Annual Changes in Levels of Plasma LH and Size of Cloacal Protrusion in Japanese Quail (*Coturnix coturnix japonica*) Housed in Outdoor Cages under Natural Conditions

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Japanese quail of the strain used in our laboratory do not show a complete decrease in levels of circulating luteinizing hormone (LH) concentrations and show no collapse of the testes following their transfer from long to short days under laboratory conditions. Thus, merely manipulating photoperiods in the laboratory does not simulate an annual breeding cycle. To see whether an annual breeding cycle does exist in "our" quail under natural conditions, mature male birds were housed in individual cages and placed on the roof of a building at 35°45′N, 139°53′E; day length and ambient temperature were not controlled at all though food and water were continuously supplied. For 16 months blood was collected every week and the area of the cloacal protrusion measured at the time of each blood collection. The results showed that levels of plasma LH and the area of the cloacal protrusion had a clear annual cycle under the natural conditions. To detect more precisely the changes in circulating LH concentrations during spring and autumn, samples were collected every other day. The first significant increase in levels of plasma LH was found when the day length exceeded 12–12.5 hr, though the increase was sporadic and not synchronized among individuals. The results also showed that circulating levels of LH declined significantly in early September starting when the day length was still about 14 hr; this downward trend continued rather steadily to nonbreeding levels. The record of ambient temperatures indicated that in early spring there was a fairly constant range of low temperatures despite some fluctuations, and in late summer to early autumn temperatures began to decrease although the daytime levels remained high. These results indicate that (1) under natural conditions our Japanese quail showed clear annual breeding cycles, (2) the increase in plasma LH in early spring was sporadic and not necessarily proportional to the increase in day length, and (3) the decrease in circulating LH in late summer is difficult to explain by a simple photoperiodic mechanism; the results indicate that other factors are involved. Our previous results (Wada *et al.*, 1990, *Gen. Comp. Endocrinol.* 80, 465–472) indicated that the decrease in ambient temperature seems to be the most probable factor causing termination of LH release from late summer to early autumn in this species.   © 1992 Academic Press, Inc.

We have been investigating photoperiodic control of luteinizing hormone (LH) release in Japanese quail to understand hormonal control mechanisms of breeding strategies in this species. So far, laboratory experiments indicate that LH release is easily inducible through manipulation of photoperiods; a change from short to long days induces a significant increase in plasma levels of LH and results in gonadal growth.

Also found is that short light pulses given during the photoinducible phase simulate long days (Wada, 1979, 1981; Hatanaka and Wada, 1988). However, termination of LH release through the manipulation of photoperiods is not always successful. Exposure to long days causes circulating LH levels to stay high enough to maintain testicular activity for months and a change of photoperiods from long to short days does not al-

ways induce a sufficient decrease in plasma LH levels to regress the testes.

Using the same species but perhaps a different strain, Follett and his collaborators demonstrated photoperiodic responses both in initiation and in termination of LH release (for reviews see Follett and Robinson, 1980; Robinson and Follett, 1982; Follett, 1984). To decrease LH secretion in this strain of quail, changes of the photoperiods from long days to relatively short days is necessary ("relative" photorefractoriness) (Urbanski and Follett, 1982). This is opposed to the case of white-crowned sparrows in which continued exposure to long days induces reduction of LH secretion and collapse of the testes ("absolute" photorefractoriness).

The discrepancy between the two study groups has been explained as the result of quail domestication. However, the quail used in the study by Robinson and Follett (1982) were also from a domesticated strain originating from stock imported into the United States nearly 30 years ago. Thus the discrepancy does not seem to come from the birds used in the two laboratories but from other factors disregarded in the experimental conditions. Recent measuring of LH plasma levels (Wada et al., 1990) demonstrated that low temperature in addition to short days was crucial in suppressing LH secretion under laboratory conditions and suggested that not only short days but also low temperature were involved in termination of the breeding activity in this species in the natural environment.

Laboratory experiments show only a part of the mechanism that actually controls the breeding cycle. Observations and experiments under natural conditions are necessary to confirm the validity of laboratory experiments. Follett and Maung (1978) and Robinson and Follett (1982) have already observed annual changes in levels of LH in male Japanese quail kept on natural photocycles at Bangor and Bristol. However, they neglect discussion of ambient temperature and discuss mainly the effects of photoperiod and photorefractoriness. Thus the purpose of the present experiment is (1) to assess whether our strain of Japanese quail does have an annual reproductive cycle (2) to test the hypothesis described in Wada et al. (1990) that low temperature in addition to short days is required to terminate LH secretion in the species under natural conditions. Our approach involves more frequent sampling intervals than the related experiments previously reported in the literature.

MATERIALS AND METHODS

Animals

Male Japanese quail (*Coturnix coturnix japonica*), 3 weeks old, were purchased from a commercial source. At first, they were raised under a short daily photoperiod of 8L:16D (lights on at 0800 hr) until 5–6 weeks of age. At that point they showed full somatic growth but were still sexually immature. They were then transferred to a long daily photoperiod of 16L:8D (lights on from 0800 to 2400 hr). After 2 months of photostimulation, they were transferred back to the original short daily photoperiod at low temperature (Wada et al., 1990) for an additional 2 months to induce regression of the gonads and then finally transferred to the experimental condition (Experiment 1 and 2–1). In Experiment 2–2, birds were transferred to the experimental condition after 2 months of photostimulation. No selection on the basis of responsiveness of gonadal activity to alternating long and short daily photoperiods was made beforehand.

Experimental Schedules

Experiment 1. The experiment started in December 1986 and finished in April 1988. In December 1986, seven mature male Japanese quail who had hatched in July 1986 and whose cloacal protrusion had already regressed were introduced into individual cages (15 × 30 × 16 cm). The cages were placed on the roof of a building on the campus of Tokyo Medical and Dental University in Ichikawa City (35°45′N, 139°53′E). Food (Nihon Haigo Siryo Co., Yokohama) and water were continuously supplied.

From January 16, 1987, about 0.4 ml of blood was collected from each bird every week between 1000 and 1200 hr using heparinized capillary tubes through venipuncture of the wing vein. The area of the cloacal protrusion was measured by ruler to the nearest millimeter at the time of blood collection. Blood samples

were centrifuged and the plasma was stored at −20° until assay.

During the experiment, two birds were accidentally killed; one in the 4th week of August and the other in the 1st week of November.

Experiment 2. To detect the changes in circulating LH concentrations more precisely, samples were collected every other day during an increasing phase (January through April 1988) and a decreasing phase (August through October 1989) of LH secretion. Experiment 2-1 started on January 12 and finished on April 25 using eight quail, while Experiment 2-2 started on August 2 and finished on October 31 using six quail.

Blood was collected from each bird between 1000 and 1200 hr through venipuncture of the wing vein and the plasma was stored at −20° until assay.

Meteorological Data Collection

Ambient temperatures were recorded continuously during the experimental periods by a self-recording thermometer placed beside the cages. Day length was calculated from a table of sunrise and sunset times at Tokyo 35°39′N, listed in "Chronological Scientific Tables," edited by Tokyo Astronomical Observatory, and published by Maruzen Co., Tokyo, as a duration of the times between the center of the sun being 7°21′ 40″ below the horizon at sunrise and sunset. In this way, the light period includes civil twilight of dawn and dusk and is longer than the period between actual sunrise and sunset. Thus the calculated day length for February 15 was 12 hr. Actual changes in light intensity received by the cages over 1 day were measured with a digital illuminometer (Model T-1, Minolta Optical Co., Tokyo) on April 23, 1987, when calculated day length was 14.5 hr (Fig. 1). The day length equaled a duration of light intensity above 0.1 lux.
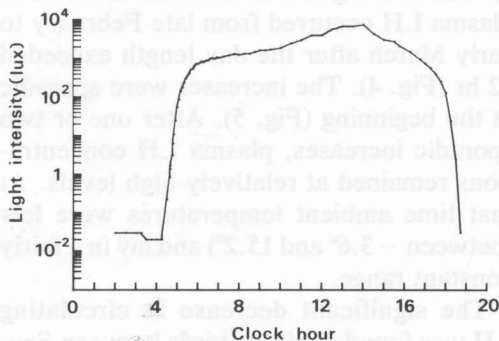

FIG. 1. Changes in light intensity of a day measured next to quail cages on the roof of a three-story building on the Kohnodai campus of Tokyo Medical and Dental University (35°45′N, 139°53′E) on April 23, 1987, at a calculated day length of 14.5 hr.

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 preparations of chicken LH (fraction AGCHDS112312A) were 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 a chicken LH fraction IRC-2, Gunma.

Intraassay and interassay coefficients of variation were 6.37 and 8.67%, respectively.

Statistics

Two-way analyses of variance followed by Kruskall–Wallis tests were used to test the statistical significance of fluctuations in the mean hormone values between sampling times. If it was proved significant, the Mann–Whitney U test was used to test the significance of the difference between the values obtained for any two sampling times. Differences were considered significant when $P < 0.05$. Throughout the text, means are given with ±1 SEM.

RESULTS

Experiment 1

Figure 2 shows the changes in plasma LH levels of the birds kept in the outdoor cages. The changes in day length and ambient temperature during the experiment period are also shown in Fig. 2. The curve of temperature change shows the highest and lowest temperatures for each day. The changes in the area of the cloacal protrusion are shown in Fig. 3.

Circulating levels of plasma LH began to increase between the middle of February and the beginning of March; the first significant increases were detected between February 9 and February 17 in 1987 and February 25 and March 3 in 1988. At first this increase was sporadic, varying from individual to individual, but by late March LH increase became steady and in early April the circulating LH levels reached more than 1 ng/ml. Thereafter plasma levels of LH continued to increase, attaining the maximal level (about 3 ng/ml) by May and
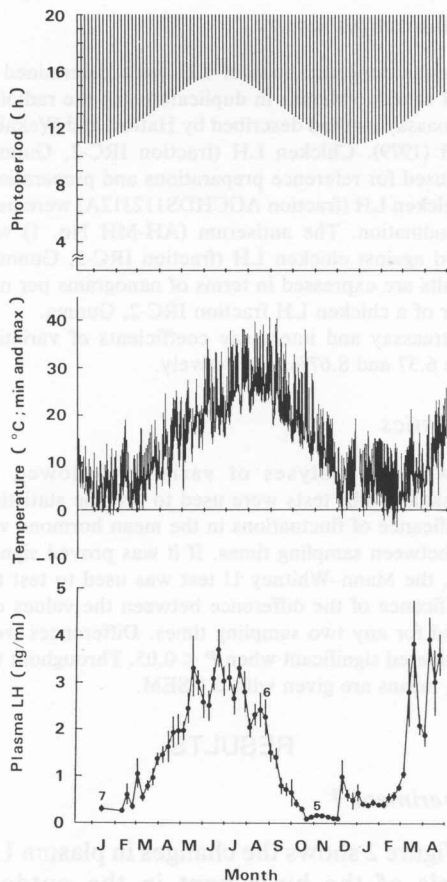
FIG. 2. Changes in plasma LH concentrations in Japanese quail kept in outdoor cages under natural conditions. The top and middle panels show changes in day length and ambient temperature, respectively. The values of LH concentrations are means and the vertical bars are SEM. The number of birds is depicted above the points. Those points lacking a number have the same value as the number to the left.

then leveling off (Fig. 2). From mid-August to September, concentrations of plasma LH began to decrease; significant decrease was found between August 27 and September 3 and by the end of September it had become less than 1 ng/ml.

The cloacal protrusion, completely regressed during winter, began to develop in March, gradually at the beginning but markedly later, and leveled off from April to May. Developed protrusion was maintained until August, began to decrease in
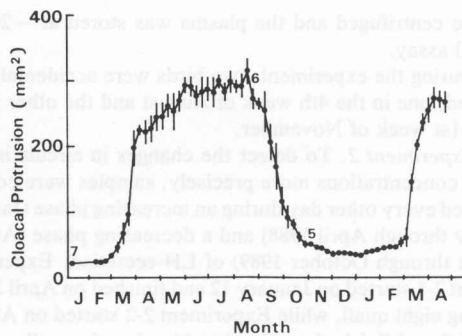

FIG. 3. Changes in size of the cloacal protrusion for the same birds as used for Fig. 1. The values are means and the vertical bars are SEM. The number of birds is depicted above the points. Those points lacking a number have the same value as the number to the left.

September, and became regressed by the end of October (Fig. 3).

The record of the temperatures at the cages showed the annual cycle of ambient temperature to have a trough in January to February and a peak in August (Fig. 2).

Experiment 2

Figure 4 shows changes in plasma LH concentrations from February to April 1988 and August to October 1989. The changes in individuals are shown in Figs. 5 and 6.

The overall profile of the changes in circulating levels of LH in Experiment 2 (Fig. 4) was similar to that in Experiment 1 (Fig. 2). The first significant increase in levels of plasma LH occurred from late February to early March after the day length exceeded 12 hr (Fig. 4). The increases were sporadic at the beginning (Fig. 5). After one or two sporadic increases, plasma LH concentrations remained at relatively high levels. At that time ambient temperatures were low (between −3.6° and 15.2°) and lay in a fairly constant range.

The significant decrease in circulating LH was found in all the birds between September 1 and 5 when the day length was still 14 hr (Figs. 4 and 6). The plasma levels of LH were constant at about 2 ng/ml before the decrease and decreased steadily to non-
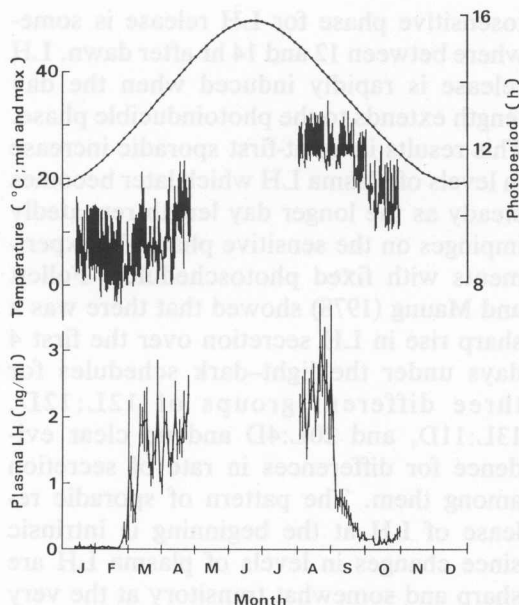
FIG. 4. Changes in concentrations of plasma LH in quail kept in outdoor cages under natural conditions. Samples were collected every other day from January 12 to April 25, 1988 in eight birds and from August 2 to October 31, 1989 in six birds. Day length and ambient temperature are shown in the top panel. The values of LH concentrations are means and the vertical bars are SEM.

breeding levels of less than 0.5 ng/ml after it occurred. The record of ambient temperatures from late summer to early autumn of 1989 indicates that temperature began to fluctuate at this time; higher temperatures were followed by consecutive days of relatively low temperature (Fig. 4).

DISCUSSION

The present experiments clearly show that our strain of Japanese quail has a distinct annual reproductive cycle under natural conditions. The increase of circulating LH from nonbreeding levels occurred from late February to early March when day length exceeded 12 hr. The decrease from the high and stable levels of circulating LH to the nonbreeding levels occurred from late August to early September while the day length was still 14 hr.
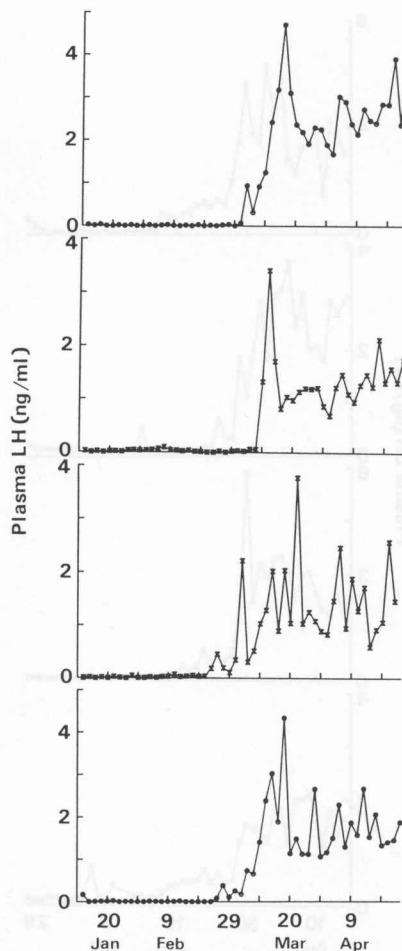

FIG. 5. Changes in concentrations of plasma LH in an individual bird randomly selected from the birds of Fig. 4. Samples were collected from January 12 to April 25, 1988.

Our results indicating LH increase in early spring confirm those of Follett and Maung (1978), Follett and Robinson (1980), and Robinson and Follett (1982) for the same species. According to Follett and Maung (1978), an increase in LH levels was observed between March 2 and 15 when the photoperiod (including civil twilight) increased from 11 hr 55 min to 12 hr 59 min. Differences from our data as to the date of the onset of LH increase are obviously due to latitudinal differences (Bangor 53° 27′N, Bristol 51°27′N, and Ichikawa 35°45′N). In
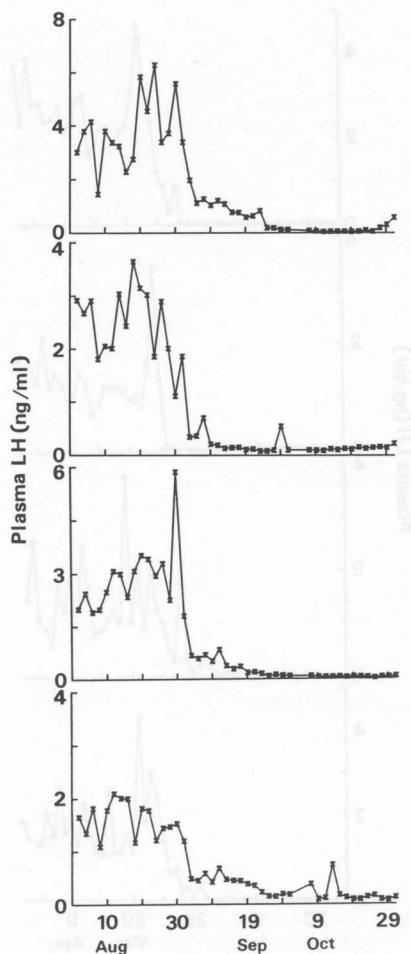
FIG. 6. Changes in concentrations of plasma LH in an individual bird randomly selected from the birds of Fig. 4. Samples were collected from August 2 to October 31, 1988.

tosensitive phase for LH release is somewhere between 12 and 14 hr after dawn. LH release is rapidly induced when the day length extends to the photoinducible phase. This results in an at-first sporadic increase in levels of plasma LH which later becomes steady as the longer day length repeatedly impinges on the sensitive phase. In experiments with fixed photoschedules, Follett and Maung (1978) showed that there was a sharp rise in LH secretion over the first 4 days under the light–dark schedules for three different groups of 12L:12D, 13L:11D, and 20L:4D and no clear evidence for differences in rate of secretion among them. The pattern of sporadic release of LH at the beginning is intrinsic since changes in levels of plasma LH are sharp and somewhat transitory at the very beginning of photostimulation even on long days of 20L:4D (Follett et al., 1977) and 16L:8D (Wada, 1979; Hatanaka and Wada, 1988), respectively, as well as the light–dark schedule of 8L:4.5D:0.5L:11D (Hatanaka and Wada, 1988).

The increase in levels of plasma LH concentration in spring shown in Fig. 2 seems to be proportional to the length of photoperiod. However, this is misleading because the data are derived from infrequent sampling (once a week) and the use of mean figures. As shown in Fig. 4 and 5, the levels of plasma LH in individuals increased rapidly to relatively high ranges within a week or so.

Inclement weather such as periods of unseasonably cold weather, sudden storms, and prolonged precipitation may cause delay of gonadal recrudescence under natural conditions in white-crowned sparrows (Wingfield et al., 1983) and song sparrows (Wingfield, 1984, 1985a,b). However, these results indicate that the delay is mostly due to reduced availability of food and not directly due to cold weather. When levels of plasma LH are increasing under natural conditions, ambient temperature seems to have little if any role. Laboratory experi-

both cases, the day length for the initiation of LH increase is 12–12.5 hr.

These data described in the previous paragraph are fairly consistent with data obtained by the laboratory experiments. Night interruption experiments using a 0.5-hr pulse (Wada, 1979, 1981) show that the time of maximum response to the inductive effects of light lies between 13 and 15 hr after dawn; Nicholls et al. (1983) also show that a single 4-hr light pulse is effective in castrated quail when given 10–16 hr after dawn. These results indicate that the pho-

ments show that circulating LH concentrations increase on 16L:8D with constant ambient temperature of 8° (Wada *et al.*, 1990). Thus day length is the main initial predictive information for the onset of LH increase in early spring with other supplementary information playing only minor roles.

The initial decrease in plasma LH was found between late August and early September when the day length was still 14 hr, quite long when compared to day length leading to the onset of LH release in early spring. The same results were also obtained by Follett and Maung (1978) and Robinson and Follett (1982); the day length at which LH begins to decrease was 15 hr in Bristol and Bangor. To explain this phenomenon, Robinson and Follett (1982) did a series of experiments using fixed day lengths and concluded that birds kept on 16L:8D read 13L:11D as long days in early spring but as short days in late summer due to a shift in the threshold of sensitivity to photoperiods. Their results showed that after 8 weeks of photostimulation on 16L:8D, levels of plasma LH in castrated birds fell significantly within 11 days of transfer to 13L:11D, while LH fell to the levels of the short-day intact birds after 10 weeks of photostimulation on 16L:8D. They also showed that quail undergoing testicular regression in late summer under natural photocycle responded rapidly to an increase in day length. This was also tested in quail that had experienced 16L:8D for 9 weeks and then were transferred to 13L:11D. The birds that were returned to 16L:8D after 7, 9, and 11 days on 13L:11D all responded with significant LH increase within 7 days of reexposure to long days (Robinson and Follett, 1982). This is designated as "relative" photorefractoriness contrary to the "absolute" photorefractoriness found in white-crowned sparrow (Wingfield and Farner, 1980).

We find difficulty in applying the above relative photorefractoriness concept for our strain of Japanese quail. Under laboratory conditions reproductive activity did not terminate following transfer of birds from long days of 16L:8D to short days of 8L:16D. Plasma LH concentrations decreased significantly to 1–2 ng/ml, but since those levels are high enough to maintain testicular activity, they also maintain the cloacal gland activity, indicating that these levels are not typical for the nonbreeding state but are typical for the basal reproductive state (Wada *et al.*, 1990). Further suppression to nonreproductive titers of less than 0.5 ng/ml requires low temperature in addition to short days. The decreased levels of circulating LH presented by Robinson and Follett (1982) are around 1 ng/ml, and sometimes even greater levels, which are not necessarily low enough to preclude breeding. Another difficulty concerns circadian rhythms which are believed to regulate the photosensitive phase for LH release (Follett and Sharp, 1969; Follett *et al.*, 1974; Wada, 1979, 1981). Circadian rhythms do not normally show a gradual phase shift over many weeks when entrained to light–dark cycles (Follett, 1984).

The alternate explanation that low ambient temperature is required as supplementary information for termination of breeding activity, at least for our Japanese quail, well explains our present data; in August plasma concentrations of LH became lower due to decreasing daily photoperiods after the summer solstice, and from late August to early September lowering temperatures induced further reduction of LH release to nonbreeding levels. Actually the temperature records in Figs. 2 and 4 indicate that there were several days of relatively low temperature after hot days. Thus we believe that unlike the increase in levels of plasma LH in spring where temperature is not significant, temperature is required as supplementary information for suppression of LH release. At the present moment, however, we do not know what variables of low temperature are significant. A number of consecutive days at lower temperatures,

high- and low-temperature variations, or some other mechanism may be the key factor.

In white-crowned sparrows, levels of plasma LH decrease long before the photoperiod becomes 14L:10D and under laboratory conditions, they fall to nonbreeding levels after 60 to 80 days of photostimulation. This indicates that no supplementary information is involved (Wingfield and Farner, 1980). It may be postulated that the difference is due to differences in migratory habitats; white-crowned sparrows are long-distant migrants while quails are short-distant migrants. Termination of reproductive activity far before the environmental conditions deteriorate will give an adaptive value for long-distant migrants in gaining the trophic resources necessary for migration. Short-distant migrants or sedentary species may not require such a long time to gain trophic resources.

Even our strain of Japanese quail had some individuals (about 30% of the population) whose circulating levels of LH decreased in response to short-day treatment only (Wada et al., 1990). In these birds plasma levels of LH decreased to nonbreeding levels when transferred from long days of 16L:8D to short days of 8L:16D with constant temperatures of 19° and 25°, respectively. It seems there are two separate lines in our Japanese quail population with sensitivity to temperature being the differentiating feature. If we had preselected those birds which respond to only photoperiodic manipulation for our experiments, we would have obtained the same results as Follett's laboratory did. At present we do not know the physiological basis of the two strains.

Photorefractoriness is still a matter of debate as no satisfactory theory to describe the physiological basis of the phenomenon has been advanced (Nicholls et al., 1988). However, Japanese quail can give an insight into this phenomenon if used in proper experiments.

ACKNOWLEDGMENTS

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