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**A domestication related mutation in the thyroid stimulating hormone receptor gene
(*TSHR*) modulates photoperiodic response and reproduction in chickens**

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Abstract

The thyroid stimulating hormone receptor gene (*TSHR*) has been suggested to be a “domestication locus” in the chicken. A strong selective sweep over *TSHR* in domestic breeds together with significant effects of a mutation in the gene on several domestication related traits, indicate that the gene has been important for chicken domestication. *TSHR* plays a key role in the signal transduction of seasonal reproduction, which is characteristically less strict in domestic animals. We used birds from an advanced intercross line between ancestral Red Junglefowl (RJF) and domesticated White Leghorn (WL) to investigate effects of the mutation on reproductive traits as well as on *TSHB*, *TSHR*, *DIO2* and *DIO3* gene expression during altered day length (photoperiod). We bred chickens homozygous for either the mutation (*d/d*) or wild type allele (*w/w*), allowing assessment of the effect of genotype at this locus whilst also controlling for background variation in the rest of the genome. *TSHR* gene expression in brain was significantly lower in both *d/d* females and males and *d/d* females showed a faster onset of egg laying at sexual maturity than *w/w*. Furthermore, *d/d* males showed a reduced testicular size response to decreased day length, and lower levels of *TSHB* and *DIO3* expression. Additionally, purebred White Leghorn females kept under natural short day length in Sweden during December had active ovaries and lower levels of *TSHR* and *DIO3* expression compared to Red Junglefowl females kept under similar conditions. Our study indicates that the *TSHR* mutation affects photoperiodic response in chicken by reducing dependence of seasonal reproduction, a typical domestication feature, and may therefore have been important for chicken domestication.

Introduction

One of the most striking characteristics of domesticated animals is the reduced seasonality in reproduction (Trut et al., 2009). While non-domesticated animals living in temperate regions typically reproduce on a seasonal basis, the ability to have offspring throughout the year is apparent in most domestic species such as sheep, cattle, pigs, cats and dogs (Faya et al.; Setchell; Trut, 1999). Seasonal reproduction depends on stimuli varying across the year, such as day length, and ensures that offspring is born at a time of year when food is abundant, thereby increasing the chances of survival of the offspring. During domestication, less strict seasonal reproduction is highly beneficial, and has therefore been under selection in most species. Furthermore, the adaptation to a captive environment tends to modify an array of other traits as well, including morphology, physiology, development and behaviour (Jensen, 2006; Price, 1999).

The Red Junglefowl (RJF, *Gallus gallus*) is the ancestor of the domestic chicken (West and Zhou, 1988). The bird lives in Southeast Asia and shows a robust photoperiodic response (Ono et al., 2009). During spring and summer RJF females lay clutches of 3-7 eggs which they incubate (Collias and Collias, 1967). The domestication of the chicken started already 6000 B.C. (West and Zhou, 1988) and has caused obvious phenotypic changes as a consequence of underlying genetic alterations. For example, the White Leghorn (WL, *Gallus gallus domesticus*) is a domesticated breed selected for high egg production, which lays eggs throughout the year, independent of season, and rarely incubates them.

In a seminal study, RJF as well as eight different populations of domestic chickens were subjected to whole-genome resequencing, in order to identify selective sweeps and candidate mutations of importance for domestication (Rubin et al., 2010). The most striking sweep was

detected at the locus for the thyroid stimulating hormone receptor (*TSHR*), and was shared among all domestic chickens in the study. A missense mutation causing a non-conservative amino acid substitution was suggested as the candidate mutation in the sweep (Rubin et al., 2010). Ancient DNA studies have shown that the mutation was present already in classical Greek chickens which indicates that it is old, but data also suggest that the sweep allele was not fixed in European chickens until much later in time, and took place only 500 years ago due to strong human-mediated selection (Girdland Flink et al., 2014).

The TSHR plays an important role in the hypothalamic-pituitary-gonadal axis, which regulates photoperiodic responses and seasonal reproduction. The photoperiodic signal transduction in birds starts with stimulation of deep brain photoreceptors (Nakane et al., 2010; Nakane and Yoshimura, 2010) and is further translated into neuroendocrine responses transmitted to the *pars tuberalis* where it induces thyroid stimulating hormone β subunit (*TSHB*) expression. TSHB associates with the common glycoprotein alpha subunit (CGA), which is rhythmically expressed, to form thyroid stimulating hormone (TSH). TSH from the *pars tuberalis* then connects to TSHR expressed in the ependymal cells and induces the expression of type 2 deiodinase (*DIO2*), that converts thyroxine (T₄) to bioactive 3, 5,3'-triiodothyronine (T₃) (Ikegami and Yoshimura, 2012; Nakane and Yoshimura, 2010; Nakao et al., 2008). *DIO2* is expressed during long day conditions, and during short days there is a reciprocal switch, where *DIO2* is down regulated and the type 3 deiodinase (*DIO3*), which inactivates T₃, is up-regulated. Long day induced T₃ production causes secretion of luteinizing hormone and follicle stimulating hormone and thereby further gonadal growth (Yasuo et al., 2005). The molecular mechanisms of seasonal reproduction in birds have mainly been investigated in Japanese quail, but studies have shown that the Red Junglefowl shows similar responses (Ono et al., 2009).

These facts together indicate that the *TSHR* mutation may be a true “domestication gene”. In previous studies, we have shown that the mutation delays hatching by about one day, and also modifies a range of behavior in a way typically associated with domestication (Karlsson et al., 2015). In the present study we focus on the effect on photoperiodic responses. To study this we have used birds generated from a Locus Controlled Advanced Intercross Line (LAIL). The chickens were bred from an F10-generation of an advanced intercross line between RJF and WL and were either homozygous for the wild type *TSHR* allele (*w/w*) or for the mutant allele (*d/d*). This allowed us to study the effect of the genotype at the *TSHR* locus against the random RJF x WL hybrid background genotype achieved by accumulating recombinations during the previous ten generations.

We observed reproductive traits as well as *TSHB*, *TSHR*, *DIO2* and *DIO3* gene expression during altered day length (photoperiod) in females and males with alternative genotypes at the *TSHR* locus in order to find consistent differences between genotypes. In addition, we included a small number of purebred WL and RJF females kept under natural day length as a proof of principle. Furthermore, the presence of the *TSHR* mutation was investigated in a number of Swedish landrace chickens, in order to evaluate the presumed age and spread of the mutation. We hypothesized that animals with different *TSHR* genotypes would differ in their reproductive responses to altered day length, and that this would be mirrored in the expression of genes involved in the photoresponse cascade. Moreover, we predicted that the mutation would be present at a high rate in Swedish landraces, reflecting our hypothesis that the mutation was selected early during domestication.

Materials and methods

Ethical note

The study was approved by Linköping local Ethical committee of The Swedish National Board for Laboratory Animals (approval no. Lkp 85–07).

Animals

Advanced Intercross: TSHR chickens

The main part of this study was performed on offspring from the F10 generation of an advanced intercross between WL and RJF. The WL-line (SLU 13) has a long history of selection for egg production traits and originated from a Scandinavian selection and crossbreeding experiment (Liljedahl et al., 1979). The Red Junglefowl originated from a Swedish zoo population; more details about the original animals used for the intercross can be found in Schütz and Jensen (2001). For the advanced intercross, approximately 100 birds were maintained in each generation. Details about the breeding of the advanced intercross line are described elsewhere (Johnsson et al., 2014).

The chickens were bred from 10 families and pedigree hatched. The parental birds were heterozygous *w/d* at the *TSHR* locus, and the homozygous offspring (*w/w* and *d/d*) were used for the study. Two batches with a total of 70 chickens were hatched. Batch 1 consisted of 8 females (*w/w*: 4, *d/d*: 4) and 13 males (*w/w*: 6, *d/d*: 7) and batch 2 of 29 females (*w/w*: 16, *d/d*: 13) and 20 males (*w/w*: 10, *d/d*: 10). In the following, these birds are referred to as the “*TSHR*

chickens”. Both batches were treated identically throughout the experiment. For details of hatching and rearing, see Karlsson et al. (2015).

At the age of six weeks the chickens were moved to the main chicken facility, 10 km outside of Linköping. They were divided by sex and housed in two identical pens measuring $3.0 \times 2.5 \times 3.0$ m ($l \times w \times h$), allowing full visual and auditory contact between pens. Food and water was available *ad libitum*, and the pens contained perches, nest boxes and wood shavings on the floor. The light was kept on a 12/12 h light:dark cycle (except during light experiments; see below) and the room temperature was 20 °C.

Parental birds under free-range conditions

For a proof-of-principle study, to describe the reproductive system under “natural” conditions, we included three pure-bred female RJF and four pure-bred female WL from the parental lines (described above). These birds were housed in a private chicken house also located 10 km outside Linköping under natural day length (NDL) (lat 58°24′). In the following, these birds are referred to as the “NDL-RJF” and “NDL-WL” respectively. The study was performed in December, and day length was 6 h and 40 min. No artificial light was available and the birds had free access to outdoor ranges at all hours. The chicken house consisted of a single pen, measuring $4.0 \times 4.0 \times 2.4$ m ($l \times w \times h$) and was equipped with perches, nest boxes and wood shavings on the floor, with food and water available *ad lib*. The birds were moved from the “Wood-Gush” research chicken house 6 months prior to this study, and were well accustomed to the new, natural environment. Moreover, we included four female RJF, housed in a separate pen at the “Wood-Gush” research chicken house, under same conditions as the *TSHR* chickens described above, in order to get data from a wild ancestral bird under stable light conditions. In the following, these birds are referred to as the “12/12-RJF”. All the NDL-

WL/RJF and 12/12-RJF individuals were genotyped (see below), and found to be homozygous at the *TSHR* locus. All these birds had received the same treatment from hatching until they were moved to the NDL-conditions (or to the separate pen) as the *TSHR* chickens, as described above.

Swedish landraces

Blood samples from nine out of eleven officially recognized Swedish landraces were obtained from the Swedish Chicken Genebank collection at the Swedish University of Agricultural Sciences and the birds were genotyped at the *TSHR* locus (see below). The 15 individuals, all females, were of the following breeds: Hedemorahöns (2 individuals), Gotlandshöns (2 individuals), Bohusläns-Dals svarthöns (2 individuals, both originating from the same population), Skånsk blommehöns (2 individuals, both originating from the same population), Ölandshöns (2 individuals, both originating from the same population), Öländskt dvärghöns (2 individuals), Åsbohöns (1 individual), Kindahöns (1 individual) and Orusthöns (1 individual).

Genotyping

DNA was extracted from blood using standard salt extraction. Fragments of 296 bp spanning the *TSHR* mutation were amplified with PCR and all primers used are listed in Table 1. Each reaction mixture contained 0.75 U DreamTaq DNA polymerase (Thermo scientific, Waltham, MA USA), 2.5 µl 10× DreamTaq Buffer (Thermo scientific, Waltham, MA USA), 2.5 µl dNTP mix 2mM (Thermo scientific, Waltham, MA USA), forward and reverse primers 5µM (1 µl of each), 1 µl DNA template (5-100 ng/µl) and 18 µl nuclease-free water. The PCR conditions were as follows: initial denaturation, 95 °C for 3 min, followed by 40 cycles of 30 s at 95 °C (denaturation), 30 s at 63-53 °C (touchdown, annealing) and 30 s at 72 °C

(extension) and final extension for 15 min at 72 °C. The PCR-products were analysed through pyrosequencing using PyroMark Q24 (Qiagen, Germantown, USA) according to manufacturer's protocol.

Photoresponse measurements

TSHR males

Photoperiodic response was measured in *TSHR* males during transfer from LD to SD conditions. At 10 weeks of age males were moved into a box, impermeable to light and measuring 1.8 × 1.8 × 1.8 m (l × w × h), and maintained under LD conditions (20/4 h light/dark cycle) for three weeks. Light was supplied by LED-lamps, delivering light within the spectrum of natural day light, with an intensity of 200 lux at the level of the bird's head. The chickens had access to food and water *ad libitum*, the floor was covered with wood shavings and the temperature was 22 °C. At the age of 13 weeks the light was changed into SD conditions (8/16 h light/dark cycle). At three occasions birds were euthanized through decapitation: 1) at the last LD, (5 *w/w* and 5 *d/d*), 2) on the 5th SD (5 *w/w* and 5 *d/d*) and 3) on the 15th SD (6 *w/w* and 5 *d/d*). Blood plasma for detection of luteinizing hormone (LH) levels (see below), and paired testis weight was collected, and the part of the midbrain containing the hypothalamus was rapidly dissected and snap-frozen in liquid nitrogen as described previously (Lindqvist et al., 2007). The sampling was performed 16 hours after dawn at all occasions.

TSHR females

Onset of lay (indicating sexual maturation) was measured in females during a constant photoperiod of 12/12 h light/dark cycle, followed by measurement of the response in laying frequency to decreased day length. At the age of 13 weeks females were moved into individual pens measuring 0.62 × 0.45 × 0.51 m (l × w × h). Each pen allowed visual and auditory contact with other birds, and was equipped with a LED-lamp delivering an intensity of 200 lux at the level of bird's head, within the spectrum of natural day. The birds had access to food and water *ad libitum*, the floor was covered with wood shavings and the temperature was 20 °C. The light was kept on a 12/12 h light/dark cycle. Daily inspection for eggs was done for each individual chicken in order to record the onset of lay.

At 30 weeks of age, when all females were active layers, the light rhythm was changed to SD conditions (8/16 h light/dark cycle). Eggs were collected on a daily basis to record the response to decreased photoperiod for each individual chicken. At 47 weeks of age the birds in batch two were euthanized through decapitation. Samples from 14 *w/w* and 12 *d/d* birds were collected. Hypothalamus from brain was rapidly dissected and snap-frozen in liquid nitrogen and blood plasma was collected for assessment of luteinizing hormone (LH) levels (see below). Visual inspection of ovaries was done for each individual in order to determine laying status. The ovary from a not reproductive hen is small with no active follicles, while an active layer show enlarged ovary and developed follicles (Bowles, 2005). The sampling was done 16 hours after dawn.

For the *TSHR* batch 1, onset of lay data was collected from the day the first egg was laid until the day the last females laid her first egg, i.e. during week 19-20. Thereafter, laying data was

collected from week 29-45 in order to record the termination of lay. For *TSHR* batch 2 laying data was consecutively collected from week 18-45.

Parental birds under natural conditions

All NDL-WL, NDL-RJF and 12/12-RJF birds were euthanized at a single occasion, 16 hours after dawn, following the procedures described for the *TSHR*-females above. By the time of sampling the birds were of following age: NDL-RJF 471 days, NDL- WL between 323- 478 days of age and 12/12-RJF between 239-291 days of age.

Assessment of luteinizing hormone (LH) levels

Blood was collected from the jugular vein in a 50 ml falcon tube together with 20 µl 0.5 M EDTA immediately after decapitation. 2,0 ml of blood was further transferred to an eppendorf tube and centrifuged at 3000 rpm at room temperature for 20 min. Plasma was stored at -20 °C until further analysis.

The concentration of luteinizing hormone in the plasma samples was determined using a commercial LH ELISA kit (MyBiosource, San Diego, USA), according to the manufacturer's instructions. All samples were tested in duplicate and the analytic range of the assay was 6.25-200 ng/ml.

Gene expression

Brain tissues were homogenized in Ambion TRI reagent (Applied Biosystems, Carlsbad, CA, USA) and total RNA was extracted according to the standard protocol from the manufacturer. The quantity and purity of samples were assessed by spectrometry using NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Freemont, CA, USA). The integrity of the RNA was verified by Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), by calculating RNA Integrity Number (Schroeder, 2006) following the manufacturer's instructions. RNA samples were then stored in -80 °C until further analysis. Maxima First Strand cDNA Synthesis Kit for RT-qPCR with dsDNase (Thermo Fisher Scientific, Freemont, CA, USA) was used to synthesize single stranded cDNA from 2 µg of total RNA according to manufacturer's protocol. Target specific primers were designed using NCBI Primer blast (Ye et al., 2012) and optimized in a gradient cycler with regard to DreamTaq DNA Polymerase (Thermo Fisher Scientific, Freemont, CA, USA). The specificity of primers was confirmed by the presence of a single band of PCR product with gel electrophoresis and through investigating the melting curve in Light Cycler.

The RT-qPCR was conducted according to LightCycler 480 System (Roche Diagnostics, Basel, Switzerland) instructions. A master mix containing 2 µl water, 1 µl forward primer (0.5 µM), 1 µl reverse primer (0.5 µM) and 5 µl LightCycler® 480 SYBR Green I Master (Roche Diagnostic) was added to each well in a 96-well PCR Plate together with 1 µl diluted cDNA (15 ng reversed transcribed total RNA) template. The RT-qPCR was performed as follows: 10 min 95 °C denaturation followed by 40 times (95 °C for 10 s, 55 °C for 10 s, 72 °C for 20 s) ending with a melting curve program with temperature rising from 72 °C to 95 °C and a final cool down to 40 °C. The efficacy of RT-qPCR for each gene was calculated by analyzing the slope of the serial cDNA dilution. The expression of target genes was normalized to three

housekeeping genes (primer sequences, see Table 1). The relative expression difference between genotypes was calculated according to Pfaffl (2001).

Statistics

Mean values and SEM were calculated within sex for all the recorded variables. For analysing genotype effects, we used either Mann-Whitney U-test (disregarding family effects when family representation was very uneven), or Generalized Linear Models (SPSS v. 22.0) with genotype nested within family as predictor. The probability distribution used was “Normal” and the link function “Identity”, and significance levels were determined with the Wald Chi-squared test with adequate degrees of freedom. The Omnibus (Likelihood Ratio Chi-Square) test was used for determining the performance of the model versus the intercept, and this was deemed acceptable when the significance level was below 0.05. This was the case for all results reported here.

Results

TSHR males

There was a significant increase in paired testicular weight from sampling session one to two for the *TSHR w/w* genotype ($U = 0.0$, $n_1 = 5$, $n_2 = 4$, $P = 0.02$), but no differences between genotypes within each sampling occasion (Fig. 1 a). As shown in Fig 1b, there was a significant decrease in *TSHB* mRNA levels from sampling occasion one to two ($U = 0.0$, $n_1 = 5$, $n_2 = 4$, $P = 0.02$) and from two to three ($U = 0.0$, $n_2 = 4$, $n_3 = 6$, $P = 0.01$) for the *TSHR w/w* genotype and from occasion two to three for the *d/d* genotype ($U = 0.0$, $n_2 = 5$, $n_3 = 5$, $P = 0.01$). No significant genotype differences within sampling occasions were observed. *TSHR* mRNA levels were significantly higher in the *TSHR w/w* genotypes in sampling occasion two ($U = 0.0$, $n_{d/d} = 5$, $n_{w/w} = 4$, $P = 0.02$) and three ($U = 0.0$, $n_{d/d} = 5$, $n_{w/w} = 6$, $P = 0.01$), but no differences between sampling occasions were seen (Fig. 1c). A significant decrease in mean *DIO2* level was observed from sampling occasion one to two for the *TSHR d/d* genotype ($U = 1.0$, $n_1 = 5$, $n_2 = 5$, $P = 0.02$), but no genotype differences were seen within sampling occasions (Fig. 1 d). The mean *DIO3* level increased significantly from sampling occasion two to three for the *THSR w/w* genotype ($U = 1.0$, $n_2 = 4$, $n_3 = 6$, $P = 0.03$), but no genotype differences within sampling occasions were observed (Fig. 1e).

There were no significant effects of genotype or sampling occasion on LH-levels in the males (Fig 2a).

TSHR females

Whilst the age at onset of egg laying did not differ between the genotypes, the laying rate (numbers of eggs per week) increased faster in *TSHR d/d* females, reaching a significantly higher number at the age of 21 weeks (Fig. 3a). Both genotypes responded to a decrease in day length with a drop in average number of eggs produced during week 34, three weeks after transfer to SD. However, both genotypes recovered at week 35 and kept a relatively stable laying throughout the experiment. No significant difference in rate of lay was seen between genotypes during the SD period (Fig. 3b). The gene expression analysis showed significantly higher expression levels of *TSHR* (Wald $\chi^2(12)=25.7$, $P=0.012$) and *DIO2* (Wald $\chi^2(12)=57.4$, $P<0.001$) in *d/d* females compared to *w/w* females, but no differences for *TSHB* or *DIO3* (Fig. 3c).

TSHR d/d females had significantly lower plasma levels of LH at culling (Fig 2 c; Wald $\chi^2(12)=45.0$ $P<0.001$).

NDL-WL/RJF and 12/12-RJF

Visual inspection of the ovaries of the newly sacrificed purebred birds showed that all three NDL-RJF females had small and inactive ovaries (shown by the black arrow, Fig. 4 a, upper picture). All 12/12-RJF females and 3 out of 4 NDL-WL females showed enlarged, active ovaries (Fig. 4 a, lower picture). One NDL-WL individual had ovaries with reduced size, indicating that laying was terminated a short time prior to sampling. From the gene expression analysis (Fig. 4 b) a significant difference was seen for the *TSHR* gene, where the NDL-WL chickens showed a significantly lower expression than the 12/12-RJF, but not the NDL-RJF ($U = 0.0$, $n_{12/12-RJF} = 4$, $n_{NDL-WL} = 4$, $P = 0.03$). Furthermore, a significant difference

was found between NDL-WL and NDL-RJF, but not NDL-WL and 12/12-RJF in *DIO3* expression ($U = 0.0$, $n_{\text{NDL-RJF}} = 3$, $n_{\text{NDL-WL}} = 4$, $P = 0.05$).

Swedish Landraces

The genotyping of the *TSHR* mutation in ancient Swedish Landrace chickens showed that all individuals were homozygous for the mutation (d/d), except the two from Bohuslän-Dals svarthöns that were both heterozygous (w/d) (Table 2).

Discussion

The results from this study showed that the domestication related mutation in the chicken *TSHR* gene affects reproductive traits. *TSHR* expression in brain differed significantly between genotypes for both males and females. Males homozygous for the “domestic” allele (*d/d*) showed a reduced phenotypic response and lower levels of gene expressions in comparison to the *w/w* genotype when exposed to alterations in day length, and *TSHR d/d* females showed a faster onset of lay in comparison to *w/w* females. Furthermore, purebred White Leghorn females kept under natural daylight conditions (NDL-WL) had active ovaries and low expression of *DIO3* compared to ancestral Red Junglefowl under similar conditions. Thirteen out of 15 birds representing Swedish Landrace breeds were homozygous for the *TSHR* mutation, underscoring its common occurrence among a variety of domesticated chickens.

In all populations and genotypes included in this study *TSHR* gene expression levels differed significantly between homozygous *w/w* and *d/d* birds. Levels of *TSHR* mRNA were consistently lower in females and males with the *d/d* genotype in comparison to birds with the *w/w* genotype for both *TSHR* birds and pure WL and RJF. This indicates either that the mutation (Rubin et al., 2010) affects the expression levels of *TSHR*, or that there is an unknown regulatory mutation in strong linkage disequilibrium to the gene. The *TSHR* mutation is a nonsynonymous substitution resulting in a glycine to arginine shift (Rubin et al., 2010). A bioinformatic analysis has shown that this amino acid substitution pushes the extracellular residue outwards from the membrane and may affect ligand interaction (Rubin et al., 2010). At present it is not known how the amino acid substitution could affect the

expression levels of the gene, or what is causing the consistently lowered *TSHR* mRNA levels in the *d/d* genotype. However, it is possible that one or more regulatory mutations in strong linkage disequilibrium with the missense mutation is causing the difference in gene expression, but mutations affecting phenotype can also affect expression levels (Johnsson et al., 2014).

Under the natural day length of approximately 6.5 h in December in Sweden (58°24'), three out of four NDL-WL females, but no NDL-RJF bird showed active ovaries. The NDL-RJF birds stopped laying already two months prior to this study (data not shown). By that time day length at this latitude was about 10 h per day. This clearly demonstrates one remarkable difference between domesticated and wild chickens; the ability of domestic chickens to lay eggs also under SD conditions. It is very likely that this trait has been selected for during domestication since a prolonged reproduction season is clearly beneficial from a production perspective, giving a potential to generate more offspring per year and thereby increasing reproductive success.

The results of the LH-analysis supports the suggestion that birds carrying the *TSHR* mutation are able to sustain laying also under suboptimal conditions. While there were no differences in LH-level in the relatively young males, *d/d*-females had significantly lower levels, in spite of maintaining egg laying under the short day-length conditions. We suggest that this indicates that domesticated genotype birds are more sensitive to LH, and can therefore sustain laying also with relatively low hormone levels.

The mutation in the *TSHR* gene did not affect expression levels of *DIO2*, but interestingly, a significant difference was seen in *DIO3* expression between NDL-RJF (all *w/w*) and NDL-

WL (all *d/d*). Photoperiodic regulation of *DIO2* and *DIO3* is complex and appears to differ between species (Watanabe et al., 2007). Often, during LD conditions the expression of *DIO2* is high and *DIO3* is low, and opposite during SD conditions (Yasuo et al., 2005). The rapid reciprocal switching of *DIO2* and *DIO3* is well described in Japanese quail (Yasuo et al., 2005), whereas, in wild male European starlings, no correlations of *DIO2* with photoperiod or with gonad size has been found (Bentley et al., 2013). Furthermore, in some species, *DIO3* has been shown to have a dominant role in the regulation of photoperiodic reproduction (Barrett et al., 2007). The significantly lower *DIO3* expression in NDL-WL birds in comparison to NDL-RJF and the obvious phenotypic difference in laying status suggest that *DIO3* could possibly play a key role in the control of seasonal reproduction in chickens. Furthermore, the mutation in the *TSHR* gene could prolong seasonal reproduction by suppressing *DIO3* expression.

The possible significance of the *DIO3* gene is also suggested by our correlational results from the *TSHR* males. It was somewhat unexpected that testicular weight increased after the transition from LD to SD, but the results are in fact in line with studies of Japanese quail males, where levels of gonadal growth-stimulating plasma LH remained high for 8-16 days after transition to SD before the levels started to decline (Nicholls et al., 1973). Moreover, studies in hamsters have shown that testicular weight in males transferred from LD to SD increased after 5 days (Prendergast et al., 2013), similar to what we observed in *TSHR* *w/w* males. The decreased *TSHB* levels for both genotypes over all sampling occasions shows that the LD to SD transition did indeed cause a physiological response, and the fact that *DIO2* levels decreased significantly for *d/d* males supports this. These results suggest that the phenotypic effect of an altered photoperiod on testicular weight is subtler in males with a mutation in *TSHR*, which therefore appear less sensitive to changes in day length.

Females homozygous for the domestic *TSHR* allele showed a faster onset of lay with a significantly higher number of eggs at week 21 of age. It is unclear what the original goals were for chicken domestication, whether chickens were solely selected for production purposes, or as suggested, were originally used mainly for cock-fighting (Crawford, 1990). However, it appears likely that a fast onset of lay would have been of selective advantage early in the domestication process allowing individuals to reproduce earlier. Since the *TSHR* gene expression differed between *w/w* and *d/d* females in the same direction as for the NDL-birds and the *TSHR* males, with significantly lower levels in the *d/d* genotype, it is likely that the mutation is related to earlier onset of lay.

Another possible effect of the mutation would be that frequent egg laying could be sustained for a longer period under short day length conditions. However, the rate of lay did not differ during the 14 weeks that the animals were kept under short photoperiod. The gene expression analysis showed that *w/w* females had significantly higher *TSHR* and *DIO2* levels in comparison to the *d/d* genotype, but no differences between genotypes were seen for *TSHB* or *DIO3*. Based on earlier studies in European hamsters (Hanon et al., 2010), Sibirian hamsters (Barrett et al., 2007) and Japanese Quail (Yasuo et al., 2005), we expected *DIO3* to be up-regulated in the short photoperiod. Contrary to this, the *DIO3* level in our *TSHR* females kept in SD was similar to that of 12/12-RJF females kept at intermediate photoperiod. However, prior photoperiodic history could also affect the expression of the gene. Hamsters transferred from LD (15/9 light/dark) to intermediate-duration (13.5/10.5 light/dark) photoperiods showed increased levels of *DIO3* expression and exhibited gonadal regression, whereas those with no prior exposure to LD showed no increased *DIO3* expression or gonadal regression in intermediate-duration photoperiods (Kampf-Lassin and Prendergast, 2013b). Hence, the

length of the intermediate photoperiod for our *TSHR* birds at 12/12 light/dark was perhaps not sufficient to induce *DIO3* expression and therefore comparable to the absent prior exposure as described for the hamsters (Kampf-Lassin and Prendergast, 2013b). If elevated expression of *DIO3* is sufficient to terminate reproduction, as suggested from the NDL-RJF/WL data, the absent induction of *DIO3* expression could explain why all birds were still in an active laying phase by the end of the study.

Taken together, our data show that the *TSHR* mutation is associated with the control of photoperiodic reproduction and a possible mechanism may be that the mutation prevents reproduction from terminating during SD conditions by suppressing *DIO3* expression. The ability to reproduce independently of season is a main characteristic of domesticated animals. Most domesticated breeds of chicken worldwide carry the mutant *TSHR* allele in at least one copy, which suggests that the mutation is relatively old (Rubin et al, 2010). Studies of ancient chicken DNA from different archeological sites in Europe have shown that the *TSHR* mutation was fixed already in Classical Greek chickens (Girdland Flink et al., 2014). However, it is unclear if these populations were founders for the chickens we have in Europe today. Another scenario is that the mutation became fixed through strong artificial selection during the past 500 years (Girdland Flink et al., 2014). Our genotyping showed that birds sampled from eight out of nine Swedish landrace breeds were homozygous for the domestic *d/d* alleles and only two from one breed were heterozygous. This suggests that the *d* mutation may have been close to fixed already in the founder populations that were brought to Sweden 2000 years ago, or, less likely, that it was introduced more recently and then spread across chicken populations across the country. The selective sweep surrounding the *TSHR* locus is thus not primarily related to traits favored by the modern chicken industry, such as age of

sexual maturity and egg weight (Arthur and Albers, 2003), but rather to more general traits related to domestication such as loss of a strict seasonal reproduction.

In previous experiments, we have shown that the *TSHR*-mutation also affects other aspects of chicken biology (Karlsson et al., 2015). For example the *d/d* genotype showed a longer incubation time, less fearful behaviours, lower aggression, and decreased levels of the thyroid hormone T4, in comparison to the *w/w* genotype. These differences between *TSHR* genotypes mirror the differences in development and behaviour between pure WL and RJF chickens. This further supports the idea that the *TSHR* mutation may explain a significant part of the phenotypic changes induced by chicken domestication. Hence, we believe that these previous results together with the present may represent the first demonstration of the tentative importance of a single mutation in the domestication of an animal species.

As a note of caution, the breeding scheme which we have used means that any gene within the same linkage disequilibrium (LD)-block as *TSHR* could differ between genotypes and hence potentially be responsible for the different phenotypic outcome. The size of the LD-block in the advanced intercross used here is less than 500 kb (unpublished data), and within this region, five genes in addition to *TSHR* are located. These are: *DIO2* (type II iodothyronine deiodinase), *CEP128* (centrosomal protein), *GTF2A1* (general transcription factor IIA, 1), *STON2* (stonin 2: adapter protein involved in endocytic machinery), *SEL1L* (sel-1 suppressor of lin-12-like). Hence, it remains a possibility that polymorphisms linked to *DIO2* are the causes of the observed effects rather than the different *TSHR* genotype, and epistatic interaction between the two genes could possibly also contribute to the phenotypic differences seen in our study. Regardless, this scenario would support the hypothesis that the hypothalamus-pituitary-thyroid (HPT)-axis has been targeted for selection during

domestication. *DIO3* is not situated in this linkage block, but may of course be affected by transcripts from the region.

In conclusion, the present study shows that chickens, which are homozygous for a mutant allele (*d/d*) at the *TSHR* locus, show lower levels of *TSHR* gene expression in comparison to chickens homozygous for the wild type (*w/w*) allele. The decreased *TSHR* levels seem to be associated with a faster onset of lay in *TSHR d/d* females, suppressed levels of *DIO3* expression and reduced testicular response to altered photoperiods in *TSHR d/d* males, and active ovary status in purebred White Leghorn *d/d* females kept under natural SD conditions. The *TSHR* mutation is apparently old and was possibly widespread already when chickens were first brought to Sweden. The mutation in *TSHR* gene affects seasonal reproduction in the direction that is characteristic for domesticated animals, which suggest that the *TSHR* mutation has been targeted for selection during chicken domestication.

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Table 1 Primers used in the study.

Gene	Forward Primer	Reverse Primer
PCR		
TSHR	ATCATGCTATAGAGTGGCAGACAG	AGCTACAGCAAAGTCAGCATCTG (biotinylated)
Pyrosequencing		
TSHR	sequencing primer:	GCCCTTTTGCCACTG
RT-qPCR		
TSHR	TATCCAAGTCACTGCTGTGCT	GGTCCTCTGATAGCTCTGAACG
TSHB	CGCTGGATTTTGCATGAC	GTGCACACGTTTTGAGACAG
Dio2	CCAGCCACGCTCTGTCAATA	ATCCCGAAGGAAGAGAGCCT
DIO3	AGGCTCTCTTCCTTCGGGAT	TAGCACTTGCTAGGCAGCAC
Beta-2 microglobulin	AAGGAGCCGCAGGTCTAC	CTTGCTCTTTGCCGTCAT
TATA-binding protein	TAGCCCGATGATGCCGTAT	GTTCCCTGTGTCGCTTGC
RNA Polymerase II	GGACTCTGTTTGCGGTGTTT	GGGGTTGATTCTTTGTCTGC

Table 2. Genotypes on the *TSHR* locus in 15 different chickens from nine old Swedish landraces. The table lists number of individuals of each breed carrying a specific genotype at the locus. *w/w*: homozygous for the domesticated mutation; *w/d*: heterozygous; *d/d*: homozygous for the wild type allele.

Breed	Genotype		
	<i>w/w</i>	<i>w/d</i>	<i>d/d</i>
Bohusläns-Dals svarthöns	0	1	1
Gotlandshöns	0	0	2
Hedemorahöns	0	0	2
Kindahöns	0	0	1
Orusthöns	0	0	1
Skånsk blommehöns	0	0	2
Åsbohöns	0	0	1
Ölandshöns	0	0	2
Öländskt dvärghöns	0	0	2

Legends to figures

Figure 1. Data from TSHR males for each sampling occasion; 1 (last Long Day), 2 (the 5th Short Day) and 3 (the 15th Short Day). a) Average paired testicular weight ($\pm$ SEM). Gene expression in hypothalamus measured as relative mRNA level ($\pm$ SEM) for b) *TSHB*, c) *TSHR*, d) *DIO2* and e) *DIO3*. * $P < 0.05$. Different letters indicate statistically significant differences between genotypes within sampling occasion at $P < 0.05$.

Figure 2. Plasma levels of Luteinizing Hormone (LH). A: Males, sampled at three occasions; 1 (last Long Day), 2 (the 5th Short Day) and 3 (the 15th Short Day). B: Purebred females kept under stable or natural light conditions; 12/12 RJF: Red Junglefowl kept under 12:12 light:dark rhythm; NDJ-RJF: Red Junglefowl kept under natural short day conditions; NDJ-WL: White Leghorns (domesticated laying hens) kept under natural short day conditions. C: Female intercross birds differing in genotype at the TSHR locus (d/d: homozygous for the domesticated mutation; w/w: homozygous for the wild type allele).

Figure 3. Egg-laying in *TSHR* females shown as average number of eggs per day ($\pm$ SEM) during one week for each genotype, and gene expression of central genes in the photoperiodic response. a) Onset of lay during sexual maturation in constant 12/12 light/dark rhythm, * $P < 0.05$. b) Laying frequency as a response to decreased day length. Dashed line represent the time of transfer from 12/12 to 6/18 light/dark cycle. c) Gene expression in hypothalamus at the end of the laying period, measured as relative mRNA level ($\pm$ SEM) for *TSHB*, *TSHR*, *DIO2* and *DIO3*. * $P < 0.05$

Figure 4. Ovaries and gene expression in the 12/12-RJF and NDL-RJF/WL chickens. a) Pictures from visual inspection of laying status, black arrows show (upper picture) inactive, small ovary, (lower picture) enlarged, active ovary with follicles. b) Gene expression in hypothalamus measured as relative mRNA level ($\pm$ SEM) for *TSHB*, *TSHR*, *DIO2* and *DIO3*. *P<0.05

a **b**