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Light wavelength dependent circadian and seasonal responses in blackheaded bunting

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Animals in the wild are exposed to daily variations in sun light, viz. duration, intensity and spectrum. Photosensitive blackheaded buntings (*Emberiza melanocephala*) were exposed to photoperiods differing in the length of light period, wavelengths and intensity. The effects of such light changes were measured on locomotor activity rhythm as well as seasonal responses like development of migratory restlessness: *Zugunruhe*, body mass and gonadal growth. The results show that the buntings are differentially responsive to light wavelengths and intensities and are indicative of a phase-dependent action of light on the circadian photoperiodic system. These birds seem to use changes in the light variables of the solar environment to regulate their circadian and seasonal responses.

Keywords: *Emberiza melanocephala,* Intensity, Locomotor activity rhythm, Photoperiodic clock, Testes, Wavelength

The temporal information from the light environment to an organism about the time-of-day and/or the time-of-year is encoded in three important variables: the duration, the quantity (irradiance/brightness) and the quality (spectrum) of light. These light characteristics change during the day as well as during the season of the year. The precision with which daily and seasonal activities are temporally organized clearly suggests the involvement of an endogenous mechanism that is fine-tuned by these inputs from the environment. In nature, animals are exposed to daily twilight transitions and to day-to-day variations in light intensity and these variables of daylight influence the physiological clock[1-3]. The photoperiodic clock, expressed as a biological rhythm, is suggested consisting of two distinct phases: the phase lying in the subjective day is the entraining phase, and the phase lying in the subjective night is the inductive phase. Thus, the first light pulse is called entraining light period, and the second light pulse an inducing light period for the endogenous circadian rhythm of photoperiodic photosensitivity (CRPP)[4-6]. Therefore, in a photoperiodic species, it is logical to assume that light intensity and wavelength, the two main characteristics of 24 h daily cycle of illumination, will have effects on the photoperiodic induction of physiological processes. However, light intensity may produce synergistic photoperiodic effects, at high light intensity a photoperiod is interpreted as being longer than at low light intensity[7,8]. Also, at the same light intensity, if the wavelength of light used is long (e.g. red light) in contrast to short (e.g. blue or green light), a stimulatory photoperiod is interpreted as being "stronger", resulting in a faster rate and greater magnitude of the gonadal response, which is clearly evident from studies on a number of birds including ducks (*Anas platyrhynchos*)[9], Japanese quail (*Coturnix c. japonica*)[10], blackheaded bunting (*Emberiza melanocephala*)[5,6,11-14]. Experiments described here clearly show that light wavelengths affect the photoperiodic induction and that the bunting is able to discriminate between long (red, 640 nm) and short (blue, 450 nm) wavelengths of light. Locomotor activity (perch hopping) of buntings was also recorded in selected experiments as a measure of the response of the circadian system as it is well established that the activity-rest cycles are the easiest and reliable marker of the circadian activity[15].

Materials and Methods

All the experiments were performed on adult male blackheaded bunting, which is a migratory finch overwintering in India, 25°N; breeding occurs at

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~40°N in west Asia and east Europe[16]. Wild caught birds from the wintering flock were acclimatized in an outdoor aviary for at least 1 week before maintaining them on short day lengths (8 h light : 16 h darkness, 8L : 16D). Thus, all birds were pretreated with short photoperiods until exposed to experimental LD conditions, which ensured their photosensitivity[17]. Food (grains of *Setaria italica* and *Oryza sativa*) and water were available *ad libitum*, and replenished during the light phase. Birds were held in light-tight boxes and the desired light intensity and wavelength were maintained by covering the CFLs with neutral density and coloured cinemoid filters (Rosco Filters: Blanchard Works, Kangley Bridge Road, Sydenham, London, England), respectively. The light intensity was measured at perch level by using Q203 Quantum Radiometer (Macam Photometrics Ltd. having accuracy of ±1 digit with a linearity error of <1%). Subsequent experiments were performed using fattening and gain in body mass, testicular growth, and development of *Zugunruhe* (migratory restlessness in caged birds) as indices of the effects on the bunting's photoperiodic clock. The observations on body mass and testes were taken at regular intervals of two or four weeks, as appropriate. The body fattening and weight gain were considered as the migratory phenotype in buntings[18]. Body mass was recorded by weighing individuals on a top-pan balance at an accuracy of 0.1 g. Testicular response was assessed by laparotomy under local anaesthesia[19]. Briefly, 2% xylocaine (Astra-IDL Ltd. Bangalore, India) was injected, sc and a small incision was made between last two ribs on the left flank and the left testis was located within the abdominal cavity with the help of a spatula. The dimensions of the left testis were recorded and testis volume was calculated from $4/3\pi ab^2$, where a and b denote half of the long (length) and the short (width) axes, respectively. Locomotor activity was recorded as general activity of a bird housed in a specially designed activity cage (size = 60 × 45 × 35 cm) that was placed singly in a photoperiodic chamber (size = 71 × 67 × 48 cm). Each activity cage was furnished with two perches and mounted with a Passive Infrared Motion Sensor 12m (400) range (C & K Systems [Intellisense XJ-413T] Conrad Electronic, Germany, Haustier PIR- Melder), which detected movement of the bird within its cage. Each sensor was connected to a separate channel, and the recording was done using a software program (Stanford Software Systems, Stanford, CA) run on an IBM-compatible computer[6]. Data were presented as mean±SE were analysed by one-way analysis of variance with repeated measures (one-way RM ANOVA) using Graph Pad Prism software. The mean values of any two groups were compared by unpaired Student's *t*-test. Significance was taken at $P < 0.05$.

Experiment I: Effects of light wavelengths on long day photostimulation—The experiment was carried out on photosensitive unstimulated (reproductively quiescent) buntings. Groups of birds (n=9-10) were exposed to a long day length (13L:11D) that was well above the threshold but was still shorter than the summer day lengths, buntings experience in nature. Long day lengths were produced at short (blue, 450 nm), mid (green, 500 nm) and long (red, 640 nm) light wavelengths, at different light intensities that were relatively weakly inductive. A group of birds exposed to white light served as control. The experiment was completed in two sets (A and B), and ran for a period of 7 weeks each time. Experiment IA compared the effects of mid light wavelength (green light) with the full spectrum (white) light, birds were exposed to white and green lights at 0.11 ± 0.01 Watt per meter square (Wm^{-2}) irradiance. Experiment IB compared the effects of short (blue light) and long (red light) light wavelengths, birds were exposed to blue and red light at 0.06 Wm^{-2} (reduced by half as compared to the experiment IA) and at 0.22 ± 0.02 Wm^{-2} (increased by two folds as compared to the experiment IA) irradiance. The experiments ran for 7 weeks and the observations were recorded at the beginning, the end and at appropriate intervals (Figs 1 and 2) for changes in body fattening (fat score) and body mass (percent change in body mass), as well as growth and development of testes (testis volume).

Experiment II: Phase-dependent effects of light wavelengths on photoperiodic induction—This study examined the phase-dependent effects of light wavelengths in a complete photoperiod (long day length, 13L:11D). In the morning, or evening, or at both times, for a few hours the photophase was given in monochromatic light of different wavelengths and the rest of the photophase composed of white light; light intensity however, was kept at the same level throughout the photophase. Six groups of birds (n=5-6) were exposed to 8L:16D and then after one week of exposure to short days they were shifted to 13L:11D (L - 37 ± 2 lux, D - 0 lux), the light phase of

the experimental regime was composed of white and monochromatic lights as under. In four groups; of the 13 h photophase, 4 h in the morning (zt 0-4; zt 0 [Zeitgeber time 0] refers to the beginning of light-on period) or in the evening (zt 9-13) was given in monochromatic light of green (500 nm) or red (640 nm) colour; the experimental light regimes were thus 4L:9L:11D or 9L:4L:11D. The remaining two groups received monochromatic light of either green or red colour, both (same wavelength) in the morning (for 4 h) and evening (for 2 h); the experimental light regimes were thus 4L:7L:2L:11D. The light intensity remained same (37 ± 2 lux) throughout the photophase. The experiment ran for 8 weeks. The observations on body fattening (fat score and percent change in body mass) and growth and development of testes (testis volume) were taken at the beginning, at the end, and at appropriate intervals during the experiments (Figs. 3 and 4).

Experiment III: Influence of light wavelengths on locomotor activity rhythm under stimulatory photoperiods—This experiment investigated whether short (blue, 450 nm) and long (red, 640 nm) light wavelengths under stimulatory photoperiods will induce differential effects on circadian activity rhythms in the blackheaded bunting. Adult male blackheaded buntings were acclimatized and maintained on short day lengths (8L:16D), before shifting to experimental facility. The experiment had two groups: in group 1, the bunting were exposed to 12L:12D and in group 2, they were exposed to 13L:11D. Each time 3 birds were exposed to blue and red light wavelengths in both the groups at 2.5 Wm^{-2} light irradiance. These birds were housed individually in activity cages to record their locomotor activity rhythm. The experiment ran for 8 weeks. Body fattening (fat score, body mass) and growth and development of testes were also recorded at periodic intervals over the duration of the experiment.

Experiment IV: Influence of light wavelengths on locomotor activity rhythm under equal energy levels—This experiment was done, to test whether at equal energy level, the migratory birds can discriminate between the two wavelengths of light: long (red, 640 nm) and short (blue, 450 nm). Here the energy level was measured in terms of PAR ($\mu Mm^{-2}s^{-1}$). Adult male blackheaded buntings (n=14), that were acclimatized and maintained on short day lengths (8L:16D), were shifted to experimental facility in the same photoperiodic schedule. After maintaining for 4 days in the same photoperiod, the photoperiod was increased to 12L:12D, with light on at 0600 hrs and light off at 1800 hrs (day time intensity: night time intensity = 13 lux or 0.33 $\mu Mm^{-2}s^{-1}$: 0.03 lux). On day 15, the lights did not turn off at 1800 hrs, thus it became free run condition. The birds remained in this schedule for 14 days after that they were divided into two groups. Group 1, (n=7 birds) was exposed to 12 h (blue): 12 h (red) light regimen and group 2, (n=7 birds) was exposed to 12 h (red): 12 h (blue) regimen (PAR = 0.33 $\mu Mm^{-2}s^{-1}$). After 12 days of exposure in the respective wavelengths, the light wavelengths were interchanged within the groups, i.e. the birds of group 1, which were exposed to 12 h (blue): 12 h (red) regimen were provided 12 h (red): 12 h (blue) and group 2 earlier exposed to 12 h (red): 12 h (blue) received 12 h (blue): 12 h (red) regimen. The experiment ran for 8.2 weeks. The observations on body mass were taken at the beginning, at the end, and at appropriate intervals during the experiments. Experimental conditions, methods of observation and statistical analyses were the same as described in the materials and methods.

Results

Experiment I: Effects of light wavelengths on long day photostimulation—In experiment 1A, at 0.11 ± 0.01 Wm^{-2} light intensity, birds exposed to full spectrum (white light) light showed bigger response than the response in birds that were exposed to monochromatic (green light; 500 nm) light, (Fig. 1). On week 2 (day 14), response to green light was slower and was significantly lower (P = 0.099, Student's t-test) in body mass than the response to white light. At the end of 4 weeks (day 28), there was a moderate increase in body fat and body mass in birds exposed to white light but not in birds exposed to green light. However, testes in both groups were found unstimulated by day 28. At the end of 7 weeks (day 49), birds exposed to white light showed significant increase in body fat and body mass, but there was only small increase in fat stores in few birds exposed to green light, (Figs 1A and B). However, by day 49, there was partial testis recrudescence in 8 of 10 birds exposed to white light and only a slight initiation of testis growth in 4 of 9 birds exposed to green light, (Fig. 1C).

In experiment 1B, birds that received short light wavelength (blue light; 450 nm) did not show photoperiodic induction both at 0.06 and 0.22 ±

0.02 Wm^{-2} light intensity (Fig. 2). Rather, there was a significant ($F_{2,8}$ = 8.912, P = 0.0025, one-way RM ANOVA, Fig. 2B) reduction in body mass at the end of the experiment in the group that received blue light at 0.06 Wm^{-2}. On the other hand, birds that received long light wavelength (red light; 640 nm) showed photoperiodic induction both at 0.06 and 0.22 ± 0.02 Wm^{-2} (Fig. 2A-F). However, the photoperiod-induced response under red light was slower at 0.06 Wm^{-2} as compared to that at 0.22 ± 0.02 Wm^{-2} (Figs 2A-C and 2D-F), but there was a significant difference in body fat and body mass between birds released in blue light and red light on day 28 (body mass: P = 0.0085; body fat: P = 0.036, Student's t-test) and day 49 (body mass: P = 0.0001; body fat: P = 0.0001, Student's t-test). The testes were not stimulated on day 28 in birds that received red light at 0.06 Wm^{-2}, and were partially stimulated in 4 of 10 birds on day 49. At 0.22 ± 0.02 Wm^{-2} red light, however, testes were stimulated in all birds on day 28 and they remained stimulated until the end of the experiment (day 49) (Fig. 2F). There was significant difference in the photoperiod-induced responses (body mass, body fat and testes volume) on days 28 and 49 in the birds exposed to red light as compared to blue light at 0.22 ± 0.02 Wm^{-2} (Figs 2A-C and 2D-F).

Experiment II: Phase-dependent effects of light wavelengths on photoperiodic induction in complete photoperiods—Birds in the group that received 4 h monochromatic light of green colour in the morning, did not show significant increase in body fat, body mass gain and testis volume as compared to day 0, however the group which received 4 h monochromatic light of green colour in the evening, the birds fattened and gained body mass by day 28

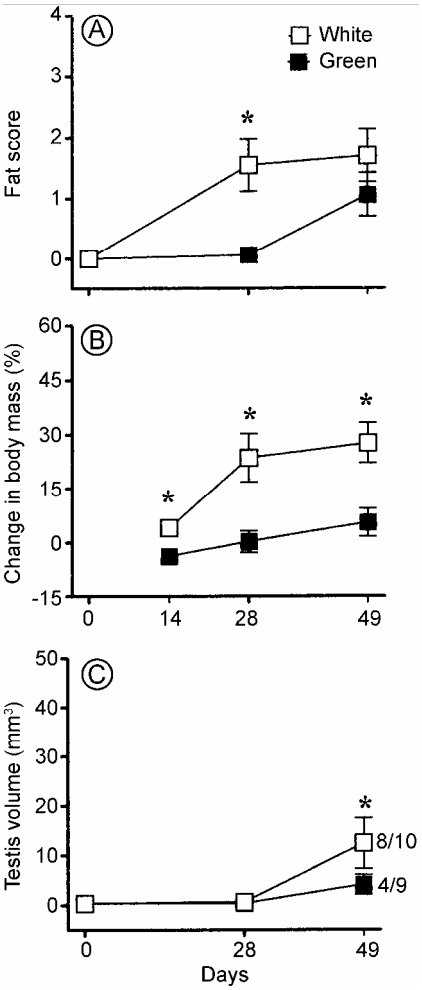

Fig. 1—Effects of light wavelengths on photoperiodic induction. Birds were subjected to a long photoperiod, 13L:11D in green and white light at 0.11 ± 0.01 Wm^{-2} for a period of 49 days. Each point symbol represents the mean ± SE response for 9-10 birds. An asterisk on the point symbol indicates significance of difference ($P < 0.05$) between groups on the same day.

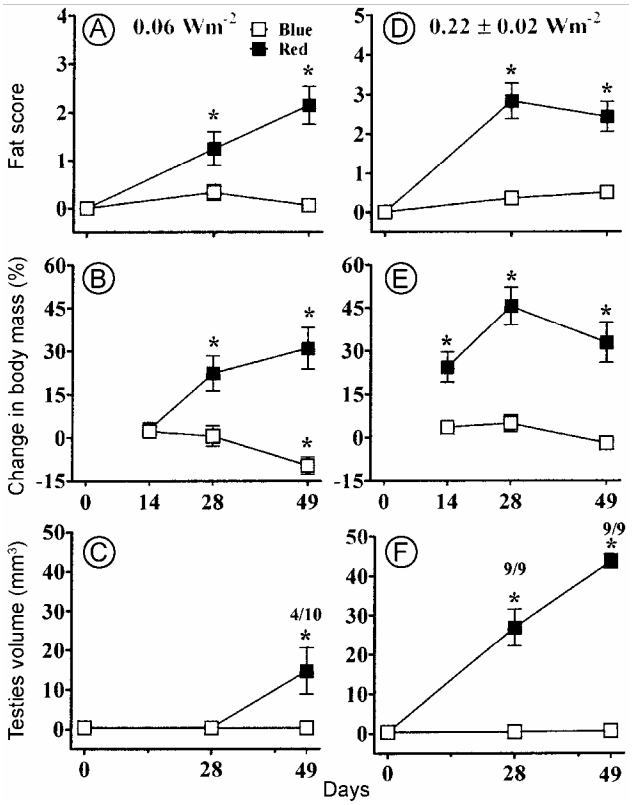

Fig. 2—Effects of short (blue; 450 nm) and long (red; 640 nm) light wavelengths on photoperiodic induction. Birds were subjected to a long photoperiod, 13L:11D at 0.06 Wm^{-2} (left hand panel) and 0.22 ± 0.02 Wm^{-2} (right hand panel) for a period of 49 days. Each point symbol represents the mean ± SE response for 9-10 birds. An asterisk on the point symbol indicates significance of difference ($P < 0.05$) between groups on the same day.

(Figs 3A and B, $F_{3,5}$ = 14.49, P = 0.0001, one-way RM ANOVA) and this remained almost at the same level until the end of the experiment, day 56. Testes also recrudesced by day 28 in both the groups (Fig. 3C). There was no significant difference between the morning and evening monochromatic light groups, but the evening light group showed slightly bigger response. The response of two groups that received red light in the morning or evening was somewhat greater than response to green light, and also that there was a decline in body fat and body mass after day 28 (Figs 3D and E), however the testes continued to grow. Similar to green light, exposure of red light given in the evening induced bigger response, and hence a significant difference in testis size was found between two groups on day 56 (P = 0.0215, Student's t-test) (Fig. 3F).

The other two groups that received monochromatic lights both in the morning and the evening for 4 h and 2 h, respectively, (4L:7L:2L:11D) did not show difference in body fattening and gain in body mass between green and red light (Figs 4A and B). However, there was significant difference in testis recrudescence on day 28 as well as on day 56, in the group that received red light than the group which received green light, (day 28; P = 0.0003 and day 56; P = 0.0032, Student's t-test); testes in green light group were slightly initiated by the end of the experiment, day 56 (Fig. 4C).

Experiment III: Influence of light wavelengths on locomotor activity rhythm under stimulatory photoperiods—Responses of circadian activity rhythm: The actograms (Figs 5 and 6) show circadian

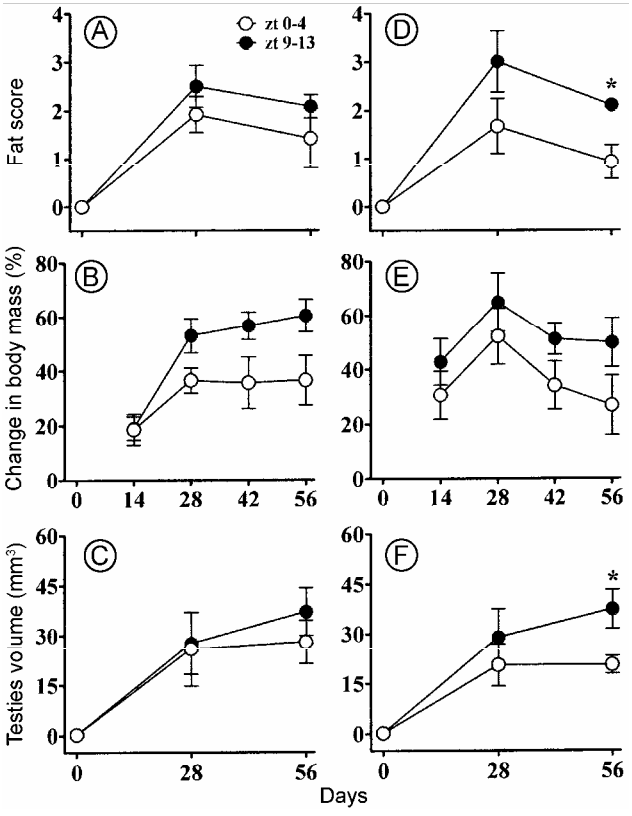

Fig. 3—Effects of long photoperiod (13L:11D) of which a portion of light phase in the morning (zt 0-4) or evening (zt 9-13) was substituted by lights of different colours: green; 500 nm (A, B, C) and red; 640 nm (D, E, F) at 37± 2 lux. Each point symbol represents mean ± SE response for 5 birds. An asterisk on the point symbol indicates significance of difference at ($P < 0.05$) between the two groups.

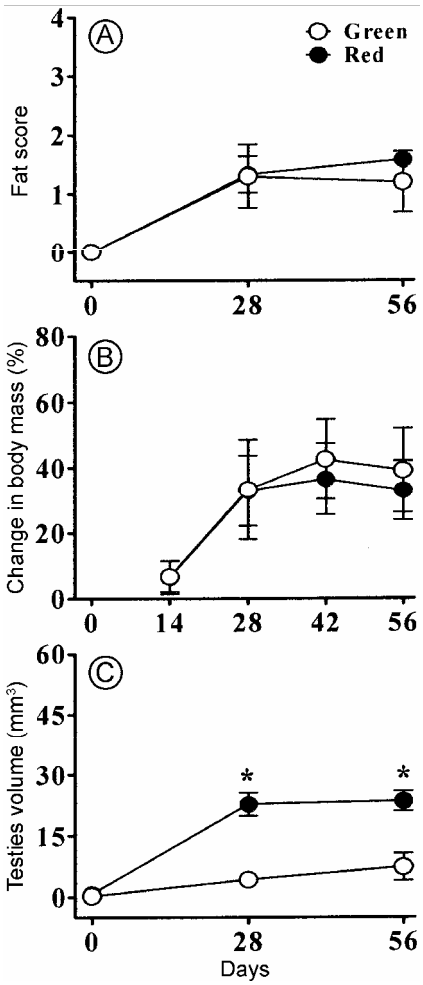

Fig. 4—Effects of long photoperiod (13L:11D) of which a portion of light phase in the morning (zt 0-4) and evening (zt 11-13) was substituted by lights of different colours: green (500 nm) or red (640 nm) at 37± 2 lux. Each point symbol represents mean ± SE response for 5-6 birds. An asterisk on the point symbol indicates significance of difference at ($P < 0.05$) between two groups.

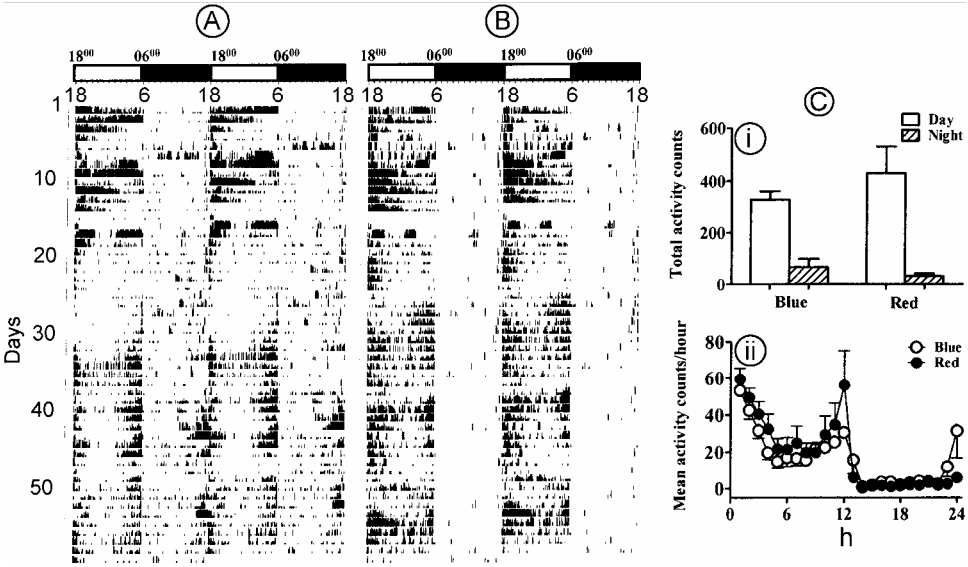

Fig. 5—Representative actograms (left panel) and rhythm profile (right panel) under 12L:12D in short (blue; 450 nm, A) and long (red; 640 nm, B) light wavelengths at equal irradiance (2.5 Wm^{-2}). The graphs in the right (C) show (i) individual total activity counts, (ii) 24 h activity rhythm profile, in two light wavelengths.

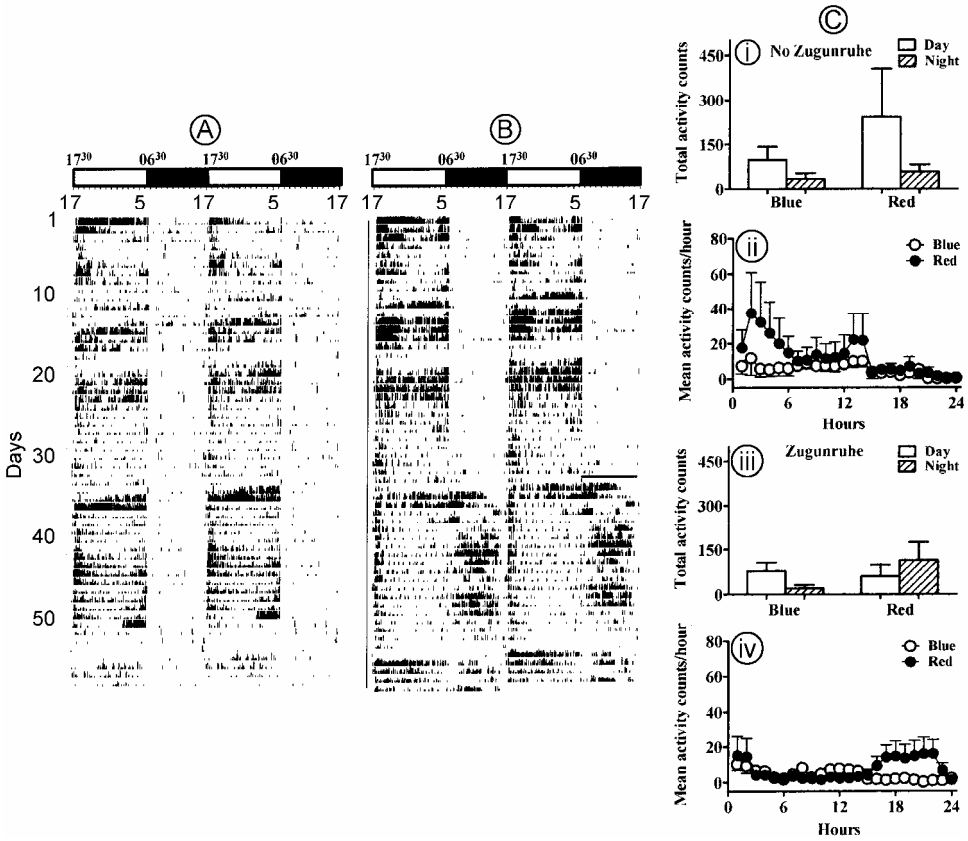

Fig. 6—Representative actograms and rhythm profile under 13L:11D in short (blue; 450 nm, A) and long (red; 640 nm, B) light wavelengths at equal irradiance (2.5 Wm^{-2}). The graphs in the right (C) show (i) individual total activity counts, (ii) 24 h mean activity rhythm profile, when Zugunruhe was absent, (iii) individual total activity counts, (iv) 24 h activity rhythm profile, when Zugunruhe appeared (red light).

distribution of locomotor activity rhythm under various LD cycles. As a result of light-induced positive masking effects, bouts of activity were confined to the light periods, irrespective of the light wavelength applied (Figs 5A, B and 6A, B). However, there was difference in the magnitude of locomotor activity between the light conditions. Under 12L:12D, the total activity counts (Fig. $5C_i$) and 24 h activity rhythm profile (Fig. $5C_{ii}$) showed higher amplitude in red light as compared to blue light (though not significant). In case of blue light one out of three birds showed bouts of activity at night also (Figs 5A and 5C), however Zugunruhe did not appear in any of the light conditions. Under 13L:11D, the overall activity was less as compared to 12L:12D, but a striking difference was seen in the development of Zugunruhe (intense activity at night). The birds developed intense Zugunruhe within 5 weeks of exposure to 13L: 11D in red light, but not in blue light (cf. 6B, A and; $6C_{iii}$, C_{iv}). Thus, to sum up, the amount of locomotor activity was more in red light (Figs 5 B, C and 6 B, C) than in blue light (Figs 5 A, C and 6 A, C) in both the photoperiodic situations. Taken together, the results suggest that light wavelengths given at equal irradiance have only small effects on circadian activity rhythms.

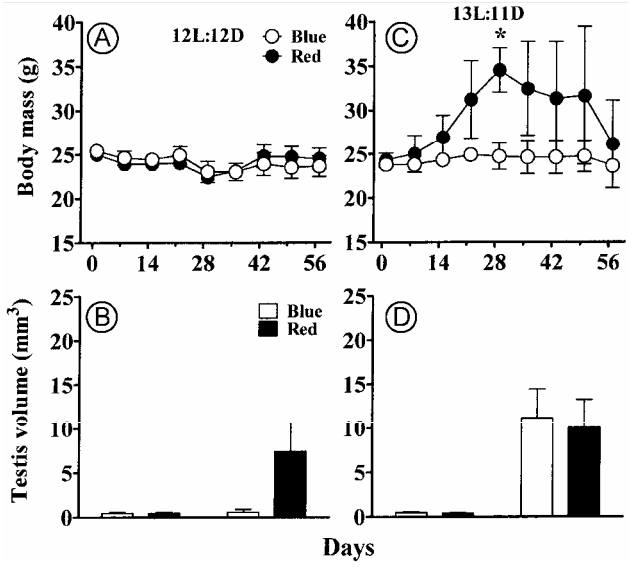

Fig. 7—Effect of wavelengths under marginally inductive (12L:12D) and highly inductive (13L:11D) photoperiods. Birds (n=3 per group) were exposed to short (blue, 450 nm) and long (red, 640 nm) light wavelengths at equal irradiance (2.5 Wm^{-2}). Each point symbol represents the mean ± SE. An asterisk on the point symbol indicates significance of difference ($P < 0.05$, unpaired t-test) in mean values at the respective days between two light treatments.

Photoperiodic induction of body mass and testis growth— Figure 7 shows the results of group 1; 12L:12D (left panel) and group 2; 13L:11D (right panel) of the experiments. In general, the response to short (blue) light wavelength was slower than to long (red) light wavelength. Under 12L:12D, by the end of 8 weeks, testes were photostimulated to about one-sixth of maximum size in red light, but were not photostimulated under blue light (Fig. 7B). Birds did not fatten under 12L:12D (Fig. 7A). On the other hand, under 13L:11D, testes grew to similar size in both the treatments, blue and red lights, although testis growth was submaximal; testes grew to about one-third of the maximum size (Fig. 7D). Birds showed significant body fattening under red light but not under blue light (day 28; Fig. 7C, $P = 0.0456$, Student's *t*-test). However, the fattening of birds to red light under 13L:11D was more variable (Fig. 7C).

Experiment IV: Influence of light wavelengths on locomotor activity rhythm under equal energy levels—The activity rest pattern in different light wavelengths at equal energy levels demonstrated that birds can differentiate the two different colours of light. The circadian locomotor activity of birds showed that both light wavelengths can entrain the clock of arrhythmic birds (Fig. 8). Moreover the amount of activity of birds was more in blue light than in red light (Fig. 8C) in both the groups, and even after inverting to regular light cycles, birds shifted their activity towards blue light (Fig. 8D). In both the groups, there was a significant difference in the activity counts of 12L and 12D periods (Figs 8A, E and F) but when the birds were released in LL, their activity was scattered throughout (Fig. 8B). In group 1: 12 h of blue light:12 h of red light (12B:12R), the locomotor activity counts in 12 h of blue light were significantly more than in 12 h of red light ($P = 0.0062$, Student's *t*-test, Figs 8C, E and F). After flipping the light schedules, 12B:12R to 12R:12B, the activity counts also shifted towards the blue light and were significantly different than in red light ($P = 0.0096$; Student's *t*-test, Fig. 8D-F). In group 2 12R:12B, there was no significant difference in activity counts of red light and blue light before as well as after reverting (12B:12R), though there were always more activity counts wherever blue light was applied (Figs 8D-F).

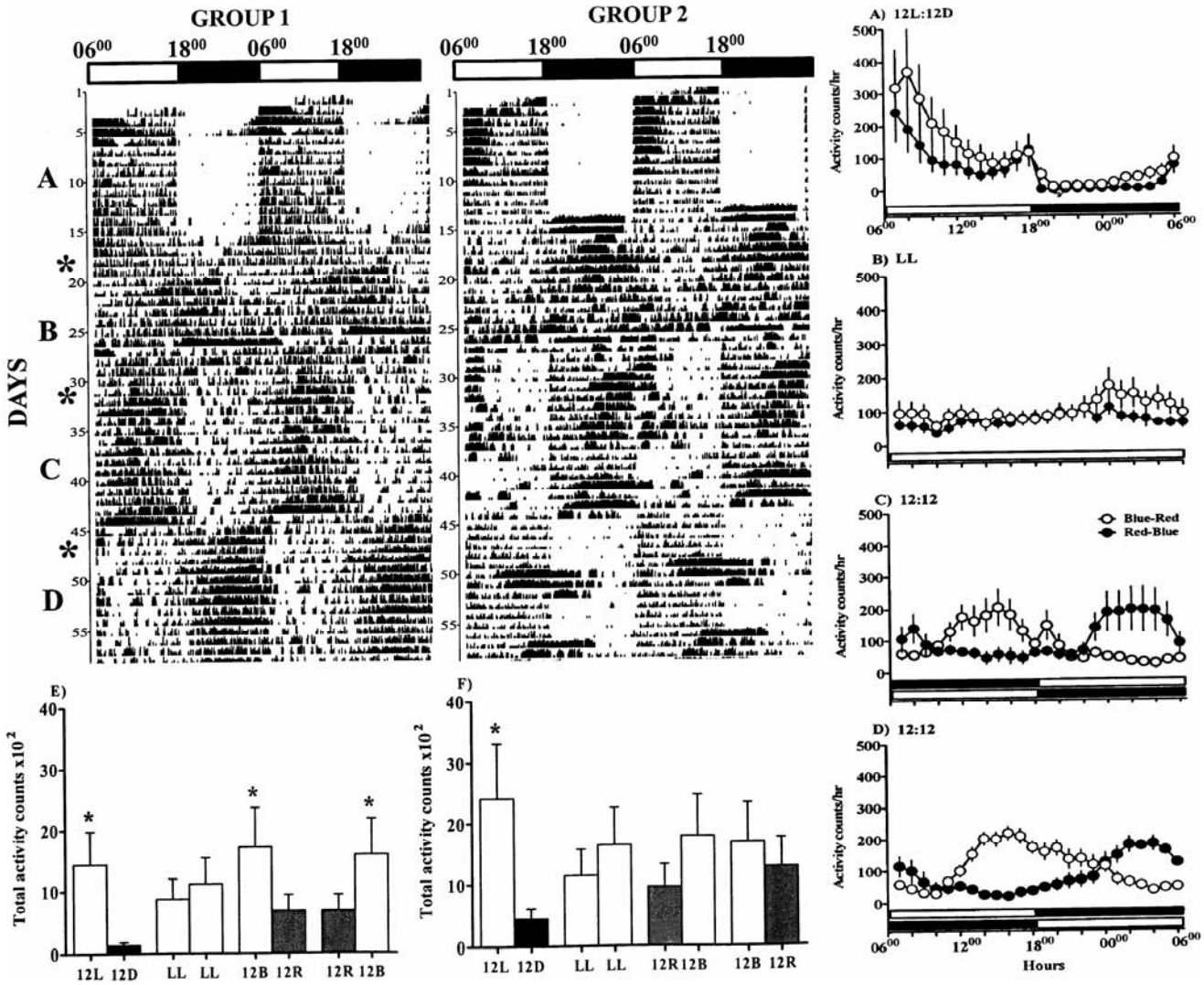

Fig. 8—Representative actograms (left panel) and 24 h activity rhythm profile (right panel) at equal energy level (0.33 $\mu Mm^{-2}s^{-1}$) under (A) 12L:12D condition (B) LL_{bright} (continuous white light) (C) Group 1: 12 h Blue:12 h Red; Group 2: 12 h Red:12 h Blue (D) Group 1: 12 h Red:12 h Blue; Group 2: 12 h Blue:12 h Red. Figures (E, F) represent the total mean activity counts in different light schedules. The asterisks (*) in the actogram show change in the light schedules and on the point symbol indicates significance of difference ($P < 0.05$, unpaired *t*-test) in total mean values.

Discussion

The results from the first experiment (Experiment I, Figs 1 and 2), which examined the influence of light wavelengths in the long day photostimulation, suggest that the wavelength of light is an important factor in determining the photoperiodic response in the blackheaded bunting. Birds fattened and gained body mass, and testes recrudesced when long light wavelength (red light) was given, but not when short light wavelength (blue light) was applied (Fig. 2). At intermediate light wavelength (green light) also, there was only slight initiation of testicular response in 4 of 9 birds (Fig. 1C). Clearly, at a given level of intensity applied from outside the skull, red light is more effective than white light and white light is more effective than blue or green light. In other words, the short wavelengths are less effective than the long wavelengths in stimulation of the photoneuroendocrine system in the blackheaded bunting. A similar response has been reported by Kumar and Rani[11] for blackheaded bunting subjected to 13L:11D at 100 lux light intensity. A recent study by Reddy *et al.*[20] states that in tropical conditions, the declining egg production in layer hens (*Gallus g. domesticus*) could be reversed by exposure to long wavelength (red spectrum) of light. Hence, because of the rapid

transmission and deeper penetration of red light to brain tissues, thereby access to extra-retinal photoreceptor-mediated hypothalamic GnRH-I mRNA expression, activation of the HPG axis is faster in red light than at other light wavelengths. A direct innervation of GnRH neurons by deep brain photoreceptors (DBPs) in ring doves (*Streptopelia roseogrisea*) have been shown by Saldanha *et al*.[21], which indicates that DBPs are necessary and sufficient for the detection of changes in day-length regulating avian reproduction, thus DBPs are not linked to the reproductive axis via the circadian system rather they themselves act as the photoperiodic clocks.

In general, the results of experiment II support the results of the experiment I. The monochromatic lights given in the evening produced greater response than in the morning and the photoperiodic induction was faster and greater in the group that received red light than that received green light. This clearly suggests that the photoperiodic oscillator in the blackheaded bunting is differentially responsive to a light wavelength at the same intensity during its different circadian phases. In this experiment, the light illumination was measured in lux. It is likely, therefore, that the two light illuminations at 37 lux intensity provided different number of photons. However, the difference in the number of photons available by a light source does not appear to be solely responsible for photoperiodic induction in the blackheaded bunting. If that were the case, both LD cycles in the experiment II in which the total amount of photons available per cycle was identical (the two LD cycles differed only in the timing of the monochromatic light; either morning or evening) should have produced similar photoperiodic response, since they did not (cf. Fig. 3), therefore, this clearly suggests that the circadian oscillator regulating photoperiodic responses in the blackheaded bunting is differentially responsive to a light wavelength at the same level of intensity during its different circadian phases.

The results from experiment III are in conformity with the earlier studies by Kumar and Rani[11], Rani and Kumar[22], that there is a clear effect of light wavelengths on photoperiodic induction under stimulatory photoperiods. This experiment was done not only to analyze the effect of light wavelengths on photoperiodic induction, but also to examine the responses of circadian system (by measuring one of the hands of the endogenous clock, the locomotor activity) to stimulatory photoperiods by using long (red, 640 nm) and short (blue, 450 nm) wavelengths of light. The actograms of representative animals from the group (Figs 5A, B and 6A, B) show that, as expected, circadian activity rhythms were entrained to stimulatory photoperiods, although there is a difference in the pattern of activity between the treatments. For example, bimodal pattern of activity suggesting two oscillator components— the morning (M) and evening (E) oscillators[23], within the circadian system of the blackheaded bunting, was more conspicuous in 12L:12D than in 13L:11D, and within 12L:12D, the birds exposed to blue light showed clearer bouts of morning and evening activity (cf. Figs 5A and 6A). This is similar to other species showing two peaks in activity, linked with dawn and dusk[24,25]. Further, a differential effect on the pattern of daytime activity and also on the development of nighttime activity in individuals of 13L:11D exposed to red light wavelength, shows that this photoperiod exerted differential effects on the bunting's photoperiodic clock. In this experiment (Figs 6B, Actogram and $6C_{iii}$), buntings showed migratory activity during the experimental test conditions, but it is still not known whether there are separate circadian oscillators controlling day-time and night-time activities in the bunting as has been evident in the study on migratory garden warblers (*Sylvia borin*)[26]. If so is the case, then one possibility can be that the two separate oscillators receive different photic inputs from the various photoreceptors, or maybe they are differentially sensitive to light of different spectral compositions. It might also be possible that the observed differences in the ability to entrain corresponded to seasonal (or circannual) changes in the sensitivity of the circadian pacemaking system to the spectral composition of light.

The results obtained from experiment IV show that artificial light cycles of different monochromatic light (short, blue and long, red) even at equal energy level can cause differential effects on circadian activity rhythms of buntings (Fig. 8, Actograms). From the above experiments it is clear that the bird's circadian system responds to changes in the spectral composition of light, hence it is expected that light cycles alternating between the red and blue range of the visual spectrum would be equally effective. This is supported by the experiments on bramblings (*Fringilla montifringilla*) and common redpolls (*Carduelis f. flammea*)[27] where 12B:12R (12 h blue:

12 h red) cycles were provided with different colour temperatures and similar energies. The activity of bramblings occurred mainly during the "blue" phase (ca 5,000 K), while under 12B:12R cycles with filtered blue and red light, the bramblings were mostly active during the red phase (at ca. 650 nm). In common redpolls that were exposed to 16.5B:7.5R cycles with similar qualitative light parameters and a B:R photons flux ratio of 0.67:1, the rhythms entrained with activity mainly within the blue phase (at 440 nm). Thus the results conclude that even at same energy level the circadian system of bunting is differentially sensitive to long and short wavelengths of light at different times of the day.

Thus results indicate that the photoperiodic response system in bunting (i) is capable of discriminating between different wavelengths of light, (ii) is differentially responsive to a light wavelength during its different circadian phases, and (iii) requires a minimum light intensity threshold for photostimulation. This might be taken to suggest that different photoreceptors are involved in photoperiodic regulation of circadian system in birds. This is not surprising in view of the results from other species. In *Gonyaulax*, Roenneberg and Hastings[28,29] have shown that different photoreceptors (photopigments) and different pathways to the pacemaker are the basis for the entrainment of circadian rhythms by qualitative changes in illumination. During the daytime, the neurons are sensitive for both inhibitory and excitatory components of the environmental light but the neuronal activity is dependent on the variation in the spectral composition. Differential sensitivity of the photoreceptors is suggested to be responsible for the photic entrainment of the circadian rhythms in birds and mammals[30-33]. Spectral sensitivity of the photoreceptors mediating photic entrainment has also been suggested in at least three bird species, the domestic canary (*Serinus canaria domestica*), the common redpolls and brambling[27,34]. In contrast to mammals, where possibly only the photoreceptors in the retinae that transduce photic information to the circadian pacemaker(s), birds possess extraretinal photoreceptors in the pineal and in the hypothalamus that may be differentially involved in the entrainment of the circadian system, as well as in the measurement of day length[35-41]. Rhodopsin-like photopigments were found to be responsible for the high spectral sensitivity near 500 nm of photoreceptor elements in the pineal and in the hypothalamus, although long wavelengths (>600 nm) reach to these receptors more easily than shorter wavelengths[36,37,42]. The mRNA expression of melanopsin (Opn 4) in the chick septal region[43], and the VA-opsin like immunoreactivity in the quail and chicken anterior hypothalamus[44] have been reported, thus melanopsin and VA-opsin have been suggested to be the candidate deep brain photoreceptors. But in 2010, Nakane *et al.*[40] have reported neuropsin (Opsin 5) as a deep brain photoreceptive molecule in quail brain, it is violet sensitive photopigment, showed peak sensitivity (λ_{max}) at ~420 nm and was localized in the paraventricular organ (PVO) with its fibres projecting in the external zone of the median eminence (ME). Primarily different neural pathways to, and/or different sensitivities of, the receptor elements within the pacemaking structures of the avian brain may accomplish the differential effects of light of different wavelengths upon the circadian system.

In conclusion, there are differential effects of the wavelength and intensity of light on the circadian processes mediating photoperiodic regulation of daily and seasonal responses in the migratory buntings. That the circadian and photoperiodic systems in birds are responsive to light wavelength in a phase-dependent manner may have adaptive implications. In a seasonal breeder like bunting, daily entrainment of clock occurs in morning and the photoperiodic induction occurs in the evening only during long day lengths of spring and summer when light is available later in the day (after ~12 h) to interact with the Φi. Although the light environment is always dominated by red light, there are very precise spectral changes during the day, especially during twilight periods[45]. It may be advantageous for a species, especially a long day breeder, to be equipped with a circadian clock, which perhaps is responsive to the spectral changes of the light environment.

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References

1 Bünning E, *The physiological clock*, (Springer-Verlag, New York, Heidelberg, Berlin) 1973.

2 Aschoff J, A survey on biological rhythms, in *Handbook of behavioural neurobiology*, edited by J Aschoff, (Plenum Publishing Corporation, New York) 1981, 3.

3 Rani S, Singh S & Kumar V, Light sensitivity of the biological clock, in *Biological rhythms,* edited by V Kumar, (Narosa Publishing House, N. Delhi/ Springer-Verlag) 2002, 232.

4 Kumar V & Follett B K, The nature of the photoperiodic clock in vertebrates, *Proc Zool Soc (Calcutta), Haldane Comm* (1993) 217.

5 Malik S, Rani S & Kumar V, The influence of light wavelength on phase dependent responsiveness of the photoperiodic clock in migratory blackheaded bunting, *Biol Rhythm Res*, 33 (2002) 65.

6 Malik S, Rani S & Kumar V, Wavelength dependency of light induced effects on photoperiodic clock in the migratory blackheaded bunting (*Emberiza melanocephala*), *Chronobiol Int,* 21 (2004) 367.

7 Bissonnette T H, Sexual periodicity, *Quart Rev. Biol,* 11 (1931) 371.

8 Bentley G E, Goldsmith A R, Dawson A, Briggs C & Pemberton M, Decreased light intensity alters the perception of day length by male European starlings (*Sturnus vulgaris*), *J Biol Rhythms,* 13 (1998) 148.

9 Benoit J, The role of the eye and of the hypothalamus in the photostimulation of gonads in the duck, *Ann New York Acad Sci,* 117 (1964) 204.

10 Oishi T & Lauber J K, Photoreception in the photosexual response of quail I. Site of photoreceptor, *Amer J Physiol,* 225 (1973) 155.

11 Kumar V & Rani S, Effects of wavelength and intensity of light in initiation of body fattening and gonadal growth in a migratory bunting under complete and skeleton photoperiods, *Physiol Behav,* 60 (1996) 625.

12 Kumar V, Rani S & Malik S, Wavelength of light mimics the effects of the duration and intensity of a long photoperiod in stimulation of gonadal responses in the male blackheaded bunting (*Emberiza melanocephala*), *Curr Sci,* 79 (2000) 508.

13 Rani S & Kumar V, Phasic response of the photoperiodic clock to wavelength and intensity of light in the redheaded bunting, *Emberiza bruniceps, Physiol Behav,* 69 (2000) 277.

14 Rani S, Singh S, Misra M, Malik S, Singh B P & Kumar V, Daily light regulates seasonal responses in the migratory male redheaded bunting *Emberiza bruniceps*, *J Exp Zool,* 303A (2005) 541.

15 Daan S & Aschoff J, Circadian rhythms of locomotor activity in captive birds and mammals: Their variations with seasons and latitude, *Oecologia*, 18 (1975) 269.

16 Ali S & Ripley S D, *Handbook of the birds of India and Pakistan*, (Oxford Univ. Press, Bombay, London, New York) 1974.

17 Kumar V, Jain N, Singh B P & Kumar B S, Plasma levels of luteinizing hormone in intact and castrated blackheaded bunting (*Emberiza melanocephala*) exposed to stimulatory and nonstimulatory photoperiods, *Reprod Nutr Dev,* 33 (1993) 143.

18 King J R & Farner D S, Premigratory changes in body weight and fat in wild and captive male white-crowned sparrows, *Condor,* 61 (1959) 315.

19 Kumar V, Singh S, Misra M & Malik S, Effects of duration and time of food availability on photoperiodic responses in the migratory male blackheaded bunting (*Emberiza melanocephala*), *J Exp Biol,* 204 (2001) 2843.

20 Reddy I J, David C G, Selvaraju S, Mondal S & Kiran G R, GnRH-1 mRNA, LH surges, steroid hormones, egg production, and intersequence pause days alter in birds exposed to longer wavelength of light in the later stages of production in *Gallus gallus domesticus, Trop Anim Health Prod*, 44 (2012) 1311.

21 Saldanha C J, Silverman A J & Silver R, Direct innervation of GnRH neurons by encephalic photoreceptors in birds, *J Biol Rhythms,* 16 (2001) 39.

22 Rani S & Kumar V, Time course of sensitivity of the photoinducible phase to light in the redheaded bunting, *Emberiza bruniceps, Biol Rhythm Res,* 30 (1999) 555.

23 Daan S, Albrecht U, van der Horst G T J, Illnerova H, Roenneberg T, Wehr T A & Schwartz W J, Assembling a clock for all seasons: Are there M and E oscillators in the genes? *J Biol Rhythms,* 16 (2001) 105.

24 Aschoff J, Spontane lokomotorische aktivität, in *Handbuch der zoologie*, edited by J G Helmcke, H V Lengerken & D Starck, (Gruyter De, Berlin) 1962, 1.

25 Aschoff J, Circadian activity pattern with two peaks, *Ecology,* 47 (1966) 657.

26 Bartell P A & Gwinner E, A separate circadian oscillator controls nocturnal migratory restlessness in the songbird *Sylvia borin*, *J Biol Rhythms*, 20 (2005) 538.

27 Pohl H, Spectral composition of light as a zeitgeber for birds living in the high arctic summer, *Physiol Behav,* 67 (1999) 327.

28 Roenneberg T & Hastings J W, Two photoreceptors influence the circadian clock of a unicellular alga, *Naturwissenschaften*, 75 (1988) 206.

29 Roenneberg T & Hastings J W, Are the effects of light on the phase and period of the *Gonyaulax* clock mediated by different pathways? *Photochem Photobiol,* 53 (1991) 525.

30 Gordon S A & G A Brown, Observations on spectral sensitivities for phasing of circadian temperature rhythms in *Perognathus penicillus*, in *Biochronometry*, edited by M Menaker, (National Academy of Sciences, Washington DC) 1971, 363.

31 McGuire R A, Rand W M & Wurtman R J, Entrainment of the body temperature rhythm in rats: Effect of color and intensity of environmental light, *Science*, 181 (1973) 956.

32 Joshi D & Chandrashekaran M K, Spectral sensitivity of the photoreceptors responsible for phase shifting the circadian rhythm of activity in the bat, *Hipposideros speioris*, *J Comp Physiol A,* 156 (1985) 189.

33 Holzhausen K & Roenneberg T, Red and blue light have qualitatively different effects on the free-running period of *Serinus canaria*, (Abst.) *VII Ann Meeting European Society of Chronobiology, Marburg, Germany* (1991).

34 Pohl H, Wavelength-specific light sensitivity of a bird's circadian system, (Abst.) *V Meeting Society for Research on Biological Rhythms, Jacksonville, Florida* (1996).

35 Deguchi T, Rhodopsin-like photosensitivity of isolated chicken pineal gland, *Nature,* 290 (1981) 706.

36 Foster R G & Follett B K, The involvement of a rhodopsin-like photopigment in the photoperiodic response of the Japanese quail, *J Comp Physiol A,* 157 (1985) 519.

37 Foster R G, Follett B K & Lythgoe J N, Rhodopsin-like sensitivity of extra-retinal photoreceptors mediating the photoperiodic response in quail, *Nature,* 313 (1985) 50.

38 Foster R G, Schalken J J, Timmers A M & DeGrip W J, A comparison of some photoreceptor characteristics in the pineal and retina. I. The Japanese quail (*Coturnix coturnix*), *J Comp Physiol A,* 165 (1989) 553.

39 Menaker M, Extraretinal light perception in the sparrow. I. Entrainment of the biological clock, *Proc Natl Acad Sci, USA,* 59 (1968) 414.

40 Nakane Y, Ikegami K, Ono H, Yamamoto N, Yoshida S, Hirunagi K, Ebihara S, Kubo Y & Yoshimura T, A mammalian neural tissue opsin (Opsin 5) is a deep brain photoreceptor in birds, *Proc Natl Acad Sci, USA,* 107 (2010) 15264.

41 Oliver J & Bayle J D, Brain photoreceptors for the photo-induced testicular responses in birds, *Experientia*, 38 (1982) 1021.

42 Hartwig H G & van Veen T, Spectral characteristics of visible radiation penetrating into the brain and stimulating extraretinal photoreceptors, *J Comp Physiol A,* 130 (1979) 277.

43 Chaurasia S S, Rollag M D, Jiang G, Hayes W P, Haque R, Natesan A, Zatz M, Tosini G, Liu C, Korf H W, Iuvone P M & Provencio I, Molecular cloning, localization and circadian expression of chicken melanopsin (Opn4): Differential regulation of expression in pineal and retinal cell types, *J Neurochem,* 92 (2005) 158.

44 Halford S, Pires S S, Turton M, Zheng L, Gonzalez-Menendez I, Davies W L, Peirson S N, Garcia-Fernandez J M, Hankins M W & Foster R G, VA opsin-based photoreceptors in the hypothalamus of birds, *Curr Biol,* 19 (2009) 1.

45 Roenneberg T & Foster R G, Twilight times: Light and the circadian system, *Photochem Photobiol*, 66 (1997) 549.