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Tonic Immobility and Open Field Responses in Nagoya, White Leghorn, and White Plymouth Rock Chicks

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Innate fear responses to tonic immobility (TI) and open field (OF) were evaluated in newly hatched chicks of three breeds with distinct breed origin and genetic relationships. The breeds studied were Nagoya (NAG), a native Japanese breed; White Leghorn (WL), a representative of layers; and White Plymouth Rock (WPR), a parental breed of common broilers. The TI test revealed that WL was the most sensitive to extensive fear evoked by the TI test among the three breeds, followed in order by WPR, and NAG. In contrast, the OF test revealed that NAG was the most sensitive to mild fear evoked by the OF test, followed in order by WPR, and WL. The different fear responses between NAG and WL were supported by minimal phenotypic correlations between TI and OF traits in each breed. These results demonstrated that NAG and WL breeds exhibit extreme and opposite responses to TI and OF fears.

Key words: Nagoya, open field, tonic immobility, White Leghorn, White Plymouth Rock

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Introduction

During the process of animal domestication, fear-related behaviors have been reduced in frequency and intensity (Agnvall *et al*., 2012; Belyaev, 1979; Brlyaev *et al*., 1985; Price, 1999). In chickens, Campler *et al*. (2009) reported that Red Junglefowl, a wild ancestor of domesticated chickens, is more sensitive to fear than White Leghorn, a domesticated breed for egg production, which was derived from the Mediterranean type of chickens developed during the 19th century (Kerje, *et al*., 2003). Furthermore, previous studies have revealed differences in fear-related behaviors between and within chicken breeds developed for meat and egg production. For example, Abe *et al*. (2013) reported that White Leghorn is less sensitive to innate fear than Nagoya, a native Japanese breed with high-quality production of meat and eggs, which was established from the Nagoya Cochin breed in Aichi Prefecture, Japan in 1912-1926 (Tsudzuki, 2003). Nagoya is reported to be a cowardly chicken breed, and crowding accidents, often leading to the death of many birds in a flock, result from fear induced by environmental stimuli such as loud noises and intensive flashes (Kato *et al*., 1991). Innate fear was investigated in chicks of Tosa-Jidori, another native Japanese breed (Nakasai *et al*., 2013). In Japanese breeds, information on innate fear is limited to the above two reports on Nagoya and Tosa-Jidori. Although aggressive behavior, leading to a large animal welfare issue, has been reported in commercial male broilers (Millman *et al*., 2000; Li *et al*., 2016), little is known about fear-related behavior in meat-type breeds, such as White Plymouth Rock, which has been used worldwide as a parental breed of commercial broilers.

Several behavioral tests have been used to evaluate fear in chickens, and reviewed by Forkman *et al*. (2007). In the present study, we used tonic immobility (TI) and open field (OF) tests, which are the most commonly used fear tests (Forkman *et al*., 2007). The TI test evokes intense fear in animals, through the application of light pressure from a human hand, while the OF test evokes mild fear in animals, indicating no immediate perceived threat, by placing them in a novel open field (Schütz *et al*., 2004).

In the present study, we evaluated innate fear responses to TI and OF in chicks of three chicken breeds, Nagoya (NAG), White Leghorn (WL), and White Plymouth Rock (WPR), which have distinct breed origin and genetic relationships among the breeds (Osman *et al*., 2006).

Materials and Methods

Animals

Hatching eggs for meat-type chickens of NAG (strain 87)

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and WPR (strain 981) were purchased from the Hyogo station, National Livestock Breeding Center, Hyogo, Japan, and those for egg-type chickens of WL (strain WL-G) were purchased from the Avian Bioscience Research Center, Graduate School of Biological Sciences, Nagoya University. Hatching eggs were incubated from 9:00 AM. Hatching was checked on the evening of the day prior to an expected hatching (3 weeks after incubation), and at 9:00 AM on an expected hatching day. Only chicks not hatched in the evening of the previous day but hatched by 9:00 AM on the expected day were used for behavioral tests. We could therefore assume that all chicks were almost the same age. All chicks were kept in an incubator at 32℃ with 24 h lighting. Until the TI test was performed, chicks at 0 days of age were not provided food and water. After the TI test, chicks were provided water before the OF test. All chicks used in this study were handled in accordance with the guidelines of the Animal Research Committee of Nagoya University.

Behavioral Tests

The TI test was performed in the afternoon of the hatching day and the OF test was performed in the morning of the next day using the same chicks. As handling of chicks was minimized as much as possible, the imprinting of chicks to the experimenter was assumed to be minimal. However, the chicks could imprint onto their companions in the same groups, because imprinting can occur within the first 4 days after hatching in chicks (Nakamori *et al*., 2013). Both tests were performed by the same person in a quiet, separate room at approximately 30℃ under fluorescent lights with an average light intensity of approximately 300 lx.

For the TI test, a cradle comprising plywood (42 cm length, 23 cm width, and 2 cm thickness) fixed at a right angle to a V shape was used. Each chick was placed on its back on the V-shaped cradle covered with black felt (polyester 100%), and light pressure was placed on its breast for 5 s by a human hand. This trial was repeated three times consecutively. The duration of TI and the number of inductions were recorded for each chick using a fixed video camera (Handycam HDR-PJ675, Sony, Tokyo). In the present study, short and long durations of TI (hereafter referred to as S-duration and L-duration, respectively) were measured for each trial. The S-duration was defined as the time taken for the chick to move its head or peep loudly with eyes open. The L-duration was defined as the time taken for the chick to completely right itself. When chicks that righted within 5 s were not considered to have entered TI status, both S- and L-durations were scored as zero. Conversely, when the TI status lasted for 600 s and more, both S- and L-durations were scored as 600 s. The first S-duration recorded and the first L-duration recorded were defined as the first S- and L-durations recorded among the three consecutive trials, respectively. Similarly, short and long inductions (hereafter, S- and L-inductions, respectively) were recorded. The S-induction was the number of trials in which a chick presented with the S-duration for the first time. The L-induction was the number of trials in which a chick presented with the L-duration for the first time. The S- and L-inductions were scored as 4 if the TI status was not attained after three trials. In total, 10 behavioral traits were recorded for the TI test (see Table 1). After the TI test, the body weight of the chicks was measured.

An arena (54 cm length, 79 cm width, and 30 cm height) was used for the OF test. The area 15 cm from the edge of the arena was defined as the periphery zone, and the inside of the periphery zone was defined as the center zone (39×64 cm). Each chick was placed at the lower left end of the periphery zone in darkness, and then the OF test began when the light was turned on. Chick behavior was recorded for 10 min from the top of the field with a video camera (Handycam HDR-PJ675). The record was analyzed using the software program SMART v3.0 (Panlab Harvard Apparatus, California). In total, 14 behavioral traits were measured during the OF test (see Table 3). Resting time was defined as the time when the moving speed of the chick was less than 2.5 cm/s. Slow time was the time when the moving speed was 2.5–15 cm/s. Fast time was the time when the moving speed was 15 cm/s and more. The parallel index was used to indicate whether the animal progressed in a direction parallel to the previous direction of progression. The value ranged from -1 to 1, and became close to 1 when the movement of animals was linear. However, it was close to -1 when the movement of animals was not linear, showing a tendency toward exploration. The body weight of chicks was measured after the OF test.

Sexing

Blood was taken from the chicks via the carotid artery. Genomic DNA was extracted from blood cells and animals were sexed by PCR amplification of the *CHD* (chromo-helicase-DNA binding protein) gene on sex chromosomes, as described by Suzuki *et al*. (2019).

Statistical Analysis

Data on behavioral traits were analyzed with the JMP Pro software version 13.2.0 (SAS Institute Japan Ltd., Tokyo). Before breed differences were compared, the raw data were tested to determine whether the data were affected by environmental factors, such as sex and room temperature, using a linear model of JMP Pro. In the model, sex and the testing order of individuals measured on the same day were included as fixed effects. Room temperature and body weight were included as random effects. The effects, and their possible interaction effects that were significant at the nominal level of 5% were used to adjust the raw data. Breed differences in adjusted data were tested by the Kruskal–Wallis test followed by the Steel–Dwass *post hoc* test. Spearman's rank correlation coefficients were computed to measure phenotypic relationships among TI traits, among OF traits, and between TI and OF traits in each breed.

Results

Table 1 presents the results of the TI test in chicks of NAG, WL, and WPR breeds. There were no significant sex differences in any of the 10 behavioral traits examined at the nominal 5% level. Data for four traits, S-duration in the 3rd

Table 1. **Means and standard errors of behavioral traits for a tonic immobility (TI) test in the Nagoya (NAG), White Leghorn (WL), and White Plymouth Rock (WPR) chicks**

Trait	NAG	WL	WPR	*P* value	Fearfulness	
					Most	Least
No. of animals	70	71 (54)[1]	47			
S-duration in the 1st trial (s)	51.0±15.0^{a}	74.7±13.9^{b}	91.4±23.1^{ab}	**6.1E-04**	WL≥WPR≥NAG	
S-duration in the 2nd trial (s)	52.1±11.7^{a}	54.4±7.3^{b}	44.6±14.0^{a}	**0.0028**	WL>NAG≥WPR	
S-duration in the 3rd trial (s)	−539.4±9.4	−536.2±6.2	−536.2±13.2	0.17	—	
First S-duration recorded (s)	−408.8±16.8	−413.4±13.7	−371.8±22.3	0.28	—	
S-induction (no)	0.4±0.1	0.1±0.1	0.2±0.1	0.097	—	
L-duration in the 1st trial (s)	99.8±21.2^{a}	173.4±24.8^{b}	180.6±31.1^{ab}	**0.0011**	WL≥WPR≥NAG	
L-duration in the 2nd trial (s)	71.9±14.8	91.0±16.5	79.3±16.3	0.090	—	
L-duration in the 3rd trial (s)	−637.3±9.4	−619.9±10.6	−636.9±13.2	0.11	—	
First L-duration recorded (s)	−462.5±22.4^{a}	−423.0±24.9^{ab}	−389.2±28.9^{b}	0.041	WPR≥WL≥NAG	
L-induction (no)	1.4±0.1^{a}	1.1±0.1^{b}	1.3±0.1^{a}	**4.3E-04**	WL>WPR≥NAG	

The S-trait denotes the short time from the trait until the chick either moved its head or peeped in a loud voice with eyes open, and the L-trait denotes the long time from the trait until the chick completely righted. The raw data were adjusted for random effects of environmental factors (see Materials and Methods). The trait differences among the three breeds were tested by the Kruskal–Wallis test, and the *P* values obtained were approximated by the chi-square value for the one-way test. The *P* values in bold exceeded the Bonferroni-corrected 5% level.

[1] 71 and 54 animals were assessed for S- and L-traits, respectively, in WL.

[a–c] Means with different letters were significantly different between breeds at $P<0.05$ by the Steel–Dwass *post hoc* test.

— =not applicable.

trial, first S-duration recorded, L-duration in the 3rd trial, and first L-duration recorded, were adjusted for room temperature. Data for S-induction were adjusted for testing order and body weight. Data for the remaining five traits were not adjusted for any environmental factor.

Significant differences among the three breeds were observed for five TI traits at the nominal 5% level, as shown in Table 1. Among them, four traits of S-duration in the 1st trial; S-duration in the 2nd trial; L-duration in the 1st trial; and L-induction exceeded the Bonferroni-corrected 5% level (0.05/10=0.005). Values for three traits of S- and L-durations in WL were significantly higher than those of NAG. In contrast, a value for L-induction in WL was significantly lower than that in NAG. Values for these four traits in WPR were approximately intermediate between NAG and WL. Therefore, the results demonstrated that WL was the most sensitive to fear evoked by the TI test among the three breeds; the second most sensitive was WPR; and the least sensitive was NAG, as chicks with higher values for TI durations and lower values for TI inductions are considered to be more fearful than those with lower values for TI durations and higher values for TI inductions (Schütz *et al*., 2004).

Table 2 presents the Spearman's rank correlation coefficients among 10 TI traits in each of the three breeds. In the same trial, S-duration was positively correlated with L-duration at the Bonferroni-corrected 5% significance level (0.05/45=0.0011) across three breeds. A positive correlation between the first S-duration recorded and the first L-duration recorded was observed at the Bonferroni-corrected 5% significance in all breeds. Likewise, a positive correlation between S-induction and L-induction was observed at the Bonferroni-corrected 5% significance level in NAG and WPR and at the nominal 5% level in WL. Conversely, the first S-duration recorded and the first L-duration recorded were positively correlated with S-duration in the 1st trial, and with L-duration in the 1st trial, respectively, at the Bonferroni-corrected 5% significance level. Negative correlations between S-induction and S-duration in the 1st trial, and between L-induction and L-duration in the 1st trial were observed at the Bonferroni-corrected 5% significance level in NAG and WPR, and at the nominal 5% level in WL. Although only NAG and WL were positively correlated between the 1st and 2nd trials of S-duration at the nominal 5% level, no correlation was observed among trials of L-durations; however, the reasons for this are unknown. In addition to the result showing that only S- and L-durations were significant among the three breeds in the 1st trial (Table 1), only two TI traits (TI duration in the 1st trial and TI induction) for either short (S-) or long (L-) TI behavior were sufficient for evaluation of TI behavior in the three breeds.

Table 3 presents the results of the OF test in NAG, WL, and WPR chicks. None of 14 behavioral traits examined revealed a significant sex difference at the nominal 5% level. Seven traits, including latency of 1st entrance to the center zone, distance in the periphery zone, total distance, resting time, fast time, mean speed, and mean speed without resting were adjusted for room temperature. Two traits, maximum speed and parallel index, were adjusted for body weight. The remaining five traits were not adjusted for any environmental factor.

Significant breed differences were observed for 13 of 14 OF traits at the nominal 5% level (Table 3). Among them, eight traits (number of entries in the center zone, latency of the 1st entrance to the center zone, total time in the center zone, distance in the center zone, fast time, mean speed without resting, parallel index, and the number of excrements) exceeded the Bonferroni-corrected 5% level (0.05/14

Table 2. **Spearman's rank correlation coefficients (ρ) between TI traits in each of the Nagoya (NAG), White Leghorn (WL), and White Plymouth Rock (WPR) chicks**

Trait 1	Trait 2	NAG		WL		WPR	
		ρ	*P* value	ρ	*P* value	ρ	*P* value
S-duration in the 2nd trial	S-duration in the 1st trial	0.28	0.017	0.30	0.0069	NS	NS
S-duration in the 3rd trial	S-duration in the 1st trial	NS	NS	NS	NS	NS	NS
	S-duration in the 2nd trial	NS	NS	NS	NS	NS	NS
First S-duration recorded	S-duration in the 1st trial	0.38	**0.00081**	0.73	**1.7E-14**	0.59	**1.2E-05**
	S-duration in the 2nd trial	0.37	0.0014	0.35	0.0016	0.45	0.0017
	S-duration in the 3rd trial	0.51	**3.4E-06**	0.29	0.0084	NS	NS
S-induction	S-duration in the 1st trial	−0.80	**2.4E-17**	−0.31	0.0048	−0.70	**3.8E-08**
	S-duration in the 2nd trial	−0.26	0.025	NS	NS	NS	NS
	S-duration in the 3rd trial	NS	NS	−0.29	0.0081	NS	NS
	S-duration first recorded	−0.26	0.029	NS	NS	NS	NS
L-duration in the 1st trial	S-duration in the 1st trial	0.84	**3.6E-20**	0.64	**9.6E-09**	0.80	**1.2E-11**
	S-duration in the 2nd trial	NS	NS	NS	NS	NS	NS
	S-duration in the 3rd trial	NS	NS	NS	NS	NS	NS
	First S-duration recorded	0.27	0.022	0.53	**6.9E-06**	0.46	0.0012
	S-induction	−0.65	**4.6E-10**	−0.26	0.041	−0.46	0.0011
L-duration in the 2nd trial	S-duration in the 1st trial	NS	NS	NS	NS	NS	NS
	S-duration in the 2nd trial	0.84	**1.1E-20**	0.75	**7.7E-13**	0.66	**4.1E-07**
	S-duration in the 3rd trial	NS	NS	NS	NS	NS	NS
	S-duration first recorded	0.24	0.040	NS	NS	NS	NS
	S-induction	NS	NS	NS	NS	NS	NS
	L-duration in the 1st trial	NS	NS	NS	NS	NS	NS
L-duration in the 3rd trial	S-duration in the 1st trial	NS	NS	NS	NS	NS	NS
	S-duration in the 2nd trial	NS	NS	NS	NS	NS	NS
	S-duration in the 3rd trial	0.86	**1.0E-22**	0.77	**1.2E-13**	0.86	**4.6E-15**
	First S-duration recorded	0.40	**0.00043**	NS	NS	NS	NS
	S-induction	NS	NS	NS	NS	NS	NS
	L-duration in the 1st trial	NS	NS	NS	NS	NS	NS
	L-duration in the 2nd trial	NS	NS	NS	NS	NS	NS
First L-duration recorded	S-duration in the 1st trial	0.33	0.0042	0.43	**0.00036**	0.62	**4.0E-06**
	S-duration in the 2nd trial	0.24	0.038	NS	NS	NS	NS
	S-duration in the 3rd trial	0.42	**0.00025**	NS	NS	NS	NS
	First S-duration recorded	0.61	**9.4E-09**	0.70	**1.2E-10**	0.61	**5.4E-06**
	S-induction	NS	NS	NS	NS	NS	NS
	L-duration in the 1st trial	0.46	**4.1E-05**	0.82	**9.1E-17**	0.76	**5.7E-10**
	L-duration in the 2nd trial	0.36	0.0018	NS	NS	0.30	0.039
	L-duration in the 3rd trial	0.43	**0.00013**	NS	NS	NS	NS
L-induction	S-duration in the 1st trial	−0.68	**4.4E-11**	−0.35	0.0048	−0.62	**2.9E-06**
	S-duration in the 2nd trial	NS	NS	NS	NS	NS	NS
	S-duration in the 3rd trial	NS	NS	NS	NS	NS	NS
	First S-duration recorded	NS	NS	NS	NS	NS	NS
	S-induction	0.68	**3.6E-11**	0.35	0.0051	0.58	**2.2E-05**
	L-duration in the 1st trial	−0.79	**1.9E-16**	−0.37	0.0029	−0.74	**4.2E-09**
	L-duration in the 2nd trial	NS	NS	NS	NS	NS	NS
	L-duration in the 3rd trial	NS	NS	NS	NS	NS	NS
	First L-duration recorded	NS	NS	NS	NS	NS	NS

The *P* values in bold exceeded the Bonferroni-corrected 5% level.
NS=not significant at the nominal 5% level.

=0.0036). The value obtained for latency of the 1st entrance to the center zone in NAG was significantly higher than that of WL. The values for the other seven traits in NAG were significantly lower than those in WL. WPR expressed fearfulness in the eight traits that was intermediate between that of NAG and WL. These results indicated that NAG was the most sensitive breed to fear evoked by the OF; the second most sensitive was WPR; and the least sensitive was WL, since chicks inactive were considered to be fearful (Schütz *et al*., 2004). Conversely, NAG and WL expressed opposing fear response to two traits, parallel index and the number of excrements, which differed in behavioral nature from the other traits. Interestingly, the lower parallel index of NAG meant that NAG had a higher tendency toward

Table 3. **Means and standard errors of behavioral traits for an open field (OF) test in the Nagoya (NAG), White Leghorn (WL), and White Plymouth Rock (WPR) chicks**

Trait	NAG	WL	WPR	P value	Fearfulness	
					Most	Least
No. of animals	25	64	23			
No. of entries in the center zone (no)	2.8 ± 1.0^{a}	6.4 ± 0.8^{b}	3.4 ± 0.9^{ab}	**8.8E-04**	NAG≧WPR≧WL	
Latency of 1st entrance to the center zone (s)	1190.6 ± 41.4^{a}	974.2 ± 24.7^{b}	1046.1 ± 46.6^{b}	**3.2E-04**	NAG＞WPR≧WL	
Total time in the center zone (%)	4.7 ± 1.8^{a}	11.9 ± 2.0^{b}	49.5 ± 9.2^{c}	**3.4E-05**	NAG＞WL＞WPR	
Distance in the periphery zone (cm)	-5964.5 ± 122^{a}	-5047.1 ± 210.0^{b}	-5625.8 ± 115.3^{ab}	0.018	NAG≧WPR≧WL	
Distance in the center zone (cm)	55.5 ± 16.9^{a}	143.4 ± 18.4^{b}	75.6 ± 19.4^{ab}	**0.0021**	NAG≧WPR≧WL	
Total distance (cm)	-6240.3 ± 135.4^{a}	-5240.5 ± 219.2^{b}	-5870.8 ± 124.0^{ab}	0.012	NAG≧WPR≧WL	
Resting time (s)	959.2 ± 12.9^{a}	899.2 ± 13.9^{b}	918.6 ± 16.1^{ab}	0.033	NAG≧WPR≧WL	
Slow time (s)	54.7 ± 9.6	85.1 ± 8.0	81.5 ± 15.0	0.16	—	
Fast time (s)	-222.6 ± 3.5^{a}	-189.9 ± 6.9^{b}	-215.0 ± 2.5^{a}	**0.0017**	NAG≧WPR＞WL	
Mean speed (cm/s)	-10.4 ± 0.2^{a}	-8.7 ± 0.4^{b}	-9.8 ± 0.2^{ab}	0.012	NAG≧WPR≧WL	
Mean speed without resting (cm/s)	-11.4 ± 0.6^{a}	-8.6 ± 0.5^{b}	-11.0 ± 0.3^{a}	**1.7E-04**	NAG≧WPR＞WL	
Maximum speed (cm/s)	66.4 ± 4.6^{ab}	79.5 ± 3.4^{a}	64.6 ± 2.7^{b}	0.0087	WPR≧NAG≧WL	
Parallel index	0.2 ± 0.1^{a}	0.8 ± 0.0^{b}	0.6 ± 0.1^{c}	**1.0E-07**	WL＞WPR＞NAG	
No. of excrements (no)	0.3 ± 0.1^{a}	0.8 ± 0.1^{b}	0.3 ± 0.1^{a}	**1.0E-04**	WL＞NAG＝WPR	

The raw data were adjusted for random effects of environmental factors (see Materials and Methods). The trait differences among the three breeds were tested by the Kruskal-Wallis test, and the P values obtained were approximated by the chi-square value for the one-way test. The P values in bold exceeded the Bonferroni-corrected 5% level.

$^{a-c}$ Means with different letters were significantly different between breeds at $P < 0.05$ by the Steel–Dwass *post hoc* test.

— ＝not applicable.

exploration than WPR in the OF arena.

Table 4 presents the Spearman's rank correlation coefficients among 14 OF traits in each of the three breeds. Over three-quarters of 91 trait combinations were significantly correlated at the nominal 5% level in each of the three breeds. However, across breeds, 22 combinations remained significant at the Bonferroni-corrected 5% significance level (0.05/91＝0.00055). Among these, we focused on correlations in OF traits with significant breed differences in Table 4, because describing all 22 combinations would be redundant. Latency of the 1st entrance to the center zone was negatively correlated with the number of entries in the center zone and the distance in the center zone, both of which were positively correlated with each other. Mean speed without resting was also positively correlated with fast time. However, neither parallel index nor the number of excrements was significantly correlated with any traits across breeds at the Bonferroni-corrected 5% significance level.

As NAG and WL displayed opposite responses to the TI and OF fears, Spearman's rank correlation coefficients were estimated between 10 TI and 14 OF traits in each of the three breeds, and the results are summarized in Table 5. Among 140 trait combinations compared, 19 were significant at the nominal 5% level in NAG. Only one and four combinations were significant in WPR and WL, respectively. However, no combination exceeded the Bonferroni-corrected 5% significance level (0.05/140＝0.00036) in each of the three breeds.

Discussion

The social hierarchy of housed chicken groups is established during the first 5 weeks after hatching (Guhl, 1958). An ordered group structure is maintained for top-ranking chicks during the first 3 weeks of life (Rogers and Astiningsih, 1991). Hence, the difference in social order between young and adult age groups can affect TI and OF behaviors, as previously noted (Jones, 1986; Buitenhuis *et al*., 2004). In fact, Buitenhuis *et al*. (2004) revealed different quantitative trait loci (QTLs) for OF behavior between chickens at 5 and 29 weeks of ages. In addition, Abe *et al*. (2013) quantified innate responses to TI fear in Nagoya and White Leghorn chicks at 1 and 2 days of age. Nakasai *et al*. (2013) reported age-dependent changes in innate TI responses in chicks of Tosa-Jidori, another native Japanese breed, at 2–15 days of age. Therefore, in the present study, newly hatched chicks were used before social order was established in order to measure innate responses to TI and OF fears.

Fear-related behavior is closely associated with a stress response that activates the hypothalamic-pituitary-adrenal (HPA) axis, leading to the release of glucocorticoids (mainly cortisol and corticosterone) from the adrenal glands of animals (Matteri *et al*., 2000). Ericsson and Jensen (2016) analyzed blood corticosterone levels and behavioral responses before and after physical restraint in Red Junglefowl and layer chicks at 1–23 days of age and revealed that the HPA axis responds to stress induced by physical restraint in chicks at 1 day of age. By analyses of behavior, hormone levels, and production traits in layer chickens at hatching to 140 days of age, Hedlund *et al*. (2019) revealed that the stress of a commercial hatchery process (separation from shells, conveying, sex sorting, vaccination, etc.) during the first hours of life has short- and long-term effects on behavior and stress reactivity, and potentially egg production in later life. For example, chicks exposed to hatchery stress presented higher corticosterone levels at hatching and were

Table 4. **Spearman's rank correlation coefficients (*ρ*) between OF traits in each of the Nagoya (NAG), White Leghorn (WL), and White Plymouth Rock (WPR) chicks**

Trait 1	Trait 2	NAG		WL		WPR	
		ρ	*P* value	*ρ*	*P* value	*ρ*	*P* value
Latency of 1st entrance to the center zone	No. of entries in the center zone	−0.80	**4.4E-07**	−0.54	**3.2E-06**	−0.79	**6.2E-06**
Total time in the center zone	No. of entries in the center zone	0.99	**4.9E-21**	0.62	**3.7E-08**	NS	NS
	Latency of 1st entrance to the center zone	−0.78	**8.9E-07**	−0.46	**0.00016**	NS	NS
Distance in the periphery zone	No. of entries in the center zone	NS	NS	0.53	**5.9E-06**	0.46	0.028
	Latency of 1st entrance to the center zone	−0.61	0.00058	−0.36	0.0034	NS	NS
	Total time in the center zone	NS	NS	NS	NS	NS	NS
Distance in the center zone	No. of entries in the center zone	0.99	**1.4E-21**	0.96	**1.9E-35**	0.94	**5.8E-11**
	Latency of 1st entrance to the center zone	−0.81	**2.1E-07**	−0.54	**4.3E-06**	−0.82	**1.7E-06**
	Total time in the center zone	0.98	**7.0E-20**	0.62	**4.9E-08**	NS	NS
	Distance in the periphery zone	0.45	0.016	0.56	**1.4E-06**	NS	NS
Total distance	No. of entries in the center zone	0.44	0.021	0.57	**6.8E-07**	0.61	0.0021
	Latency of 1st entrance to the center zone	−0.69	**4.3E-05**	−0.38	0.0017	NS	NS
	Total time in the center zone	0.44	0.018	NS	NS	NS	NS
	Distance in the periphery zone	0.99	**2.0E-23**	1.00	**1.2E-66**	0.96	**3.0E-13**
	Distance in the center zone	0.54	0.0030	0.61	**9.6E-08**	0.57	0.0044
Resting time	No. of entries in the center zone	−0.53	0.0047	−0.61	**8.9E-08**	−0.67	**0.00045**
	Latency of 1st entrance to the center zone	0.72	**1.6E-05**	0.46	**0.00016**	0.45	0.032
	Total time in the center zone	−0.53	0.0040	NS	NS	NS	NS
	Distance in the periphery zone	−0.94	**8.7E-14**	−0.97	**1.1E-38**	−0.85	**2.5E-07**
	Distance in the center zone	−0.61	**0.00052**	−0.64	**1.2E-08**	−0.67	**0.00042**
	Total distance	−0.97	**2.8E-17**	−0.97	**5.9E-40**	−0.93	**6.6E-11**
Slow time	No. of entries in the center zone	0.61	0.00080	0.61	**6.6E-08**	0.69	**0.00028**
	Latency of 1st entrance to the center zone	−0.62	**0.00048**	−0.47	**0.00010**	−0.50	0.015
	Total time in the center zone	0.60	0.00069	NS	NS	NS	NS
	Distance in the periphery zone	0.73	**1.2E-05**	0.84	**6.8E-18**	0.84	**4.8E-07**
	Distance in the center zone	0.67	**0.00011**	0.65	**4.6E-09**	0.70	**0.00019**
	Total distance	0.78	**1.1E-06**	0.84	**2.0E-18**	0.93	**1.2E-10**
	Resting time	−0.88	**4.8E-10**	−0.93	**2.1E-28**	−1.00	**1.4E-23**
Fast time	No. of entries in the center zone	NS	NS	0.52	**1.2E-05**	NS	NS
	Latency of 1st entrance to the center zone	−0.63	**0.00036**	−0.30	0.016	NS	NS
	Total time in the center zone	NS	NS	NS	NS	0.49	0.018
	Distance in the periphery zone	0.94	**2.8E-13**	0.98	**2.0E-43**	0.76	**2.8E-05**
	Distance in the center zone	0.37	0.050	0.55	**3.2E-06**	NS	NS
	Total distance	0.92	**3.2E-12**	0.97	**5.6E-42**	0.66	**0.00054**
	Resting time	−0.83	**5.4E-08**	−0.92	**3.7E-26**	−0.48	0.022
	Slow time	0.51	0.0057	0.75	**1.0E-12**	0.46	0.027
Mean speed	No. of entries in the center zone	0.44	0.023	0.57	**7.0E-07**	0.61	0.0021
	Latency of 1st entrance to the center zone	−0.69	**5.1E-05**	−0.38	0.0018	NS	NS
	Total time in the center zone	0.44	0.019	NS	NS	NS	NS
	Distance in the periphery zone	0.99	**1.2E-23**	1.00	**7.6E-67**	0.96	**3.0E-13**
	Distance in the center zone	0.54	0.0032	0.61	**9.8E-08**	0.57	0.0044
	Total distance	1.00	**7.5E-48**	1.00	**1.4E-145**	1.00	0
	Resting time	−0.97	**2.4E-17**	−0.97	**5.3E-40**	−0.93	**6.6E-11**
	Slow time	0.78	**1.1E-06**	0.84	**1.9E-18**	0.93	**1.2E-10**
	Fast time	0.92	**3.1E-12**	0.97	**6.6E-42**	0.66	**0.00054**

Table 4. **Spearman's rank correlation coefficients (*ρ*) between OF traits in each of the Nagoya (NAG), White Leghorn (WL), and White Plymouth Rock (WPR) chicks (continued)**

Trait 1	Trait 2	NAG		WL		WPR	
		ρ	*P* value	*ρ*	*P* value	*ρ*	*P* value
Mean speed without resting	No. of entries in the center zone	0.62	0.00056	0.58	**4.9E-07**	0.57	0.0043
	Latency of 1st entrance to the center zone	−0.71	**2.2E-05**	−0.38	0.0019	NS	NS
	Total time in the center zone	0.63	**0.00032**	NS	NS	0.55	0.0064
	Distance in the periphery zone	0.76	**2.8E-06**	0.89	**2.2E-22**	0.76	**2.6E-05**
	Distance in the center zone	0.70	**3.8E-05**	0.60	**1.6E-07**	0.48	0.021
	Total distance	0.78	**7.6E-07**	0.90	**9.0E-24**	0.75	**4.4E-05**
	Resting time	−0.77	**1.9E-06**	−0.85	**5.1E-19**	−0.61	0.0018
	Slow time	0.60	0.00069	0.74	**2.7E-12**	0.62	0.0018
	Fast time	0.76	**2.6E-06**	0.89	**7.5E-23**	0.88	**2.1E-08**
	Mean speed	0.78	**7.8E-07**	0.90	**1.2E-23**	0.75	**4.4E-05**
Maximum speed	No. of entries in the center zone	0.64	**0.00030**	0.62	**5.3E-08**	0.49	0.017
	Latency of 1st entrance to the center zone	−0.61	0.00061	−0.42	0.00057	NS	NS
	Total time in the center zone	0.66	**0.00013**	NS	NS	0.48	0.021
	Distance in the periphery zone	0.49	0.0083	0.70	**8.9E-11**	0.57	0.0045
	Distance in the center zone	0.69	**5.0E-05**	0.64	**1.7E-08**	0.43	0.042
	Total distance	0.55	0.0027	0.71	**3.3E-11**	0.60	0.0025
	Resting time	−0.57	0.0017	−0.72	**1.3E-11**	−0.50	0.016
	Slow time	0.55	0.0024	0.70	**1.1E-10**	0.51	0.014
	Fast time	0.45	0.015	0.67	**1.3E-09**	0.73	**7.2E-05**
	Mean speed	0.55	0.0027	0.71	**3.5E-11**	0.60	0.0025
	Mean speed without resting	0.83	**5.8E-08**	0.81	**6.7E-16**	0.86	**1.4E-07**
Parallel index	No. of entries in the center zone	0.45	0.018	0.52	**1.0E-05**	0.65	0.00089
	Latency of 1st entrance to the center zone	−0.53	0.0035	−0.35	0.0047	−0.49	0.017
	Total time in the center zone	NS	NS	NS	NS	NS	NS
	Distance in the periphery zone	NS	NS	0.76	**5.3E-13**	0.47	0.025
	Distance in the center zone	0.39	0.039	0.54	**4.1E-06**	0.66	0.00068
	Total distance	0.44	0.020	0.76	**5.0E-13**	0.59	0.0028
	Resting time	−0.51	0.0060	−0.80	**2.9E-15**	−0.72	**9.6E-05**
	Slow time	0.53	0.0039	0.77	**1.2E-13**	0.74	**6.2E-05**
	Fast time	0.37	0.050	0.72	**1.9E-11**	NS	NS
	Mean speed	0.44	0.020	0.76	**5.0E-13**	0.59	0.0028
	Mean speed without resting	NS	NS	0.74	**3.6E-12**	NS	NS
	Maximum speed	NS	NS	0.66	**2.6E-09**	NS	NS
No. of excrements	No. of entries in the center zone	0.40	0.037	0.29	0.018	NS	NS
	Latency of 1st entrance to the center zone	NS	NS	NS	NS	NS	NS
	Total time in the center zone	NS	NS	NS	NS	NS	NS
	Distance in the periphery zone	0.47	0.011	NS	NS	0.42	0.044
	Distance in the center zone	0.38	0.048	0.34	0.0059	NS	NS
	Total distance	0.47	0.012	NS	NS	NS	NS
	Resting time	−0.43	0.023	NS	NS	NS	NS
	Slow time	0.42	0.026	NS	NS	NS	NS
	Fast time	NS	NS	NS	NS	0.49	0.018
	Mean speed	0.47	0.012	NS	NS	NS	NS
	Mean speed without resting	0.39	0.039	NS	NS	NS	NS
	Maximum speed	NS	NS	NS	NS	NS	NS
	Parallel index	NS	NS	NS	NS	NS	NS

The *P* values in bold exceeded the Bonferroni-corrected 5% level.
NS=not significant at the nominal 5% level.

more fearful to novelty at 1 day of age than control chicks at the same age not exposed to the stress. When the exposed chicks became adults at 140 days of age, the adults had more feather damage and injuries on their wattle and comb than

Table 5. **Spearman's rank correlation coefficients (ρ) between traits for TI and OF tests in each of the Nagoya (NAG), White Leghorn (WL), and White Plymouth Rock (WPR) chicks**

TI trait	OF trait	NAG		WL		WPR	
		ρ	*P* value	ρ	*P* value	ρ	*P* value
S-duration in the 1st trial	Mean speed without resting	−0.50	0.010	NS	NS	NS	NS
S-duration in the 2nd trial	No. of entries in the center zone	−0.50	0.013	NS	NS	NS	NS
	Latency of 1st entrance to the center zone	0.56	0.0036	NS	NS	NS	NS
	Total time in the center zone	−0.51	0.010	NS	NS	NS	NS
	Distance in the center zone	−0.51	0.010	NS	NS	NS	NS
	Mean speed without resting	−0.52	0.0072	NS	NS	NS	NS
	Maximum speed	−0.49	0.012	NS	NS	NS	NS
	Parallel index	−0.47	0.018	NS	NS	NS	NS
S-duration in the 3rd trial	Distance in periphery zone	0.41	0.042	NS	NS	NS	NS
	Fast time	0.51	0.0095	NS	NS	NS	NS
	Latency of 1st entrance to center zone	NS	NS	0.29	0.036	NS	NS
First S-duration recorded	Distance in the periphery zone	0.43	0.033	NS	NS	NS	NS
	Fast time	0.50	0.010	NS	NS	NS	NS
S-induction	Distance in the center zone	NS	NS	−0.29	0.030	NS	NS
	Parallel index	NS	NS	−0.41	0.0021	NS	NS
	Total time in the center zone	NS	NS	NS	NS	0.42	0.044
L-duration in the 2nd trial	No. of entries in the center zone	−0.45	0.026	NS	NS	NS	NS
	Total time in the center zone	−0.51	0.0088	NS	NS	NS	NS
	Distance in the center zone	−0.48	0.015	NS	NS	NS	NS
	Mean speed without resting	0.40	0.045	NS	NS	NS	NS
L-duration in the 3rd trial	Fast time	0.46	0.022	NS	NS	NS	NS
	Mean speed without resting	0.47	0.017	NS	NS	NS	NS
	No. of excrements	NS	NS	0.34	0.012	NS	NS
L-induction	Mean speed without resting	0.40	0.045	NS	NS	NS	NS

No *P* value exceeded the Bonferroni-corrected 5% level.
NS = not significant at the nominal 5% level.

control animals at the same age, suggesting that feather pecking and aggression occurred more often in the adults. Therefore, the results of those studies clearly demonstrated that the innate fear responses of chicks can be precisely measured even at hatching, and showed that stressful or fearful experiences at hatching and 1 day of age are linked to injurious pecking and other aggressive behaviors in later life, which are importantly related to poultry welfare.

We performed the TI test and then the OF test on the following day. Due to the order of these tests, it is possible that the first behavioral test affected the results of the second behavioral test. We considered this possibility to be minimal, because no phenotypic correlations between TI and OF behavioral traits were observed in each of our NAG, WL, and WPR breeds at Bonferroni-corrected 5% significance levels. The no phenotypic correlations were consistent with a previous report in which no phenotypic correlations were observed between TI and OF traits in other White Leghorn chickens (Heiblum *et al*., 1998). Ours and previous findings suggest that two kinds of fear evoked by the TI and OF tests differ in nature. Therefore, the opposite fear responses observed in our NAG and WL breeds reflected differences in the nature of TI and OF fears.

Previously, Abe *et al*. (2013) reported that the TI duration of the Nagoya breed is longer than that of the White Leghorn breed. The finding of that previous report did not support our TI result with the NAG and WL breeds. This discrepancy may result from differences in genetic backgrounds reflecting differences in the breeding histories of Nagoya and White Leghorn used in our study and the previous report. The previous report used strains of Nagoya and White Leghorn, both of which were developed at the Aichi Agricultural Research Center, Aichi, Japan. Our WL was the WL-G line, which has been maintained as a closed breeding colony at Nagoya University since 1969, and our NAG was the 87 strain developed at the Hyogo station, National Livestock Breeding Center, Hyogo, Japan.

Several studies have reported QTLs for TI and OF fears in chickens. For example, Schütz *et al*. (2004) identified a QTL for TI duration on chromosome 1 in an F_2 intercross population between White Leghorn chickens and Red Junglefowl. Fogelholm *et al*. (2019) reported 18 QTLs for TI behavior on chromosomes 1, 2, 4, 6, 7, 10, 12, 15, 20, and 24, and identified five candidate genes for behavior in an advanced intercross population between White Leghorn chickens and Red Junglefowl. For OF behavior, Buitenhuis *et al*. (2004) revealed 11 QTLs on chromosomes 1, 2, 4, 9, and 10 in an F_2 hen population between two White Leghorn lines selected for egg production traits. Johnsson *et al*. (2016) reported 34 QTLs for OF behavior on chromosomes 1–4, 6–8, 10, 13, and 17, and identified 10 candidate genes for the behavior in an advanced intercross population between White Leghorn chickens and Red Junglefowl. In the present study, we demonstrated that NAG and WL breeds exhibit extreme and opposite behavioral responses to TI and OF. It is thus suggested that the two breeds may be animal resources useful for identification of new QTLs governing the opposing TI and OF behaviors. Some causal genes for QTLs may be HPA-related genes (Matteri *et al*., 2000). Genetic selection for chicks with low fear genes may be beneficial for poultry production, because selected chicks can, for example, increase the ease of human handling and decrease the risk of injurious pecking (feather pecking and vent pecking), which often leads to cannibalism, all of which result in improved poultry welfare.

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Conflicts of Interest

The authors declare no conflicts of interest.

References

Abe H, Nagao K, Nakamura A and Inoue-Murayama M. Differences in responses to repeated fear-relevant stimuli between Nagoya and White Leghorn chicks. Behavioural Processes, 99: 95–99. 2013.

Agnvall B, Jöngren M, Strandberg E and Jensen P. Heritability and genetic correlations of fear-related behaviour in red junglefowl–possible implications for early domestication. PLoS One, 7: e35162. 2012.

Belyaev DK. Destabilizing selection as a factor in domestication. Journal of Heredity, 70: 301–308. 1979.

Belyaev DK, Plyusnina IZ and Trut LN. Domestication in the silver fox (*Vulpes fulvus Desm*): Changes in physiological boundaries of the sensitive period of primary socialization. Applied Animal Behaviour Science, 13: 359–370. 1985.

Buitenhuis AJ, Rodenburg TB, Siwek M, Cornelissen SJB, Nieuwland MGB, Crooijmans RPMA, Groenen MAM, Koene P, Bovenhuis H and van der Poel JJ. Identification of QTLs involved in open-field behavior in young and adult laying hens. Behavior Genetics, 34: 325–333. 2004.

Campler M, Jöngren M and Jensen P. Fearfulness in red junglefowl and domesticated White Leghorn chickens. Behavioural Processes, 81: 39–43. 2009.

Ericsson M and Jensen P. Domestication and ontogeny effects on the stress response in young chickens (*Gallus gallus*). Scientific Reports, 6: 35818. 2016.

Fogelholm J, Inkabi S, Höglund A, Abbey-Lee R, Johnsson M, Jensen P, Henriksen R and Wright D. Genetical genomics of tonic immobility in the chicken. Genes, 10: 341. 2019.

Forkman B, Boissy A, Meunier-Salaün M. C, Canali E and Jones RB. A critical review of fear tests used on cattle, pigs, sheep, poultry and horses. Physiology & Behavior, 92: 340–374. 2007.

Guhl AM. The development of social organisation in the domestic chick. Animal Behaviour, 6: 92–111. 1958.

Hedlund L, Whittle R and Jensen P. Effects of commercial hatchery processing on short- and long-term stress responses in laying hens. Scientific Reports, 9: 2367. 2019.

Heiblum R, Aizenstein O, Gvaryahu G., Voet H, Robinzon B and Snapir N. Tonic immobility and open field responses in domestic fowl chicks during the first week of life. Applied Animal Behaviour Science, 60: 347–357. 1998.

Johnsson M, Williams MJ, Jensen P and Wright D. Genetical genomics of behavior: a novel chicken genomic model for anxiety behavior. Genetics, 202: 327–340. 2016.

Jones RB. The tonic immobility reaction of the domestic fowl: a review. World's Poultry Science Journal, 42: 82–97. 1986.

Kato S, Ohguchi H, Ando I, Kawamura T and Ohsuga A. Studies on feeding and management for Nagoya breed: effects of bird density and debeaking treatment on productivity of birds, and factors causing crowding accidents when rearing birds in a big flock on floor pens. Research Bulletin of the Aichi-Ken Agricultural Research Center, 23: 453–457. 1991. (In Japanese).

Kerje S, Carlborg Ö, Jacobsson L, Schütz K, Hartmann C, Jensen P and Andersson L. The twofold difference in adult size between the red junglefowl and White Leghorn chickens is largely explained by a limited number of QTLs. Animal Genetics, 34: 264–274. 2003.

Li Z, Zheng M, Abdalla BA, Zhang Z, Xu Z, Ye Q, Xu H, Luo W, Nie Q and Zhang X. Genome-wide association study of aggressive behaviour in chicken. Scientific Reports, 6: 30981. 2016.

Matteri R, Carroll J and Dyer C. Neuroendocrine responses to stress. In: The biology of Animal Stress (Moberg GP and Mench JA eds.). pp: 43–76. CABI Publishing, Wallingford. 2000.

Millman ST, Duncan IJH and Widowski TM. Male broiler breeder fowl display high levels of aggression toward females. Poultry Science, 79: 1233–1241. 2000.

Nakamori T, Maekawa F, Sato K, Tanaka K and Ohki-Hamazaki H. Neural basis of imprinting behavior in chicks. Development, Growth & Differentiation, 55: 198–206. 2013.

Nakasai E, Tanizawa H, Takawaki M, Yanagita K, Kawakami S-I, Oka T, Tsudzuki M and Bungo, T. Age-dependent change of tonic immobility response in chicks of a native Japanese chicken breed, Tosa-Jidori. Journal of Poultry Science, 50, 321–325. 2013.

Osman SAM, Sekino M, Nishihata A, Kobayashi Y, Takenaka W, Kinoshita K, Kuwayama T, Nishibori M, Yamamoto Y and Tsudzuki M. The genetic variability and relationships of Japanese and Foreign chickens assessed by microsatellite DNA profiling. Asian-Australasian Journal of Animal Sciences, 19: 1369–1378. 2006.

Price EO. Behavioral development in animals undergoing domestication. Applied Animal Behaviour Science, 65: 245–271. 1999.

Rogers LJ and Astiningsih K. Social hierarchies in very young chicks. British Poultry Science, 32: 47–56. 1991.

Schütz KE, Kerje S, Jacobsson L, Forkman B, Carlborg Ö, Andersson L and Jensen P. Major growth QTLs in fowl are related to fearful behavior: possible genetic links between fear responses and production traits in a red junglefowl×White Leghorn intercross. Behavior Genetics, 34: 121–130. 2004.

Suzuki S, Kobayashi M, Murai A, Tsudzuki M and Ishikawa A. Characterization of growth, fat deposition, and lipid metabolism-related gene expression in lean and obese meat-type chickens. Journal of Poultry Science, 56: 101–111. 2019.

Tsudzuki M. Japanese native chickens. In: The Relationships between Indigenous Animals and Humans in the APEC Region (Chang H-L and Huang Y-C eds). pp. 91–116. The Chinese Society of Animal Science. Chinese Taipei. 2003.