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Domestication related variation in social preferences in chickens is affected by genotype on a growth QTL

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Abstract

A growth related QTL on chicken chromosome 1 has previously been shown to influence domestication behaviour in chickens. In this study we used Red Junglefowl (RJF) and White Leghorn (WL) as well as the intercross between them to investigate whether stress affects the way birds allocate their time between familiar and unfamiliar conspecifics in a social preference test ("social support seeking"), and how this is related to genotype at specific loci within the growth QTL. RJF males spent more time with unfamiliar chickens before the stressful event compared to the other birds, whereas all birds except WL males tended to spend less time with unfamiliar ones after stress. A significant QTL locus was found to influence both social preference under undisturbed circumstances and social support seeking. The WL allele at this QTL was associated with a preference for unfamiliar individuals but also with a shift towards familiar ones in response to stress (social support seeking). A second, suggestive QTL also affected social support seeking, but in the opposite direction; the WL allele was associated with increased time spent with unfamiliar individuals. The region contains several possible candidate genes, and gene expression analysis of a number of them showed differential expression between RJF and WL of *AVPR2* (receptor for vasotocin), and possibly *AVPR1a* (another vasotocin receptor) and *NRCAM* (involved in neural development) in the lower frontal lobes of the brains of RJF and WL animals. These three genes continue to be interesting candidates for the observed behavioural effects.

Introduction

The chicken is an excellent model species for studying evolution in the form of animal domestication. Domestication changes the physiology and behaviour of animals, and these changes can be interpreted as a process of adaptation to the captive environment with its specific selection pressures (Price, 1998). Compared to the wild, captivity is signified by a lack of predators, higher numbers of animals on a smaller space and a continuous presence of humans. In addition, humans have introduced new selection pressures, often for production traits such as high milk yield, egg production and growth rate. Given these simultaneous changes in multiple selection pressures, it is not unexpected that domestic animals often are less fearful of predators and more tolerant to unfamiliar conspecifics and humans (Price, 1998) and at the same time have more favourable production traits. However, domestication experiments (e.g. the classic silver fox experiment (Trut *et al.*, 2009)) have

shown that selecting animals for only one trait (e.g. tameness) can yield a correlated response in others, such as earlier sexual maturation, altered coat colour and later onset of fear response. This reoccurrence of a set of correlated traits in domestic species has been called the "domestic phenotype" (Price, 2002). This phenomenon may be explained by either pleiotropy or linkage of several genes affecting different traits.

We have earlier reported that a growth related QTL on chromosome 1 of an intercross line between the domestic White Leghorn layer (WL) and the Red Junglefowl (RJF, main ancestor of domestic chickens) simultaneously affects emotionality and social behaviours (sociality and tolerance of social novelty). WL genotypes in this locus are associated with less exploration of novel environments and more time spent with conspecifics (Wirén *et al.*, 2009, Wirén & Jensen, 2011, Väisänen, 2005, Väisänen *et al.*, 2005). The region has also been found to be involved in fear reactions, for example tonic immobility and open field behaviour (Schütz *et al*, 2001). A region spanning 8 MB around the QTL includes behaviourally potent genes such as *AVPR1a, AVPR2, NRCAM* and *Contactin-1*. However, whether pleiotropy or linkage is responsible for the correlation between traits and which parts of the QTL region that affect which traits remains unknown.

Stress is the response to a challenging situation. This response can be alleviated by the presence of familiar conspecifics (Kaiser *et al.*, 2003, Kirschbaum *et al.*, 1995), so called social support. Because of the greater tolerance of domestic birds to unfamiliar individuals (Wirén & Jensen 2011) we hypothesized that WL chickens depend less on social support from familiar individuals, than RJF. To test this hypothesis and elucidate the genetic basis for such a difference, we subjected purebred WL and RJF as well as animals from an RJF×WL intercross line to a social preference test, where birds had a choice of spending time with familiar or unfamiliar stimulus birds before and after a stressful episode of physical restraint. The two pure breeds were chosen, since they have previously been extensively studied with respect to genetic mapping of behavioural traits, and because the intercross line is based on precisely the two lines used here. We then performed a refined QTL study using markers limited to the region of the growth QTL. In addition we examined differential expression of a number of genes in the region in brain tissue from purebred birds.

Material and Methods

Animals

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

The birds used for behavioural testing included purebred RJF and WL as well as birds from the F_9 generation of an advanced intercross line between the two.QTL-analysis was performed on the intercross birds and gene expression analysis on the purebred lines. For a detailed description of the origin of the animal material, see Schütz *et al.* (2001). The purebred animals used for behavioural testing were hatched in one batch (PB1), whereas the intercross birds were hatched in three batches during a time span of 3 months (AIL). The purebred animals used in gene expression analysis constituted a separate batch (PB2). PB1 included 14 males and 14 females of each breed, AIL included 68 birds from 19 different families (36 males and 32 females), and PB2 12 RJF (5 males, 7 females) and 10 WL (5 males, 5 females). All animals were hatched at Kruijt animal facility at Linköping University, Sweden, and kept in mixed sex groups of 30-60 individuals. The rearing pens measured 1.4×0.7×1.6 m (length×width×height) and were supplied with food and water *ad libitum*, as well as perches. The temperature ranged between 25 and 30 °C, and the birds experienced a 12/12 hour dark/light cycle with a light level of 11 Lux. At 28-35 days of age (depending on batch) the birds were moved to Wood-Gush research facility, where they were kept in single sex groups in pens measuring 3m×2.5m×3m (l×w×h). Pens were equipped with perches,nest boxes and a bedding of wood chips. Food and water was available *ad libitum*. The animals experienced a light regime of 12 h light and 12 h dark with a light intensity of 5–8 Lux during the light period, and ambient temperature ranging between 19 and 27 °C.

Behaviour test

All birds were 250-334 days old at the time of testing and each animal was tested only once. The test arena (figure 1) was a runway, measuring 300×90×180 cm (l×w×h) and consisted of cardboard and wire mesh on wooden frames. Two compartments, S1 and S2 (60×90×180 cm, l×w×h) at opposite ends of the arena each housed two adult stimulus animals, which

were visible to the single test chicken, but physically separated by means of wire mesh. Both S1 and S2 were provided with food and water, and the test birds were placed in the central position of the runway at the start of each test. The two stimulus birds in S1 were familiar to the test individual, whereas the other two (in S2) were not, and all the birds were of the same sex. Light levels in the arena were 50 Lux in the stimulus compartment and 20 Lux in the runway, and the temperature ranged between 20 and 23 °C. The arena floor was covered with wood chips.

A test session started when a test bird was placed in the runway, which was divided into three zones of equal size (60×90×180 cm, l×w×h); a familiar zone ("F") adjacent to the familiar stimulus birds, an unfamiliar zone ("U") adjacent to the unfamiliar stimulus birds, and a neutral zone ("N") between the other two zones. The test bird was allowed to explore the arena freely and the durations in seconds in each zone were recorded by means of direct observations. After 300 s the bird was exposed to an acute stressor by being caught and restrained for 180 s in a net suspended from the roof of the arena. This stressor has been shown to induce a significant increase in corticosterone levels in chickens (Karlsson *et al.*, 2011). After this the bird was released in the centre of the arena and again allowed to explore the arena for another 300 s, and the recording of time in the different zones continued. The location of the familiar stimulus animals was balanced between tests and individuals (in half the trials S1 was to the right and in the other half to the left).

The total time spent in each zone before and after the restraint (Duration in unfamiliar zone before restraint = DurB-U, Duration in familiar zone before restraint = DurB-F, and Duration in neutral zone before restraint = DurB-N, the corresponding variables after restraint; DurA-U, DurA-F, DurA-N) was recorded, and differences calculated between time spent in each zone after compared to before restraint (Diff-U, Diff-F, and Diff-N).

The data for the parental birds were sufficiently close to normal distribution in order to warrant a univariate ANOVA to detect breed and sex effects on social preference before stress and repeated measures ANOVA to test how birds allocated their time between the different zones in the time period before and after restraint (again using breed and sex as independent variables). Analyses were performed using SPSS 19.0.

Genotyping

Genotyping was performed following standard procedures. In short, blood was collected in EDTA and genomic DNA isolated using the DNeasy Blood & Tissue Kit (Qiagen GmbH, Hilden, Germany) according to the instructions of the manufacturer, with minor modifications. Four microsatellite markers and two SNPs in the growth QTL region were genotyped.

The microsatellites (UG0006, UG0002, UG0022, MCW0106) were PCR amplified and fragment length was analyzed on a MegaBACE 500 instrument (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The SNPs, 1_36652477 and 1_37164711, were genotyped using HRM (high resolution melt) analysis on a Rotorgene 6000 thermal cycler (Corbett Research, Mortlake, Australia). Primer sequences and annealing temperatures are listed in table 1.

QTL analysis

Map generation and QTL analysis was performed using R/qtl (Broman *et al.*, 2003). QTL analysis was performed using Haley-Knott regression. Fixed factors of sex, rearing batch (there were 3 batches in total) and family (19 families) were included in the initial analysis and then excluded if non-significant for a particular trait. Significance was determined through permutation, as outlined in (Churchill & Doerge 1996), with 1000 random permutations of the phenotype data resulting in a threshold LOD score of ~2.1 for the 5% genome-wide significance and 1.0 for the 20% genome-wide suggestive level. The linkage map constructed using the six markers was 169 cM in long in total, with an average marker spacing of 34 cM. Although a 1.8 LOD drop is required for a true 95% confidence interval in an F_2-type analysis (Broman *et al.*, 2003), in the case of this highly localised region, we used a 1 LOD drop for a suggestive confidence threshold.

Gene expression analysis

RJF and WL birds used for gene expression analysis were decapitated at 35 (WL) and 36 (RJF) days of age and the lower frontal lobes of their brains were immediately removed, frozen in liquid nitrogen and then stored at -80°C. The tissues were homogenized in TRI Reagent Solution (Applied Biosystems, Foster City, CA) and Lysing Matrix D (MP Biomedicals, Solon, OH) on a FastPrep®-24 instrument (MP Biomedicals, Solon, OH), according to the

instructions of the manufacturers. cDNA was synthesized using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA), according to the instructions supplied by that manufacturer. Using 1 μg of this cDNA as template, qRT-PCR was performed in duplex (2 genes amplified in the same tube) with standard PCR primers and TaqMan probes labelled with either TET or FAM and BHQ (sequences are listed in table 1). Two reference genes were used in each amplification, *GAPDH* (*Glyceraldehyde-3-phosphate dehydrogenase*) and *TBP* (*TATA box binding protein*), and a standard curve was constructed for each gene to allow relative quantification of transcripts. The following temperature program was used; 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C (15 s) and annealing/elongation (60 s). Annealing/elongation temperature was varied depending on the genes being amplified, and is listed in table 1. The *AVPR1a* gene was an exception, as its transcript was amplified in monoplex using standard PCR primers and Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA), with *GAPDH* as a reference gene. Standard curves, threshold cycles and relative expression were determined using Rotor-Gene™ 6000 Series software. Each individual's expression level of a gene was normalized to the mean level of the two reference genes for that individual. This was done in Microsoft® Office Excel® 2007 (Microsoft Corporation, 2007). Normalized relative expression of each gene was analyzed in Statistica v 9.1 (Statsoft Inc., 2010) using a general linear model with breed (RJF/WL) as a fixed factor (sexes were analyzed separately) and a significance level of $p<0.05$.

Results

Behaviour of purebred birds (PB1)

There were no significant differences between breeds or sexes in the time spent with familiar birds before restraint (DurB-F) (figure 2a). However, before the restraint episode, females spent significantly less time with unfamiliar birds (DurB-U)($F_{1,52}$=8.86; P=0.04), and there was also a significant breed×sex interaction, where RJF males, but not WL males, spent more time than females with unfamiliar animals (DurB-U)($F_{1,52}$=11.3; P=0.001) (figure 2b).

After the restraint episode, there were no breed effects on time spent with either familiar or unfamiliar birds (DurA-F, DurA-U) (P>0.1), but females spent significantly less time than males with unfamiliar birds (DurA-U)($F_{1,52}$=16.9; P<0.001) and more with familiar (DurA-F)($F_{1,52}$=8.1; P=0.006) (figure 2a & b). There was no significant interaction between sex and breed.

The stress episode tended to cause the birds to increase the time spent with familiar birds (Diff-F)($F_{1,52}$=2.9; P=0.09) (figure 2a & b). The effect was significant for females, which responded more strongly than the males ($F_{1,52}$=4.6; P=0.03), but there was no effect of breed (figure 2a & b). The time spent with unfamiliar individuals (Diff-U) accordingly decreased after stress, again with a significant sex effect (females reacting more strongly) ($F_{1,52}$=4.6; P=0.03) but no effect of breed.

QTL analysis

There was a suggestive QTL for duration in the familiar zone prior to restraint (DurB-F), with its peak at marker 1_366542477. The RJF allele was associated with increased time spent with familiar conspecifics prior to restraint, with this allele apparently fully dominant over the WL allele (DurB-F; LOD=1.95, a=-31.13+/-21.4, d=97.52+/-33.07) (figure 3). Unsurprisingly, a corresponding, though somewhat weaker, QTL was also identified for duration spent with unfamiliar birds prior to restraint (DurB-U). Here the WL allele was associated with a longer duration in the unfamiliar zone, with the peak once again at 1_36652477 (DurB-U; LOD=1.63, a=36.81+/-21.54, d=-85.73+/-32.29) (figure 3). Phenotypic means for behavioural variables affected by the markers described above can be found in table 2. Using 1 LOD drop as confidence interval, both the above QTL were positioned above marker MCW0106 and up to and including the marker at 1_37164711.

The QTL analysis for Diff-F and Diff-U identified one significant and one suggestive QTL for each trait. In the case of Diff-U, the significant QTL was located at 148 cM, with the WL allele associated with spending less time with unfamiliar conspecifics post-restraint. The WL allele appears to be fully dominant over the RJF allele. (LOD=2.7, a=-104+/-33, d=115+/-49). An additional suggestive QTL was located at 70 cM though in this case the WL allele was

associated with spending more time with unfamiliar conspecifics after restraint than before (LOD=1.7, a=71+/-27,d=-20+/-40). Therefore these two QTL are actually in repulsion with regard to social support seeking behaviour, though the lesser effect of the WL allele at the 70 cM QTL will lead to a net decrease from a WL genotype over the entire region. A similar mirror-effect can also be seen in Diff-F. The significant QTL is located at 149cM, with the WL allele associated with an increase in time spent with familiar conspecifics after restraint (LOD=3.3, a=116+/-30, d=-90+/-46). Similarly, the suggestive QTL is located at 70 cM (LOD=2.0, a=-72+/-25, d=29+/-37), with the WL allele associated with decreased time associating with familiar conspecifics post-restraint. Using a one-LOD drop for a suggestive QTL confidence interval (C.I.) separates these two QTL at either end of the interval analysed, though with a small overlap for Diff-F. Confidence intervals for Diff-U were; suggestive C.I. from 0-129cM, Diff-U, significant C.I. 109-160cM. For Diff-F the confidence intervals were; suggestive C.I.=5-83cM, significant C.I.=104-161cM. No sex interaction effects were found with any of the significant or suggestive QTL.

In an advanced intercross population, family substructure may be present due to non-random mating in the preceding generations, and can lead to problems of over-inflation of LOD scores and issues with non-syntenic association (Cheng *et al.*2010). In this experiment individuals were bred from a large number of families (n=18) to reduce or remove this issue, as the smaller the number of individuals used per family, the more analogous the population is to a standard RIL (Peirce *et al.* 2008). To check that this substructure was not a problem, we firstly included a family covariate in the QTL analysis and this was found to be non-significant. Secondly, it is possible to fit a relatedness matrix within a QTL analysis framework using the package QTLRel (Cheng *et al.* 2011). When this was performed, the QTL for associating with familiar individuals prior to restraint ('Bef-Fam') was unchanged (LOD=1.66). For the QTL for Diff-U, the first (suggestive) QTL is unchanged (LOD=1.49, therefore still suggestive) whilst the second QTL goes from significant to suggestive (LOD=1.70). Given the extreme non-significance of the family covariate and the general lack of changes to the QTL, we believe this shows that family substructure is not a confounding factor in our analysis.

Gene expression analysis

Of the analysed genes, *AVPR2* was significantly differentially expressed in females, where RJF individuals had higher levels than WL birds with a fold change of 1.58 ($F(1,12)=6.34$, $p=0.03$,) (figure 4). In male chickens RJF showed tendencies towards a higher expression of *NRCAM,* with a fold change of 1.59 ($F(1,10)=4.34$, $p=0.07$) and *AVPR1a* with a fold change of 1.24 ($F(1,10)=4.12$, $p=0.08$). None of the other genes were differentially expressed in any of the sexes.

Discussion

Our results show that social preference of familiar over unfamiliar birds may have been modified by domestication in chickens, and the breed effect was most clear in males. Furthermore, the tendency to associate with conspecifics, and to seek social support after a stressful event is affected by loci within a major growth QTL on chromosome 1. This indicates that the QTL-region may contribute to domestication effects on social behaviour and stress coping in addition to its already documented effect on growth and reproduction (Schütz *et al.*, 2004).

The social preference test showed that undisturbed birds preferred to stay close to familiar conspecifics, as has been demonstrated earlier (Väisänen & Jensen, 2003), and a brief stress experience made this tendency stronger, particularly in females. However, purebred RJF males associated more with unfamiliar birds, which may be related to territory defence rather than social affiliations. Seeking of social support is also a well known response to fear and stressful stimuli in other species (Kaiser *et al*, 2003), and the present results may therefore be closely linked to the previously shown effect on fear reactions in this cross (Schütz *et al*, 2001).

After a brief period of restraint stress, this preference increased, in line with the known importance of social support (Kaiser *et al.*, 2003, Kawachi & Berkman, 2001, Kirschbaum *et al.*, 1995). The notable exception was the WL males, which appeared not to be socially

affected by the stress experience. Among the intercross birds, there was a large individual variation in the preference for familiar over unfamiliar birds for this social support, which made it possible to investigate the role of the growth QTL on chromosome 1 in this respect. Although no heritability estimates are known for the exact phenotypes measured here, we have previously reported moderate to high heritability in social reinstatement tendency in chickens, which is a closely related behavioural response (Agnvall *et al*, 2012).

The present level of resolution does not allow us to distinguish whether we are dealing with one single locus or two separate QTL within the examined region, since one of the peaks is only suggestive. This could eventually be resolved with a larger animal material and higher marker density. However, opposite direction of effect of the two QTL peaks for Diff-U suggest the presence of two separate loci. Assuming that this is correct, a QTL peaking at marker 1_36652477 affected the tendency to spend more time with familiar conspecifics before restraint, which may indicate that this locus is related to the preference for social familiarity under undisturbed circumstances. After a stressful episode of restraint, birds with at least one RJF allele at this locus did not change this preference for social familiarity – if they sought social support from conspecifics, they did so from familiar individuals. However, birds with two WL alleles shifted their previous preference for social novelty (which may also be interpreted as high social tolerance) to a preference for familiar birds. It therefore appears that this locus affects social preference as well as support seeking.

The QTL locus at 69-70 cM, however, affected the shift in social preference in response to restraint in the opposite direction: if birds homozygous for a WL allele preferred social novelty under undisturbed circumstances, they did so even more after an episode of stress. This indicates that one or more genes within the confidence interval make WL genotype birds more interested in social novelty and more socially tolerant than RJF genotype birds.

Comparing the additive and dominance effects (a and d) at the two QTL loci affecting Diff-U and Diff-F (the shift in social preference in response to stress), the locus at 149 cM had a greater effect than that the one at 70 cM. If there were little recombination between these two loci, there would still be a small net effect on the behavioural outcome. In the case of WL alleles this net effect would be to make birds shift their social preference towards familiar birds in response to stress. Hence, if birds are selected for being more tolerant to

unfamiliar individuals under non-stressed circumstances (which could be considered adaptive in a captive environment), they would get a small net tendency to shift social preference towards familiar individuals when stressed.

It is interesting to note that the locus peaking at 149 cM was associated with the tendency to seek social contact both before and after stress, whereas the one at 70 cM only affected social behaviour after restraint stress. Hence, the 149 cM locus may be a general sociality associated locus, whereas the 70 cM locus may be more related to stress coping ability and stress recovery.

The QTL-region investigated here has been shown to affect several domestication related traits in chickens, for example growth (Kerje *et al.*, 2003), fearfulness (Schütz *et al.*, 2004), reproduction (Carlborg *et al.*, 2003) sociality (Väisänen & Jensen, 2003) and comb size (Wright *et al.*, 2010). It has been argued that the locus represents an ancient selection signature, perhaps from the early ages of domestication, since the effects of this locus are much less pronounced in QTL-crosses involving different strains of domesticated chickens (Schütz, 2002). Our present results show that the locus also affects social tolerance and support seeking following stress, and regardless of which of the QTL-related phenotypes that were the target of early selection, genetic linkage would cause a correlated response in the rest. Hence, together with earlier studies of the effects of this locus, this shows that a complex of domesticated phenotypes may emerge due to genetic architecture.

The effect of individual loci within the QTL region are not directly reflected in the phenotypic differences between breeds (purebred RJF males spend more time with unfamiliar individuals than other birds do). One possibility is that this is because of spurious QTL effects in a small sample size. However, it could also be that additional fixed loci affect the overall phenotype in RJF and WL, masking variation in the specific region examined in this study - variation that has been freed by several generations of intercrossing.

It is still only possible to speculate about the genes causing the individual QTL found in this study . Some candidates may be identified by visual inspection of gene content in the chromosome region, e.g. *NRCAM*, which is associated with autism in humans (Marui *et al.*, 2009) and impaired sociability in mice (Moy *et al.*, 2009), *Contactin-1*, which may be involved in neuronal development (Chung *et al.*, 2008, Stoeckli, 2010, Suter *et al.*, 1995) and

AVPR2, a homolog of the Arginine vasotocin receptor. In order to provide a first examination of which genes that may be involved in the observed behavioural difference between RJF and WL, gene expression analysis was performed on a number of tentative candidates in the QTL-region. *AVPR2* was significantly differentially expressed, and a tendency was found for *AVPR1a* and *NRCAM*. It should be remembered that the gene expression analysis was performed on a fairly large part of the brain, and this could easily "dilute" a signal originating from differential expression in a smaller area or cell population. These three genes therefore remain candidates for causing the observed effects on behaviour, but other possible candidates need to be examined as well in future experiments.

Arginine vasotocin receptor density in this brain region has previously been found to correlate with gregariousness in a comparison between several species of finches (Goodson *et al.*, 2006). Another vasotocin receptor homolog, *AVPR1a*, is known to affect social behaviour in many other species (Bielsky *et al.*, 2005, Goodson *et al.*, 2006, Walum *et al.*, 2008). It is interesting to note that *AVPR1a* is positioned in the overlapping confidence intervals of the two QTL-regions discovered here, and therefore could be an interesting candidate for both QTL-effects.

At this point, it is not possible to assess with certainty whether the two loci are driven by a single polymorphism or by two or more. One possibility is that a single mutation affects the expression of several genes in the interval, but it is equally likely that there are at least two different mutations, appearing in each of the QTL-regions. This could be addressed by marker assisted selection in a further advanced intercross, where recombination break-up would be even more intense, or by breeding of introgression lines, where small regions of the QTL-locus are bred into a background of either WL or RJF.

The genes in the QTL-region may have been selected independently, or they may be hitch-hiking on some unknown mutation in one of them, or in non-coding parts of the QTL. With the present resolution, it is not possible to resolve which of these suggestions that are more likely. The expression differences could indicate that non-coding regulatory regions have been selected, but the sex differences complicate this suggestion. They may result from different selection pressures in the two sexes, or by genetic effects from trans-located loci (for example on the sex chromosomes). However, the overexpression of *AVPR1a* in males is

in line with what has been observed in other species (for example, Bielsky *et al*, 2005; Walum *et al*, 2008). Phenotypically, we have earlier shown that stress reactions differ between the sexes, where males often have a stronger fear and stress reaction, and this may be an effect of the genetic differences (Campler *et al*, 2009).

In conclusion, the present experiment revealed two loci with significant effects on social preference and social support seeking following stress. Since the QTL-region has earlier been found to affect a range of domestication related traits, the results indicate that changes in social behaviour may be genetically linked to these. *AVPR1a*, *AVPR2* and *NRCAM* are three possible candidate genes for the observed QTL effects.

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Table legends

Table 1. Primers used for genotyping and gene expression analysis.

Tm = melting temperature. TET = tetrachlorofluorescein.

Primer name	Sequence (5'-3')	Tm (°C)
UG0006-for	TET-TGCTTCTTGGCTCATATCTATTCAC	56 (preceded by 6 cycles
UG0006-rev	AGTTTTCCTCCCACCTTTCTTG	touch down 61-56°C)
UG0002-for	TET-AATAACATCTCTTTGAGTTCCACA	52 (preceded by 7 cycles
UG0002-rev	TET-GAACCAATTCAAGTAAAATCTTCTA	touch down 58-52°C)
UG0022-for	TET-ATGCCAGCCTAGAGGAAGC	54 (preceded by 6 cycles
UG0022-rev	AAATGACTCAAAGACTCTGACTCAA	touch down 60-54°C)
MCW0106-for	TET-GGCAACTAAGTTGTGGACTG	50 (preceded by 11 cycles
MCW0106-rev	GCAGCATTCAGTGGGATAAT	touch down 60-50°C)
1_36652477-for	TTTTTGAGCTTAGGATCTGTCAC	51
1_36652477-rev	TCGTTCCTTTCTGGTTCTTACA	
1_37164711-for	GCACGTAGGAATGTGTATTTCCA	54
1_37164711-rev	AACCGAGGCCACATCAGAAG	

Table 2. Means and standard errors of phenotypic values for selected variables at each of six markers.

Diff-U = time spent in zone close to unfamiliar stimulus birds, after-before restraint, Diff-F = time spent in zone close to familiar stimulus birds, after-before restraint, DurB-U = time spent in zone close to unfamiliar stimulus birds, before restraint, DurB-F = time spent in zone close to familiar stimulus birds, before restraint, S.E.M = standard error of the mean.

		DurB-U		DurB-F		Diff-U		Diff-F	
Marker	Genotype	Mean	S.E.M	Mean	S.E.M	Mean	S.E.M	Mean	S.E.M
UG0006	RJF	148,4	33,3	121,5	30,3	-77,7	27,0	71,0	28,8
	HET	143,5	19,4	113,0	18,9	-55,2	23,5	28,1	22,7
	WL	106,1	26,2	149,4	28,2	52,5	41,2	-60,9	37,3
UG0002	RJF	131,1	27,5	132,1	26,5	-70,1	24,8	45,4	27,6
	HET	150,5	21,6	97,4	19,1	-36,1	29,0	20,8	26,9
	WL	117,3	25,9	152,5	27,5	6,5	37,3	-22,4	34,4
UG0022	RJF	133,8	31,9	138,2	29,5	-77,6	31,5	53,1	35,4
	HET	122,0	31,7	136,1	32,9	-96,0	24,6	78,4	29,4
	WL	137,8	18,2	117,3	18,0	-1,9	25,0	-14,1	22,7
MCW0106	RJF	113,6	25,8	138,9	25,0	-31,4	28,9	4,3	30,5
	HET	146,0	22,4	107,6	21,8	-53,2	31,0	33,9	29,8
	WL	147,4	27,8	120,9	27,6	-22,5	39,3	9,7	35,8
1_36652477	RJF	135,5	18,1	114,8	16,9	-25,3	23,3	-1,4	21,1
	HET	98,6	24,6	172,2	26,9	6,2	35,5	-7,1	35,5
	WL	210,9	38,8	59,7	35,8	-140,6	38,5	122,7	40,2
1_37164711	RJF	148,5	26,2	99,4	22,0	-30,6	29,2	0,1	25,4
	HET	114,2	20,6	153,9	23,3	-22,1	31,6	4,3	32,1
	WL	142,4	26,9	119,0	26,8	-43,2	36,2	40,1	33,8

Figure Legends

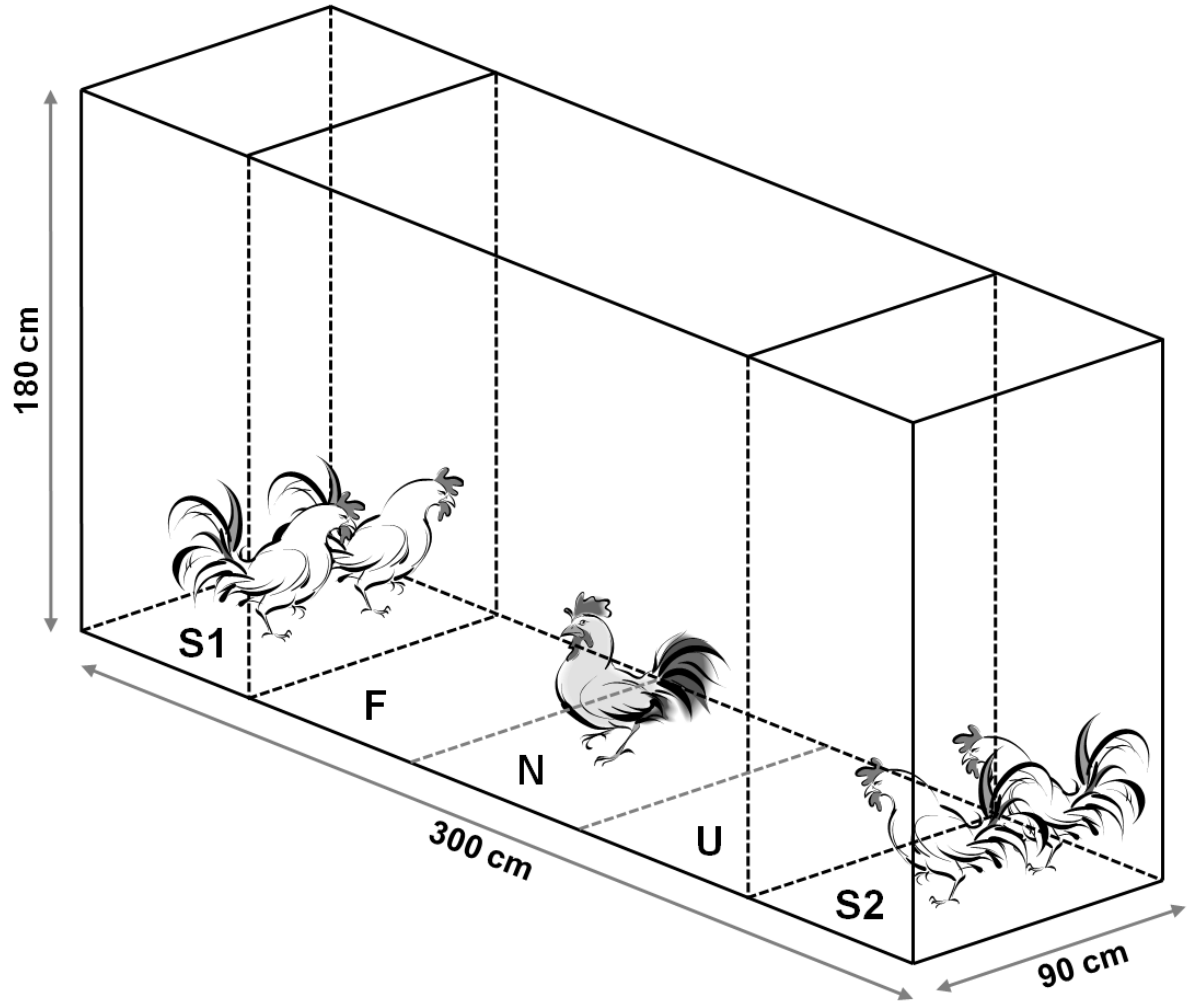

Figure 1. The runway test arena. S1 = familiar stimulus animals, S2 = unfamiliar stimulus animals, F = zone close to familiar stimulus animals, U = zone close to unfamiliar stimulus animals, N = neutral zone.

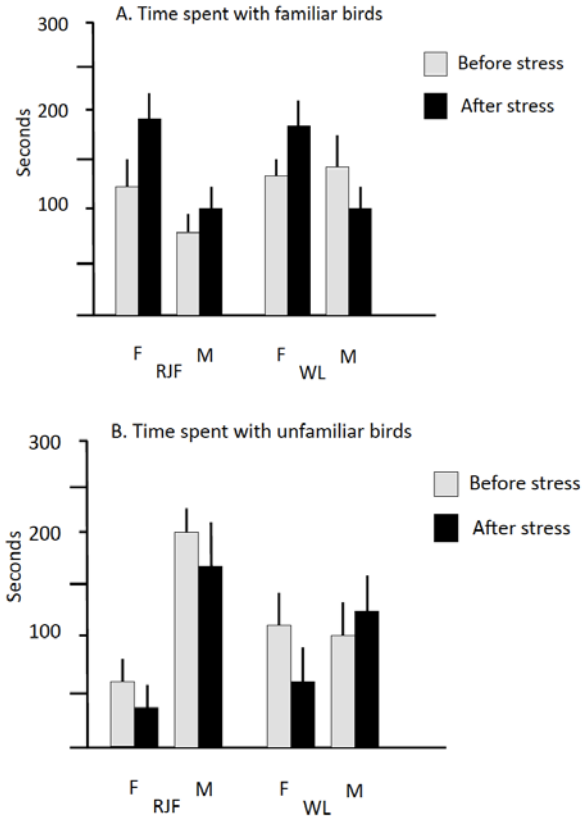

Figure 2. Time spent by purebred Red Junglefowl and White Leghorns close to familiar and unfamiliar birds, before and after restraint. (A) Average nrs of seconds (+ 1 SEM) spent close to familiar birds before and after restraint. (B) Average nrs of seconds (+1 SEM) spent close to unfamiliar birds before and after restraint. RJF= Red Junglefowl, WL= White Leghorn, M=Males, F=Females.

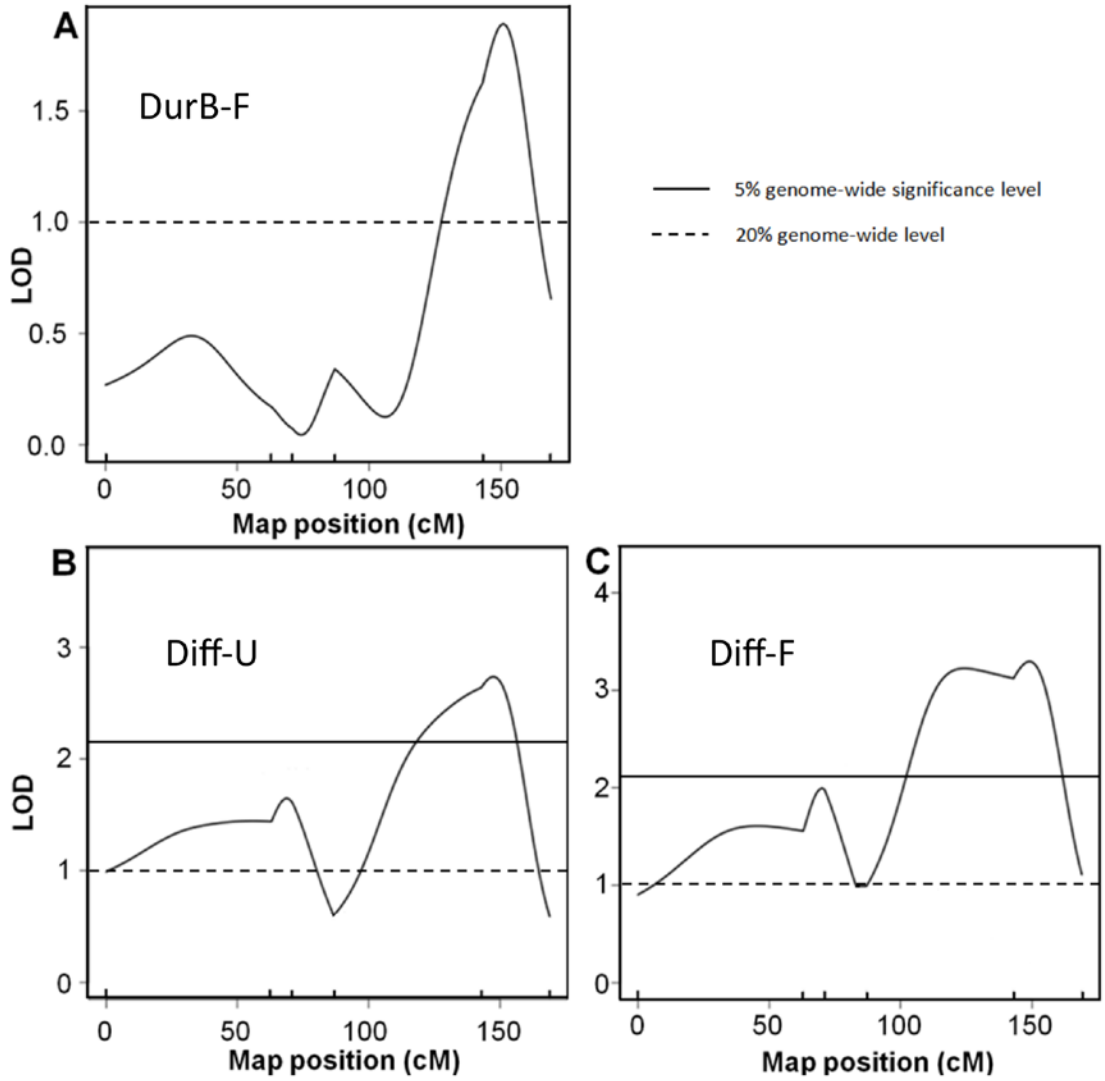

Figure 3. LOD scores for behaviour QTLs. The portion of the chromosome shown here represents the area between markers UG0006 (at 0 cM) and 1_37164711 (at 169 cM). Markers positions are shown as short vertical lines above the x-axis. A) LOD scores for variable DurB-F (time spent in zone close to familiar birds, before restraint) B) LOD scores for variable Diff-U (time spent in zone close to unfamiliar birds, after-before restraint) C) LOD scores for variable Diff-F (time spent in zone close to familiar birds, after-before restraint). Solid vertical line represents the 5% genome-wide significance level, dashed line represents the 20% genome-wide suggestive level.

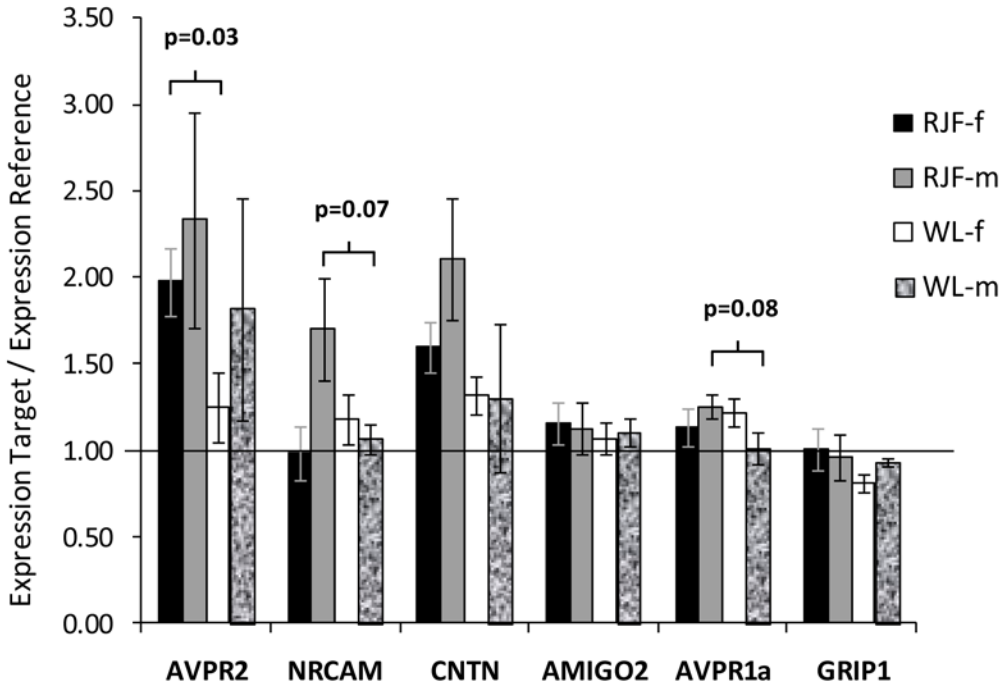

Figure 4. Relative expression levels of six genes in the Growth 1 QTL-region. Means and standard errors for each breed and sex, normalized to the mean expression level of GAPDH and TBP (horizontal line at 1.00). RJF-f: Red Junglefowl females, RJF-m: Red Junglefowl males, WL-f: White Leghorn females, WL-m: White Leghorn males.