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—Original Article—

Effects of Sulfamethazine on Induction of Precocious Puberty in Japanese Quails (*Coturnix japonica*) Assessed through Monitoring the Hormonal Changes and Gonadal Development

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Abstract. To investigate the effect of dietary supplementation of sulfamethazine (SMZ) on growth performance, gonadal development and hormonal changes, male and female Japanese quails (*Coturnix japonica*) were fed a control diet with or without SMZ (0.2%) from one day post hatching until 6 weeks of age. In male quail, the deviation in growth performance between SMZ and control chicks started at the 3rd week, and the disparity was significant at the 5th and 6th weeks. Hormonal analysis revealed a substantial increase in the pituitary and circulating LH (at the 5th and 6th weeks), testicular and circulating testosterone (at the 6th week) and plasma ir-inhibin (at 5th week) levels following feeding of the diet containing SMZ. The testicular size and weights were significantly larger at the 5th week, and histological analysis demonstrated an enlargement of seminiferous tubules, filling of the luminal fluid with spermatozoa and a number of interstitial cells. In female quail, the body and ovarian weights were considerably increased at the 6th week. The SMZ supplemented group showed a significant elevation in pituitary LH content (from the 4th week), plasma LH (at the 5th and 6th weeks), ir-inhibin (at the 3rd and 6th week) and progesterone (at the 2nd, 5th and 6th weeks) as compared with control chicks. These results indicated that SMZ was able to stimulate the secretion of gonadotropins and accordingly the gonadal hormones and that was associated with an early gonadal function in male (at the 5th week) and female (at the 6th week) Japanese quail.
Key words: Ir-inhibin, Luteinizing hormone, Puberty, Quail, Sulfamethazine

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Quail belong, along with chickens, pheasants and partridges, to the family phasianoidea of the order galliformes of the class aves of the animal kingdom. The Japanese quail (*Coturnix japonica*), like the chicken, is an interesting domesticated economic species for commercial egg and meat production [1]. Also, Japanese quail are an ideal biological and experimental model due to their fast development, small size, early sexual maturity and relatively small food consumption [2, 3].

Sulfonamides are a group of complex synthetic organic chemical compounds that have a common chemical nucleus and chemotherapeutic activity. These compounds diffuse very widely into tissues, penetrating into all fluids. Sulfamethazine (SMZ) is a sulfonamide widely used in veterinary medicine to treat a variety of bacterial diseases and prevent coccidiosis, as well as to promote growth performance in cattle, sheep, pigs and poultry. SMZ is acetylated more easily and in a greater quantity than sulfadiazine and sulfamerazine, and it is also bound to protein to a greater degree. It is a lipid-soluble and poorly ionized drug of low molecular weight and has been found to penetrate the blood-brain barrier differently in various species of animals [4].

Adding SMZ at a concentration of 0.2% to a chick starter diet induced precocious puberty in broilers [5]. The SMZ effects on fertility were found to be rapid in onset (1 to 4 weeks) and cumulative in nature [6]. However, neither the SMZ effect on gonadal development nor its mechanism of action, which is still unknown, has been investigated in quail. Therefore, the aim of this study was to elucidate the effect of dietary supplementation of 0.2% SMZ on the growth performance, puberty, reproductive function and interrelated hormonal changes in Japanese quail (*Coturnix japonica*) of both sexes.

Materials and Methods

Animals and experimental design

Japanese quail (*Coturnix japonica)* from low antibody response (L) selected lines were used in this study from hatching until sexual

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maturity. Fertilized eggs were incubated in a hatcher (Showa Incubator Laboratory, Tokyo, Japan) at 38.7 C with 55 ± 10% humidity and continuous controlled turning (once every hour) and were candled on Day 16 of incubation (one day before hatch). Hatched chicks were tagged and brooded under standard environmental conditions (lights on, 0500 h to 1900 h; temperature, 23 ± 2 C; humidity, 55 ± 10%; air exchanged 20 times hourly). All procedures were carried out in accordance with the guidelines established by Tokyo University of Agriculture and Technology, for use of laboratory animals.

Quail chicks were randomly separated into four groups consisting of 5–12 birds each in each week of the treatment period as follows: i) control male group, ii) SMZ male group, iii) control female group and iv) SMZ female group. Control groups were fed a control diet (Kanematsu quail diet; Kanematsu Agri-Tech, Ibaraki, Japan). SMZ groups were fed a control diet supplemented with 0.2% SMZ. All Chicks were provided with food and water *ad libitum* from one day old to six weeks of age.

The live body weights of 5–12 quail chicks from each group were recorded before decapitation at 1-week intervals beginning at the start of first week from dietary supplementation. Samples of blood, gonads and pituitary glands from each bird were collected at each time point.

Tissue sampling and preparation

After dissection, the chicks were sexed, and organs collected and weighed; the diameters of the organs were then measured (means of the long- and short-axis diameters at right angles). The pituitary and one testis were washed in ice-cold saline and placed in 0.5 ml saline for hormone measurement. The other testis was fixed in 0.4% PFA and kept in 70% ethanol for histology.

For the hormonal assay, the pituitary or testes were sliced into small pieces and homogenized at a ratio of 1:10 (w/v) with saline solution for 30 sec. All homogenates were centrifuged at 20,800× *g* for 30 min at 4 C. The supernatants were collected and stored at –20 C until hormone assay.

For the histological examination, the testes were dehydrated through a series of graded concentrations of ethanol and xylene, embedded in paraffin, sectioned serially at 4 µm, mounted on glass slides coated with 3-aminopropyltriethoxysilane (APS) (Sigma Diagnostics, St. Louis, MO, USA) and dried overnight at 37 C.

Blood sampling and hormonal assay

Blood samples were collected into heparinized tubes by cardiac puncture until the 3rd week of age, and by decapitation thereafter. Plasma was collected after centrifugation at 1,700× *g* for 15 min at 4 C and were stored at –20 C until hormone assay by a double-antibody radioimmunoassay (RIA) system using ^{125}I-labeled radioligands.

Concentrations of LH were measured using a USDA-ARS RIA kit (Beltsville, MD, USA) for chicken LH. The antiserum used was anti-avian LH (HAC-CH27-01 RBP75). Chicken USDA-cLH-I-3 and USDA-cLH-K-3 [kindly provided by Dr John A Proudman, Biotechnology and Germplasm Laboratory, Animal and Natural Resources Institute, Beltsville, MD, USA] were used for iodination and reference [**7**]. The intra- and interassay coefficients of variation were 5.2 and 11.2%, respectively. The antiserum against avian LH was kindly provided by the Biosignal Research Center, Institute for Molecular and Cellular Regulation, Gunma University, Gunma, Japan [8].

Plasma concentrations of ir-inhibin were measured as described previously [9], and 32 kDa bovine inhibin was used for iodination and reference. The antiserum used was rabbit antiserum against bovine inhibin (TNDH-1). The intra- and interassay coefficients of variation were 8.8 and 14.4%, respectively.

Concentrations of testosterone and progesterone were determined by a double-antibody RIA system with ^{125}I-labeled radioligands as described previously [10]. The antiserum against testosterone (GDN 250) and progesterone (GDN 337) were kindly provided by Dr GD Niswender (Colorado State University, Fort Collins, CO, USA). The intra- and interassay coefficients of variation were 3.1 and 9.1% for testosterone and 6.8 and 17.1% for progesterone, respectively.

Statistical analysis

A general linear model procedure was used for analysis of variance. Data were tested for homogeneity of variance. Significant differences were analyzed with the Student's *t*-test using the Statistical Package for the Social Sciences (SPSS) program. A value of $P<0.05$ was considered to be significant.

Results

Effects of SMZ on growth performance and gonadal development in male Japanese quail

Growth performance represented by body weight showed parallel changes in the control and SMZ treated groups. However, differences in body weights between the two groups were observed from the 3rd week after treatment, and the divergence increased with age and became noticeably high at the 5th (101.38 ± 1.85 *vs*. 95.12 ± 1.21 g) and 6th (117.16 ± 1.20 *vs*. 110.96 ± 1.89 g) weeks post treatment (Fig. 1A); however, the difference was not statistically significant.

Development and testicular size paralleled each other in the control and SMZ treated groups until the 4th week of age. Yet, the testicular sizes and weights of the SMZ treated group were significantly larger and heavier as compared with the control group at the 5th week after treatment (1.62 ± 0.15 cm *vs*. 1.20 ± 0.15 cm, $P<0.05$, and 1.11 ± 0.17 g *vs*. 0.53 ± 0.10 g, $P<0.01$, respectively). On the other hand, at the 6th week of age, the disparity in testis weight and size between groups showed a tendency ($P=0.06$) to be significantly high as compared with the control groups (Fig. 1B).

Effects of SMZ on the pituitary-testicular hormonal secretory activity in male Japanese quail

Although the concentration of LH in the pituitary homogenate (Fig. 2A) showed a mild increase at the 3rd week in the SMZ group, the pituitary LH content was noticeably higher at 5th and 6th weeks after dietary supplementation of SMZ (13.30 ± 1.77 *vs*. 8.47 ± 1.21, $P<0.05$, and 23.50 ± 0.61 *vs*. 7.51 ± 0.84 µg/g, $P<0.001$, respectively). Furthermore, it was positively correlated with plasma LH during the treatment period ($r=0.77$, $P=0.06$). Therefore, concurrent significantly higher serum LH levels (Fig. 2A) were observed in the SMZ treated group as compared with the control group of the same age (5.94 ± 0.92 *vs*. 2.83 ± 0.17 and 4.37 ± 0.66 *vs*. 2.56 ± 0.19 ng/ml).

Testicular and plasma testosterone concentrations showed a high

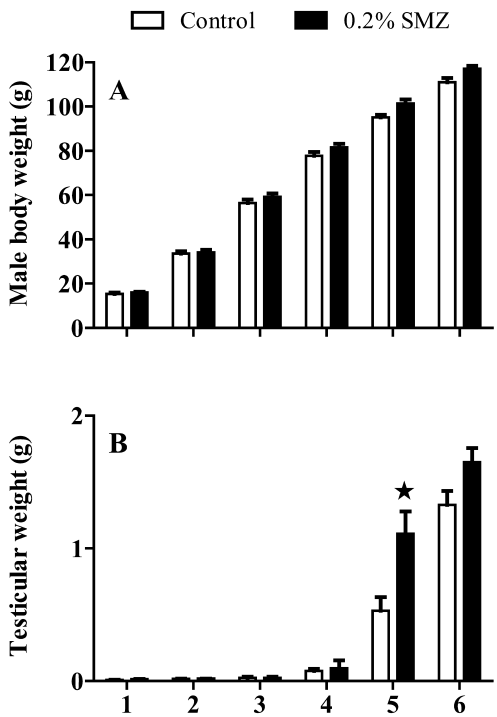

Fig. 1. Weekly changes in body (A) and testes (B) weights in male quails following intake of a control (□) or 0.2% SMZ (■) diet. Each bar represents the mean ± SEM of (5–12) quails per group. $P<0.05$ was considered to be a significant difference between control and treatment birds.

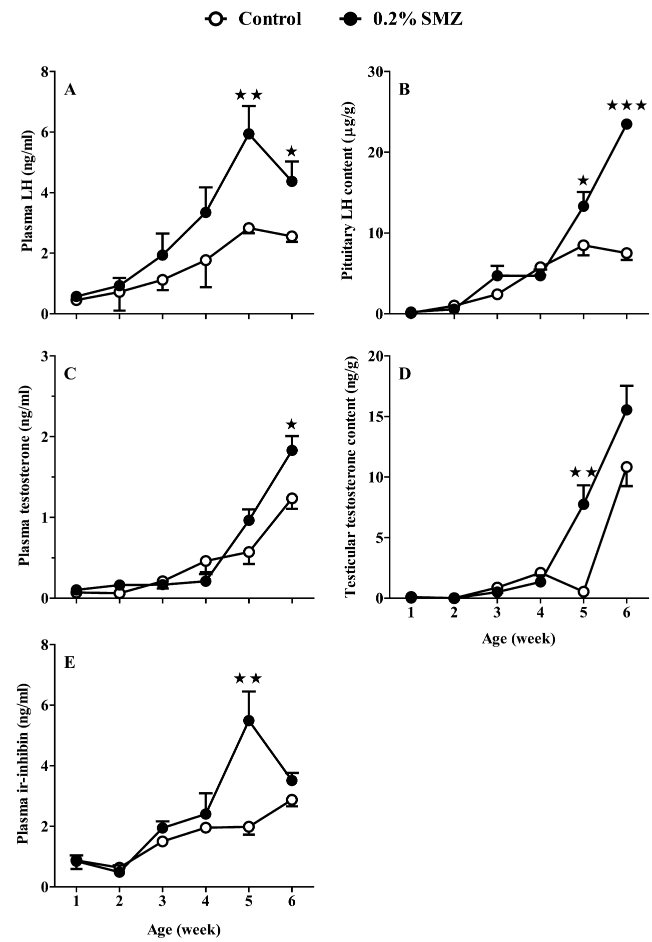

Fig. 2. Weekly changes in plasma (A) and pituitary (B) LH, plasma (C) and testicular (D) testosterone and ir-inhibin (E) concentrations in male quails following intake of a control (○) or 0.2% SMZ (●) diet. Each bar represents the mean ± SEM of (5–12) quails per group. $P<0.05$ was considered to be a significant difference between control and treatment birds.

correlation ($r=0.99$, $P<0.001$) in the SMZ-treated group. Nevertheless, the testicular content of testosterone (Fig. 2D) and plasma ir-inhibin (Fig. 2E) were significantly higher at the 5th week and tended to be higher ($P=0.09$) at the 6th week in the SMZ-treated group as compared with the control group. On the other hand, the plasma testosterone levels (Fig. 2C) tended to increase ($P=0.08$) at the 5th week and rose significantly ($P<0.01$) at the 6th week in the SMZ group.

Effects of SMZ on testicular morphology in male Japanese quail

Treatment with SMZ had little or no effect on testicular morphology until the 4th week of age. However, coincident with the onset of puberty at the 5th week of age, the histological and morphological analysis of SMZ group testes with light microscopy clearly showed a decrease in the spaces between seminiferous tubules, an enlargement of seminiferous tubules, thinning of seminiferous tubular basement membranes and a higher number of cells in the interstitial spaces. Moreover, there were alterations in the seminiferous tubule architecture characterized by increased active spermatogenesis, fewer multinucleated giant cells per testis and filling of the luminal fluid with spermatozoa (Fig. 3).

Effects of SMZ on growth performance and gonadal development in female Japanese quail

As shown in Fig. 4, the growth performance of the female Japanese quail chicks was significantly different between the SMZ-treated and control groups at the 6th week post treatment. Body weight showed a tendency ($P=0.06$) to be different at the 5th week and significantly ($P<0.01$) increased at the 6th week after SMZ treatment (112.02 ± 2.30 *vs.* 117.06 ± 2.45 g). On the other hand, ovarian weight showed a significant ($P<0.01$) increment only at the 6th week in SMZ chicks (0.57 ± 0.13 *vs.* 0.35 ± 0.13 g).

Effects of SMZ on the pituitary-ovarian hormonal secretory activity in male Japanese quail

While pituitary LH content (Fig. 5B) was significantly different between groups from the 4th week post treatment, the plasma LH levels (Fig. 5A) exhibited highly significant increases at the 5th and the 6th weeks in the SMZ group. In the mean time, plasma ir-inhibin (Fig. 5C) showed higher levels at the 3rd and 6th weeks after SMZ treatment. Furthermore, plasma progesterone levels (Fig. 5D) were significantly increased at the 2nd, 5th and 6th weeks of age in the

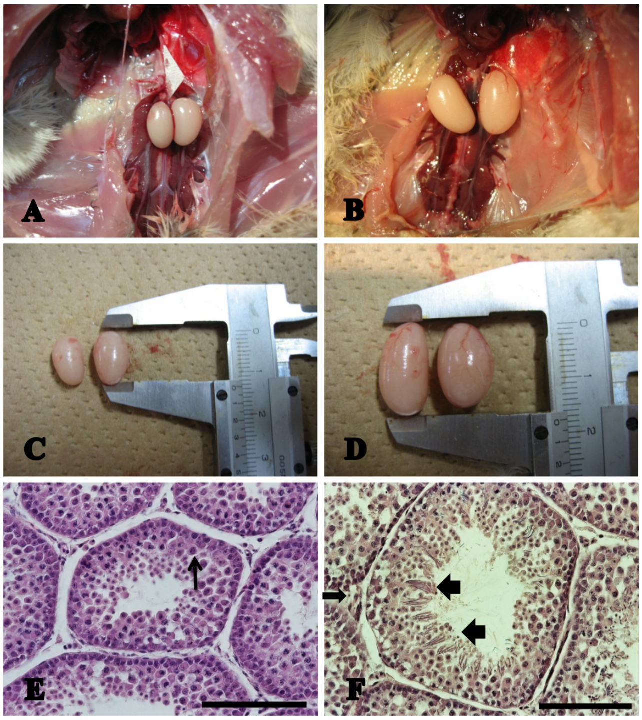

Fig. 3. Gross and microscopic examination of the testes in control (A, C and E) and 0.2% SMZ-treated (B, D and F) quails at 5 weeks of age. The scale bar represents 100 µm. Note the enlargement of the seminiferous tubules, fewer multinucleated giant cells (narrow arrow, ←) per testis, filling of the luminal fluid with spermatozoa (left wide arrow, ⬅) and higher number of Leydig cells in the interstitial spaces (right wide arrow, ➡).

SMZ treatment group as compared with the control group and the latter two increments were at the approximate time of the onset of egg production in this species.

Discussion

Research with Japanese quail, an ideal avian model, has expanded from avian science-related topics to biology and medicine. This bird can be genetically controlled and kept in relatively large numbers in an environmentally controlled animal facility. The present study clearly demonstrated the feasibility of dietary supplementation of SMZ to induce early gonadal function and onset of precocious puberty in male (at the 5th week) and female (at the 6th week) Japanese quail kept under a long-day photoperiod (14 h light/ 10 h dark) through stimulation of pituitary secretion of LH and accordingly gonadal secretion of testosterone, progesterone and ir-inhibin hormones.

In male Japanese quail, chicks fed a diet containing 0.2% SMZ showed an exponential increase in body weight. From 3 weeks of age, birds under treatment started to be heavier than controls, and the differences between the groups became significantly high at the 5th and 6th weeks post treatment. The current results also showed that the testicular weight was significantly heavier as compared with the control group at the 5th–6th weeks after SMZ treatment

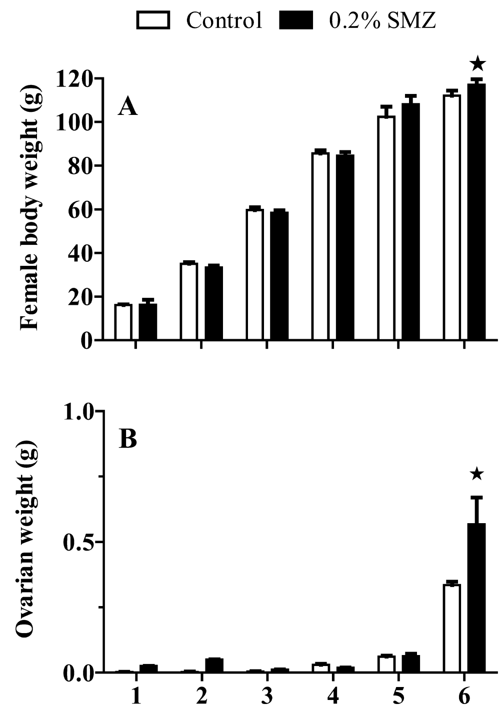

Fig. 4. Weekly changes in body (A) and ovarian (B) weights in female quails following intake of a control (□) or 0.2% SMZ (■) diet. Each bar represents the mean ± SEM of (5–12) quails per group. P<0.05 was considered to be a significant difference between control and treatment birds.

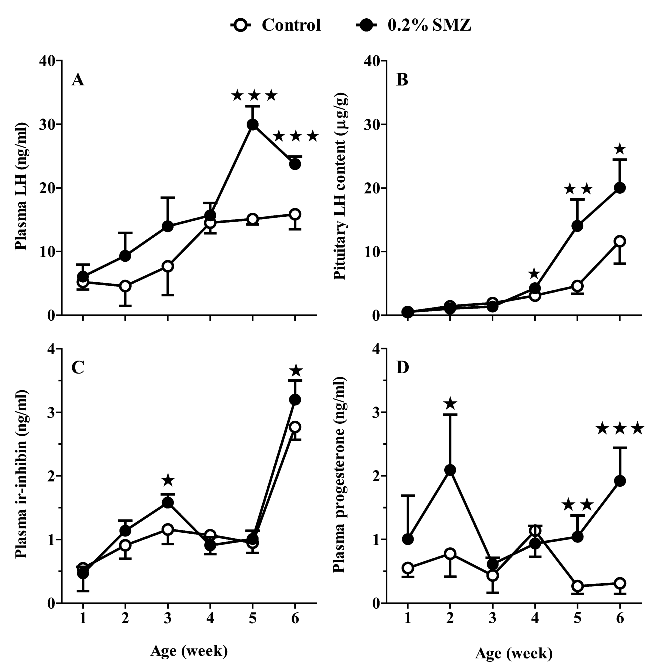

Fig. 5. Weekly changes in plasma (A) and pituitary (B) LH, ir-inhibin (C) and progesterone (D) concentrations in female quails following intake of a control (○) or 0.2% SMZ (●) diet. Each bar represents the mean ± SEM of (5–12) quails per group. P<0.05 was considered to be a significant difference between control and treatment birds.

and showed active spermatogenesis and filling of the luminal fluid with spermatozoa from 5th week. To clarify the possible mode of action of SMZ in stimulating testis development, circulatory and pituitary concentrations of LH were measured in the present study. The concentration of LH in the pituitary homogenate was positively correlated with plasma LH during the treatment period. It increased slightly at the 3rd week and noticeably at 5th and 6th weeks after consuming a diet containing SMZ. It would appear that the rate of testicular growth was proportional to the concentration of LH in the circulation, suggesting that the compound is acting at the hypothalamus and pituitary level to stimulate early gonadal development in quails. In a previous study in the male chicken (1 week of after hatching) body weight did not differ significantly between control and treated groups at 3 and 6 weeks of age after feeding a ration containing 0.2% SMZ for 2 weeks or 5 weeks, respectively [5]. Earlier studies suggest that SMZ is a substrate of the mixed function oxidase system and that induction is dependent on dosage, age and sex of the animals [11]. Kuenzel *et al.* [12] showed that chicks exposed to a continuous photoperiod and fed a diet containing 0.2% SMZ had an exponential increase in testes size. At 6 weeks of age (5 weeks on the SMZ diet), the treated group had a significantly heavier testes weight than controls. The same authors [12] recorded elevated LH in chicks treated with SMZ in diet at the initial 1-week sampling point, and the LH level remained elevated throughout the entire experiment (5 weeks). This finding is similar to those when the photoperiod was changed for domestic and wild birds. It has been shown that long-day photoperiod significantly increased the plasma levels of LH and that there was rapid development of the gonads as compared with a short-day photoperiod in birds [13].

In the present results, testicular and circulating testosterone concentrations were positively correlated in the SMZ-treated group. Besides, the high level of testicular testosterone at the 5th week followed by the significant increase in the plasma testosterone levels at the 6th week was probably due to the stimulating effect of gonadotropins produced from the pituitary gland in response to the SMZ treatment [12]. High levels of circulating LH were associated with an increase in testosterone and ir-inhibin secretion from gonads. It is well known that Leydig cells or interstitial cells play a crucial role in synthesizing testosterone and regulating the process of spermatogenesis under the control of LH [14].

In female Japanese quail, addition of SMZ at a concentration of 0.2% to the chick diet from hatching to puberty affected the body and ovarian weights at the 6th week age, which is the approximate time of the onset of egg laying. Body weight, a key morphological characteristic, is correlated with robustness in birds; therefore, ascertaining growth performance and developmental stages could provide valuable information on their physiology and behavior [15]. To clarify the possible mode of action of SMZ in female quail, pituitary and plasma concentrations of LH and the ovarian secretory activity of progesterone and ir-inhibin were measured in the current study. LH content in pituitary homogenate was significantly different between treated and control groups from the 4th week post treatment, yet the plasma LH levels were highly increased at the 5th and the 6th weeks in the SMZ group. Furthermore, the significant alternation in the plasma progesterone levels (at the 2nd, 5th and 6th weeks of age) preceded the elevation in plasma ir-inhibin (at the 3rd and 6th weeks) after SMZ treatment. The increased progesterone levels from the 5th week might be due to gonadal response to elevated pituitary LH secretion at the 3rd week. On the other hand, the elevated progesterone levels from the 5th week were probably associated with egg laying. Steroids are lipophilic, low-molecular weight compounds derived from cholesterol and are extensively metabolized peripherally, notably in the liver. The clearance rate of steroid hormones is influenced by compounds that alter liver enzyme activity involved in hormone clearance. Long-term administration of SMZ has been found to inhibit the liver enzyme P450C2 [11], which might result in decreased clearance of progesterone.

Earlier work suggested that SMZ activated thyroid function, resulting in alterations in thyroid hormone concentrations and increased secretion of thyroid–stimulating hormone (TSH), thereby influencing gonadal development [16]. SMZ action involves inhibition of thyroid peroxidase, resulting in alterations in thyroid hormone concentrations and increased secretion of thyroid–stimulating hormone (TSH). In rats, the incidence of thyroid gland hyperplasia was greater in males than in females among groups receiving lower concentrations of compound [17]. Intraperitoneal administration of SMZ to chickens for 3 days showed significant induction of cytochrome P450 levels [11]. Our previous paper clearly demonstrated that hypothyroidism caused both gonadal and adrenal disturbances in the male Japanese quail [18]. Reproduction of the Japanese quail is under photoperiodic control. A previous paper [19] reported that light-induced expression of type 2 iodothyronine deiodinase, which catalyses the intracellular deiodination of thyroxine (T4), prohormone to the active 3,5,3'-triiodothyronine (T3), in the mediobasal hypothalamus may be involved in the photoperiodic response of gonads in Japanese quail. In addition, another previous paper [20] reported that the expression of TSH β-subunit in the *pars tuberalis* is the key molecular event involved in the regulation of the long-day reproductive photoperiodic response and identified *pars tuberalis* TSH as a key factor controlling photoperiodic signal transduction.

In summary, SMZ most likely stimulates the hypothalamus and pituitary axis by increasing gonadotropins secretion, and the increased gonadotropins stimulate testicular and ovarian activities, resulting in induction of precocious puberty in male and female Japanese quail. Activation of the function of the hypothalamus-pituitary-thyroid gland axis may be one of the mechanisms responsible for induction of gonadal activation in Japanese quail by SMZ. Further research is required to clarify the mechanisms responsible for the action of SMZ on gonadal function.

Acknowledgments

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