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Low Temperature and Short Days Together Induce Thyroid Activation and Suppression of LH Release in Japanese Quail

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We have found in Japanese quail that low ambient temperature is required to terminate breeding activity in the presence of short days (Wada *et al.*, 1990, *Gen. Comp. Endocrinol.* **80**, 465–472; Tsuyoshi and Wada, 1992, *Gen. Comp. Endocrinol.* **85**, 424–429). To elucidate the mechanism for photoperiodic and temperature regulation of the release of luteinizing hormone (LH), several serum variables were measured in three groups of mature male birds: (a) birds kept on long days of 16L:8D, 24 hr at 19° as an initial control group (Group IC), (b) birds transferred from long days to short days of 8L:16D, 24 hr at 19° (Group S) for 14 days, and (c) birds transferred to short days of 8L:16D and low temperature cycles of 12 hr, 19°:12 hr, 9° (Group SL) for 14 days. Testicular mass and plasma concentrations of LH significantly decreased to nonbreeding levels in Group SL, but not in Group S, confirming our previous results. Hematocrit, serum osmolarity, and concentrations of Na^+, K^+, Ca^{2+}, Mg^{2+}, and Cl^- ions were not different among the three groups. Serum concentrations of free fatty acids were increased in Group SL, but the increase was not statistically significant. On the other hand, plasma concentrations of thyroid hormones changed significantly; thyroxine (T_4), but not triiodothyronine (T_3), increased in Group S, and T_3, but not T_4, increased in Group SL. To follow the changes in plasma levels of LH, T_4, and T_3 during the treatments, blood samples were collected every other day for 2 weeks from birds in the three groups of mature male birds described above. In Group C, plasma concentrations of LH, T_4, and T_3 did not change during the experimental period. In Group S, plasma concentrations of LH remained at relatively high levels and T_4 increased after an initial decrease; T_3 showed a slight increase. In Group SL, plasma concentrations of LH decreased rapidly to the nonbreeding level and T_3 showed a great increase, while changes in T_4 were basically similar to those in Group S. These results indicate that thyroid hormones are involved when a breeding season terminated by short days and low temperature, and they suggest that T_3 and 5′-monodeiodination of T_4 are at least a part of the mechanism.

Field observations indicate that Japanese quail start breeding in the spring and terminate breeding in late summer to early autumn (Takatsukasa, 1967). This annual breeding cycle can be simulated in caged quail kept under approximately natural environmental conditions (Follett and Maung, 1978; Robinson and Follett, 1982; Wada *et al.*, 1992). The annual reproductive cycle is basically controlled by the alternate increase and decrease of gonadotropin release. The mechanism for the increase of luteinizing hormone (LH) at the beginning of the breeding season is well-established (see for review Follett, 1984). Birds measure day length by receiving light in the photoinducible phase in early spring (Wada, 1979, 1981; Hatanaka and Wada, 1988). If the day length is increasing at early spring in the natural conditions (Wada *et al.*, 1992) or is 16L:8D in laboratory conditions (Wada *et al.*, 1990), levels of plasma LH increase even though the ambient temperature is very low.

However, the mechanism for termination of LH release at the end of the breeding season appears to involve two factors, the decrease in day length and ambient temper-

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ature in late summer to early autumn (Wada *et al.*, 1992). Under laboratory conditions, we could terminate breeding activity by transferring the bird from 16L:8D to 8L:16D at a continuous temperature of 8° (Wada *et al.*, 1990). Such extreme exposure to low temperature was not necessary, however. Short days (8L:16D) with 12 hr, 19°:12 hr, 9°, or with 16 hr, 19°:8 hr, 9° also effectively reduced circulating LH to a nonreproductive level (Tsuyoshi and Wada, 1992).

Until now the role of ambient temperature has not been carefully evaluated in reproductive endocrinology of avian species. However, the data obtained in our laboratory indicate that ambient temperature has a crucial role in terminating reproductive activity at the end of the breeding season. When blood was collected in the above mentioned experiments, it appeared that plasma had changed in its viscosity and clarity after temperature treatment. Thus, in the present study, we have measured several variables in blood which are related to physical properties of plasma, and also measured thyroid hormones which are involved in thermoregulation. The aim of the experiment was to seek possible mediators through which low temperatures decrease plasma levels of LH in Japanese quail.

MATERIALS AND METHODS

Animals

Male Japanese quail (*Coturnix japonica*), 3 weeks old, were purchased from a commercial source. The birds were kept in environmental chambers at a constant ambient temperature (19 ± 1°). They were reared under a daily photoperiod of 8L:16D (lights on at 0800 hr) until 5 weeks of age. At that point they showed full somatic growth but were still sexually immature. They were then transferred to a daily photoperiod of 16L:8D (lights on at 0800 hr) until 10 weeks of age. Food and water were continuously available.

The birds were then used in the experiments described below. Fluctuations of the regulated temperatures were within 0.5° under the experimental conditions. No selection on the basis of responsiveness of gonadal activity to alternating long and short photoperiods was made beforehand.

Experiment Schedules

Experiment 1. To identify a possible mediator through which low temperatures decrease LH, several variables in blood that possibly relate to physical properties of plasma and thyroid hormones which are involved in thermoregulation were measured in three groups of Japanese quail.

Prior to the changes of photoperiod and temperature, 6 birds were killed by decapitation between 1000 and 1200 hr and trunk blood was collected into a test tube for the initial control group (Group IC). The plasma was separated and stored at −20° until assay. At blood collection, body mass was measured to the nearest gram and area of the cloacal protrusion was measured to the nearest millimeter by use of a ruler. A small amount of blood was collected from the wing vein into a hematocrit capillary tube to estimate the hematocrit value. Combined testicular weight was measured with an analytical balance to the nearest milligram. Seven birds were transferred to 8L:6D at a constant temperature of 19° (Group S). Another group of seven birds was transferred to 8L:16D at a temperature cycle of 19° for 12 hr and 9° for 12 hr (low temperature from 1800 to 0600 hr) (Group SL). After 14 days, the birds were killed and assessed by the same procedures as were the birds of the initial control group.

Molting was also assessed by checking fallen body feathers and primaries on the cage floor.

Experiment 2. The result obtained in Experiment 1 indicated that the variables in blood, possibly related to physical properties of plasma, were not changed significantly, but plasma concentrations of thyroid hormones changed significantly after the treatments. Thus, temporal patterns of the changes of thyroid hormones over 14 days were assessed in relation to the changes of plasma concentration of LH.

Seven birds each were transferred from 16L:8D to either 8L:16D (19°) (Group S) or to 8L:16D (12 hr, 19°:12 hr, 9°) (Group SL). Another seven birds remained in the initial condition of 16L:8D (19°) (Group C). Blood samples were collected 2 days before the transfer, on the day of the transfer, the first and second days after transfer, and every other day thereafter for 14 days.

Blood samples of 0.2–0.3 ml were taken into heparinized capillary tubes at 1000–1100 hr by venipuncture of the wing vein. The plasma was separated and stored at −20° until assay.

The area of the cloacal protrusion was estimated by use of a ruler to the nearest millimeter, at the time of blood collection. The onset of molting was also as-

sessed by checking fallen body feathers and primaries on the cage floor.

Radioimmunoassay

Plasma concentrations of LH were determined in 50-μl sample volumes, in duplicate, using the radioimmunoassay method described by Hattori and Wakabayashi (1979). Chicken LH (fraction IRC-2, Gunma) was used for reference preparations and a preparation of chicken LH (fraction AGCHDS112312A) was used for iodination. The antiserum (AH-MH No. 1) was raised against chicken LH (fraction IRC-2, Gunma). Results are expressed in terms of nanograms per milliliter of chicken LH fraction IRC-2 (Gunma).

Intraassay and interassay coefficients of variation were 4.87% and 5.94%, respectively.

Plasma concentrations of thyroxine (T_4) and triiodothyronine (T_3) were determined according to Tasaki *et al.* (1986) with slight modification; sample volumes were 1-μl for T_4 and 5-μl for T_3 and the first incubation was carried out for 3 hr at room temperature and the second overnight at 4° instead of 1 hr at 37°. Barbital buffer solution did not contain Triton-X.

Analysis of Ion Concentrations, Osmolarity and Nonesterified Fatty Acids

Ion concentrations were determined by use of atomic absorption spectrophotometry (Hitachi 180-50) and osmolarity was determined by use of a 5550 Vapor pressure osmometer (Wescor).

Plasma nonesterified fatty acids were determined by an acylCoA synthetase–acylCoA oxidase method using a commercial kit (NEFAzyme-S, Eiken Chemicals Co., Inc., Tokyo).

Statistics

For Experiment 1, one-way analysis of variance, followed by Duncan's multiple range test, were used to test the statistical significance between groups. If it was proved significant, the *t* test was used to determine the significance of the difference between the values obtained for any two groups. If the variance was proved not to be equal, the Kruskall–Wallis test and Mann–Whitney *U* test were applied. For Experiment 2, two-way analysis of variance were used to test the statistical significance of fluctuations in the mean hormone values between sampling times and groups. If is was proved significant, the Mann–Whitney *U* test was used to estimate the significance of the difference between the values obtained for any two sampling times and for any two group at the same sampling time.

Differences were considered significant when $P < 0.05$. Throughout the text, means are given with ±1 SEM.

RESULTS

Experiment 1

Body weight and hematocrit values were not different among the three groups (Fig. 1). The mean combined testicular weights of Group S decreased somewhat but were not significantly different from the mean of Group IC; however, the testes of Group SL were greatly involuted (Fig. 1). The areas of the cloacal protrusion was maintained in Group S, but it regressed to almost a nonbreeding level in Group SL. Concentrations of plasma LH decreased significantly in Group S, and those of Group SL decreased further to the nonbreeding level (Fig. 1).

There were no apparent differences in serum osmolarity and serum concentrations of several biologically important ions (Fig. 2). Total nonesterified fatty acids were 400 ± 51.4 in Group IC, 431 ± 135.2 in Group S, and 493 ± 111.8 (μ EQ/liter) in Group SL. No statistically significant differences were detected among the groups, although the level in Group SL increased compared to the control ($0.08 < P < 0.09$).

Serum T_4 increased in Group S but was unchanged in Group SL. Serum T_3 increased to a certain extent in Group S, and it increased greatly in Group SL (Fig. 3).

Molting was observed six out of seven birds in Group SL. One bird in Group S showed weak molt. In this specimen testicular mass, the area of cloacal protrusion and plasma LH were all decreased to the levels found in Groups SL.

Experiment 2

Changes in the area of cloacal protrusion and in plasma concentrations of LH after transfer from 16L:8D (19°) to either 8L:16D (19° C) (Group S) or 8L:16D (12 hr, 19°:12 hr, 9°) (Group SL) are shown in Fig. 4. In the control (Group C) in which the birds

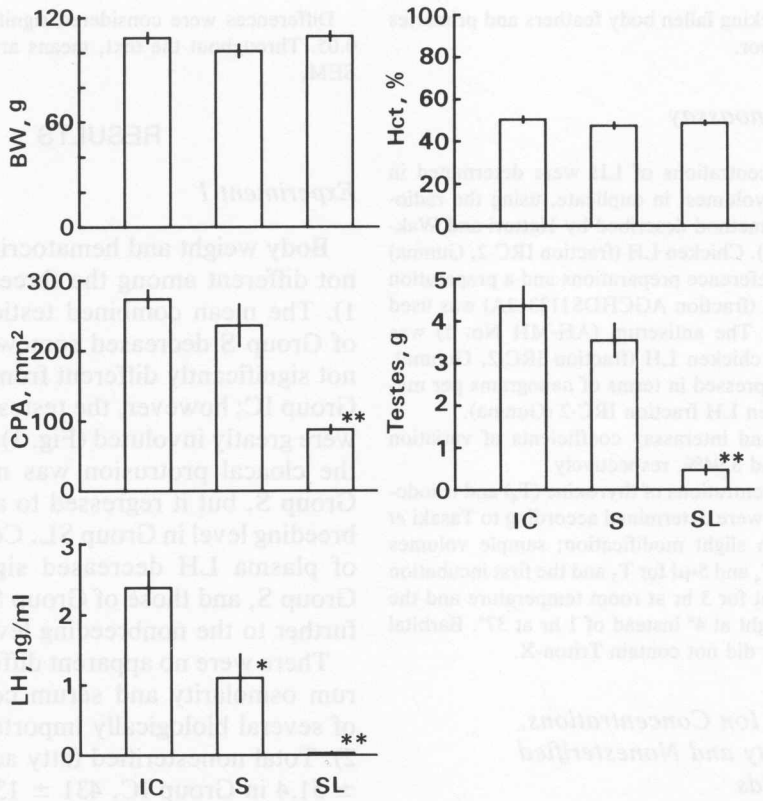

FIG. 1. Body weight (BW), hematocrit (Hct), area of the cloacal protrusion (CPA), combined testicular weight (Testes), and plasma concentration of LH (LH) in birds of the initial control group (IC, $n = 6$), on short days for 2 weeks (S, $n = 7$), and on short days with 12 hr low temperature (SL, $n = 7$). Significantly different from the initial control at $P < 0.05$ (*) and $P < 0.01$ (**).

remained on 16L:8D (19°), both the area of the cloacal protrusion and concentrations of plasma LH were maintained at the initial level, although significant fluctuations were detected in the concentrations of plasma LH. In Group S, the plasma LH concentrations decreased to some extent by the end of 2 weeks on short days (significantly different from the initial value and from that of Group C, $P < 0.05$), but the area of the cloacal protrusion was maintained at the initial level. In Group SL, plasma LH decreased rapidly to the nonreproductive level and the area of the cloacal protrusion decreased progressively.

Changes in plasma concentrations of T_4 and T_3 in the experimental birds mentioned above are shown in Fig. 5. In Group C, plasma T_4 and T_3 levels were essentially constant although there were small fluctuations. In Group S, plasma T_4 increased after an initial decrease (Days 2 and 4 were significantly different from the initial levels of Days -2 and 0, $P < 0.05$). The level on Day 14 was not significantly different from the initial level but it was different from that of Group C on Day 14 ($P < 0.05$). Changes in plasma T_3 concentrations of Group S were almost the same as those of Group C. In Group SL, plasma T_4 also showed the initial decrease (Days 2 and 4 were significantly different from the initial values of Days -2 and 0, $P < 0.05$) and then increased gradually. However, the value on

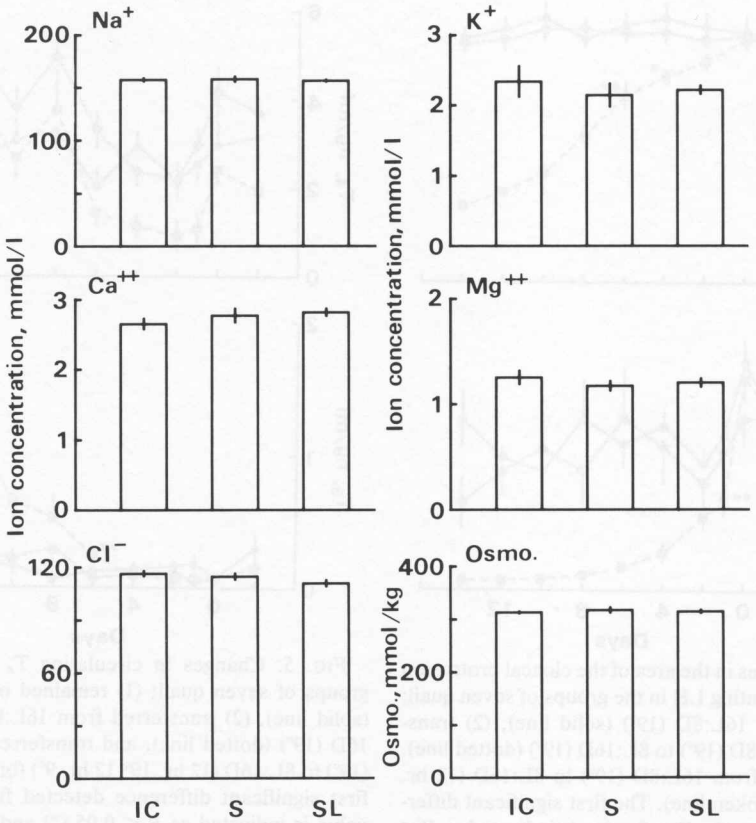

FIG. 2. Serum concentrations of sodium (Na^+), potassium (K^+), calcium (Ca^{2+}), magnesium (Mg^{2+}) and chloride (Cl^-) ions and osmolarity (Osmo.) in birds of the initial control group (IC, n = 6), on short days for 2 weeks (S, n = 7), and on short days with 12 hr low temperature (SL, n = 7).

Day 14 was not different from the initial levels or that of Group C on day 14. On the other hand, plasma T_3 increased gradually and reached a level eight-fold higher than the initial level.

The plasma concentrations of T_4 in Group S and SL were different from the beginning ($P < 0.05$ up to Day 6), but the changes were parallel to each other.

In group SL, molting started in two birds

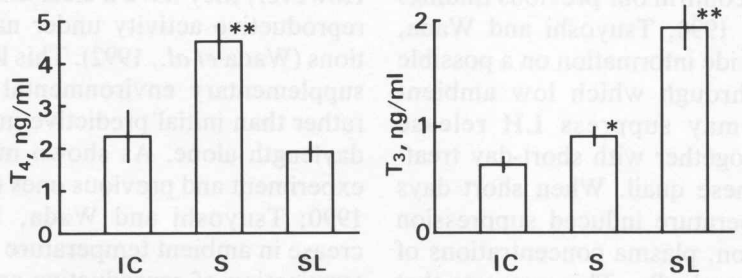

FIG. 3. Serum concentrations of T_4 and T_3 in birds of the initial control group (IC, n = 6), on short days for 2 weeks (S, n = 7), and on short days with 12 hr low temperature (SL, n = 7). Significantly different from the initial control at $P < 0.05$ (*) and $P < 0.01$ (**).

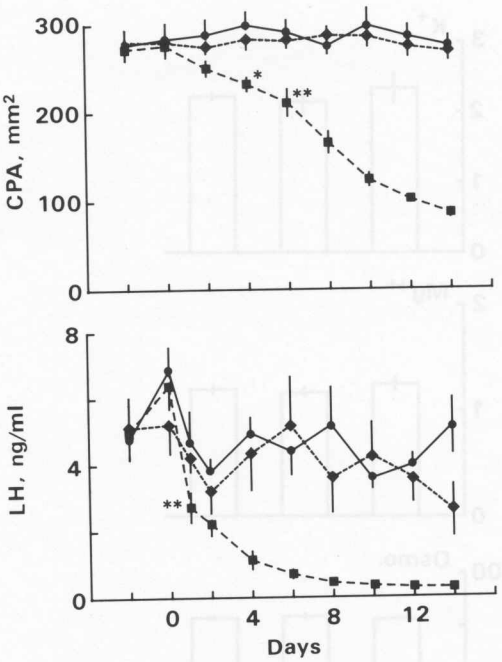

FIG. 4. Changes in the area of the cloacal protrusion (CPA) and circulating LH in the groups of seven quail; (1) remained on 16L:8D (19°) (solid line), (2) transferred from 16L:8D (19°) to 8L:16D (19°) (dotted line), and transferred from 16L:8D (19°) to 8L:16D (12 hr, 19°:12 hr, 9°) (broken line). The first significant difference detected from the Day 0 value is indicated as $P < 0.05$ (*) and $P < 0.01$ (**). Significant differences are the same thereafter.

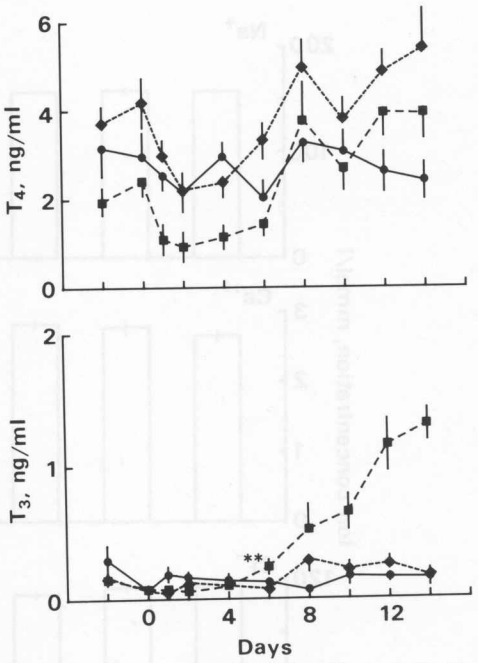

FIG. 5. Changes in circulating T_4 and T_3 in the groups of seven quail; (1) remained on 16L:8D (19°) (solid line), (2) transferred from 16L:8D (19°) to 8L:16D (19°) (dotted line), and transferred from 16L:8D (19°) to 8L:16D (12 hr, 19°:12 hr, 9°) (broken line). The first significant difference detected from the Day 0 value is indicated as $P < 0.05$ (*) and $P < 0.01$ (**). Significant differences are the same thereafter.

on Day 10 after transfer, in two birds on Day 12, and in one bird on Day 14. No bird showed molting in Group S.

DISCUSSION

The results confirm our previous findings (Wada *et al.*, 1990; Tsuyoshi and Wada, 1992) and provide information on a possible mechanism through which low ambient temperature may suppress LH release when it acts together with short-day treatment in Japanese quail. When short days and low temperature induced suppression of LH secretion, plasma concentrations of T_3 increased markedly. This suggests that effect of lowered temperature may be relayed through a primary thyroid activation.

We have shown that Japanese quail do not show photorefractoriness under long photoperiod (Figs. 1 and 4) as do passerine birds like white-crowned sparrows and starlings; they do not even show gonadal regression after transfer from long days to short days, if temperature remains high. However, they have a clear annual cycle of reproductive activity under natural conditions (Wada *et al.*, 1992). This led us to seek supplementary environmental information rather than initial predictive information of daylength alone. As shown in the present experiment and previous ones (Wada *et al.*, 1990; Tsuyoshi and Wada, 1992), a decrease in ambient temperature is crucial for termination of reproductive activity in this species (Figs. 1 and 4).

This is an important consideration in view of the fact that Japanese quail have a

very long breeding season (Takatsukasa, 1967; Wada *et al.*, 1992). Timing of termination of breeding season may in fact be different among groups of quail at different localities and it may be determined by local climatic changes, especially temperature decreases. Long-distance migrants, such as white-crowned sparrows, utilize only photoperiod to end their reproductive activity at an appropriate time since they must migrate southward before the environment deteriorates (for review see Wingfield *et al.*, 1992). Japanese quail as short-distance migrants have more options; thus photoperiod only indicates the possibility of end of the breeding season, and apparently low temperatures finally end the breeding activity. It would be of interest to determine the dependence on different environmental cues in species in different habitats.

It is also interesting to note that short days and the decrease of ambient temperature induced thyroidal activation (Figs. 3 and 5), resulting in an increase in circulating T_3. Thyroid hormones have been suggested to be involved in onset of photorefractoriness of starlings (Wieselthier and van Tienhoven, 1972), Baya weaver (Chandola *et al.*, 1974), and spotted munia (Pandha and Thapliyal, 1964) since thyroidectomy blocks the onset of photorefractoriness, or administration of T_4 blocks gonadal development. Starlings maintained on 11 hr of light per day underwent testicular recrudescence and remained sexually mature indefinitely. Administration of T_4 to these birds results in rapid testicular involution, onset of molt and onset of prolactin secretion. These are typical accompaniments for photorefractoriness (Goldsmith and Nicholls, 1984; Goldsmith *et al.*, 1985). Only short periods of T_4 injection are sufficient just after photostimulation to induce a spontaneous regression in thyroidectomized birds several weeks later (Boulakoud and Goldsmith, 1991).

In Japanese quail thyroidal involvement in photorefractoriness was suggested by Follett and Nicholls (1984; 1985). In turkeys, Lien and Siopes (1989) measured plasma thyroid hormones and prolactin concentrations throughout an egg-laying cycle in relation to photorefractoriness and suggested that elevated plasma T_4 involved in development of photorefractoriness and increase in plasma T_3 involved in gonadal regression. In laying hens, forced molting by deprivation of food and water caused thyroid hormone levels to increase and circulating LH levels to decrease (Hoshino *et al.*, 1988; Sekimoto *et al.*, 1990). Large doses of thyroxine suppress circulating LH levels (Sekimoto *et al.*, 1987). The present results are in agreement with the above-cited studies. Our data show clearly that one feature of environmental information, temperature, can induce thyroidal activation, resulting in suppression of circulating LH and consequently regression of gonadal function. This agrees also with the results of Oishi and Konishi (1978) which suggested that combined photoperiodic and temperature changes influence testicular activity. The studies in starlings also indicate that changes in thyroidal hormone are the probable cause of LH suppression through the hypothalamo–hypophysial axis, even though the nature of the physiological mechanism is not clear at the moment (Boulakoud and Goldsmith, 1991).

In starlings, T_4 was administered by adding it to drinking water and the effectiveness of T_3 was not tested. However, recent studies of monodeiodination of T_4 to T_3 in chickens (Kühn *et al.*, 1987) indicate that peripheral conversion of T_4 to T_3 is crucial for expression of biological activity. Moreover, acute low temperature cause an increase of thyroid hormones level (Bobek *et al.*, 1980; Rudas and Pethes, 1986) and enhance the conversion of T_4 to T_3 (Rudas and Pethes, 1986), resulting in an increase of plasma T_3 concentrations. Plasma T_3 increase in the present experiments (Figs. 3 and 5) may be explained by this mechanism. However, it is not known whether

sustained low temperature enhances the enzyme activity and thus increases the plasma level of T_3 concentrations for prolonged periods.

Perhaps our data suggest that short days activate thyroid gland to secrete T_4, and low temperature activates enzymatic conversion of T_4 to T_3; both together necessary for termination of the reproductive activity in Japanese quail. Low temperature do not suppress plasma levels of LH in birds remains on long days (Wada *et al.*, 1990).

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