

Genotype on the Pigmentation Regulating *PMEL17* Gene Affects Behavior in Chickens Raised Without Physical Contact with Conspecifics

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Abstract Chickens homozygous for the *Dominant white* or *wild-type* allele of *PMEL17* were subjected to a broad phenotyping in order to detect consistent differences between genotypes. To exclude feather pecking, the chickens were individually housed without physical contact, from the day of hatching, and tested for social, aggressive, fear and exploratory behaviors, and corticosterone and testosterone levels were assessed. In a principal component analysis, 53.2% of the behavior variation was explained by two factors. Factor one was an activity and social factor, and there was a significant effect of genotype on the factor scores. On factor two, related to aggressive behavior, there were significant effects of genotype, sex and their interaction. There were no genotype effects on hormone levels or any other measured non-behavioral phenotypes. Hence, differences in behavior between *PMEL17* genotypes remained when negative social experiences were excluded, indicating a direct pleiotropic effect of the gene on behavior.

Keywords Chicken · Behavior · *PMEL17* · *Dominant white* · Pigmentation

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Introduction

Pigmentation is associated with behavior and stress responses in a variety of species, (reviewed by Ducrest et al. 2008; Hayssen 1997; Kittilsen et al. 2009; Loehr et al. 2008). In general, pigmented individuals are more aggressive, and show a stronger corticosteroid reactivity than non-pigmented of the same species. Interestingly, reduction of pigmentation of fur or plumage is among the earliest responses to selection during domestication (Price 1998), perhaps indicating that low pigmented phenotypes adapt easier to coping with the stress of captivity. This is further supported by the experiments of Belyaev in the 1950s, where foxes (*Vulpes vulpes*) were selected for tameness. In the selected lines, loss of pigmentation occurred as side effects within only a few generations (Trut 1999). Coat color genes have furthermore been found to have pleiotropic effects on domesticated behavior in minks and sables (Keeler and Moore 1961; Trapezov et al. 2008), and in rats (Cottle and Price 1987; Keeler 1942; Keeler and Dean King 1942). These observations indicate that color phenotype is linked to behaviors preferred during domestication and thus may be favored, either as side-effects of selection for behaviorally suited phenotypes, or sometimes even directly selected (Fang et al. 2009).

Domesticated chickens also demonstrate a wide array of plumage color alterations compared to the wild ancestor, the red junglefowl (Kerje et al. 2004; Sheppy in press). Chickens differing in genotype at the pigmentation related *PMEL17* locus (a mutation causing the well-known *Dominant white* phenotype (Kerje et al. 2004)) have been shown to differ in social and exploratory behavior (Karlsson et al. 2010; Nätt et al. 2007), and the *Dominant white* allele has also been linked to the risk of being victim of feather-

pecking, a detrimental behavior disorder in chickens (Bright 2007; Keeling et al. 2004; Nätt et al. 2007).

PMEL17 is not directly involved in the melanin synthesis (Theos et al. 2005), a pathway which has been suggested as one possible part of a mechanism for the connection between pigmentation and behavior in other species (Ducrest et al. 2008). In chickens, this mutation therefore most likely exerts its behavioral effect in some other way. One possibility would be a direct effect on CNS function, and another that the gene affects endocrine target organs. Since the gene does not seem to be expressed in the brain (Karlsson et al. 2010), direct CNS effects appear less likely. An alternative explanation could be that the color phenotype affects the social experiences of the individual and thereby indirectly the behavior. The logic of this alternative would be that wild type colored chickens are predisposed to being pecked solely because the dark plumage may attract feather pecking, and the experience of being a victim of this may in turn affect various aspects of the behavior of the victimised bird, such as increased fear. Hence, the previously recorded behavioral differences between the *PMEL17*-genotypes (Karlsson et al. 2010; Nätt et al. 2007) could be a secondary effect of negative social experiences rather than a direct effect of the gene.

In the present work we attempted to exclude indirect effects by studying birds raised in physical isolation from each other, hence removing any experience of being feather pecked. We used birds from an F₆-generation of an advanced intercross line between red junglefowl and White Leghorn chickens, homozygous for alternative *PMEL17* alleles, thus creating what we refer to as Locus Controlled Advanced Intercross Line (LAIL).

The chickens were either homozygous for the wild type *PMEL17* allele (*i/i*)—hence with a wild type plumage color—or homozygous for the *Dominant white* allele (*I/I*), and therefore with a white plumage. With this method, we could study the effect of the genotype on one locus against a random background hybrid red junglefowl × White Leghorn genotype achieved by accumulating recombinations in the previous five generations. From the day of hatching the chickens were housed individually, with full auditory and visual contact but not allowed physical contact, to avoid behavioral differences due to feather pecking. We then exposed the birds to a battery of different behavioral tests which measured different aspects of social behavior, exploration and fearfulness, and propensity to carry out feather pecking. Moreover, we collected weights at different ages and sampled blood for corticosterone and testosterone analyses. This broad phenotyping was done in order to detect consistent differences between genotypes both in behavior and physiology.

The aim of this study was to analyse effects of the *PMEL17* genotype on behavior in chickens where we could exclude effects of different social experiences.

Materials and methods

This study was approved by the local Ethical Committee of The Swedish National Board for Laboratory Animals.

Animals

The chickens used were offspring from the F₆ generation of an advanced intercross between White Leghorn and Red junglefowl. The White Leghorn line used for this cross (SLU 13) has a long history of selection for egg production traits and originated from the Scandinavian selection and crossbreeding experiment (Liljedahl et al. 1979). The red junglefowl originated from a Swedish zoo population; more details about the original animals used for the intercross can be found in (Schütz and Jensen 2001). The same cross has been used and described earlier by Karlsson et al. (2010).

The chickens used in this study were pedigree hatched and bred from two wild type families (two males and three females) and two white families (two females and two males). All the parental birds were selected based on visual inspection and further genotyped. The genotyping confirmed all wild type (*i/i*) individuals and one white (*I/I*) male to be homozygous. The remaining white parents were heterozygous (*I/i*). Therefore, all offspring were further genotyped (for details on genotyping, see below) and only homozygous chickens were used for this study. The total sample size was 30 wild type birds (*i/i*; 14 females; 16 males) and 15 white (*I/I*; 9 females; 6 males). The unbalance in the dataset was taken account of in the statistical analyses (see below).

Genotyping

For the genotyping, blood samples were obtained and DNA was purified using standard protocols according to manufactures recommendations. The polymorphic repeat in exon 7 of the *PMEL17* gene was amplified with PCR using forward primer (5'-CCTCAGTGTCTCAGGTGGTGA-3') and reverse primer (5'-CAGAAGATACAGCTGTGGCTC-3') (Kerje et al. 2004). The PCR was performed with reagents from the AmpliTaq Gold kit (Applied Biosystems, Foster City, CA, USA) according to manufacturer's protocol. Fragments of 490 and 574 bp associated with the wild type (*i*) and Dominantwhite (*I*) alleles respectively were displayed on an agarose gel. Data were compared to

photographs of the genotyped animals revealing a perfect match between genotypes and phenotypes.

Housing conditions of animals

The chickens were hatched in an automatic incubator (Masalles 25 DIGIT) at the “Kruijt” hatchery at Linköping University. At hatching, the chickens were marked with wing-tags and vaccinated against Marek’s disease. The chickens were housed individually in wire mesh cages with full visual and auditory contact, but no physical contact, to prevent them from pecking at each other. Every cage measured $32 \times 26 \times 18$ cm (W \times L \times H) and was equipped with a food trough and a drinking bottle and the floor was covered with wood shavings. The cages were arranged in rows and tiers so each chick had two or three adjacent neighbour chickens, of which at least one was of the same genotype and one of the alternative one. Room temperature was maintained at 28°C with fan heaters and the light (5–8 lux) was kept on a 12:12 h light:dark cycle. At an age of 45 days, the chickens were moved to the “Wood-Gush” chicken research facility, 10 km outside of Linköping. The birds were again housed in separate wire-mesh cages $40 \times 100 \times 50$ cm (W \times L \times H) with full visual and auditory contact with one or two neighbours, but no physical contact was possible. The birds had access to food *ad libitum*, free access to water, perches and wood shavings on the floor. They were kept at a 12:12 h light:dark cycle (5–8 lux) and the room temperature was maintained at 22°C.

Behavioral tests

All chickens were tested both as young (14–31 days old) and adults (163–294 days old) in a battery of tests, of which some were identical at the two ages except for scaling of the arenas. However, in the resident-intruder test only males were tested (see below). The test order was balanced between genotypes but randomized within each genotype. When tested as adults, the test order was balanced for sex within genotype as well. As young, all tests were carried out in the same room as the chickens were housed (described above). The adult birds were tested in a separate room, with similar conditions as for the housing room (described above), but with no other birds present. For all tests, except for the aggressive reactivity test and tonic immobility test, the chickens were carefully caught one by one in their cage and carried to the test arena where they were placed before the start of test. During tests, the behavior was either recorded manually during the test, or recorded by a roof mounted video camera and scored either directly by using the software Ethovision XT from Noldus, or afterwards manually from the video tapes (as detailed in

the descriptions of the tests below). Except for the tonic immobility and aggressive reactivity test (see below), no observer was visible to the chickens during testing. The animals were weighed at the time of hatch and at 8, 46 and 112 days of age.

Aggressive behavior tests

Aggressive reactivity test

To estimate the general aggressiveness of the birds, the propensity to attack a neutral stimulus in the home cage was measured when the birds were 192 days old in the aggressive reactivity test (AR). As stimulus, a hand covered in cloth was used, and the observer moved the hand back and forth in front of the cage of the tested bird for 60 s, using a standardised movement. The behavior of the bird in the cage was recorded on video, and the frequency of aggressive behaviors (outlined in Table 1) were scored manually from the video tapes using continuous recording.

Resident-intruder test

In order to estimate the aggressive behavior towards an unfamiliar intruder, a resident-intruder test was carried out when the birds were 220 days old. Pilot experiments on birds not included in the experiment showed that females rarely or never behaved aggressively in this test, so only males were included. The arena measured 120×150 cm (W \times L). The test chicken was placed in the arena and allowed to habituate for 2 min. In darkness, an unfamiliar heterozygous male from the same cross (but not part of the experiment) was introduced into the arena. The test started when the light was turned on and the latency until the first aggressive attack was manually measured using a stop watch. The light was turned off directly after the first encounter (this immediately inhibited further aggression), or—if no aggressive attacks were observed—after a maximum time of 5 min. The test was then immediately repeated in the same way two times with new intruder males at each test occasion.

Social behavior tests

Mirror test

To measure the reactions towards a perceived unfamiliar conspecific, the chickens were subjected to the mirror test (MT) twice (Y:31 days old, A: 191 days old). The Y arena was made of hardboard and measured $40 \times 80 \times 95$ cm (W \times L \times H), and the A arena measured $70 \times 140 \times 160$ cm (W \times L \times H). One short end of the arena was covered by a mirror. For recording purposes, the arena was

Table 1 Behavior variables recorded in the complex environment (CE) and aggressive reactivity test (AR)

Categories	Behaviors	Description	Tests used
Social	Peck	Aggressive or non-aggressive pecking at other	CE
	Other	Other social interactions	CE
Exploratory/foraging	Ground peck	Pecks at items (visible or not) on ground	CE
	Drink	Drinking from water bath	CE
	Ground scratch	Scratching at ground often followed by one-two steps backwards	CE
	Bill rake	Wiping of the beak, in feed, ground or against objects	CE
	Passive	Stand/sit alert	Immobile in a standing or sitting posture with eyes open and an alert body stance
Stand/sit relaxed		Immobile in a standing or sitting posture with eyes fully or partially closed and a relaxed body stance	CE
Locomotion	Locomotion	Walking or running	CE
	Fly/jump	Moving without touching ground	CE
	Other	Other unspecified not passive behaviors	CE
Comfort	Preen	Uses beak to trim and arrange feathers	CE
	Dustbathing	Forcing fine, dry material into the plumage with the body, wings and legs	CE
Perching	Water	Perching on edge of water zone	CE
	Perch	Perching on branch in forest zone	CE
	Sand	Perching on edge of sand zone	CE
	Hackle threat	Head towards observer hand, hackles raised	AR
	Following hand	Follows the movements of the observer hand with the head	AR
	Close to hand	Spends time within a range of 20 cm from the observer hand	AR
	Aggressive attacks	Bird gives a fast peck, or jumps towards the observer hand	AR

divided into two equally sized zones, the social zone and non-social zone. The chicken was placed in the non-social zone in darkness, and a 5 min behavioral recording was started by turning the light on and finished by turning the light off. All tests were recorded by a roof mounted video camera and the time the chickens spent in each of the two zones was measured using the software Ethovision XP from Noldus. Total aggressive behaviors displayed towards the mirror were manually scored from the video, using 1/0 sampling in intervals of 15 s.

Sociality test

In order to measure social propensity versus explorative behavior, a sociality test (ST) was performed at 175 days of age (due to technical failure data from the same test carried out when the birds were 24 days old were lost). The arena (Fig. 1) was circular shaped with a diameter of 2.2 m, and built of cardboard. Four hardboard screens (0.6 m × 0.6 m) were situated opposite to each other, on an inner circle of 1.4 m diameter. A circular pen (diameter; 0.6 m) containing three familiar chickens was placed in the centre of the arena and covered with a plastic cone to prevent the test chicken from sitting on top of the pen. The inner circle area, next to the centred holding pen was considered as the social zone, and there the test chicken

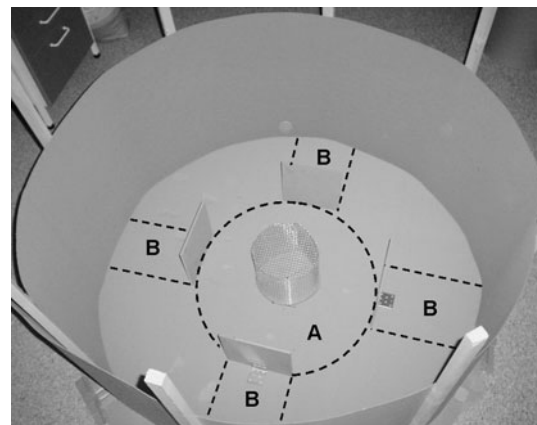


Fig. 1 The sociality test (ST) arena divided into A: social zone, B: explorative zones, used to measure social propensity versus explorative behavior

had unlimited visual contact with the familiar chickens. The areas behind the screens towards the arena wall were considered as explorative zones, since no visual contact was possible between the test chicken when in this area, and the chickens in the holding pen. The chickens were habituated for 10 min in the central holding pen in groups of four (two from each genotype) before the test started. In darkness, one chicken was then taken from the pen and placed between two of the hardboard screens, but with full

visual contact with the birds in the holding pen. Once the bird had been tested, it was returned to the holding pen and another chicken from the group was used as test animal. This was repeated until all four birds had been tested. The starting location was the same for all tested animals. Each test lasted for 10 min and was started by turning the light on and ended by turning the light off. Duration in zones, distance moved and latency to enter the social zone were measured using the software Ethovision XP from Noldus.

Exploration and emotionality tests

Complex environment

To estimate emotionality when exposed to a novel and unknown environment paired with frightening stimuli, the chickens were tested in a complex environment-test (CE) twice: The first time as young (Y) chickens, 14 days old and the second time as adults (A), 163 days old. The Y chicken arena measured 70 × 140 × 160 cm (W × L × H), and the A chicken arena measured 150 × 300 × 180 cm (W × L × H). The complex environment arena was divided into 6 equally sized zones and furnished with different materials (Fig. 2). The chickens were tested in pairs of the same genotype, to exclude social isolation as a factor in the test. Before the test started the chickens were habituated in a circular pen (Y: height 25 cm and Ø 25 cm, A: height 60 cm and Ø 60 cm) placed in zone E for 5 min. The test started by removal of the pen, allowing the birds free access to the entire area. Behaviors (outlined in Table 1) were recorded with interval sampling at 10 s intervals and frequencies of zone entries were counted afterwards from video recordings. After 10 min a model of a predator bird (measuring; Y: 25 cm × 15 cm, A: 50 cm × 30 cm) was pulled along a string over the arena during a time of 5 s. The predator was not visible for the

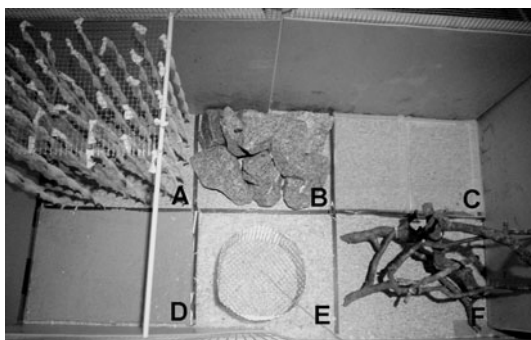


Fig. 2 The complex environment (CE) arena measuring emotionality in chickens when exposed to a novel and unknown environment paired with frightening stimuli. The arena is divided into 6 different furnished zones; A: textile strings, B: rocks, C: water, D: sand, E: wood shavings, F: tree branches

chickens before or after the exposure. After the simulated predator exposure, the same behaviors as before were recorded for 5 more minutes.

Tonic immobility

At the age of 168 days a tonic immobility (TI) test was performed. This is a well-established fear test in chickens (Forkman et al. 2007; Jones 1986). The test was carried out in the same room as the chickens were housed (see above). Each chicken was carefully taken out from its pen, and placed on its back in a wooden cradle. To induce TI, a light pressure with the observers hand was applied to the chest of the bird for 10 s and thereafter slowly removed. TI was considered successfully induced if the bird did not move within the first 5 s. The time to rightening of the bird and numbers of induction attempts were recorded. If tonic immobility was not induced in five attempts the bird was assigned a maximum value of 7. This was done to differentiate between birds that did enter TI after 5 attempts and those that did not. The test lasted for a maximum of 10 min and if the bird had not righted itself within that time the test was interrupted.

Hormone analyses

Blood samples for corticosterone and testosterone (males only) analyses were obtained from the wing veins at 273 days of age. Samples were taken immediately before and after 3 min of physical restraint, during which the chicken was placed in a free hanging net. Corticosterone was measured from both samples, and testosterone from the first one only.

Testosterone was assayed using a RIA kit (Spectria Orion, 68626) after diethyl ether extraction. Inter assay CV were 10.1 and 11.8% for 1.5 and 12.8 nmol/ml, respectively. Total corticosterone in plasma was measured after ethyl alcohol extraction with an in-house RIA using a highly specific antibody provided by Dr. H Vaudry (University of Rouen, France). Cross reactivity with related compounds such as cortisol was <3%. Intra-assay and inter-assays variations were <6% and 10%, respectively for 288 ng/ml.

Statistics

Mean values and standard errors were calculated for each genotype within sex for all the recorded variables. Visual inspection of normality plots indicated that the variables were sufficiently normally distributed to allow the use of ANOVA to estimate the effects of genotype, sex and family, as well as interactions between genotype and sex, on behavioral variables. Since the dataset was unbalanced

with respect to genotype and sex the data were analysed using GLM in Statistica v. 8.0. Variables with significant effects of family were neither analyzed for the interaction between genotype and sex, nor considered for further analyses. This was done to reduce the risk that differences in behavior that appeared to be genotype related in fact were due to effects of family.

In order to analyze for consistent reaction patterns across tests at various ages a multi variate principal component analysis (PCA) was done in the following way. One variable from each of the six behavioral tests where values were obtained for all birds (CE as young and adult, MT as young and adult, TI, ST) were selected for subsequent PCA. The variables chosen were those showing the strongest effect of genotype based on visual inspection of bar graphs. The analysis was limited to one variable per test in order not to bias the PCA towards any specific test. Based on visual inspection of a scree plot and the explained variance, the number of components to retain for further analysis was decided. Principal component scores were calculated for each individual and analysed with ANOVA, with the same model as above, using the GLM procedure in Statistica v. 8.

Results

The means of the weight data and the behavioral variables from the different tests, and the results of the ANOVA are summarized in Table 2. The weight data showed a significant effect of genotype for hatch weight, but there was also a significant effect of family for this variable.

For the behavioral variables significant effects of genotype were seen in four variables. In general, the wild type genotype seemed to be more active and explorative since both males and females entered significantly more zones in total in the CE test both as young and adults. Furthermore, wild type females and males also showed significantly more locomotion as adults in the CE test, in comparison to the white females and males. A tendency to significant genotype effects was seen in the social variable of the CE test as young where both white males and females had a higher frequency of social interactions than the wild type males and females. Moreover, a tendency of genotype but also a clear effect of sex was shown in passive behavior in the CE test as adults. Of the white genotype both males and females had more passive behavior than the wild type and furthermore, the males in general scored higher than the females for this variable. Further, a tendency of being significant for genotype was seen in total aggressive behaviors in the MT as adults, where wild type females and males showed more aggressive behaviors than

white males and females. In the TI test, wild type females required more induction attempts than white females.

Other variables that showed a significant effect of sex were weight at day 50 and 200 where the males were heavier, and the variables exploratory/foraging and comfort in the CE test as adults, where the females scored higher than the males for both variables.

Based on inspection of the scree plot from the PCA and the variance explained, three factors were retained for further analysis. These three factors together explained 73.5% of the variance in the dataset. The factor loadings of the 6 variables included are shown in Table 3. The first factor, explaining 27.6% of the variance, was mainly spanned by high loadings of total zone entries (CE) both as young and adult, total aggressive behaviors (MT) as young, latency to enter social zone (ST) and induction attempts (TI). Hence this factor seems to reflect variance in active/exploratory and social behavior across tests. The second factor, explaining 25.6% of the variance, was mainly spanned by high loadings of total aggressive behaviors (MT) both as young and adult and total zone entries (CE) as adults. Therefore, this factor appears to mainly reflect variance in aggressive behaviors across tests. The third factor, explaining 20.3% of the variance, did not show any consistent pattern allowing interpretation. A significant effect of genotype was found on the factor score for factor 1 ($F_{1,30} = 8.68$, $p < 0.01$), where the wild type scored higher than the white genotype (Fig. 3a). On scores for factor 2 (Fig. 3b), there were significant effects of genotype ($F_{1,30} = 6.39$ $p < 0.05$) and of sex ($F_{1,30} = 6.09$ $p < 0.05$) and of the interaction between genotype and sex ($F_{1,30} = 6.79$ $p < 0.01$). There were no effects of family on the factor scores for either factor 1 or 2. On factor 3 there were no effects of either genotype, sex or their interaction.

In the aggressive reactivity test, only males showed aggressive responses, while all females behaved mildly fearful, withdrawing slightly from the stimulus and being vigilant towards it during the course of the test. Therefore only results for the males are presented in Fig. 4. The wild type males spent significantly more time actively following the stimulus than the white males. Wild type males also showed a tendency to spend more time close to the hand and to have a higher average number of aggressive attacks (Fig. 4). No differences between genotypes were seen in the average latency to attack in the resident-intruder test. The results from the hormone analyses are shown in Table 4. There were no significant differences in testosterone levels between males of the two genotypes. Physical restraint caused a significant increase in corticosterone levels, but there were no differences between genotypes in either basal or stress corticosterone levels.

Table 2 Means (\pm SEM) of weight data and all variables measured in the six behavioural tests which formed the basis for the principal component analysis (PCA)

		Females				Males				Effect of (<i>p</i> -value)		
		Wild-type	SEM	White	SEM	Wild-type	SEM	White	SEM	Sex	Genotype	Sex ^a Genotype
Weight	Hatch	27.1	± 0.6	30.1	± 0.8	28.6	± 0.9	30.7	± 0.9	0.36 ^a	0.07 ^a	
	Day 8	36.9	± 1.4	39.2	± 2.2	39.0	± 1.2	41.4	± 3.3	0.28	0.23	0.97
	Day 50	279	± 10	269	± 15	324	± 10	307	± 19	0.00	0.31	0.78
	Day 200	878	± 37	819	± 28	1262	± 31	1224	± 97	0.00	0.31	0.83
Young	Complex environment											
	Social (% of obs.)	0.2	± 0.1	1.3	± 0.8	0.6	± 0.4	1.7	± 1.3	0.51	0.07	0.98
	Exploratory/foraging (% of obs.)	0.6	± 0.6	0.6	± 0.4	2.9	± 1.9	3.6	± 2.5	0.15	0.86	0.84
	Passive (% of obs.)	35.4	± 6.4	59.0	± 12.8	36.9	± 5.4	58.8	± 12.1	0.53 ^a	0.28 ^a	
	Locomotion (% of obs.)	8.1	± 1.5	7.2	± 0.6	9.9	± 1.8	10.8	± 5.0	0.24	0.99	0.69
	Comfort (% of obs.)	2.0	± 1.0	2.0	± 1.4	0.7	± 0.4	1.9	± 1.1	0.49	0.55	0.56
	Perching (% of obs.)	53.7	± 7.4	29.9	± 12.7	48.9	± 7.1	23.2	± 13.7	0.19 ^a	0.24 ^a	
	Total zone entries (nmb.)	39.7	± 6.8	10.3	± 2.4	28.9	± 6.5	21.5	± 6.4	0.98	0.02	0.17
Mirror test												
Duration mirror zone (% of obs.)	7.4	± 2.2	8.8	± 4.0	11.5	± 2.2	9.8	± 4.1	0.37 ^a	0.90 ^a		
Total aggressive behaviours (% of obs.)	1.1	± 0.3	2.2	± 0.5	3.6	± 0.6	2.0	± 0.7	0.07	0.68	0.05	
Adult	Complex environment											
	Social (% of obs.)	0.1	± 0.1	0.4	± 0.2	0.2	± 0.1	0.0	± 0.0	0.22	0.69	0.08
	Exploratory/foraging (% of obs.)	20.2	± 2.4	18.2	± 3.4	2.9	± 1.0	5.1	± 3.0	0.00	0.95	0.41
	Passive (% of obs.)	54.3	± 4.3	59.0	± 11.6	73.1	± 4.3	91.7	± 4.8	0.00	0.09	0.30
	Locomotion (% of obs.)	4.5	± 0.7	3.7	± 2.1	9.1	± 1.4	2.8	± 2.0	0.26	0.03	0.09
	Comfort (% of obs.)	6.5	± 1.5	3.6	± 1.6	0.3	± 0.1	0.4	± 0.1	0.00	0.29	0.24
	Perching (% of obs.)	14.4	± 4.3	15.1	± 9.1	14.4	± 4.6	0.0	± 0.0	0.23	0.28	0.23
	Total zone entries (nmb.)	13.3	± 3.0	5.8	± 1.6	23.8	± 3.8	5.3	± 4.3	0.24	0.00	0.19
	Sociality test											
	Duration explorative zone (% of time)	12.9	± 5.4	8.6	± 5.5	6.5	± 6.5	24.8	± 24.8	0.60	0.46	0.24
	Duration social zone (% of time)	58.1	± 10.4	51.8	± 13.7	35.5	± 9.9	47.7	± 27.6	0.36	0.84	0.52
	Distance moved (cm)	1931	± 346	2083	± 525	2498	± 720	1008	± 209	0.06 ^a	0.00 ^a	
	Latency to enter social zone (s)	77.1	± 53.6	208.0	± 95.4	305.8	± 66.3	312.7	± 166.2	0.07	0.44	0.49
	Mirror test											
	Duration mirror zone (% of obs.)	9.7	± 2.3	9.2	± 3.1	10.9	± 2.4	5.0	± 5.0	0.65	0.32	0.41
	Total aggressive behaviours (% of obs.)	2.0	± 0.7	1.5	± 0.6	6.2	± 2.0	0.0	± 0.0	0.47	0.07	0.13
Tonic immobility												
Rightening time (s)	154	± 55	164	± 73	117	± 43	207	± 132	0.97	0.48	0.57	
Induction attempts (nmb.)	3.8	± 0.8	1.0	± 0.0	1.8	± 0.3	1.8	± 0.5	0.33	0.04	0.04	

p-values from ANOVA analysis. Showing the effects of genotype. Sex and their interactions are given for all variables

^a Denotes that the ANOVA showed a significant effect of family

Discussion

The results from this study show that differences in social, active/exploratory and aggressive behaviors between homozygous wild type (*ii*) and white (*II*) *PMEL17* genotype chickens were clearly demonstrable in a situation

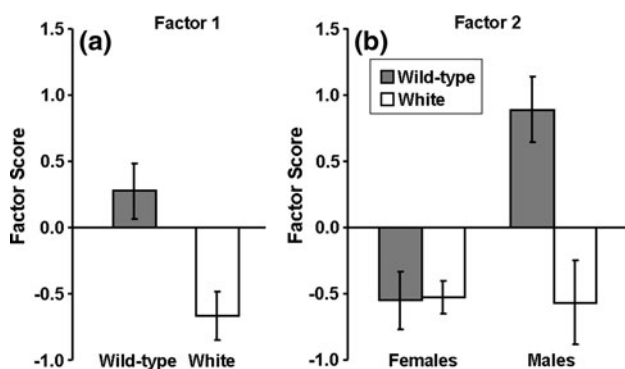
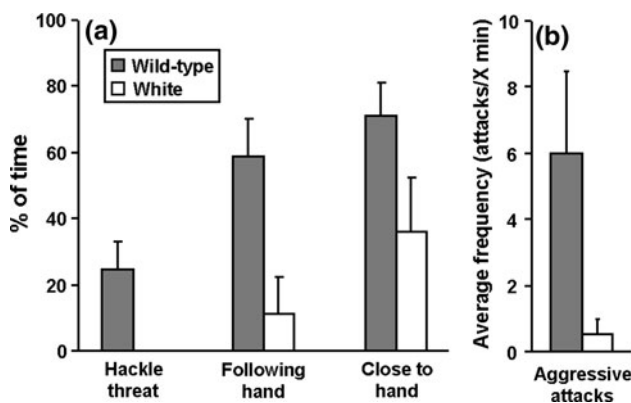
where negative social experiences were excluded as possible cause. This strongly indicates that the genotype on the *PMEL17* gene has a direct effect on behavior in chickens.

To investigate the effects of the gene of interest, we utilized what we call a Locus Controlled Advanced Intercross Line. By controlling for genotype at one locus in an

Table 3 Factor loadings on the first three principal components (PC) for each variable from the six behavioral tests included, and the variance explained by each component

Variable	PC 1	PC 2	PC 3
Total zone entries (nr.) CE Y	0.50	0.43	-0.52
Total aggressive behaviors (% of obs.) MT Y	-0.68	0.56	-0.00
Total zone entries (nr.) CE A	0.55	0.52	0.41
Total aggressive behaviors (% of obs.) MT A	0.31	0.74	-0.64
Latency to enter social zone (sec.) ST A	-0.53	0.33	0.01
Induction attempts (nr.) TI A	0.51	-0.35	-0.60
% variance explained by factor	27.58%	25.58%	20.27%

CE complex environment, MT mirror test, ST sociality test, TI tonic immobility, Y young chickens, A adult chickens

**Fig. 3** The average factor scores (\pm SEM) on the first two factors extracted from the principal component analysis (PCA); **a** scores of wild type and white genotype on factor 1 and **b** scores of females and males of both genotypes on factor 2**Fig. 4** Average frequencies of **a** threatening behaviors (hackle threat, following hand and close to hand) and **b** aggressive attacks recorded in the aggressive reactivity test (means \pm SEM)

advanced intercross line, we aimed to study the effect of this genotype against a background of random hybrid genotypes, achieved by accumulation of recombinations. There were essentially two families of each genotype, so

there is a possibility that observed effects could be due to genetic variation at loci not controlled for. This should be detectable by significant family effects, but such were rare. For example, hatch weight differed significantly between genotypes but a significant effect of family was also found for this variable, indicating that this particular difference may be due to spurious genetic effects. It has been suggested that the effects of *PMEL17* allele on weight may be sex dependent (Karlsson et al. 2010; Nätt et al. 2007), which was not supported by our findings when we could control for family effects.

Although the difference in specific response variables in the behavioral tests only rarely reached statistical significance, the Principal Component Analysis (PCA) showed that individual birds had some consistency in their reaction patterns across different tests. In fact, 73.5% of the between test variation was explained by three factors, where the two-first could be interpreted as an activity/exploration and an aggression related factor respectively. This indicates that individual chickens differed in their personality traits (Bouchard and Loehlin 2001). The factor score distribution on the first factor suggests that chickens high in exploration are also more social and less fearful, while the second suggests that more aggressive birds were also more fearful, as indicated by the TI-test. Furthermore, the PCA showed a significant effect of genotype on both the two-first factor scores. This supports earlier studies showing that *PMEL17* has an effect on social and exploratory behaviors (Karlsson et al. 2010; Nätt et al. 2007), and indicates that the gene somehow affects basic personality traits. It is unfortunate that the data from the sociality test as chicks were lost. Data from this may have strengthened the PCA results further. However, a mirror is considered to be perceived as a social companion (Feltenstein et al. 2002) and therefore the mirror test, performed both in young and adult chickens, gave a measure of sociality in the chickens also at young age.

Factor two was mainly related to aggressive behavior and the group of wild type males was the only group that scored positive. It is important to distinguish between defensive aggressive behaviors and offensive aggressive behaviors, which differs with respect to underlying functions and factors (Blanchard and Blanchard 2003; Blanchard and Caroline Blanchard 1977; Blanchard et al. 2003). Defensive aggression is described as an attack in defense of the subject's own bodily integrity (Blanchard et al. 2003) and is related to fearful behavior. Defensive aggression was measured in the aggressive reactivity test where wild-type males showed more aggressive behaviors than white males. Offensive aggression is described to involve response to challenge over adaptively important resources (Blanchard et al. 2003) and was measured in the resident-intruder test. Genotype differences were found in the aggressive reactivity test but not in the resident-intruder

Table 4 Results from hormone analysis showing mean (\pm SEM) plasma concentrations of corticosterone and testosterone

	Corticosterone basal levels (ng/ml)		Corticosterone stress levels (ng/ml)		Testosterone (nmol/l)	
	Mean	SEM	Mean	SEM	Mean	SEM
Wild-type	1.17	0.11	2.45	0.24		
Females	1.23	0.20	2.81	0.42		
Males	1.12	0.11	2.11	0.23	5.38	0.88
White	1.26	0.20	2.58	0.33		
Females	1.56	0.34	3.31	0.43		
Males	0.92	0.10	1.73	0.18	5.70	2.13

The results for corticosterone measurements show basal levels before and stress levels after physical restraint for males and females of both genotypes. Testosterone was measured in males only

test, indicating that *PMEL17* genotype appears to mainly affect defensive aggressive behavior. Individuals with a dark eumelanic coat or plumage color have been described as more aggressive in a various number of species. In a literature review of available data, Ducrest et al. (2008) found that in 18 of 20 species dark eumelanic males were more aggressive than less pigmented individuals in the same population. The increased aggressive behavior has been suggested to be induced by binding of melanocortins to the melanocortin 5 receptor (Ducrest et al. 2008).

The melanocortin system has been suggested to account for pleiotropic effects on behavior and physiological traits in a variety of species (Cottle and Price 1987; Ducrest et al. 2008; Hayssen 1997; Keeler 1942; Schmutz and Berryere 2007). However, it is important to note that *PMEL17*, the causative gene for the lack of pigmentation in White Leghorn chickens, is not involved in melanogenesis and is not assumed to affect the melanin pathway directly. The *PMEL17* protein is located in the vesicular structures of premelanosomes and is essential in the amyloid fibril formation during premelanosome maturation where the striations serve as the site of melanin deposition (Huff et al. 2003; Raposo et al. 2001; Yasumoto et al. 2004). The *Dominant white* mutation constitutes a nine basepair insertion leading to an insertion of three amino acids (WAP) in the transmembrane region of *PMEL17* (Kerje et al. 2004), and chickens homozygous for the mutation have no mature eumelanosomes and show a completely white plumage. It is at present not clear how this could be related to behavioral differences, but speculatively, (Nätt et al. 2007) suggested that preventing melanin to bind to the premelanosome striations may perhaps cause an accumulation of intracellular melanin which could alter the biochemical equilibrium and affect catecholamine levels. However, *PMEL17* appears not to be expressed in the brain of adult chickens (Karlsson et al. 2010), but it is of course not impossible that similar events may occur in other organs for example endocrine glands such as adrenals or sensory organs. Furthermore, studies of mouse neural

tissue have shown that *PMEL17* is expressed during embryonic development (Baxter and Pavan 2003), and this could possibly be the case in chickens as well.

The hormone analysis showed no differences between testosterone levels in males, which might have been expected given the difference in aggressive behavior. In spite of the fact that corticosterone response was measured after only 3 min of restraint there was a strong and significant effect of the treatment. However, there were no genotype effects on corticosterone baseline or stress levels following physical restriction, an indicator of HPA-axis sensitivity. Since peak of corticosterone response in chickens has been shown to occur about 15 min after start of test (Fraise and Cockrem 2006) it is possible that a genotype difference had not yet developed in our test. In salmon and trout, it has been shown that low pigmentation is related to high levels of cortisol (Kittilsen et al. 2009), and in barn owl studies have shown that corticosterone-implanted nestlings produce feathers with less phaeomelanic coloration (Roulin et al. 2008).

There are two main lines of theories on why coat color phenotypes have changed during domestication. Firstly, coat color may be linked to behaviors preferred during domestication and therefore selected as a side effect, which has been observed in earlier studies (Price 1998; Trut 1999). Secondly, it could be that coat color has been intentionally selected by humans, as suggested by Fang et al. (2009), and behavior changes may have occurred secondarily. Regardless of the historical process, there is strong evidence from several species showing that genes regulating coat color have pleiotropic effects on behavior (reviewed by Ducrest et al. 2008) and this is further supported by our results.

Earlier findings have shown that *PMEL17* wildtype birds are more susceptible to becoming victims of feather pecking (Keeling et al. 2004), and that this may partly depend on differences in behavior that predispose some chickens to become victims (Nätt et al. 2007). However,

previous studies have not been able to tear apart the direct effects of genotype on behavior from indirect effects caused by different social experience. An alternative explanation to the genotype related behavioral differences could be that the wild color phenotype attracts feather pecking, and therefore wild type birds will suffer from the experience of being a victim of feather pecking which in turn may affect various aspects of the behavior of the victimised bird. Several studies have shown that dark birds are more feather pecked than white birds when kept in mixed groups (Bright 2007; Nätt et al. 2007; Savory and Mann 1999). Bright (2007) suggested that black or grey Oakham Blue hens are more susceptible to feather pecking because black and grey birds appear more different inside the house due to the lack of UV reflectance, than white birds, and Savory and Mann (1999) found that pecks were often directed at contrasting particles on the plumage. On the other hand, behavior differences may predispose for feather pecking regardless of plumage color. For example, inactive individuals are more likely than active ones to become victims (Riber and Forkman 2007), and locomotor activity was higher in a line of chickens selected for high feather pecking in comparison to birds selected for low feather pecking (Kjaer 2009). Because of the difficulty to tear apart behavior effects caused by the negative social experiences of being feather pecked from possible direct behavior effects of the predisposing genotype, the present experiment studied birds of different genotype with similar experience. Our results suggest *PMEL17* genotype does exert a direct effect on behavior, since the genotype differences remained even when negative social experiences from being feather pecked are excluded. However, due to the genetic selection method used, we cannot exclude possible effects of linked genes, or spurious family effects from fixation of alleles in other regions in a particular family, caused either by unintentional selection or genetic drift. Nevertheless, principal component scores were analysed for family effects with no significant results, supporting that our findings of genotype differences are due to effects of the *PMEL17* gene rather than family effects. Theoretically it is possible that other alternative alleles than *PMEL17* have been fixated in the 9 parental birds used for breeding, causing the genotype differences in our study. Though this possibility seems highly unlikely, it can not be entirely excluded.

In conclusion, we have used chickens with alternative homozygous genotypes on the *PMEL17* gene in order to investigate if behavioral differences between genotypes remain even when negative social experiences are excluded. The results show that wild type genotypes differ from white birds in social, active/exploratory and aggressive behavior even when the chickens are raised separate from

each other, suggesting that the *PMEL17* gene has pleiotropic effects on behavior.

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