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Association between Temperament and Stress-related Gene Expression in Day-old Chickens

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Stress in day-old chickens from commercial hatcheries is associated with problematic behavior in adult animals. Recently, we developed a new behavioral handling test for day-old chickens and demonstrated that it assessed temperament differences between seven breeds of native Japanese and Western chickens. In this study, we used 2-day-old male chicks from five of the above breeds to investigate the relationship between temperament and mRNA levels of three stress-related genes (nuclear receptor subfamily 3 group C member 1 (*NR3C1*), cytochrome P450 family 11 subfamily A member 1, and hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1) involved in the hypothalamic-pituitary-adrenal axis. Principal component analysis of 10 behavioral traits for the handling test revealed that the Fayoumi breed and Hiroshima line of the Chabo breed, both of which exhibited boisterous temperament, clustered separately from the other breeds. Only *NR3C1* expression showed a significant positive correlation with two behavioral traits (general vocalization and approaching the wall), and a negative correlation with movement. These results suggest that the complex temperament of day-old chickens is regulated, in part, by stress-related genes along the hypothalamic-pituitary-adrenal axis.

Key words: chickens, gene expression, hypothalamic-pituitary-adrenal axis, stress, temperament

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Introduction

In the poultry industry, pre- and post-hatching management exposes day-old chicks to a variety of stressors, including incubation sounds, sexing, vaccination, and transportation[1,2]. Hatchery-induced stress can have lasting effects and is associated with behavioral and physiological issues later in life. For example, chickens placed in stressful environments during hatching show an increased incidence of feather pecking and comb injuries at 20 weeks of age[1]. Similarly, laying hens that experienced fear in the tonic immobility test at 7 days of age showed a moderate genetic correlation with feather pecking and aggressive pecking behavior at 40 days of age[3]. These behaviors not only affect animal welfare, but lead also to economic losses.

As reviewed by Forkman *et al*.[4], several behavioral tests, such as the tonic immobility and open field tests, have been developed and conducted on young and adult chickens to evaluate their stress and fear responses. However, using aged chickens for these tests may fail to capture their natural temperament, as social hierarchies are established within the first 5 weeks after hatching[5]. Recently, we explored temperament differences among six native Japanese chicken breeds using the tonic immobility and open field tests in day-old chickens, and compared them to two Western chicken breeds. The results revealed breed differences in the innate fear responses[6,7]. Quantitative trait locus (QTL) analysis in an F2 cross between the Japanese Nagoya breed with a timid temperament and the G line of the control White Leghorn breed revealed the presence of four QTLs for tonic immobility on chicken chromosomes 1–3 and 24, as well as three QTLs for open field behavior on chromosomes 2, 4, and 7. QTLs for tonic immobility and open field behaviors are located in different chromosomal regions, indicating a distinct genetic base for these two traits[8]. In a subsequent study, the neuropeptide Y receptor Y5 (*NPY5R*) gene and an uncharacterized *LOC101749214* gene were identified as candidates for a major QTL of open field behavior on chromosome 4[9]. Furthermore,

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Table 1. **List of five breeds of native Japanese and Western chickens used in this study.**

Breed	Abbreviation	No. of males	Source
Chabo H line	CHB-H	6	JABPC
Chabo N line	CHB-N	5	ABRC
Oh-Shamo	OSM	6	JABPC
Fayoumi	PNP	7	ABRC
Ryujin-Jidori	RYU	5	LPLESWP

ABRC: Avian Bioscience Research Center, Graduate School of Bioagricultural Sciences, Nagoya University, Japan; JABPC: Japanese Avian Bioresource Project Research Center, Hiroshima University, Japan; LPLESWP: Laboratory of Poultry, Livestock Experimental Station, Wakayama Prefecture, Japan.

in an F2 cross between the aggressive Japanese Oh-Shamo breed and the docile White Leghorn T line, two QTLs for open field behavior were mapped to chromosomes 2 and 4[10].

The hatchery-related stress and fear experienced by day-old chickens during commercial handling are likely to be more complex and intense than those evoked by the traditional behavioral tests described above. Accordingly, a single behavioral test is insufficient to measure hatchery stress and the natural temperament of chickens. Recently, Ishikawa *et al*.[11] developed a new handling test designed for day-old chickens that could simultaneously assess multiple aspects of chicken temperament. In this handling test, three stimuli (human proximity, cotton swab contact, and human handling) were applied to the tested chicks. Although the handling test was not designed to mimic hatchery-related stress and fear, it can effectively assess the natural temperament and behavioral responses of day-old chicks. The handling test successfully characterized the complex behavioral temperaments of day-old chickens from seven native Japanese and Western breeds[11].

In general, stress and fear activate the hypothalamic-pituitary-adrenal (HPA) axis and increase the levels of glucocorticoids, including cortisol and corticosterone, in the adrenal glands[12]. In chickens, the development of the HPA axis begins early in the embryonic stage and becomes fully functional several days before hatching[13]. A significant increase in plasma corticosterone levels was observed after physical restraint at 0, 9, 16, and 23 days post hatching in White Leghorn and Red Junglefowl chickens, the ancestors of domesticated animals[14].

The present study aimed to investigate whether temperament characteristics evaluated by the handling test were influenced by the HPA axis in day-old chicks from five breeds of native Japanese and Western chickens. These breeds were selected from those used in our previous study because they exhibited distinctive temperaments[11]. We assessed the mRNA levels of three stress-related genes along the HPA axis and investigated the relationship between temperament and gene expression. The three genes studied were nuclear receptor subfamily 3 group C member 1 (*NR3C1*) (synonym: glucocorticoid receptor), cytochrome P450 family 11 subfamily A member 1 (*CYP11A1*), and hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1 (*HSD3B1*) (synonym: 3 beta-hydroxysteroid dehydrogenase 2). *NR3C1* is activated in specific brain regions and regulates the HPA axis through glucocorticoid feedback[15]. *CYP11A1* and *HSD3B1* are involved in the biosynthesis of steroid hormones including glucocorticoids by the adrenal glands[16,17].

Materials and Methods

Ethical approval

All animal experiments were conducted in compliance with the Guidelines for the Care and Use of Laboratory Animals from Nagoya University, Japan. The Nagoya University Animal Research Committee approved this study (approval no. AGR2019016).

Animals

A total of 29 2-day-old male chickens from five breeds were used, as summarized in Table 1. Three were native Japanese chicken breeds: Chabo (CHB), Oh-Shamo (OSM), and Ryujin-Jidori (RYU). The CHB breed was divided into two separate lines: one from Hiroshima University (CHB-H) and the other from Nagoya University (CHB-N). The remaining, Western breed was PNP, a highly inbred line derived from the Fayoumi breed. Fertilized eggs of all breeds were obtained from the resource centers listed in Table 1 and hatched at Nagoya University. Incubation began at 9:00 AM, and egg candling was performed approximately 18 days later to monitor hatching progress. Only chicks that hatched on the morning of the scheduled hatching day (21 days after incubation) were used. These chicks were reared in a small brooder at 32 °C and were given water but no food from hatching until 2 days of age.

Handling test

The handling test in 2-day-old males was performed as described previously[11] in an arena (40 cm in length, 21 cm in width, and 29 cm in height) enclosed on three sides with a wire mesh. Three different stimuli (human proximity, cotton swab contact, and human handling) were applied to chicks using a human hand and a homemade cotton swab. The behaviors of the birds were recorded for 7 min using a video camera (Handycam HDR-PJ675; SONY, Tokyo, Japan). The recordings were analyzed based on the criteria for 10 behavioral traits (Table 2), with the occurrences of each trait counted by one of the authors (TT).

Tissue collection

After the handling tests, the chicks were weighed and blood

Table 2. **Criteria for 10 behavioral traits in the handling test.**

Trait abbreviation	Criterion
Distress vocalization	Number of distress vocalizations. More vocalizations indicate more clamor.
Moving	Number of times the bird crossed the line during a 30-s observation period after stimulation. More moves indicate more noise.
Escaping cage	Number of escape attempts from the cage. More attempts indicate more clamor.
General vocalization	Number of general vocalizations other than distress vocalizations. More vocalizations indicate relatively fewer distress vocalizations and less noise.
Sleeping	Number of sleeping periods in each stimulus phase. More sleep indicates less bustle.
Floor/cage pecking	Number of times floor or cage was pecked. More pecks indicate more aggression.
Biting	Number of times the hand was bitten. More bites indicate more aggression.
Surprised voice	Number of times surprised voices were raised when stimulated. More voices indicate more timidity.
Escaping stimulus	Number of escape attempts when stimulated. More attempts indicate more timidity.
Approaching the wall	Number of times approaching the cage wall. More approaches indicate more timidity.

Table 3. **Primer pairs used for real-time PCR analysis.**

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Reference
NR3C1	GCCATCGTGAAAAGAGAAGG	TTTCAACCACATCGTGCAT	18
CYP11A1	ACTTCAAGGGACTGAGCTTTGGGT	AGTTCTCCAGGATGTGCATGAGGA	19
HSD3B1	TGCTGGAAGAAGATGAGGC	TGTGGATGACGAGCGAGAC	20
Pol II	AAGGAGCCGCAGGTCTAC	CTTGCTCTTTGCCGTCATAC	21
TBP	TAGCCCGATGATGCCGTAT	GTTCCCTGTGTCGCTTGC	21

NR3C1, nuclear receptor subfamily 3 group C member 1; *CYP11A1*, cytochrome P450 family 11 subfamily A member 1; *HSD3B1*, hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1; *Pol II*, RNA polymerase II subunit; *TBP*, TATA-box-binding protein.

was collected from the carotid artery using a microtube containing heparin. The diencephalon, pituitary gland, adrenal glands, and liver were collected from each chick, which was euthanized by decapitation after anesthesia with isoflurane. The tissues were soaked in RNAlater solution (Thermo Fisher Scientific, Tokyo, Japan) for at least 1 day at room temperature and stored at -80 °C.

Sexing

Genomic DNA was extracted from blood samples. Each chick was sexed using the PCR amplification method described previously[11].

RNA extraction

Total RNA was extracted from the diencephalon, pituitary gland, and adrenal gland using TRI Reagent (Cosmo Bio, Tokyo, Japan). The concentration of extracted RNA was measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific), and the RNA was stored at -80 °C.

Real-time PCR analysis

Briefly, cDNA was synthesized from 1.0 μg of total RNA using the PrimerScript RT Reagent Kit with gDNA Eraser (Takara Bio, Shiga, Japan) according to the manufacturer's instructions. Real-time PCR was conducted using an Applied Biosystems StepOnePlus Real-Time PCR system (Thermo Fisher Scientific) with THUNDERBIRD SYBR qPCR Mix (TOYOBO, Osaka, Japan) according to the manufacturer's instructions. The primer sequences for the three stress-related genes and two endogenous control genes, RNA polymerase II subunit (*Pol II*) and TATA-box-binding protein (*TBP*), are listed in Table 3. *Pol II* was used to normalize the expression levels of *CYP11A1* and *HSD3B1*; whereas *TBP* for *NR3C1*. All samples were analyzed in triplicates. Expression was measured using the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

All data on body weight, behavioral handling traits, and gene expression (Supplemental Table S1) were analyzed using the software package JMP Pro version 17.2.0 (SAS Institute, Tokyo, Japan). Raw data for handling traits were tested using a linear regression model in JMP Pro to determine whether they were affected by body weight. Breed differences were assessed using one-way analysis of variance (ANOVA), followed by Tukey's honest significant difference (HDS) *post hoc* test. Principal component analysis using a correlation matrix was conducted on the handling trait data to gain a comprehensive understanding of the temperament of each breed. Linear regression analysis was used to investigate correlations between handling traits and gene expression.

Results

Body weight

The mean body weight of male chickens at 2 days of age differed significantly among the five breeds ($P = 1.2 \times 10^{-9}$) (Table 4). The OSM breed had the highest body weight, followed by

Table 4. **Differences among five chicken breeds in body weight, 10 behavioral handling traits, and expression levels of three stress-related genes.**

Trait or gene	CHB-H	CHB-N	OSM	PNP	RYU	*P* value
Body weight (g)	16.7 ± 1.2 [a]	17.0 ± 1.3 [a]	32.7 ± 1.2 [b]	27.1 ± 1.1 [c]	19.5 ± 1.3 [a]	1.2×10^{-9}
Distress vocalization	497.3 ± 61.8 [ab]	108.2 ± 67.7 [c]	266.3 ± 61.8 [bc]	564.1 ± 57.2 [a]	517.6 ± 67.7 [ab]	1.1×10^{-4}
Moving	4.2 ± 1.5 [a]	0.4 ± 1.6 [a]	2.8 ± 1.5 [a]	10.9 ± 1.4 [b]	3.2 ± 1.6 [a]	4.5×10^{-4}
Escaping cage	0.3 ± 0.3 [a]	0.2 ± 0.3 [a]	0.0 ± 0.3 [a]	1.0 ± 0.2 [a]	0.4 ± 0.3 [a]	0.080
General vocalization	0.7 ± 0.8 [a]	6.4 ± 0.9 [b]	7.3 ± 0.8 [b]	0.1 ± 0.8 [a]	1.0 ± 0.9 [a]	6.7×10^{-7}
Sleeping	0.2 ± 0.5 [a]	2.6 ± 0.6 [b]	0.2 ± 0.5 [a]	0.0 ± 0.5 [a]	0.2 ± 0.6 [ab]	0.018
Floor/cage pecking	7.7 ± 2.2 [a]	0.2 ± 2.4 [a]	4.2 ± 2.2 [a]	7.8 ± 3.0 [a]	1.9 ± 1.9 [a]	0.19
Biting	0.0 ± 0.7 [a]	0.0 ± 0.7 [a]	2.2 ± 0.7 [a]	0.0 ± 0.6 [a]	0.0 ± 0.7 [a]	0.11
Surprised voice	5.3 ± 0.9 [a]	0.6 ± 1.0 [b]	3.7 ± 0.9 [ab]	3.6 ± 0.8 [ab]	2.0 ± 1.0 [ab]	0.018
Escaping stimulus	4.8 ± 0.7 [a]	2.4 ± 0.8 [a]	1.8 ± 0.7 [a]	3.0 ± 0.7 [a]	3.0 ± 0.8 [a]	0.77
Approaching the wall	1.2 ± 0.4 [ab]	1.2 ± 0.7 [ab]	0.8 ± 0.4 [ab]	0.0 ± 0.4 [b]	2.0 ± 0.5 [a]	0.047
NR3C1	1.0 ± 0.1 [a]	1.2 ± 0.1 [a]	1.9 ± 0.1 [b]	1.1 ± 0.1 [a]	1.6 ± 0.1 [b]	8.2×10^{-7}
CYP11A1	1.0 ± 0.2 [a]	0.6 ± 0.2 [a]	0.9 ± 0.2 [a]	0.5 ± 0.2 [a]	0.9 ± 0.2 [a]	0.39
HSD3B1	1.0 ± 0.2 [ab]	0.6 ± 0.2 [b]	1.0 ± 0.2 [ab]	0.8 ± 0.1 [b]	1.5 ± 0.2 [a]	0.014

Data are presented as means ± standard error. *P* values were obtained using one-way ANOVA.
[a-c] Means with different superscript letters for each trait are significantly different between the two breeds at $P < 0.05$ (HSD *post hoc* test).

PNP. These two breeds were distinct from the remaining three breeds (CHB-H, CHB-N, and RYU), which had nearly the same body weight.

Handling test

Despite the body weight differences noted above, none of the 10 behavioral traits measured during the handling test were affected by body weight ($P = 0.12$–0.95). Therefore, raw trait data were used for the statistical analyses presented below.

Six handling traits (distress vocalization, moving, general vocalization, sleeping, surprised voice, and approaching the wall) showed significant differences among breeds ($P = 6.7\times10^{-7}$–0.047); whereas the remaining four traits were not significantly different ($P = 0.080$–0.77) (Table 4). Interestingly, the frequency of distress vocalization was the highest in CHB-H, PNP, and RYU; whereas the frequency of general vocalization was the highest in CHB-N and OSM. PNP exhibited the greatest movement.

Principal component analysis was performed for six traits that showed significant breed differences. The first and second principal component axes accounted for 43.1% and 19.6% of total variance, respectively (Fig. 1A). For the first principal component axis, three of the six traits had positive factor loadings, with distress vocalization and moving showing nearly the highest loading values (0.88 and 0.79, respectively). The remaining traits had negative factor loadings, with general vocalization showing the lowest loading value (-0.74) (Fig. 1B). Hence, the first principal component axis explained the bustling behavior of chickens (Table 2), associating positive scores with more bustling behavior. The mean first principal component scores for the CHB-H and PNP breeds were significantly higher than those for the other breeds at least at $P < 0.05$ (Fig. 1C).

For the second principal component axis, only approaching the wall had a high loading value (0.88); while the other traits had much lower absolute values (0.01–0.51). The second principal component axis explained a timid chicken temperament (Table 2). A significant difference in the mean second principal component scores was observed between the PNP and RYU breeds ($P < 0.05$) (Fig. 1D).

Gene expression

Based on one-way ANOVA (Table 4), the levels of *NR3C1* in the diencephalon and *HSD3B1* in the adrenal glands differed significantly among the five breeds ($P = 8.2 \times 10^{-7}$ and 0.014, respectively). *CYP11A1* expression in the adrenal glands did not differ significantly ($P = 0.39$). *NR3C1* expression levels were significantly higher in OSM and RYU than in the other three breeds ($P < 0.05$). *HSD3B1* expression in the RYU breed was not significantly different from that in the CHB-H and OSM breeds, but was the highest in the group. Therefore, RYU was characterized by higher expression of both *NR3C1* and *HSD3B1*.

Relationships between behavioral handling traits and gene expression levels

Linear regression analysis was conducted to explore the correlations between the levels of *NR3C1* and *HSD3B1* genes, which differed significantly among breeds, the six individual behavioral traits, and the first and second principal component scores that summarized the variances of these six traits. The scatter plots depicting gene-trait combinations (Fig. 2) revealed no outliers in most cases, except for two instances involving two genes and the sleeping trait, whereby one outlier was observed. However, neither including nor removing this outlier correlated significantly with the expression of the two genes (Fig. 2 and Supplemental Fig. S1).

HSD3B1 expression in the adrenal gland did not correlate with either principal component scores. In contrast, *NR3C1* ex-

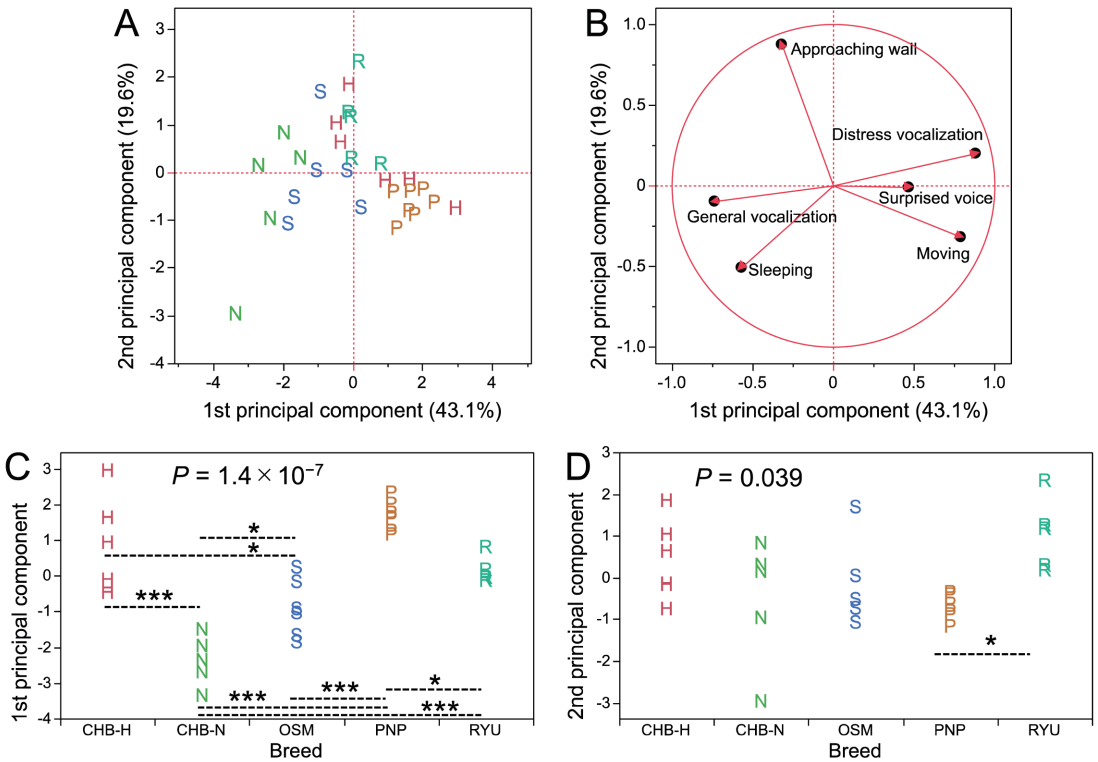

Fig. 1. **Principal component analysis of six behavioral handling traits in five chicken breeds.** (A) Score plot for the first and second principal components. Percentages of total variance explained by each principal component axis are shown in parentheses. (B) Vector plot of factor loadings for the six traits. (C) Breed comparisons for the first principal component scores. (D) Breed comparisons for the second principal component scores. *P* values in (C) and (D) were obtained from one-way ANOVA of principal component scores for the five breeds. Dashed lines indicate significant differences in mean principal component scores between the two breeds at $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***) (HSD *post hoc* test). A single letter in (A), (C), and (D) indicates the abbreviations of the breed, and each letter represents one individual of the breed.

pression in the diencephalon correlated significantly with both principal component scores ($P < 0.05$), albeit in opposite ways. Specifically, *NR3C1* expression correlated positively with general vocalization ($r = 0.50$) and approaching the wall ($r = 0.46$), but negatively with movement ($r = -0.42$). These three traits are associated with the bustling aspect of chicken temperament, as noted earlier.

The scatter plots (Fig. 2) summarize the breed-specific characteristics of gene-trait relationships. Specifically, the OSM and RYU breeds, characterized by the highest *NR3C1* expression, were classified into a group that exhibited less locomotion but more general vocalization and wall-approaching behavior. In contrast, the PNP and CHB-H breeds, with the lowest *NR3C1* expression, formed a distinct group characterized by high mobility, lower levels of general vocalization, and a tendency to approach the walls.

Discussion

In this study, only male chicks at 2 days of age were used. Females were not examined to exclude the influence of two possible confounding factors in understanding the obtained data. One factor was the limited number of females among the five breeds used; the CHB-H and PNP breeds had fewer than five individuals for handling trait data[11]. Another factor was the presence of sex-specific QTLs for behavior in day-old chicks. Sex-specific QTLs refer to genetic loci that have significant phenotypic effects in one sex but not in the other. In our previous QTL analysis of an F2 cross between the Nagoya breed and the White Leghorn G line, we identified a male-specific QTL and a female-specific QTL for tonic immobility on chromosomes 1 and 2 on hatching day[8]. In an F2 cross between the OSM breed and White Leghorn T line, we found two male-specific QTLs for open field behavior and a female-specific QTL for tonic immobility on chromosomes 4, 7, and 10 at 1 and 2 days of age[10]. These

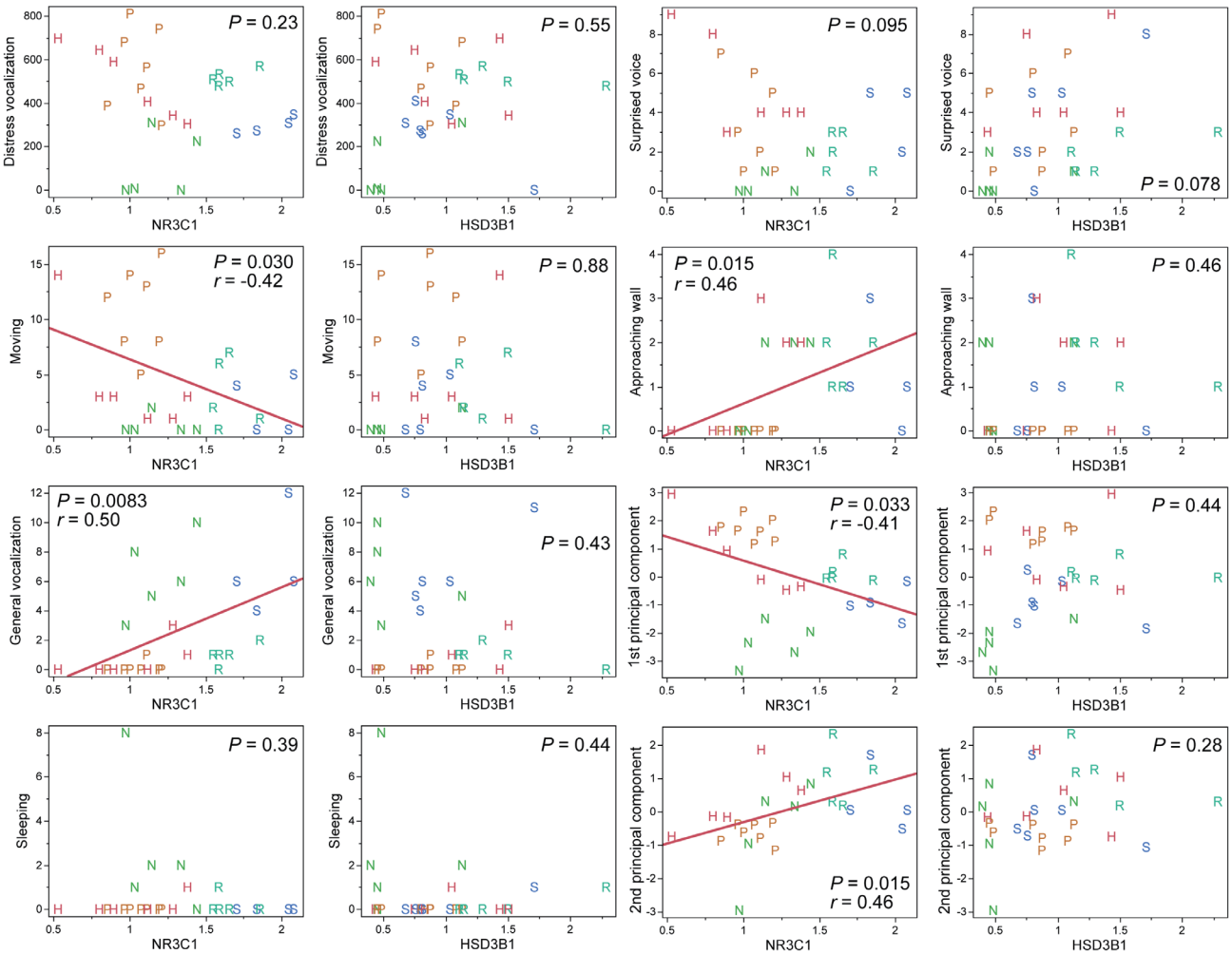

Fig. 2. **Scatter plots of *NR3C1* and *HSD3B1* expression levels versus six behavioral handling traits and the two principal components in five chicken breeds.** Each letter in the scatter plots indicates the abbreviations of the breed, and each letter represents one individual from the breed. *P* values were obtained from linear regression analysis.

findings suggest that QTLs that affect handling traits may exhibit sex-specific effects. Furthermore, because the HPA becomes fully functional several days before hatching[13], sex-specific QTL effects may result from sex-specific differences in the expression of HPA genes. Therefore, to simplify the interpretation of results, we focused only on male chicks in this study. This approach is the first attempt to explore the relationships between temperament characteristics revealed by handling tests and expression levels of stress-related genes along the HPA axis.

Principal component analysis of six significant behavioral traits obtained from the handling test revealed that the CHB-H and PNP breeds exhibited more bustling behavior than the CHB-N, OSM, and RYU breeds. Our previous study[11] had already highlighted the bustling nature of PNP breeds. However, in the present study, we were able to provide a clearer insight into the temperament of the CHB-H breed. Specifically, our findings suggest that, although CHB-H and CHB-N belong to the same breed, they possess distinct temperaments. While the precise reason for this dichotomy remains unclear, it is plausible that temperament differences reflect genetic variations between the ancestral base populations of these two CHB lines, as discussed previously[11].

The *NR3C1* gene used in the present study was reported to regulate HPA axis activity through glucocorticoid feedback[15]. *CYP11A1* and *HSD3B1* genes contribute to steroidogenesis, ultimately leading to glucocorticoid synthesis in the adrenal glands[16,17]. Real-time PCR revealed significant differences among breeds with respect to *NR3C1* expression in the diencephalon and *HSD3B1* expression in the adrenal glands. Instead, *CYP11A1* expression in the adrenal glands was similar across breeds. Linear regression analysis between *NR3C1* and *HSD3B1* levels and behavioral traits revealed somewhat surprisingly that only diencephalon *NR3C1* expression correlated significantly

with behavioral traits. This suggests that the brain, particularly the paraventricular nucleus of the hypothalamus[15], is a potent tissue regulating HPA activity in native Japanese chickens. The above finding is supported by two previous gene expression studies, in which the adrenal level of key steroidogenic genes did not differ between Red Junglefowl and White Leghorn[22], but *NR3C1* expression in the hypothalamus was higher in White Leghorn than in Red Junglefowl[23].

In the present study, significant differences in *NR3C1* expression and general vocalization were observed among different breeds, highlighting notable breed-specific variations. In particular, the OSM and CHB-H breeds showed the highest and lowest levels of *NR3C1* expression and general vocalization, respectively, with a significant correlation coefficient ($r = 0.50$). The scatter plots depicting gene-trait relationships provided a visual confirmation of consistent trends across breeds, pointing to the robustness of the observed correlations. Although biases such as data outliers or small sample sizes can potentially affect correlation outcomes, the clear breed-specific patterns observed in the present study indicate that the correlation between *NR3C1* and general vocalization is driven not only by biased data. It is unlikely that these breed-specific characteristics are significantly influenced by small sample sizes or outliers. However, to enhance the reliability of our findings, increasing sample size would provide a more comprehensive understanding of gene-trait relationships among breeds.

Interestingly, the RYU breed exhibited significantly higher expression levels for both *NR3C1* and *HSD3B1* genes. This suggests that the RYU breed may be more strongly governed by genes involved in the HPA axis, indicating a possible breed-specific difference in HPA activity. In mammals, in addition to the HPA axis, the hypothalamic-spinal-adrenocortical axis is involved in the regulation of glucocorticoid secretion[24] and pro-opiomelanocortin (POMC)-derived peptides play crucial roles in various aspects of adrenal function[25]. Indeed, selection for tameness in foxes has been demonstrated to significantly alter *POMC* expression in the anterior pituitary[26]. In the present study, we attempted to measure *POMC* expression in the pituitary glands of chicken breeds, but failed to obtain the corresponding values for unknown reasons.

In conclusion, this study confirmed that handling tests provided a detailed investigation of breed differences in the complex temperaments of day-old chickens. This also indicates that temperamental differences in stress and fear responses may reflect variations in reactivity within the HPA axis. To gain deeper insights into the reactivity of the HPA and hypothalamic-spinal-adrenocortical axes to stress and fear, it is necessary to measure blood corticosterone levels and conduct a comprehensive transcriptome analysis of the brains of the studied chicken breeds.

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Author Contributions

Akira Ishikawa conceived, designed, and supervised this study. Tomoka Takanuma performed all experiments. Tomoka Takanuma and Akira Ishikawa analyzed data. Norikazu Hashimoto and Masaoki Tsudzuki prepared eggs. Akira Ishikawa wrote the manuscript. All the authors contributed to the discussion and editing of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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