Internal coincidence of serotonergic and dopaminergic oscillations modulates photo sexual responses of Japanese quail, *Coturnix coturnix japonica*

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Specific temporal phase relation of neural oscillations appears to be the regulator of gonadal development in many seasonally breeding species. To find out the specific phase angle of two neural oscillations that triggers gonado-inhibitory or gonado-stimulatory response, and to test the internal coincidence model, sexually immature male Japanese quail were administered with the serotonin precursor, 5-hydroxytryptophan and the dopamine precursor, L-dihydroxyphenylalanine at hourly intervals of 6, 7, 8, 9, 10, 11 and 12 h (5 mg/100 g body weight/day for 12 days under continuous condition of light, LL). Thereafter all the groups were shifted to long photoperiod (LD16:8). During post-treatment period, in general, a significant suppression of gonadal activity was seen in the 7 h and 8 h quail and an increase in the 11 h and 12 h quail compared to the control and these effects were maintained until 105 days post treatment when the study was terminated. These findings suggest that, in addition to the effects of photoperiod, the gonadal development of Japanese quail may be also modulated by internal coincidence of serotonergic and dopaminergic oscillations (induced by the administration of their precursor drugs) and the gonadal response varies depending on the time interval between the administrations of two drugs on a circadian basis. These results also demonstrate inversion of gonadal response from 7/8 h (suppressive) to 11/12 h (stimulatory) phase relation of the two oscillations and suggest that similar to photoperiodic time measurement, Japanese quail may also detect changes in the phase angle of circadian oscillations to modulate its gonadal activity.

**Keywords:** Cloacal gland, 5-hydroxytryptophan, Internal Coincidence model, Japanese quail, L-dihydroxyphenylalanine, Temporal phase relationship, Testis

Circadian rhythms play a fundamental role in the effective functioning of complex organisms by allowing them to anticipate changing environments in ways that enhance their survival. Timing and rhythms are important during reproduction and development. In vertebrates, the existence of circadian variations in endocrine secretions, hypothalamic factors and neurotransmitters is well documented. Various models have been proposed to describe the system of circadian organization in the photoperiodic time measurement (PTM) of seasonally breeding animals, including external and internal coincidence models. In the external coincidence model, the coincidence of photosensitive/photo inducible phase (oi) of the photosensitive rhythm with the external photoperiod leads to a photo inductive response under long days, whereas the non-stimulation of gonad under short days is due to non-coincidence of light with oi. In the internal coincidence model, photoperiodic stimulation under long days occurs due to a specific phase relationship (and thereby coincidence) between the two circadian rhythms/oscillators and non-stimulation is due to a different relationship between these rhythms. This model assumes that the photoperiodic clock depends on the internal coincidence of two (or more) circadian rhythms whose relative phase relationship alters with the annual changes in day-length. The attractiveness of the Pittendrigh model is that it is based on entrainment theory and does not require a special photo inducible oscillator.

The strongest physiological evidence for an internal coincidence device comes from Meier and his colleagues in migratory sparrows and Syrian hamster and Moshkin et al. in water vole and later from numerous studies on many seasonally breeding subtropical avian and mammalian species investigated by Chaturvedi and her group. In these studies, initially six phase relations of four hour interval (0, 4, 8, 12, 16 and 20 h) were induced to study gonadal responses. This experimental approach based on numerous studies from our laboratory has demonstrated that, the administration of serotonergic and dopaminergic precursor drugs (5-hydroxytryptophan, 5-HTP and L-dihydroxyphenylalanine, L-DOPA) at 8 h

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interval suppressed and at 12 h interval stimulated gonadal activity in many seasonally breeding avian (spotted munia\textsuperscript{15,16}, lal munia\textsuperscript{17}, redheaded bunting\textsuperscript{18-20}, Indian weaver bird\textsuperscript{21}) and mammalian species (Indian palm squirrel\textsuperscript{22-24}) while other relationships were found to be ineffective. Specific phase relation of neural oscillations is also reported to modulate reproduction in continuous breeder i.e. laboratory mice\textsuperscript{25-27}. Temporal phase relation of these oscillations is reported to modulate reproduction and metabolic performance of Japanese quail, \textit{Coturnix coturnix japonica} also\textsuperscript{28-30}. It was postulated that seasonally breeding species possess daily rhythms in the secretion of hormones and neurotransmitters and when these rhythms exist in a particular phase relationship to each other, it initiates or terminates seasonal breeding and related events. This assumption is supported by the existence of different phase relationship in the circadian hypothalamic serotonin and dopamine contents of breeding versus non-breeding quail under both control and simulated conditions\textsuperscript{32,33} and in spawning and non-spawning fish\textsuperscript{34}.

On the basis of these reports, it is evidently not the amount of the serotonergic or dopaminergic drug that is injected but, it is the specific temporal phase relation between the two neural oscillations (entrained by their precursor drugs, 5-HP and L-DOPA injected at specific time intervals) which triggers different gonadal response. However, it is still unclear that at what phase angle gonadal growth starts changing from having no effect to having an effect or from an inhibitory to a stimulatory effect. Hence, the present study has been undertaken to test the internal coincidence model with respect to specific phase angle/relationship of neural oscillations which determines the change in gonadal response i.e. from no effect to inhibitory/stimulatory or vice-versa. For this i.e., to pinpoint the exact circadian time which induces change in the trend of gonadal response, the two drugs were injected at hourly intervals instead of 4 h interval tested in earlier studies.

**Materials and Methods**

Three week old sexually immature male Japanese quail purchased from Central Avian Research Institute (CARI), Izatnagar, Bareilly (UP), were housed under hygienic conditions in a well-ventilated, photoperiodically controlled room and were provided with commercial food — Chicken ration (starter upto 6 weeks and finisher grower thereafter) and tap water \textit{ad libitum}. All the experiments were conducted in accordance with institutional practices and within the framework of the revised Animals (Scientific Procedures) Act of 2002 of the Government of India.

The quail were weighed and randomly divided into 8 groups (seven experimental groups and one control) of 6 each. Quail of all the seven experimental groups were injected with 5-HP at 0800 h, followed by injections of L-DOPA at different times in the different groups, i.e. at 1400, 1500, 1600, 1700, 1800, 1900 and 2000 h, so as to establish 6, 7, 8, 9, 10, 11 and 12 h phase relations between the two injections of serotonin and dopamine precursors (5 mg/100 g body weight/day). Control received two daily injections of normal saline at 0800 and 1600 hrs. Food and water was provided \textit{ad libitum}. During the treatment period, the birds were maintained under continuous dim light (dim LL-1 lux) to avoid any possible photoperiodic interference from the light–dark cycle during the entrainment of the neural oscillations by the drug injections. After 12 days of treatment, birds of all the groups were transferred to long days (LD 16:8) in photoperiodically controlled room (lights on at 0600 h and off at 2200 h by an automatic timer).

For cloacal gland volume, its length and width was measured \textit{in situ} with dial calipers and the volume was calculated using Bissonett’s formula $\frac{4}{3}\pi ab^2$ ($a$ = half of the long axis; $b$ = half of the short axis)$^{23,35}$. These observations were recorded weekly (before, during and after the treatment). At the termination of the study (after 105 days post treatment), blood was collected from the alar vein into a heparinized tube and centrifuged at 4000 rpm for 20 min at 4 °C to separate the plasma and stored at -20 °C for testosterone assay. Thereafter, birds were anaesthetized, weighed, sacrificed by decapitation and dissected quickly. Both the testes were excised, weighed and gonado-somatic index-GSI was calculated (paired testes weight $\times$100/body weight).

For histological study, both the testes were fixed in Zamboni’s solution. Twenty-four hours after fixation, the testes were dehydrated in an ascending series of alcohol, treated with xylene and then embedded in paraffin wax. The 6 μm thick sections were cut by a Weswox rotary microtome (Western Electric and Scientific Works, Ambala Cantt, India), and stained with hematoxylin-eosin. Histological sections of the testes were viewed under a microscope (Axioskop 2 Plus; Carl Zeiss AG, Oberkochen, Germany) and images were captured with a digital camera. The diameter of the seminiferous tubules was determined in 10 sections from each testis by using the ocularmeter and micrometer.
For plasma testosterone level, enzyme immunoassay (EIA) was performed using commercial testosterone kit (DSI s.r.l., Italy) following manufacturer’s protocol. The antiserum used in the assay was 100% specific for testosterone (cross reactivity/specificity with testosterone was 100%); the cross reactivity of the assay was 0.056% with progesterone, 0.004% with cortisol, 0.005% with estradiol, 4.8% with dihydrotestosterone, 3.6% with androstenedione, 0.048% with androsterone, 0.004% with cortisone, 0.002% with estriol and 0.007% with estrone. The analytical sensitivity of the assay was 0.0576 ng/mL. The intra-assay coefficient of variation (CV) was 5.6% whereas inter-assay CV was 7.1%. Accuracy for this assay was 99%.

All the numerical data (cloacal gland volume, GSI, seminiferous tubule diameter and plasma testosterone concentration in plasma) were analyzed by one-way analysis of variance (ANOVA), followed by post-hoc Dunnett test for the comparison of group means. Significance was calculated at the level of \( P < 0.05 \).

Results

During post treatment period in general, compare to control, a trend of suppression of cloacal gland volume was observed in the 6 h quail and further regression was noted in 7 h and 8 h quail. However, a reverse trend i.e. increased development started in 9 h and 10 h quail, followed by a significantly increased degree of cloacal gland development in 11 h and 12 h quail compared to control. In general, more or less similar trend was maintained up to the termination of the study i.e. 105 days post treatment but the other experimental groups i.e., 9 h and 10 h were not different from the control (Fig. 1). Similar trend was seen with respect to GSI (Fig. 2). Plasma testosterone concentration also decreased in 6, 7, and 8 h quail but increased significantly in the 11 h and 12 h quail as compared with the control (Fig. 3). On the basis of cytometric measurement in the transverse section of the testes, similar changes were observed in the diameter of seminiferous tubules with significant suppression in 7 h and 8 h quail as compared to control (Fig. 4).

Histologically, when observed at the termination of the study, the testes of the control and all other groups, except the 6, 7 and 8 h groups, showed more or less active spermatogenesis, with spermatozoa attached to spermatids or lying in the lumen. The seminiferous tubules of the testes of 6, 7 and 8 h quail showed more or less degenerative changes. Seminiferous tubules...
of 6 and 7 h quail exhibited depletions and loosening of germ cells (other than spermatogonia) with lumen containing either exfoliated cells or debrises. Maximum atrophy was evident in 8 h quail testes with dramatically reduced size of seminiferous tubules having only inactive spermatogonial cells. Further, while testes of 9 h and 10 h were more or less similar to control, increased degree of gonadal development/spermatogenesis was evident in 11 h and 12 h quail with bunches of spermatozoa in the lumen (Fig. 5).

Discussion

Administration of 5-HTP followed by L-DOPA at hourly interval induced variable effects as a function of their phase relation, on the gonadal responses of Japanese quail maintained under LD 16:8. In the present study, as the relative position of the two oscillations changes as a function of their specific phase relation, it may stimulate or suppress gonadal growth. Thus, unlike internal coincidence model, where light entrains the oscillations; in the present model, these cycles were experimentally entrained by the drug injections given at specific time intervals. But, in both the cases, the sum of the effects i.e. gonadal induction or suppression is the outcome of coincidence/non-coincidence/specific phase angle of the two cycles.

In the present study, a trend of gonadal suppression started from a phase relation of 6 h with maximum suppression at 8 h and reverse trend i.e. maximum gonadal stimulation was evident during 11 and 12 h phase relation, while 9 and 10 h relation were ineffective. However, depending on the different sensitivity of the parameters used, in some 6 h effects were significantly different from control and in some not, although a trend of suppression was quite evident.

Fig. 2—Gonadosomatic index (GSI) of Japanese quail receiving 5HTP and L-DOPA at the interval of 6, 7, 8, 9, 10, 11 and 12 h. Values are mean ± SE. F-value: F(7, 24)=48.598. Significance of difference from control- ** P<0.01.

Fig. 3—Plasma testosterone level of Japanese quail receiving 5HTP and L-DOPA at the interval of 6, 7, 8, 9, 10, 11 and 12 h. Values are mean ± SE. F-value: F(7, 24)=83.156. Significance of difference from control- * P<0.05, ** P<0.01 and *** P<0.001.

Fig. 4—Seminiferous tubule diameter of the testes of Japanese quail receiving 5HTP and L-DOPA at the interval of 6, 7, 8, 9, 10, 11 and 12 h. Values are mean ± SE. F-value: F(7, 24)=31.242. Significance of difference from control- **P<0.01, ***P<0.001.

Fig. 5—Transverse sections of testes of Japanese quail administered with 5HTP and L-DOPA at the interval of 6, 7, 8, 9, 10, 11 and 12 h. Control (con) received two daily injections of normal saline. Note active spermatogenesis in control group testis having enlarged somniferous tubules with many layers of spermatogonia (SG), all the stages of spermatogenesis and bunches of spermatozoa (SZ) in the lumen. 6 h and 7 h quail testis exhibit depletion and loosening of germ cells and lumen contains exfoliated cells/ or debrises. The 8 h quail testis exhibits complete atrophy with reduced seminiferous tubule diameter having only 2-3 layers of inactive spermatogonial cells. The testes of 9 h and 10 h quail are more or less similar to control. 11 h and 12 h quail testes exhibit increased degree of gonadal development with enlarged seminiferous tubules containing many bunches of spermatozoa in the lumen.
Thus a variety of responses starting from regressing to completely regressed condition to no effect (i.e., similar to control) as well as increased degree of gonadal activity as compared to the control was evident under the long photoperiod, which in classical sense is always gonado-stimulatory in Japanese quail. These observations support the hypothesis that gonadal development occurs in response to specific phase angle of circadian oscillations which changes seasonally. Moreover, the effect of these specific phase relations also appears to modulate classical photo sexual response of birds.

Although dose and duration of neurotransmitter precursors administered (5 mg/100 g body weight/day over a period of 12 days) in this experimental study are same in all the groups, it induced different quantitative effects on gonadal growth, depending on the specific interval between the two precursor injections. Obviously it is not the amount of the neurotransmitters (serotonin and dopamine) that are important but the specific circadian phase relation between the two neural oscillations that modulates gonadal development as well as expression of GnIH. Different phase relation between circadian variation in serotonin and dopamine/catecholamine in the reproductively active and quiescent quail and a teleost fish provide further support to this hypothesis.

In all the other studies reported so far, specific phase relations were tested at the interval of four hours i.e. at 0, 4, 8, 12, 16 and 20 h. Since in general, 8 h induced gonadal suppression and 12 h induced stimulation, the next question was at what specific point the trend of effect changes? Hence in this study, phase relation was altered at hourly interval starting from 6 to 12 h. The specific gonadal responses based on the hourly interval of the two injections led us to conclude that, similar to photoperiodic time measurement, bird’s gonadal system can recognize even one hour change in the phase relation of circadian neural oscillations and responds accordingly. Further, in addition to the regulation of seasonal gonadal cycles in the species tested so far, the relative position of the two circadian oscillations may not only determine the rate and the trend of gonadal development in Japanese quail but may also modulate its photo sexual responses.

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