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Circadian rhythms of testosterone-dependent behaviors, crowing and locomotor activity, in male Japanese quail

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Summary. An apparatus was devised to record crowing (mate calling by males) together with locomotor activity and recorded data was analyzed by several methods for rhythm analysis. Crowing and locomotor activity of Japanese quail held on long days were recorded during sexual development as estimated from circulating gonadotropins and testosterone. Both behaviors were testosterone-dependent but commencement of crowing preceded the increase in locomotor activity. When the two behaviors attained their maximum levels, crowing showed consistent daily rhythms in which two peaks were apparent, a major one at the onset of light and a broader one 8 hours later. Locomotor activity also showed a clear daily rhythm with a peak between the two peaks of crowing rhythm suggesting a fixed phase relationship between the two rhythms.

Both rhythms free-ran under constant dim light with periods shorter than 24 h. They persisted in birds which had been castrated and then supplied with exogenous testosterone via implanted Silastic capsules. The durations of both rhythms were quite comparable to each other and they maintained a fixed phase relationship similar to that found under LD cycles.

The results indicate that testosterone is essential for the induction of crowing and for the enhancement of locomotor activity but the formation of the rhythms in behavior was strictly dependent on a circadian oscillatory mechanism.

Introduction

Accumulated evidence indicates that a number of biological phenomena, from cell division to locomotor activity, are regulated or influenced by a circadian oscillatory mechanism (review Aschoff 1981). Locomotor activity has been analyzed extensively so far due to the relative ease of data collection for long periods of time without disturbing the animals. It has proved to be a valuable measure of biological clock studies. However, multiple criteria are required to study mechanisms underlying the circadian oscillatory system. We know little except in man about circadian phenomena other than locomotor activity in vertebrates.

Field studies show that singing in birds fluctuates in a diel pattern with most species singing frequently before and around dawn. Such pattern have also been observed in Japanese quail (Guyomarc'h and Thiboult 1969; Ottinger 1983; Wada 1981), but precise studies of the temporal patterns of quail calling are still lacking. Wada (1981, 1982) devised a non-intrusive method for recording both crowing and activity from quail over extended periods of time and this technique provides quantitative analysis of both crowing behavior and locomotor activity.

Crowing in quail is androgen-dependent (Adkins and Adler 1972; Beach and Inman 1965; Ottinger and Brinkley 1978; Wada 1981, 1982). The level of locomotor activity is also regulated by androgen; castration in adult birds reduces locomotor activity to the level found in immature individuals while testosterone administration reestablishes activity (Wada 1981, 1982). However, there is no short-term relationship between the fluctuation of plasma androgen and either crowing or locomotor activity (Ottinger 1983; Wada 1982). The purposes of this study were (1) to describe the basic characteristics of the rhythms in crowing and locomotor activity during the reproductive development in quail and (2) to look at these two rhythms under constant conditions.

Abbreviations: *LH* luteinizing hormone; *FHS* follicle-stimulating hormone; *LD* light-dark; *LDim* light-dim light

Materials and methods

Animals. Male Japanese quail (*Coturnix coturnix japonica*) were purchased from a commercial source at the age of 3 weeks and kept on short days of LD 8:16 (lights on from 0800 to 1600) or long days of LD 16:8 (lights on from 0800 to 2400). They were allowed to free access to pelletized food and water.

Apparatus. Each bird was kept singly in a recording cage (15 × 30 × 16 cm) in which the floor moved as a seesaw so that each deflection of the floor triggered an installed microswitch. The cage was placed in a light-tight box (inner dimensions, 30 × 38 × 30 cm), which was ventilated through a light-tight trap with a motor-driven fan. Illumination was provided inside the box by an overhead white fluorescent lamp through frosted glass which produced a light intensity of about 200 lx at the floor level. The lamp was covered with a water jacket with continuous water flow. A small neon lamp was installed beside the main lamp which provided a light intensity of approximately 0.1 lx on the cage floor, if the main light was turned off. The ambient temperature was 25 °C. The 24-h light-dark cycle was regulated by an external timer.

A small microphone was placed in the box and connected to an electronic device (Kokusai Electronics Co., Ltd., Tokyo) composed of a bandpass filter, a comparator, and a timer. Sounds between 1 and 10 kHz frequency and a certain duration were detected through the microphone and pulses emitted. In quail the crow is composed of three notes with an upper frequency range of 6 or 7 kHz and a duration of 0.5 s (Potash 1974) so that only a crow can induce a pulse while calls other than the crow or background noises are eliminated. Numbers of floor deflections and pulses by crowing, respectively, were recorded by a counter (Kokusai Electronics Co., Ltd., Tokyo). Every hour, cumulative numbers of floor deflections (activity) and calls (crows) were printed automatically. Each event was also recorded as a single pen deflection on a 15-channel event recorder (Shimadzu Denki Keisokuki Co., Ltd,. Kyoto). For each animal, the activity and crowing records from a single day were pasted, respectively, beneath those of the previous day. They were photographed and reduced in size to produce a double-plotted activity record.

Experimental schedules

Experiment I. A group of sexually immature birds (3 week) of 50–60 g body weight was purchased which had been held under constant light since hatching. The birds were separated into 2 groups. Six birds of the first group were transferred to the recording cages and maintained under a long day of LD 16:8 until 70 days of age. Forty-six birds of group 2 were placed into individual cages and kept in a room of long days. Every 4 to 7 days, 4 to 6 birds were killed by decapitation and trunk blood collected. Serum was separated after centrifugation and stored frozen until assay.

Experiment II. Five mature birds were castrated and kept in recording cages under light-dim light cycles (16 h light and 8 h dim light, LDim 16:8). Two weeks after castration, when crowing was abolished and activity reduced, Silastic implants (3.18 mm o.d. and 1.57 mm i.d.; 2 × 30 mm) filled with crystalline testosterone (Sigma Chem., St. Louis) and sealed with Silastic Adhesive type A at both ends were subcutaneously implanted. The capsules were washed overnight in saline solution at room temperature before implantation. Two weeks after implantation, the main light was turned off resulting in continuous dim light (0.1 lx at the floor level) so allowing the behaviors to free-run.

Ten days after continuous dim light, the main light was given for 8 h to make light-dim light cycles (LDim 8:16). After 2 weeks, the main light was again turned off producing constant dim light. Ten days after continuous dim light, the lighting schedule was returned to LDim 8:16. Ten days after entraining under LDim 8:16, birds were killed by decapitation collecting trunk blood. Sera were separated after centrifugation and stored at −20 °C until radioimmunoassay of testosterone.

Radioimmunoassay. Immunoreactive LH and FSH concentrations were estimated by double antibody radioimmunoassay using 50 µl sample in duplicate. LH concentrations were expressed in terms of ng of the chicken LH fraction IRC-2 (Gunma) per milliliter (Hattori and Wakabayashi 1979) and FSH was expressed in terms of ng of the chicken FSH (AGCHDS111135A) per milliliter (Sakai and Ishii 1983).

Testosterone concentrations were also estimated in duplicate with radioimmunoassay after extraction of the serum by ether using ammonium sulphate precipitation for bound and free separation. The assay was performed without previous chromatography and the antiserum (Teikoku Zoki Pharmaceutical Co. Ltd., Tokyo) used in the present experiment cross-reacts with 5α-dihydrotestosterone at about 13.5%. Thus the values here reflect the total concentrations of androgens, testosterone and 5α-dihydrotestosterone.

Data analysis. All the data from the system mentioned above were analyzed by a microcomputor (PC-8001 and its peripherals, Nippon Electronics Co., Tokyo) with BASIC programms. Hourly data from each bird were computed to detect rhythm components by autocorrelation, cross-correlation between crowing and locomotor activity, power spectrum method, least square spectrum method, and periodogram method (review Enright 1981).

Results

Experiment I

In birds held on LD 16:8 circulating FSH and LH increased from day 30 to day 42. Circulating testosterone also increased by day 30 and reached a maximum level by day 49 (Fig. 1).

Crowing was first recorded on day 30, but only a few times around at the onset of light. The number of crows then increased gradually until to reach a plateau by day 50 (Fig. 2). Locomotor activity increased in parallel with crowing. However, the onset of the increase of crowing preceded that of locomotor activity.

When both crowing and activity had reached their maxima they showed steady daily rhythms (Fig. 3). The birds began to crow a few hours before the onset of light and crowed most frequently around the onset of light. Crowing then decreased to almost one-third of the maximum level, but a smaller peak occurred in the afternoon around 8 h after the onset of light. The quails did not move much at night but began to be active after the light had come on. They moved most frequently 5–7 h after the onset of light at the time when crowing decreased.

The rhythms of both behaviors were very stable

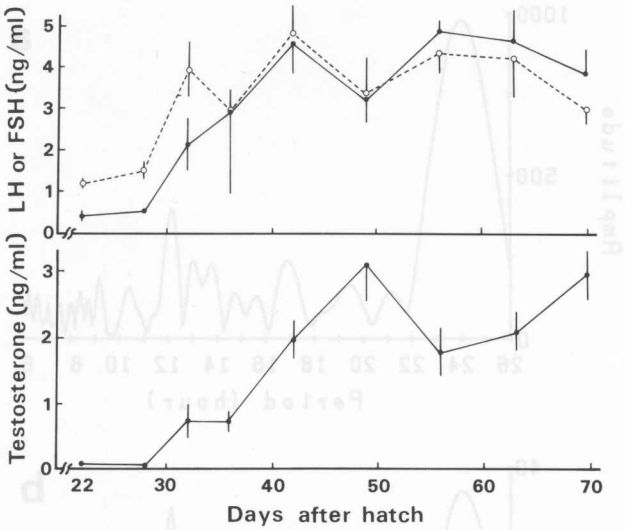

Fig. 1. Changes in circulating LH (solid line in upper panel), FSH (broken line in upper panel), and testosterone (lower panel) in male Japanese quail kept on LD 16:8. Each point represents the mean of 4 to 6 birds and SEM

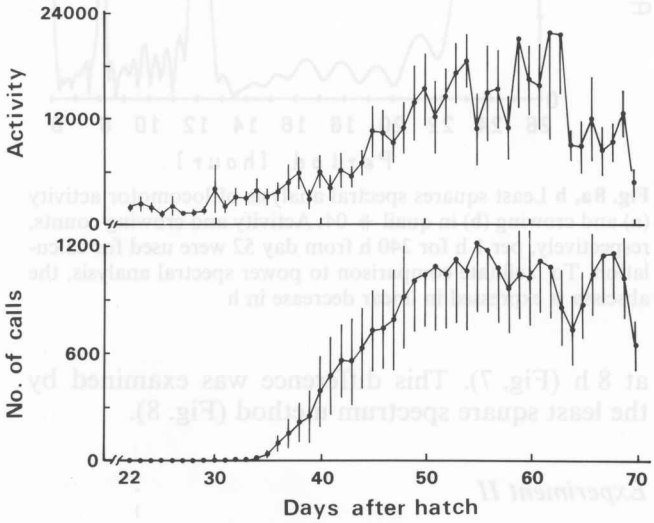

Fig. 2. Changes in daily locomotor activity (upper panel) and the number of calls (crows) (lower panel) in quail kept on LD 16:8. Each point represents the mean of the same 6 birds and SEM

in light-dark cycles of LD 16:8. Figure 4 was constructed from the hourly data for the amount of crowing in consecutive days and shows the pattern of the rhythm during the entire experimental period. It clearly shows two peaks, steep one at the onset of light and the other about 8 h after the first peak, both of which persisted. An autocorrelogram (Fig. 5) of each behavior indicated 24-h cyclicity.

From Fig. 3 it appeared that the two rhythms, the number of crowing and locomotor activity,

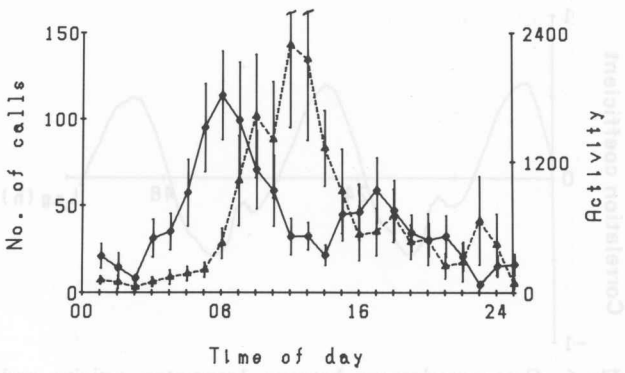

Fig. 3. Changes in locomotor activity (broken line) and the number of calls (crows) in a long day of LD 16:8 (lights on from 0800 to 2400, day 60 after hatch). Points represent the mean of 6 birds and SEM

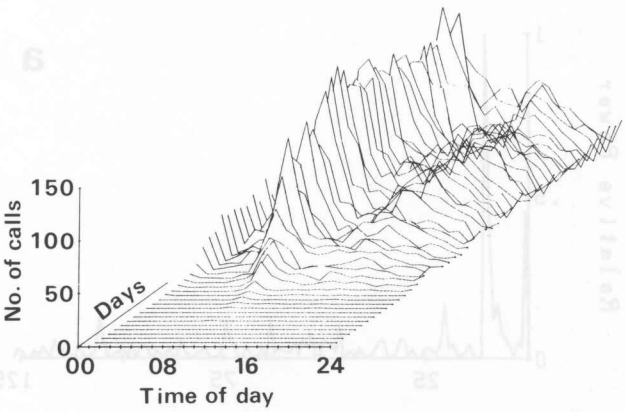

Fig. 4. A 3-dimensional reconstruction of ontogenetical development of crowing from daily data in quail kept on LD 16:8 from day 22 after hatch. Each line connects the hourly mean of 6 birds but SEM is omitted

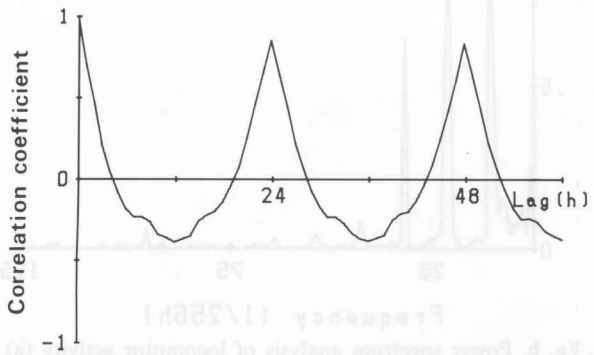

Fig. 5. Autocorrelogram of a crowing rhythm in quail # 02. Autocorrelation coefficients were calculated from sequential time series data of 1 h intervals (240 h from day 52) against 60 lags (lag/h). Autocorrelogram for locomotor activity was almost the same and not shown here

bore a fixed relationship to each other. Cross-correlograms of the two behaviors showed a strong relationship with 5–7 h phase difference (Fig. 6). However, the components of the 24-h rhythms

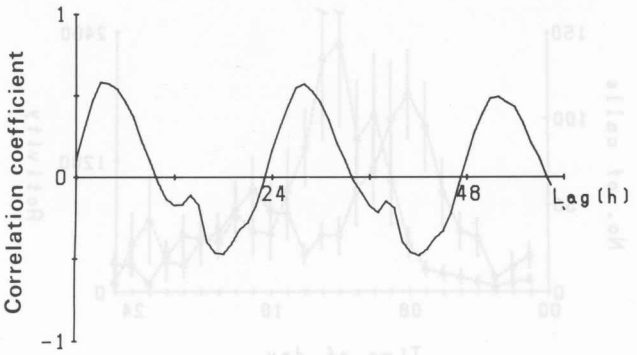

Fig. 6. Cross-correlogram between locomotor activity and crowing in quail # 02. Cross-correlation coefficients were calculated from sequential time series data of 1 h intervals in locomotor activity and crowing (240 h from day 52) against 60 lags (lag/h)

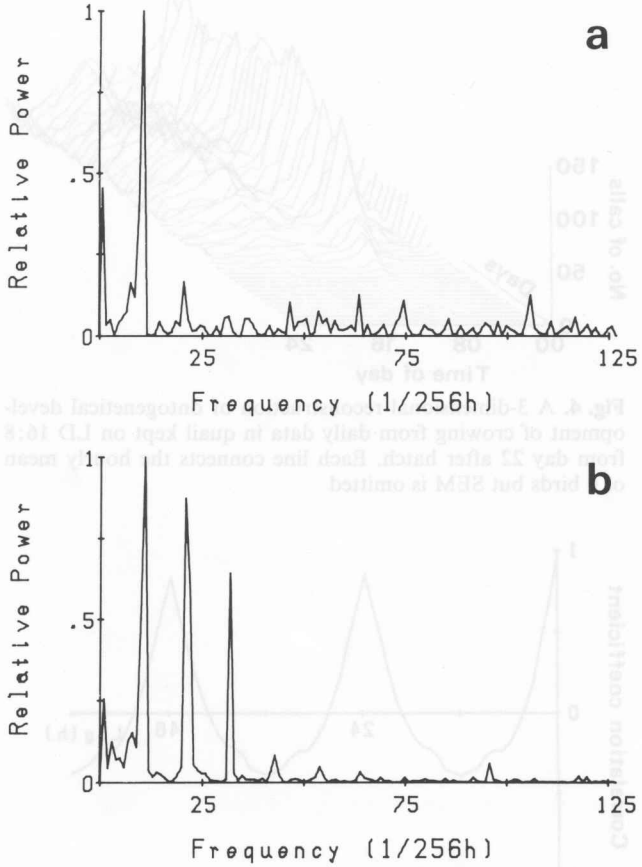

Fig. 7a, b. Power spectrum analysis of locomotor activity (**a**) and crowing (**b**) in quail # 06. Each profile means changes in relative power calculated from sequential time series data of 1 h intervals (240 h from day 52) by fast Fourier transform against linear increase in frequency (i.e. 24 h is located at about 11 on abscissa).

were different. Power spectrum analysis indicated that the locomotor activity rhythm had a major cycle of 24 h whereas that of crowing had two additional components; one at 12 h and the other

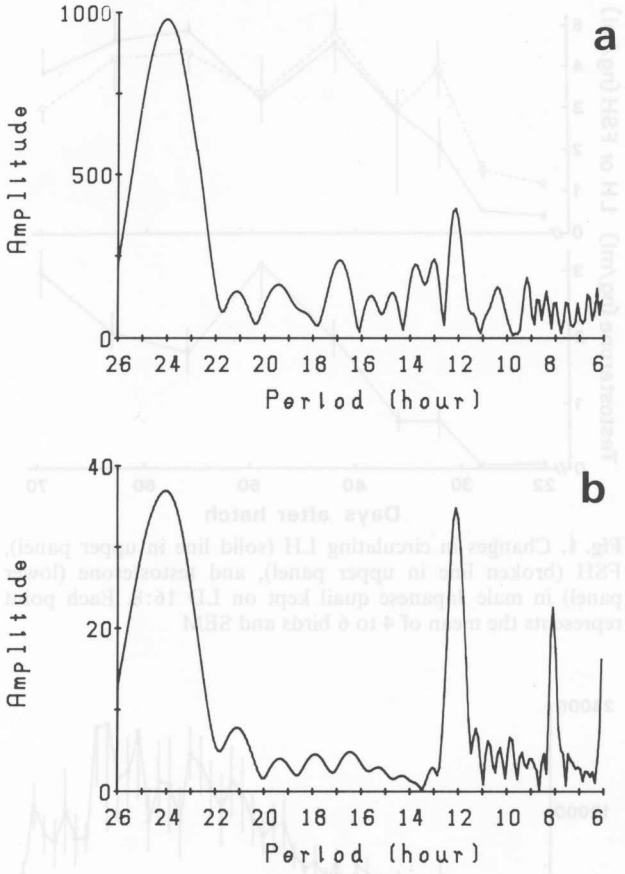

Fig. 8a, b Least squares spectral analysis of locomotor activity (**a**) and crowing (**b**) in quail # 04. Activity and crowing counts, respectively, per 1 h for 240 h from day 52 were used for calculation. To facilitate comparison to power spectral analysis, the abscissa is expressed in linear decrease in h

at 8 h (Fig. 7). This difference was examined by the least square spectrum method (Fig. 8).

Experiment II

Castrated birds with subcutaneous implants of testosterone showed daily rhythms of locomotor activity. As shown previously (Wada 1981) castration abolished calling completely and reduced locomotor activity while testosterone recovered crowing to the precastration level and enhanced the locomotor activity. In Fig. 9, the arrows indicate the day of testosterone implantation. After several days under LDim 16:8, crowing was restored and activity enhanced. However, the appearance of both behaviors was slightly different from those seen under LD cycles (Fig. 3). In particular, crowing commenced several hours prior to the onset of light and continued to increase until lights-on, at which time crowing declined. Throughout the light period, crowing was intermittent. Rhythm

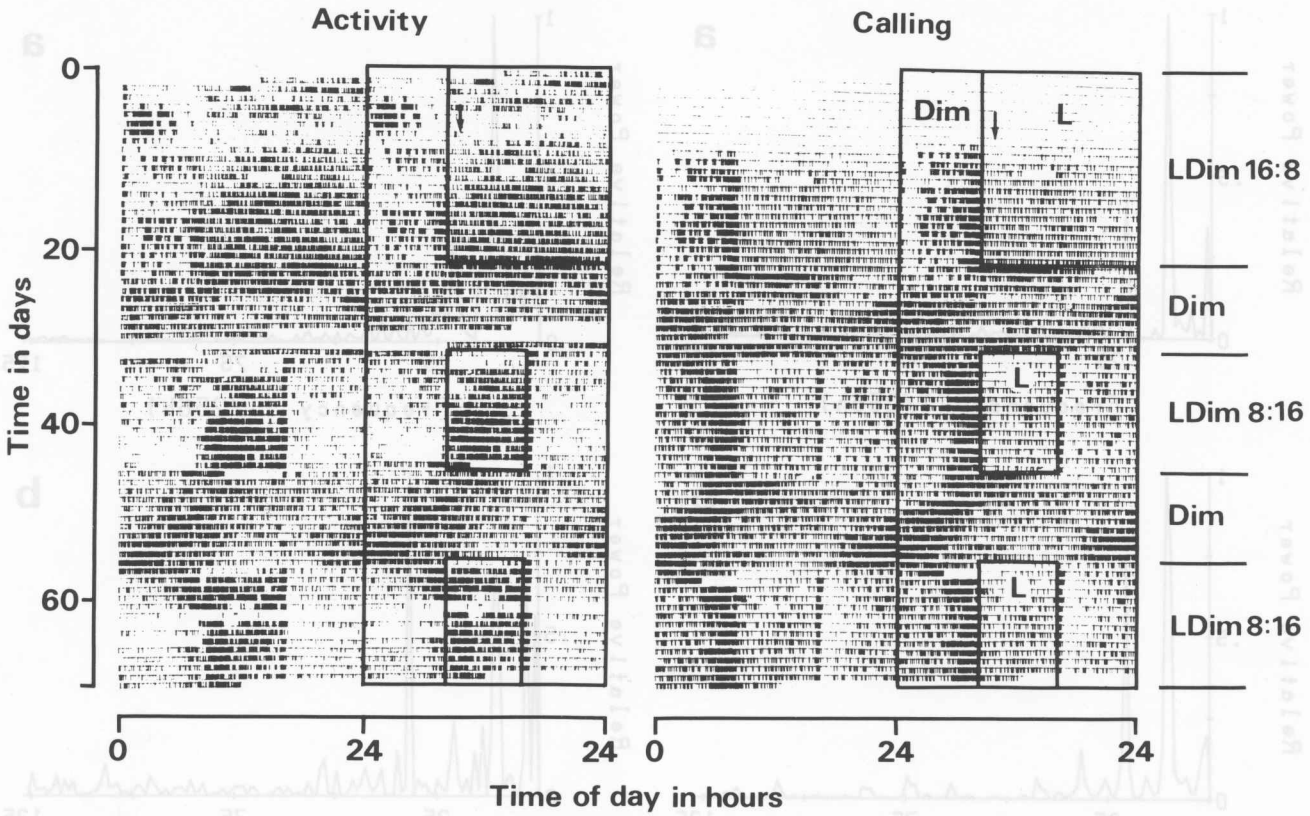

Fig. 9. Daily record of locomotor activity (left) and calling (crowing) (right) in quail maintained in LDim cycles or in constant dim light. Successive days are plotted from top to bottom and the records have been double plotted over 48-h time interval to facilitate visualization of the rhythms. Quail was castrated before the start of recording and was implanted with Silastic implants of testosterone on the day indicated by arrows. Free-running with shorter period than 24 h is clearly shown in constant dim light after LDim 8:16 in both activity and crowing

analyses of crowing and locomotor activity under LDim 16:8, however, showed that the two rhythms were basically similar to those under LD 16:8 (Fig. 10), even though the 8 h crowing component was somewhat reduced. Under LDim 8:16, the 8 h component became clear again (Fig. 11). Patterns of the activity rhythms under LDim 8:16 were basically similar to those under LD 16:8 and LDim 16:8

Under constant dim light, both rhythms free-ran with periods of less than 24 h (Fig. 9). The period of the free-running rhythms after LDim 16:8 seemed to be shorter than those after LDim 8:16 but statistical analyses would not be undertaken because the free-running rhythms after LDim 16:8 in most cases tended to be less clear; this was particularly true for locomotor activity. Both rhythms were particularly stable under constant dim light after being released from LDim 8:16 (Fig. 9). The period estimated from periodograms was 22.66 ± 0.22 h in locomotor activity and 22.94 ± 0.22 h in crowing ($n = 5$ by periodogram method) (Fig. 12). The two values were very similar to each other. Cross-correlogram between the two rhythms under constant dim light after LDim 8:16 showed a phase relationship with a 5–7 h phase difference, similar to that found in LD cycles.

Serum testosterone at the end of the experiment was 1.34 ± 0.15 ng/ml ($n = 5$). The Silastic implants withdrawn from each bird still contained crystalline steroid, and mean loss of the weight during 62 days implantation was 6.6 ± 0.2 mg ($n = 5$).

Discussion

The results showed that the rhythms of crowing and locomotor activity were regulated by an endogenous circadian oscillatory mechanism and were not dependent upon the fluctuations of circulating levels of testosterone.

The apparatus used to detect crowing seems reliable. In a preliminary test, tape-recorded calls activated the apparatus and triggered a pulse. Calls

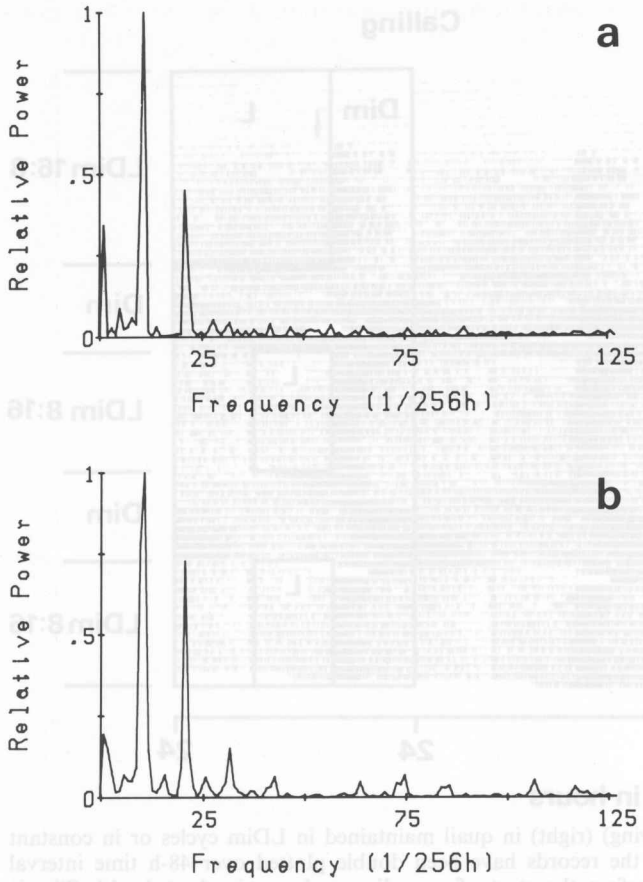

Fig. 10a, b. Power spectrum analysis of locomotor activity (**a**) and crowing (**b**) in quail # 103. Each profile means changes in relative power calculated from sequential time series data of 1 h intervals under LDim 16:8 (240 h from day 12) by fast Fourier transform against linear increase in frequency

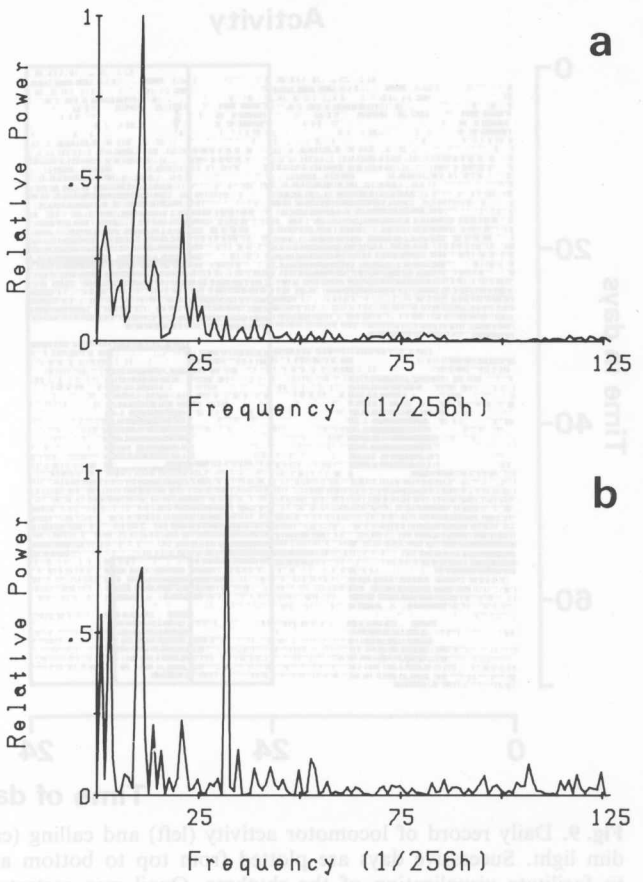

Fig. 11a, b. Power spectrum analysis of locomotor activity (**a**) and crowing (**b**) in quail # 101. Each profile means changes in relative power calculated from sequential time series data of 1 h intervals under LDim 8:16 (240 h from day 36) by fast Fourier transform against linear increase in frequency

such as the contact call or other indifferent vocalizations did not activate the apparatus. Background noises of low frequency were not counted. In the recording box, a motor was placed for ventilation. Sound from the motor, which was continuous with a low frequency, gave a white noise. Although the boxes were not perfectly soundproofed the white noise did interfere with crows from quail in the neighboring boxes. Crows from the adjacent boxes never activated the apparatus.

In quail maintained on long day photoperiod (LD 16:8) from hatching, crowing appeared around day 30, somewhat earlier than the increase in locomotor activity. Ottinger and Brinkley (1978) showed crowing to appear on day 31.8 when circulating testosterone was 3.3 ng/ml, whereas mating was first observed on day 34.9 when testosterone was 4.7 ng/ml. Crowing was initiated earlier than mating and the onset of both behaviors was associated with significantly different levels of serum testosterone. In this experiment, initiation of crowing was associated with the first rise of circulating testosterone which was preceded by an increase of circulating gonadotropins. Elevation of locomotor activity seemed to be initiated by a higher level of circulating testosterone than that required for commencement of crowing (Figs. 1 and 2).

Testosterone enhanced locomotor activity in this species (Fig. 9) as shown previously (Wada 1981, 1982). Gwinner (1974) reported in starlings that testosterone lengthened the duration of activity, perhaps resulting in an increase in activity (review Turek and Gwinner 1982). It is difficult to separate the locomotor activity into general locomotor activity and locomotor activity related to androgen increment. However, the fact that level of activity decreases after castration to that in sexually immature birds indicates there is residual ac-

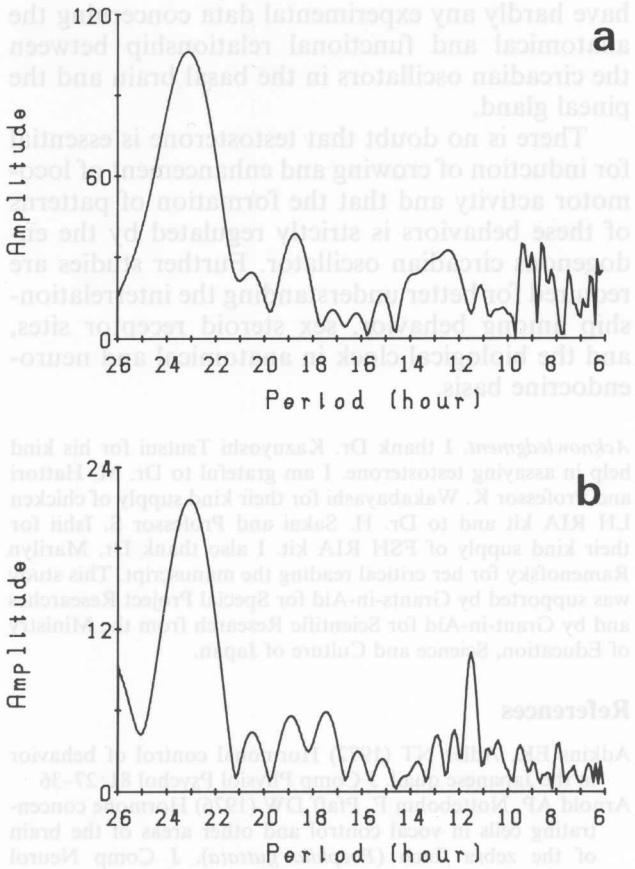

Fig. 12a, b. Least squares spectrum analysis of locomotor activity (**a**) and crowing (**b**) in quail # 102. Activity and crowing counts, respectively, per 1 h for 240 h from day 36 in Fig. 9 were used for calculation. To facilitate comparison to power spectral analysis, abscissa is expressed in linear decrease in h

tivity after castration that is general locomotor activity. The daily activity time of free-living male birds of several species is considerably longer during the spring breeding season than on days with similar photoperiods in autumn (Daan and Aschoff 1975). Enhanced locomotor activity by androgen, and also by estradiol, will be related to sexual activity such as maintaining a territory. Males of white-crowned sparrows defend territory and sing intensively at pair formation and courtship, and at this time testosterone approaches maximum values (Wingfield and Farner 1980).

It is also true that locomotor activity is enhanced by estradiol, which is an aromatized form of testosterone; however crowing is not induced by estradiol (Wada 1982). These data seem to indicate that enhanced locomotor activity is related to sexual activity and crowing to courtship behavior from the viewpoint of its steroid dependency. Separate areas of the brain may regulate courtship behavior (crowing) and mating behavior (locomotor activity in this experiment), each with its own specific activity in steroid metabolism (Nottebohm et al. 1976; review Balthazart 1983).

A site in the central nervous system responsible for reproductive behavior is assumed to be the preoptic area in the most vertebrate species studied so far. Autoradiographic studies in chickens and in other avian species indicate that the preoptic area is one of the sites which accumulate tritiated testosterone and estradiol (Arnold et al. 1976; Barfield et al. 1978; Kim et al. 1980; Martinez-Vegas et al. 1976). Other sites are the ventromedial hypothalamus related for the feedback mechanism and the areas responsible for vocalization such as the nucleus intercollicularis, hyperstriatum ventrale pars caudalis and nucleus nervi hypoglossi pars tracheosyringealis (Arnold et al. 1976; Zigmond et al. 1973). At least, one of these sites should be responsible for crowing in quail. Testosterone implants into the third ventricle of castrated birds restored crowing to the levels found in intact mature birds (Wada 1984). Testosterone implantation into the preoptic area in chickens (Barfield 1971; Gardner and Fisher 1968) and in ring doves (Barfield 1971; Hutchison 1971) restored mating behavior and courtship behavior, respectively. However, testosterone implanted into the preoptic area and the midbrain vocal areas could not induce male vocalization in capons (Phillips and Barfield 1977). Cohen and Cheng (1982) showed in ring doves that testosterone, 5α-dihydrotestosterone and estradiol induced nest-coo when implanted into the nucleus intercollicularis. At present it is difficult to draw a general conclusion but the preoptic area and/or the midbrain vocal area are activated with androgens to make courtship vocalization.

In LD cycles, the pattern of crowing was quite stable as was locomotor activity. Quail crowed most frequently around the onset of light. Ottinger et al. (1982) and Ottinger (1983) showed a similar pattern in the same species, but the second peak that appeared in the present study (Fig. 3) is lacking in her results. This peak is apparent in all individuals and contributes to the 8 h cyclicity shown in Figs. 7b and 8b. At present I do not know how this component is derived.

Circulating testosterone is usually fluctuating, comprising several peaks in a day, if sample collection was made at 15 min intervals (Ottinger 1983). Crowing is not directly dependent on this fluctuation of testosterone levels; castrated and testosterone implanted birds showed the same daily rhythms as found in intact birds. One could argue

that the clearance rate of circulating testosterone fluctuates so as to produce the daily rhythm of circulating testosterone in castrated and testosterone implanted birds which is associated with changes in crowing. However, a previous study by myself in which testosterone was implanted into the third ventricle of castrated quail showed the same patterns of crowing as in intact birds indicating that circulating testosterone is not directly associated with the patterns of crowing (Wada 1984).

A rhythm of locomotor activity was affected by testosterone implantation in starlings; the steroid induced splitting of the free-running activity rhythms under constant conditions (Gwinner 1974). In the present study, splitting was not observed under constant dim light, but ten days may not be enough.

What drives the rhythms of crowing and locomotor activity? A circadian oscillatory mechanism is a highly plausible candidate. Already it is clear that the locomotor activity in quail is truly circadian and the present results confirm this and also show that crowing of castrated quail with Silastic testosterone implants free-ran under a constant condition as well as locomotor activity (Fig. 9). In this free-running state, the two rhythms had almost the same cycle duration. The values estimated were quite comparable to that estimated by Szafarczyk et al. (1978) and by Simpson and Follett (1982) in locomotor activity. Moreover, both rhythms free-ran with the same fixed phase relationship to each other as in the LD cycles. This suggests that both behaviors may be controlled by a common oscillator. The crowing rhythm had several components with different durations. Some of these, for example that of 8 h under LDim 8:16 (Fig. 8b), might be only a reflection of lights on and lights off. However, there were 2 peaks in the crowing rhythm even under LD 16:8 which were quite consistent (Fig. 4). This may indicate that the crowing rhythm is controlled by multiple, at least two, oscillators not by one oscillator. A model of multiple oscillators controlling rodent locomotor activity was discussed by Pittendrigh and Daan (1976).

It has been suggested that the circadian oscillator(s) is located in the pineal gland in avian species (review Underwood et al. 1984). Recent studies showed that the suprachiasmatic nucleus is involved in the circadian mechanism also in avian species (Ebihara and Kawamura 1981; Simpson and Follett 1981; Takahashi and Menaker 1982). The eye is also suggested to be involved in circadian rhythms (Ebihara et al. 1984) as a source of melatonin (Underwood et al. 1984). So far, we have hardly any experimental data concerning the anatomical and functional relationship between the circadian oscillators in the basal brain and the pineal gland.

There is no doubt that testosterone is essential for induction of crowing and enhancement of locomotor activity and that the formation of patterns of these behaviors is strictly regulated by the endogenous circadian oscillator. Further studies are required for better understanding the interrelationship among behavior, sex steroid receptor sites, and the biological clock in anatomical and neuroendocrine basis.

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