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Animal behaviour

Evidence for the circadian gene *period* as a proximate mechanism of protandry in a pollinating fig wasp

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Protandry in insects is the tendency for adult males to emerge before females and usually results from intra-sexual selection. However, the genetic basis of this common phenomenon is poorly understood. Pollinating fig wasp (Agaonidae) larvae develop in galled flowers within the enclosed inflorescences ('figs') of fig trees. Upon emergence, males locate and mate with the still galled females. After mating, males release females from their galls to enable dispersal. Females cannot exit galls or disperse from a fig without male assistance. We sampled male and female *Ceratosolen solmsi* (the pollinator of *Ficus hispida*) every 3 h over a 24 h emergence period, and then measured the expression of five circadian genes: *period* (*per*), *clock* (*clk*), *cycle* (*cyc*), *pigment-dispersing factor* (*pdf*) and *clockwork orange* (*cwo*). We found significant male-biased sexual dimorphism in the expression of all five genes. *per* showed the greatest divergence between the sexes and was the only gene rhythmically expressed. Expression of *per* correlated closely with emergence rates at specific time intervals in both male and female wasps. We suggest that this rhythmical expression of *per* may be a proximate mechanism of protandry in this species.

1. Introduction

Protandry, the tendency for adult males to emerge prior to females, is common in insects and can be explained by sexual selection [1,2]. Males that emerge early gain a mating advantage, especially in monandrous species, and these males can mate as females emerge or even when females are still within their pupae [2,3]. The underlying proximate genetic causes of this important, sexually dimorphic life-history trait are poorly understood. This lack of understanding persists despite extensive investigations into the 'circadian clock' genes that regulate the processes of insect migration [4], diapause [5] and mating behaviours [6]. However, the timing of adult emergence from pupae in insects has been reported to be influenced by at least one gene, *per*, associated with the 'circadian clock.' In *Drosophila*, increased or decreased doses of *per* can lead to earlier or delayed emergence from pupae, respectively [7].

Agaonid wasps are best known as the obligate pollinators of fig trees (*Ficus* spp.) and are ideal for investigating protandry. In addition to pollinating the trees, fig wasps gall individual flowers within the enclosed *Ficus* inflorescence (syconium, hereafter 'fig'), in which each larva develops. The wingless adult males do not leave their natal fig, are much smaller than females, and eclose

while the females are still in their galls [8]. The males then chew small holes in the walls of galls containing females to enable mating. After mating, males return to the females and enlarge the mating holes to enable the females to emerge into the central cavity of the fig. At the same time, some males chew holes in the fig wall to enable the fertilized (and now pollen laden) females to disperse. In addition to intra-sexual competition for matings, understanding protandry in pollinating fig wasps thus has further biological significance. Without male benevolence based on protandry, females cannot escape their galls and natal fig to carry pollen to, and reproduce within, the figs of other trees in the population [9]. However, until now data on how circadian clock genes may influence protandry in pollinating fig wasps are lacking.

To resolve this problem, we measured over a single day after eclosion the expression patterns of five major circadian genes, including *per*, in male and female *Ceratosolen solmsi*, the pollinator wasp species of *Ficus hispida*. This enabled us to identify whether any of these five genes contributed significantly to the overall molecular mechanism(s) promoting protandry in this species.

2. Material and methods

All fig and wasp samples used for this work were collected from Danzhou (19°52′ N, 19°52′ E), Hainan province, China, between July and August 2012. Initially, we identified five male *F. hispida* trees, each with a crop of receptive figs. We haphazardly selected 20 pollinated figs per tree (identified by the detached wasp wings on the outside of the entrance tunnel, the ostiole) and covered them with fine-mesh bags to prevent future colonization by parasitic wasps. All figs were left to mature, and then removed from the tree and brought to a laboratory room lit only with red light for wasp collection.

For three mature figs, we recorded male and female emergence patterns over time. Each fig was bisected and placed into a plastic container with a ventilated lid, and checked every hour for wasp emergence. After the first males were seen to emerge, each fig was then checked every 3 h. At the same time, the numbers of male and female wasps were counted. Disturbance to the emerging wasps was therefore minimized.

A total of 100 figs were used to collect wasps for gene expression analysis. Each fig was placed individually into a ventilated plastic container to allow the wasps inside to emerge from their galls naturally. Because male wasps release the females from their galls, different figs were used to collect male or female wasps ($N = 40$ and 60 figs used for males and females, respectively). Each fig was bisected just prior to male wasp emergence, and then checked hourly to determine the point when the first males eclosed from their galls. After the first males had emerged, they were removed. For male collection, figs were subsequently checked and all wasps that had eclosed were removed every 3 h. All wasps were immersed in liquid nitrogen within 15 min of collection, and then stored in Sample Protector (an RNA enzyme inhibitor; TAKARA, China) prior to processing for genetic analysis. Any females that had eclosed were also removed but these were discarded.

For the collection of female wasps, the same process was used as for males except that only the females were removed. Within figs used to collect females, males were not collected because males are needed to enable the females to eclose. To control for random effects between wasps emerging from different figs, wasps of each sex for each time emergence period (eight in all) were stored collectively, with 100 haphazardly chosen individuals of each sex collected for further investigation.

For gene expression measurements of male and female wasps that emerged at the eight different time points, we obtained partial coding sequences of five circadian genes: *per*, *clk*, *cyc*, *pdf* and *cwo* by PCR amplification. The 100 wasps of each sex collected for each time point since first male eclosion were haphazardly separated into three groups. This was because approximately 30 wasps were needed for RNA extraction owing to their small size. Each group was thus treated as a unit of replication for further real-time quantitative PCR (RT-qPCR) analyses. The primers used for PCR were designed using Primer Premier v. 5.0 (sequences are shown in the electronic supplementary material, table S1) [10]. For each gene, we used RT-qPCR to analyse the daily transcript levels (primers are listed in the electronic supplementary material, table S2). Partial coding sequences of the five *C. solmsi* genes investigated were deposited in GenBank (see the electronic supplementary material).

3. Results

(a) Emergence patterns of male and female wasps

Most males eclosed soon after the first male was seen moving freely in the fig cavity, with emergence rates declining gradually for 24 h, after which eclosion ceased (figure 1*a*). As expected, females mainly emerged later than males with an emergence peak 24 h post first male eclosion (figure 1*a*).

(b) Sexual dimorphism in gene expression

The expression levels of all five genes were significantly higher in males than in females (table 1). In addition, the expression ratios between males and females, which are likely to reflect levels of sexual dimorphism in gene expression, varied among the five genes, with values of 4.42, 2.46, 76.80, 7.67 and 8.55 for *clk*, *cyc*, *per*, *cwo* and *pdf*, respectively (electronic supplementary material, figure S1). Among these five genes, *per* thus had the largest gene expression divergence between the sexes.

(c) Expression of *per* in males and females that eclosed at different times

Of the five genes we examined, only *per* was expressed significantly differently according to time after first male eclosion in both males (repeated measures ANOVA: $F_{2,7} = 369.80$, $p < 0.001$) and females (repeated measures ANOVA: $F_{2,7} = 23.66$, $p = 0.001$). The other four genes, *clk*, *cyc*, *pdf* and *cwo*, were expressed arrhythmically (electronic supplementary material, figure S2). In other words, only *per* was expressed rhythmically. Mean relative mRNA levels for *per* correlated closely with the timing of eclosion since first male eclosion negatively in males and positively in females. This resulted in peak expression 3 h post eclosion in males, and at 24 h post first male eclosion in females (figure 1*b*).

4. Discussion

We show in a pollinating fig wasp a distinct male-biased sexual dimorphism of the expression of five genes associated with insect circadian rhythms. This is especially pronounced for *per*, previously linked to time-to-eclosion in *Drosophila* [7]. Our data are thus consistent with *per* being under stronger selection in male fig wasps than in females owing to sexual competition between males for the earliest access to females
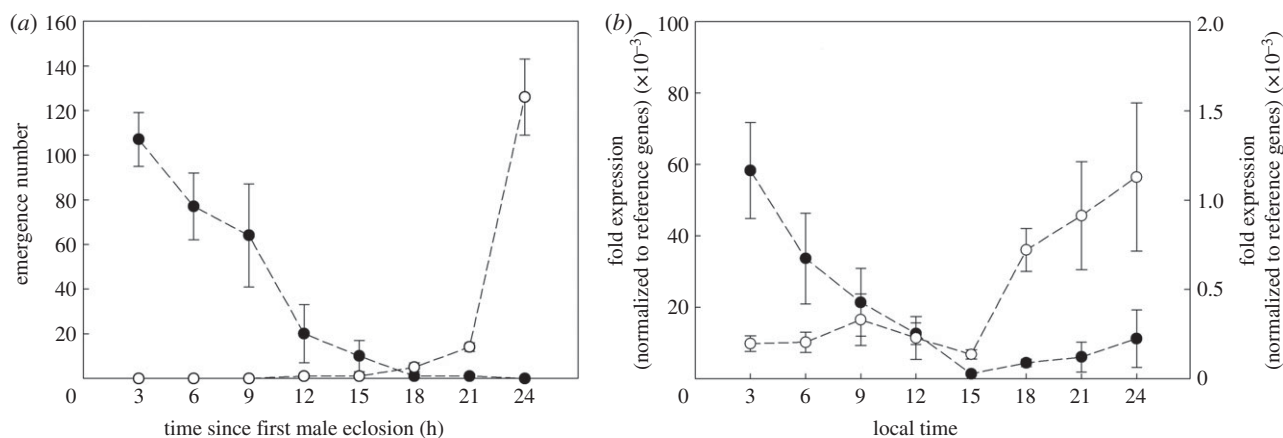
Figure 1. (a) The emergence frequency and (b) the transcript abundance of *per* at eight time points spread over 24 h, starting with the point (time 0) the first male was seen to eclose. Data points are: (a) mean ($\pm$ s.e.) numbers of wasps that eclosed in the 3 h since the previous counts were recorded, and (b) mean ($\pm$ s.e.) relative mRNA levels. Solid circles represent values for males (refer to the left vertical axis scale for (b)), and open circles represent values for females (refer to the right vertical axis scale for (b)).

Table 1. Sexual dimorphism for the expression of five circadian genes in *C. solmsi*. Values (fold expression (normalized to reference genes)) given are overall means $\pm$ s.e. *F*-values are ANOVA results (d.f. = 1, 47 for all tests) and all are significant ($p \leq 0.001$).

	males	females	F
clk	1.125 $\pm$ 0.116	0.367 $\pm$ 0.069	29.33
cyc	0.516 $\pm$ 0.056	0.243 $\pm$ 0.052	12.62
per	0.0186 $\pm$ 0.0044	0.0005 $\pm$ 0.0001	16.80
cwo	0.321 $\pm$ 0.038	0.054 $\pm$ 0.006	46.56
pdf	0.238 $\pm$ 0.039	0.036 $\pm$ 0.006	25.94

[2,11], which are likely to mate only once [12]. Maximum *per* expression concurs with maximum eclosion rate in both sexes, even though females rely on males to eclose, suggesting that *per* expression may reflect development rate rather than influence mechanisms facilitating efficient emergence from galls.

The specialist adaptations of male pollinating fig wasps (small size; reduced legs, eyes and pigmentation; lack of wings) are likely to allow faster development than for females [8]. This dimorphism may thus increase any effects of different *per* expression between the sexes. Moreover, because females disperse into the wider environment and males do not, they will be exposed to more agents of selection than males. For instance, there may be disruptive or stabilizing selection in females for time-of-day emergence owing to costs associated with diurnal/nocturnal dispersal, or owing to pleiotropic effects of selection acting on other traits. The specialized biology of fig wasps may therefore result in the effects of selection on some traits shared by males and females (e.g. development time as larvae; time of emergence) being more constrained in females, which may contribute to

the overall levels of sexual dimorphism in circadian gene expression we found.

Of the five tested circadian rhythm genes in *C. solmsi*, only *per* was rhythmically expressed. Expression of *per* closely matched the different patterns of male and female emergence over time, especially the emergence peaks, which were spaced by 21 h. This probably reflected a time lag between males emerging, finding galls containing females and mating, and then returning to the mated females to ensure their eclosion. Our data thus support previous findings that increased doses of *per* reduce time-to-eclosion in *Drosophila* [7]. Moreover, because male emergence rates and *per* expression were more variable than those of females which eclosed soon after the first males (figure 1*a*,*b*), this may reflect different competitive capabilities of individuals. We expect that future investigations into any relationship between *per* expression and variation in the mating success of male pollinating fig wasps will be informative. Finally, previous studies have shown that circadian clock genes in insects can be driven by diverse mechanisms [13]. For instance, the circadian clock of honeybees *Apis mellifera* is affected by loss of the *cry1* gene. Circadian gene expression in pollinating fig wasps may therefore be influenced by similar processes and thus demand more detailed attention in the future.

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