A mutation in the TSHR gene – how does it affect social and fear related behaviours in chickens?

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**Sammanfattning/Abstract:**
Thyroid hormones are well known important to be in development and growth in birds and that signaling of thyrotropin (TSH) regulates the photo induced seasonal reproduction. A mutation at the thyroid stimulating hormone receptor (TSHR) gene in domestic breeds of chicken could be involved in the release of the photoperiodic regulation. Furthermore, TSH can affect a wide range of domestication related phenotypes, such as behaviour, growth rate and pigmentation. The aim of this study was to investigate the behaviours expressed in the different genotypes on the TSHR gene in chickens. Four standard tests were conducted, aerial predator, fear of human, social dominance and tonic immobility. An advanced intercross line of chickens between red junglefowl and White leghorn was used. Male domestic type chickens explored more, showed more less fear behaviours and showed least fear behaviour in the fear of human test. Increased activity and flight response has been interpreted as a lower fear response, which is in line with this study. The wild type chickens showed more social dominance than domestic type chickens which are in line with previous results. In tonic immobility there was a difference between the wild type male and heterozygous male chickens in latency until first head movement. The conclusion of this study is that there is a difference between the wild type and domestic type chickens. This indicates that the TSHR gene is involved in behavioural changes during domestication, but whether it is due to passive or active selection is the question.

**Nyckelord/Keyword:**
Domestication, Chicken, Fear, Dominance, Thyrotropin, Thyroid stimulating hormone receptor, TSHR
1 Abstract

Thyroid hormones are well known important to be in development and growth in birds and that signaling of thyrotropin (TSH) regulates the photo induced seasonal reproduction. A mutation at the thyroid stimulating hormone receptor (TSHR) gene in domestic breeds of chicken could be involved in the release of the photoperiodic regulation. Furthermore, TSH can affect a wide range of domestication related phenotypes, such as behaviour, growth rate and pigmentation. The aim of this study was to investigate the behaviours expressed in the different genotypes on the TSHR gene in chickens. Four standard tests were conducted, aerial predator, fear of human, social dominance and tonic immobility. An advanced intercross line of chickens between red junglefowl and White leghorn was used. Male domestic type chickens explored more, showed more less fear behaviours and showed least fear behaviours in the fear of human test. Increased activity and flight response has been interpreted as a lower fear response, which is in line with this study. The wild type chickens showed more social dominance than domestic type chickens which are in line with previous results. In tonic immobility there was a difference between the wild type male and heterozygous male chickens in latency until first head movement. The conclusion of this study is that there is a difference between the wild type and domestic type chickens. This indicates that the TSHR gene is involved in behavioural changes during domestication, but whether it is due to passive or active selection is the question.

2 Introduction

2.1 Domestication

Domestication is the process when animals adapt to a life with humans (Price 2002). During the process of domestication there were genetically changes in a population due to a selection by human (Schütz and Jensen 2001). The first target of selection, intentionally or unconsciously, was probably reactivity against humans and other potential threats. The animals with a lower fear response would cope with captivity better and thereby had a higher fitness during early domestication (Campler et al. 2009). Throughout domestication, increased resources provided by human have been utilized by domestic animals (predator and weather protection, food provision). This has altered the selection pressures and might have caused modified behaviours (Schütz and Jensen 2001). It is generally accepted that it is only the frequency of behaviours, by threshold changes, which have
changed during domestication and artificial selection, rather than eliminating or adding behaviours (Price 1997). Price (1997) states that there are three processes that are central to domestication. The first is selection by humans, so called artificial selection, with behaviours preferred by humans. Secondly, a natural selection occurs in captivity which leads to adaptation. Thirdly, a relaxation of natural selection occurs, certain factors such as reproduction and predation decrease. With comparative studies of domestic species and their wild ancestors, evidence of typical domestication changes have been found, summarized as “the domestic phenotype” (Jensen 2006, Jensen 2010), such as behaviour, internal and external morphology and physiology (Rubin et al. 2010). Examples of behavioural changes are reduced fear, increased sociability and reduced antipredator response. In morphological changes there are altered fur and plumage colours, body size and growth pattern and adapted relative sizes of internal organs. For physiological changes there are endocrine responses and reproductive cycles and earlier sexual maturity (Jensen 2006).

All chicken breeds have been domesticated during a long time, for at least 8000 years, and the ancestor is the Red junglefowl, RJF, which live wild in Southeast Asia. During the last 100 years the domesticated chicken diverged into broilers and layers respectively, for meat and egg production (Havenstein et al. 2003). There are numerous differences between the ancestor, Red junglefowl, RJF, and the domesticated chicken, White leghorn, WL. The WL has a large increase in growth, body size, egg production (in both egg mass and egg number), a decrease in fear-related and social behaviours and plumage color alterations when compared to the RJF (Wright et al. 2010). There are mainly four behaviour aspects that differ between the RJF and WL: first WL was in general less active, with a reduced foraging and exploratory behaviour. Secondly, their social behaviour were less intense, with a reduced frequency of social interactions. Thirdly, the antipredator behaviours was modified and less intense. Fourthly, they showed a modified foraging strategy, the WL were less inclined to explore unknown food sources (Jensen and Andersson 2005).

The environment of the chickens can be controlled from the point of egg laying, which makes them excellent as behaviour genetic models. During the last 50 years a study has been going on with silver foxes (Vulpes vulpes) in Russia in which the individuals showing an aggressive-avoidance response towards humans were selected (Trut et al. 2009). After only a few generations the selected foxes showed more dog-like
behaviours, such as eagerly seeking contact with humans, whining and dog-like tail wagging. The research team only selected the silver fox for tameability but they got correlated changes in features of behaviour, morphology and physiology.

To better understand the domestication the genetic design has to be solved (Wright et al. 2010). To do this a mapping of quantitative loci with molecular markers is performed, referred to as quantitative trait locus analysis (QTL-analysis). The definition of a QTL is a locus that contains alleles that in different ways affect the expression of a phenotypic trait that is continuously distributed (Jensen 2006). Several different domesticated species have a number of similarities in common in the domesticated genetic design, when it comes to QTL distribution, location and effect size (Wright et al. 2010). When the frequency of a new beneficial mutation increases in a population due to natural selection, the neighboring regions genetic variation will be affected. The variability level will be reduced, level of linkage disequilibrium will be increased and the allele frequency pattern will be skewed. The elimination of a standing variation in a region that is linked to a beneficial mutation that has recently been fixed is known as a “selective sweep” (Nielsen et al. 2005). Due to the short evolutionary history of animal domestication the genomic footprints of major selective sweeps is believed to largely remain (Andersson and Georges 2004). To find the selective sweep for this mutation Rubin et al. (2010) searched the genome for regions with high degrees of fixation. The first domestic animal to have its genome sequenced was the chicken. Early studies show that bird genomes are roughly one-third of mammalian genomes (Eltanany and Distl 2010).

2.2 TSHR

The thyroid stimulating hormone receptor (TSHR) gene on chromosome 5 (Tixier-Boichard et al. 2011) has mutated in domestic breeds and is homozygote at the TSHR locus (Yoshimura et al. 2003). The strongest selective sweep in all domesticated chicken occurred at the TSHR gene, which indicates that it is involved in the release of seasonal reproduction in domestic chicken. This can also affect development, behaviour and growth (Rubin et al. 2010). A missense mutation in the TSHR gene causing a change in glycine to arginine is the most obvious candidate mutation, since glycine is conserved at this position in all known vertebrate TSHR sequences (Rubin et al. 2010).

Thyrotropin-releasing hormone (TRH), which is stimulatory to the anterior pituitary production and release of thyrotropin, thyroid-stimulating
hormone (TSH), is present in the hypothalamus in both mammals and birds (McNabb 2007). The thyroid gland produces the thyroid hormones and is located with one lobe on either side of the trachea (Grommen et al. 2011). Thyroid hormones controls some processes, e.g. embryonic differentiation and maturation, development and function of the central nervous system, embryonic postnatal growth, hair and skin pigment production, stress response, behaviour correlation and daily and seasonal thyroid hormone variation (Crockford 2006).

One thing that all domesticated species have in common is the loss of seasonal reproduction, which likely has been favoured during domestication. All domesticated species carry a mutant allele on the TSHR locus, which could be the domestication locus. Taking into account that most of the wild animals are homozygous for this mutation might indicate that it plays a central role, perhaps already in early stages of domestication (Rubin et al. 2010). In birds and mammals the photoperiodic control of reproduction is regulated by the THSR gene signaling between the pars tuberalis, in the pituitary gland, and ependymal cells in the hypothalamus, which is well established now (Rubin et al. 2010). It is known that TSHR is expressed in several extrathyroidal tissues, e.g. bone, kidney, ovary, testis and cells of the immune system. However, TSHR regulatory role in these tissues is not yet understood (Haas et al. 2011).

The core feature of the “first day release model” of avian reproductive photoperiodism is when the plasma luteinizing hormone (LH) increases at the end of the first long day. The components that are needed for birds for photoperiodic signal transduction are located in the mediobasal hypothalamus (MBH). Under shortday conditions it is the type 2 deiodinase (DIO2), which translates the prohormone thyroxine (T4) to bioactive triiodothyronine (T3), at a low level. At the end of the first long day a rapid switch in DIO2 expression occurs which results in a local increase in T3 concentration (Nakao et al. 2008). Ono et al. (2009) showed that long day induced TSH expression in the pars tuberalis triggers DIO2 expression in MBH through the TSHR-cAMP signals pathway, which leads to secretion of LH from the pituitary gland. The earliest event in the photoinduced process is the long day induction of expression of thyrotropin beta subunit (TSHB) in the pars tuberalis.

The results by Nakao et al. (2008) show a comprehensive analysis of changes in the gene expression of the hypothalamus, which are thought to be involved in the long-day regulation of the reproductive photoperiodic response. They also point out the pars tuberalis TSH as a key factor that
controls the photoperiodic processes. Considering the lack of strict regulation of seasonal reproduction, which is found in natural populations, the TSHR sweep could be related to a classic feature of all domestic animals (Rubin et al. 2010).

When performing genetic studies of phenotypic evolution domestic animals are excellent models. They have developed genetic adaptations to the new domestic environment, the farm, and have been in focus of a very strong human-driven selection (Rubin et al. 2010). Since the environment of birds can be controlled, direct from egg laying, there is a benefit with using birds as a genetic model (Jensen 2006). In the present study an F9-generation of an advanced intercross line between White leghorn and red junglefowl was used. Which being homozygous for alternative TSHR alleles, creates what is referred to as Locus Controlled Advanced Intercross Line (LAIL). With this method the effect of the genotype on one locus could be studied. Advanced intercross line is based on the principle that continued intercrossing of a population will reduce the genetic variation and strengthen the existing alleles (Darvasi and Soller 1995).

The loss of fear related behaviours have been favoured during domestication and are common for all domestic species, as like seasonal reproduction. It could be that the loss of fear is due to a mutation in the TSHR gene, it is also involved in the loss of seasonal reproduction as suggested by the finding of the TSHR sweep.

2.3 Aim and hypothesis
The aim of this project is to see if the TSHR gene is involved in the domestication effect of fear related and social behaviours. My hypothesis is that the wild type chickens will show more fear related behaviours and be more social than domestic type chickens. Since the domesticated chicken show less fear and social behaviours than their ancestor, the red junglefowl, and are homozygote at the mutation of the TSHR gene I hypothesize that fear and social behaviours are controlled by this locus.

3 Material & methods

3.1 Animals
Two batches with a total of 164 chickens from an advanced intercross line between red junglefowl and White Leghorn were used. Originally one red
junglefowl male and four White leghorn females was paired to generate 36 F₁ females and 4 males. These were intercrossed to generate over 1000 F₂ and thereafter each generation was by random intercrossed to produce at least 100 birds in each following advanced generation. The F₈ generation was genotyped and the birds that were heterozygous for the TSHR-mutation were chosen and crossed. The F₉ generation was genotyped and either homozygous for the wild type allele (w/w), 25%, or the domestic type allele (d/d), 25%, or heterozygote at the TSHR allele (w/d), 50%, see Table 1.

<table>
<thead>
<tr>
<th>m</th>
<th>f</th>
<th>w</th>
<th>d</th>
</tr>
</thead>
<tbody>
<tr>
<td>w</td>
<td>w/w</td>
<td>w/d</td>
<td></td>
</tr>
<tr>
<td>d</td>
<td>w/d</td>
<td>d/d</td>
<td></td>
</tr>
</tbody>
</table>

Table 1. A schematic figure over the alleles at the TSHR locus.

3.2 Housing

The chickens were hatched and kept at “Kruijt” hatchery at Linköpings University until six weeks of age and then moved out to “Wood-Gush” research chicken house, about 15 km outside of Linköping. They were housed together in nearby, identical pens (L x W x H: 2.5 m x 3.1 m x 3.0 m), with full visual and auditory contact between the pens. The pens contained food and water ad libitum, perches, nest boxes and cutter shavings on the floor. The chickens were kept on a 12 hour light and dark cycle and the temperature in the room was 20°C.

3.3 Behaviour tests

Each behavioural test was conducted blindly, not knowing which bird had which gene, except for the social dominance test when the different genotypes were tested against each other. All tests, except for tonic immobility that was conducted in total darkness, were recorded with a video camera. The aerial predator test was conducted during the chicken’s 11ᵗʰ and 12ᵗʰ week of life, tonic immobility during their 13ᵗʰ week, fear of human during their 26ᵗʰ week and social dominance during their 27ᵗʰ week of life.

3.3.1 Aerial predator test

In the aerial predator test the chicken’s response to a hawk silhouette was recorded, the behaviours that occurred before and after the release of the silhouette were measured, as well as their reaction to the silhouette. A test arena, made by plywood, was used (L x W x H; 1500 mm x 500 mm x 500 mm) and the chickens had two minutes to habituate to it. A see-through
mesh was used as a roof to make sure the chickens couldn’t escape. The test arena was divided into three different zones, A, B and C. The silhouette was hidden behind a plastic curtain in each end of the room, before and after crossing above the arena. Every test lasted for ten minutes and nine behaviours were recorded, see Table 2. 1/0 sampling was used with 10 seconds interval for every behaviors except the attempt to escape which was recorded with continuous sampling. After five minutes the silhouette was released, flying from zone A to C and about 170 cm above the chickens for about 1.5 seconds, see Figure 1. A five-graded scale was used to measure the chickens’ response to the silhouette: 0 = no response, 1 = lift head once, 2 = lift head once and look around for more than three seconds, 3 = attempts to run or fly away, 4 = intense reaction, jump high. After the release of the silhouette the chickens were observed for additionally five minutes. The mean difference in percentage was calculated. In every behaviour I took the number of times observed after the predator minus the number of times observed before the predator to get the differentiation. Exploratory behaviour, ground pecking, preening and walk alert were pooled together as behaviours with low fear. Freezing, lying and standing were pooled together as behaviours with high fear. The mean difference in percentage was calculated.

![Figure 1. A figure over the aerial predator test.](image)
<table>
<thead>
<tr>
<th>Behaviour</th>
<th>Aerial predator test</th>
<th>Fear of human test</th>
<th>Social dominance test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aggressive peck</td>
<td>Gives peck</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Attack</td>
<td>Running attack with beak</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Attempt to escape</td>
<td>Jumping with wing movements</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Chase</td>
<td>Follows another bird in an aggressive context</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Explore</td>
<td>Walk or stand with eyes on objects</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Feed</td>
<td>Feed from hand</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Flight</td>
<td>Tries to fly from another bird</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Freeze</td>
<td>Stiff body posture</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Ground peck</td>
<td>Pecks at feed or ground</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Hide</td>
<td>Put head in corner trying to hide it from another bird</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Ly</td>
<td>Lying on ground</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Preen</td>
<td>Trimming of plumage with beak</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Raised hackle threat</td>
<td>Threat with raised hackle, neck feathers</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Stand</td>
<td>Stand with eyes open</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Stand and walk on human</td>
<td>Standing and/or walking on human hand</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Threat</td>
<td>Stiff body posture towards another bird standing within 25 cm from each other</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Threat with wing flap</td>
<td>Threats with wing flapping</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Vocalize</td>
<td></td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Walk alert</td>
<td>Movement of legs in normal speed</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>
3.3.2 Fear of human test

The fear of human test investigated the chickens’ fear response to a human. Six birds was placed in a plastic box and kept there for 30-60 minutes being deprived from food and water. Every chicken was tested individually. A test arena (L x W x H; 1500 mm x 500 mm x 500 mm) was used, which was divided into three equal zones, start zone, middle zone and human zone, see Figure 2. The chickens were placed in the start zone which had a solid sliding door that opened as soon as the test started. Outside the human zone was a human placed facing the test arena, with a hand containing standard chicken food in the middle of the human zone. The roof of the test arena and the open side of the human zone were covered with a see-through mesh. Each chicken had two minutes of habituation in the start zone before the test started, when the sliding door was opened. Five minutes of behavioural recording was conducted using 1/0 sampling with 10 seconds interval. The latency until the chicken left the start box, the latency until the chicken started to feed and behaviours expressed during the test were measured and the behaviours are shown in table 2. A mean percentage of every behaviour was calculated. Escape, exploratory behaviour, feeding, ground pecking, preening, stand and walk on human and walk alert were pooled behaviours with low fear. Freezing, lying and standing were pooled together as behaviours with high fear. In the two pooled groups a mean percentage was calculated.

3.3.3 Social dominance test

The social dominance test was conducted to assess which one of two individuals of the same sex that accessed a limited resource, meal worms, first to be the most dominant one, combined with observation of antagonistic behaviours. The different genotypes of the TSHR gene were tested against each other; every individual was tested against each
other except the ones with the same genotype. The number of chickens that were tested was decided by the genotype group with least numbers of individuals. The chickens were selected randomly in the two groups with more individuals than the limited group. During the week prior to the test the chickens had access to meal worms in feeding troughs covered with plexiglas with holes in their home pen for habituation. Four test boxes (L x W x H; 1000 mm x 1000 mm x 1700 mm) were constructed in a testing room, with three solid walls and the front with a see through mesh so the chickens could be observed. The chickens had 15 minutes to habituate in the test box, separated by a solid wall so they couldn’t see each other. During the test they had access to the meal worms, through a hole in the plexiglass for five minutes and the winner, the one to eat first, combined with antagonistic behaviours determined the dominant individual, see table 2. The test was conducted a second time with 30 minutes of separation and no access to the meal worms after the first session. The behaviours threat, threat with wingflap, walzing agonistic, raised hackle threat, aggressive peck, attack, chase and fight were pooled together to one variable called aggression. Behaviours escape and hide were pooled into fear.

3.3.4 Tonic immobility test

The tonic immobility test is a well-established fear test in chickens (Forkman et al. 2007). In the tonic immobility (TI) test the chicken were placed upside down in a cradle, seen in Figure 4. The latency until the chicken’s first head movement and the latency until the chicken righted itself was observed. Ten chickens were collected and put in a plastic box that was placed right outside their home pen. The light was out in the stable and the chickens had 20 minutes of habituation in the plastic box before the test started. The test was performed outside the home pen so the chickens were in a familiar environment. Each bird was placed on its back in a wooden cradle with a light pressure on the breast for ten seconds and then a decreasing pressure for five seconds, until the hand was completely removed. If the chicken righted itself during the first ten seconds of the test it was not considered to be in TI. Each chicken got three chances to enter TI and got scored by thereafter. If the chicken never entered TI it got a five in attempts to be able.
to differentiate the birds that entered TI after three attempts. The observer stood absolutely still during the test with a headlamp directed away from the chicken until the chicken righted itself or ten minutes had passed. A mean time was calculated and mean number of attempts.

3.4 Data analysis and statistics

Mean values between the genotypes, sexes and families were analyzed by ANOVA. Mean values between genotypes within each sex were analyzed by multivariate General Linear Model Post Hoc. Where a family effect was found the genotypic effect was analyzed within every family by ANOVA. All variations are given as standard errors of the mean.

A principal component analysis (PCA) was used to look at the factor effect and ten variables with significant effects on genotype and sex were selected from the tests aerial predator, fear of human and tonic immobility. In the aerial predator test were predator reaction, freezing, walk alert and activity in zone B. In tonic immobility variables latency until first head movement and difference between first head movement until righted were used and in fear of human explore, standing, vocalizing and latency until eat from hand. For each individual the principal component scores were calculated and analyzed with ANOVA, with the same model as above. The statistical program that was used was IBM SPSS Statistics 19.

4 Results

4.1 Aerial predator test

There were a significant difference between the different genotypes in freezing where the wild type showed more freezing behaviour after the predator than both the heterozygous and domestic type (F(2,102)=3.04; P=0.05). There was a trend between the different genotypes within sexes in escape attempts. Heterozygous females showed more than both wild type and domestic type (F(2,118)=2.63; P=0.08) and heterozygous males showed less than both wild type and domestic type (F(2,118)=2.63; P=0.08). A significant difference was found between the different genotypes within the different families in freezing behaviour (F(12,102)=2.19; P=0.02) and vocalizing (F(12,102)=2.59; P=0.005). When pooling the genotypes the males were lying more before the predator than after compared to females (F(1,102)=7.36; P<0.01). Pooled females walked more before the predator than after compared to pooled males (F(1,118)=15.89; P<0.001). Pooled males tried to escape more after the predator than pooled females (F(1,118)=4.6; P=0.03). Pooled males spent more times in zone A (F(1,118)=9.58; P=0.002)
and zone B ($F_{(1, 118)}=9.86; P=0.002$) than pooled females after the predator. In the group with less fear behaviours there were a significant difference between the pooled sexes, females showed more of the pooled behaviours before the predator ($F_{(1, 118)}=7.03; P<0.01$) than the males. Also in the group with stronger fear behaviours there were a sex difference, pooled females showed more of the behaviours after the predator ($F_{(1, 118)}=7.75; P<0.01$) than the pooled males. In the predator intensity there was a trend between the pooled sexes, males showed a higher intensity than females ($F_{(1, 118)}=3.34; P=0.07$). There were also a trend in the activity in zone C, pooled males spent more time in it before the predator than pooled females ($F_{(1, 118)}=3.26; P=0.07$).

![Figure 5. Mean difference percent in vocalizing between genotypes within families in aerial predator test. ** p<0.01](image-url)
Figure 6. Mean difference percent in freezing between genotypes within families in aerial predator test. * p<0.05

Figure 7. Mean difference percent in lying and walk alert between sexes in aerial predator test. Males lied more before the predator and walked more after the predator than females. ** p<0.01 *** p<0.001
Figure 8. Mean number of times escape in aerial predator test. Males tried to escape more times than females. * p<0.05

Figure 9. Mean difference in total number of times in each zone in aerial predator test. Males spent significantly more times in zone A and B than females. There was a trend for males to spend more time in zone C than females. ** p<0.01
**Figure 10. Mean difference percent between sexes in the group with low fear and high fear behaviours. Females performed more low fear behaviours before the predators than males. Males performed more high fear behaviours after the predators than females. ** p=0.01

### 4.2 Fear of human

A significant difference was found between the different genotypes within sexes in exploratory behaviour, domestic type males showed more exploratory behavior than both wild type males (F(2, 85)=3.7; P=0.01) and heterozygous type males (F(2, 85)=3.7; P=0.02). A significant difference was found between the genotypes within families in walk and stand on human (F(12, 69)=2.07; P=0.03). In the group with pooled low fear response behaviours domestic type males showed more of them than both wild type males (F(2, 85)=4.08; P=0.05) and heterozygous type (F(2, 85)=14.04; P=0.01). In the group with pooled stronger fear behaviours the domestic type males showed less than both heterozygous males (F(2, 85)=5.28; P=0.01) and wild type males (F(2, 85)=5.28; P=0.02). There was a significant difference between the pooled sexes in ground pecking, females showed more than males (F(1, 85)=9.83; P=0.002). In preening pooled females showed significantly more than pooled males (F(1, 85)=3.88; P=0.05). In vocalization pooled females performed it significantly more than pooled males (F(1, 85)=32.11; P<0.001). Pooled males performed standing significantly more than pooled females (F(1, 85)=18.68; P<0.001). Walk alert was performed significantly more by pooled females than pooled males (F(1, 85)=9.47; P=0.003). Freezing was performed significantly more by pooled males than pooled females (F(1, 85)=7.5; P=0.008). Pooled females fed significantly
more than pooled males ($F_{(1, 85)}=9.63; P=0.003$). Pooled males spent significantly more time in start zone than pooled females ($F_{(1, 85)}=16.51; P<0.001$) whilst pooled females spent significantly more time in human zone than pooled males ($F_{(1, 85)}=26.75; P<0.001$). Pooled females left both start zone ($F_{(1, 81)}=20.13; P<0.001$) and middle zone ($F_{(1, 85)}=49.66; P<0.001$) faster than pooled males. Pooled females ate from human hand significantly faster than pooled males ($F_{(1, 85)}=38.68; P<0.001$). There were a significant difference between the sexes in both the group with less fear behaviours and the group with stronger fear behaviours. Pooled females showed more of the pooled less fear behaviours than pooled males ($F_{(1, 85)}=53.82; P<0.001$) and males showed more of the pooled stronger fear behaviours than pooled females ($F_{(1, 85)}=35.82; P<0.001$).

![Figure 11. Mean percent of exploratory behaviour in sexes within genotypes. Domestic males showed more exploratory behaviour than both wild type and heterozygote males. * $p<0.05$ **$p<0.01$](image-url)
Figure 12. Mean percentage of pooled behaviours with less fear. Domestic type males showed more than both wild type and heterozygote males. * $p<0.05$ ** $p<0.01$

Figure 13. Mean percentage of pooled behaviours with more fear. Domestic type males showed less than both wild type and heterozygote males. * $p<0.05$ ** $p<0.01$
Figure 14. Mean percent of walk and stand on human in fear of human test between genotypes within families. * p<0.05

Figure 15. Mean percent in ground peck, preen, vocalization, stand, walk alert, freeze and feed. Females performed more ground peck, preen, vocalization, walk alert and feed than males. Males performed stand and freeze more than females. * p<0.05 ** p<0.01 *** p<0.001
Wild type chickens ate from the meal worms significantly more times than the domestic type ($F_{(2, 39)}=5.74; P=0.002$). In aggression behaviours there was a significant difference where the wild type chickens showed more than the domestic type ($F_{(2, 39)}=4.28; P=0.01$). The domestic type chickens showed significantly more fear behaviours than the wild type ($F_{(2, 39)}=4.31; P=0.01$). There was a trend where the domestic type showed more fear behaviours than the heterozygous type ($F_{(2, 39)}=4.31; P=0.08$).

4.3 Social dominance

Wild type chickens ate from the meal worms significantly more times than the domestic type ($F_{(2, 39)}=5.74; P=0.002$). In aggression behaviours there was a significant difference where the wild type chickens showed more than the domestic type ($F_{(2, 39)}=4.28; P=0.01$). The domestic type chickens showed significantly more fear behaviours than the wild type ($F_{(2, 39)}=4.31; P=0.01$). There was a trend where the domestic type showed more fear behaviours than the heterozygous type ($F_{(2, 39)}=4.31; P=0.08$).
Figure 17. Mean number of times the chickens ate from the meal worms, performed aggressive behaviours and fear behaviours. Wild type chicken ate more from the mealworms and performed more aggressive behaviours, whilst domestic type chicken performed more fear behaviours. ** p<0.01

4.4 Tonic immobility

A significant difference was shown between the wild type males and the heterozygous males in mean time seconds until first head movement, where the wild type males took longer time ($F_{(2, 112)}=2.47; P=0.04$). Pooled males tended to lie longer until righted than pooled females ($F_{(1, 112)}=3.27; P=0.07$). In the mean time between first head movement until righted pooled males stayed longer than pooled females ($F_{(1, 112)}=4.93; P=0.03$).
Figure 18. Mean seconds until first head movement between genotype within sex in tonic immobility. Wild type males took longer time until first head movement than heterozygous males. *p<0.05

Figure 19. Mean seconds until righted and difference from first head movement until righted in tonic immobility. Males stayed longer in the cradle from the first head movement until righted than females. A trend was seen if females to stay shorter in TI until righted. *p<0.05
4.5 Principal component analysis

Four factors, with eigenvalues greater than one, were extracted from the Principal Component Analysis. Based on the scree plot combined with the eigenvalues and the explained variance, four factors were chosen for further analysis. Together, these four factors explained 67.88% of the variance of the data set. The ten variables used in the factor loadings are shown in Table 3. The first factor, explaining 27.24% of the variance, contained active related behaviours from both the aerial predator test and fear of human. The pooled male chickens had a significantly higher score than pooled females ($F_{(1, 85)}=18.91; P<0.001$). The second factor, which explained 18.06% of the variance, had the highest loading value for explorative behaviours, such as explore and vocalization. Pooled females had a significantly higher score than pooled males ($F_{(1, 85)}=31.67; P<0.001$). The third factor explained 12.45% of the variance and contained high loadings of TI behaviours. The fourth factor explained 10.13% of the variance and contained predator behaviours. A significant difference was found between the sexes, where pooled males got a higher score than the pooled females ($F_{(1, 85)}=6.22; P=0.02$).

![Scree Plot](image)

*Figure 20. Scree plot showing the variance in the data represented by each factor. The first four factors were chosen since they had an eigenvalue greater than one, they explained 67.878% of the variance.*
Table 3. Factor loadings on the four principal components and the variance explained by each component. AP= aerial predator, FH= fear of human, TI= tonic immobility.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Factor 1 Active behaviours</th>
<th>Factor 2 Explorative behaviours</th>
<th>Factor 3 TI behaviours</th>
<th>Factor 4 Predator behaviours</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP Difference activity zone B</td>
<td>0.718</td>
<td>0.480</td>
<td>-0.225</td>
<td>0.060</td>
</tr>
<tr>
<td>AP Difference freezing %</td>
<td>-0.660</td>
<td>-0.344</td>
<td>-0.183</td>
<td>0.241</td>
</tr>
<tr>
<td>AP Difference walk alert %</td>
<td>0.776</td>
<td>0.415</td>
<td>-0.196</td>
<td>0.118</td>
</tr>
<tr>
<td>AP Intensity predator</td>
<td>0.464</td>
<td>0.128</td>
<td>0.198</td>
<td>-0.575</td>
</tr>
<tr>
<td>FH Explore</td>
<td>-0.274</td>
<td>0.706</td>
<td>-0.089</td>
<td>0.371</td>
</tr>
<tr>
<td>FH Latency to eat from hand</td>
<td>0.460</td>
<td>-0.304</td>
<td>-0.262</td>
<td>0.542</td>
</tr>
<tr>
<td>FH Standing</td>
<td>0.516</td>
<td>-0.593</td>
<td>0.025</td>
<td>0.068</td>
</tr>
<tr>
<td>FH Vocalization</td>
<td>-0.582</td>
<td>0.534</td>
<td>-0.140</td>
<td>-0.045</td>
</tr>
<tr>
<td>TI Difference head movement until righted</td>
<td>0.236</td>
<td>0.054</td>
<td>0.691</td>
<td>0.390</td>
</tr>
<tr>
<td>TI First head movement</td>
<td>-0.082</td>
<td>0.192</td>
<td>0.713</td>
<td>0.128</td>
</tr>
</tbody>
</table>

5 Discussion

The results from this study show that there are behavioural differences between the ancestor, RJF, and the domestic breed, WL, which have changed during the domestication. Eklund and Jensen (2011) saw that the fundamental aspects of animals’ behaviour, such as form and frequency, has not changed during the domestication. The loss of social interactions in chickens could be due to a relaxation of protective behaviour during domestication. In a study conducted by Eklund and Jensen (2011) the White leghorns had less synchronized perching than the red jungle fowl and significantly longer distances during perching. I could see that the domestic type chickens were less social towards other individuals in the social dominance test.

Forkman et al. (2007) pointed out that fear related behaviours like freezing and immobilization have been interpreted as stronger responses of fear than increased activity and flight response. The results from the fear of human test supports the statement from Forkman et al. (2007), when pooling the behaviours with high or low fear, the wild type chickens showed more of the behaviours with high fear than domestic type. It has been observed that RJF walk alert and vocalize significantly more after the predator than the WL, whereas WL stand alert more (Schütz et al. 2001). This was not observed in this study, which could be due to different measurements of the behaviours.

To conclude the results from the social dominance test in this study the wild type chickens are more dominant than the domestic type chicken.
Domestic chickens are less inclined to explore unknown food sources (Jensen 2006). Even though the mealworms were presented to the chickens in their home pen during one week prior to the test, for habituation, the domestic chickens were less inclined to explore the new food when they came into the new environment. Schütz et al. (2001) observed that RJF spent more time feeding from a novel food site with a mirror whilst WL spent more time feeding the easily obtained familiar food with a mirror. This is in line with my results in the social dominance test. As a personal observation I could, however, see that the domestic type chickens ate more from the mealworms after some sessions in the arena. They were only habituated to the arena before the sessions and not to the mealworms in the arena. The results might have been different if this had been done.

Erhard et al. (1999) states that the variation of tonic immobility in birds reflects the different levels of fear, where long durations of immobility is a high level of fear. This would be seen by that the wild type chickens in this study would stay longer in TI, as they represent the red junglefowl, but, however, I did not get any significant results. Schütz et al. (2001) observed that it is easier to induce TI in White leghorn than in red junglefowl, whereas in this study there was no significant result in amount of attempts. This could be due to different environmental setup and how the handler is with the chickens. Heiblum et al. (1998) saw that chickens can be induced in tonic immobility as early as at the first day of life and that the duration of TI decreased by habituation. A stressor, e.g. isolation or physical stress, prior to the TI could increase the response (Heiblum et al. 1998). The tonic immobility test conducted in this study was performed outside the home pen to minimize the stress, if it had been conducted in another room and or with a stressor prior to the test the results might have been stronger.

There are both physiological and behavioural reactions that characterize fear-related reactions, which prepare the animal to deal with the situation (Forkman et al. 2007). Cockrem (2007) defines fear as the state or situation in which an animal interprets a stimulus to be a threat, and generating a behavioural and/or physiological response. Even though domestic animals are protected against predators and rarely meet them, the mechanisms and the emotion remain, with the behavioural responses (Forkman et al. 2007). Fear is complex, two individuals may show very different behavioural and physiological responses, depending on the context of the stimuli, or the inherent genetic differences in coping strategies, or both (Forkman et al. 2007). When an environmental stimulus is perceived as threatening, the stress response is initiated, with an activation of the hypothalmo-pituitary-adrenal axis which releases corticosterone from the adrenal gland. The
animal also experiences the fundamental emotion of fear during a stress response. Behavioural responses and corticosterone responses to stimuli vary distinctly between individuals (Cockrem 2007).

Different personalities may be associated with different stress responses (Korte et al. 2005). Cockrem (2007) defines coping styles, characteristic patterns of behaviour that the animal uses to cope with demands from their environment, as personalities. When quails were challenged with a mild stressor rather than a strong, there were individual differences in the corticosterone responses (Cockrem 2007). The stressors that the chickens in this study were exposed to can be regarded as mild, since they were not real and/or a bit habituated, e.g. humans. This might explain the variation in the PCA, no genotypic differences were found there. Cockrem (2007) divided the personalities into two, proactive and reactive. The proactive personality has a bold and fast behavioural response and relatively low corticosterone stress response to a stimulus. The reactive personality has a shy and slow behavioural response and a large corticosterone response. Individual corticosterone and behavioural responses, which are linked, depend on every individual’s personality (Cockrem 2007).

During domestication several behaviours altered, due to both natural and artificial selection (Price 1997). A passive selection might have occurred in traits that carry a fitness cost that at the same time won’t give significant fitness benefits. An example of this is that high levels of aggressive behaviours could be costly both in energy and risk of injury, but if the more aggressive animals weren’t provided the reproductive opportunities under captive conditions increased (Eklund and Jensen 2011). This might explain why the results from this study were not stronger, the fear related behaviours might have changed during a passive selection during an active selection for the release of seasonal reproduction. It is well-known and also supported by the results in this study that domestic animals show less aggressive behaviours but if it is due to a passive selection is still unclear.

As stated by Crockford (2006), there can be differences in the physiology in individual thyroid hormone, which could potentially be responsible in stress response in individuals and differences in social dominance that is seen in animals like primates, wolves and whales. This might explain the variation of the results in this study, if there are individual levels of the thyroid hormones during the test. It would be interesting to measure that prior to every test to see if there is a correlation. There are indications that this mutation plays an early role in the domestication considering that a large number of animals are homozygote for the mutation. There are
different developmental methods in precocial and altricial birds and mammals, and due to that the developmental pattern of the thyroid function might differ. Regarding domesticated species, the TSHR sweep would therefore benefit some further research in precocial species to fully look into the TSH effect. The prenatal development might have a large impact on behaviours with fluctuating levels of thyroid hormone (Crockford 2006).

5.1 Conclusion
The results from this study supports my hypothesis, there are behavioural differences between the wild type and domestic type chickens. Domestic type chickens show less fear related behaviours and are less dominant than the wild type chickens. From this I can draw the conclusion that the mutation of the TSHR gene is involved in the behavioural changes during domestication.

6 Acknowledgement
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