Plumage colour and feather pecking – behavioural effects of *PMEL17* genotypes in fowl (*Gallus gallus*)

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LITH-IFM-EX--05/1478--SE

Avdelning, Institution
Division, Department

Datum
Date

Språk
Language

☐ Svenska/Swedish
☒ Engelska/English

☐ _____

Rapporttyp
Report category

☐ Licentiatavhandling
☒ Examensarbete
☐ C-uppsats
☐ D-uppsats
☐ Övrig rapport

☐ _____

ISBN
LITH-IFM-EX—05/1478--SE

ISRN

Serietitel och serienummer **ISSN**
Title of series, numbering

URL för elektronisk version

https://cms.ifm.liu.se/biology/master_projects/2005/danis102/

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Sammanfattning
Abstract

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Nyckelord
Keyword

Fear, Feather preference, Melanin, Openfield, Red jungle fowl, Tameness, Victimization, White leghorn,

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1 Abstract

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Key words: Plumage colour, Fear, Feather pecking, Feather preference, Melanin, Openfield, PMEL17, Red jungle fowl

2 Introduction

Severe feather pecking is the abnormal behaviour seen in different species of fowl where feathers are forced loose from one individual by another. This behaviour is one of the causal factors leading to cannibalism, and could result in death of the victim (Cloutier et al. 2000). Recently, after stronger legislation against cages and the gradual reposition to free range systems in the EU, feather pecking has become one of the largest welfare problems in the poultry industry (Yngvesson 2002; Elson 2004).

Numerous causal theories of feather pecking are present, where a redirection of substrate pecking in neonates is the most prominent (Blokhuis 1986, Huber-Eicher & Wechsler 1997, Johnsen et al. 1998). Blokhuis (1986) argued for a misdirected foraging behaviour. This have further been strengthened by studies showing that feather pecking decreases if hens are provided with litter substrates that elicit foraging

(Huber-Eicher & Wechsler 1998). In contradiction Bilcik and Keeling (2000) reported no redirection of ground pecks to feather pecks. An alternative explanation is that feather pecking is a redirection of substrate pecking during dust-bathing (Vestergaard et al. 1993). Lately studies also suggest that this behaviour could have an underlying social component (Jones et al. 1995). Bilcik and Keeling (2000) reported that feather pecking in laying hens was positively correlated with increased group size and Riedstra and Groothuis (2002) showed that if unfamiliar birds are introduced in an established flock feather pecking increases.

Correlations between genotype and phenotype in relation to performing or receiving feather pecks have been reported (Kjaer & Sørensen 1997) and inheritance of both perspectives is considered to be polygenic (Buitenhuis et al. 2003). Differential selection for high and low feather pecking behaviour, from a performer perspective, has been successful (Kjaer et al. 2001) and many traits such as open-field behaviours and physiological responses of induced stress diverge between these two lines (Jones et al. 1995, Korte et al. 1997, van Hierden et al. 2002). To date no examination of open-field response of victims to feather pecking has been conducted. A good review in the topic of feather pecking has been written by Rodenburg et al. (2004).

Recently, a QTL-analysis on an inter-cross between White leghorn (*Gallus gallus domesticus*) and Red jungle fowl (*Gallus gallus*) was conducted by Keeling et al. (2004). It resulted in the discovery of a QTL on the linkage group E22C19W28 that was responsible for as much as 14.9 % of the residual phenotypic variance in plumage damage. Further examinations revealed that black birds of this cross had poorer feather condition then white birds. Furthermore, a coat colour gene, called *PMEL17* (homologous to the *Silver* and *gp100*), showed perfect alignment with the QTL. *PMEL17* has previously been characterized both to genomic position and mutations (Kerje et al. 2004). It codes for a transmembranous protein associated with melanosomes, which is critical for the intercellular production of the black/brown pigment eumelanin. The plumage colour of a wild type homozygous (illustrated *i/i*) chick carrying two functional *PMEL17* alleles is black or dark brown. The classical *Dominant white* loci usually illustrated with an *I* (Bateson 1902: cited by Kerje et al. 2004), have been associated to a mutation in exon 6 and 10 of the *PMEL17* gene. This causes a functional failure of the eumelanosome, probably due to the inability of the gene product to incorporate properly into the melanosome membrane, making the mutant homozygote (*I/I*) unable to express eumelanin and therefore shows a white phenotype (Kerje et al. 2004). Sturm et al. (2001) have written a good review on cellular pigmentation.

Another surprising relationship is that feather pecking is increased in a flock with increased numbers of wild typed homozygous (*i/i*) relative homozygous mutant (*I/I*) individuals (Keeling et al. 2004).

An association of coat colour and behaviour has been observed in many species that been domesticated. Clyde Keeler reported behaviour differences in colour mutants of the Norway rat, *Rattus norvegicus* (Keeler 1942, Keeler C & King H D 1942), and the Mink, *Mustela vision* (Keeler & Moore 1961). The Russian scientist Dmitry Belyaev began in 1959 to select silver foxes, *Vulpes vulpes*, for one criterion only, namely tameness (Trut 1999, Rekilä et al. 1997). After 40 generations of selection the result was a fox-breed that differed clearly both in behaviour and physiology (Harri et al. 2003). One of the earliest changes was the appearance of a more whitish coat colour (Trut 1999).

Even though large aggregates of evidence saying that coat colour might have an influence on behaviour, attempts to reveal the molecular mechanisms of this peculiar correlations has so far been absents.

The aim of this work was to confirm and reveal some of the mechanisms of the relationship between having the Red jungle fowl *PMEL17* genotype (*i/i*) and the increased susceptibility of being pecked. By measuring behavioural traits of individuals that differ solely on one allele of one gene in an otherwise randomized genome the expectations were to find behavioural features that are dependent on the respective alleles.

Firstly, a strictly observational study (Box observation) of 20 small groups differing in gender and *PMEL17* genotype composition was preformed. Roosters were included in the study to investigate if the phenomenon of black victimization also is present among males. Otherwise the main purpose was to strengthen earlier results based on feather damage with real-time observations of the feather pecking behaviour. I hypothesized that *i/i* would receive significantly more feather pecks then *I/I* birds and that there would be more feather pecking in groups inhabiting a high proportion of *i/i* individuals. I also expected that the increased feather pecking directed to *i/i* birds should be reflected in a plumage damage evaluation at the end of the study.

Secondly, a series of tests that mostly concerned females and was investigating genotype specific differences were performed. Three of these tests were conducted repeatedly during a greater time period. A Feather preference test was utilized to investigate the preference to feathers of black and white colour. The hypothesis was that black *i/i* feathers would receive more pecks then white *I/I* feathers. This test also measured reactions to a novel object. An Open-field arena thought to measure fearful, explorative and social reinstatement behaviours, as well as a Fear-for-

human test, estimating tameness and anxiety, was also conducted. At one occasion a Tonic immobility test was investigating predatory fearfulness. I hypothesized that white *I/I* birds would behave differently in the Open-field arena, Feather preference, Novelty, Anxiety, Tonic immobility test, and show less fearful behaviours toward a human being then black *i/i* birds do.

3 Materials and Methods

3.1 Animal material

A F_4 generation of a cross between White leghorn and Red jungle fowl were used as parents in this study. The cross has recently been utilized in genetic and behavioural studies (Schütz et al. 2002, Carlborg et al. 2003, Keeling et al. 2004, Kerje et al. 2004). The White leghorn line (SLU13) has a long history of selection for layer production traits, while the Red jungle fowl comes from a Swedish zoo population. Parents, as well as offspring were maintained at Götala research station, Skara, Sweden.

3.2 Genotyping the parents

To acquire *PMEL17* homozygous offspring (*i/i* and *I/I*) all parents were genotyped on the position of the *PMEL17* gene. The method has been used in a previous study (Kerje et al. 2004). Blood samples were taken and DNA was purified from the parents using an AGOWA DNA PLUS-kit (AGOWA). Firstly, a polymorphic repeat in exon 7 was primed, amplified with PCR and visualized with MegaBACE^{TM} 1000 (Amersham Bioscience) – a fluorescence-based DNA system utilizing capillary electrophoresis with up to 96 capillaries operating in parallel. For visualization an ET900-R size standard (Amersham Bioscience) was used and displayed by the software Genetic Profiler (Amersham Bioscience). The programme showed two amplified fragments of 490 and 574 bp that earlier been associated with the *Dominant white* (I) and wild type (i) alleles respectively (Kerje et al. 2004).

Comparisons to the photographs of the parents revealed some uncertain combinations and another approach with different markers was therefore conducted. The new primers annealed around the polymorphic insertion/deletion on *PMEL17* exon 10, which is partly responsible for the Dominant White phenotype (Kerje et al. 2004). Except of the markers, use of a new thermo cycling scheme and an ET400-R size standard (Amersham Bioscience) this analysis was performed with the same method as that on exon 7. Fragments of 198 and 206 bp were displayed and associated to the wild type (i) and *Dominant white* (I) allele respectively. Conclusively, the

exon 10 marker showed much more confidence with the phenotype (Appendix 1).

3.3 Mating procedure

Genotyping data was used to construct breeding groups. Firstly, 5 recessive homozygous males (*i/i*) were mated with 8 recessive homozygous females (*i/i*) yielding 39 offspring (13 males and 26 females) and 3 dominant homozygous males (*I/I*) were mated with 5 dominant homozygous females (*I/I*) yielding 27 offspring (16 males and 11 females).

Secondly, because of a shortage of female *I/I* parents three of the *I/I* males were mated with six of the *I/i* females yielding 34 offspring. A blood sample of the offspring was taken and genotyped on the *PMEL17* exon 10 region with the same method that was used for their parents. This yielded 20 chicks with *I/I* genotype (13 males and 7 females).

In total the mating procedure resulted in 39 *i/i* (13 males and 26 females) and 47 *I/I* offspring (29 males and 18 females). One *i/i* and one *I/I* female died due to a large outbreak of coccidiosis at five weeks of age. Three females, two *i/i* and one *I/I* were withdrawn from the experiment due to abnormal developmental patterns, most probably due to genetic disorders.

3.4 Housing conditions for the offspring

The first five weeks after hatching all offspring were housed in two separate groups. At five weeks of age 60 individuals were randomly divided into 20 groups - 11 all female and 9 all male - with 3 individuals in each and put into boxes measuring: 2.0 (length) x 1.3 (width) x 2.1 (height) m. Each sex was further divided in relation to genotype composition: half had two *i/i* (black) and one *I/I* (white); the other half had one *i/i* and two *I/I* individuals (Figure 1). This yielded 4 different group compositions divided by sex and *PMEL17* genotype frequency. All boxes had automatic feeders and drinkers, where standard commercial pellets and water were provided *ad libitum*. Litter of wood shavings was provided and changed gradually to prevent sudden changes of the environmental background colour. Light scheme was set on a 12 h cycle.

3.5 The experiments

From 6 to 23 weeks of age the birds were observed and tested to evaluate any genotype, configuration and/or sex differences. Weights were measured on two occasions: 10 and 24 weeks after hatching. Temperature varied between 25 (summer) and 15 (winter) ^{o}C and observations took place between 8:30-15:30. All tests were performed by the same experimenter/observer.

a **b**

Figure 1. Genotype configurations of the home-boxes. Half of the boxes had a) black i/i majority and half b) had white I/I majority. Classification of plumage colour according to Kerje et al 2003, from the right: female wild type, female black, female white, male white with red/brown colour, male white and male barred.

3.5.1 Box observation

Observations in the home-boxes were conducted on five occasions: 6, 11, 15, 19 and 23 weeks of age. An observation of the group included that all birds was observed 2 x 60 s seven times evenly distributed over the day during a four day period. In total each bird was observed 14 min (120 s x 7 observations) each week, yielding 70 min for all five weeks.

Social pecks were observed with focal animal sampling and continuous recording, partly according to an earlier study (Bilcik & Keeling 1999):

1. Gentle feather pecking (allopreening and stereotypic pecking)
2. Severe feather pecking (pulling feathers)
3. Aggressive pecks
4. Pecks on non feathered areas (beak, toe, eyes, ring tags).

Activity variables were observed with 1/0 focal animal sampling with intervals of 60 s:

5. Object pecks (walls, perch, metallic part of feeder, wired mesh)
6. Manipulation of feathers on the floor
7. Ground pecks
8. Pellet pecks (in feeder)
9. Drinking
10. Comfort behaviours (stretching, preening)

11. Sleeping
12. Dust bathing

A 60 s habituation period was conducted before the start of each observation and during the observation if any sign of stress activity due to human disturbance was sighted in the target box.

If cannibalism or bleeding wounds were observed on the birds the victims were immediately removed and isolated until they healed.

3.5.2 Open-field arena

On three occasions females were tested in an open-field arena: week 12, 16 and 20 after hatching. A hardboard arena on concrete floor measuring 1.50 x 1.50 x 0.40 m covered with wired mesh was used. It was divided into 36 zones measuring 0.25 x 0.25 m by black tape on the floor.

Variables recorded were:

1. Latency to vocalize
2. Latency to first step
3. Latency to first peck
4. Number of vocalizations
5. Number of pecks
6. Number of defecations
7. Number of zones entered (zones may be entered more then once).
8. Number of zones visited (zones may only be visited once).

An observer was located on an elevated point hidden behind two spotlights. Vocalizations, pecks, defecations and latency to first step were measured by the observer: latencies were measured continuously and frequencies with 1/0 sampling al a 10 s intervals. Entered and visited zones were recorded by a video camera and analyzed later. Before the test, birds were caught in their home-boxes, transported a short distance in a wooden container and lifted into the arena in complete darkness. After 90 s of habituation a 600 s test period began by turning on four spotlights directed to the arena.

3.5.3 Feather preference test

Females were tested in a feather preference test that included a novelty test at 13, 17 and 21 weeks after hatching. Each bird was deprived of food for 120 min (± 15 min) in an adjusted commercial cage with wired floor and automatic drinkers. The cages had a dimension of 0.82 x 0.48 x 0.45 m,

was provided with a perch and a cardboard litter box of 0.38 x 0.47 x 0.10 m containing wood shavings.

A panel with a body of hardboard measuring 0.45 x 0.12 m covered with tape of grey colour was presented to the bird. Three equally large zones were separated on the panel by black tape and contained one out of three stimuli: familiar pellets in the middle zone, and in each of the edge zones, white (*I/I*) and black feathers (*i/i*) respectively. Stimuli of ten flight and ten down feathers of each colour were collected fresh from the home-boxes and glued to the panel. The presentation of the panel varied so that black and white feathers were presented equally on the right and left side of the bird.

Following a 60 s habituation period a 600 s novelty test was conducted, where the test panel functioned as a novel object. Variables recorded were:

1. Latency to put two claws on perch (far from the novel object)
2. Latency to put two claws in the litter box (close to the novel object)
3. Latency to the first peck directed to the test panel.

Immediately following the first peck toward the penal the novelty test was executed and a 300 s preference test began. Variables measured were:

4. Latency to peck at food,
5. Latency to peck at black feathers
6. Latency to peck at white feathers
7. Number of pecks directed to black
8. Number of pecks directed to white

If the bird directed its first peck toward the test panel during habituation the preference test started immediately. These individuals also gained the highest score in the novelty test.

During the test the observer stood about 1 m from the cage, hidden behind a wall with a mirror glassed window.

3.5.4 Fear-for-human test

Fearful behaviour toward humans in females was investigated on three occasions: 13, 17 and 21 weeks after hatching. This test is corresponding to the titbit test used in foxes (Harri et al. 2003). A hardboard box measuring 0.80 x 0.50 x 0.40 m was utilized. The top and one of the short side walls of the box (the front) was made of wired mesh, while the other sides were solid. Underneath the wired meshed front a small opening was provided.

The box was divided into two equally large zones separated by a guillotine door: one close to and one far from the front.

Birds were food deprived with the method of the Feather preference test. After deprivation the birds were caught, put in a wooden box and transported to a nearby test room. In darkness they were lifted into the far zone. This was followed by light and a 120 s habituation period. At this time the experimenter was located 0.3-0.4 m from the wired meshed front. Before the onset of the test he reached his hand filled with familiar pellets through the opening beneath the front and into the close zone. The test started when the experimenter pulled a rope, which resulted in the opening of the guillotine door, allowing visual interaction between bird and human.

Movements were recorded by a video camera and number of pecks was counted directly by the observer. Variables measured were:

1. Latency to first step
2. Latency to enter close zone
3. Duration in close zone
4. Number of entered zones
5. Latency to first peck directed to the palm of the experimenter
6. Numbers of pecks directed toward the palm.

The test was conducted during 600 s, but numbers of pecks to the palm was measured from the first peck and 120 s onward independent on the maximum length of the test.

3.5.5 Tonic immobility

At 24 weeks of age a tonic immobility test was preformed to reveal any differences in predatory fearfulness. It was conducted in front of the test subject's own home-box and was including males. Birds were individually caught and laid on their back in a wooden cradle. To induce tonic immobility the experimenter used his hand to deliver a light pressure over the breast of the bird for 10 s. The test period started when the hand was lifted from the bird and was proceeded for 600 s. If the test subject moved during the first 10 s the test was cancelled and the induction procedure repeated. Five repeats were considered to be the upper limit, after that the bird was withdrawn from the experiment. Variables measured were:

1. Latency to stand upright
2. Number of induction attempts.

3.5.6 Feather Score

An evaluation of the plumage condition of each bird (both sexes) was preformed at 28 weeks of age (see Gunnarsson et al. 2000 for full description of the method). Three areas of the body – rump, tail and belly – were evaluated on a six classed scale, where 0 represented perfect feather condition and 5 an almost completely nude bird. The scores were set by an outside observer, previously well experienced with this particular feather score method, but not familiar to the birds and the hypothesis of this study.

3.6 Statistical methods

General Linear Models were used to compare the between and within sample effects of genotype, configuration and/or sex on the different behavioural variables. Tests with a time factor (Box observation, Open-field, Feather preference and Fear-for-human test) were analysed with repeated measure, while the other tests (Tonic immobility, Feather score and weights) used multivariate or univariate procedures. Independence and linearity/additively were assumed. Levene's test for equal variance and Shapiro-Wilkins test for normality (of the standardized residuals) was used to investigate if the data were in accordance with the remaining GLM assumptions. If not in accordance, a suitable transformation (square-root, logarithmic, inverse or arcsine) were conducted. If the data still were not sufficient enough to meet the assumptions a Mann-Whitney U-test for unrelated samples were used. For those comparisons that exclusively were dealing with dependent variables (Feather preference – white verses black feathers and Box observation – genotype comparison of received pecks) a Wilcoxon sign rank test were used. All deviations from the mean were given as ± 1 SEM.

3.7 Ethical consideration

This study was approved by the local Ethical Committee of The Swedish National Board for Laboratory Animals. The committee has the task to evaluate welfare of the animal in relation to the purpose of the study, possibilities of alternative methods and to determine if the study is not a repetition of an already conducted experiment.

4 Results

In the genotype comparison within the home-boxes (Table 1), black *i/i* females (n = 11) received more severe pecks than white *I/I* females (n = 11)(Z = -2.312), while any differences in other plumage directed pecking behaviours were absent. Most difference in severe pecking was seen at

Table 1. Genotype comparison of received pecks in the Box observation. Wilcoxon two tailed sign rank test for related samples were used.

		Black *i/i*		White *I/I*		
Peck	Sex	Mean	±SEM	Mean	±SEM	sign
gentle	♀	5.20	3.73	1.98	0.79	NS
severe	♀	0.27	0.06	0.09	0.04	0.02
aggressive	♀	0.11	0.02	0.13	0.02	NS
gentle	♂	1.55	0.62	1.02	0.17	NS
severe	♂	0.18	0.05	0.09	0.02	NS
aggressive	♂	0.19	0.31	0.12	0.09	NS

* level of significance: p = 0.05

early age (Figure 2). No differences in bird directed pecking between genotypes were present in males.

There were no effects of group configuration or gender on feather pecking behaviours. Of the other variables aggressive pecks ($F_{1,14} = 17.58$, $p = 0.001$) and feeding in males ($F_{1,14} = 5.33$, $p = 0.05$) were the only ones that diverged. Numbers of aggressive pecks in female groups with black majority were from the start very high but declined with time (Figure 3a), while male groups with white majority were high all through time (Figure 3b). In total the frequency of feather pecks in the Box observation was 80 pecks h^{-1}, where severe feather pecking represented 5 pecks h^{-1}.

The feather preference test showed no preference to black immobile feathers, nor were there any genotype effect in feather preference or reaction to novelty. A frequency of 19 pecks h^{-1} was directed to feathers on the test panel.

Black *i/i* individuals vocalized both earlier (Figure 4a) and more (Figure 4b) in the Open-field arena. At 20-21 weeks of age white *I/I* individuals tended to enter more zones in the arena (Figure 5a) and differed significantly in the Fear-for-human test (Figure 5b). The Fear-for-human test also showed tendencies that white *I/I* birds pecked earlier ($F_{1,29} = 3.98$, $p = 0.06$) and more to the experimenters palm ($F_{1,29} = 3.66$, $p = 0.07$).

Black *i/i* females (n = 16) in relation to white *I/I* females (n = 15) showed higher Feather score at the rump location (1.06 ± 0.37 verses 0.13 ± 0.09, $Z = -2.023$, $p = 0.04$), but not on other areas of the body or among males. Weights of white *I/I* males were higher at both 10 (637 ± 24 verses 708 ±18 g, $F_{1,25} = 5.55$, $p = 0.02$) and 23 weeks of age (1295 ± 49 verses 1445 ± 37

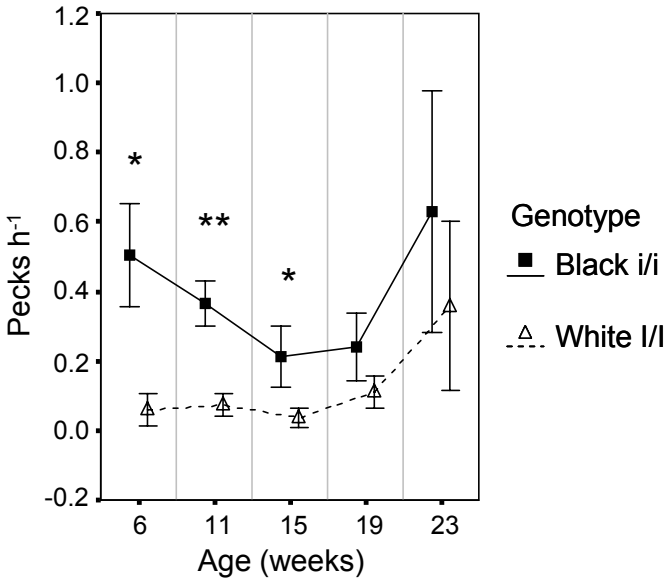

Figure 2. Genotype comparison of received severe pecks in female groups. At 6 (Z = -2.10, p = 0.04), 11 (Z = -2.82, p = 0.005) and 15 (Z = -2.12, p = 0.03) weeks of age there are significant differences. Wilcoxon two tailed sign rank test.

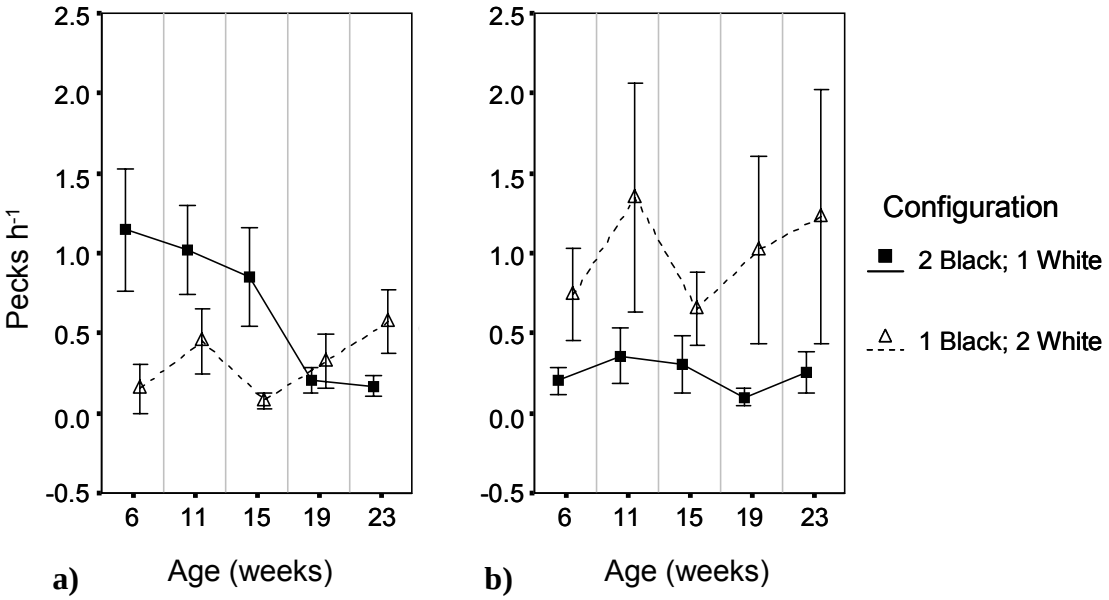

Figure 3. Received aggressive pecks by group configuration. In (a female groups there were a significant effect between subjects ($F_{1,7}$ = 5.93; p = 0.05) and a tendency within time ($F_{4,28}$=2.49; p = 0.07). In (b male groups there was a significant effect between subjects ($F_{1,7}$ = 12.13, p = 0.01).

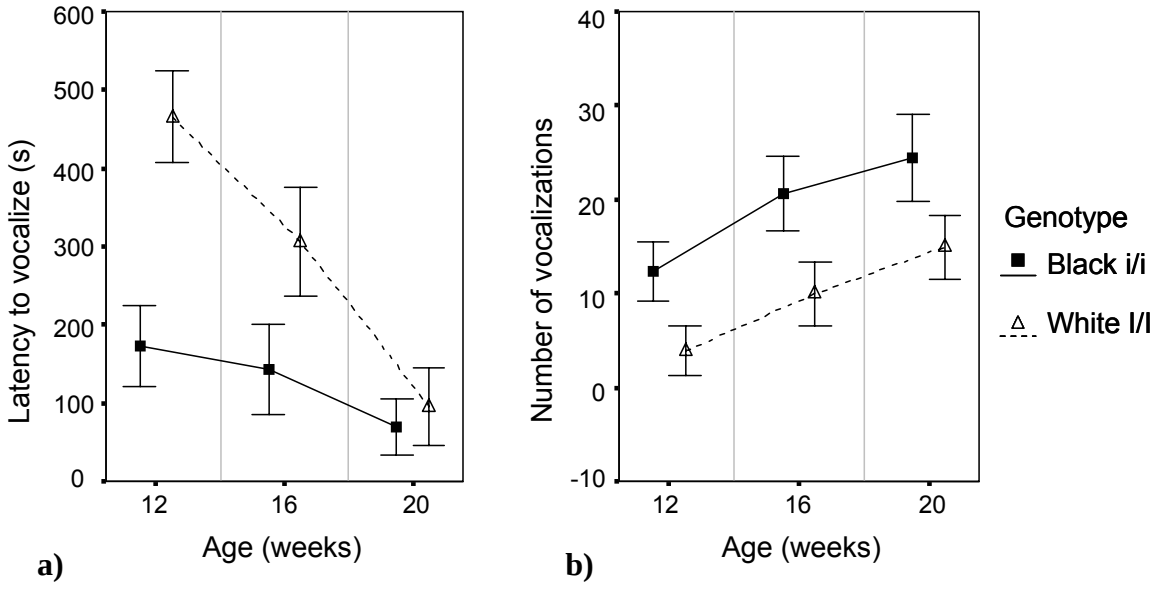

Figure 4. Differences of vocalization in an open-field arena between genotypes. In a) Latency to vocalize showed significant effect between subjects ($F_{1,30}$ = 6.867, p = 0.01) as well as within time ($F_{2,60}$ = 4.018, p = 0.02). Numbers of vocalizations b) differed between subjects ($F_{1,30}$ = 6.635, p = 0.02).

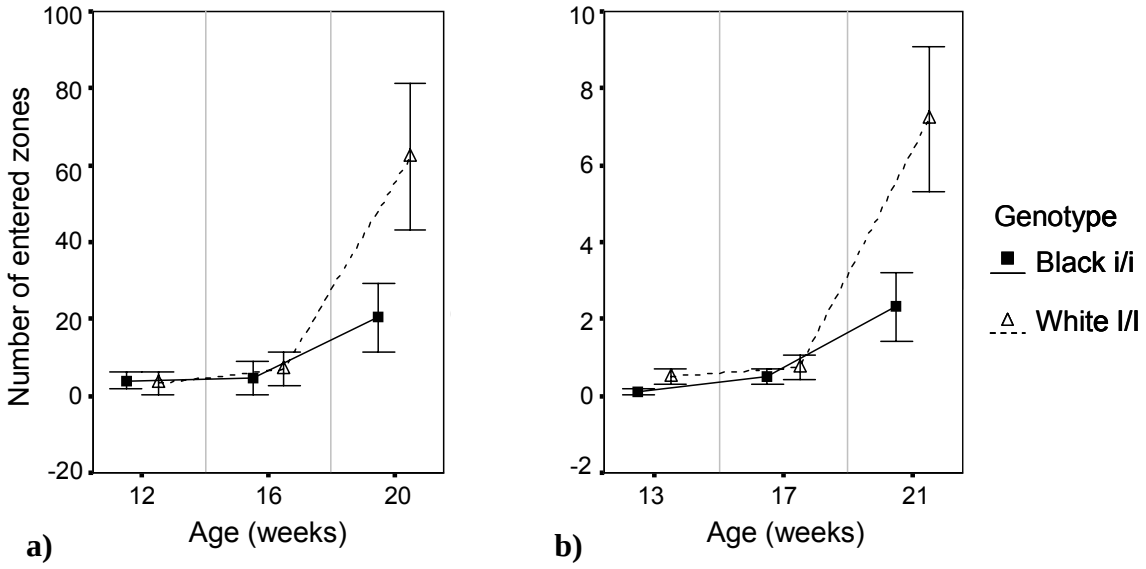

Figure 5. Differences of activity variables between genotypes. The Open-field a) revealed a tendency ($F_{1,30}$ = 2.756, p = 0.11) between subjects and a significant effect within time ($F_{2,60}$ = 3.676, p = 0.03). The fear for human test b) showed significant effects both between subjects ($F_{1,29}$ = 6.424, p = 0.02) and within time ($F_{2,58}$ = 13.772, p = 0.03).

g, $F_{1,25} = 6.09$, $p = 0.02$), but not among females. There was no difference between genotypes in tonic immobility.

Except of the Box observation none of the tests showed any effect by group configuration. In Appendix 2 a table is presented containing a majority of the results from all the analysis.

5 Discussion

I have verified that female black birds of en inter-cross between Red jungle fowl and White leghorn carrying a wt *PMEL17* homozygous genotype (*i/i*) are more feather pecked and show poorer feather conditions than white female birds carrying a mutated *PMEL17* homozygous genotype (*I/I*). I have also given the first evidence that a mutation in the *PMEL17* gene directly or indirectly give rise to behavioural changes. Female black *i/i* individuals was more prominent to vocalize in an open-field arena than their white *I/I* counterparts. In addition white *I/I* individuals showed more activity at around 20 weeks of age.

5.1 The home-box observations

Data from the Box observation and Feather score fall into accuracy with earlier results found by Keeling et al. (2004). This strengthen the findings that female black *i/i* individuals receives more severe pecking then white *I/I*. It also shows that this relationship can be seen in low crowded conditions, which according to Bilcik & Keeling (1999) should contribute as an inhibiting factor on feather pecking.

The absence of any differences between genotypes in the male groups could be explained by the ability of testosterone to inhibit feather pecking (Hughes 1973). To conclude that the preference to peck at black *i/i* individuals does not occur in male groups, a study with higher crowding conditions should be conducted.

Keeling et al. (2004) also showed that feather conditions of black *i/i* birds got worse if the frequency of black *i/i* relative to white *I/I* birds increased in a group. This was not confirmed by the observation of feather pecking or the feather evaluation of the present study.

Even though there were no differences in feather pecking between groups differing in their genotype composition, aggressive pecks were diverged both in female and in male groups. In males feeding and weights also diverged. Aggressive pecks, food competition and individual weights are all features that are related to dominancy, which indicate that *PMEL17* have a sex dependent effect on the social stability of the flock.

5.2 The black preference

Savory and Mann (1999) suggested that black feathers may increase the contrast of light particles on the plumage and therefore make it easier for flock mates to detect and peck at them. In the present study, the higher frequency of black directed pecks in females of younger ages than of older ages is in agreement with this hypothesis; the litter turns darker by time due to heavier loads of defecation. In contrast, an argument for it to be rejected here is that pecking toward whites is not counter adjusted when the litter turns darker. Additionally, the pattern does not appear in males, even though feather pecking is present there too.

The feather preference test in my study suggests that immobile black *i/i* feathers alone are not enough to trigger the preference toward black, because there is no preference seen to any of the two colours. This could indicate that mobility, and thereby behaviour, is needed for the black preference observed in the home-boxes.

Comparing the frequency of feather pecks in the Feather preference test (19 pecks h^{-1}) to the Box observation (80 pecks h^{-1}) shows disturbingly low frequency in the preference test. But most pecking in the boxes were gentle pecking (92 %), which is related to allopreening – a social behaviour by definition (Vestergaard et al 1993) – and should be inhibited in the preference test where no social attributes were present. On the other hand, severe feather pecking that is thought to be a misdirected food search behaviour (Blokhuis 1986; Huber-Eicher and Wechsler 1997) should be reinforced in the test due to the food deprivation of the tested birds. The frequency of severe pecking in the Box observation is much lower (5 pecks h^{-1}), which means that the peck frequency in the preference test is high.

5.3 Behavioural differences

Black *i/i* birds showed through the whole experiment a higher degree of vocal based social reinstatement behaviour under open-field conditions than white *I/I* birds, but most apparently at early age. A consideration of the statement that this difference may originate from a difference in the treatment of the genotypes is important here and is reinforce by data from the observations of black victimization in the home-boxes. But from a logical point of view the reverse relationship should appear; why develop higher social motivation if close contact to pen mates causes pain?

At around 20 weeks of age, at the onset of puberty, white *I/I* started to enter more zones then black *i/i* birds in the Openfield arena and especially in the Fear for human test. The higher significance in the Fear for human test implies that the behaviour seen is related more to anxiety then to

exploration, because higher number of entered zones in this test means that the test bird is running in a back and forth fasion.

Anxious pre-laying behaviours have earlier been observed many times in fowl and are considered to be related to the increased motivation to find a nesting place before the first lay (Freire et al. 1997). There are two possible interpretations of the results: first the white *I/I* individuals are more anxious in general at the first weeks of sexual maturation, or they enter sexual maturation earlier then black *i/i* birds. Hypothetically speaking there are arguments for both this explanations. Freire et al. (1997) found that an individual's position in a pecking order could have influence on how much search behaviour it performs during the pre-laying period. On the other hand, in some social species low ranked individuals enter sexual maturation later because of puberty repression (Ågren et al. 1989, Bercovitch & Strum 1993). Basically the most dominant birds could be the ones that enter sexual maturation first and express less pre-laying anxiety. Results from received aggressive pecks in the present study suggest that both of these arguments should be overthrown because neither of the genotypes dominates the other.

Data from comparative studies between Red jungle fowl and White leghorn reinforce the indication of a genetic difference. Earlier sexual maturation, lower vocal based social reinstatement motivation and high number of entered zones in an open-field-like arena at adult age are characteristics that have been coupled to the leghorn relative to the wild type genotype (Schütz et al 2001 and 2002). Early sexual maturation and hyper-active laying tendencies are desirable traits in the poultry industry and have been selected for a very long time (Groen 2003). Decreased social reinstatement motivation is harder to explain by domestication. One reflection is that in flocks of several thousand individuals, which is the common scenario in the free ranged systems to day, selection for increased social reinstatement should be inhibited because each bird is not more then inches from their flock mates at any time. More social studies are needed, especially when open-field vocalizations as a measure for sociality in hens could be questioned (Schütz et al. 2001 in contradiction to Väisänen & Jensen 2003).

5.4 Criticism to a genetic origin

I have already touched the problem with stating if a behaviour trait is genetically or environmentally derived. There are three possible sources of error here: 1) Black birds are treated differently because they have received more pecks then white; 2) Individuals are treated differently because they

have been living in different configurations; 3) An undetected trait that is derived genetically may cause the observed behavioural differences.

Black individuals have received more feather pecks then white, but in the Feather score only the rump position diverged with low significance between genotypes. This suggests that black *i/i* individuals, even though received more pecks, have not been victimized that extensively to express a much worse feather condition then white *I/I* individuals. This makes it more evident to believe that the treatment of black *i/i* birds is not enough to trigger those large and very specific behavioural differences between the genotypes that have appeared in the individual tests.

Another treatment problem is that some birds have been living in black majority groups while others have been living in white. To avoid this reliability problem all individual tests comparing genotypes were analysed considering the effect of the configuration. No effect could be detected, which should give strong evidence that the birds behaved independent of their genotype group composition.

Further more, findings on zebra fish, rats, guinea-pigs and humans have revealed that retinal dysfunction might be coupled to albinism (O'steen et al. 1995, Biswass & Louyd 1999, Bui & Vingry 1999, Ren et al. 2002). This eye deficiency is explained by an abnormal expression of melanin-specific photoreceptors, which could cause behavioural changes due to poor eye sight. The non-red reflection of the retinal epithelium noticed in white *I/I* birds suggests that it still express eumelanin and should be fully functional. Nevertheless, a poorer eye sight of the white *I/I* birds could explain some of the behavioural differences seen between the genotypes. While a lot of research have been done on poultry vision (reviewed by Prescott et al 2004), a comparative study of strains with different coat colours is missing. That is why a reactivity test of the retina in black and white strains, preferably by an electroretinogram (Bui & Vingrys 1998), must be stressed.

Genomic linkage is also a possible feature that could affect the results of this study. According to the Ensamble Contig web-tool (2005) there are three genes within a 40 Kb distance of *PMEL17*: a precursor to an activin receptor, a GLI1 zinc finger protein and a Hcc-1 protein (Uniprot 2005). At least the products of the two later genes could influence behaviour, due to their general function as transcription factors that often interact with lipophilic hormones like estrogens (Berg 2002, page 880-881). But the possibility of these sequences to be linked with the *PMEL17* gene is weak. The E22C19W28 linkage group are considered to be a highly recombinant

region of the chicken genome[1] and even in the present study it has turned up evidence that support this. The phenotypic reliability problem of the exon 7 primer relative to the exon 10 primer (Appendix 1) have not been detected before (Kerje et al. 2004) and indicates that recombination have occurred inside the gene. This should reject the criticism saying that neighbouring genes are responsible for the behavioural differences.

5.5 New hypothesis and future experiments

Could PMEL17, a protein associated to pigmentation, directly affect behaviour? A functional *PMEL17* gene is needed for the proper formation of amyloid fibrils in premelanosomes and without amyloid stratiations in the mature eumelanosome the eumelanin molecules can not be stabilized and expressed (Huff et al. 2003). The pathway for the synthesis of melanin uses dopamine as a close precursor, which means that melanin is closely related to catecholamines (Hemmer 1990, pp 115-120). Both dopamine and the catecholamines have major influence on behaviour due to their active contribution to the diffuse modulatory system (rewarding system), in stress responses and there general function as neurotransmitters (Bear et al. 2001, chp 15). The *PMEL17* gene are scarcely expressed in adult murine tissues other then melanocyte specific (Kawakami et al. 1994), but some evidence based on human cell cultures suggests that it is expressed in the pigmented brain structure of *Substantia nigra* (Wagner et al. 1997). Further more, resent studies also shows that it is expressed in or close to neural tissue in murine embryos (Baxter et al. 2003). If an accumulation of eumelanin or its metabolites is the result of a non-functional PMEL17 protein, it could alter the biochemical equilibrium in the cell and increase the concentrations of dopamine and/or catecholamines in *I/I* birds. It is likely that this would have a crucial implication on both the adult and the developing brain and the manifestation of behaviour. Bear in mind that this is only hypothetical reasoning, but it would not be difficult to investigate if there are higher amounts of dopamine, catecholamines and/or their metabolites in the brains of white *I/I* then in black *i/i* birds; the methods are already in practise (Beuving & Blokhuis 1997, van Hierden et al. 2002).

Further behavioural studies are needed to investigate how these behavioural differences between the genotypes are coupled and how it effects feather pecking. More social tests must be preformed to examine the development of the behaviours. Aggression in males must be investigated more in detail. Studies should be concentrated on early ages, preferably as

[1] Pers. comm., Leif Andersson, Department of Medical Biochemistry and microbiology, Uppsala University

soon as possible after hatching, to eliminate some of the environmentally induced errors. Sexual maturation is another important period that needs to be better studied. Correlation studies between neonatal and pubertal behaviours could be preformed, as well as physiological measurements to see if there are hormonal differences between genotypes. The tendency seen in the Fear for human test that white *I/I* is tamer then black *i/i* females must be further investigated before any conclusions could be drawn.

5.6 Conclusions

1) This study confirms that black birds homozygous on a functional *PMEL17* gene are more severely pecked then white birds that are homozygous on a non-functional, mutated *PMEL17* gene.
2) The first evidence that the preference to peck at black birds having functional *PMEL17* gene has a behavioural component is put forward.
3) If the behavioural differences between *PMEL17* functional and non-functional individuals are derived from a genetic or an environmental origin is still to be investigated, but there are many arguments that speaks for a genetic.

6 Acknowledgments

This is a part of an ongoing research project called FUNchick (Functional Genomics in chicken) that is funded by Wallenberg Courtium Norta. First of all I want to thank my supervisor professor Per Jensen especially for his ability of being a never expiring source of inspiration. Then Susanne Kerje for her indispensable help and support at the molecular lab, Ann Larsen for her feather scoring and Alf Blomqvist for practical help. Lastly, I put my regard to some of the FUNchick scientists that have giving me some splendid ideas, especially Finn Hallböök and Leif Andersson.

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Appendix

Appendix 1. Results obtained from the genotyping of the parents. I = dominant white allele; i = recissive black allele; W = white leghorn genotype; J = Red Jungle fowl genotype; R = Recombinant genotype; Wi = White phenotype; Bl = Black phenotype. X = Marks birds that were finally used in the experiment.

	Exon 7		Exon 10					
Bird	Allele 1	Allele 2	Allele 1	Allele 2	Genotype	Phenotype	Sex	Choice
520	I	I	I	I	W	Wi	♀	X
552	I	I	I	I	W	Wi	♀	X
555	I	I	I	I	W	Wi	♀	X
566	I	I	I	I	W	Wi	♀	X
589	I	I	I	I	W	missing	♀	X
512	i	i	i	i	J	Bl	♀	X
518	i	i	i	i	J	Bl	♀	X
534	i	i	i	i	J	Bl	♀	X
537	i	i	i	i	J	Bl	♀	X
582	i	i	i	i	J	missing	♀	X
588	i	i	i	i	J	missing	♀	X
595	i	i	i	i	J	missing	♀	X
601	I	i	I	I	R	Wi	♀	X
535	i	i	i	I	R	Wi	♀	
523	i	i	_	_	?	Bl	♀	X
504	I	I	I	I	W	Wi	♂	X
533	I	I	I	I	W	Wi	♂	X
538	I	I	I	I	W	Wi	♂	X
543	I	I	I	I	W	Wi	♂	
553	I	I	I	I	W	Wi	♂	
565	I	I	I	I	W	Wi	♂	X
592	I	I	I	I	W	Wi	♂	X
603	I	I	I	I	W	Wi	♂	X
602	_	_	I	I	W	missing	♂	
563	i	i	i	i	J	Bl	♂	
519	i	i	i	i	J	Bl	♂	X
579	i	i	i	i	J	Bl	♂	X
594	i	i	i	i	J	Bl	♂	X
605	i	i	i	i	J	Bl	♂	X
607	i	i	i	i	J	Bl	♂	X
564	_	_	i	i	J	Bl	♂	
598	I	i	I	I	R	Wi	♂	
600	I	i	I	I	R	Wi	♂	
516	i	i	i	I	R	Wi	♂	
532	I	I	i	I	R	Wi	♂	
578	I	i	I	I	R	missing	♂	
604	I	I	i	I	R	missing	♂	
599	I	i	I	I	R	missing	♂	
587	i	i	_	_	?	Bl	♂	

Appendix 2 - Summary of results

Observation/test	Subject	Gender	Variable	Analysis	df	F/Z	Sign
Box observation	between configuration	♀♂	Gentle feather peck	GLM repeated	1,14	2,077	NS
			Severe feather peck	GLM repeated	1,14	0,426	NS
			Total feather peck	GLM repeated	1,14	1,408	NS
			Aggressive peck	GLM repeated	1,14	17,567	0.001
			Non feather peck	GLM repeated	1,14	0,312	NS
			Feather manipulation	GLM repeated	1,14	0,045	NS
			Object peck	GLM repeated	1,14	0,628	NS
			Ground peck	GLM repeated	1,14	0,078	NS
			Feed	GLM repeated	1,14	5,085	0.04
			Drink	GLM repeated	1,14	0,267	NS
			Preen	GLM repeated	1,14	0,519	NS
			Sleep	GLM repeated	1,14	2,590	NS
			Dustbath	GLM repeated	1,14	0,008	NS
Box observation	between genotype	♀	Gentle feather peck	Wilcoxon		-0.051	NS
			Severe feather peck	Wilcoxon		-2.312	0.02
			Aggressive peck	Wilcoxon		-0.867	NS
Feather preference	between feathers	♀	Latancy to peck - black vs white	Wilcoxon		-1,065	NS
			Numbers of pecks - black vs white	Wilcoxon		-0,078	NS

Fether preference	between gentoype	♀	Latancy to peck at black feathers	GLM repeated	1,28	0,091	NS
			Latancy to peck at white feathers	GLM repeated	1,28	0,373	NS
			Latancy to peck at food	GLM repeated	1,28	0,501	NS
			Numbres of pecks black feathers	GLM repeated	1,28	0,441	NS
			Numbres of pecks white feathers	GLM repeated	1,28	0,005	NS
Novelty test	between genotype	♀	Latancy to two claws at pearch	GLM repeated	1,28	0,316	NS
			Latancy to two claws in litter box	GLM repeated	1,28	0,154	NS
			Latancy to peck at plank	GLM repeated	1,28	1,678	NS
Openfield	between genotype	♀	Latancy to walk	GLM repeated	1	0,948	NS
			Latancy to vocalize	GLM repeated	1	4,189	0,05
			Latancy to peck	GLM repeated	1	2,315	NS
			Numbre of vocalizations	GLM repeated	1	4,600	0,04
			Numbre of pecks	GLM repeated	1	0,615	NS
			Numbre of defications	GLM repeated	1	1,484	NS
			Entered zones	GLM repeated	1	3,856	NS
			Visited zones	GLM repeated	1	2,523	NS
Fear for human	between genotype	♀	Latancy to first step	GLM repeated	1,27	1,532	NS
			Latancy to enter close zone	GLM repeated	1,27	1,585	NS
			Duration close zone	GLM repeated	1,27	0,927	NS
			Latancy to first peck to palm	GLM repeated	1,27	3,231	NS
			Numbre of pecks to palm	GLM repeated	1,27	3,135	NS
			Numbre of entered zones	GLM repeated	1,27	6,531	0,02

Tonic immobility	between genotype	♀♂	Numbre of trails	GLM multi	1	0,509	NS
			Latancy to move head	GLM multi	1	1,369	NS
			Latancy to standing position	GLM multi	1	0,020	NS
Feather score	between genotype	♀	Damage to rump	Mann-Whitney		-2.023	0.04
			Damage to tail	Mann-Whitney		-0.346	NS
			Damage to belly	Mann-Whitney		-0.912	NS
			Total Damage	Mann-Whitney		-1.419	NS
		♂	Damage to rump	Mann-Whitney		0	NS
			Damage to tail	Mann-Whitney		-0.267	NS
			Damage to belly	Mann-Whitney		-0.855	NS
			Total Damage	Mann-Whitney		-0.101	NS
Weight	between genotype	♀	Weight week 10	GLM multi	1,31	2,363	NS
			Weight week 24	GLM multi	1,31	4,044	NS
		♂	Weight week 10	GLM multi	1,25	5.550	0.02
			Weight week 24	GLM multi	1,25	6.087	0.02