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Changes in pituitary gene expression may underlie multiple domesticated traits in chickens

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Running title: Effects of domestication on pituitary transcriptome

Abstract

Domesticated animals share a unique set of morphological and behavioral traits, jointly referred to as the domesticated phenotype. Striking similarities amongst a range of unrelated domesticated species suggest that similar regulatory mechanisms may underlie the domesticated phenotype. These include color pattern, growth, reproduction, development and stress response. Although previous studies have focused on the brain to find mechanisms underlying domestication, the potential role of the pituitary gland as a target of domestication is highly overlooked. Here, we study gene expression in the pituitary gland of the domesticated *White Leghorn* chicken and its wild ancestor, the Red Junglefowl. By overlapping differentially expressed genes with a previously published list of functionally important genes in the pituitary gland, we narrowed down to 34 genes. Amongst them, expression levels of genes with inhibitory function on pigmentation (*ASIP*), main stimulators of metabolism and sexual maturity (*TSHB* and *DIO2*), and a potential inhibitor of broodiness (*PRLR*), were higher in the domesticated breed. Additionally, expression of 2 key inhibitors of the stress response (*NR3C1*, *CRHR2*) was higher in the domesticated breed. We suggest that changes in the transcription of important modulatory genes in the pituitary gland can account not only for domestication of the stress response in domestic chickens, but also for changes in pigmentation, development, and reproduction. Given the pivotal role of the pituitary gland in the regulation of multiple shared domesticated traits, we suggest that similar changes in pituitary transcriptome may contribute to the domesticated phenotype in other species as well.

Keywords: Domestication, pituitary, transcriptome, chicken

Introduction

A number of animal species have undergone domestication, and thereby adapted to living in captivity and in proximity to humans (Price, 1999). The domesticated phenotype is a collection of similar traits evolving in many unrelated species, encompassing changes to morphology, physiology and behavior (Price, 1999; Jensen, 2014). These traits include, e.g., changes in color pattern, increased docility, higher growth rate, earlier sexual maturity, lack of strict seasonal reproduction, alteration of craniofacial morphology, and lowered stress response (Künzl and Sachser, 1999; Price, 1999).

The domesticated chicken originated from the Red Junglefowl (RJF) around 8000 years ago in Asia (Tixier-Boichard, Bed'Hom and Rognon, 2011). Today, the chicken is the most numerous domesticated species on earth, and hundreds of diverse breeds have evolved as a result of selection for meat production, egg laying, research, and hobby (Mirkena *et al.*, 2010). In comparison with the ancestral Red Junglefowl, domesticated chickens reach sexual maturity earlier, grow faster, lay more and bigger eggs throughout the year, are less fearful, and have an attenuated stress response (Schütz *et al.*, 2002; Kerje *et al.*, 2003; Ericsson *et al.*, 2014; Fallahsharoudi *et al.*, 2015).

The genetic basis for the majority of the modified traits during animal domestication is unknown (Wiener and Wilkinson, 2011). Genetic changes causing differences in gene expression have been suggested to underlie most phenotypic differences between closely related species (Albert and Kruglyak, 2015). Gene expression differences between domesticated and wild animals could therefore underlie the traits that are associated with domestication (Albert *et al.*, 2012). Given the importance of the brain in regulation of physiology and behavior, several studies have focused on the effects of domestication on brain gene expression (Saetre *et al.*, 2004; Tymchuk, Sakhrani and Devlin, 2009; Albert *et al.*,

2012; Nätt *et al.*, 2012; Heyne *et al.*, 2014). However, other tissues, with as much or even more relevance to behavior and physiology have seldom been studied. For instance, the pituitary, a key structure in the hypothalamic-pituitary-adrenal (HPA) axis as well as in the hypothalamic-pituitary-gonadal (HPG) axis, has so far largely been overlooked, and we therefore decided to focus this experiment on the pituitary gland, using chickens as a suitable model species.

The pituitary gland is a neuroendocrine organ that plays a fundamental role in the regulation of multiple vital biological processes, including pathways affecting several domesticated traits (Ferris, 2006; Scanes, 2015). Inhibitory and stimulatory peptides with hypothalamic origin regulate the production and release of pituitary hormones. The pituitary consists of two distinct structures (anterior and posterior) that have different embryonic origins. The anterior pituitary originates from Rathke's pouch and consists of cells that produce adrenocorticotrophic hormone (ACTH), luteinizing hormone (LH), follicle stimulating hormone (FSH), prolactin, growth hormone (GH), thyroid stimulating hormone (TSH), and the melanocyte-stimulating hormones (MSH). The posterior adrenal gland originates from the infundibulum and serves as the site for secretion of oxytocin and vasopressin (and the homologue vasotocin in birds) into the blood. The pituitary hormones can either have direct effects on other tissues (i.e. GH), or modulate activity of other endocrine tissues (i.e. adrenal and thyroid glands) (Scanes, 2015).

One major trait shared by many domesticates is an attenuated stress response (Weiler *et al.*, 1998; Künzl and Sachser, 1999; Gulevich *et al.*, 2004; Trut, Oskina and Kharlamova, 2009; Plyusnina, Solov'Eva and Oskina, 2011; Ericsson *et al.*, 2014). During stress, the hypothalamic-pituitary-adrenal axis is activated. Neural signals reach the hypothalamus, which secretes corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) into the portal vessel. These hypothalamic peptides reach their designated receptors in the anterior

pituitary and stimulate the release of adrenocorticotrophic hormone (ACTH) into the general circulation. ACTH in turn is transported to the adrenal glands, and stimulates release of stress mediators such as glucocorticoids (Joëls and Baram, 2009; Herman *et al.*, 2016).

The activity of the HPA axis is mainly regulated by the negative feedback loop through the action of corticosteroids at glucocorticoid receptors (GR) and mineralocorticoid receptors (MR) that are located in the brain and pituitary gland (Keller-Wood, 2015). Given its central role in regulating many biological processes, we hypothesize that gene expression changes at the level of the pituitary gland may influence multiple domesticated traits. We therefore used microarray analysis of pituitaries of ancestral Red Junglefowl and domesticated White Leghorns to obtain a broad picture of gene expression differences.

Materials and Methods

Ethical statement

All experimental protocols were approved by Linköping Council for Ethical Licensing of Animal Experiments, ethical permit no 50-13. Experiments were conducted in accordance with the approved guidelines.

Animals and housing

We studied a captive population of ancestral Red Junglefowl and one population of domesticated White leghorn (WL). The RJF population that was used in this experiment was originally brought to Sweden from wild population in Thailand to a Zoological park in northern Sweden (Frösö Zoo). Based on the previous habitat and the appearance of the acquired RJF, these birds most likely were of the subspecies *G. g. gallus* or *G. g. spadiceus* (Schütz, Forkman and Jensen, 2001). We acquired 1-day-old chickens from Frösö Zoo, kept, and bred them in our animal facility in Linköping Sweden for 16 generations with a

population size of around 100 with pedigree breeding. SLU13 originates from the Scandinavian selection and crossbreeding experiment (Liljedahl *et al.*, 1979) and was maintained at the Swedish University of Agricultural Sciences. SLU13 line developed for research purposes and selected for egg mass but does not represent any commercial strain of birds (Schütz, Forkman and Jensen, 2001). We currently have a population size of around 100 individuals per generation of SLU13 at our facility at Linköping, Sweden. For this study, we collected and incubated fertile eggs from floor-housed flocks of 30-40 females and 6 males for both populations simultaneously. The population of RJF that was used in this study has a relatively long history of living in captivity, and therefore we can speculate that factors such as genetic drift and unintentional selection might have influenced it. However, compared to the domesticated egg layer breeds, the RJF birds are smaller, show more fearful behavior, have a lower HPA axis reactivity, lay less and smaller eggs and show seasonal reproduction behavior such as broodiness (Schütz and Jensen, 2001; Ericsson *et al.*, 2014). Therefore, although the studied RJF population does not represent the true ancestral population, it is more like wild-living Red Junglefowl than to WL. However, to be able to generalize the findings of this study to other chicken breeds, more wild populations as well as other domesticated breeds selected for diverse production traits, and landrace chickens should be studied.

RJF and WL chicks were hatched, and thereafter kept under 12h light and dark periods with ad libitum access to food and water in pen sized 1m x 2m. Due to the distinct phenotypic and behavioral differences between domesticated WL and RJF, when kept together in a single pen, WL and RJF form separate groups based on their breed (previous observations), and therefore, one group may systematically affect the other group, for instance, by pecking or preventing them to access food and water. Thus, we kept the breeds in separate pens divided into two mixed-sex groups per breed.

Tissue collection

We chose the age of 6 weeks for this study because this is when phenotypic differences between the breeds and the sexes become obvious. A random sample of 12 animals from each breed, six of each sex, were culled and sampled under calm conditions, and an additional 12 animals from each breed, also six of each sex, were exposed to 15 minutes of stress by means of physical restraint in a net before culling (in total 48 chickens). Culling was performed by decapitation, and dissection took place immediately after. The whole brain was removed, and the pituitary was retrieved. The tissues were frozen in liquid nitrogen within ten minutes of sacrifice, and subsequently stored at -80° C until further processing.

Gene expression analysis

Total RNA was isolated from each individual sample using TRIzol™ reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. RNA purity and integrity were checked in a Bioanalyzer 2100 system (Agilent Technologies, Palo Alto, CA, USA). RNA integrity number (RIN) was larger than 8.0 in all samples used for microarray analysis. RNA was standardized in concentration, and samples were pooled so that each pool contained RNA from two birds. The two birds were from the same breed, sex and treatment. Since the animals had been kept divided into two mixed-sex groups per breed, we selected one bird from each group for each pool. Six samples resulted in a very poor yield and were not used for microarray analysis, thus six microarray samples could not be pooled and consisted of just one individual each. In total, we therefore had 24 microarray samples, out of which 18 were pools with RNA from two individuals, and six contained RNA from only one individual. The information regarding each sample as well as the details of pooling are provided in **Supplementary Table 1**.

Microarray probe sequences are originally designed based on RefSeq mRNA or Ensembl transcripts (WASHUC2.1/galGal3) as previously described (Johnsson *et al.*, 2016). However the probe sets were later updated to Ensembl (version 85; (Yates *et al.*, 2016)) according to previous description (Johnsson *et al.*, 2018). Each probe set gives the average value of 2 – 3 probes, and in total the array included 20,771 probe sets. Amongst all included probe sets 16,360 were annotated to a genomic location and the rest were probe sequences from a chicken brain cDNA library (Boardman *et al.*, 2002). The probe sequences were controlled for presence of known SNP in the founder populations by masking all known SNP positions based on resequencing of Red Junglefowl and domesticated chickens (Rubin *et al.*, 2010).

Total RNA was labelled with Agilent Low Input Quick Amp Labeling Kit One Color (Agilent) and hybridized to 8x60K SurePrint G3 Custom Gene Expression Microarrays (Agilent). Scanning was performed with a MS200 Microarray scanner (Roche NimbleGen, USA). Array data were extracted using the Feature Extraction Software v12.0 (Agilent Technologies, USA). We chose to use microarrays over RNA sequencing in this experiment, since our lab has extensive experience with this method. Gene expression profiles generated by microarrays are highly correlated with that observed with RT-QPCR, although RNA sequencing is superior in detecting low abundance and novel transcripts. We also validated the expression of 10 functionally important genes using RT-PCR. Six biological replicates for each breed, sex and treatment (n = 48) were used for cDNA synthesis using Maxima first strand cDNA synthesis kit for RT-qPCR with dsDNase (Thermo Fisher Scientific, USA). The reactions were performed in the Light Cycler 480 (Roche Diagnostics, Basel, Switzerland). We previously have published the details for performing the RT-qPCR (Fallahsharoudi *et al.*, 2015; Løtvedt *et al.*, 2017). The primer sequences for RT-qPCR can be found in Supplementary Table 2.

Data analysis

All data analysis was performed using R (<http://www.r-project.org>) and Bioconductor (www.bioconductor.org). All expression data was normalized, and control probes were subsequently removed from the following analyses. Mean values from the probes for each gene were calculated and used in further analyses. The quality of the data was assessed through logarithmic box plots of overall expression signals, as well as with PCA analysis. No outlying samples were detected (**Supplementary Figure 1**).

To find differently expressed genes, we used linear modelling with the *limma* package for R (Ritchie *et al.*, 2015). The model used included breed, sex and treatment as fixed predictors, as well as the interaction between breed and treatment (gene expression \sim breed + sex + treatment + breed \times treatment). We adjusted p-values for multiple testing using the FDR approach (Benjamini and Hochberg, 1995). We also set a threshold of 0.5 for the log fold change. For hierarchical clustering and generation of heatmaps, we used the *hclust* function with default parameters from the *stats* package in R, in combination with the *ggplot2* package (Wickham, 2009). Ensembl transcript IDs extracted from all probe IDs that were differentially expressed between breed, sex and treatment groups were used for gene ontology analysis through the Database for Annotation, Visualization and Integrated Discovery (DAVID version 6.8, Oct. 2016) (Huang, Sherman and Lempicki, 2008). Next, we overlapped the list of the annotated genes that were differentially expressed (DE) between the breeds with a published list containing 407 “pituitary-important” genes in chickens (Pritchett *et al.*, 2017). This list was based on RNA sequencing of multiple tissues (pituitary, abdominal fat pad, heart fat pad, breast muscle, cerebellum, heart, liver, duodenum, jejunum, ileum, spleen, retina, pineal and hypothalamus) in chickens. These genes were either exclusively expressed in the chicken pituitary gland at day 42 or were more abundantly expressed in the pituitary compared to the other tissues at this age (Pritchett *et al.*, 2017). We also overlapped the breed

DE genes with the list of 265 genes that were located within domestication related selective sweeps (Rubin *et al.*, 2010).

Results

When comparing WL and RJF (gene expression \sim breed + sex + treatment + breed \times treatment), 489 annotated genes were found to be differentially expressed (DE) between the breeds (FDR corrected p-value threshold 0.05 and log fold change $>$ 0.5). Of these, 289 transcripts were found to be expressed at higher levels in RJF and 200 genes were expressed at higher levels in WL (**Figure 1** and **supplementary Table 3**).

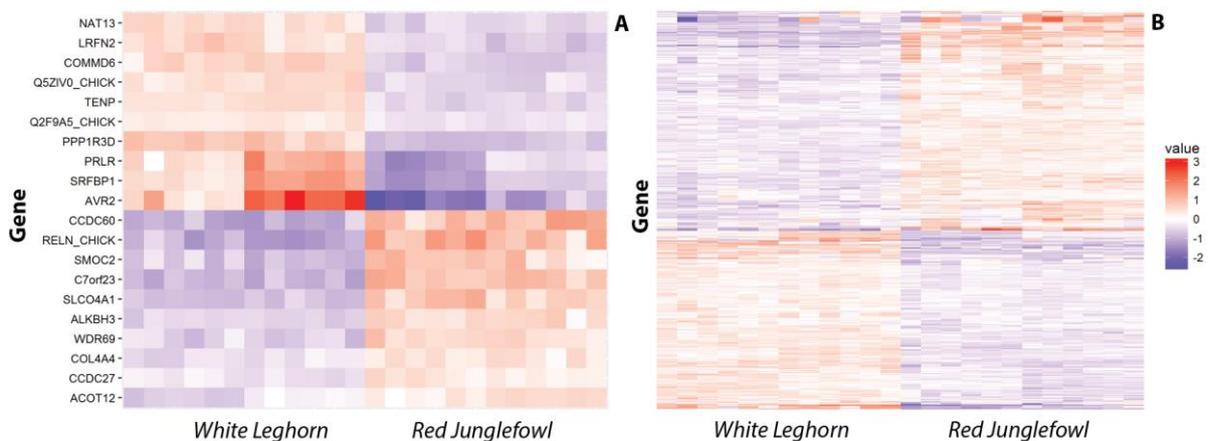


Figure 1. Normalized gene expression profile of the pituitary gland of domesticated *White Leghorn* and Red Junglefowl. **1A** shows the 10 top genes that are expressed higher in WL and in RJF. **1B** shows all differentially expressed genes between the two breeds (FDR corrected p-value threshold 0.05 and log fold change $>$ 0.5). The first six samples (from left to right) for each breed are females and the rest are males. The full list of DE transcripts can be found in the **Supplementary Table 3**.

Out of the 489 DE annotated genes, 430 were included in UniProt Knowledgebase (UniProtKB) database. GO analysis identified a significant enrichment (Benjamini corrected $P < 0.01$) of genes in four functional categories, “Signaling”, “Disulfide bond”, “Glycoprotein” and “Secreted” (**Figure 2** and **Supplementary Table 4**). It is worth noting that an additional 412 transcripts from expressed sequence tags (dbEST) were found to be DE

between breeds. DbEST is a collection of short single-read transcript sequences from GenBank (Boguski, Lowe and Tolstoshev, 1993), that are not annotated and therefore were not included in any downstream analysis.

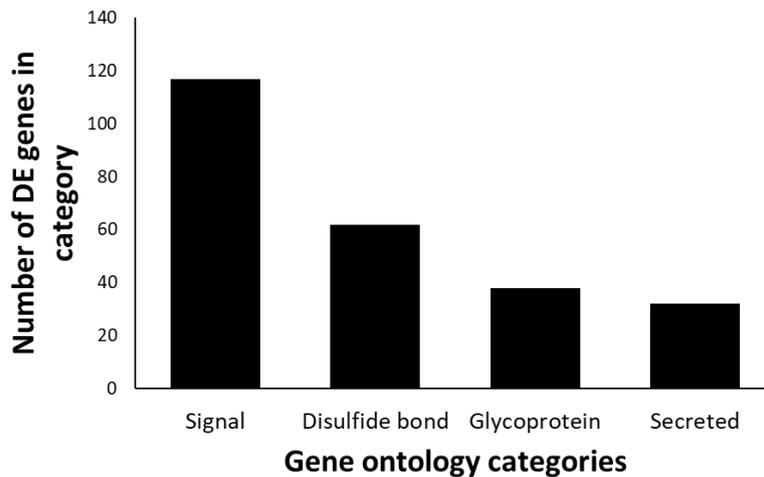


Figure 2. Number of annotated genes differentially expressed between breeds that fell within each of the four significantly enriched gene ontology terms. Amongst the 430 genes with a known annotation, around 250 genes (including the genes which fell in more than one categories) fell within the significant categories. The uniprot ID for Signal is KW-0732, Disulfide bond is KW-1015, Glycoprotein is KW-0325 and for Secreted is KW0964. The list of genes within each category is presented in **Supplementary Table 4**.

Next, we overlapped the breed DE list (489 genes) with a list of 407 specific genes. Based on differential tissue expression patterns, these genes have been suggested to have important functions in the pituitary gland of chickens at day 42 (Pritchett *et al.*, 2017). Thirty-four genes were common to both lists, suggesting their importance in explaining the phenotypical and physiological differences between WL and RJF (Table 1). Overlapping breed DE genes with selective sweeps revealed 12 genes that were in both lists (**Supplementary Table 3**).

Table 1. The list includes the subset of differently expressed genes between the domesticated White leghorn and Red Junglefowl based on the ANOVA model (~breed + sex + treatment), that overlaps with the list of pituitary-important genes at day 42 (Pritchett et al., 2017). Direction indicates whether the expression of the genes is higher in the domesticated White Leghorn or in the Red Junglefowl.

Abbreviation	Gene name	Direction	
PRLR	Prolactin receptor	Higher in WL	
KCNIP2	Kv channel interacting protein 2		
ENO4	Enolase family member 4		
OTOG	Otogelin		
SIX1	SIX homeobox 1		
ASIP	Agouti signaling protein		
TCERG1L	Transcription elongation regulator 1 like		
ADAMTS18	ADAM metalloproteinase with thrombospondin type 1		
KCNH7	Potassium voltage-gated channel, subfamily H, member 7		
PSD	Pleckstrin and Sec7 domain containing		
TMEM215	Transmembrane protein 215		
LOC42098	HCG1657980		
CCNA1	cyclin A1		
TSHB	Thyroid stimulating hormone beta		
HESS	Hes family bHLH transcription factor 5		
FOXP1	Forkhead box G1		
MMRN1	Multimerin 1		
SNTN	Sentan, cilia apical structure protein		
AVPR1A	Arginine vasopressin receptor 1A		
FAM46D	Family with sequence similarity 46-member D		
DIO2	Deiodinase, iodothyronine, type II		
SSTR5	Somatostatin receptor 5		
LOC428967	Gamma-aminobutyric acid receptor sub pi-like		
COL4A4	Collagen, type IV, alpha 4		Higher in RJF
ZNF414	Zinc finger protein 414		
CDHR3	Cadherin related family member 3		
TOM1L1	Target of myb1 like 1 membrane trafficking protein		
PENK	Proenkephalin		
CLCA2	Chloride channel, calcium activated, member 2		
CCDC39	Coiled-coil domain containing 39		
SIX2	SIX homeobox 2		
SOX3	SRY (sex determining region Y)-box 3		
GPC3	Glypican 3		
NR4A3	Nuclear receptor subfamily 4, group A, member 3		

Subsequently, we selected 9 breed DE genes and 1 important pituitary gene (*POMC*) that was not amongst the DE gene list for validation with RT-qPCR. The expression patterns of 9 out of 10 genes agreed with the microarray results (**Figure 3 and Supplementary Table 5**).

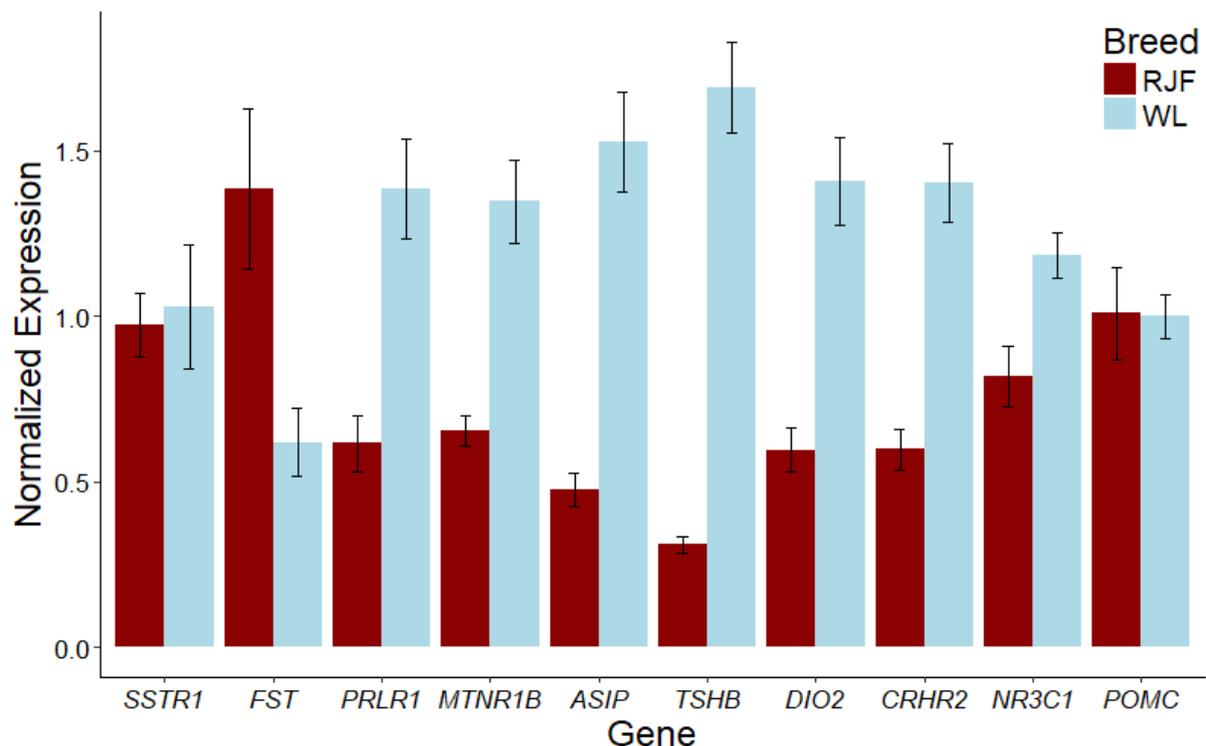


Figure 3. Normalized relative expression (Delta C_t) levels of genes showing differential expression between Red Junglefowl and White Leghorn, selected on grounds of overlapping with a list of pituitary-important genes or having known functions in processes relevant for the domesticated phenotype. Genes shown are *somatostatin receptor type 1 (SSTR1)*, *folliculin (FST)*, *Prolactin receptor 1(PRLR1)*, *melatonin receptor 1B (MTNR1B)*, *agouti signaling protein (ASIP)*, *thyroid stimulating hormone beta (TSHB)*, *deiodinase iodothyronine type II (DIO2)*, *corticotropin releasing hormone receptor 2 (CRHR2)*, *glucocorticoid receptor (NR3C1)*, and *proopiomelanocortin (POMC)*. The used primers for RT_qPCR and results from statistical analysis can be found in **Supplementary Table 2 and 5** respectively.

Comparing males and females, including breed and stress treatment in the model, 294 annotated genes were differently expressed, out of which, 37 were expressed higher in

females, whereas 257 genes (165 Z-linked and 92 autosomal genes) were expressed higher in males. Overlapping sex DE gene list with the list of pituitary-important genes (407 genes) resulted in identification 23 genes that may play important sex specific role in chicken (**Supplementary Table 6**).

Stress treatment, affected expression of 6 genes, 3 of which were expressed higher after stress, whereas 3 were downregulated after stress. Two of these stress DE genes also overlapped with the list of pituitary-important genes (**Table 2 and Supplementary Table 7**).

Table 2: Annotated genes that were differentially expressed between control and stress treatment. The asterisk indicates that the gene is amongst the published pituitary-important genes (Pritchett *et al.*, 2017). Direction indicates whether the gene is up or downregulated after 15 minutes of restraint stress.

Abbreviation	Gene name	Direction
<i>PPP1R14D</i>	<i>Protein phosphatase 1, regulatory (inhibitor) subunit 14D</i>	Higher after stress
<i>CYR61</i>	<i>Cysteine rich angiogenic inducer 61</i>	
<i>RGS1</i>	<i>regulator of G-protein signaling 1</i>	
<i>SLCIA3*</i>	<i>Solute carrier family member 3</i>	Lower after stress
<i>G3BP2</i>	<i>Ras GTPase-activating protein-binding protein 2</i>	
<i>NDRG1*</i>	<i>N-myc downstream regulated 1</i>	

Only one gene was found to show a significant breed x treatment interaction when correcting for multiple testing, the *dual specificity protein phosphatase 4 (DUSP4)* gene (adj. p = 0.04). In RJF, expression of DUSP4 increased after stress, whereas it decreased in WL.

Discussion

We have used cDNA microarray to assess the effects of domestication and stress on the gene expression pattern in the pituitary gland of domesticated *White Leghorn* and Red Junglefowl. Around 500 annotated genes were differentially expressed between the breeds. Overlapping the breed DE list with the list of genes that are uniquely or more abundantly expressed in chicken pituitary gland (Pritchett *et al.*, 2017) narrowed down the candidate gene list to 34 genes (Table 1). This candidate gene list contained genes that are known to affect central aspects of the domesticated phenotype, such as coat or plumage color, growth and sexual maturity, reproduction, and stress. The expression differences were in the direction expected based on the phenotypic differences between RJF and domesticated chickens.

Changes in body size and modification of coat or plumage color are generally associated with animal domestication (Price, 1999). The gene coding agouti signaling peptide (ASIP), a key modulator of plumage or coat color was amongst the list of candidate genes (**Table 1**, and **Figure 3**). Acting as an inverse agonist on melanocortin receptor 1 (MCR1), ASIP inhibits melanogenesis and promote yellow pigmentation (Norris and Carr, 2013b). ASIP also promote feeding by blocking the appetite-suppressing effects of α -MSH in the hypothalamus, and therefore, over-expression of this gene leads to obesity as well as dominance of yellow fur in mammals (Dreger and Schmutz, 2011; Norris and Carr, 2013a). Thus, it is likely that the observed higher expression of *ASIP* in WL is indeed associated with the well-known differences in color pattern and body size between domesticated WL and RJF.

Reaching sexual maturity at a younger age as well as a lack of strict seasonal reproduction are other shared characteristics in domesticated animals (Price, 1999; Trut, Oskina and Kharlamova, 2009; Shinomiya *et al.*, 2014). Thyroid stimulating hormone β (TSHB) and deiodinase iodothyronine type II (DIO2) are localized in the pars tuberalis of the anterior pituitary and function as master regulators of puberty as well as seasonal reproduction in birds

(Nakao *et al.*, 2008; Karlsson *et al.*, 2016; Nishiwaki-Ohkawa and Yoshimura, 2016). Both *TSHB* and *DIO2* were expressed at higher levels in WL compared with RJF (**Table 1**, and **Figure 3**), consistent with the earlier puberty and higher metabolic rate in the domesticated WL.

Prolactin receptor (PRLR) was amongst pituitary-important DE genes (Table 1) and was also amongst the top 10 breed DE genes (**Figure 1A** and **3**). In birds, prolactin and PRLR are involved in regulation of maternal behavior and broodiness (Grommen *et al.*, 2006; Dawson, 2014). Prolactin secretion from the pituitary gland is controlled by the hypothalamus as well as by a negative feedback loop. This negative feedback is mediated through action of prolactin on *PRLR* at the level of the hypothalamus and pituitary gland (Chiu, Koos and Wise, 1992). Thus, higher expression of PRLR in the pituitary gland of WL may inhibit prolactin secretion and subsequently contribute to the lack of broodiness in this domesticated breed.

Besides the aforementioned genes that overlapped with the list of pituitary important genes, searching known stress modulatory genes revealed that *CRHR2* and *NR3C1*, genes encoding hormone receptors involved in stress response, were also differentially expressed between breeds (Figure 3). The glucocorticoid receptor (GR) is the main mediator of negative feedback of the HPA axis, and as we found a higher expression of the gene encoding GR, *NR3C1*, in WL. *CRHR2* is also involved in modulation of the stress response. Investigations of mice deficient in *CRHR2* have shown that these animals have higher levels of ACTH and corticosterone shortly after stress exposure (Preil *et al.*, 2001). Thus, the higher expression of *NR3C1* and *CRHR2* we observe in WL may also be implicated in the reported dampened hormonal stress response in domesticated chickens (Soleimani *et al.*, 2011; Ericsson *et al.*, 2014).

Based on their biological roles in the regulation of seasonality and reproduction, we also selected *FST* and *MTNR1B* for RT-QPCR validation. Expression levels of *FST* were higher in

RJF, whereas, *MTNR1B* was expressed at higher levels in WL (**Figure 3**). FST inhibits biosynthesis and secretion of FSH from the pituitary and thus inhibits puberty and reproduction (Herbison, 2016). Lower gene expression levels of the inhibitory *FST* in the pituitary gland of the WL is consistent with higher egg laying capacity of this egg layer breed. Additionally, *MTNR1B*, which contributes to seasonally activated behavior such as molting (Chiu and Wise, 1994; Wilkanowska *et al.*, 2014), was expressed differently between the breeds, which may be related to the observed domestication effects on reproductive behavior.

To identify genomic regions associated with domestication, previous research has analyzed selective sweeps, i.e. chromosome regions with reduced genetic variation caused by selection of certain gene variants coupled with linkage (Andersson, 2012). Overlapping breed DE genes (489 genes) with annotated genes within selective sweeps (261 genes) showed that 12 genes were present in both list (**Supplementary Table 3**). Only one of these genes, *nuclear receptor subfamily 4, group A, member 3 (NR4A3)* was also present in the list of pituitary-important genes. In human, NR4A3 is suggested to play a key role in regulation of cell proliferation, differentiation, and metabolism (Pearen *et al.*, 2006; You *et al.*, 2017).

In the functional analysis, genes that were expressed differently were related to membrane components, post-translational modification of secretory proteins, or secretion into the cell surroundings (Frاند, Cuzzo and Kaiser, 2000; Sevier and Kaiser, 2002). Therefore, there was an overrepresentation of genes involved in different aspects of hormone secretion, suggesting that the activity of the pituitary gland differs in domesticated WL compared with RJF. However, the potential function of most genes that were DE between the breeds remains unknown. Domesticated chickens have diverged from the ancestral RJF at least 6000 years ago (Tixier-Boichard, Bed'Hom and Rognon, 2011), and therefore, we can expect neutral fixation as well as divergent selection pressure to underlie the observed changes in the expression of hundreds of genes (Khaitovich *et al.*, 2004).

Male and female birds usually differ extensively in a range of traits, including appearance and morphology, physiology and behavior. The hypothalamus and pituitary gland play key roles in modulation of physiological and morphological differences between the sexes (Scanes, 2015). Around 165 Z-linked and 130 autosomal genes were differentially expressed between sexes. The fact that all DE Z-linked genes were expressed at a higher level is consistent with the well-known incomplete dosage compensation in chicken (Warnefors *et al.*, 2017). Overlapping sex DE genes with the list of genes with important function in the pituitary gland revealed 23 genes which may contribute to development of sex specific characteristics in chickens (**supplementary Table 6**). Some of these genes such as *gonadotropin-releasing hormone receptor (GNRHR)*, *follicle stimulating hormone beta subunit (FSHB)*, and *PRLR* have a known function in regulation of reproduction, whereas the others present novel candidate genes for functional characterization. In the present study, the time from the onset of the stress treatment to sample collection was only 15 minutes. We therefore expected the number of differently expressed transcripts to be low. Only 6 transcripts were found to differ between the treatments (**Table 2** and **Supplementary Table 7**). Among the genes that were upregulated by the stress treatment were *PPP1R14D* and *RSG1*. *PPP1R14D* encodes a protein that binds to and inhibits the activity of protein phosphatase 1 (PP1) (Liu *et al.*, 2004; Eto, 2009). PP1 is associated with both pre-mRNA slicing (Shi, Reddy and Manley, 2006) and glycogen metabolism and regulation (Karthik and Ravikumar, 2011), among other things. Inhibiting PP1 could impede slicing of RNA and thus play a role in gene regulation, but could also increase glycogen breakdown and reduce glycogen synthesis (Karthik and Ravikumar, 2011). Currently, we do not know which of these roles the upregulation of *PPP1R14D* after stress has in the stress response.

RSG1, also upregulated after restraint, encodes a protein that regulates G protein signaling. *RSG1* has also previously been shown to be upregulated in the pituitary of mice after 2 hours

of restraint stress treatment (Kim *et al.*, 2010). Several G protein-coupled receptors are located in the pituitary, including AVPR1A, AVPR1B, CRHR1, CRHR2, MTNR1B, GnRH and SSTR1 (Alexander *et al.*, 2013), and it is possible that *RGS1* may interact with any or several of these.

Only one gene, *DUSP4* (also known as *MAPK phosphatase, MKP-2*), showed a significant interaction between breed and treatment. When the pituitary gland is stimulated by GnRH, MAPK pathways are activated, in turn increasing the expression of genes encoding subunits of the gonadotropins (Zhang and Roberson, 2006). GnRH stimulation also increases the expression of *DUSP4*, mediated by early growth hormone 1 (EGR1) (Zhang, Wolfe and Roberson, 2001). *DUSP4* is a MAPK phosphatase that inactivates MAPK through dephosphorylation (Zhang, Mulvaney and Roberson, 2001; Zhang, Wolfe and Roberson, 2001). Thus, the stimulation of *DUSP4* expression by GnRH constitutes a negative feedback mechanism to desensitize the gonadotrophs to further stimulation by GnRH (Zhang, Wolfe and Roberson, 2001; Zhang and Roberson, 2006). This mechanism has been implicated in relaying GnRH pulse frequencies to the secretion rhythms of FSH and LH from gonadotrophs. Thus, the increase of *DUSP4* after stress in the Red Junglefowl could indicate a desensitization of the gonadotrophs to stimulation by GnRH, whereas an opposite effect is observed in WL.

We have previously studied gene expression pattern in the hypothalamus of 21 days old and mature WL and RJF using microarrays (Nätt *et al.*, 2012). Around 280 genes were DE between the breeds at both ages, however, we did not detect genes with known association with the domesticated phenotype in this list. Using RNA sequencing, (Albert *et al.*, 2012) studied gene expression in brain frontal cortex in three pairs of mature domesticated and wild species (wolves and dogs, wild boars and pigs, and wild and domesticated rabbits). There was no overlap between the DE genes in the different domestication events (Albert *et al.*, 2012).

Since previous studies of domestication effects on gene expression failed to show any consistent patterns in different parts of the brain (Saetre *et al.*, 2004; Tymchuk, Sakhrani and Devlin, 2009; Albert *et al.*, 2012; Nätt *et al.*, 2012; Heyne *et al.*, 2014), we suggest that domestication may primarily have acted on the pituitary. In that sense, the pituitary stands out as a potential central regulator of the domestication effect. We suggest that gene expression profiling of the hypothalamus and pituitary at different developmental stages in other populations of chickens as well as other domesticated-wild species pairs should be made to test whether our results are generalizable across species.

In conclusion, our results suggest that modified gene expression in the pituitary may account for phenotypic differences seen between domesticated chickens and ancestral RJF. We identified differential expression of genes involved in pigmentation, stress responses, growth, metabolism and reproduction, and the direction of the observed differences were in line with the known phenotypic differences between the breeds. Our results therefore increase our current understanding of how biological pathways have been modulated by the domestication process.

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Conflict of interest

The authors declare no conflict of interest.

Data Archiving

The present experiment is uploaded on the Array Express website and is available under <http://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-6269>

Username: Reviewer_E-MTAB-6269, Password: nmmqJJKn

Supplementary information is available at Heredity's website.

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Supplementary Figure and Tables

Supplementary Figure 1. 1A. Gene expression distribution. **1B and C.** PCA plots.

Supplementary Table 1. 1A. Information regarding all chickens used in the present experiment. ID shows the individual’s given id. RJF represents Red Junglefowl and WL represents domesticated White Leghorn. Sex shows the sex of individuals where M stands for

males and F stands for females. Treatment Baseline is when the individual is sacrificed immediately after capture, whereas treatment Stress is when the individuals are kept in a hanging net for 15 minutes before being sacrificed. Cage represent the id of the cage where each chicken was kept. **1B.** Information regarding the pooling of the samples. Pool ID represents the id given to each pool of the individuals when pooled. ID shows the id of individuals from **Supplementary Table 1** that were pooled together. The ID column contains only 1 ID for the samples that were not pooled.

Supplementary Table 2. List and sequences of the used primers for RT-qPCR.

Supplementary Table 3. The list of genes in the pituitary glands which are differently expressed between the domesticated White leghorn and Red Junglefowl based on the ANOVA model (\sim breed + sex + treatment). The reported values include: Transcript ID, log₂ fold change (logFC), t value, P.Value, BH adjusted P.Value (adj.P.Val), B Value, Entrez Gene ID, and gene annotation according to DAVID 6.7. The positive logFC values suggest that the gene is up regulated in White Leghorn and negative logFC values suggest up-regulation of the gene in Red Junglefowl. Overlap Sweep column shows if the gene overlaps selective sweeps.

Supplementary Table 4. List of the annotated genes differentially expressed between breeds that fell within each of the four significantly enriched gene ontology terms.

Supplementary Table 5. Results from linear regression with gene expression as response variable, breed and sex as fixed predictors, and the interaction between breed and sex (gene expression \sim breed + sex + breed:sex). P < 0.05 is coded by '*', P < 0.01 is '**' and P < 0.001 is coded by '*''.

Supplementary Table 6. The list of genes in the pituitary glands which are differently expressed between sexes on the ANOVA model (\sim breed + sex + treatment). The reported

values include: transcription ID, log2 fold change (logFC), t value, P.Value, BH adjusted P.Value (adj.P.Val), B Value, Entrez Gene ID, and gene annotation according to DAVID 6.7. The positive logFC values suggest that the gene is up regulated in males and negative logFC values suggest up-regulation of the gene in females. Overlap Pituitary-important column shows the genes that overlap a list of pituitary-important genes (Pritchett *et al.*, 2017) . Chromosome shows the location of the genes based on Gallus_gallus-5.0 chicken genome assembly.

Supplementary Table 7. The list of genes in the pituitary glands which are differently expressed between baseline and 15 minutes after restraint based on the ANOVA model (~breed + sex + treatment). The reported values include: transcription ID, log2 fold change (logFC), t value, P.Value, BH adjusted P.Value (adj.P.Val), B Value, Entrez Gene ID, and Gene name according to DAVID 6.7. The positive logFC values suggest that the gene is up-regulated in the stressed individuals and negative logFC values suggest a higher gene expression in baseline samples.