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Blood Testosterone Concentration and Testosterone-induced Aggressive Behavior in Male Layer Chicks: Comparison between Isolated- and Grouped-Raising

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Testosterone (T) is known to induce aggressive behavior, mainly in male animals. Subcutaneous implantation of T-filled silastic tubes, rather than intramuscular injection of T, is generally recommended for long-term treatment using exogenous T. However, the effect of T implantation on chicken aggressive behavior has not been investigated. In addition, the concentration of T required to induce aggressive behavior or whether rearing conditions such as isolated- or grouped-raising affect T-induced aggressive behavior in chickens is not known. The present study aimed to examine the relationship between the lengths of T-filled tubes, blood T concentration, and aggressive behavior in group- and isolation-raised male layer chicks. The testes were bilaterally removed and silactic tubes of various lengths filled with crystalline T were subcutaneously implanted at 14 days of age. A social interaction test was performed to quantitatively assess chick aggressive behavior at 32 days of age. Comb weight and size were used to assess the activation of endogenous androgen receptors. Total aggression frequencies (TAF) and aggression establishment rate (AER) were used to evaluate aggressiveness. Significant positive correlations ($P<0.001$) were observed between the comb parameters and plasma T concentration. In the isolation-raised chicks, the TAF and AER were high irrespective of the lengths of the implanted T tubes or the corresponding plasma T concentrations. However, in the group-raised chicks, the AER tended to differ between the T-implanted aggressors ($P=0.0902$), and the AER significantly increased with implantation of 1.0-cm-long T-filled tubes ($P<0.05$), which corresponded to approximately 47 pg/mL plasma T concentration. These results suggest that both grouped raising and approximately 47 pg/mL plasma T concentration are required for the induction of T-dependent aggressive behavior, and that isolation-induced aggressive behavior is T-independent in male layer chicks.

Key words: aggressive behavior, male layer chicks, social interaction test, testosterone

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Introduction

Aggressive behavior is a social behavior that is associated with conflict between two individuals (Scott and Fredericson, 1951). In general, aggressive behavior is classified into two types: offensive and defensive aggression (Veroude *et al*., 2016). Offensive aggression is characterized by an unprovoked act by the aggressors, and defensive aggression is caused by the perception of threat from other individuals with the purpose of eliminating the threat. Ethological studies have illustrated that aggressive behavior is essential for winning a competition for limited resources, such as mates, territories, and feed (Oakeshott, 1974; Balshine *et al*., 2005; Suwanvecho and Brockelman, 2012). Aggressive encounters are also visible in the formation of dominant hierarchies between conspecifics (Issa *et al*., 1999), and in anti-predator defense (Díaz-Uriarte, 1999). As chickens are social animals that naturally live in groups and hold a determined territory (McBride *et al*., 1969), they show high frequency of aggressive behavior towards conspecifics (Craig *et al*., 1969). Severe aggression, however, leads to serious economic problems in the poultry industry: male broilers display high levels of aggression against females, and the sustained fearfulness and injuries of females inflicted by males result in reduced fertility and increased mortality (Millman *et al*., 2000). Therefore, it is necessary to elucidate the mechanisms of chicken

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aggressive behavior and adopt countermeasures against severe aggression of chickens in the poultry industry.

Sex steroid hormones are known to induce aggressive behavior of animals. Testosterone (T) is one of the most studied androgen. It is mainly produced in the testes and transferred to the brain and other target tissues through blood circulation. Castrated male chicks showed reduced aggressive behavior toward male conspecifics (Berthold and Quiring, 1944), and intramuscular injection of T increased their frequencies of aggressive behavior (Andrew, 1975; Astiningsih and Rogers, 1996). In the target organs, T directly binds to the androgen receptors (ARs), or is converted to 5α-dihydrotestosterone (DHT) by the enzyme 5α-reductase. DHT also binds to the ARs and triggers AR-mediated intracellular activities. Aromatization is another important metabolic pathway of T: aromatase converts T into 17β-estradiol (E2), which binds to and activates the estrogen receptors (ERs). In male Japanese quails, subcutaneous administration of T or E2, not DHT, resulted in a significant increase in aggression display (Tsutsui and Ishii, 1981), and administration of 4-hydroxyandrostenedione, an aromatase inhibitor, blocked T-induced aggressive behavior (Schlinger and Callard, 1990). In addition, aromatase activity was detected in the anterior parts of the quail hypothalamus, and it was significantly higher in males than in females (Balthazart *et al*., 1990). These reports show that circulating T is mainly converted to E2, which promotes T-induced aggressive behavior in the brain of quails. However, in male chickens, administration of T or DHT was reported to induce aggressive behavior, whereas E2 administration did not (Young and Rogers, 1978; Clifton *et al*., 1986; Clifton and Andrew, 1989), which suggests that activation of ARs, not ERs, is essential for the induction of aggressive behavior in chickens. Therefore, to understand the effect of T on aggressive behavior in chickens, it is necessary to determine the concentration of T that is sufficient to induce aggressive behavior in chickens. However, the reports assessing the relationship between blood T concentration and aggressive behavior in chickens are lacking. It is noteworthy that subcutaneous implantation of a silastic tube filled with crystalline T, rather than intramuscular injection of T dissolved in oil, is generally recommended for long-term treatment using exogenous T (Schlinger and Callard, 1990; Albert *et al*., 1990); however, studies investigating the effect of subcutaneous T implantation on aggressive behavior of chickens are lacking. Therefore, to improve our understanding of the effect of T on aggressive behavior of chickens, it is necessary to elucidate the relationship between the length of the implanted T-filled tubes, blood T concentration, and the occurrence of aggressive behavior.

In general, two behavioral models (i.e., social interaction (SI) and resident-intruder (R-I) tests) have been used to evaluate aggressive behavior in animals. The SI test is used to monitor various kinds of social behaviors, such as sniffing, grooming, and attacking (Silverman *et al*., 2010). The R-I test is used to study the territorial aggression induced by intrusion of another conspecific to the experimentally reproduced territory of animals (Koolhaas *et al*., 2013). To develop effective behavioral models that quantitatively estimate aggressive behavior in chickens, we previously monitored the aggressive behavior of male layer chicks from 8 to 24 days of age using the SI and R-I tests (Raihan *et al*., 2017). The chicks in the R-I test showed aggressive behavior more frequently than those in the SI test, indicating that the R-I test, rather than the SI test, is more effective in monitoring the aggressive behavior of male layer chicks. However, it is likely that the low frequency of aggressive behavior in the SI test is due to low T concentration in the chick blood in our previous study. As mentioned previously, the SI test can monitor isolation-induced aggression, and the R-I test can detect territorial aggression. Whether testosterone is essential for inducing isolation-induced aggression of avian is not known, although several reports have showed that avian territorial aggression, especially in the non-breeding season, was T-independent (Schwabl and Kriner, 1991; Canoine and Gwinner, 2002; Marasco *et al*., 2011; Apfelbeck *et al*., 2012). In addition, the plasma concentration of T in male chicks is reported to be low until 28 days of age (Tanabe *et al*., 1979). These observations suggest that exogenous T supplementation promotes higher frequencies of aggressive behavior in male layer chicks in the SI test, which affects investigations regarding the mechanisms of aggressive behavior in chickens using this test.

The present study aimed to investigate aggressive behavior and blood T concentration of the T-implanted male layer chicks using the SI test. Silactic tubes of various lengths filled with crystalline T were subcutaneously implanted into castrated male layer chicks, and the frequencies of aggressive behavior and plasma T concentration were measured. The comb area and weight in the T-implanted chicks were also measured as indices of T-sensitive tissue growth (Zeller, 1971). As a previous report showed that chicks raised in isolation were more aggressive than those raised in groups (Guhl, 1958), we also compared the aggressive behavior of the chicks between isolated- and grouped-raising.

Materials and Methods

Animal Management and Experimental Design

One-day-old male layer chicks (Julia Lite) were obtained from a local hatchery (Akita Co., Ltd., Hiroshima, Japan). The chicks were maintained in a room (3.4×3.5×2.1 m, length×width×height) with 20-h lighting and 4-h dark cycle with lights on at 3 AM. The temperature was set at 30℃ for the first few days and gradually lowered to 26℃ according to the growth of the chicks. They were given free access to a commercial starter diet (Chubushiryo Co., Ltd., Aichi, Japan) and water during the experimental period. All experimental protocols were approved by the Animal Experiment Committee of Hiroshima University.

The chicks were reared in groups (3–4 chicks per cage) until 13 days of age in the home cages (30×20×25 cm, length×width×height). On 14 days of age, the chicks were bilaterally caponized under isoflurane anesthesia according to the method of Rikimaru *et al*. (2011), and various lengths of silastic tubes (Laboratory Tubing 508-007, O.D.=2.41

mm, I.D.＝1.57 mm, Dow Corning, MI, USA) filled with crystalline T (Sigma-Aldrich, MO, USA) were subcutaneously implanted. In Experiment 1, the chicks were divided into three groups: chicks in which one blank 2-cm-long silastic tube was implanted (TB, n＝11); chicks in which one 2-cm-long T-filled silastic tube was implanted (T 2 cm×1, n＝9); chicks in which two 2-cm-long T-filled silastic tubes were implanted (T 2 cm×2, n＝8). The chicks were reared in isolation in the home cage up to the time of the SI test. In Experiment 2, the chicks were divided into three groups: TB (n＝15); T 2 cm×1, (n＝9); chicks in which 1 1-cm-long T-filled silastic tube was implanted (T 1 cm×1, n＝9). The chicks were reared in groups (three chicks per cage) in the group cage (30×50×25 cm, length×width×height) up to the time of the SI test.

SI Test

The SI test was performed with 32-day-old male layer chicks as described by Raihan *et al*. (2017). After measuring body weight with an electronic scale (HF-2000, A&D Co. Ltd., Tokyo, Japan), a pair of chicks, T-implanted (as an aggressor) and intact (as an opponent), were simultaneously transferred by hand to the diagonal corners of the observation cage (44×30×24 cm, length×width×height), and aggressive behavior of the aggressor and opponent was recorded using a video camera (GZ-R470, JVC KENWOOD Corporation, Kanagawa, Japan). Total aggression frequencies (TAF) were determined for the indices of aggressive behavior of the chicks (Raihan *et al*., 2017). TAF are defined as the sum of the frequencies of pecking, biting, kicking, threatening, and leaping. Brief descriptions of each aggression display are as follows (Xie *et al*., 2010): pecking: the male chick pecks the opponent's body or head; biting: the male chick bites the opponent's body, head, or legs; kicking: the male chick kicks the opponent's body; threatening: the male chick stands in front of another male with its neck and head raised and wings slightly extended; leaping: the male chick jumps toward his opponent while the opponent flees. All tests were conducted between 9 AM and 1 PM.

Aggression Establishment Rate (AER)

To compare the aggressiveness of the chicks in the SI tests, we calculated the AER (Raihan *et al*., 2017), which is equal to the number of aggressors showing high aggressive behavior per total behavioral trials. The criterion of high aggressive behavior was defined as the TAF, where aggressors showed more than 30 times TAF and the opponents showed less than one-third the TAF of the aggressors. Thus, the AER is defined as a rate of aggressors showing high aggressiveness with few counterattacks from opponents.

Measurement of Comb Weight, Comb Area, and Plasma T Concentration

After the SI test, blood samples were collected from the wing vein of the T-implanted aggressor chicks. Blood was centrifuged at 5,000×g for 15 min, and plasma was stored at −20℃ for analyzing of plasma T concentration. After blood sampling, the aggressor chicks were sacrificed and the combs were removed with surgical scissors. Comb weight was measured with an electronic scale (FZ-300iWP, A&D Co., Ltd., Tokyo, Japan). The digital images of the combs were captured using the video camera (GZ-R470), and analyzed with ImageJ 1.46r (Schneider *et al*., 2012) for measurement of comb area.

Plasma T concentration was determined using enzyme immunoassay, as described by Isobe *et al*. (2005a, b). One hundred microliters of plasma was extracted with 3 mL of diethyl ether. The ether phase was decanted into another tube and evaporated. Borate buffer (0.05 M boric acid, 0.1% bovine serum albumin (BSA), and 0.05 mg/mL potassium dichromate) was added to the tube for suspending T. The extracted sample was added to the well of plates that were previously coated with goat anti-rabbit IgG antibody (Sato *et al*., 2011). Then, antibody against testosterone-3 (E) - carboxymethyloxime conjugated to BSA (Cosmo Bio Co. Ltd., Tokyo, Japan) and horseradish peroxidase-labeled T (Cosmo Bio) were applied to the wells, followed by incubation at room temperature for 2 h. After washing, a substrate solution containing 0.25 mg/mL 3,3',5,5'-tetramethylbenzidine and 0.05 M citric acid was added to the wells, followed by incubation for 30 min at room temperature. The optical density was measured at 650 nm using a microplate reader (Multiskan FC, Thermo Fisher Scientific Inc., MA, USA). The test of parallelism revealed that the sequential dilution of the chick plasma samples were parallel to the T standard curve (data not shown).

Statistical Analyses

For comparisons of body weight, comb weight, comb area, plasma T concentration, and TAF between the experimental groups, we performed one-way analysis of variance (ANOVA) using the GLM procedure of SAS for Windows software version 9.4 (SAS Institute Inc., Cary, NC, USA). The significance of the differences between means was assessed using a Tukey-Kramer test. For correlation analysis between the comb parameters and plasma T concentration, we calculated Pearson's correlation coefficient using the CORR procedure of SAS. For comparing AERs between the experimental groups, we performed Pearson's chi-square test using the FREQ procedure of SAS, and the significance of the differences between AERs was assessed using analysis of the residuals with js-STAR version 8.9.7j. Statistical significance was set as $P<0.05$.

Results

One-way ANOVA revealed that there were no significant differences in body weight between the T-implanted aggressors in both experiments (P＝0.2029 in Experiment 1; P＝0.1523 in Experiment 2). One-way ANOVA also revealed that an increase in tube length was associated with a significant increase in comb weight (Fig. 1a, $P<0.05$), comb size (Fig. 1b, $P<0.05$), and plasma T concentration (Fig. 1c, $P<0.05$) of the male layer chicks. The average plasma T concentration (± standard error of the mean) in each experimental group was as follows (pg/mL): TB: 24.3±2.85; T 1 cm×1: 47.1±6.47; T 2 cm×1: 85.9±5.64; T 2 cm×2: 154.5 ±23.40 (Fig. 1c). Pearson's correlation analysis revealed a strong positive correlation between the comb weight and

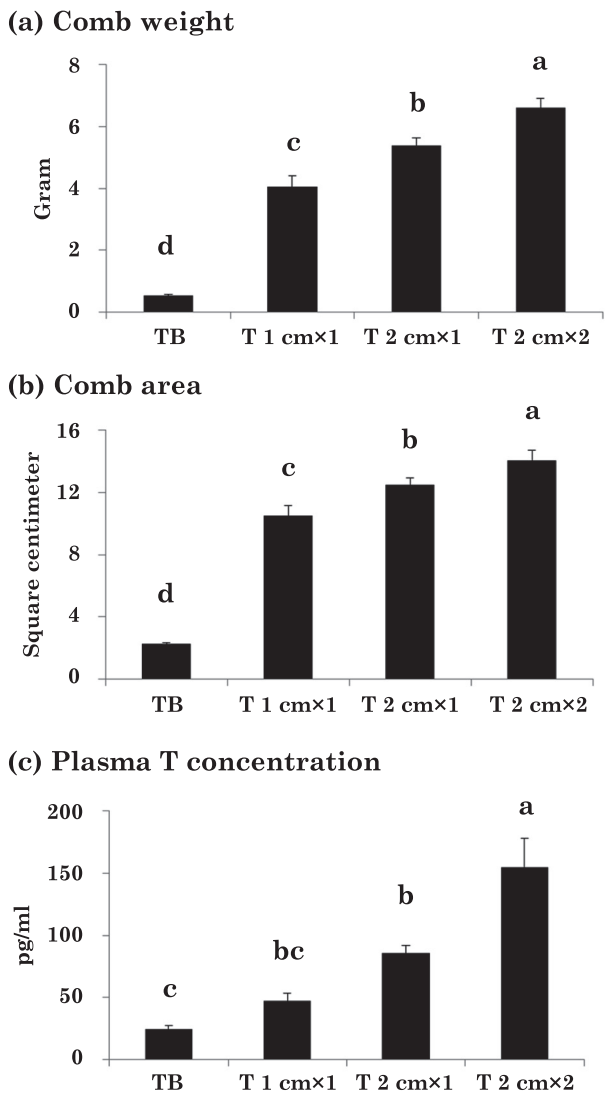

Fig. 1. **Comb weight (a), comb area (b), and plasma testosterone (T) concentration (c) in the male layer chicks in which silactic tubes of various lengths filled with crystalline T were subcutaneously implanted.** TB: chicks in which one blank 2-cm-long silastic tube was implanted; T 1 cm×1: chicks in which one 1-cm-long T-filled silastic tube was implanted; T 2 cm×1: chicks in which one 2-cm-long T-filled silastic tube was implanted; T 2 cm×2: chicks in which two 2-cm-long T-filled silastic tubes were implanted. Different letters above the bars denote significant differences ($P<0.05$).

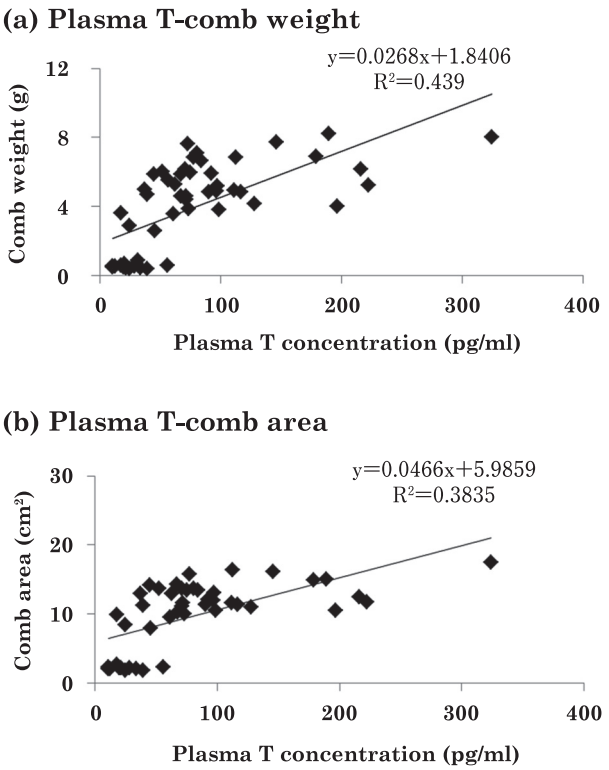

Fig. 2. **Correlation between the comb weight and plasma T concentration (a), and between the comb area and plasma T concentration (b).**

plasma T concentration (Fig. 2a, $R^2=0.439$, $P<0.001$), and between the comb area and plasma T concentration (Fig. 2b, $R^2=0.3835$, $P<0.001$).

In the isolation-raised male layer chicks, one-way ANOVA revealed that there were no significant differences in the TAF between the aggressors in TB, T 2 cm×1, and T 2 cm×2 groups (Fig. 3a, $P=0.9073$). Pearson's chi-square test showed that there were no significant differences in the AER between the aggressors in TB, T 2 cm×1, and T 2 cm×2 groups (Fig. 3b, $P=0.5239$). In the isolated raising, high TAF and AER were observed in chicks of the TB group in which the silastic tubes containing no T were implanted (Fig. 3).

In the group-raised male layer chicks, one-way ANOVA showed that there were no significant differences in the TAF between the aggressors in TB, T 1 cm×1, and T 2 cm×1 groups (Fig. 4a, $P=0.1216$). Pearson's chi-square test, however, revealed that there was a trend towards differences in the AER between the aggressors in TB, T 1 cm×1, and T 2 cm×1 groups (Fig. 4b, $P=0.0902$), and analysis of the residuals showed that the AER significantly increased in the T 1 cm×1 group, compared to TB and T 2 cm×1 groups (Fig. 4b, $P<0.05$).

Discussion

In the present study, T implantation significantly increased the comb weight and size of the male layer chicks in a dose-dependent manner (Fig. 1). Our results showed that T implantation induced aggression in the chicks. It was apparent that the sex steroid receptors of the chicks in the present study were active in both peripheral and central tissues, and that it could mediate sex steroid-dependent biological actions, such as comb growth and induction of aggressive behavior. Previous reports on chickens strongly indicated that

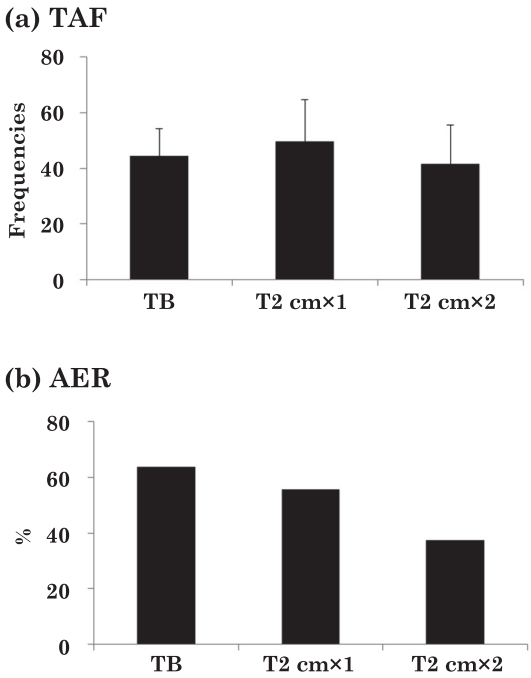

Fig. 3. **Total aggression frequencies (TAF, a) and aggression establishment rate (AER, b) in the isolation-raised male layer chicks.**

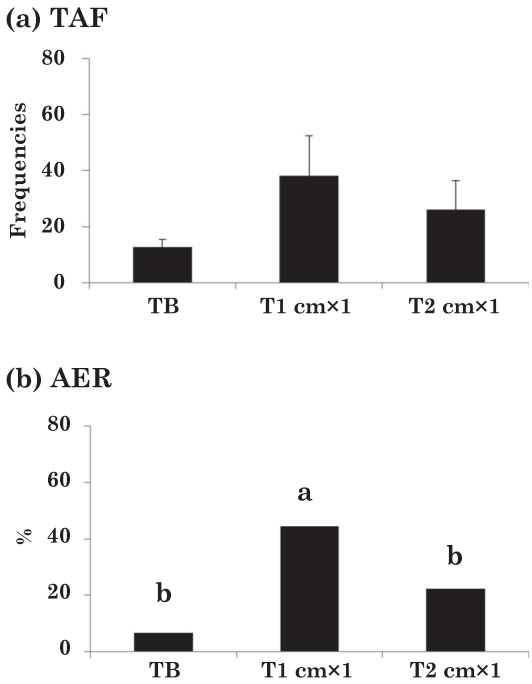

Fig. 4. **TAF (a) and AER (b) in the group-raised male layer chicks.** Different letters above the bars denote significant differences ($P<0.05$).

ARs, not ERs, mainly mediated T-dependent comb growth; in other words, the administration of DHT, which binds to ARs and is not converted to E_2, increased the comb weight of the castrated male layer chicks (Zeller, 1971). Oral administration of ICI176334, a nonsteroidal anti-androgen, suppressed T-induced comb growth (Fennell e*t al*., 1996). The activity of 5α-reductase, which converts T to DHT, and AR-immunoreactivity were also observed in the combs of chickens (Gloyna and Wilson, 1969; Shanbhag and Sharp, 1996). These reports suggest that circulating T is conveyed in the comb tissue and converted to DHT, which binds and activates ARs and promotes comb growth in chickens. Our results also revealed a significant positive correlation between comb size and plasma T concentration (Fig. 2). T concentration in the blood is an important index that determines the extent of sexual maturation in male domestic animals; however, conducting T assays in the poultry industry is time- and money-intensive. Our results showed that plasma T concentration of chickens can be determined easily by calculating the size of the combs from digital images, which offers the poultry industry an easy and useful T assay that is non-invasive and inexpensive.

Our results revealed that the isolation-raised chicks in the TB group showed higher levels of TAF and AER (Fig. 3), which suggests that the rearing condition, such as isolated-raising, influences the aggressiveness of chickens irrespective of the plasma T concentration. Therefore, isolated-raising is not suitable for monitoring T-induced aggression in chickens. Consistent with our results, previous reports also showed that chicks in isolated-raising displayed higher frequencies of aggressive behavior than group-raised ones (Guhl, 1958), and that long-term isolation increased aggressive behavior in male mice (Valzelli, 1973) and rats (Wongwitdecha and Marsden, 1996). Although the reason underlying the increase in aggressive behavior in isolated animals is not known, isolated-raising is widely used for experimental induction of aggressive behavior, and a gamma-aminobutyric acid (GABA)-mediated mechanism for isolation-induced aggression has been proposed for rodent models. Socially-isolated male mice showed reduced responsiveness to sedatives such as pentobarbital, which act by potentiating the action of GABA at the GABA-A receptors (Matsumoto *et al*., 1999). Furthermore, lower binding capacity of GABA was observed in the synaptosomal fraction of isolated mice brains than in those of group-raised ones (DeFeudis *et al*., 1976). The increase in the duration of aggressive behavior of isolated mice was inversely related to the content of endogenous olfactory 3α, 5α- tetrahydroprogesterone, a neurosteroid that is known to suppress isolation-induced aggressive behavior in mice and is endowed with potent positive allosteric modulatory activity of GABA at the GABA-A receptor (Pinna *et al*., 2003). As intraperitoneal administration of muscimol, a GABA-A receptor agonist, is known to inhibit isolation-induced aggressive behavior in mice (Puglisi-Allegra and Mandel, 1980), it is suggested that isolated-raising attenuates GABA-A-mediated neurotransmission and consequently promotes aggression in rodents. However, information regarding the relationship between GABA neuro-

transmission and avian aggressive behavior is lacking. Oral administration of diazepam, a benzodiazepine that binds to the GABA-A receptors and enhances the action of GABA, suppressed feed competition behavior of female pigeons (Fachinelli *et al*., 2003), and mRNA of glutamic acid decarboxylase-65, a GABA synthesizing enzyme, was localized in the chick hypothalamus, such as the preoptic nucleus, paraventricular nucleus, and mammillary body (Sun *et al*., 2005). Therefore, it is necessary to elucidate whether GABA-A-mediated neurotransmission plays an important role in isolation-induced aggression in the brain of chickens, and whether T is really required to promote chicken aggressive behavior.

In the present study, T-induced aggressive behavior was observed in the T 1 cm×1 group in the group-raised chicks (Fig. 4). T is well-known to play an important role in facilitating aggressive behavior of male animals; for example, castration decreased the frequencies of aggressive behavior in rodents, and subcutaneous replacement of T restored the behavior in castrated animals (Beeman, 1947; Barfield *et al*., 1972). Aggressive behavior in adult male Japanese quails (*Coturnix coturnix japonica*) was suppressed after castration and subcutaneous injection of T recovered aggressive behavior in the castrated birds (Tsutsui and Ishii, 1981). Similarly, castration of immature male chicks decreases their male-typical behaviors, such as crowing and aggressive fighting with other males (Berthold and Quiring, 1944). Furthermore, intramuscular administration of T induces aggression in male chicks, but not in females (Andrew, 1975; Astiningsih and Rogers, 1996). However, the amount of T in blood that is sufficient to induce aggressive behavior in chickens is not known. In the present study, blood T concentration of the chicks in the T 1 cm×1 group was approximately 47 pg/mL (Fig. 1), which suggests that this concentration in the blood is required to facilitate aggressive behavior in chickens. On the other hand, aggressive behavior appears to be suppressed in the T 2 cm×1 group in the group-raised chicks (Fig. 4). The present study showed that the blood T concentration of the chicks in the T 2 cm×1 group was approximately 86 pg/mL (Fig. 1), and this concentration might cause ligand-induced AR inactivation which has already been reported for other receptors, such as those of insulin (Carpentier, 1994). In addition, the expression of the gene encoding AR was suppressed by high concentration (100 nmol/L) of T in human megakaryocytes and erythroleukemia cells *in vitro* (Khetawat *et al*., 2000), suggesting that higher concentrations of blood T inhibit AR gene expression in chicken brains. Further studies are required to elucidate the relationship between AR and blood T concentration in chicken brains.

Previous reports using laboratory rodents suggested that various neurotransmitters play an important role in the regulation of aggressive behavior. Serotonin (5-HT) mediates adaptive and pathological forms of aggressive behavior. Intraperitoneal administration of parachlorophenylalanine, an inhibitor of 5-HT synthesis, was found to increase offensive, but not defensive, aggression in male rats (Vergnes *et al*., 1986), which suggests that 5-HT suppresses the aggressive behavior induced by anger and impulse. GABA is one of the major inhibitory neurotransmitters in the brain. Injection of bicuculline methiodide, a GABA-A receptor antagonist, into the ventral parts of the hypothalamus elicited aggressive behavior in male rats, showing that GABA-A receptors play an important role in suppressing aggressive behavior (Roeling *et al*., 1993). Dopamine is another major neurotransmitter that is also involved in mediating animals' motivated behavior, such as reproductive and feeding behaviors. A previous study using microdialysis revealed that an increased dopamine level was detected in the nucleus accumbens of male rats that anticipated the next aggression episode (Ferrari *et al*., 2003). These previous reports on rodents have revealed candidate neurotransmitters that regulate aggressive behavior in the brain, although the mechanisms of aggression in other species, such as chickens, remain unknown. Further studies are required to elucidate the role of neurotransmitters that play important roles in regulation of aggressive behavior in chickens.

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