Genetics and Genomics of Social Behavior in a Chicken Model

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ABSTRACT The identification of genes affecting sociality can give insights into the maintenance and development of sociality and personality. In this study, we used the combination of an advanced intercross between wild and domestic chickens with a combined QTL and eQTL genetical genomics approach to identify genes for social reinstatement, a social and anxiety-related behavior. A total of 24 social reinstatement QTL were identified and overlaid with over 600 eQTL obtained from the same birds using hypothalamic tissue. Correlations between overlapping QTL and eQTL indicated five strong candidate genes, with the gene *TTRAP* being strongly significantly correlated with multiple aspects of social reinstatement behavior, as well as possessing a highly significant eQTL.

KEYWORDS behavior; eQTL; QTL; sociality

IDENTIFICATION of the genes responsible for behavioral variation has numerous ramifications, ranging from medical research to evolutionary theory on personality syndromes. The identification of these genes is challenging for the same reasons as for all complex traits, and additionally because of the problems involved in defining and measuring behavioral traits. Quantitative trait loci (QTL) mapping has been successful in finding QTL associated with numerous behavioral traits, but isolating the actual genes underlying these QTL has been considerably more difficult (reviewed by Flint (2003)).

Sociality is an extremely diverse behavioral category and can range from communication behavior to the interactions between individuals of the same (and even different) species. Examples can range from dogs seeking out human contact and support, to honeybee foraging strategies and nursing behavior. Several genetic mapping studies have found associations with social behavior in mammals (Brodtkin *et al.* 2002; Donaldson and Young 2008; McGraw and Young 2010; Takahashi *et al.* 2010; Persson *et al.* 2016; vonHoldt *et al.* 2017), fish (Wright

et al. 2006a,b; Kowalko *et al.* 2013; Greenwood *et al.* 2016), and fruit flies (Wu *et al.* 2003; Shorter *et al.* 2015). A few genes have been found that determine certain aspects of sociality. For example *Avpr1*, a vasopressin receptor gene, is involved in promiscuity in voles (Lim *et al.* 2004) and social behavior in dogs (Kis *et al.* 2014). The neuropeptide receptors *npr-1* (de Bono and Bargmann 1998) and *exp-1* (Bendesky *et al.* 2012), as well as the sensory globin gene *glb-5* (McGrath *et al.* 2009), all regulate aggregation propensity in nematodes. The *Gp-9*-linked supergene affects colony organization in fire ants (Krieger and Ross 2002; Wang *et al.* 2013). The *for* gene protein kinase affects behavioral maturation in social insects (Ingram *et al.* 2005), while alterations in behavior in the honeybee are controlled via insulin/insulin-like growth factor (Ament *et al.* 2010) and juvenile hormone (JH). JH also has numerous other effects on social behavior in insects (Bloch *et al.* 2009). Other pathways relating to insect sociality are reviewed in Weitekamp *et al.* (2017).

Social reinstatement, the tendency of an animal to seek out conspecifics, is both a sociality and anxiety-related behavior (Mills and Faure 1991). It has been classically used in a variety of bird species to measure the strength of sociality at a base level, typically using a runway or treadmill test to assess an animal's social motivation (Suarez and Gallup 1983; Jones *et al.* 1991). Given the links to anxiety, the question of how

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doi: <https://doi.org/10.1534/genetics.118.300810>

Manuscript received February 9, 2018; accepted for publication March 4, 2018; published Early Online March 12, 2018.

Supplemental material is available online at www.genetics.org/lookup/suppl/doi:10.1534/genetics.118.300810/-/DC1.

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social reinstatement relates to sociality in general is pertinent. In the case of the social reinstatement assay, perhaps the largest body of works concerns two lines of Japanese quail selected for high and low social reinstatement. These selected bird lines have then been assessed for a wide variety of social assays to determine the extent to which selection for social reinstatement can affect other aspects of sociality. Launay *et al.* (1991) found that high social reinstatement birds spent longer associating with conspecifics when given a paired goal box (one box empty, one box containing conspecifics). Two more studies found that when looking at pairs of high social reinstatement individuals in an open field arena they had a significantly shorter interindividual distance as compared to the low social reinstatement birds (Mills *et al.* 1992). High social reinstatement birds will even associate with conspecifics at the expense of food and water access and will also use social facilitation to learn to eat a novel food source by copying a conspecific “teacher” (Mills *et al.* 1997). Furthermore, high social reinstatement birds show a nonspecific attraction for social conspecifics (Schweitzer *et al.* 2009) and have a consistently stable emotional reactivity, even in the face of high social instability (Schweitzer and Arnould 2010). The above studies therefore indicate that such social reinstatement tests do measure a component of sociality, but that anxiety-related behavior is also involved in the assay.

In this study we identify a number of putative quantitative trait genes underlying phenotypic differences in a social reinstatement behavioral assay between Red Junglefowl and domesticated White Leghorn chickens. Chickens are a social species that typically live in groups of between 6 and 10 individuals in the wild and display a range of social behaviors (Johnson 1963). The domestic chicken exhibits a wide range of behavioral as well as morphological differences, as compared to its wild-derived progenitor, the Red Junglefowl. This includes anxiety, sociality (Schütz *et al.* 2001), and feather-pecking behavior, among others (Jensen and Wright 2014). Previous behavior QTL work in the chicken has included mapping of feather-pecking behavior (Buitenhuis *et al.* 2003) and open field behavior (Buitenhuis *et al.* 2004) in layer chickens as well as fear-related behaviors and feeding behavior in the F₂ generation of the intercross line used in this work (Schütz and Jensen 2001; Schütz 2002; Schütz *et al.* 2002, 2004). The study presented here continues on from this work using an advanced intercross to generate small confidence intervals for mapping (Darvasi 1998). This intercross has already been used to identify genes affecting anxiety-related behavior in an open field test (Johnsson *et al.* 2016).

We use a three-phase approach where we map social reinstatement QTL and expression QTL for hypothalamic gene expression, and then integrate them to search for candidate quantitative trait genes. By integrating QTL and eQTL analysis in the same cross, we can identify eQTL that overlap phenotypic QTL, and correlate gene expression with the trait values in the same individuals. This can identify genes that exhibit a close correlation with the phenotypic trait and underlie a particular QTL. In the first phase, we performed QTL mapping

of a social reinstatement behavior ($n = 572$) in an eighth generation advanced intercross of Red Junglefowl $\times$ White Leghorn chickens. In the second phase, a subset of the cross tested for behavior ($n = 129$) that has already been used in an expression QTL (eQTL) mapping of hypothalamic gene expression using a 135k probe array for each of these individuals, was used to overlap the previously detected eQTL with the newly detected behavioral QTL. Any gene that overlapped with a behavioral QTL was then checked for a correlation between gene expression and the behavioral trait in question and finally used in causation analysis using a Network Edge Orientation approach. In addition to this, genome-wide gene expression data were also correlated directly with the social reinstatement trait values. This allowed us to assess genes with a high correlation with the behaviors, regardless of location, and also assess gene regulatory networks involving all those genes that were correlated with behavior.

Materials and Methods

Chicken study population and cross design

The intercross population used in this study was an eighth generation intercross between a line of selected White Leghorn chickens maintained from the 1960s and a population of Red Junglefowl originally from Thailand (Schütz *et al.* 2002, 2004). One male Red Junglefowl and three female White Leghorn were used to found the intercross and generate 41 F₁ progeny. The intercross was maintained at a population size of ~ 100 birds per generation until the F₇ generation. The F₂ intercross has previously been used to identify QTL for a number of different behavioral, morphological, and life history traits (Schütz *et al.* 2002; Kerje *et al.* 2003; Wright *et al.* 2008, 2010, 2012). A total of 572 F₈ individuals were generated from 118 families using 122 F₇ individuals (63 females and 59 males) and assayed for social reinstatement behavior. Average family size was 4.76 ± 3.1 (mean, SD) in the F₈. One hundred twenty-nine of the 572 F₈ were used in an eQTL experiment, with the hypothalamus/thalamus dissected out at 212 days of age, RNA extracted, and run individually on a 135k probe microarray [see Johnsson *et al.* (2016)]. For further details on feed and housing see Johnsson *et al.* (2012).

Ethics statement

The study was approved by the local Ethical Committee of the Swedish National Board for Laboratory Animals.

Phenotyping

Social reinstatement assay: The social reinstatement test, or “runway test” (Suarez and Gallup 1983), measures social coherence and anxiety, with stressed chicks exhibiting a stronger social cohesion response (Marin *et al.* 2001). The individual is placed at one end of a narrow arena, with conspecifics located at the far end. The amount of time the focal individual spends associated with the conspecifics as opposed to exploring the remainder of the arena is considered a measure of sociality/anxiety. A more social (or potentially more

anxious) animal will spend more time associating with conspecifics, will approach the conspecifics more rapidly (decreased latency to approach), and will spend less time in the start zone of the arena (Marin *et al.* 2001).

Trials were performed in a 100 cm × 40 cm arena. The stimulus zone measured 20 cm × 40 cm and was adjacent to a wire mesh compartment containing three unfamiliar conspecific birds of the same age. The start zone (where the birds were placed prior to the start of the experiment) also measured 20 cm × 40 cm. Birds were placed in the start zone of the arena in the dark, prior to the lights being turned on and the trial beginning. Eight separate arenas were available, allowing up to eight individuals to be analyzed simultaneously. Measurements were taken using the Ethovision software and continuous video recording (Noldus Information Technology, www.noldus.com). For each trial, total distance moved, velocity, length of time spent in the stimulus zone (adjacent to the three conspecific birds), latency to first enter the stimulus zone, and length of time in the start zone (the starting position of each bird, farthest away from the stimulus zone) were measured.

Trials were replicated twice per individual, with each trial being 5 min in length. Trials were performed at 3 weeks of age. There was ~1 week between an individual's first and second test. Individuals were immediately removed from the arena upon the completion of the test to reduce potential habituation. Repeated testing reduces environmental noise and allows additional variables to be extracted for each individual. For four measures (time in start zone, time in stimulus zone, latency to enter the stimulus zone, total distance moved), we performed QTL mapping of the value in trial 1, the value in trial 2, the average value of the two trials, the minimum value, and the maximum value. We also performed QTL mapping of total velocity in trial 1 and total velocity in trial 2, for a total of 22 traits.

Correlations between the two trials were found to be extremely significant (see Supplemental Material, Table S1 in [File S1](#)), indicating that the tests were strongly repeatable. Similarly, there were strong correlations between different traits measured during the tests (see Table S1 in [File S1](#)). In total, 217 of the 231 pairwise Spearman correlations between the 22 different traits derived from the two social reinstatement trials were significant. A Principal Component Analysis (PCA) of these 22 traits found two significant eigenvalues, with the first explaining 78% of the variation present in the data and the second explaining 21% of the variation in the data. These two principal components (PCs) were also used as traits. Figure S1 in [File S1](#) shows the distributions of the traits. Traits were nonnormally distributed, and no outliers were present.

The hypothalamus and its role in sociality: The hypothalamus is one of several regions involved in the regulation of social behavior and sociality, and therefore was selected as the basis for an expression QTL mapping experiment for use in this study [eQTL are reported previously in Johnsson *et al.* (2016), which also contains details of extraction, dissection, and the

arrays used]. It has a central role in the distribution of several key neurotransmitters involved in sociality (Donaldson and Young 2008). Oxytocin is one of the most well known of these neurotransmitters and is known to affect multiple social behavior phenotypes (Carter *et al.* 2008). Oxytocin emerges from the paraventricular and supraoptic nuclei in the hypothalamus, with the hypothalamus being the primary oxytocinergic region, along with the amygdala (Lee *et al.* 2009). The neurotransmitters arginine vasotocin and arginine vasopressin are also regulated in the hypothalamus and correlate with sociality in birds (Goodson 2008). Neuroimaging shows differences in hypothalamus structures between carriers of OXTR risk alleles correlates with social temperament in humans (Tost *et al.* 2010). In birds, differences in sociality as measured by immediate early gene responses were found in both the extended medial amygdala and the hypothalamus (Goodson *et al.* 2005).

Genotyping, QTL, and eQTL mapping: DNA preparation was performed by Agowa (Berlin, Germany), using standard salt extraction. A total of 652 SNP markers were used to generate a map of length ~9267.5 cM, with an average marker spacing of ~16 cM [see Johnsson *et al.* (2012) for a full list of markers]. QTL analysis was performed using the R/qtl software package (Broman *et al.* 2003), with standard interval mapping and epistatic analyses performed. Interval mapping was performed using additive and additive + dominance models. In the behavioral QTL analysis, batch, sex, and arena were always included as covariates. In addition, body weight measured at 42 days of age was also included as a covariate as a precaution in case size affected movement speed (though appeared to be nonsignificant). A PCA of the first 10 principal components of the genotypic data were fitted to account for population substructure [see Johnsson *et al.* (2016) for details], with all significant principal components retained in the final model (the final number retained varying from trait to trait). A sex-interaction effect was added, where significant, to account for a particular QTL varying between the sexes. Digenic epistatic analysis was performed according to the guidelines given in Broman and Sen (2009). A global model was constructed for each trait that incorporated standard main effects, sex interactions, and epistasis. The most significant loci were added to the model first, followed by the less significant loci.

eQTL mapping was performed on the cross using R/qtl, as has already been documented previously (Johnsson *et al.* 2016). A local, potentially *cis*-acting, eQTL (defined as a QTL that was located close to the target gene affected) was called if a signal was detected in the closest flanking markers to the gene in question, to a minimum of 100 cM around the gene (*i.e.*, 50 cM upstream and downstream of the gene). A distance of 50 cM was used to ensure that at least two markers up and downstream from the gene location were selected to enable interval mapping to be performed. The actual physical distance that corresponds to 50 cM varies depending on the chromosome and location but would

typically be ~5 Mb. The *trans*-eQTL scan encompassed the whole genome and used a genome-wide empirical significance threshold. In total, 535 local eQTL and 99 *trans*-eQTL were identified previously.

Significance thresholds: Significance thresholds for the social reinstatement QTL analysis were calculated by permutation (Churchill and Doerge 1994; Doerge and Churchill 1996). A genome-wide 20% threshold was considered suggestive [with this being more conservative than the standard suggestive threshold (Lander and Kruglyak 1995)], while a 5% genome-wide level was significant. The ~5% significant threshold was LOD ~4.4, while the suggestive threshold was ~3.6. To account for the number of phenotypes that were tested and the potential issue of multiple testing correction, a combined permutation test was performed that tested all 22 behavioral traits simultaneously. During each round of permutation, the highest LOD score generated from these 22 traits was retained, with a total of 1000 permutations being performed. This led to a suggestive threshold of 3.9, and a significant threshold of 4.8. Confidence intervals for each QTL were calculated with a 1.8 LOD drop method (*i.e.*, where the LOD score on either side of the peak decreases by 1.8 LOD), with such a threshold giving an accurate 95% confidence interval for an intercross type population (Manichaikul *et al.* 2006). The nearest marker to this 1.8 LOD decrease was then used to give the confidence intervals in megabases. Epistatic interactions were also assessed using a permutation threshold generated using R/qtl, with a 20% suggestive and 5% significant genome-wide threshold once again used. In the case of epistatic loci, the approximate average LOD significance threshold for pairs of loci were as follows [using the guidelines given in Broman and Sen (2009)]: full model ~11, full vs. one ~9, interactive ~7, additive ~7, additive vs. one ~4.

Analysis of candidate genes (eQTL genes falling within QTL intervals): Significant social reinstatement QTL were overlapped with the previously identified eQTL, and all significant eQTL genes were then tested as candidate genes for the specific social reinstatement QTL with which they overlapped. These candidate genes were then modeled using the gene expression value on the behavioral trait for the QTL of interest. For example, if an eQTL overlapped a QTL for time spent in the stimulus zone, the eQTL gene expression trait would be correlated with the amount of time spent in the stimulus zone. The linear model for this analysis used the behavioral trait as the response variable and the expression trait as the predictor, and included sex and batch as factors, and weight at 42 days as a covariate. The *P*-values for the regression coefficient were Bonferroni corrected for the number of uncorrelated eQTL in the QTL region. eQTL that were present within a QTL confidence interval and that were also significantly correlated with the QTL trait were then considered to be candidates. Network Edge Orientation (NEO) analysis and conditional QTL models were then used to

further assess causality (see below). One issue with this approach is that the behavioral QTL were based on up to 572 individuals, whereas the eQTL/expression phenotypes were only available for 129 individuals. Therefore, causality testing was only applied where the behavioral QTL was detectable in the smaller data set ($n = 129$).

Causality analysis consisted of a conditional genomic QTL scan and Structural equations modeling with the NEO software (Aten *et al.* 2008). For the conditional genomic QTL scans we fit models of the form *behavior trait* = *QTL* + *gene expression trait* + *covariates* + *error* and compare it with *behavior trait* = *QTL* + *covariates* + *error* to test whether the inclusion of gene expression as a covariate reduces the QTL effect. If there is a causal relationship, the gene expression trait should act as a mediator of the QTL effect (Le Bihan-Duval *et al.* 2011; Leduc *et al.* 2011). In essence, both the QTL genotype factor and the gene expression are explaining the same variation for the behavior in the model, so the inclusion of both should weaken the genotypic effect. We illustrate this by showing the fold change decrease in the *P*-value of the QTL factor in the linear model when the gene expression covariate is included. In addition, the Akaike Information Criterion (AIC) was calculated for each model. However, this gives the fit of the overall model in each case, so it is less useful than the specific fold change of the QTL genotype factor in the model for the purposes of causality testing.

Single-marker analysis was performed with NEO comparing a causal model (where the genotype affects behavior by means of changing gene expression) to four alternative models, reflecting other possibilities:

- CAUSAL: Genotype modifies gene expression which in turn modifies behavior (genotype → expression trait → behavior).
- REACTIVE: Genotype modifies behavior which in turn modifies the expression trait (genotype → behavior → expression trait).
- CONFOUNDED: Genotype modifies both the expression trait and the behavior separately (expression trait ← genotype → behavior).
- COLLIDER (behavior is the collider): Genotype and the expression trait both independently modify behavior (genotype → behavior ← expression trait).
- COLLIDER: (expression is the collider): Genotype and behavior both independently modify the expression trait (genotype → expression trait ← behavior).

The NEO software uses the *P*-value associated with a χ^2 statistic as an index of model fit. A higher *P*-value indicates a better fitting model. The support for the causal model is described by a ratio of its *P*-value to the *P*-value of the second best model. As a score, NEO uses the logarithm (base 10) of this ratio, called the local edge orienting (leo.nb) score. A positive score indicates that the causal model fits better than any competing model. Aten *et al.* (2008) use a single-marker score of 1, corresponding to a 10-fold higher *P*-value of the causal model, as their threshold. They also suggest users to inspect the

P-value of the causal model to make sure the fit is good. If this *P*-value is nonsignificant ($P >> 0.05$), this indicates that only the causal model fits the data (other models are rejected). A significant *P*-value ($P < 0.05$) despite a high *leo.nb* score would mean that none of the models fit the data very well. For each gene, we report *leo.nb* score and *P*-value of the causal model.

Global gene expression correlations with behavior: A further analysis was performed for each behavioral trait whereby global gene expression (each gene, in turn, covering all 36,000 probesets) was correlated with behavior. We used a linear model with the behavior trait as response variable and expression, sex, and batch as predictors. To control for the large numbers of probes tested, we performed a permutation test. For any given behavior, the behavioral variable was permuted, with this permuted phenotype then tested against all 36,000 probesets. The top 0.1% value was then retained from this permutation. A total of 500 permutations were performed for each behavioral measure. The top 5% of these generated an experiment-wide threshold for significance of $\sim 4 \times 10^{-4}$ for individual traits. To account for multiple testing of behavioral traits, a similar procedure to the QTL mapping threshold estimation was used. In this case, all 22 traits were permuted simultaneously, with the highest LOD score from any of the 22 traits retained during each round of permutation. Five hundred permutations were performed, with a 5% experiment-wide cut-off then giving a threshold of 2.3×10^{-5} . Therefore, this value was used to give an experiment-wide significant value, while the individual cut-offs (*i.e.*, the permutations based on individual traits) were used to give an experiment-wide suggestive value.

Gene network

We constructed a binary correlation network of the correlational candidate genes. We used a threshold of a pairwise Pearson correlation coefficient of 0.50. All probesets that were significantly correlated with one or more social reinstatement behavior were included, as well as the five candidates that were identified using the combined QTL/eQTL overlap and trait correlation. We used the R package igraph (v1.0.1) for network visualization.

Pleiotropy vs. linkage tests: We used qxpak 5 (Pérez-Enciso and Misztal 2011) to perform pairwise pleiotropy vs. linkage tests of overlapping behavior QTL. A model with two separate QTL was compared with one with a single pleiotropic QTL, using a likelihood ratio test, with the χ^2 distribution with two degrees of freedom. Nominal *P*-values are reported, a low *P*-value signifying the rejection of the pleiotropic model.

Data availability

Microarray data for the chicken hypothalamus tissue are available at E-MTAB-3154 in ArrayExpress. Full genotype and phenotype data are available on figshare with the following doi: 10.6084/m9.figshare.1265060.

Results

Social reinstatement QTL

We performed quantitative trait locus mapping of behavior in the social reinstatement test in an advanced intercross of Red Junglefowl and White Leghorn chickens. The 22 different measures of sociality from the social reinstatement test were mapped individually. By combining these overlapping QTL, a total of 24 social reinstatement QTL were identified (see Figure S2 and Table S2 in File S1), spread over 16 chromosomes. The average variance explained was 4%. The average confidence interval for the behavioral QTL was ~ 3 Mb, indicating that the advanced intercross generated far tighter confidence intervals than a standard F_2 intercross. F_2 QTL intervals in the same cross for behavioral traits were typically over 10 Mb in length (Wright *et al.* 2010).

There was a strong degree of overlap between the QTL and those previously identified for open field measures, most notably on chromosomes 2 and 10 (see Table S2 in File S1). Pleiotropy vs. linkage tests indicated that the cluster on chromosome 2 is the result of linkage (*i.e.*, the social reinstatement and open field QTL are separate but linked, LR test statistic = 8.11, $P = 0.017$, pleiotropy rejected), while the cluster on chromosome 10 at 99 cM was inconclusive (LR test statistic -0.21 , $P < 0.05$, pleiotropy and close linkage were indistinguishable).

eQTL candidate genes within QTL intervals

Five genes are plausible candidates for causative genes, based on the presence of an eQTL and the correlation between gene expression and behavior. In total, eQTL for 139 genes overlapped social reinstatement behavior QTL. There were five genes that had an eQTL overlapping a QTL interval, and a significant correlation between gene expression and trait value (see Figure 1 and Table 1). These five genes represent four different QTL regions (on chromosomes 1, 2, and 10). Of the significant candidates, *TTRAP* was very strongly correlated ($P < 0.001$) with two of the behavioral traits (see Figure 2 and Table 1), and significantly correlated ($P < 0.05$) with three more traits, and is therefore the strongest candidate for the behavioral QTL on chromosome 2 at 658 cM. Three different genes were correlated with the QTL “minimum time spent in the start zone” on chromosome 1 at 1417 cM – *ACOT9*, *SRPX*, and *PRDX4*. This behavioral QTL (minimum time in start zone) on chromosome 1 also had an epistatic interaction with a second QTL on chromosome 10 at 138 cM. Interestingly, there is a cis eQTL for *ACOT9* on chromosome 1, as well as a trans-eQTL for *ACOT9* on chromosome 10. This trans-eQTL locus on chromosome 10 also overlaps the “minimum time in start zone” QTL. Therefore, *ACOT9* expression not only correlates well with this QTL, but also has both local and trans-eQTL at the same locations as the social reinstatement QTL. For the QTL “minimum time in the start zone” on chromosome 2 at 505 cM, two genes were candidates – *TTRAP* and the probeset based on the EST 603866246F1.

Figure 1 LOD profiles for *TTRAP*, *ACOT9*, and *PRDX4* and their associated social reinstatement behaviors. Map distance in centimorgans is shown on the x-axis, with LOD score shown on the y-axis. Colored bars below the x-axis indicate the confidence interval of each QTL. QTL significance thresholds are marked with horizontal lines (orange for behavioral QTL, black for eQTL).

Table 1 Candidate genes and causality scores

Trait	QTL chromosome and position (cM)	eQTL	Gene		QTL P-value	Fold change QTL		NEO				
			P-value	QTL		model	P-value	AIC.gene	AIC.QTL	AIC.combined	leo.n score	Model P-value
SR_minimum_time_in_start_zone	chr 1 - 1417	ACOT9	0.005**		0.004**	5		-72.34	-75.36	-75.66	0.736	0.316
	chr 1 - 1417	SRPX	0.008**		0.004**	3		-70.96	-75.36	-75.66	0.595	0.229
	chr 1 - 1417	PRDX4	0.02*		0.004**	5		-69.12	-75.36	-73.82	0.777	0.348
SR_minimum_time_in_start_zone	chr 10 - 138	ACOT9	0.009**		0.1	6		-71.88	-66.2	-69.26	1.1*	0.455
SR_minimum_time_in_start_zone	chr 2 - 505	TTRAP	0.008**		0.06	4		-74.64	-67.12	-73.4	1.17*	0.672
SR_minimum_time_in_start_zone	chr 2 - 505	603866246F	0.006**		0.06	2		-75.56	-67.12	-76.16	1.2*	0.26
SR_time_in_start_zone_average	chr 2 - 657.6	603866246F	0.03*		0.002**	3		-70.5	-75.4	-77.08	0.68	0.539
SR_latency_to_enter_stimulus_zone_average	chr 2 - 658	TTRAP	0.02*		0.01**	5		-24.04	-24.8	-26.48	0.74	0.663
SR_latency_to_enter_stimulus_zone_trial2	chr 2 - 657.6	TTRAP	0.04*		0.05*	2		-21.74	-17.9	-22.34	0.85	0.772
SR_maximum_time_in_start_zone	chr 2 - 657.6	TTRAP	0.001**		0.006**	5		-54.4	-50.56	-57.76	1.17*	0.672
SR_time_in_start_zone_average	chr 2 - 658	TTRAP	0.0002**		0.002**	5		-80.16	-74.94	-85.36	1.21*	0.935

The behavioral QTL location (chromosome) and position (in cM) is provided. Gene P-value shows the significance between gene expression and behavioral trait (corrected for multiple tests). QTL P-value indicates the significance between the genotype and the behavioral trait for this reduced sample size subset, calculated during causality modeling. Fold change indicates the degree to which the QTL significance drops upon inclusion of the gene expression covariate, with a higher value indicating the greater potential for causation between gene and behavior. The AIC is also shown for the model predicting behavior incorporating just gene expression, just the genotype, and both. The NEO causality score (leo.nb) and NEO model P-value are also shown. A leo.nb score above 1 is considered to be significant, while a model P-value >0.05 indicates that the causal model is the most significant. All significant values are indicated with an asterisk (* indicates a P-value significant to $P < 0.05$, ** indicates a P-value significant to $P < 0.01$).

Causality testing of candidate genes

Conditional QTL models and structural equations modeling with the Network Edge Orientation software support candidate genes *TTRAP*, *ACOT9*, and *PRDX4* as potentially causative (Table 1, leo.nb scores >1 , P-value $>> 0.05$, fold change >5). For the QTL “minimum time in the start zone” on chromosome 1 locus, conditional modeling results support *ACOT9* (fold change = 5) and *PRDX4* (fold change = 5). NEO analyses were not significant for *ACOT9*, *PRDX4*, or *SRPX* at this locus, but were higher for *ACOT9* (leo.nb = 0.736) and *PRDX4* (leo.nb = 0.777). *SRPX* had even less support from the NEO analysis and is not consistent with causality at this locus (leo.nb = 0.595, fold change = 3). The QTL “minimum time in the start zone” on chromosome 10 has support for *ACOT9* from both methods (leo.nb = 1.1, fold change = 6). The QTL on chromosome 2 for “maximum time in start zone” and “average time in the start zone” both show support for the gene *TTRAP* as being causal, with the strongest support from the NEO analysis for a causal effect (leo.nb 1.17 and 1.21, respectively) for any of the genes considered here. In comparison, one other probe on chromosome 2 also had a correlation with time in the start zone, 603866246F1; however, NEO only supported this probeset for the QTL “minimum time in the start zone” (leo.nb = 1.2, fold change = 2) and not for the QTL “average time in the start zone” (leo.nb = 0.68, fold change = 3).

Correlations between gene expression and social reinstatement

A total of 61 genes were significantly or suggestively correlated at an experiment-wide level with a variety of different measurements from the social reinstatement test (see Table S3 in File S1), with 11 of these genes correlating with multiple traits. Of these 61 genes, 11 fall within the confidence interval for one of the behavioral QTL and are thus also putative candidate genes (*ANKRD29*, *CALB2*, *Gga.50063*, *HERPUD1*, *RDM1*, *SEPN1*, *TMEM57*, *TTRAP*, *TYMS*, *ENSGALG00000007103*, and *EST* probeset 603602419F1). With the exception of *ANKRD29*, the behavioral trait QTL overlap was different from the actual trait that was correlated with the gene in this analysis (see Table 2). The gene *TTRAP* possessed an eQTL as well as overlapped with several behavioral QTL, whereas none of the other 11 genes had both an eQTL and overlapped with behavioral QTL. A gene network was constructed using all 61 of the genome-wide significantly or suggestively correlated genes, plus the four candidate genes (see Figure 3). The gene *TTRAP* had a total of five connections, with several other candidates also having relatively high numbers of connections (see Figure 3, *ANKRD29* 12 connections, *SEPN1* 17 connections, *TMEM57* 21 connections, *TYMS* 4 connections, *CALB2* 12 connections, *HERPUD1* 8 connections, *Gga.50063* 8 connections).

Discussion

Using a combination of QTL and eQTL mapping, followed by further correlations and causality analyses, we have identified a number of high confidence candidate genes that affect social

Figure 2 Scatter plot of the average time in the start zone (note that residuals are plotted to control for sex and batch effects) against *TTRAP* expression. Correlation coefficient = 0.76.

reinstatement, a social and anxiety-related behavior. Of the candidates, the five genes with the highest support and overlapping QTL and eQTL (*TTRAP*, *PRDX4*, *ACOT9*, *SRPX*, and 603866246F1) all showed a correlation between gene expression and behavior and had evidence of causality. Three of these genes (*TTRAP*, *PRDX4*, and *ACOT9*) have previously been identified as affecting either neuronal development or behavior. Ten other candidate genes were also identified; these genes were significantly correlated at a genome-wide level with an aspect of social reinstatement behavior and also overlapped a QTL interval. However, the QTL trait was different from the correlative trait for these 10 genes, so causality testing could not be used, and these genes therefore have less support as candidates.

Three of the principal candidate genes (*ACOT9*, *PRDX4*, and *TTRAP*) have known links with anxiety and stress behavior, cognitive processes, and neurogenesis and degeneration (Poletto *et al.* 2006; Levine *et al.* 2013; Gómez-Herreros *et al.* 2014). The best candidate, based on a wide number of measures, is the gene *TTRAP*. This was identified in both the QTL/eQTL overlap and had the highest correlation between trait and gene expression of any of the five main candidates. In addition, the causality tests (both conditional mapping and NEO) both indicate it as being the best candidate gene. It is correlated with numerous aspects of social reinstatement behavior. *TTRAP* is a DNA phosphodiesterase, involved in DNA repair (Zeng *et al.* 2011). It has associations with early-onset Parkinson's disease (Zucchelli *et al.* 2008) and might serve in a protective role against neurodegradation. Its human ortholog (*TDP2*) is required for normal neural function (Gómez-Herreros *et al.* 2014). *PRDX4*, a cytoplasmic antioxidant

enzyme, is linked with stress and social isolation in piglets (Poletto *et al.* 2006), anxiety-related measures in mice after treatment with paroxetine (an antidepressant) (Sillaber *et al.* 2008), and with atypical frontotemporal lobar degeneration in humans (Martins-de-Souza *et al.* 2012). Both *TTRAP* and *PRDX4* are involved in the cellular response to reactive oxygen species and in the regulation of NF κ B signaling (Jin *et al.* 1997; Pye *et al.* 2000). NF κ B signaling, in turn, is connected both to neurogenesis in response to stress (Madrigal *et al.* 2001; Koo *et al.* 2010) and to contextual fear memories (Lubin and Sweatt 2007). *ACOT9* is likely an enzyme involved in hydrolyzing acyl-coenzyme A thioesters. Loss of function of Acyl-CoA thioesterases may be involved in neuronal degradation (Kirkby *et al.* 2010). Of the additional 10 genes identified using global correlations, *CALB2* has previously been linked with schizophrenia and neuronal growth (Arion *et al.* 2010), while *HERPUD1* has also been linked with neuronal apoptosis (Ding *et al.* 2017) and *TMEM57* has been associated with neurocognitive impairment in Alzheimer's disease (Levine *et al.* 2013).

How causal genes interact with other genes in networks is often complex. It is still not clear if such causal genes act as "hubs" (i.e., connected/correlated with many other genes) or if they act more in isolation. Many of the candidates discovered in the study presented here had a relatively high connectivity, though the best candidate *TTRAP* had a low connectivity. Gene networks can be highly conserved, and genes with a high number of connections within these networks can potentially be less evolutionarily labile [see review in Weitekamp *et al.* (2017)]. For example, honeybee transcriptional regulatory networks that are highly correlated are under strong negative selection (Molodtsova *et al.* 2014), while those with the least connections, when comparing over multiple tissue types, are under positive selection (Jasper *et al.* 2014). Gene networks can also correspond to specific phenotypes over multiple species, for example such traits as worker sterility and queen number are controlled by similar networks over multiple different ant species (Morandin *et al.* 2016). In the gene networks presented here the relatively high connectivity for some genes may indicate that their effects could be conserved over different species. The candidate gene *TTRAP* had relatively few connections, so it is potentially easier to modify its expression without perturbing a preexisting highly conserved network.

The genetic architecture of behavior and its association with behavioral syndromes (where multiple character aspects are correlated across different situations) is yet to be fully explored. In this study, animals were measured for both social reinstatement and open field activity. Although the open field arena has long been considered as a measure of anxiety, it has also been posited that the test contains separate elements of fear/anxiety (leading to the inhibition of activity) and also the search for social companions (Faure *et al.* 1983; Suarez and Gallup 1983; Mills *et al.* 1993). In the experiment presented here, we find that there is a strong overlap between QTL for social reinstatement and open field phenotypes, with a large

Table 2 Global gene correlation candidates

Gene	Trait	Correlation significance	eQTL LOD	SR QTL overlaps
ANKRD29	SR_time_in_start_zone_trial2	0.0003	NA	SR_minimum_latency_to_enter_stimulus_zone; SR_maximum_time_in_stimulus_zone
ANKRD29	SR_minimum_latency_to_enter_stimulus_zone	0.0001	NA	SR_minimum_latency_to_enter_stimulus_zone; SR_maximum_time_in_stimulus_zone
ANKRD29	SR_minimum_time_in_stimulus_zone	0.0001	NA	SR_time_in_stimulus_zone_trial1;
CALB2	SR_time_in_start_zone_trial2	0.0003	NA	
CHD5	SR_time_in_start_zone_average	0.0001	8.2	
CHD5	SR_time_in_start_zone_trial1	0.0002	8.2	
ENSGALG00000007103	SR_start_zone_max	0.0002	NA	SR_time_in_stimulus_zone_trial1
ENSGALG00000025912	SR_start_zone_min	0.0008	10.2	
ENSGALT00000001485_Q5SZRL_Ast7a_rCt_HzloCnKe_min	SR_start_zone_min	0.0001	5.5	
GGA.50063	SR_maximum_latency_to_enter_stimulus_zone	0.0003	NA	SR_minimum_latency_to_enter_stimulus_zone
HCCS	SR_minimum_latency_to_enter_stimulus_zone	0.0003	4.3	
HERPUD1	SR_time_in_start_zone_average	0.0001	NA	SR_time_in_stimulus_zone_trial1;
HERPUD1	SR_time_in_start_zone_trial2	0.0001	NA	SR_time_in_stimulus_zone_trial1;
RANBP17	SR_minimum_latency_to_enter_stimulus_zone	0.0009	4.2	
RDM1	SR_time_in_start_zone_trial2	0.0004	NA	SR_minimum_latency_to_enter_stimulus_zone
SEPN1*	SR_latency_to_enter_stimulus_zone_trial2	0.0002	NA	SR_latency_to_enter_stimulus_zone
TMEM57	SR_time_in_start_zone_trial2	0.0003	NA	SR_latency_to_enter_stimulus_zone
TTRAP	SR_time_in_start_zone_average	0.0003	7.4	SR_minimum_latency_to_enter_stimulus_zone; SR_maximum_time_in_stimulus_zone
TTRAP	SR_time_in_start_zone_trial2	0.0004	7.4	SR_maximum_latency_to_enter_stimulus_zone;
TYMS	SR_time_in_start_zone_trial2	0.0004	NA	SR_maximum_time_in_stimulus_zone
X603602419F1	SR_start_zone_max	0.0004	NA	SR_latency_to_enter_stimulus_zone_average; SR_maximum_latency_to_enter_stimulus_zone

Genes significantly correlated with behavior at a genome-wide threshold that also possessed either an eQTL or overlapped with a behavioral QTL. All values are suggestive (P -value $< 4 \times 10^{-4}$). In the case of gene names marked with an *, these genes were the closest to the custom EST probeset used on the array.

Figure 3 Gene network for all genes significantly correlated with social reinstatement (SR) behavior. The number of connections each gene shares with other genes in the network is provided in the legend, as well as the key to identify specific genes within the network. Genes that were significantly correlated at the genome-wide level with SR and also overlapped an SR QTL are in orange, genes that were identified using the full overlap between an overlap of eQTL and QTL and were significantly correlated are in blue, while *TTRAP*, which fulfilled both these requirements, is marked in green. In the case of gene names marked with an asterisk, these genes were the closest to the custom EST probeset used on the array.

number of significant correlations between the two. Our study therefore shows a stable (*i.e.*, not disrupted by recombination) behavioral syndrome for sociality/anxiety exists in this experimental cross. Current work on behavioral syndromes uses statistical genetic correlations (Dingemanse *et al.* 2012) to link syndromes with their underlying genetics. Here, we show that many of the same loci affect both of our measured behavioral traits. While the species of animal and the test battery differ, our conclusions on the genetic architecture of sociality and anxiety behaviors are similar to results from mice (Turri *et al.* 2001; Henderson *et al.* 2004), with overlapping architectures for different behavioral tests. Like

the present work, there were some shared loci, where pleiotropy cannot be excluded, and some independent loci for specific test situations.

In conclusion, a combination of behavior QTL mapping and transcriptome-wide eQTL mapping identified five primary candidates for social behavior in the chicken. The overlap between QTL and eQTL, behavior–gene expression correlations, and structural equations modeling all support these candidates’ quantitative trait genes. Most of the candidate genes are previously known to affect behavior or nervous system function; however, this is the first time that at least four of the genes have been implicated in sociality. The

advanced intercross design gives us the high resolution needed to detect multiple QTL of modest effect. Additionally, the intercross demonstrates that overlapping loci underlie correlated behaviors at the phenotypic level, with evidence of a modular basis for a behavioral syndrome, with both pleiotropic and linkage effects.

Acknowledgments

The research was carried out within the framework of the Swedish Centre of Excellence in Animal Welfare Science and the Linköping University Neuro-network. SNP genotyping was performed by the Uppsala Sequencing Center. The project was supported by grants from the Carl Tryggers Stiftelse, Swedish Research Council (VR), the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (FORMAS), and European Research Council (advanced research grant GENEWELL 322206).

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Communicating editor: K. Peichel