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Adaptive evolution of the circadian gene *timeout* in insects

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Most insects harbor two paralogous circadian genes, namely *timeout* and *timeless*. However, in the
Hymenoptera only *timeout* is present. It remains unclear whether both genes, especially *timeout* in
hymenopteran insects, have distinct evolutionary patterns. In this study, we examine the molecular
evolution of both genes in 25 arthropod species, for which whole genome data are available, with addition of
the daily expression of the *timeout* gene in a pollinating fig wasp, *Ceratosolen solmsi* (Hymenoptera:
Chalcidoidea: Agaonidae). *Timeless* is under stronger purifying selection than *timeout*, and *timeout* has
positively selected sites in insects, especially in the Hymenoptera. Within the Hymenoptera, the function of
timeout may be conserved in bees and ants, but still evolving rapidly in some wasps such as the chalcids. In
fig wasps, *timeout* is rhythmically expressed only in females when outside of the fig syconium but
arrhythmically in male and female wasps inside the syconium. These plastic gene expressions reflect
adaptive differences of males and females to their environment.

T imeless[1] and timeout[2] are paralogous genes in animals. Phylogenetic analyses suggest that *timeless* origi
nated as a duplication of *timeout* around the time of the Cambrian explosion[3]. In insects, both *timeless* and
timeout occur in fruit flies, mosquitoes, butterflies, moths, *Tribolium* beetles and pea aphids, whereas
hymenopteran insects have only *timeout*[4]. *Timeless* is reported to function as a canonical circadian clock gene in
Drosophila and some other insects[5–7]. In contrast, *timeout* is a multifunctional gene, which plays an essential role
in the maintenance of chromosome integrity, light entrainment of the circadian clock, embryonic development,
and regulation of DNA replication[8–10]. Recent studies have provided evidence that *timeout* also plays a critical role
in the circadian clock[11]. This functional divergence suggests that *timeout* and *timeless* may have distinctive
evolutionary patterns. In addition, hymenopterans have lineage-specific loss of *timeless*, and to compensate
for the function of *timeless*, natural selection may have driven the evolution of *timeout*.

In this study, we used gene structure analysis on *timeout*, and evolutionary selection analysis on the *timeout/
timeless* family, in 25 species of arthropods to gain further insights into the evolutionary history of this gene
family. We also characterized the daily expression of *timeout* in a pollinating fig wasp, *Ceratosolen solmsi*
(Hymenoptera: Agaonidae), to examine in detail plasticity of gene expression according to the ecology of this
species.

Results

Gene structure and evolutionary analyses on *timeout*. We obtained orthologous data for the *timeout* gene from 25
arthropod species which have had their entire genomes sequenced. Our gene structure analysis detected that *timeout*
has varied gene lengths, with the maximum length of 305.6 kb in *Megachile rotundata* (Hymenoptera: Apoidea:
Megachilidae) and a minimum length of 3.09 kb in *Tetranychus urticae* (Arachnida: Trombidiformes:
Tetranychidae). Hymenopteran insects tended to have longer *timeout* genes than non-hymenopteran insects
(Fig. 1a, orange bar vs. blue bar). This divergence was due not to exon length (Table S1), but longer introns
(average total-intron-length-of-*timeout* of 141,932 bp) in hymenopterans compared with all other arthropods
(average total-intron-length-of-*timeout* of 16,395 bp). We also detected one actively transcribing gene "nested"
in the intron of *timeout* in all hymenopterans as well as in *Tribolium* and *Drosophila* (species in red branches, Fig. 2).
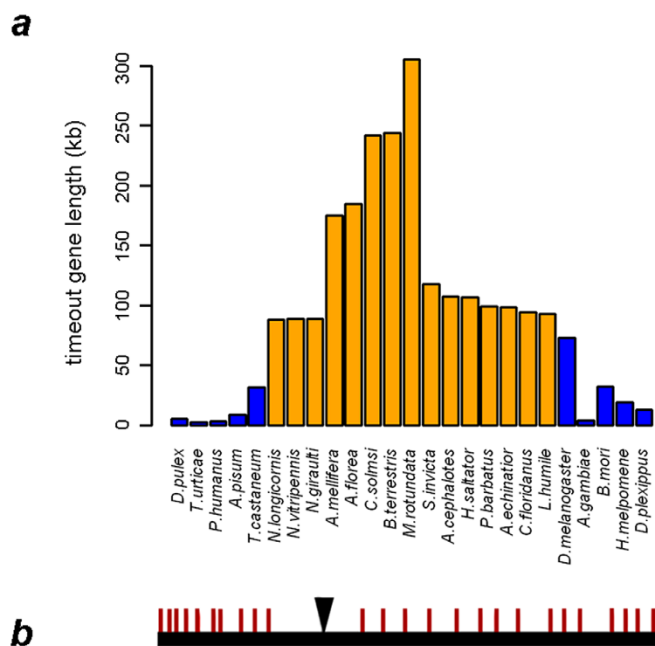
Figure 1 | Gene structure analyses on *timeout*. (a) Variation of gene lengths among arthropods. The orange columns represent hymenopteran species, and the blue columns represent non-hymenopteran species. (b) The genomic structure of *timeout* in *Ceratosolen solmsi*. The red bars represent exons, and the black reversed triangle denotes the nested gene, putatively encoding an insulin growing banding protein.

The gene structure of *timeout* of *C. solmsi* was shown in Fig. 1b. This spans 241.73 kb in the scaffold and comprises of 25 exons and 24 introns, with the tenth intron harboring one actively transcribing gene.

A phylogeny was constructed based on *timeout* sequences for all 25 species. The hymenopterans, which have no *timeless* gene, clustered into a single clade (Fig. 2, branch a). Based on this gene tree, we tested if *timeout/timeless* had distinct evolutionary patterns by using site- and branch-site models nested in PAML (Table 1)[12]. Potential positive selection was tested based on the ratio (ω) of non-synonymous (Ka) to synonymous (Ks) substitutions rates (ω = Ka/Ks). Generally, if ω = 1 amino acid substitutions were assumed to be largely neutral; $\omega > 1$ is evidence of positive selection, $\omega < 1$ was consistent with purifying selection. Five models were used to test for positively selected sites: M1, M2, M7, M8, and M8a. We employed site models M7 vs. M8 to test for positive selection of specific codons. The results showed that both genes were under purifying selection, but selection pressure on *timeless* was stronger than that on *timeout* (one-ratio ω value of 0.00925 for *timeless*, and 0.08332 for *timeout*, table S2). The results from the site-model (M7 vs. M8) indicated that *timeout* had one positively selected site (242 V, ω = 1.10056) (Table S2). Our branch-site model results further demonstrated that *timeout* had positively selected sites on almost all branches (Fig. 2, branches a, b, c, f, g, h, and i) except those of ants and bees (Fig. 2, branches d and e). In general, more significant positive sites were present in the Hymenoptera than other lineages (Table S2). For example, the fig wasp *C. solmsi* had one significant positively selected site (45 S, Table S2).

Daily expression of *timeout* in the fig wasp *C. solmsi*. We measured daily levels of *timeout* mRNA in both female and male fig wasps using real time qPCR. At the early stage of development, both males and females develop within the fig syconium, and after mating, only the females leave the fig syconium. Taking the ecological microcosm of the fig syconium into consideration, three sample groups were allocated: 1) female wasps collected from within the syconium cavity; 2) male wasps collected in the same manner, and 3) female wasps that had successfully emerged from their natal syconium, and had been exposed to natural light for at least three hours. Repeated measures ANOVAs were employed to show the expression values of *timeout* fluctuate over time (Fig. 3). In addition, we performed cosinor analysis[13] (http://www.circadian.org/softwar.html) to reveal if *timeout* is rhythmically expressed. The parameters used for these tests on cosinor software were: start at period 8 h, and end at period 34 h, with incremental steps being set at 0.1. The results showed that *timeout* is only rhythmically expressed in females that had successfully dispersed from their syconium ($F_{2, 5}$ = 6.35, P = 0.043), but arrhythmic in both males ($F_{2, 5}$ = 5.77, p = 0.051) and females ($F_{2, 5}$ = 5.66, p = 0.050) still within the syconium.

Discussion

Phylogenetic analysis for the *timeless/timeout* family suggest that these two paralogous genes evolved from gene duplication in early animals[3]. Therefore, possession of only one of these genes in some insects represents lineage-specific gene loss events. Among the arthropods we examined, only hymenopterans, including ants, bees and wasps, possessed *timeout* but not *timeless*. It is likely that the loss of *timeless* occurred in their common ancestor. Moreover, we discover that *timeout* is significantly longer in the Hymenoptera compared to the other insects mainly due to long introns. These long introns may harbor undetected information in regulating the expression of this gene[14].

Our analyses of the *timeless/timeout* family in arthropods reveals distinct evolutionary patterns of each gene. *Timeless* is under stronger purifying selection than *timeout*, and both site- and branch-site models provide evidence of positive selection sites for *timeout*. The strong purifying selection for *timeless* is predictable, because the function of *timeless* as a component of the canonical circadian clock is clearly conservative. However, considering its multifunctional nature, *timeout* should be subject to stronger positive selection to maintain its variable function in different lineages. In the Hymenoptera, and in the absence of the function conferred by *timeless*, *timeout* may have been subject to stronger positive selection to compensate for the absence of *timeless*. The stronger signal of positive selection in this lineage supports this possibility. Previous studies have also supported this hypothesis for another circadian gene, *cryptochrome*[15]. Although in general hymenopterans had more positively selected sites than did other lineages, evidence of positive selection in taxa that diverged from the common ancestor of ants and bees was absent. We suggest that the function of TIMEOUT has been conserved in these lineages. However, for parasitoid fig wasps and *Nasonia*, *timeout* may have evolved rapidly under positive selection for adaptations associated with their distinct ecological conditions[16]. For example, we detected strong signals of positive selection at one site in *C. solmsi*. We further examined the functional role of the only positive selection site, Serine (S), in *C. solmsi* by mapping it onto the predicted secondary structure of TIMEOUT (Fig. S1). In this amino acid position, compared with the amino acids Leucine (L), Isoleucine (I), Tyrosine (Y) or Asparagine (N), which occur in all the other arthropods, Serine is a polar amino acid, which readily forms a random coil structure[17]. This random coil structure usually includes the active sites of enzyme and functional sites of proteins[18,19]. All of our results suggest that *timeout* may show plastic expressions associated with adaptations to ecology or life-history, especially in wasps, all of which lack *timeless* but have the positively selected *timeout*.

Fig trees (*Ficus*: Moraceae) have an obligate mutualism with their pollinating wasps (Hymenoptera: Agaonidae)[20]. Wasps gall individual flowers and develop within the characteristic enclosed inflorescences (syconia) of the trees. Male wasps do not leave their natal syconium. Only females disperse from the syconium upon maturation, through exit tunnels chewed by males[21]. The ecological and life-
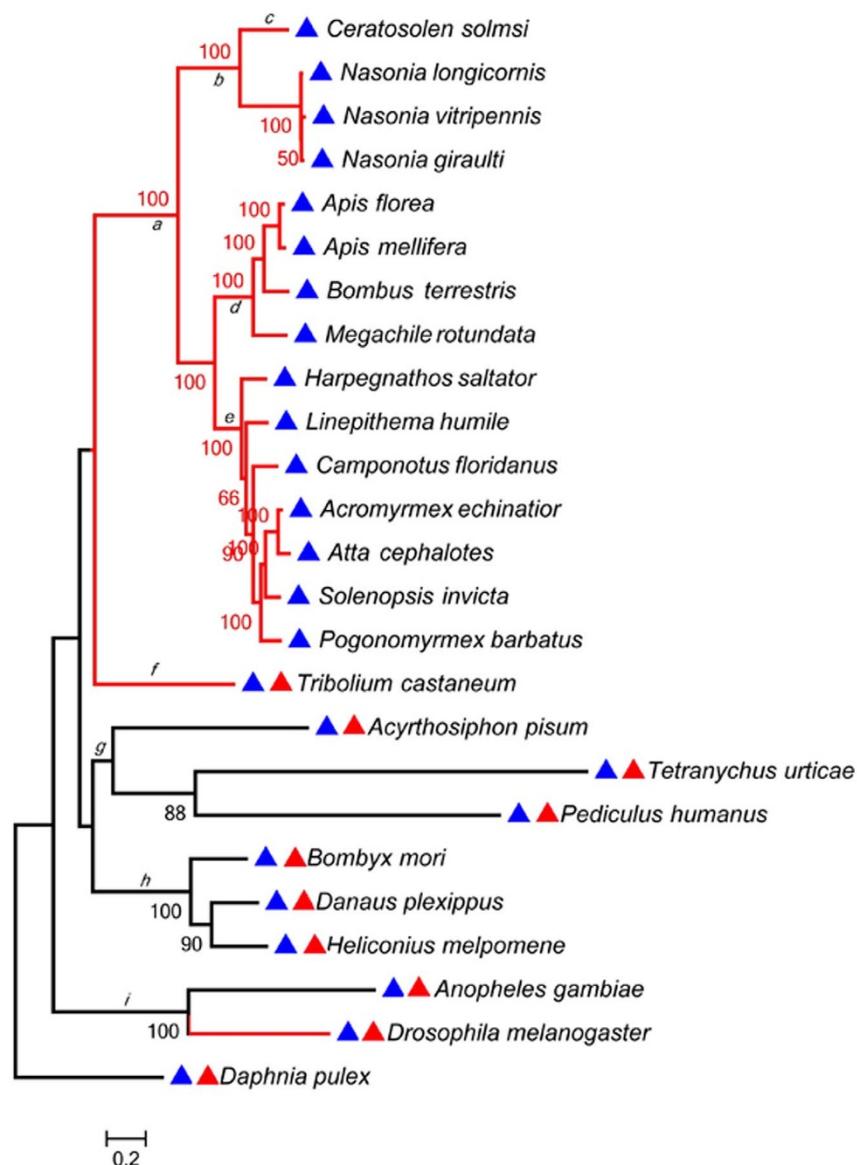
Figure 2 | The phylogeny of *timeout* in 25 species of arthropod. A red triangle before each species denotes the presence of *timeless* in a species, with a blue triangle denoting the existence of *timeout*. The branches *a* to *i* are the lineages tested for positive selection by a branch-site model using PAML software. The red colored branches denote the species in which a ''nested'' transcribed gene was detected in the intron of *timeout*.

history differences between the sexes may thus explain the expression patterns of circadian genes. Our results show that in both males and females still inside the syconium cavity arrhythmically expressed *timeout*. However, females that have successfully dispersed from their natal syconium show rhythmic expression of *timeout*. This

may reflect differences in exposure to light because light is absent within a syconium. Previous studies of other insects have shown that the expression of *timeout* is light-dependent. For example, *timeout* is needed for circadian photoreception in *Drosophila*[8] and has circadian expression in the mammalian retina[22,23]. We suggest that

Table 1	Tests for positive selection using site- and branch-site models				
Model	Models Compared	-2ln∆L	df	p value
site model	M7 versus M8	19. 568712	2	p < 0.001
branch-site model	Branch-site a (Hymenoptera)	54.141658	2	p < 0.001
	Branch-site b (wasp)	9.816718	2	p = 0.007
	Branch-site c (*C. solmsi*)	10.708974	2	p = 0.0047
	Branch-site d (bee)	13.471594	2	p < 0.001
	Branch-site e (ant)	2.762636	2	p = 0.25
	Branch-site f (*T. castaneum*)	35.287362	2	p < 0.001
	Branch-site g (Hemiptera)	7.42465	2	p = 0.012
	Branch-site h (Lepidoptera)	40.693252	2	p < 0.001
	Branch-site i (Diptera)	31.38715	2	p < 0.001
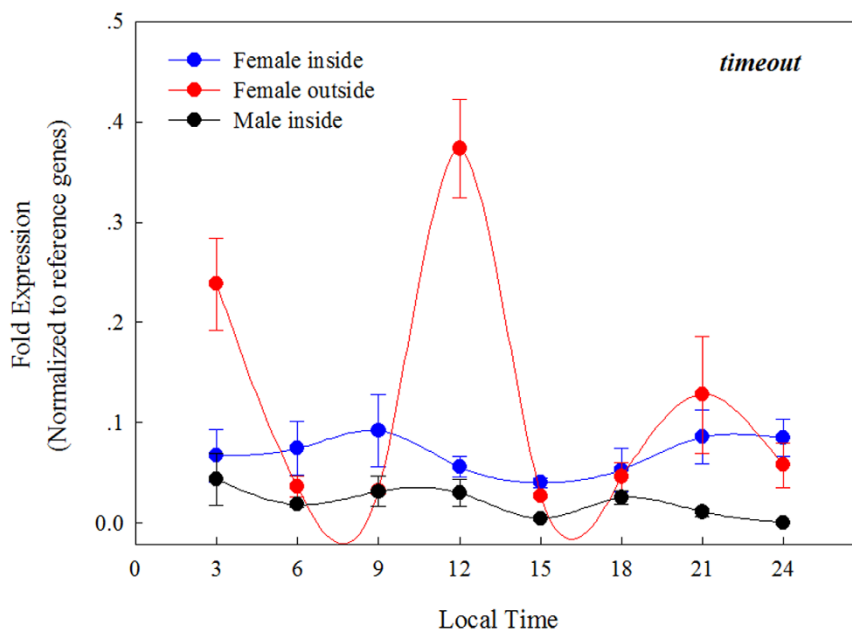
Figure 3 | The expression of *timeout* in the fig wasp *Ceratosolen solmsi* over a 24 h period in males (that do not leave their syconium), females still within their syconium, and females that had naturally dispersed from their syconium.

the expression of *timeout* may thus affect the light input pathway in *C. solmsi*, which is adaptive due to light changes between the inside and the outside of the syconium. However, another factor, temperature also exerts an important influence on the expression of circadian genes[24]. Thus, differences in temperature within and outside the syconium may also affect the expression of *timeout* in *C. solmsi*. Contrary to our previous studies on the expression of opsin genes[25], the clear shift from arrhythmic to rhythmic expression of *timeout* in female fig wasps as they move from the syconium cavity to the outside of the syconium, suggests an environmental determinant of *timeout* expression. In addition, our closer examination of *timeout* expression in *C. solmsi* provides a good model system to test in the future if evolutionary compensation affects the loss of *timeless*. Further studies of additional hymenopteran species are needed to clarify the role *timeout* plays in the absence of *timeless*.

Methods

Phylogenetic analysis. We searched public genomic DNA databases at NCBI[26], FlyBase[27], *Bombyx mori* genome[28] and BeeBase (http://racerx00.tamu.edu/bee_resources.html) for genes encoding homologs of known *timeout/timeless* proteins using TBLASTN[29]. The FGENESH+ gene predictor (http://linux1.softberry.com/berry.phtml) was occasionally used to improve the translation. The searched and selected gene sequences (all the sequences used in this study are shown in Table S3) were aligned using ClustalW implemented in MEGA 5.0[30]. We improved the alignment based on the translated protein sequences. We translated the codon sequences into protein sequences, and performed an alignment based on these protein sequences. We then re-translated the aligned protein sequences into codon sequences, which were used to instruct the improvement of the real alignment of the codon sequences. The alignment was manually edited using BioEdit[31] (For the manually edited sequences before and after deleting gap, see supplementary information). A maximum likelihood (ML) tree was constructed using PhyML3.0[32], with the best-fit model of nucleotide substitutions of GTR + G, according to the Akaike Information Criterion in jModeltest[33]. One thousand ML bootstrap replicates were obtained to assess clade robustness. *Timeout* data from a crustacean species, *Daphnia pulex*, were set as the outgroup.

Selective pressure analysis. Maximum likelihood methods were used to explore the selective pressure acting on *timeout*, and all tests were conducted using the CodeML in PAML 4[12]. Potential positive selection was tested based on the ratio (ω) of non-synonymous (Ka) to synonymous (Ks) substitutions rates (ω = Ka/Ks). To evaluate positive selection on *timeout* across the examined arthropod species, we first used the site-specific models, M7 restricted sites with ω ≤ 1, whereas models M8 included a class of sites with ω > 1. The sites with a posterior probability >0.9 were considered as candidates for selection. Positive selection was further detected with the improved branch-site likelihood method[34]. Test 1 (M1a vs. Branch site model) and test 2

(Branch site null model vs. Branch site model), which could differentiate positive selection from the relaxation of selective constraints, were used. The branch leading to the Hymenoptera (a), parasitoid wasps (b), *Ceratosolen solmsi* (c), bees (d), ants (e), *Tribolium castaneum* (f), Hemiptera (g), Lepidoptera (h), and Diptera (i), were labeled as the foreground lineage to test whether positive selection occurred along these branches. For each model, the ratio of ω was estimated, and likelihood ratio tests (LRT) were performed to compare pairs of nested models. We calculated twice the difference in log-likelihood values between the two models against a chi-square distribution. When the LRT was significant, a Bayes Empirical Bayes (BEB) analysis was used to identify positively selected sites. In addition, we obtained the secondary structure of TIMEOUT in *C. solmsi* by using the SOPMA Server (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html)[35]. This positively selected site was mapped onto the resulting figure (Fig. S1).

Sample collections, RNA isolation, cDNA synthesis and real time qPCR expression analysis of the fig pollinator wasp, *Ceratosolen solmsi*. We made field collections of the fig wasp *C. solmsi*, the pollinator of *Ficus hispida*, from Danzhou (19°30′ N, 109°29′ E), Hainan province, China between July and August 2012. Adult females and males were collected from naturally growing syconia when the loose female wasps were yet to emerge. (Male wasps do not emerge from the syconium.) We also collected adult females from different syconia. These females were exposed to natural light for at least three additional hours prior to processing.

After collection, all wasps were flash frozen in liquid nitrogen every 3 hr over a 24 h period. We then isolated total RNA from each wasp, with the first cDNA strand synthesized using TransScript II First Strand cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China). Ultimately, we used a Real Time-qPCR (RT-qPCR) technique to obtain daily transcript levels of *timeout*. *RPL13a* and *UBC* were selected as the reference genes for normalizing the RT-qPCR data[15]. The detailed methods of samples collection, RNA isolation and cDNA synthesis, and RT-qPCR expression analysis are provided as supplement material.

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Acknowledgments

Author contributions

H.F.G., J.H.X. and D.W.H. designed the work and wrote the main manuscript text. H.F.G., L.M.N. and G.C.M. collected the sample. H.F.G. demonstrated the molecular experiments. H.F.G., J.H.X. and B.W. analyzed the data. D.W.D. participated in the writing and revisions. All the authors have reviewed and approved the manuscript.

Additional information