

《Research Note》

Effects of Testicular and Non-Testicular Testosterone on Territorial and Isolation-induced Aggressive Behavior of Male Layer Chicks

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Testosterone (T) is known to induce aggressive behavior, particularly in male animals. However, our recent results showed that a certain kind of aggressive behavior is T-independent; moreover, the role of T in chicken territorial and isolation-induced aggressive behavior has not yet been investigated. In addition, castration alone is insufficient to evaluate the role of T in aggressive behavior because we found that non-testicular T concentration, probably derived from the adrenal gland, in the blood of castrated chicks was low, but not zero. In the present study, therefore, the role of testicular T in chicken aggressive behavior was evaluated through castration, and the role of non-testicular T was assessed using the subcutaneous implantation of flutamide, a non-steroidal antiandrogen, in the castrated male layer chicks. Resident-intruder (R-I) and social interaction (SI) tests were used to quantitatively monitor territorial and isolation-induced aggressive behavior, respectively. Castration and drug implantation of the chicks were performed at 14 days of age. The R-I test was performed at 29 and 30 days of age, and the SI test was performed at 31 and 32 days of age. The total aggression frequencies (TAFs) and aggression establishment rate (AER) were used as indices of chick aggressive behavior. In the R-I test, castration significantly decreased the TAFs but the AER was not affected by castration or flutamide implantation. In the SI test, on the other hand, there were no significant differences in the TAFs, but the AER tended to increase in the intact chicks and decrease in the flutamide-implanted, castrated male chicks. These results suggest that the role of T in chicken aggression depends on the differences in social context of the behavior, and that both testicular and non-testicular T play an important role in the occurrence of isolation-induced aggression in male layer chicks.

Key words: aggressive behavior, flutamide, male layer chicks, resident-intruder test, social interaction test, testosterone

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Introduction

Testosterone (T) is known to induce aggressive behavior, particularly in male animals. Castration decreases the frequency of aggressive behavior in rodents, and the subcutaneous replacement of T restores the behavior of castrated ones (Beeman, 1947; Barfield *et al.*, 1972). Castration also successfully decreases aggressive behavior in male chicks (Berthold and Quiring, 1944), and the frequency of aggressive

behavior is increased through the intramuscular injection of T in male chicks (Andrew, 1975; Astiningsih and Rogers, 1996). On the other hand, several reports have shown that for animals in which the action of T is pharmacologically blocked, aggressive behavior can also be expressed. The subcutaneous administration of non-steroidal antiandrogen, such as flutamide, in castrated male mice reduces the stimulatory effect of T on ventral prostate weight but fails to suppress T-induced territorial aggression (Clark and Nowell, 1980). Similarly, the subcutaneous implantation of flutamide in intact male European robins suppresses aggressive defense of breeding territories during the breeding season but not the defense of feeding territories during the non-breeding season (Schwabl and Kriner, 1991). These studies suggest that a certain kind of aggressive behavior, such as territorial aggression, is T-independent in both rodent and avian species.

In order to establish effective behavioral models that

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quantitatively monitor chicken aggressive behavior, we observed the aggressive behavior of male layer chicks using the resident-intruder (R-I) and social interaction (SI) tests (Raihan *et al.*, 2017). In general, the R-I test is used to monitor territorial aggression induced by invasion of another conspecific to the experimental territory of an animal (Koolhaas *et al.*, 2013), and the SI test is used to study social behavior between two animals, such as sniffing, grooming, and isolation-induced aggressive behavior (Silverman *et al.*, 2010). In our behavioral trials, the resident chicks in the R-I test showed aggressive behavior more frequently than the intruder ones from eight days of age (Raihan *et al.*, 2017). These results suggest that territorial aggression in chickens is T-independent, because the plasma concentration of T in male chicks is reported to be low until 28 days of age (Tanabe *et al.*, 1979). In addition, our latest results showed that the chicks which were castrated and reared in isolation for more than two weeks expressed high frequencies of aggressive behavior (Yan *et al.*, 2019), suggesting that isolation-induced aggressive behavior is also T-independent in chickens. However, the concentration of blood non-testicular T in the castrated chicks, probably derived from the adrenal gland (Tanabe *et al.*, 1979), is approximately 24 pg/ml (Yan *et al.*, 2019). Therefore, it is impossible to completely exclude the effect of a lower concentration of blood T on chick aggressive behavior by castration alone. In addition, our preliminary experiment showed that the surgical removal of the adrenal gland resulted in a high mortality rate of the male chicks ($n=22$), suggesting that it is difficult to examine the role of non-testicular T in chick aggressive behavior by adrenalectomy.

The aim of the present study, therefore, was to clarify the role of both testicular and non-testicular T in chicken aggressive behavior. The role of testicular T was evaluated through castration, and that of non-testicular T was assessed using the subcutaneous implantation of flutamide, a non-steroidal antiandrogen, into the castrated male layer chicks. The R-I and SI tests were used to quantitatively monitor territorial and isolation-induced aggressive behavior, respectively. The total aggression frequencies (TAFs) and aggression establishment rate (AER) were used as indices of aggressive behavior of the chicks (Raihan *et al.*, 2017; Yan *et al.*, 2019).

Materials and Methods

White Leghorn layer chicks were purchased from the National Livestock Breeding Center (Okazaki, Japan). The direct polymerase chain reaction method for the sexing of the chicks was performed according to Takenouchi *et al.* (2018). The male chicks were maintained in a room (3.4×3.5×2.1 m, length×width×height) with 14-h lighting and 10-h dark, with the lights being turned on at 7 am. The temperature was set at 30°C from the first days and gradually lowered to 26°C according to the growth of the chicks. The chicks were given free access to water and a commercial starter diet (Chubushiryō Co., Ltd., Aichi, Japan) 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) in home cages (30×20×25 cm, length×width×height) until 13 days of age. At 14 days of age, the chicks were bilaterally castrated under isoflurane anesthesia according to the method of Rikimaru *et al.* (2011), and silastic tubes (Laboratory Tubing 508-007, O.D.=2.41 mm, I.D.=1.57 mm, Dow Corning, MI, USA) filled with flutamide (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) were subcutaneously implanted.

In the present experiment, the chicks were divided into three groups: (1) Sham-castrated intact male chicks in which two 2.0-cm-long blank tubes were subcutaneously implanted (intact, $n=7$); (2) castrated chicks in which two 2.0-cm-long blank tubes were subcutaneously implanted (Cas+B, $n=7$); and (3) castrated chicks in which two 2.0-cm-long flutamide-filled tubes were subcutaneously implanted (Cas+F, $n=8$). After surgery at 14 days of age, the chicks were reared in isolation in an isolation cage (30×50×25 cm, length×width×height) for the remainder of the experiment. The R-I test was performed at 29 and 30 days of age and the SI test at 31 and 32 days of age. The TAFs and AER were calculated according to the protocols of Raihan *et al.* (2017) and Yan *et al.* (2019). The TAFs are defined as the sum of the frequencies of pecking, biting, kicking, threatening, and leaping. The AER is defined as a rate of aggressors showing highly aggressive behavior with few counterattacks from opponents. The criterion of highly aggressive behavior was defined as the TAFs, where aggressors showed more than 30 times the TAFs and the opponents showed less than one-third the TAFs of the aggressors. Body weight of the chicks was measured at 14, 29, and 32 days of age with an electronic scale (HF-2000, A&D Co. Ltd., Tokyo, Japan). After the SI test at 32 days of age, blood samples were collected from the wing veins of the aggressor chicks and plasma was used to determine T concentration using an enzyme immunoassay as described by Isobe *et al.* (2005a, b). 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).

For comparisons of body weight between the experimental groups, we performed two-way repeated measures analysis of variance (ANOVA) using the MIXED procedure of SAS for Windows software version 9.4 (SAS Institute Inc., Cary, NC, USA). The statistical model included fixed effects for treatment, day, and treatment×day interaction, with chick as a random effect. For the comparisons of plasma T concentration, comb weight, and TAFs between the experimental groups, we performed an one-way ANOVA with the GLM procedure in SAS. The significance of the differences between means was assessed using a Tukey-Kramer test. For the comparison of the AERs between the experimental groups, we performed Pearson's chi-square test with the FREQ procedure in SAS, and the significance of the differences was assessed using the analysis of the residuals with js-STAR version 8.9.7j. Statistical significance was set as $P<0.05$.

Results

The average body weight during the experimental period (\pm standard error of the mean) was measured in grams for each of the three experimental groups: intact: 290.23 ± 7.422 ; Cas+B: 253.75 ± 7.422 ; and Cas+F: 257.21 ± 6.943 . A two-way repeated measures ANOVA revealed significant differences in the body weights between the experimental groups ($P=0.0018$). The effects of the day and treatment \times day interaction were also significant ($P<0.0001$ and $P=0.0427$, respectively). The Tukey-Kramer test revealed that the body weight in the intact chicks was significantly higher than that in the Cas+B ($P=0.0036$) and in the Cas+F chicks ($P=0.0067$).

The average comb weight (\pm standard error of the mean) was measured in grams for each of the three experimental groups: intact: 1.21 ± 0.197 ; Cas+B: 0.39 ± 0.062 ; and Cas+F: 0.38 ± 0.027 . A one-way ANOVA revealed significant differences in comb weight between the experimental groups ($P<0.0001$). The Tukey-Kramer test revealed that the comb weight in the intact chicks was significantly higher ($P<0.001$) than that in the Cas+B and Cas+F chicks. The average plasma T concentration (\pm standard error of the mean) in each experimental group was measured in pg/ml: intact: 94.31 ± 8.600 ; Cas+B: 114.83 ± 19.195 ; Cas+F: 148.54 ± 15.902 . The test of parallelism revealed that the sequential dilution of the chick plasma samples was parallel to the T standard curve (data not shown). A one-way ANOVA revealed that the plasma T concentration tended to differ between the experimental groups ($P=0.060$). The Tukey-Kramer test revealed that the plasma T concentration in the Cas+F chicks tended to increase when compared to that in the intact chicks ($P=0.052$).

In the R-I test, a one-way ANOVA revealed significant differences in the TAFs between the experimental groups (Fig. 1, $P=0.0288$). The Tukey-Kramer test revealed that the TAFs in the intact chicks significantly increased when compared to that in the Cas+F chicks (Fig. 1a, $P=0.0329$) and they tended to increase when compared to that in the Cas+B chicks (Fig. 1a, $P=0.0907$). A Pearson's chi-square test showed no significant differences in the AER between the treatments (Fig. 1b).

In the SI test, a one-way ANOVA showed no significant differences in the TAFs between the experimental groups (Fig. 2a, $P=0.1386$). A Pearson's chi-square test, however, revealed a trend towards differences in the AER between the experimental groups (Fig. 2b, $P=0.0955$); analysis of the residuals showed that the AER tended to be higher in the intact chicks (Fig. 2b, $P<0.1$) and lower in the Cas+F chicks (Fig. 2b, $P<0.1$).

Discussion

In the R-I test, castration significantly decreased the TAFs but did not affect the AER in the male chicks (Fig. 1). The results suggest that testicular T increases the frequency but does not affect the occurrence of territorial aggression in male chicks. In general, songbirds use aggressive songs in-

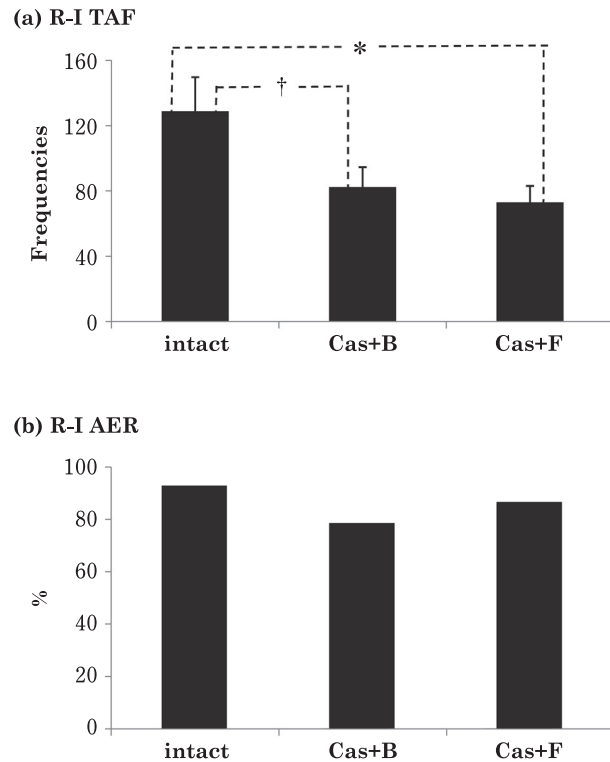


Fig. 1. **The total aggression frequencies (TAFs, a) and aggression establishment rate (AER, b) of the male layer chicks in the resident-intruder (R-I) test.** intact: sham-castrated intact male chicks in which two 2.0-cm-long blank silastic tubes were subcutaneously implanted; Cas+B: castrated chicks in which two 2.0-cm-long blank silastic tubes were subcutaneously implanted; Cas+F: castrated chicks in which two 2.0-cm-long flutamide-filled silastic tubes were subcutaneously implanted. *: $P<0.05$; †: $P<0.1$, determined using a Tukey-Kramer test.

stead of pecking and biting for territorial defense and the behavior is known to be T-dependent (Soma, 2006). Previous reports have shown that the simultaneous implantation of flutamide and 1-4-6 androstatrien-3,17-dione (ATD), an aromatase inhibitor, decreased the numbers of aggressive songs in the territorial aggression of tropical spotted antbirds in the breeding season (Hau *et al.*, 2000) and of song sparrows in the non-breeding season (Soma *et al.*, 1999). These findings support our results and show that T affects aggression frequency in the territorial aggression of avian species. On the other hand, in the SI test, castration did not affect the TAFs but tended to decrease the AER in the male chicks (Fig. 2). The results suggest that testicular T affects the occurrence of isolation-induced aggression in male chicks. Previous reports have shown that castration decreased isolation-induced aggressive behavior and subcutaneous replacement of T restored the behavior in male mice (Luttge, 1972). Although the relationship between testicular T and isolation-induced aggressive behavior has not been fully examined in chickens, castration of immature male chicks decreases their male-typical behavior such as crowing and

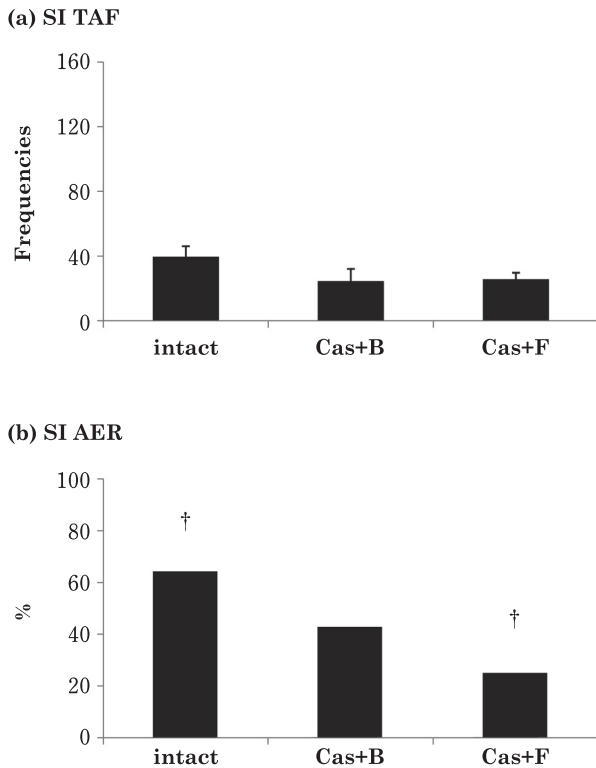


Fig. 2. The TAFs (a) and AER (b) of the male layer chicks in the social interaction (SI) test. †: $P < 0.1$, determined using an analysis of the residuals.

aggressive fighting with other males (Berthold and Quiring, 1944), and intramuscular administration of T into isolated, intact male chicks induced aggressive behavior that was monitored by a hand-thrust test (Young and Rogers, 1978). These reports together with our present results suggest that testicular T plays an important role in the occurrence of isolation-induced aggressive behavior in both mammalian and avian species.

In the SI test, flutamide implantation tended to lower the AER in the castrated male chicks (Fig. 2). The results suggest that not only testicular but also non-testicular T plays an important role in the occurrence of isolation-induced aggressive behavior in male chicks. The previous reports using wild birds also show that non-testicular steroid hormones regulate the aggressive behavior of avian species. Song sparrows express vigorous territorial aggression during the non-breeding season when the testes are completely regressed and the concentration of plasma T is undetectable (Wingfield and Hahn, 1994), and castration does not diminish the aggressive behavior of these birds during this season (Wingfield, 1994). In addition, the subcutaneous implantation of flutamide and ATD reduced non-breeding aggression in song sparrows (Soma *et al.*, 1999). These findings suggest that non-testicular T or its precursors facilitate the aggressive behavior of avian species when the amount of testicular T is small or lacking. Dehydroepiandrosterone (DHEA), a T precursor which is thought to be synthesized mainly in the adrenal

gland, is one of the candidates of steroid hormones to regulate testis-independent aggressive behavior. During the non-breeding season of song sparrows, the blood concentration of DHEA is detectable, unlike that of T and estradiol (Soma and Wingfield, 2001), and subcutaneous implantation of DHEA increases territorial behavior such as aggressive singing (Soma *et al.*, 2002). Further studies are needed to clarify the relationship between DHEA and aggressive behavior in chickens.

In the present study, the plasma T concentration tended to increase during the subcutaneous implantation of flutamide into the castrated male chicks. A recent report also showed that the subcutaneous implantation of flutamide and ATD increased the plasma T concentration in castrated male canaries (Shevchouk *et al.*, 2018). This report together with our present result suggests that flutamide binds to the androgen receptors and blocks the negative feedback effect of T on the adrenal gland because the organ is thought to be a source of steroid hormones such as T in perinatal chickens (Tanabe *et al.*, 1979). A previous report showed that the expression of androgen receptors was observed in the adrenocortical cells of male turkeys (Kiezun *et al.*, 2015), but the information about the negative feedback effect of T on the adrenal gland is lacking in both mammalian and avian species. On the other hand, it is unexpected in the present study that the plasma T concentration in the intact male chicks was not significantly higher than that in the castrated ones. Because the average comb weight in the intact male chicks was significantly higher than that in the castrated ones, it is obvious that the combs of the intact chicks were exposed to a higher concentration of blood T during our experiment. In the present study, behavioral tests were performed for four consecutive days from 29 to 32 days of age. Wingfield and Wada (1989) reported that intermale aggressive interactions over territory boundaries stimulated T release in song sparrows. The report suggests that repeated behavioral tests for four consecutive days facilitated T release which was induced by aggressive behavioral cues, causing depletion and low concentration of blood T in the intact chicks in the present study.

Guhl (1958) showed that the dominance hierarchy in group-raised chickens was completely constructed during the first five weeks of age, and Rogers and Astiningsih (1991) reported that the dominance hierarchy is maintained for the top-ranking birds but not in the low-ranking ones during the first three weeks of age. In the present study, the male layer chicks were maintained in groups from birth until the 13 days of age. These findings suggest that the dominance hierarchy was partly established in the chicks in our experiments during group-raising. This might affect our results of the behavioral tests performed from 29 to 32 days of age. However, Mench and Ottinger (1991) showed that the chicken dominance hierarchy, which was established by more than 32 weeks of group-raising, was disrupted when the top-ranking bird was removed only for a week and returned again to the group. This suggests that the dominance hierarchy that might be constructed during two weeks of group-

raising was disrupted by two weeks of isolated-raising, and did not affect our behavioral tests in the present study.

In conclusion, our results suggest that the role of T in chicken aggression depends on differences in the social context of the behavior and that both testicular and non-testicular T play an important role in stimulating isolation-induced aggression in male layer chicks.

Conflicts of Interest

The authors declare no conflict of interest.

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