVPR1B(99G>A)							0.014					0.013
AVPR1B(−938T>C)					0.050		0.054					
STAR(m.1662G>T)												
CREM(9160C>T)												
NR3C1(12905C>T)										0.010		
NR3C1(29517G>A)											0.020	
NR3C2(36328T>C)		0.030										

SNP, single nucleotide polymorphism.
Only SNPs with significant associations ($P < 0.05$) are listed. See Table 1 for the meaning of trait abbreviations and Table 2 for gene full names and SNP annotation.

is known about the regulatory effect of the dopamine receptor D3 on HPA axis and sympathetic activity. In humans, an SNP in *DRD3* was shown to be associated with a reduced ACTH and cortisol response to apomorphine in psychiatric patients [52]. More research will be necessary to explore dopaminergic mechanisms in stress responses and related traits [53].

Molecular variants of genes of the serotonin system showed little association with neuroendocrine and quality measures. Although this neurochemical system does control stress neuroendocrine responses directly [50], we expect that their main influence will come from their modulation of emotional and aggressive behaviors [49], which are main contributors to the pm neuroendocrine stress profile and meat quality. As mentioned earlier, the present experiment was conducted with minimal preslaughter stress (no mixing of animals from different pens, gentle handling), so the effect of different behavioral reactivity may have been masked. Specific experimental protocols, more directly aiming at the study of behaviors, are under study.

In conclusion, behavioral and neuroendocrine responses to stress are important contributors to production traits and animal welfare. These traits are largely influenced by genetic factors but precise phenotyping is difficult. The present results will be extended to larger populations and more candidate genes to confirm and extend the associations described. They show that the study of candidate genes selected from an abundant literature on this topic in experimental species is a promising strategy to explore the genetic architecture of behavioral and neuroendocrine stress responses and related production, carcass composition, and meat quality traits. They also open the way toward efficient marker-assisted selection.

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