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# Timed food availability affects circadian behavior but not the neuropeptide Y expression in Indian weaverbirds exposed to atypical light environment

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## HIGHLIGHTS

- First study on food effects on circadian disruption in aberrant light condition
- Daily timed food presentation synchronized the circadian activity behavior.
- Fos expression was affected by aberrant light condition as well as time-of-day.
- NPY expression was affected by food availability but not the light condition.
- Probable separate mechanisms for food-induced homeostatic and circadian effects

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## ABSTRACT

We tested the hypothesis whether daily food availability period would restore rhythmicity in individuals with disrupted circadian behavior with no effect on appetite regulation. Particularly, we investigated the effects of timed food availability on activity behavior, and Fos and neuropeptide Y expressions in Indian weaverbirds (*Ploceus philippinus*) under atypical light conditions. Initially, weaverbirds in 3 groups of 7–8 each were entrained to 7L:17D (25: <0.3 lx) with food ad libitum. Thereafter, food availability was restricted for 7 h such that it overlapped with the light period. After a week, 7L:17D was replaced with 3.5L: 3.5D (T7, group 1), 3.5L: 20.5D (T24, group 2) or constant dim light, $LL_{dim}$ (<0.3 lx, group 3) for 5 weeks. Food cycles synchronized the circadian activity behavior, albeit with group differences, but did not affect body mass, blood glucose levels or testis size. Further, Fos, not NPY mRNA or peptide, expression measured at ZT2 and ZT14 (ZT0 = time of food given) showed significant group differences in the hippocampus, dorsomedial hypothalamus and infundibular nuclear complex. Another identical experiment examined after-effects of the 3 light conditions on persistence of the circadian rhythms. Weaverbirds exposed for 4 weeks to identical food but different light conditions, as above, were released into the free-running condition of food ad libitum and $LL_{dim}$. Circadian rhythms were decayed in birds previously exposed to T7 LD cycle. Overall, these results show that timed meal restores rhythmicity in individuals with circadian rhythm disruptions without involving neuropeptide Y, the key appetite regulatory molecule.



## 1. Introduction

Rhythmic biological activities are phased to occur at an optimal time of the day, as synchronized by predictable changes in the abiotic (e.g. photoperiod) and biotic (e.g. food) cues of the surrounding environment. An overriding importance of the photoperiod as key zeitgeber (*zeit*-time; *geber*-giver) for circadian rhythm regulated functions has been documented in many vertebrates [1]. Daily and seasonal photoperiod changes are intimately tied with the food availability period and eating time preferences in animals including birds. Thus, the role of food availability appears to be far more important for daily and seasonal timekeeping than has been originally envisaged. Indeed, cycles of the presence and absence of food (P:A food cycles) have been shown to act as the zeitgeber for circadian activity rhythms in both the nocturnal rodents and diurnal birds [2–6]. Particularly among birds, the role of P:A food cycle in synchronization of circadian rhythms has been demonstrated in house sparrows, *Passer domesticus* [3], pigeons, *Columba livia* [7], Indian weaverbirds, *Ploceus philippinus* [5], redheaded buntings, *Emberiza bruniceps* [6] and blackheaded buntings, *Emberiza melanocephala* [8]. Further, mouse and *Drosophila* studies have established the importance of eating times in the synchronization of

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tissue-level circadian clocks and in the control of body weight gain and metabolism viz. high cholesterol and blood sugar levels [9–11]. However, the light-dark (LD) cycles often mask the effects of food availability in synchronization of circadian rhythms in the natural environment. This was revealed by an experiment in which the activity period was found synchronized with LD cycle when Indian weaverbirds were exposed to antiphased LD and food cycles, and with the food availability period when they were exposed to the constant dim light, $LL_{dim}$ [5].

Both the non-hypothalamic (e.g. hippocampus, HP) and hypothalamic (lateral hypothalamus, LH; dorsomedial hypothalamus, DMH; arcuate nucleus, Arc) brain regions have been shown to participate in the regulation of energy intake and homeostasis in mammals [12–14]. In birds, the infundibular nucleus complex (INc) functionally represents the mammalian Arc [15]. And, the VMN – SNS – PVN (ventromedial hypothalamic nucleus – sympathetic nervous system – paraventricular nucleus) neural pathway exerts a direct control over the food intake in birds [15]. At molecular level, neuropeptide Y (NPY) is the key candidate molecule of appetite regulation and energy homeostasis and has often been used to show the appetite regulation [16–19]. The infundibular hypothalamic neurons were also found with NPY mRNA in Japanese quails (*Coturnix c. japonica*) fasted for 24 h [19]. Wang et al. [20] found NPY mRNA expression distributed in the chicken HP, INc and PVN. Further, dense NPY mRNA and peptide immunoreactive (-ir) cells and fibers in INc, and dense NPY-ir fibers in the VMN and PVN support the involvement of INc – VMN – PVN pathway in the regulation of feeding behavior in redheaded buntings, *Emberiza bruniceps* [21].

The expression of Fos, the protein product of the c-fos immediate early gene, has been used as a reliable marker of the neural activity. Thus, Fos and NPY expressions can be used to assess the activity and functional state of brain feeding centers, respectively. In several studies, Fos-like immunoreactivity (Fos-lir) seems to occupy much of the NPY field. For example, about 70% of NPY-lir cells express Fos during the non-photic entrainment of rodent circadian rhythms [22]. Also, majority of NPY neurons express Fos in rat Arc [23]. In an experiment on mouse, about 90% of ghrelin (an orexigenic peptide) induced Fos-positive expression was found confined to the NPY synthesizing neurons of the ventromedial part of the hypothalamic Arc, but not the other hypothalamic and hindbrain nuclei [24].

The extended light hours during the night or exposure to an aberrant light condition may disturb the established relationship between the daily food availability and light conditions. This could result in mismatch of internal rhythms with the external environment, and in turn have physiological consequences. In fact, light at the wrong time of day has been shown to disrupt internal (circadian) timekeeping in many species, including birds, rodents and human [25–27]. Here, we hypothesized that daily food availability period (timed meal) would restore rhythmicity in individuals with disrupted circadian rhythm regulated functions. The present study tested this by providing food for an identical period to Indian weaverbirds (*Ploceus philippinus*) exposed to varying light conditions, which may or may not disrupt circadian rhythms. We continuously monitored activity behavior as the circadian response indicator, and quantified neurons immunoreactive to Fos and NPY as well as those containing NPY mRNA in the HP, DMH and INc, in order to gain better insights into the effects on appetite regulatory brain centers.

## 2. Methods

Indian weaverbirds (*Ploceus philippinus*) are a songbird species, with breeding season spread over May to August. Adult male birds were procured during the reproductively inactive phase in March and kept in an outdoor aviary (size = 3 × 2.5 × 2.5 m). After a week of acclimation, these birds were moved indoors and individually housed in the activity-recording cages (size = 60 × 35 × 45 cm) that were placed inside light-tight wooden chambers (size = 75 × 50 × 70 cm) lit by compact fluorescent lamps (CFL; 14W, 230V, Phillips, India) and maintained at 24 ± 2 °C temperature during the experimentation. Birds were exposed to 7 h light: 17 h darkness (7L:17D; L = 25 lx, D = ~0.3 lx) for 2 weeks, with ad libitum food (*Setaria italica* and *Oryza sativa* seeds) and water. Two experiments were performed as per the guidelines of the Institutional Ethics Committee at the University of Lucknow, India, where the study was done.

### 2.1. Experiment 1

This experiment tested the role of food availability as the key synchronizer and examined its physiological effects on blood glucose levels and brain centers implicated in the feeding behavior. It used a protocol in which weaverbirds were exposed to a consistent P:A food cycle under light conditions that might differentially affect the activity-rest pattern. After initial 2 weeks of food ad libitum under 7L:17D, the availability of food was restricted to a 7 h period, overlapped with the light hours (7 h food present: 17 h food absence, 7P:17A); thus birds were exposed to concurrent 7L:17D and 7P:17A cycles for a week. Beginning on day 8, LD condition was changed to yield a T = 7 h cycle (T7, 3.5L: 3.5D; group 1, n = 7) or modified to have a reduced light period (3.5L: 20.5D, T24; group 2, n = 8), or replaced with the constant dim light period ($LL_{dim,}$ ~0.3 lx, nighttime intensity; group 3, n = 7). We used a T7 light protocol in view of the demonstration of the direct effects of such an aberrant light on impairment of mood and learning in mice [28]. Thus, weaverbirds were exposed to an identical food cycle (7P:17A) but different light conditions in terms of the duration and light intensity for 4 weeks. Per 24 h during this 2-week exposure, T7 birds received 3.4 times more bright light than the T24 group; $LL_{dim}$ birds received no bright light. Because the light and food availability periods were out-of phase except once a week under T7, unlike under T24, we expected differences in the circadian rhythm synchronization between these 2 LD cycles. Also, we predicted synchronizing effects of the food cycle in free-running condition under the $LL_{dim}$.

Further, we recorded changes in body mass, food intake and blood glucose levels, as measures of the physiological effects, and measured the expression of mRNA and peptide of NPY and Fos (only peptide) to show effects regulatory processes underlying the feeding behavior. Whereas body mass was recorded at the beginning and end, the food intake and blood glucose levels were measured once after 3 weeks after change in the LD condition. Birds were provided with a weighed quantity of food on the day when the onsets of light and food availability period coincided, and food recovered at the end of 7 h food availability period was subtracted to give the amount of food consumed by an individual. Glucose levels were monitored 1 h before and after 7 h of food availability period in a drop of blood placed from the punctured wing vein, by an Accu-chek active strip, which measures glucose in the wide range of 10 mg/dL to 600 mg/dL and has been used for measurement of glucose in birds [29].

Finally, brains were sampled for the measurement of mRNA and peptide expressions of Fos peptide and NPY mRNA and peptide at the zeitgeber times 2 (n = 4) and 14 (n = 3 or 4; ZT 0 – beginning of the food availability period). For this, weaverbirds were deeply anaesthetized by injecting ketamine/xylazine solution (0.03 mL/g body weight) and perfused transcardially sequentially with the ice-cold saline (pH 7.4) and 4% paraformaldehyde solution (PFA in 0.1 M phosphate buffer, pH 7.4). Brains were post-fixed in the PFA over-night at 4 °C, cryoprotected by processing for 2 h through 10%, and overnight through 20% and 30% sucrose solutions, embedded in the 15% PVP solution (polyvinylpyrrolidone; PVP40T, Sigma), and stored at −20 °C, until further processed.

### 2.2. Experiment 2

This experiment used an identical protocol except that birds in 3 groups (n = 7–9) were exposed to 7P:17A food cycle under T7, T24 and $LL_{dim}$ light conditions for only 2 weeks and subsequently released