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Involvement of endogenous circadian rhythm in photoperiodic ovarian response of subtropical tree sparrow, *Passer montanus*

Namram Sushindrajit Singh[1*] and Anand S. Dixit[2]

[1,2]Avian endocrinology and Chronobiology Laboratory, Department of Zoology, North- Eastern Hill University, Shillong-793022, Meghalaya, India

Abstract

Investigations on the mechanism of photoperiodic time measurement in regulation of ovarian growth and function have been done in the subtropical population of tree sparrow (*Passer montanus*). Photosensitive female tree sparrows were subjected to various intermittent light dark cycles of different durations such as: 2L/ 2D, 3L/ 3D, 4L/ 4D, 6L/ 6D, 8L/ 8D and 12L/ 12D along with two control groups: one under short days (9L/ 15D) and the other under long days (14L/ 10D) for 30 days. Birds, under short days, did not show follicular enlargement while those experiencing long days exhibited ovarian growth confirming their photosensitive at the beginning of the experiment. Further, the birds under all the intermittent light regimes showed gonadal response except the birds under 8L/ 8D. The mean follicular diameter was significantly greater in the birds under 2L/ 2D as compared to the birds under 12L/12D. On the other hand, no significant variation in follicular diameter was observed among the birds maintained under 2L/ 2D, 3L/ 3D, 4L/ 4D and 6L/ 6D light dark cycles. Further, no significant variation was observed between the groups of birds exposed under 2L/ 2D and14L/10D (gonadostimulatory control group). Histomorphometric analysis of the ovary revealed a significant increase in the thickness of the follicular wall and follicular differentiation in the birds under gonadostimulatory light dark cycles. No significant different in body weight was observed in the birds under any of the light regimes. The above results are in agreement with the avian external coincidence model of photoperiodic time measurement and indicate that an endogenous circadian rhythm is involved during the initiation of the gonadal growth in the female tree sparrow. They further suggest that multiple flashes of light are more effective in inducing follicular growth than a broad pulse of light of same duration.

Keywords: circadian rhythm**,** intermittent light cycle, photoperiod, tree sparrow

INTRODUCTION

To effect seasonal reproduction and related events, many birds use predictable and stable environmental cues of which day length is the most consistent and reliable. Day length has been shown to control the annual cycle of reproduction in many species of birds [1, 2, 3, 4, 5]. In order to use the day length as a cue for seasonal reproduction, birds would require some mechanism to measure its duration [6, 7]. Since Rowan [8] first discovered that day length was a primary environmental signal regulating the seasonal reproductive cycles in birds, a great deal of attention has been directed towards understanding the mechanism(s) by which day length is measured. Some birds tend to adapt to daily light dark cycle using their endogenous time measuring device(s) called "clocks" because of their great precision in the timing of various physiological and behavioural events. Day length interacts with the clock and induces seasonal responses [9, 10]. Timing of reproduction and other seasonal events in captivity approximate their timing in the wild [11, 12] indicating the presence of internal timing program. This endogenous program enables the birds to identify the time when to switch on (photoinduction) and when to switch off (photorefractoriness) its physiological mechanisms so that the seasonal events occur at the most suitable time of the year. In photoperiodic birds, a circadian rhythm of photoperiodic photosensitivity (CRPP) mediates photoperiodic regulation of reproductive responses [13, 3]. CRPP responds to light in a phase dependent manner [14]. Although several hypotheses have been proposed regarding the mechanism of photoperiodic time measurement scheme [15], the discussion has been mostly confined to involvement of endogenous circadian rhythm [16]. According to Bunning, day length is measured by a circadian clock whose function is based on endogenous rhythm of about 24 h duration. The endogenous circadian rhythm consists of two halves. The first twelve hour is the subjective day or photoinsensitive phase while the latter twelve hour is the subjective night or photosensitive phase. The photogonadal stimulation is the result of direct or indirect, repeated (daily or otherwise) illumination of the photosensitive phase by external photophase [14]. According to external coincident modal, a photoperiodic response occurs due to the direct coincidence between the photoinducible phase of circadian rhythm and the environmental photoperiod. This model characterizes the dual role of light. First, light entrains the circadian periodicity in photosensitivity which is presumed to be an endogenous free running circadian rhythm and second, when of sufficient duration to extend into the photoinducible phase it induced photoperiodic gonadal response [17]. The internal coincidence model, on the other hand, predicts that an external photophase brings two or more circadian oscillator, present in the photoperiodic clock and coupled separately to dawn and dusk, into a particular phase relationship with one another resulting in a

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*Corresponding Author

Namram Sushindrajit Singh
Avian endocrinology and Chronobiology Laboratory, Department of Zoology, North- Eastern Hill University, Shillong-793022, Meghalaya, India

Tel: +91-9856137458; Fax: + 91-364-2722309
Email: ssdrajt@gmail.com

photoperiodic response. Thus, while the external coincidence model embraces the dual function of light as entrainer as well as inducer, light serves only as an entertainer agent and does not directly induce a photoperiodic response in the internal coincidence model [18]. One of the powerful experimental paradigms that can be used to test for the involvement of the circadian system in photoperiodic time measurement is the use of intermittent light-dark cycles experiment. In this experiment birds are subjected to different programmed photoperiods like 2L/2D, 3L/3D, 4L/4D, 6L/6D, 8L/8D besides a control groups held on 9L/15D and 14L/10D [19, 20]. Such types of investigation are limited [21, 22, 23]. These experiments are designed to test whether multiple light flashes given during the photosensitive phase are more effective than a single broad light pulse of same or greater duration or whether the gonadal growth depends upon the total amount of light during photosensitive phase. Further, they show that the photoperiodic responses results only when the light coincides with the photosensitive phase of an entrained endogenous circadian rhythm [24, 18]. Whether this timing mechanism is still operative in all photoperiodic birds in both the sexes is still inclusive [25]. Investigations involving the mechanism of photoperiodic time measurement in birds have mainly been confined to temperate species and have used exclusively on male birds [26, 27]. Thus, more investigation on females, especially in the regions of tropics and subtropics, are needed to validate the generalisation regarding the photoperiodic system of a bird species. Therefore, in the present study, we sought to use the intermittent light cycle experiment to test the involvement of the circadian rhythm in photoperiodic time measurement and also to know whether multiple light flashes are more effective than the single light of same duration in inducing photoperiodic response in the tree sparrow.

The tree sparrow, *Passer montanus* is a resident bird distributed abundantly in the hilly regions of the North-Eastern part of India and confine mostly along with the human habitat. It is a passerine bird in the family passeridae with distinctive characters of rich chestnut crown and nape, and a black patch near ear covert. Sexual dimorphism is not distinct while the young birds can be easily differentiated from adults as they are dull in colouration [28]. It is photosensitive, and its photoperiodic responses are similar to those of many north temperate birds [29, 30] in that long days induce gonadal development which is followed by rapid regression and photorefractoriness, whereas short days are inhibitory or ineffective [31]. Our investigations on the wild tree sparrow at Shillong (Latitude 25°34´ N, Longitude 91°53´ E) have established that (i) they are annually breeding species exhibiting seasonal changes in testicular and follicular size and bill colour that correspond to annual variation in day length at Shillong (3h and 15 min). The initiation of gonadal growth begins during February-March reaching to peak in May which is followed by gonadal regression in June and the birds regain minimum gonadal size in September that is maintained till December. Annual changes in bill colour runs almost parallel to annual gonadal cycle. Birds show annual cycles of moult in their wing primaries and body feathers that begin with gonadal regression in June and complete in September (ii) both the sexes have a photoperiodic threshold for their gonadal sensitivity that seems to lie between 10-11 h/day. Thus, in spite of significantly different environmental condition in the subtropics, this bird shows photoperiodic responses relatively similar to those of its populations living in the temperate zone [2, our unpublished data].

METERIALS AND METHODS

Capture, maintenance and pretreatment

Adult female tree sparrows were captured in and around the hills of Shillong (Latitude 25°34ºN, Longitude 91°53ºE) in the fall of 2009 and kept in an outdoor aviary. This aviary is located in the vicinity of our department in an open area surrounded by natural vegetation and receiving natural light and temperature conditions. Ovaries at this time were completely regressed and the birds had no conspicuous subcutaneous fat. These birds were then acclimatized to laboratory conditions for a fortnight. There they were subjected to natural variations of photoperiod, temperature and humidity. The birds were then transferred to the short day length (9L/15D) for eight weeks to eliminate photorefractoriness if they had any in nature and to ensure their photosensitivity at the time of commencement of the experiment. Laparotomy (surgical opening of abdominal wall between the last two ribs) at four weeks intervals during the pretreatment period revealed that they had regressed testis. These photosensitive birds were used in the present investigation.

Experimental procedure

Photosensitive adult tree sparrows were divided into eight groups (n=6 each): two controls and six experimental. Experimental groups were then subjected to different intermittent light cycles viz. 2L/2D, 3L/3D, 4L/4D, 6L/6D, 8L/8D, 12L/12D while control groups were maintained under 9L/15D (near to the shortest day length of Shillong) and 14L/10D (near to the longest day length of Shillong) for a period of 30 days. Observations on body weight and follicular sizes were recorded at the beginning and end of the experiment. A group of six birds in the beginning and all the birds at the end of the experiment were ovariectomised and their ovaries were fixed in Bouin´s fluid for histological purposes.

Experimental protocol

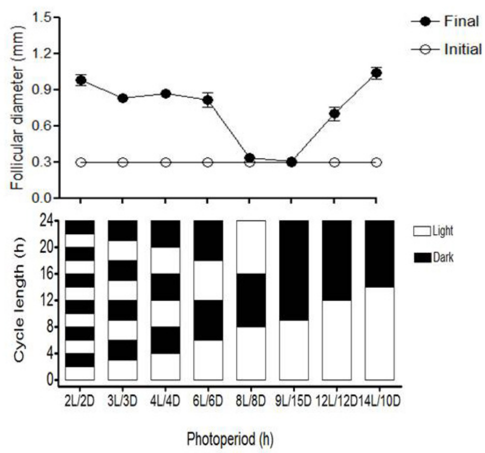
The gonadal development was measured in terms of follicle diameter. Briefly, the follicular size was recorded *in situ* by performing laparotomy under local anaesthesia using subcutaneous injection of 2% xylocaine (Astra-IDL Ltd. Bangalore, India) as per the procedure described in Kumar et al., [32]. Laparotomy was performed by surgical opening of abdominal wall between the last two ribs on the left side and ovary was located within the abdominal cavity with the help of a spatula. The ovarian growth was measured in terms of the diameter of the largest follicle. The regressed ovary with an indistinct follicle was assigned a follicular diameter of 0.3 mm to make the data statistically comparable with the stimulated follicles. Ovary was removed, freed of extra tissues and pressed gently on folded filter paper to get rid of surface fluid and then fixed in the Bouin's fluid for histological preparations [33]. The fixed ovaries were washed in 70% alcohol till the yellow colour disappears. They were then dehydrated in ascending series of alcohol grades and cleared in xylene. The tissue was then embedded in paraffin wax and sectioned at 5-7μ in thickness. Sections were stained with haematoxylin and counterstained with eosin and then mounted with DPX and covered with cover slip to observe under the microscope. Follicles were classified as primordial, primary, secondary and tertiary follicles [34]. The follicular wall thickness and number of large (>1000μm), medium (300-1000 μm), small (< 300μm) and atretic follicles were recorded in different sections (n = 5) of the histological slides of the ovary. All the measurements were taken using Motic images plus version 2 analyser. Body weight was measured using a top pan balance to an accuracy of 0.1g.

Statistical analysis

Data obtained from the experiment are presented as mean ± S.E.M. They were analyzed using one-way analysis of variance (One-way ANOVA) followed by post hoc Newman-Keul´s Multiple range't' test if ANOVA indicated significance at 95% confident limit. Also, Student's t-test was used while comparing only two means.

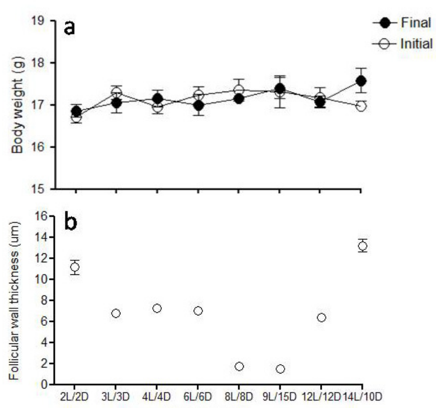
RESULTS

The results are presented in the figure 1. Birds of control group maintained under 9L/15D failed to show ovarian growth (P = 0.3632; Students t-test) while those experiencing 14L/10D exhibited significant follicular development (P < 0.0001; Student´s t-test) indicating that all the birds used in the present study were photosensitive when they were exposed to different experimental schedules. ANOVA revealed significant variation ($F_{7,47}$=49.30, P < 0.0001; One way ANOVA) in follicular diameter in tree sparrow. Significant increase in follicular diameter (P < 0.001) was evidence in birds exposed to the cycles of 2L/2D, 3L/3D, 4L/4D, 6L/6D , 12L/12D and 14L/10D (control), whereas birds under cycles of 8L/8D and 9L/15D (control) maintained regressed ovaries. The mean follicular diameter was significantly greater (P < 0.05) in the birds under 2L/2D when compared with 12L/12D birds. Further, no significant variation in follicular diameter was observed among the birds held under 2L/2D, 3L/3D, 4L/4D and 6L/6D. The difference in ovarian response between the birds exposed to 2L/2D and14L/10D was found to be insignificant. No significant change in body weight ($F_{7,47}$ = 0.8144*, P* = 0.5808; one way ANOVA) was evident in the tree sparrows which maintained their body weight (Fig 2, a) throughout the course of the experiment.



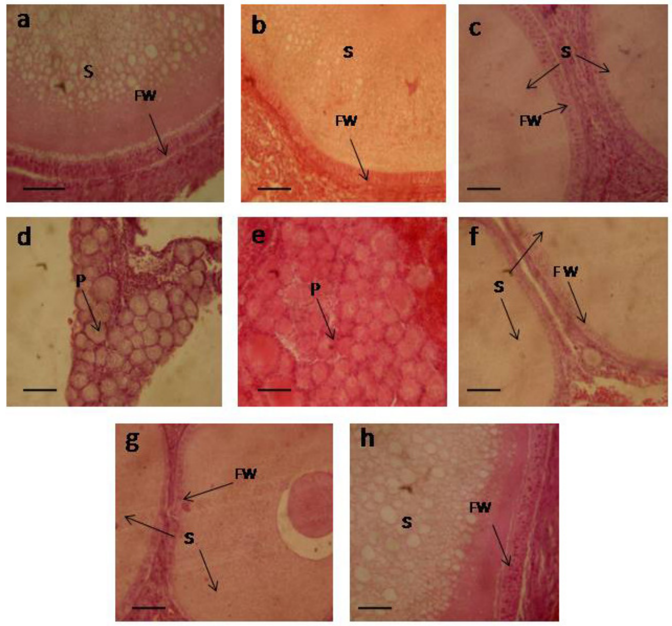
Gonadal response under different intermittent light cycles. Birds (n = 6) were recorded on the changes in follicular diameter at the beginning and end of the experiment. The data are presented in means ± s.e.m. Birds subjected to different intermittent light cycles viz. 2L/2D, 3L/3D, 4L/4D, 6L/6D, 12L/12D and 14L/10D show gonadal response while the light cycles under 8L/8D and 9L/15D do not. The mean follicular diameter was significantly greater (P<0.05) in the birds under 2L/2D when compared with 12L/12D birds. Further, the failure of birds to respond to light cycle of 8L/8D suggests that the circadian rhythm in these birds is not entrained by zeitgebers period of 16 h. Thus, the multiple flashes of light when given during the photosensitive phase of an entrained endogenous circadian rhythm are more effective in inducing gonadal growth than a single broad light pulse of same duration.

Fig 1.



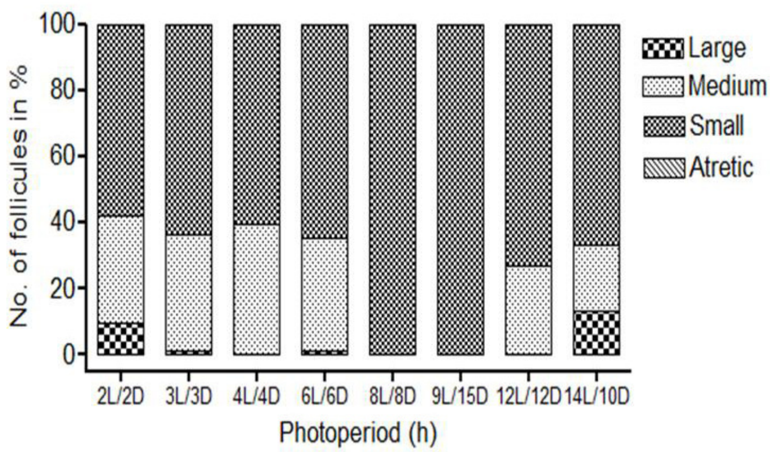
a) Body weight does not show significant change in the 30 days of exposure under different intermittent light cycles. b) Birds showed significant changes in follicular wall thickness only under stimulatory light regimes while those under 9L/15D and 8L/8D failed to respond suggesting the existence of photoperiodic mechanism in their control of ovarian growth.

Fig 2.



Histological section of the ovary under the intermittent light cycle of a) 2L/2D, b) 3L/3D, c) 4L/4D, d) 6L/6D, e) 8L/8D, f) 9L/15D, g) 12L/12D, h) 14L/10D. Scale bar=20µm, P=Primary follicle, S=Secondary follicle, FW= Follicular wall.

Fig 3.



Numbers of large, medium, small and atretic follicles are presented in the percentage. Maximum number of large follicles was observed in 2L/2D and 14L/10 (gonadostimulatory control group) while small follicles were observed in the 8L/8D and 9L/15D.

Fig 4.

Histomorphometric analysis of the ovaries of tree sparrow revealed a significant increase in the thickness of follicular wall ($F_{7, 159}$=108.2, P < 0.0001; One way ANOVA: Figure 2 b, 3). Significant increase in follicular wall thickness (P < 0.001) was noted in 2L/2D, 3L/3D, 4L/4d, 6L/6D, 12L/12D and 14L/10D (Fig 3 a ,b, c, f, g, h). Further, the follicular wall thickness was much more (P < 0.001) in 2L/2D while compared with 12L/12D. Significant variation in the percentages of large ($F_{7, 39}$ = 12.08, P < 0.0001; one way ANOVA), medium ($F_{7, 39}$=18.03, P < 0.0001; one way ANOVA) and small ($F_{7, 39}$ = 80.17, P < 0.0001; one way ANOVA) follicles was observed only under gonadostimulatory light-dark cycles (Fig 4). On the other hand, birds maintained small and quiescent follicles under non-gonadostimulatory cycles (Fig 3 d, e).

DISCUSSION

Results obtained from the experiment show that the subtropical female tree sparrows possesses a time measuring system that utilizes an endogenous circadian rhythmicity for reproductive function. They further suggest that multiple flashes of light when given during the photosensitive phase were more effective in inducing gonadal growth than a single broad light pulse of same duration. Though, all the birds used in different intermittent light dark cycles were provided with same length of light i.e., 12 h (except in 8L/8D and control groups). The ovarian growth was different among the groups depending on the numbers of pulses of light coincident with the photoinducible phase. A decreasing trend of ovarian response was observed in tree sparrow held under 2L/2D, 3L/3D and

4L/4D receiving 3, 2 and 1 light pulses in the photoinducible phase, respectively. This suggests that the photoperiodic response decreases with the decrease in number of light pulse of same duration in the photosensitive phase. Birds under 4L/4D, 6L/6D and 12L/12D light regimes showed almost similar response as they received only one pulse of light in the photoinducible phase. Ovarian response was observed in the tree sparrow under 12L/12D as the threshold period for ovarian response is about 11h per day [2]. The failure of birds to respond to light cycle of 8L/8D (even though 1 pulse of light falls in the photosensitive phase) suggest that the circadian rhythm in this bird is not entrained by zeitgebers period of 16 h. Our results on tree sparrow are consistent with the male *Coturnix coturnix japonica* [35, 24], *Zonotrichia atricapilla* and *Zonotrichia leucophrys pugetensis* [36], *Gymnorhis xanthocolis* [19] and female *Passer domesticus* [20]. However, the increasing number of light pulses does not have any effect in the gonadal growth in the male migratory finch, *Carpodacus erythrinus* [37]. A study by Wilson and Abplanalp [38], involving the effects of intermittent light on laying hens indicated that egg production can be maintained with very small amount of light energy, provided it is given intermittently (less than six one-minute intervals in 24 hours). Those chicken maintained under intermittent light of 12 h as total illumination is shown to be more effective than continuous light of same duration. The growth performance in the commercial broilers reared under intermittent lighting was found to be better than in those reared under continuous lighting [39, 40, 41, 42]. Furthermore, Van Tienhoven and Ostrander [43] reported that that intermittent light appears to be as good as 14L/10D for egg production in chicken. Light experienced by the tree sparrows during photoinsensitive phase of an endogenous circadian rhythm (as in 9L/15D) produces no gonad stimulating effect. On contrary, light falling in the photosensitive (= photoinducible) phase (as in cycles of 2L/2D, 3L/3D, 4L/4D, 6L/6D, 12L/12D and 14L/10D) of an endogenous circadian rhythm induces stimulatory effect. Thus, these results are in consistent with Bunning hypothesis and are in agreement with an external coincidence model [44, 14]. According to this model, the coincidence of the environmental photoperiod with the photoinducible phase of an entrained circadian rhythm within the bird, is the basic for a photoperiodic response and photoinduction fails to occur in absence of such coincidence [45, 18, 14].

The result of the present study besides providing evidence that females, like males of photoperiodic species, use circadian rhythm(s) to measure the photoperiodic time, also reveals that the ovary undergoes considerable follicular differentiation under gonadostimulatory intermittent light dark cycles. This is evident with the increase in follicular diameter, thickness of follicular wall and increased percentages of medium and large follicles in the photostimulated ovary. In a different study by Bhavna and Geeta [46] larger follicles were seen maximum in the breeding female jungle babbler and least in the non breeding while percentage of small follicles were more in the non breeding season than the breeding season. Further, the ovary does not grow to full breeding condition in the tree sparrow under artificial photostimulation alone while testes reach to spermatogenic level under long days (our unpublished data). The majority of investigations concerning the effects of photoperiod on avian reproduction and related functions have been limited to studies on males [26, 27]. In the bulk of literature, particularly little description has been given on the photoperiodic regulation of the ovarian growth and function as it is generally believed that biological timing will not tend to differ between males and females of the same species. However, males and females differ in relation to many aspects of reproduction related to physiology, morphology and behaviour [47]. One reason for less data in the females is that they require cues supplementary to photoperiod to move to the exponential growth phase of ovarian development and ovulation. Although the female partners have been paid less attention, the evidence that the female reproductive system is also stimulated by long days in spring is interesting [48]. The females of the species in which the males show strong photoperiodic gonadal response, usually exhibit progressive ovarian changes that are almost equivalent in rate and magnitude to the prenesting changes observed in feral females [49, 50]. The substantial reduction in the ovarian response in the photoperiodic birds is due to failure of long daily photoperiods to induce vitellogenesis and the culminative stages of follicular development [51, 52, 53]. The absence of final gonadal maturation is generally limited to one sex: testes growth is often complete while ovaries remain undeveloped under similar conditions [54, 51]. Therefore, it is quite understandable that besides the primary regulator (light in the photoperiodic species), some essential supplementary factors are required to bring about vitellogenesis and culminative stages of follicular development [55], at least some of which are species specific and may involve environmental information such as active mate, or nesting materials or nest site etc. [56, 57]. Further, birds in all the groups maintained (Fig 2 a) their body weight throughout the experiment indicating that fattening is generally lacking in non-migratory birds and if fattening occurs, it is only to a limited extend [58] that accounts for minor but insignificant changes in the body weight in the tree sparrows. Since the fat serves the purpose of stored food and fuel during migratory flights, its deposition in tree sparrow (a non-migratory resident bird) that has easy access to food in the surrounding, would hamper its flight activity [2].

CONCLUSION

Results obtained of the present experiment suggest the involvement of endogenous circadian photoperiodic time measurement during reproductive responses in the tree sparrow and are consistent with the Bunning hypothesis and the external coincidence model. Thus, in tree sparrow, entrained endogenous circadian rhythm of photosensitivity seems to possess a distinct photoinducible phase, which when illuminated, leads to a photoperiodic response. Further, the multiple flashes of light when given during the photosensitive phase of an entrained endogenous circadian rhythm are more effective in inducing gonadal growth than a single broad light pulse of same duration.

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