RESEARCH ARTICLE

Diel rhythms and sex differences in the locomotor activity of hawkmoths

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ABSTRACT

Circadian patterns of activity are considered ubiquitous and adaptive, and are often invoked as a mechanism for temporal niche partitioning. Yet, comparisons of rhythmic behavior in related animal species are uncommon. This is particularly true of Lepidoptera (butterflies and moths), in which studies of whole-animal patterns of behavior are far outweighed by examinations of tissue-specific molecular clocks. Here, we used a comparative approach to examine the circadian patterns of flight behavior in *Manduca sexta* and *Hyles lineata* [two distantly related species of hawkmoth (Sphingidae)]. By filming isolated, individual animals, we were able to examine rhythmic locomotor (flight) activity at the species level, as well as at the level of the individual sexes, and in the absence of interference from social interaction. Our results confirmed classic descriptions of strictly nocturnal behavior in *M. sexta* and demonstrated a dramatically different activity pattern in *H. lineata*. Furthermore, we showed distinct species and sex-specific differences in the maintenance of the endogenous rhythm under conditions of constant darkness. In both species, female activity peaked in advance of males whereas male activity coincided with periods of female sexual receptivity. This suggests a role for circadian patterns of locomotor activity in synchronizing periods of sexual receptivity between the sexes.

INTRODUCTION

Circadian rhythms are commonly described as ubiquitous and adaptive, occurring across all domains of life, coordinating internal metabolic events and synchronizing whole-animal behavior with favorable external conditions. A primary advantage of an endogenous time-keeping mechanism is the facilitation of behaviors requiring a sense of elapsed time – particularly anticipatory behaviors, which may confer a significant competitive advantage over purely exogenous, reactive behaviors (Horton, 2001; DeCoursey, 2004). One situation in which these anticipatory behaviors may be advantageous is in the daily activity patterns of flower-foraging insects.

Insect pollinators experience some of the highest metabolic demands on a per-weight basis (Heinrich, 1993). They regularly encounter fluctuations in resource availability due to daily rhythms of flower opening and reward distribution that regularly occur across communities of flowering plants (Feinsinger, 1978; Armbruster and McCormick, 1990; Stone et al., 1998; Willmer

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and Stone, 2004). Some of the highest recorded in-flight metabolic rates occur in hawkmoths [Lepidoptera: Sphingidae (Bartholomew and Casey, 1978; O'Brien, 1999)], which possess hovering flight and are renowned long-distance fliers that commonly forage for nectar at highly dispersed patches of floral resources (Alarcón et al., 2008). This adult-acquired nectar is known to fuel flight in hawkmoths (O'Brien, 1999) and can enhance lifetime fitness through increased longevity and fecundity (von Arx et al., 2013). Many night-blooming hawkmoth-pollinated flowers exhibit clear rhythms of flowering, scent emission and nectar secretion that coincide with periods of moth activity (Haber and Frankie, 1989; Hoballah et al., 2005); however, it is unknown whether this pattern of moth activity is under endogenous control or if moths become active and begin foraging in response to exogenous cues, such as the onset of darkness. Endogenous regulation of locomotor activity could confer a significant advantage if peaks of activity coincide with floral rhythms by allowing an insect to arrive at newly opened flowers, often the time of highest nectar availability (Gregory, 1963; Martins and Johnson, 2007), thus increasing profitability (caloric intake per unit foraging time or effort) while reducing flight costs incurred by foraging at times when resource availability is low. This type of endogenous control of locomotor activity has been shown for honeybees and other hymenopteran pollinators (Spangler, 1972; Stelzer et al., 2010) but endogenous control of adult locomotor activity remains undetermined for the majority of the Lepidoptera (Merlin and Reppert, 2010).

The hawkmoths *Manduca sexta* (Linnaeus) and *Hyles lineata* (Fabricius) provide an opportunity to test the endogenous nature of locomotor activity in two distantly related and ecologically important hawkmoth species. Both species are broadly distributed across the Americas and visit many of the same floral nectar sources (Raguso et al., 2003; Alarcón et al., 2008). As caterpillars, the activity of both species appears largely to be a response to temperature, showing consistent levels of activity regardless of time of day or photoperiod when kept in isothermal conditions (Casey, 1976). However, rhythmic patterns of behavior in these moths may develop later in life, as has been demonstrated for honeybees and *Drosophila* (Bloch et al., 2002; Malpel et al., 2004). In the better-studied *M. sexta*, clear rhythms of pheromone release and flight behavior were originally described by Sasaki and Riddiford (1984). Also, it is well established that *M. sexta* only forages for nectar at dusk and into the evening across the Americas (Bertin, 1982; Raguso et al., 2003; Moré et al. 2006). While the endogenous nature of these behaviors was not tested, further work confirmed a circadian rhythm of female calling behavior (Itagaki and Conner, 1988) and more recent studies suggest the presence of pheromone-sensitive circadian pacemaker cells in the antennae of male *M. sexta* (Schuckel et al., 2007). These results, combined with clear evidence of circadian rhythms of visual sensitivity in adult moths (Bennett,

1983), suggest that locomotor activity for this species may also be under circadian control. Yet, because the eye pigments of *M. sexta* show light-sensitive responses and visual sensitivity is severely reduced under brightly lit conditions (Höglund and Struwe, 1970), it is also possible that flight behavior in *M. sexta* is regulated by the moth's visual capabilities. Conversely, adults of *H. lineata* retain color vision at light intensities that span the full spectrum of strictly diurnal to strictly nocturnal hawkmoth species (Kelber et al., 2003). While *H. lineata* is most often described as crepuscular and commonly pollinates night-blooming flowers (Raguso et al., 1996), numerous daytime observations of floral visitation, especially in high elevation settings (Aldridge and Campbell, 2007; Thairu and Brunet, 2015), have led to the suggestion that its adult flight activity may be arrhythmic.

Endogenous rhythms of flight behavior have been evaluated in relatively few species of Lepidoptera, possibly due to the suppression of activity by constant light or constant dark conditions (Merlin and Reppert, 2010). In this study, we assessed the endogenous control of locomotor activity in both hawkmoth species using individually housed, visually isolated moths exposed to both constant light and constant darkness. In this way, we evaluated two hypotheses for both hawkmoth species: (1) locomotor (flight) behavior is under endogenous control in both species; and (2) *M. sexta* is strictly nocturnal (as originally described by Sasaki and Riddiford, 1984) (Fig. 1A) whereas *H. lineata* is crepuscular rather than arrhythmic (Fig. 1B).

Monitoring the behavior of isolated individual animals in addition to population rhythms allowed us to investigate sex-specific patterns of endogenous behavior that emerged during the course of our experiments.

MATERIALS AND METHODS
Animal care

Experiments were conducted between September 2013 and December 2014 at Cornell University, Ithaca, NY, USA. Adult *M. sexta* moths used in these experiments were the offspring of animals acquired from a laboratory colony maintained at the University of Arizona, Tucson, AZ, USA (Stillwell and Davidowitz, 2010). The *H. lineata* colony was derived from the progeny of eggs and larvae collected from *Oenothera harringtonii* plants in Colorado Springs, CO, USA, during the summer of 2009 (see von Arx et al., 2013). Larvae of both species were fed *ad libitum* on an artificial diet adapted for *M. sexta* (Bell and Joachim, 1976; Goyret et al., 2009) and were maintained at 24°C on a 16 h:8 h light:dark photoperiod in a humidified (ca. 50%) incubator. Wandering larvae were transferred to wooden pupation boxes (Yamamoto et al., 1969).

Entrainment to light:dark cycles

Pupae nearing eclosion were sorted by sex and transferred to 45 cm×45 cm×45 cm screen cages (BioQuip, Inc., Rancho Dominguez, CA, USA) in separate incubators (Precision 818, Winchester, VA, USA) for entrainment under 16 h:8 h light:dark conditions at 24°C. Male and female moths were entrained in separate incubators to eliminate the effects of pheromones or social cues on adult activity (see Figs S1 and S2; Levine et al., 2002; Silvegren et al., 2005). The screen cages were monitored daily and all experimental moths were marked with permanent ink at 17:00 h on the day of eclosion (day 0). Moths were allowed to feed *ad libitum* on 10% (by mass) sucrose solution and were maintained under these conditions for a further 48 h (days 1 and 2) before transfer to experimental conditions. Lighting conditions were maintained using white fluorescent lighting (3500 K soft white fluorescent bulbs, Sylvania, LEDVANCE, Wilmington, MA, USA) at 1022.63±97.58 lx for both experimental and entrainment conditions. Lighting systems were equipped with a Philips Advance Mark III Energy Saver magnetic ballast (Philips, Amsterdam, Netherlands) with an 'A' sound rating (20–24 dB), producing a flicker rate of 120 Hz on standard North American alternating current. This rate is above the critical flicker fusion frequency reported in *M. sexta* (Sprayberry, 2009) and below the ultrasound hearing sensitivity (20 kHz) of *H. lineata* (Roeder, 1972), and thus should not elicit chronic stress responses to sound or light (Morgan and Tromborg, 2007). Preliminary studies (see Figs S1 and S2) were conducted in a greenhouse under natural lighting conditions with a 13 h:11 h light:dark cycle.

Experimental conditions

Following entrainment, 2–4 moths of the same sex were transferred to experimental incubators identical to the entrainment incubators but were maintained under experimental photoperiods. During the experiment, moths were housed separately in 30 cm×30 cm×30 cm cages with cotton cloth walls so that the moths were visually isolated. Each moth was allowed unlimited access to 10% sucrose solution and its activity was monitored during exposure to the experimental photoperiods for 48 h (days 3 and 4). This duration was determined by preliminary experiments on *H. lineata* kept under similar conditions, which showed noticeable deterioration of wing condition and considerable mortality after day 5 (von Arx et al., 2013). This protocol made it impossible to study rhythmic activity over a longer time period, which is often done for longer-lived organisms (e.g. Giannoni-Guzmán et al., 2014).

The activity of the adult moths was monitored under three experimental light cycles and one control treatment. The control treatment was continued exposure to the 16 h:8 h light:dark
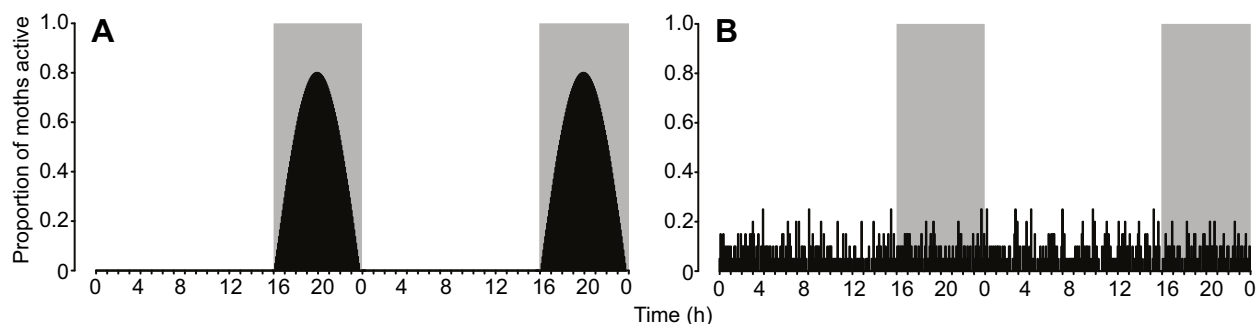

Fig. 1. Idealized representations of nocturnal and arrhythmic activity. (A) Nocturnal behavior and (B) arrythmic behavior based on a random distribution of the total number of movements made by *H. lineata* within a 48 h period. Horizontal axes are shown in zeitgeber time, where ZT 0=lights on.

conditions under which the moths were entrained [*M. sexta* N=22 (11 females, 11 males); *H. lineata* N=24 (13 females, 11 males)] whereas experimental moths were exposed to one of three treatments: 48 h of constant darkness [*M. sexta* N=20 (10 females, 10 males); *H. lineata* N=21 (10 females, 11 males)]; 48 h of constant light [*M. sexta* N=23 (12 females, 11 males); *H. lineata* N=20 (11 females, 9 males)]; or a rapid light:dark cycle alternating every 4 h [*M. sexta* N=21 (11 females, 10 males); *H. lineata* N=22 (12 females, 10 males)]. Trials were separated by species and sex but the order was randomized to allow for within-treatment comparisons.

Mating trials for *H. lineata* were conducted using identical experimental conditions. Male and female moths were maintained separately for 48 h (days 1 and 2) under 16 h:8 h light:dark conditions before mating trials began. At the end of day 2, one female and five males were placed in the experimental cages under the same lighting conditions and were observed for 48 h (days 3 and 4) or until mating occurred.

Response variables

The activity of experimental moths was monitored constantly by means of USB webcams (Logitech L600, Logitech, Newark, CA, USA) and Handy AVI time-lapse software (AZcendant Software, Mesa, AZ, USA) programmed to capture still frames at 5 min intervals. Infrared lighting was used to allow filming during the dark phase of the experimental light cycles. The resulting videos were analyzed on a frame-by-frame basis and activity was scored as a categorical variable with 0 indicating no movement and 1 indicating that the body of the moth had shifted position since the preceding frame or that the moth was captured in-flight. Mating trials were monitored in the same way, and mating was determined by visual inspection.

Statistical analyses

All analyses were conducted in R (http://www.R-project.org/). Activity patterns of moths under experimental conditions were visualized graphically by creating an activity plot showing the proportion of moths active at a given timepoint. The timing of peak activity and synchrony between individuals was evaluated during the final 24 h of the experimental treatment at the species and sex-specific level using circular statistics to determine the mean timing of activity (θ) and concentration around the mean($\bar{R}$). The Rayleigh test of uniformity was used to evaluate the mean (θ), and comparisons between sex-specific means were conducted using Watson's two-sample test of homogeneity. Mean mating time for *H. lineata* was also evaluated using circular statistics. These analyses were conducted using R-packages 'circular' and 'CircStats' (https://r-forge.r-project.org/projects/circular/; https://CRAN.R-project.org/package=CircStats). These results were confirmed using autocorrelation analyses to detect rhythmicity and periodicity of activity patterns, as well as cross-correlation analysis to detect phase-shifted relationships (e.g. see Crofford et al., 1996) between male and female diel patterns. Sex differences in total activity level within treatments were evaluated *post hoc* using a two-tailed Welch's *t*-test.

RESULTS

Establishment of baseline activity patterns

Under control conditions (16 h:8 h light:dark) the recorded activity of *M. sexta* and *H. lineata* showed distinct rhythmic patterns. The movements of *M. sexta* conformed to the expectation of strictly nocturnal behavior, with movements distributed across the majority of the scotophase (Figs 1A and 2B). Movements of *H. lineata* were

not confined to the scotophase and showed two distinct peaks of activity after lights on and lights off (Fig. 2A). The mean timing of activity (directional mean, θ) for *M. sexta* and *H. lineata* was determined to be 3.50 h and 4.36 h after the onset of scotophase, respectively. Means of individual moths were highly concentrated around the species-level mean and deviated significantly from a uniform distribution in both species (*M. sexta*: $\bar{R}$=0.97, P<0.001; *H. lineata*: $\bar{R}$=0.85, P<0.001) (Fig. 3A,B). As expected, for the light:dark treatment autocorrelation analyses of both species showed peaks of activity repeating on a near perfect 24 h cycle (Fig. 5).

Activity in the absence of external cues

In both species, moths exposed to 48 h of constant darkness post-entrainment exhibited significant, but shifted, rhythmicity in the final 24 h of the experimental treatment. In *M. sexta*, the mean activity shifted 2.78 h later in the subjective night and the concentration of individuals around the mean decreased marginally (θ=6.28 h, $\bar{R}$=0.88, P<0.0001). *Hyles lineata* experienced a similar shift of 2.70 h with a greater decrease in the clustering of individual moths around the species mean (θ=7.06 h, $\bar{R}$=0.42, P=0.02). Autocorrelation analysis of these treatments revealed similar results, showing a slight shortening of the period in both species and a dramatic reduction in the autocorrelation values for *Hyles* even within just the initial 24 h of the constant darkness treatment (Fig. 5).

Moths of both species exposed to 48 h of constant light exhibited reduced locomotor activity (Fig. 2E,F and Fig. 8). In the case of *M. sexta*, only five individual moths were recorded as active during the trial period (N=22 moths), which precluded statistical analysis. In *H. lineata* the mean timing of activity was shifted later by 0.37 h when compared with the control trials (θ=4.73 h) and the concentration of individuals around the species mean was reduced such that the distribution was not significant ($\bar{R}$=0.26, P=0.47).

Plasticity of rhythmic behavior

When transferred from control conditions (16 h:8 h light:dark) to a 4 h:4 h rapidly alternating light:dark cycle, moths of both species showed rapid synchronization to the light:dark cycle (Fig. 6). After approximately 24 h, locomotor activity patterns resembled the species-typical baseline, albeit condensed.

Sex differences

Manduca sexta

Under control lighting (16 h:8 h light:dark) conditions, male and female *M. sexta* exhibited significant differences in the timing of locomotor activity (overall P<0.01), with the female mean occurring 1.38 h before the male mean (females: θ=2.81 h, $\bar{R}$=0.98, P<0.0001; males: θ=4.19 h, $\bar{R}$=0.99, P<0.0001). Moths of both sexes exposed to the constant darkness treatment exhibited a similar shift, with the peak of activity occurring later in the subjective night. Differences between the sexes were maintained under these conditions with the female mean of activity occurring 2.31 h in advance of the male mean (P<0.01; females: θ=5.04 h, $\bar{R}$=0.85, P<0.001; males: θ=7.35 h, $\bar{R}$=0.99, P<0.0001). These patterns of offset peaks of locomotor activity between the sexes were confirmed for both the control light:dark and constant darkness treatments using cross-correlation analysis (Fig. 7). Unlike the control or constant light treatments, constant darkness elicited a significant difference in total activity with female moths moving a mean of 42 times less than males during the 48 h treatment period (P<0.001).
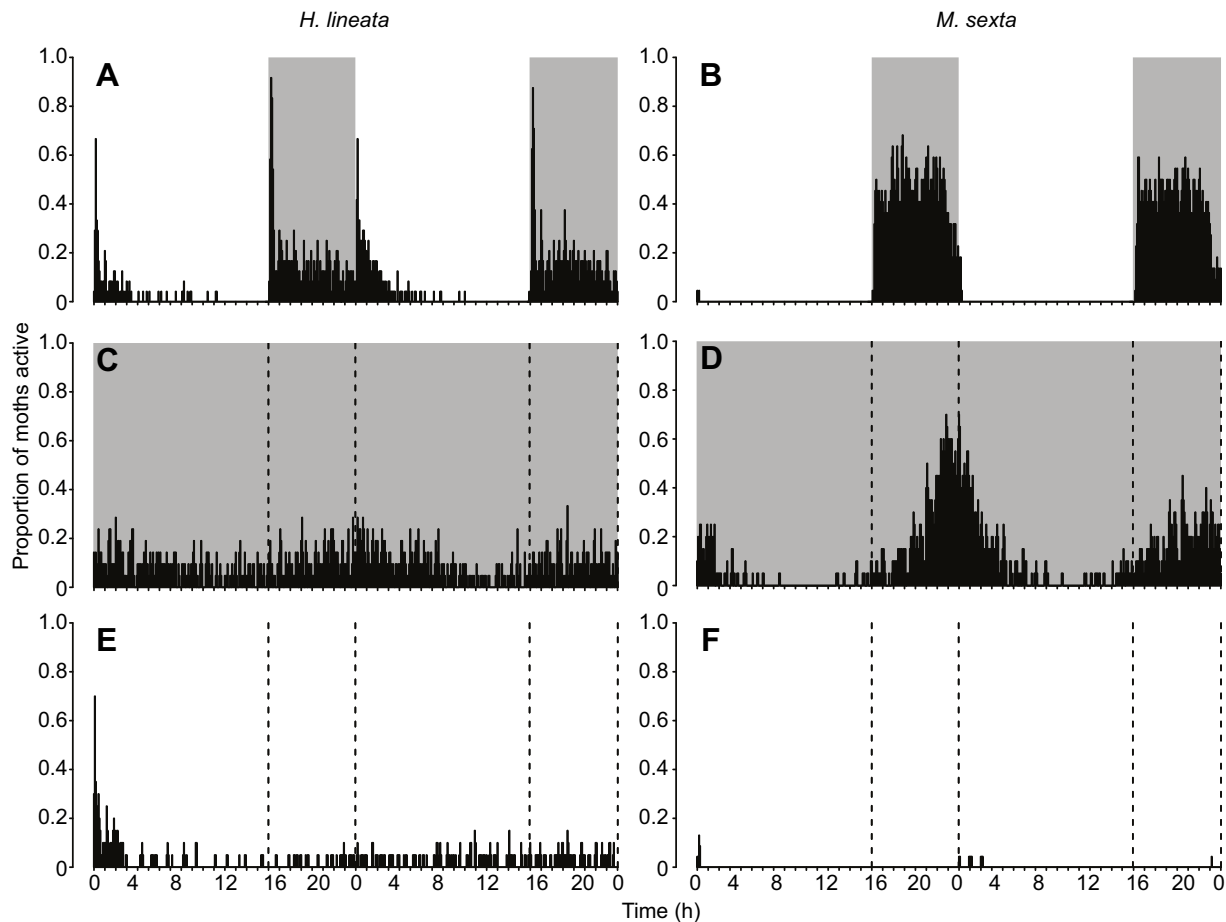
Fig. 2. Observed activity patterns in control and experimental treatments. (A) Activity plot of *H. lineata* exposed to a 16 h:8 h light:dark light cycle (*N*=13 females, 11 males), (B) *M. sexta* exposed to a 16 h:8 h light:dark photoperiod (*N*=11 females, 11 males), (C) *H. lineata* under conditions of constant darkness (*N*=10 females, 11 males), (D) *M. sexta* under conditions of constant darkness (*N*=10 females, 10 males), (E) *H. lineata* exposed to conditions of constant light (*N*=11 females, 9 males) and (F) *M. sexta* exposed to conditions of constant light (*N*=12 females, 11 males). Horizontal axes represent zeitgeber time for light:dark photoperiods (ZT 0=lights on) and circadian time for constant lighting conditions (CT 0=beginning of subjective day).

Hyles lineata

Male and female *H. lineata* also exhibited significant differences in the mean timing of activity under control conditions, with a 2.67 h separation between the means ($P<0.05$; females: $\theta=3.20$ h, $\bar{R}=0.93$, $P<0.0001$; males: $\theta=5.87$ h, $\bar{R}=0.86$, $P<0.0001$). In the constant darkness treatment, the separation between the sexes widened to 6.67 h ($P<0.01$). These results were again confirmed using cross-correlation analysis (Fig. 7). Additionally, the concentration of males around the sex-specific mean was reduced such that the distribution was no longer statistically non-random (females: $\theta=4.74$ h, $\bar{R}=0.82$, $P<0.001$; males: $\theta=11.41$ h, $\bar{R}=0.47$, $P=0.086$). Unlike *M. sexta*, differences in total activity of *H. lineata* occurred in the control and constant light treatments, and males were the more active sex ($P<0.05$) (Fig. 8). Furthermore, mating in *H. lineata* occurred exclusively during the photophase (Fig. 4) ($\theta=8.77$ h, $\bar{R}=0.98$, $P<0.0001$) rather than during the scotophase, as is the case for *M. sexta* (Sasaki and Riddiford, 1984).

DISCUSSION
Natural rhythms

Our observations of locomotor activity in socially isolated, individual moths revealed consistent diel patterns that differed between the species. When maintained under a 16 h:8 h light:dark

cycle, *M. sexta* showed a distinct nocturnal pattern of activity, consistent with our expectations and corresponding to patterns originally described by Sasaki and Riddiford (1984). Additionally, while exposure to constant illumination suppressed *M. sexta* flight activity, under conditions of constant darkness moths were able to maintain a characteristic rhythmic pattern of activity peaking during the subjective night, which suggests the presence of an endogenous, nocturnal rhythm. In contrast, these patterns were not observed in *H. lineata*. When exposed to identical experimental conditions (along with identical rearing conditions), *H. lineata* moths displayed a bimodal distribution with distinct periods of activity around lights on and lights off, suggestive of crepuscular behavior. Again, unlike the results observed in *M. sexta*, the pattern of activity demonstrated by *H. lineata* under control conditions (16 h:8 h light: dark) was lost in the absence of zeitgebers and became unimodal with activity concentrated during the subjective night. This transition from a bimodal to unimodal pattern of activity in free-running animals is also observed in the mosquito *Aedes aegypti* and may indicate a weakly nocturnal endogenous rhythm (Taylor and Jones, 1969; Beeston and Morgan, 1979).

Species means

Under both semi-natural and constant-darkness lighting conditions, each moth species exhibited distinctly different overall patterns of
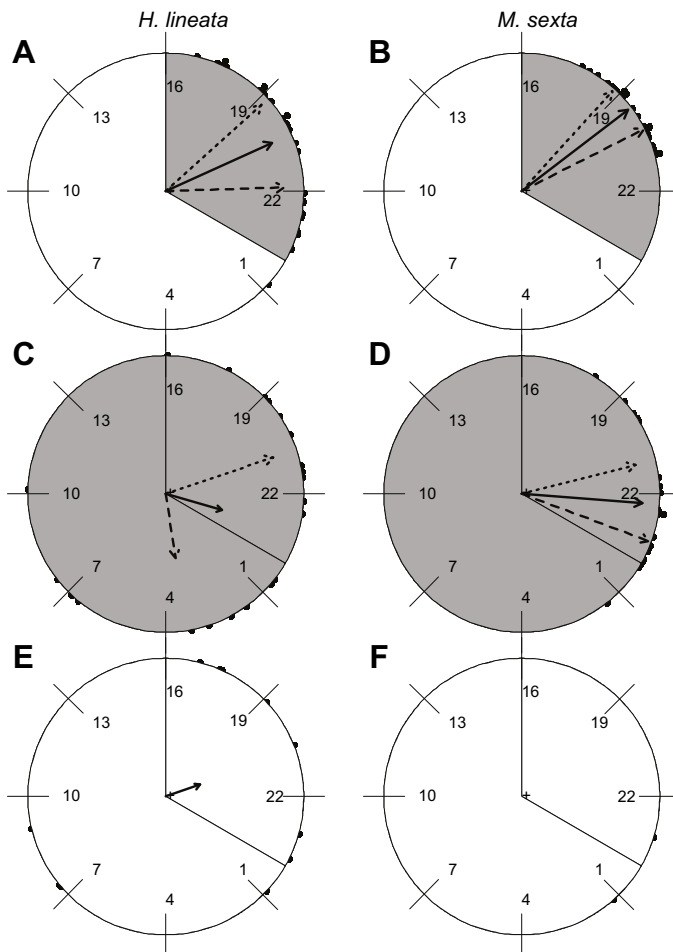
Fig. 3. Circular plots of the mean timing of activity for individual moths in the final 24 h of the experimental light cycle. Solid arrows are used to indicate the species mean and arrow length is proportional to the degree of clustering around the mean. Dotted and dashed arrows indicate female and male means, respectively. Shading indicates the experimental photoperiod. (A,B) *Hyles lineata* and *M. sexta* activity under control conditions, (C,D) constant darkness and (E,F) constant light. Sex-specific means for both species and the species mean for *M. sexta* in constant light conditions are not shown due to extremely low activity levels. Hours are shown in zeitgeber time for light:dark photoperiods (ZT 0=lights on) and circadian time for constant lighting conditions (CT 0=beginning of subjective day). Activity plots of original sex-specific data are shown in Fig. S3.

Fig. 4. Mean mating time for *H. lineata*. Mean mating time (8.77 h or 46 min into the early photophase) of *H. lineata* is indicated by the solid arrow. All matings (*N*=9) were tightly clustered in the early photophase, unlike *M. sexta* which mates during mid-scotophase (Sasaki and Riddiford, 1984). Hours are shown in zeitgeber time (ZT 0=lights on).

the sex-specific means. Applied to our observations of natural activity, these sex-specific patterns explain the consistently high activity level of *M. sexta* for the duration of the scotophase, as well as the bimodal pattern seen in *H. lineata*. In the former, the two overlapping peaks combine to form a single broad band of activity, while in the latter the bimodal pattern is entirely driven by male activity during the photoperiod (Fig. 8C).

The hawkmoths (family Sphingidae) are an interesting lineage to study with respect to niche diversification, due to their global distribution and phyletic diversity (ca. 1400 species) and their phylogenetically derived condition of adult feeding from a non-feeding ancestry in the superfamily Bombycoidea (Kawahara et al., 2009). Extant hawkmoth diversity includes a full spectrum of diurnal, crepuscular and strictly nocturnal activity patterns (Herrera, 1992; Kelber et al., 2003) along with trophic niches spanning exclusive floral nectar or rotting fruit/sap to a combination thereof (O'Brien et al., 2000; Raguso and Willis, 2003). Although *M. sexta* and *H. lineata* are sympatric over much of their distribution in the Americas, they belong to different subfamilies of the Sphingidae with a deep phylogenetic split (Kawahara et al., 2009) and thus are not closely related. Indeed, the most recent phylogenetic analysis unambiguously places the day-active genera *Hemaris* and *Cephanodes* as sister to the remainder of the Macroglossinae (the lineage that includes the genus *Hyles*), suggesting that the full diversity of diel activity patterns in this clade may be derived from a strictly diurnal ancestry. In contrast, the large genus *Manduca* and most of its relatives are more consistently nocturnal in adult activity patterns across the Sphinginae (Hodges, 1971; Moré et al., 2005).

Sexually dimorphic behavior

While there are numerous reports of diel rhythms of foraging behavior in flower-visiting insects, as well as rhythms of floral scent emission and nectar secretion, the sex-specific sub-structuring of activity patterns in *M. sexta* and *H. lineata* suggests an additional factor influencing rhythms of locomotor activity. If male activity in both species was to peak more than an hour after that of females, or even as female activity declines, then males would consistently miss the period of greatest food resource availability – the window of time immediately following anthesis in night-blooming flowers (Thom et al., 2004; von Arx et al., 2012).

In experiments using an identical 16 h:8 h light:dark light cycle, both Itagaki and Conner (1988) and Sasaki and Riddiford (1984) observed that pheromone release and calling behavior in virgin

activity as well as different mean times of peak activity. Taking natural history observations into account, the differences between *M. sexta* and *H. lineata* are not unexpected; differences in peaks and duration of activity have been reported even between species of the same genus (Lamarre et al., 2015). This divergence in flight time has been discussed in terms of temporal niche partitioning, a possible mechanism for reproductive isolation between related species, or simply a result of species-specific responses to light (Janzen, 1984; Beck and Linsenmair, 2006).

Under controlled laboratory conditions and in the absence of social cues, our results suggest that while there are species-specific responses to light, the underlying endogenous contribution to locomotor rhythms remains distinctly different between *M. sexta* and *H. lineata*. Furthermore, our results indicate that the species mean timing of peak activity does not describe the behavior of any single average moth. In both *M. sexta* and *H. lineata*, the species-level mean is the approximate halfway point between
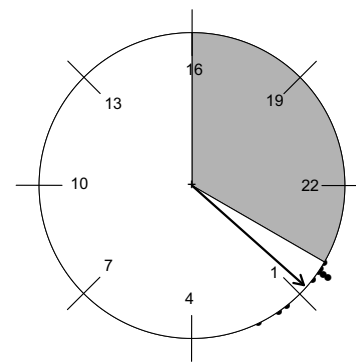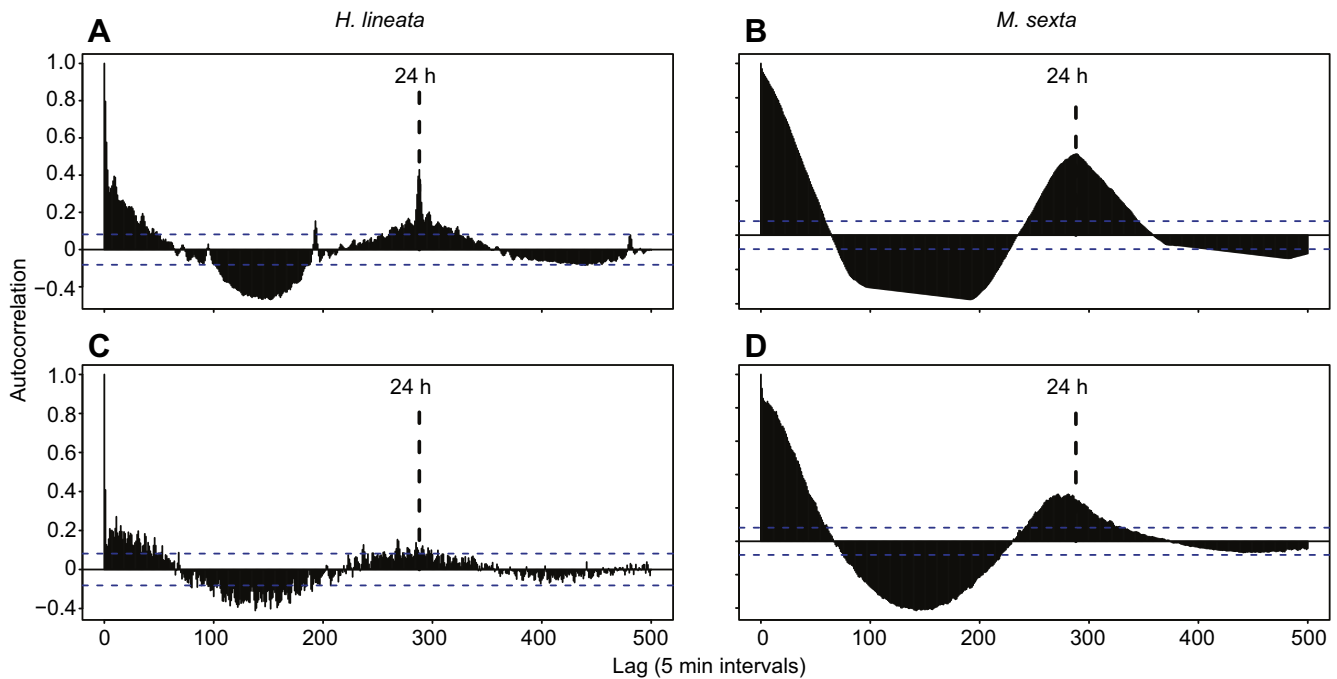
Fig. 5. Autocorrelation analyses of *H. lineata* and *M. sexta* demonstrating reduced periodicity in constant darkness. (A,B) Control (16 h:8 h light:dark) light cycle and (C,D) constant darkness treatment.

female *M. sexta* occurs approximately 5 h after the onset of scotophase. This timing directly corresponds with a decline in female activity and overlaps the mean time of greatest male activity. In this species, female calling is under circadian control (Itagaki and Conner, 1988) and is a stationary rather than in-flight behavior. Further, Itagaki and Conner (1988) observed that under conditions of constant darkness, unmated *M. sexta* females spent increasingly more time calling (emitting pheromone) on each successive night. This increased calling behavior necessitates a progressive decrease in flight activity, much like that observed in our experiments (Fig. 8B,D). The female activity patterns described by Itagaki and Conner (1988) and Sasaki and Riddiford (1984) are consistent with our own findings and may represent the interaction of two internal pacemaker mechanisms. To this, our study has added the finding that the pattern of male activity peaks as female activity wanes, and is maintained in the absence of light or social cues.

In a pattern similar to that of *M. sexta*, *H. lineata* exhibits strongly sexually dimorphic behavior, in which male activity peaks several

hours after that of females. However, the precise timing of events is quite different between the species. While female *H. lineata* activity peaks several hours after the onset of scotophase, similar to *M. sexta* females, the degree of separation between *H. lineata* males and females is greater than that of *M. sexta* (2.67 h versus 1.38 h, respectively). This is primarily due to male *H. lineata* exhibiting a second peak of activity during the early photophase – a peak that is responsible for the bimodal pattern of activity seen in the species (Fig. 2A), as females are not active during the photophase (Fig. 8A,C). Yet, in *H. lineata*, female sexual receptivity coincides with the onset of photophase and the majority of mating occurs during the first 1–2 h of the photophase (see Fig. 4 and Fig. S2). Thus, despite differences in timing, the phenomenon of male flight activity peaking at the time of greatest female sexual receptivity is consistent between the two species. This result complements the findings of Schendzielorz et al. (2015), which demonstrate that octopamine-mediated pheromone sensitivity peaks in synchrony with flight behavior in male *M. sexta*.
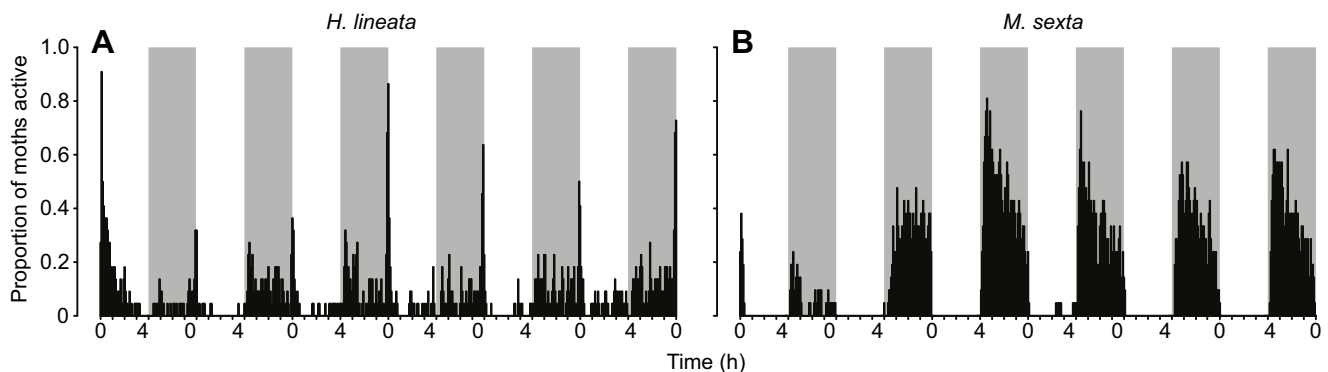

Fig. 6. Activity patterns of *H. lineata* and *M. sexta* exposed to a rapidly alternating light:dark cycle. (A) Response of *H. lineata* to a 4 h:4 h light:dark cycle (*N*=12 females, 10 males) and (B) response of *M. sexta* (*N*=11 females, 10 males). Shaded regions indicate periods of darkness while filming. Horizontal axes are shown in zeitgeber time, where ZT 0=lights on and ZT 4=lights off.
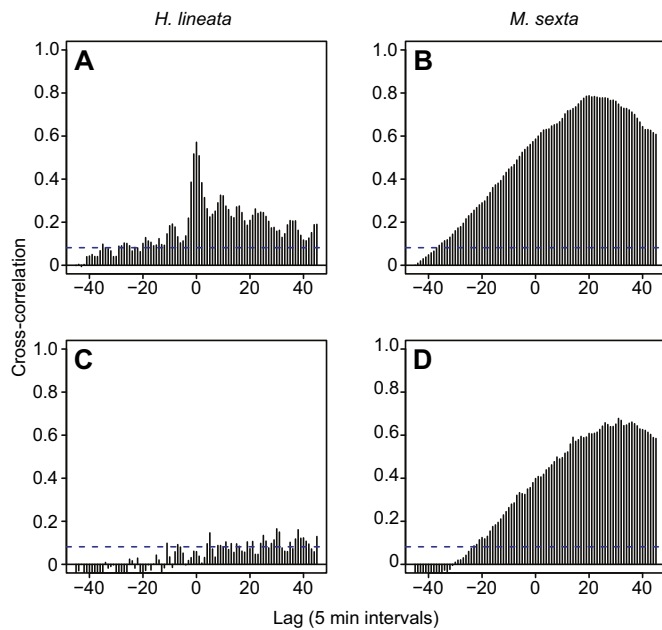
Fig. 7. Cross-correlation analyses of offset patterns in male and female activity. (A,B) *Hyles lineata* and *M. sexta* under control conditions, respectively, and (C,D) constant darkness, respectively.

Rapid light:dark cycle

As is evident in Fig. 6, both *M. sexta* and *H. lineata* show rapid acclimatization to dramatically altered light cycles, demonstrating a marked degree of plasticity in response to external light cues. Similarly, plasticity in response to extreme time periods has been previously documented in the primary mammalian circadian pacemaker (suprachiasmatic nucleus) (Patton et al., 2016), which suggests an unexpected level of adaptability in circadian systems.

Notably, in *H. lineata*, the transition between scotophase and photophase is delineated by a sharp burst in activity much like that seen in males under normal 16 h:8 h light:dark conditions (contrast Fig. 6A and Fig. 2A). Abrupt peaks of similar amplitude were not observed under conditions of constant darkness (Fig. 2C), which may suggest that this sudden male-specific burst in activity is a response to lights on rather than a component of the species' endogenous rhythm. Yet, recent studies using *Drosophila* clock-less mutants have shown that animals with functional vision are capable of maintaining normal diel rhythms in the absence of endogenous control, provided that light cues are available (Schlichting et al., 2015). Therefore, as female receptivity coincides with the onset of photophase (or sunrise) in *H. lineata* (Fig. S2), the male response to lights on may be sufficient to synchronize sexual receptivity.

Synchrony in sexual receptivity between males and females of the same species can have dramatic fitness consequences. For example, in the songbird *Parus major* (Great Tit), delaying the onset of male activity by as little as 15 min increases the probability that a male will be cuckolded by his mate (Greives et al., 2015). Similarly, male solitary bees in Israel are under strong selective pressure to search for mates early in the morning, and *Oncocyclus* iris flowers with dark petal chambers are thought to provide a thermal advantage for males that sleep in such flowers (Sapir et al., 2006). In nocturnal species or species in which individuals must locate one another across long distances, these complementary periods of activity can gain additional importance. Indeed, Silvegren et al. (2005) demonstrated that in the absence of alternative zeitgebers, exposure to sex pheromone itself may serve as a weak cue to synchronize receptivity between males and females of the noctuid moth, *Spodoptera littoralis*. Silvegren et al. (2005) demonstrated that when the circadian rhythms of the sexes are in direct opposition, mating success and, ultimately, fitness are drastically reduced.
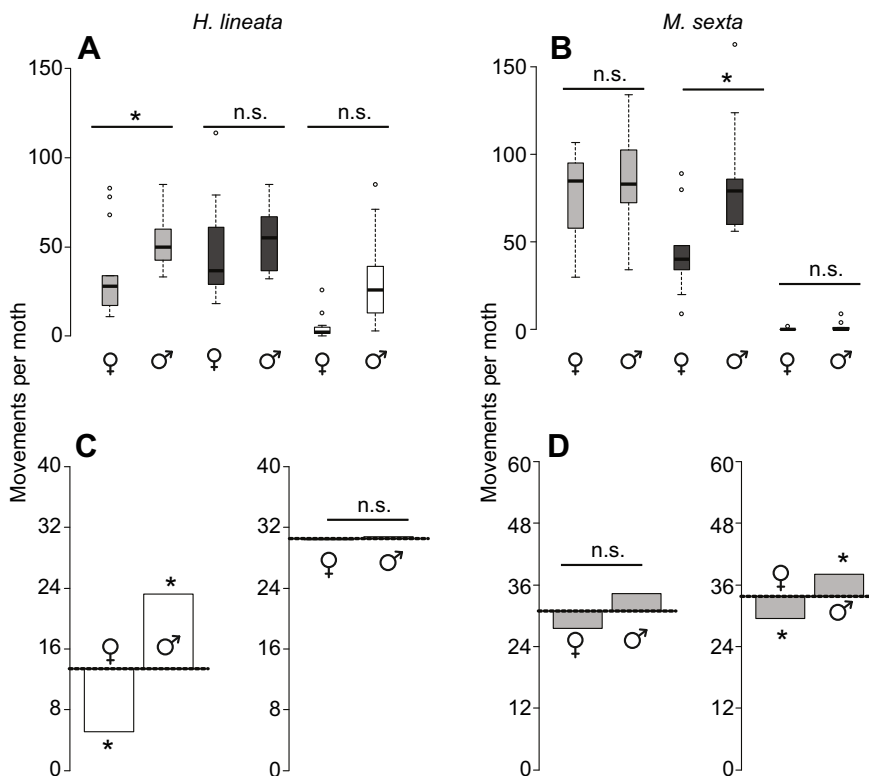

Fig. 8. Sex differences in total activity by treatment for both hawkmoth species. (A) *Hyles lineata* showed significant sex differences under control conditions as well as during exposure to constant light (LD, *P*=0.039; LL, *P*=0.02). (B) *Manduca sexta* showed significant sex differences in the constant dark treatment (DD, *P*=0.0055). (C) Analysis of total activity in male and female *H. lineata* compared with the expected mean during the light (left, 12.79 movements per moth) and dark (right, 30.21 movements) periods of the 16 h:8 h light:dark control treatment. (D) Analysis of total activity in male and female *M. sexta* compared with the expected mean during day 1 (left, 30.15 movements) and day 2 (right, 35.1 movements) of the constant darkness treatment. Expected means in C and D were determined as the mean values if the total number of movements made during the time period of interest was distributed equally regardless of sex. Treatment is indicated by shading: control, light gray shading; constant darkness, dark gray shading; constant light, white. n.s., not significant.

Collectively, our results suggest that synchronizing sexual receptivity between the sexes may be the underlying influence in shaping the circadian rhythmicity of *M. sexta* and *H. lineata*, rather than the proposed influence of periodic food availability. However, upon mating, female hawkmoths typically cease calling (Sasaki and Riddiford, 1984). As the stationary calling behavior appears to influence periods of female flight activity, this suggests that mated and virgin females may exhibit different patterns of behavior. While this is not observed in *M. sexta* (Sasaki and Riddiford, 1984), possibly due to the restricted time window of an 8 h scotophase, post-mating shifts in behavior have been reported in *Drosophila melanogaster* as well as the invasive *Drosophila suzukii* (Isaac et al., 2010; Ferguson et al., 2015) and the noctuid moth *S. littoralis* (Saveer et al., 2012). In the case of *H. lineata*, oviposition by mated females occurs throughout the day (Fig. S2), suggestive of a similar phenomenon in this species. Furthermore, the extensive temporal differences in the behavior of male and female *H. lineata* raise the possibility that the two sexes may exploit different floral resources while foraging, as floral rewards also are subject to species-specific diel rhythms (Stone et al., 1998; Martins and Johnson, 2007).

Author contributions
M.V.A. performed pilot studies that revealed the patterns explored here experimentally, and the importance of collecting data from individual moths. Experiments were designed by T.B., G.T.B. and R.A.R., data were collected by T.B. and G.T.B. and were analyzed by G.T.B. The manuscript was written by T.B. and G.T.B. and was edited by R.A.R. All coauthors have read and approved of the final text.

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