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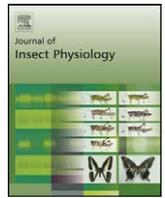
Circadian rhythms of adult emergence and activity but not eclosion in males of the parasitic wasp Nasonia vitripennis

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ABSTRACT

An endogenous circadian system is responsible for the rhythms observed in many physiological and behavioural traits in most organisms. In insects, the circadian system controls the periodicity of eclosion, egg-laying, locomotor and mating activity. The parasitoid wasp Nasonia vitripennis has been extensively used to study the role of the circadian system in photoperiodism. In this study, behavioural activities expected to be under the control of the endogenous circadian system were characterized in Nasonia. Male emergence from the host puparium is rhythmic under light–darkness conditions while eclosion from the own pupal integument is not rhythmic but continuous. Following entrainment in light–dark conditions, males show robust free-running circadian activity rhythms with a period (τ, tau) of approximately 25.6 h in constant darkness. While the endogenous circadian system is enough to trigger male emergence in Nasonia, light seems to have a modulatory effect: when present it induces more males to emerge. Our results add to the understanding of chronobiological phenotypes in insects and provide a basis towards the molecular characterization of the endogenous circadian system in Nasonia.

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1. Introduction

Day–night cycles evoked by the rotation of the earth around its axis have had profound influence on shaping physiological and behavioural patterns in organisms. Activities requiring a circadian (~24 h) regulation became controlled by an endogenous genetic mechanism, the molecular clock, whose functioning has been characterized in various organisms (Bell-Pedersen et al., 2005). In insects, the clock controls the circadian rhythms of major traits (Hall, 2003; Saunders et al., 2002), like eclosion (Pittendrigh, 1954), oviposition (Howlader and Sharma, 2006), mating (Sakai and Ishida, 2001), and locomotor activity (Petersen et al., 1988). Besides setting the pace of the near-24 h rhythm, the endogenous circadian system (or some of its components) also affects cyclic activities that have a period longer or shorter than 24 h. Examples are the circannual regulation of physiological states, like luteininzing hormone (LH) and prolactin secretion in mammals (Malpaux et al., 2001) or diapause in insects (Denlinger, 2002). A well known example for ultradian rhythms (rhythms whose frequency is significantly shorter than 24 h) is the frequency by which wings vibrate during male courtship behaviour in different Drosophila species (Kyriacou and Hall, 1980).

Two classical traits in insects displaying circadian rhythmicity are activity-rest patterns and eclosion. In Drosophila melanogaster, activity cycles are bimodal and concentrated around light-on and light-off (Helfrich-Förster, 2001). Different insect species display activity patterns that reflect their diverse life-history characteristics (Lewis and Taylor, 1965). In the absence of external entrainment, as for instance in constant darkness, the rhythm free-runs according to the insect own endogenous circadian system with a period (tau, τ) characteristic of the species (Saunders et al., 2002). Eclosion rhythms have been characterized in different insect species. In most cases, eclosion from the own pupal integument is gated by the endogenous circadian system to a restricted period of the day (Myers, 2003). In Drosophila pseudoobscura, eclosion occurs in the hours immediately preceding or following the onset of light, depending on the photoperiod (Pittendrigh, 1954). Individuals that reach maturity outside the gate will eclose at the next following gate, usually 24 h later. In general, eclosion in insects shows a circadian rhythm, is endogenously controlled, and is temperature-compensated, all features typical of a circadian output (Myers, 2003). As already observed for activity rhythms, also eclosion displays species-specific characteristics. In A. pernyi, the silkmoth, eclosion occurs later in the afternoon (Truman and Riddiford, 1970). In the hymenopteran Trichogramma embryophagum instead, eclosion from the own pupal integument is not rhythmic while emergence from the host puparium follows a circadian rhythm (Reznik et al.,

2008). These data indicate that the influence of the circadian system on traits is very plastic and can be shaped by natural selection according to the particular life-history needs of each species. In some instances, particular phenotypic adaptations have been linked to specific variations in the use or sequence of the genes underlying the endogenous clock. For example, in addition to determining species-specific wing vibration frequency, different *period* alleles cause two *Drosophila* species to mate at different times of the day (Tauber et al., 2003). In honey bees, circadian fluctuations of *period* transcript levels are associated with division of labour (Toma et al., 2000). Cryptochrome 1 and 2 are central components of an antennal clock that enables the monarch butterfly *Danaus plexippus* to show sun compass orientation (Merlin et al., 2009). Although these are well studied traits, the genetics underlying the adaptation of the clock to specific ecological needs is still poorly understood. With the advent of molecular and genomic techniques, a broader range of organisms becomes amenable to address these issues.

The hymenopteran parasitoid *Nasonia vitripennis* has a long-standing tradition in chronobiological research. Through the work of Saunders (1968, 1974), the study of diapause in *Nasonia* provided crucial insights into photoperiodic time measurement. However, the genetic underpinnings of photoperiodism have only recently started to be uncovered (Wolschin and Gadau, 2009). Beside diapause, other *Nasonia* traits that are expected to be affected by the endogenous circadian system have hardly been investigated. Examples include locomotor activity, parasitization activity, pupal eclosion, emergence from the host puparium, and mating behaviour. The sequenced and annotated genome of *Nasonia* (Werren et al., 2010) makes it now possible to efficiently link studies of behaviour with its underlying genetic architecture. As other parasitoids, *Nasonia* wasps, after having eclosed from their own pupal integument, have to emerge also from the host puparium (Whiting, 1967). In order to maximize fitness, relevant life-history traits in many animal species are typically associated with particular periods of the day–night cycle. Considering that *Nasonia* wasps are confronted with similar selection pressures (i.e. finding a mate, avoiding predators), it may be that eclosion from its own pupal integument (from now on termed simply *eclosion*) and/or emergence from the host puparium (from now on indicated as *emergence*) is under circadian control as known for similar traits in other insects (Saunders et al., 2002). In this study we analyze circadian activity, eclosion, and emergence in males of *N. vitripennis* under light–darkness and constant darkness conditions. These behavioural assays serve as starting point for genetic analyses of chronobiological traits.

2. Materials and methods

2.1. Animals stocks and rearing

N. vitripennis (Hymenoptera: Pteromalidae) is a parasitoid wasp that parasitizes fly pupae of different genera. *Nasonia* wasps have haplo-diploid reproduction (females are diploid and males are haploid), and mated females produce broods with females and males, whereas unmated females can produce only males. After having drilled a hole into the fly puparium, females inject venom into the fly pupa onto which eggs are subsequently laid. At 25 °C offspring hatch within 48 h and begin feeding from the fly pupa. Approximately 8 days after oviposition the larvae pupate and 6 days later adults eclose from the pupal integument. Next, males chew an exit hole through the host puparium and emerge. If present, like in mixed broods, *N. vitripennis* females emerge after males have emerged.

The laboratory inbred strain *N. vitripennis* AsymC, whose genome has now been sequenced (Werren et al., 2010), was used for all the experiments. This wild-type strain was collected in The Netherlands and maintained in the lab since 1971 (van den Assem and Jachmann, 1999). Wasps were reared in mass culture vials (70 mm × 20 mm) at 25 °C, constant light and around 45% relative humidity. For standard maintenance, about 25-30 wasps (females with some males) were transferred to new vials containing about 50 *Calliphora* sp. fly pupae, on which *N. vitripennis* females parasitize. After 14 days (at 25 °C) the emerging progeny was rehosted on fresh pupae in a similar way. *Calliphora* flies were obtained as last instar larvae from a commercial manufacturer (Kreikamp & zn, Hoevelaken, The Netherlands). After pupation at room temperature in the lab, fly pupae were maintained at 4 °C and used within 4 weeks.

2.2. Recording circadian activity rhythms

Activity rhythms were measured with the actometer depicted in Fig. 1. Adult *Nasonia* wasps from the mass culture were placed individually in a well of a 96-wells plate (Greiner Bio-One, Alphen a/d Rijn, Netherlands). Wells were closed with the lid obtained from a 0.5 ml polypropylene tube (Sarsted, Etten-Leur, The Netherlands). Approximately 7 µl of a 10% (w/v) honey/distilled water solution (8% final sugar concentration) were pipetted into the internal part of the lid at the beginning of the recording period to feed the wasps. Entrainment light (approximately 30 lx) for the animals was provided by a standard 8 W TL (tube light). Activity was recorded with a black/white CCD camera (Conrad Electronics, Hirschau, Germany) by constantly illuminating the set-up with infrared (IR) light. An IR filter on the CCD camera objective lens allowed recording only IR input from the set-up. A white light filter prevented TL light to directly illuminate the CCD camera and increased the image contrast (i.e. wasps are black). The actometer was placed in an incubator that allowed light, temperature, and humidity programming. Activity measurements were performed at 20 °C and 50% relative humidity. The CCD camera was connected to a PC and operated by in-house software which calculated, for a defined image sector (i.e. occupied by a well), the number of pixels that changed in light intensity between two consecutive images

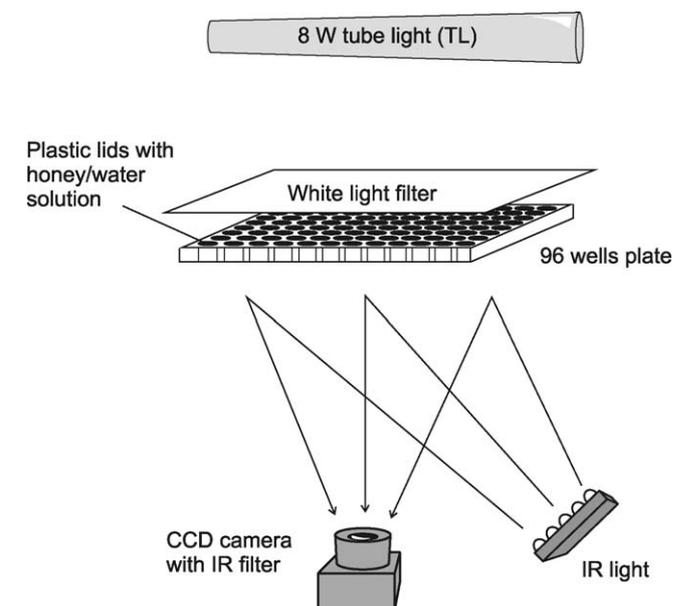**Fig. 1.** Actometer set-up used in the experiments. Wasps are kept inside a 96 wells plate (one wasp per well) with a plastic lid and entrained to light-darkness rhythms by a 8 W TL light (30 lx). A b/w CCD camera records only objects illuminated by IR light. An IR filter on the CCD camera lens blocks TL light. A white light filter prevents TL light from dazzling the CCD camera. See materials and methods for details.

taken over a specified time interval (i.e. 1 ms) by the CCD camera. The output was the average of pixel changes over a specified time interval (i.e. 1 min). Each wasp was recorded individually. Activity rhythms data were plotted and analyzed with ChronOSX v1.0.7β (Roenneberg and Taylor, 2000).

2.3. Parasitization, lighting and counting

For measuring emergence and eclosion, single virgin female wasps were placed in a cotton-plugged 60 mm × 10 mm polystyrene tube (Greiner Bio-One, Alphen a/d Rijn, Netherlands) with two or four hosts (as specified below) and allowed to parasitize for consecutive periods of 8 h (01:00–09:00, 09:00–17:00, 17:00–01:00 h) in constant light (approximately 250 lx inside the incubator). Every 8 h period of collection of parasitized hosts is called a "parasitization set", so that there are three parasitization sets per 24 h. After every 8 h parasitization round, parasitized hosts were replaced by fresh ones and transferred to experimental conditions. Offspring males emerging from the hosts were counted (and removed thereupon) at regular intervals. Counting in darkness was performed under IR vision as Nasonia wasps are sensitive to light intensities even in the far-red range (Saunders, 1975). For measuring eclosion from the own pupal integument, male wasps at the black pupal stage (just before eclosion) were removed from inside the host puparia, left inside the polystyrene tube, and counted upon eclosion. For all experiments, the 8 h darkness period in 16 h light:8 h darkness (LD 16:8) conditions was from 01:00 to 09:00 h. Temperature was constantly kept at 25 °C. Because of the large variation among females in total offspring produced, in order to be able to visualize the temporal distribution of eclosion and emergence events, mean and SEM were used instead of median and quartiles.

3. Experimental settings

3.1. Experiment 1: circadian rhythms in N. vitripennis males

Previous work has established the presence of an endogenous circadian system in Nasonia (Saunders, 1974). In order to determine its influence on activity, eclosion and emergence, circadian activity rhythms in LD and constant darkness (DD) were first assessed. The individual activity of 15 newly emerged virgin males was recorded for 4 days in LD 16:8 (entraining phase) followed by 7 days in DD, while the temperature was maintained constant (20 °C).

3.2. Experiment 2: emergence in LD 16:8 conditions

In this experiment, emergence from host puparia was investigated under LD 16:8. Thirty 1–2-day-old virgin females reared at constant light (LL) and temperature (25 °C) were allowed to parasitize on four hosts (one female per 4 hosts) for consecutive 8 h periods. Parasitized hosts were transferred to LD 16:8 (25 °C) conditions. Emergence of the male progeny was recorded at regular intervals.

3.3. Experiment 3: emergence in constant darkness

To assess the role of light, emergence in LD 16:8 and DD conditions was compared. 60 Nasonia virgin females were allowed to parasitize 4 hosts (one female per 4 hosts) during consecutive periods of 8 h in constant light (25 °C). Parasitized hosts were transferred immediately to LD 16:8 (25 °C). One week after parasitization, the 4 hosts from every tube were randomly divided into two (2 hosts per tube). Both sets were further incubated at LD 16:8 (25 °C), but the second set was shifted to DD (25 °C) after the

light phase of day 12 since the first parasitization event. Emergence was scored as done for emergence in LD (experiment 2). The rationale for this experiment was that if the endogenous circadian system would be sufficient to drive emergence in DD conditions, first, peaks of emergence would still be apparent in DD despite the missing light-on signal and, second, since the endogenous circadian system has a τ (tau, i.e. the endogenous period) greater than 24 h, in DD these peaks would be delayed in time compared to corresponding peaks in LD.

3.4. Experiment 4: eclosion versus emergence

To understand whether eclosion could influence emergence, eclosion rhythmicity was analyzed. 60 virgin females were allowed to parasitize hosts as described for the previous experiment. Parasitized hosts were kept in LD 16:8 (25 °C) conditions . One week after parasitization, the 4 hosts from every tube were randomly divided into two tubes, as described for experiment 3. Parasitized hosts in one set were opened just before eclosion, when Nasonia pupae are completely black. Eclosion (from the opened hosts set) and emergence (from the closed hosts set) were recorded in the same way as for experiment 2. Eclosing and emerging offspring originate hence from the same batch of parasitized hosts.

4. Results

4.1. Activity rhythms in N. vitripennis males

In Fig. 2 the activity rhythms of three representative males are depicted as "double plots". N. vitripennis males possess a robust endogenous circadian activity rhythm with an average τ of 25.6 h (n = 15, 0.44 SD) in constant darkness. The activity of males begins clearly after light-on without any sign of anticipatory behaviour. The onset of activity on the first day in constant darkness is usually more delayed in time than on following days. During the dark phase, males only rarely show activity, while during the light phase, activity appears uniform and does not usually last throughout the entire light phase (16 h).

4.2. Emergence in LD 16:8 conditions

Under LD 16:8, males emerge at regular intervals during 24 h, preferentially around light-on or shortly thereafter (Fig. 3A). This is even more apparent when the data are plotted only according to emergence time, not taking into account when the hosts (from which these males originate) were parasitized (Fig. 3B): emergence remains rhythmic. To further confirm that in LD conditions males emerge preferentially at the same moment of the day, data of consecutive days for males emerging at the same time of the day were pooled, as if males would have emerged within one and the same day. The result (Fig. 3C) shows clearly that even after this operation, male emergence remains periodic with a peak around light-on. In conclusion, males emerge preferentially around light-on. Moreover, there is a clear increase in emergence toward the darkness–light transition (e.g. Fig. 3B and C: compare emergence between 23–5, 5–8 and 8–11 h) indicating that males anticipate the light-on signal.

4.3. Emergence in constant darkness

Male emergence in constant darkness is shown in Fig. 4, where representative sets of parasitized hosts are compared with the corresponding set of males emerging in LD conditions. Both in LD and DD, males emerge in bouts but the first bout in time of DD males is shifted later compared with the corresponding bout in LD males. This result indicates that at least the first emergence bout in
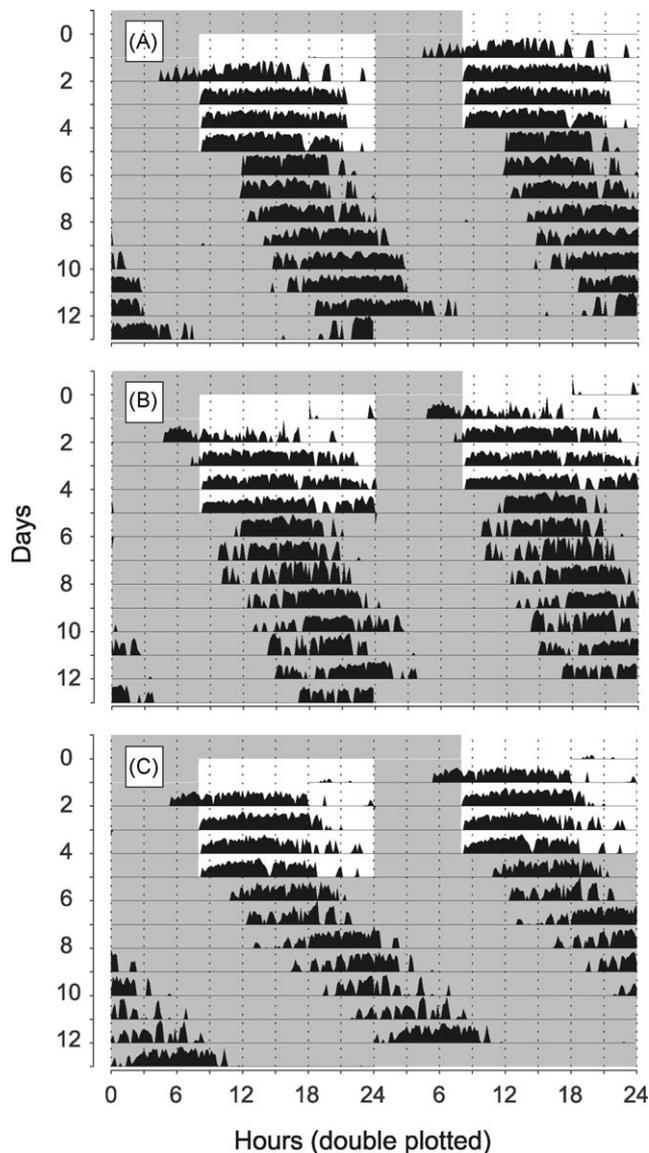
Fig. 2. Circadian activity rhythms of Nasonia males. Circadian activity of three representative males. Days are indicated on the y-axis, hours of the day on the x-axis (double plotted). Newly emerged males are subjected to entrainment for 4 days under LD 16:8 conditions and shifted to DD on day 5. Tau in DD is 24.5 h for male in A, 25.2 h in B, and 26.3 h in C. Grey background represents darkness, white background represents light.

DD, in the absence of a light-on signal, is likely endogenously controlled. This is also to be expected from the anticipation in emergence already observed (Fig. 3). However, besides the clear shift in time, there are other differences between both male sets. The first emergence bout in LD is usually more pronounced than the corresponding bout in DD (e.g. Fig. 4A and E) but subsequent bouts in DD are frequently stronger (or as strong as those in LD) and, interestingly, not as much delayed as are first bouts (e.g. Fig. 4A, C and E).

4.4. Eclosion and emergence

The results of this experiment are shown in Figs. 5 and 6. The comparison of eclosion (Fig. 5A) and emergence (Fig. 5B) in 3D graphs, in which every parasitization set is depicted independently, indicates that in LD conditions emergence is rhythmic, as seen before, but eclosion is not. This becomes explicit when, as in Fig. 3B, all parasitization sets are pooled and eclosion and
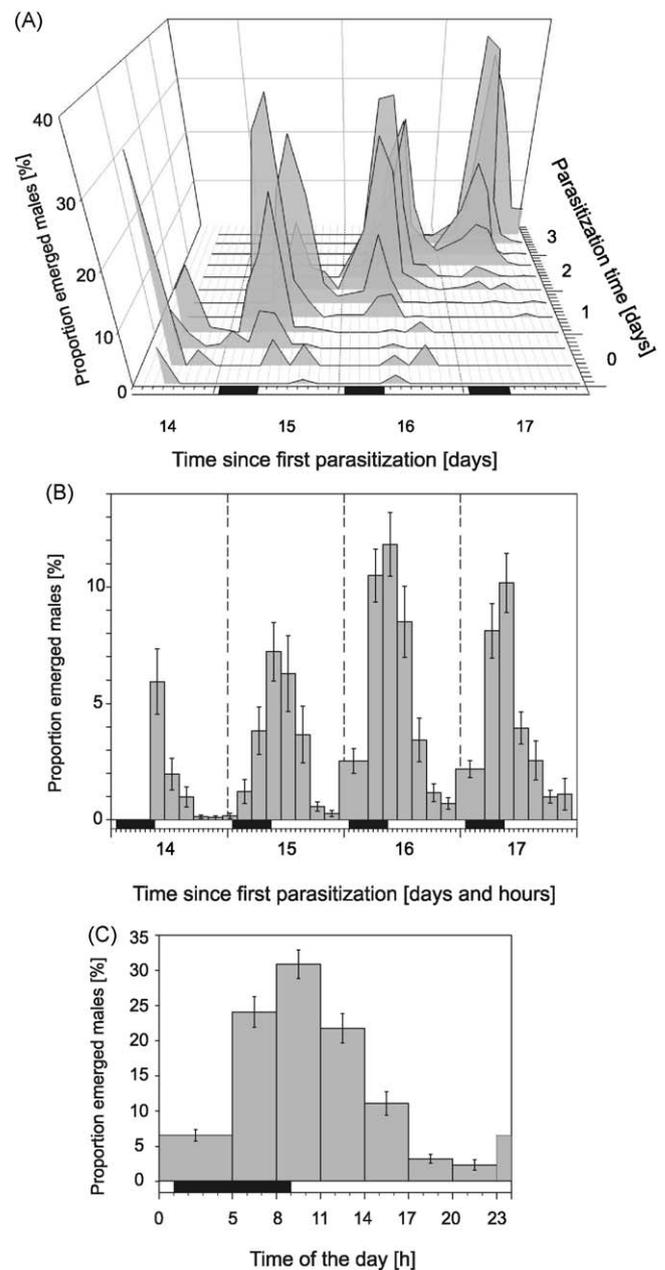

Fig. 3. Emergence of Nasonia males under LD 16:8 conditions. (A) 3D representation of emergence for all parasitization sets. Emergence days indicated on the x-axis are counted from the first parasitization day (day 0). On the y-axis, parasitization days are indicated, during each of which, 3 parasitization sets were usually produced (01:00–09:00, 09:00–17:00, and 17:00–01:00). For every parasitization set, emergence is plotted as proportion of the total number of males emerged from that parasitization set. (B) Emergence data from different parasitization sets are pooled. Emergence is plotted as proportion of the total number of males emerged in the pool. (C) Males emerged at the same moment of the day on consecutive days are pooled as if males from all parasitization sets would have emerged within 1 day. Emergence is again expressed as proportion of the total number of males emerged. Dark and light bars at the bottom of the graphs indicate darkness (01:00–09:00) and light (09:00–01:00) intervals, respectively. Plotted are means. Error bars represent standard errors.

emergence plotted in the same graph (Fig. 5C): in LD conditions, Nasonia males emerge in bouts (peaks in Fig. 5C) but eclose continuously. This means that if males, even if eclosed, do not reach the appropriate physiological state for emergence during the nearest emergence gate (around light-on), they wait inside the host puparium. This behaviour was confirmed by seeing males moving inside the host puparium in the second part of the light
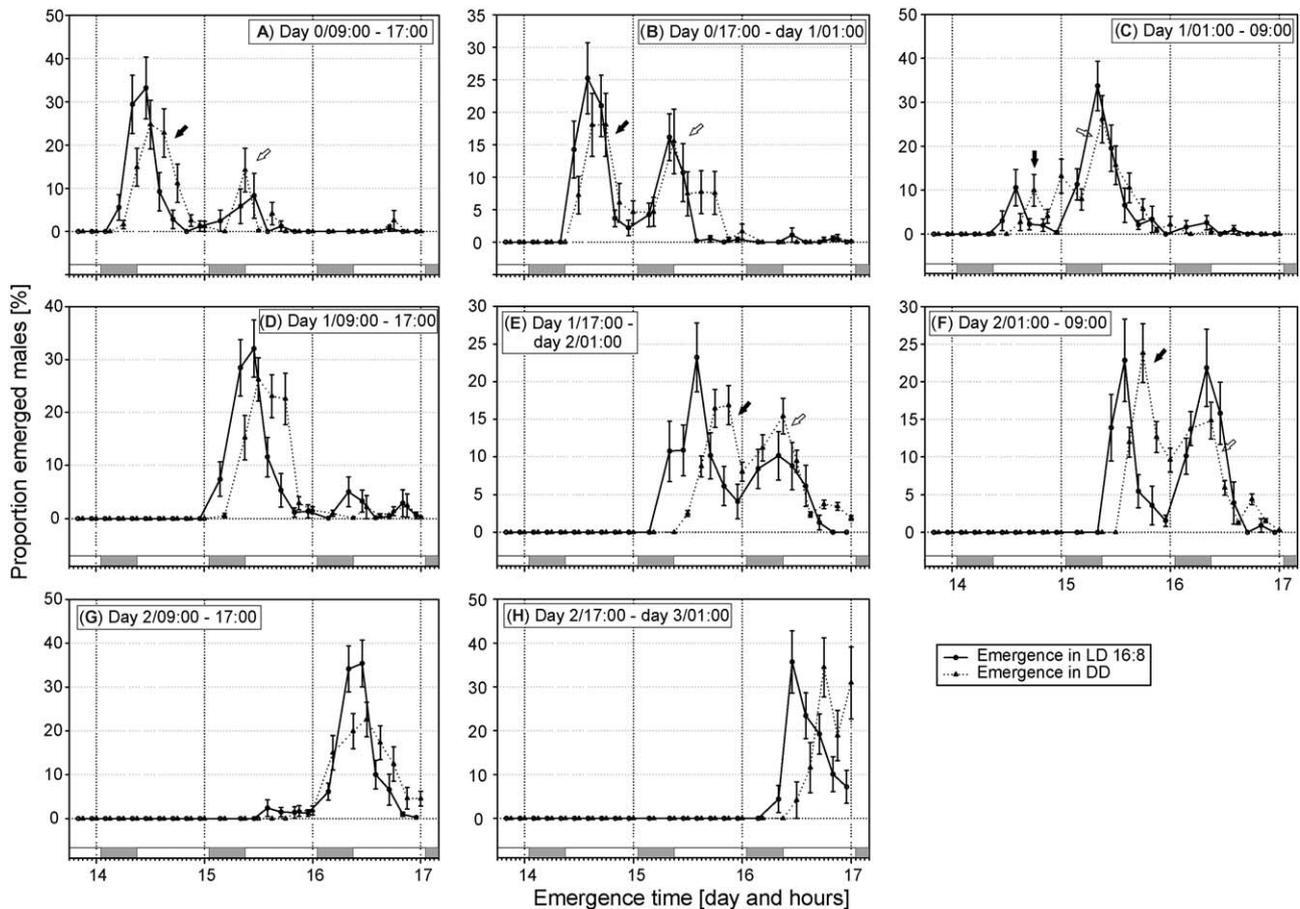
Fig. 4. Male emergence in constant darkness. Emergence in LD (straight line) and DD (dotted line) for males of the same parasitization set are compared (parasitization day and period is indicated in the inset). Note that the first emergence peak in DD (indicated with the filled arrow) is usually delayed as compared to the corresponding first peak in LD, and that subsequent peaks (open arrow) are frequently not. The more days have passed since the shift to DD, the more the first emergence peak in DD is delayed as compared to the same peak in LD (compare e.g. B, E and H). Grey shading at the bottom of the graphs represents the dark phase (01:00–09:00 h) for males in LD conditions. Means with standard errors are plotted.

phase or in the dark hours preceding light-on. To better illustrate this phenomenon, eclosion and emergence data are plotted and compared for some individual parasitization sets (Fig. 6). It appears that males can wait as long as 24 h inside the host puparium before emerging (e.g. Fig. 6A). The shortest observed time between major eclosion and emergence peaks was 16 h (Fig. 6G). Males usually emerge during the emergence gate that follows the moment of their eclosion (e.g. Fig. 6B or F). However, when a critical eclosion time is passed, emergence shifts to 24 h later (e.g. Fig. 6, A or E). In the LD scheme used, if males eclosed before 23 h, then the first emergence peak was usually observed on the following day, but when eclosion was after 23 h, the major emergence peak resulted more than 24 h later. Parasitization sets showing this duality are for instance C and D in Fig. 6 (related eclosion and emergence peaks are indicated by the same arrow).

5. Discussion

Eclosion rhythms have frequently been monitored in insects (Saunders et al., 2002). However, the differential temporal regulation of eclosion (i.e. pupal–adult ecdysis) and emergence ("eclosion" from the host puparium) in insects such as parasitic wasps, has been only addressed in *Trichogramma* (Reznik et al., 2008). In the present study we analyzed rhythms in activity, emergence, and eclosion in males of the parasitic wasp *N. vitripennis*, an organism previously used to study photoperiodism and which is now gaining much attention as an insect model system (Werren et al., 2010).

Nasonia males show unimodal activity patterns concentrated in the first part of the light interval, typical for diurnal insects (Saunders et al., 2002). Males activity begins usually with light-on without any sign of anticipation at the darkness–light transition, in contrast to what is for instance observed in *D. melanogaster* (Helfrich-Förster, 2001). This can be interpreted by light having a strong masking effect on the action of the endogenous circadian system: the onset of activity is mainly a direct response to light-on, by which the direct effect of the endogenous circadian system remains "masked" (Mrosovsky, 1999). In fact, as soon as males are shifted to DD, they do show well defined free-running activity rhythms with an average τ of 25.6 h. Another indication for a strong triggering effect of light comes from the first DD cycle, where activity onset is usually more delayed in time than would be expected if it would be solely controlled by the endogenous circadian system. It seems in fact as if males, in the first DD cycle, would wait as long as possible for the light-on signal before getting active. This phenomenon occurs sometimes and is explained by the requirement of the endogenous circadian system for some cycles – called "transients" – before adapting completely to the new conditions (Saunders et al., 2002). A different interpretation is that onset of activity in light would be controlled by the endogenous circadian system and that the delayed activity onset on the first day of darkness would result from a negative masking effect of darkness. However explained, the increase (or onset) of activity in response to light-on and the delay in the first DD cycle may help to later interpret observations in emergence behaviour.
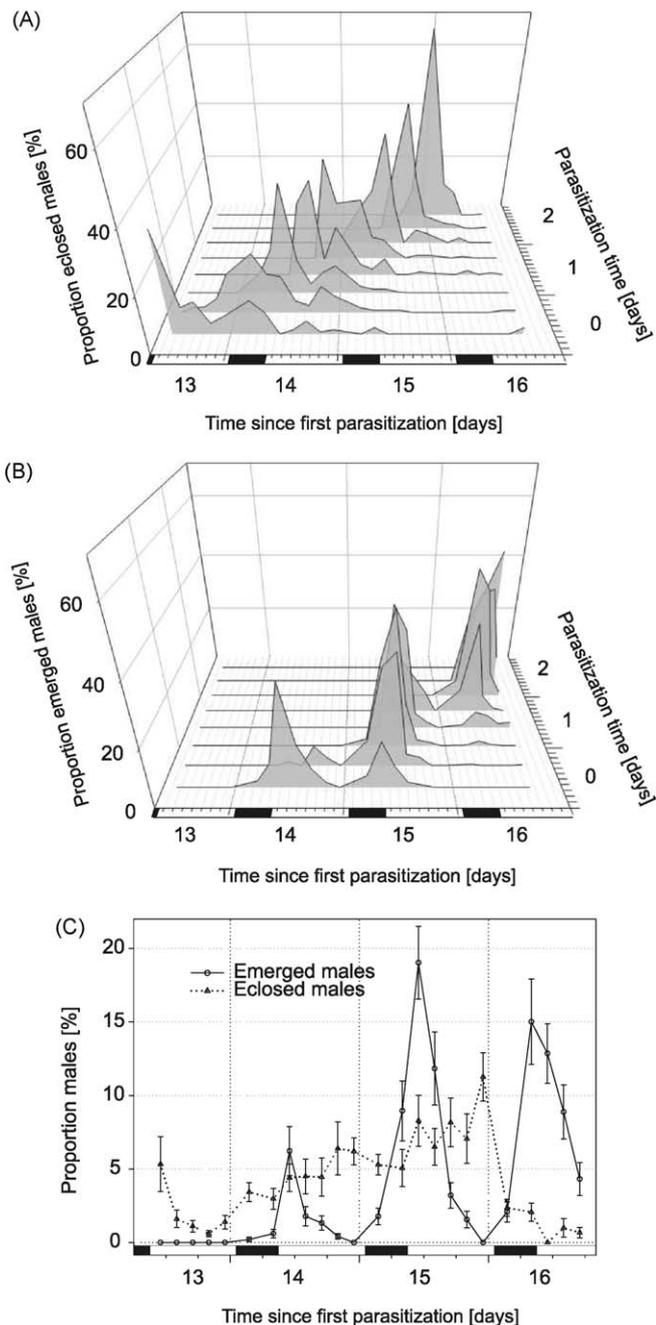
Fig. 5. Male eclosion versus emergence in LD. (A) Male eclosion and (B) emergence are compared in 3D graphs for each parasitization set. (C) Eclosion and emergence from different parasitization sets are pooled and plotted in the same graph. Notice the peaks in emergence as opposed to continuous eclosion. Other details as for Fig. 3.

Activity patterns were measured with a self-developed actometer, different from that typically used for recording activity rhythms in *Drosophila* (Rosato and Kyriacou, 2006). In the latter, activity is recorded when a light beam is crossed by a fly in the culturing vial. Such a device only measures locomotor activity without considering other types of active behaviour as e.g. grooming. The device used in our study, by measuring the change in pixels intensity from consecutive images, has the advantage of recording all types of active behaviour, including locomotor activity, and hence has a broader application to organisms whose activity is less dependent on locomotion.

N. vitripennis males emerge preferentially around light-on. Contrary to what is observed in activity rhythms, emergence does show anticipation towards the darkness–light transition, which can be an additional indication of the action of an underlying clock. Anticipation of eclosion is also observed in *D. melanogaster* (Qiu and Hardin, 1996) but not in *T. embryophagum* where emergence from the host chorion is rhythmic but does not show anticipation (Reznik et al., 2008).

In the absence of a light-on signal, the endogenous circadian system appears sufficient to trigger emergence, which is more delayed the more days have passed since the shift to DD, in agreement with a τ greater than 24 h. In other insects, free-running is observed for eclosion. In the fly *Sarcophaga argyrostoma*, for instance, the eclosion rhythm free-runs in DD with a τ close to 24 h (Saunders, 1979), while in *Antherea pernyi* it is 22 h (Truman, 1971). Taking into consideration that the shift to DD happened after the light phase of the 12th day of development, the data also indicate that an entrainable clock is already present before wasps eclose from their own pupal integument, also in agreement with what is known from other insect species (Saunders et al., 2002). The same explanations as for activity hold to explain why the first emergence bout in DD is usually less pronounced and delayed than the corresponding bout in LD. While light does not seem to be necessary *per se* for males to emerge in bouts, light could exert positive masking and induce even more males (which would otherwise not emerge) to emerge after light-on. Alternatively, the delay may be caused by a negative masking effect of darkness in the first DD cycle.

Explaining the observation that in DD emergence bouts after the first one are similar to corresponding bouts in LD (in time and intensity) is more difficult. This is probably due to different overlapping factors. On the one hand, a phenomenon as observed in the first DD cycle in circadian rhythms (delay in activity) may be present: the first emergence bout in DD would be delayed more than would be expected if driven by the endogenous circadian system, but subsequent ones would follow more precisely the endogenous rhythm and appear so anticipated compared to the bout in the first DD cycle. However, this argument would strictly apply only to differences observed between day 13 (the first day in DD) and day 14, and not later. Other influences may come from males that, in the first bout, could but did not emerge (because of the missing light-on signal, see above) and are now "eager" to emerge and start to do so earlier than expected; or from the arrival of newly eclosed males inside the host. Either possibility should be studied more in detail for a definitive explanation.

Rhythms in physiology and behaviour in insects are thought to be adaptive. For instance, eclosion in *Drosophila* happens in the early morning hours, when the conditions of dawn facilitate freshly emerged flies to spread their wings (Skopik and Pittendrigh, 1967). Also egg-laying shows a circadian pattern in a number of insect species. Eggs laid during the night would more easily escape desiccation or parasite infestation (Howlader and Sharma, 2006). Eclosion of *N. vitripennis* males from the own pupal integument does not follow a circadian rhythm. As a consequence, eclosed wasps may wait up to more than 24 h inside the host puparium before emerging. The same phenomenon was observed in *Trichrogramma* wasps and explained by the fact that, in contrast to many other insects, the ecdysed adults are within a well protected space inside the host chorion and hence the exact timing of eclosion is almost adaptively neutral. In contrast, synchronous emergence of *Trichrogramma* wasps from the host during the morning hours minimizes the risk of desiccation and optimizes the reproductive success (Karpova, 2006). Although a similar scenario could hold for the patterns in eclosion and emergence observed in *Nasonia*, additional arguments may come from the mating system which is strongly dependent on different emergence times between males and females (Drapeau and Werren, 1999).
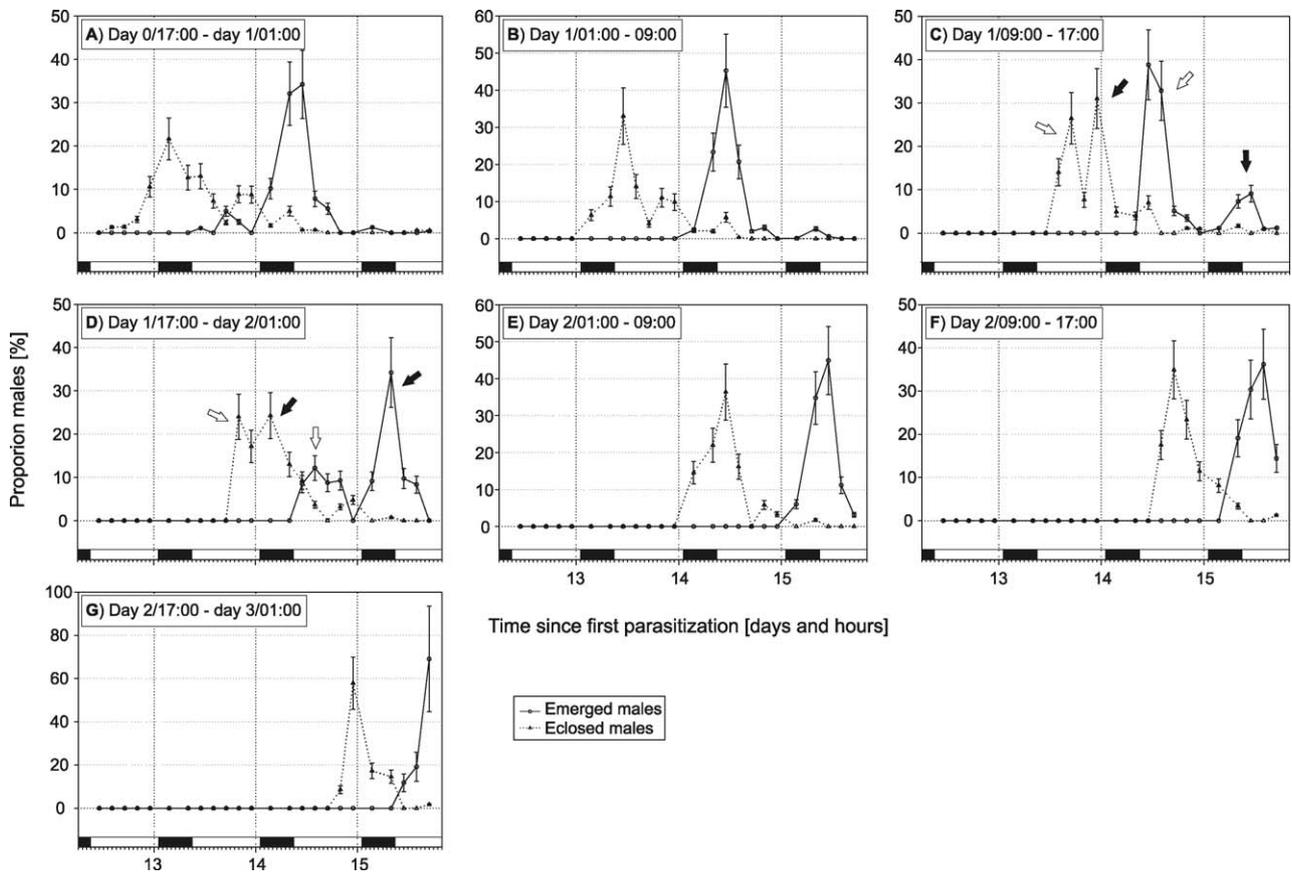
Fig. 6. Delay between eclosion and emergence. For some parasitization sets, eclosion (dotted line) and emergence (straight line) under LD 16:8 conditions are plotted in the same graph. Same type arrows indicate correlations between peaks in eclosion and emergence (see text for details). Parasitization day and period is indicated in the inset. Time elapsed since the first parasitization is indicated on the x-axis. Other details as for Fig. 4.

In the present study we only used males to obtain an initial picture of eclosion and emergence rhythms in *N. vitripennis* and to avoid additional influencing factors, such as the presence of females together with males within the host. As known for other insects (Fantinou et al., 1998), *N. vitripennis* males chew a hole in the host puparium, emerge first and wait at the exit hole to mate females as soon as they emerge. The observed anticipated emergence of *N. vitripennis* males may support this behaviour and females may only emerge after receiving the light-on signal. This behaviour is different from that of the closely related species *Nasonia giraulti*, where males emerge only once they have mated females inside the host (Drapeau and Werren, 1999). These differences in mating behaviour may be related to species-specific adaptations of the endogenous circadian system and add to the use of the *Nasonia* species complex for studying the genetics of adaptive chronobiological traits.

Acknowledgments

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