Daily Eclosion Patterns in Nymphalid Butterflies and Their Causes

by

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ABSTRACT

The molt from pupae to adult stage, called eclosion, occurs at specific times of the day in many holometabolous insects. These events are not well studied within Lepidopteran species. It was hypothesized that the eclosion timing in a species may be shaped by strong selective pressures, such as sexual selection in the context of male-male competition. The daily timing of eclosion was measured for six species of nymphalid butterflies. This was done by rearing individuals to pupation, placing the pupa in a greenhouse, and video recording eclosion to obtain the time of day at which it occurred. Four species exhibited clustered eclosion distributions that were concentrated to within 201 minutes after sunrise and were significantly different from one another. The other two species exhibited eclosion times that were non-clustered. There were no differences between sexes within species. The data support a relationship between the timing of eclosion each day and the timing of mating activities, but other as of yet undetermined selective pressures may also influence eclosion timing.

Dedicated to my mother and family

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INTRODUCTION

In many holometabolous insects, the process of ecdysis occurs at specific times of day. These events include eclosion, the pupal-imaginal ecdysis. For example, in the common stick insect, *Carausius morosus*, ecdysis at all developmental stages is extremely rhythmic, beginning at or immediately before dawn (Wadsworth et al., 2014). Eclosion in *Drosophila melanogaster* is also restricted to a period within a few hours of sunrise (Sheeba et al., 2001; Tataroglu & Emery, 2014).

Daily timing of eclosion has been understudied in Lepidoptera, especially in comparison to Dipterans (Emery et al., 1997; Peabody & White, 2013). Past experiments have shown that *Battus philenor* has an extremely restricted eclosion time, with adult emergence occurring within 1.5 hours of sunrise (Sencio et al. 2015). It is not known if other Lepidopteran species share this restricted eclosion behavior, but temporal restrictions on other behaviors, such as mate-finding in *Acrobasis nuxvorella*, *Lethe diana*, and *Pseudopidorus fasciata*, are well known (Stevenson & Harris, 2009; Takeuchi, 2010; Wu et al., 2014).

Temporal restrictions on diel eclosion patterns are suggested to be proximately maintained by an internal circadian clock that is regulated by the daily photoperiod (Emery et al., 1997; Vafopoulou & Steel, 1991; Sencio et al., 2015). Light periods strongly influence the daily, as well as seasonal, pattern of eclosion within insects. Seasonal changes in photoperiod may also affect eclosion timing as well; some lepidopterans enter diapause and greatly delay eclosion if day length decreases below a certain threshold (Bell, Rasul, & Joachim FG, 1975).

The adaptive advantages of cyclically timed eclosion patterns are not known in insects. For *B. philenor* it was hypothesized that eclosion time coincides with the daily pattern of mate-finding behaviors, which occur during early morning in this species (Sencio et al., 2015). Predator avoidance, prime feeding times, and other temporally restricted factors could also serve as selective pressure shaping diel eclosion patterns. Many Lepidoptera have specific patterns of daily and seasonal behavior; specific eclosion times could be beneficial by increasing behavior timing accuracy and thus fitness, especially in the context of temporally-restricted life events like mate-finding.

In this study, the diel pattern of eclosion in several species of Nymphalid butterflies was investigated. This specific clade was chosen because of local availability in the Sonoran Desert and because of their relatively close phylogenetic relationships, which allowed for stronger interspecific comparisons. *B. philenor* was included in this study to explore their eclosion pattern in a natural light environment and compare results to the control group in Sencio et al. (2015).

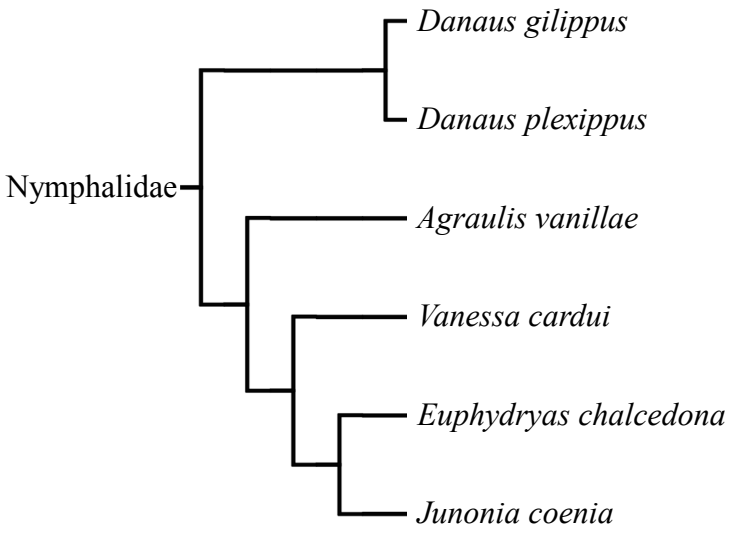


Figure 1: Phylogeny of the Nymphalid butterflies included in the experiment (Wahlberg et al., 2009).

It was hypothesized that butterfly eclosion time coincides with mate-finding behavior. This hypothesis predicts that eclosion will occur a few hours before mate-finding in response to mate-finding competition, allowing time for males' wings to fully harden and become flight-ready (Stevenson & Harris, 2009). Based on observed interspecific differences in when mate-finding behavior occurs during the day (Table 1), *E. chalcedona, A. vanillae*, *J. coenia,* and *D. gilippus* were predicted to eclose in the early morning. *D. plexippus,* and *V. cardui* were predicted to emerge at midday.

Table 1: Mate-finding behaviors of the experimental species.

Species	Mate-Finding Behavior; Courtship	Source
Euphydryas chalcedona	Perching; Morning Patrolling; Afternoon	Rutowski et al., 1988
Danaus gilippus	Patrolling; Afternoon	Brower et al., 1965
Danaus plexippus	Patrolling; Afternoon	Hill et al., 1976
Vanessa cardui	Perching; Afternoon	Shields, 1967; Scott, 1974; Brown & Alcock, unpublished data
Agraulis vanillae	Patrolling; All day	Rutowski & Schafer, 1984
Junonia coenia	Perching; All day	Scott, 1975

METHODS

Individuals of all species were reared from eggs or larvae to pupation in an incubator that mimicked the daily cycle of light and temperature in the Sonoran Desert environment. From February to May of 2016, day length was set to 12 hours (lights on at 0630 hrs. and off at 1830 hrs.) and temperature was set to a daytime constant of 27°C and nighttime constant of 18°C (rising or falling, respectively, over a 1.5-hr. time period with each temperature change). From May to December of 2016, day length was set to 16 hours, with all lights on for 12 of these hours (during the other four hours - 0500-0800 hrs. and 1800-2100 hrs. - only half of the room lights were on, signifying dawn and twilight, respectively). Temperature was set to 24°C during nighttime hours and 30°C during daytime, with temperature ascending and descending for the 3 hours of dawn and twilight. Larvae were fed *ad libitum* on cuttings from the appropriate host plants and reared in translucent 24 oz. cups measuring 4.6” wide at the top, 4.3” tall, and 3.6” at the base.

A. vanillae eggs and larvae were collected in Southeastern Tempe, AZ and in Phoenix, AZ at the Desert Botanical Garden. They were reared on various species of *Passiflora* vine including *P. vitifolia*, *P. incarnata*, and *P. arizonica*, with the highest larval consumption and rearing success with *incarnata*. Larvae were not cannibalistic towards other larvae, but consume pupae despite large amounts of food plant; care was taken to quickly remove pupae from larval containers.

D. gilippus eggs and larvae were collected at the Desert Botanical Garden and ASU Tempe Campus; adult females were collected at Mesquite Wash in the Tonto National Forest, (33.7309° N, 111.5149° W) and were placed in cup with larval food plant to collect eggs. Larvae were reared on *Asclepias subulata* and *A. angustifolia*, with the greatest rearing success on *A. augustifolia*. Larvae were aggressive and cannibalistic; they were reared with a maximum of two fifth-instar larvae per larval cup.

D. plexippus larvae were collected at the Desert Botanical Garden and ASU Tempe Campus. They were raised on the same food plants as *D. gilippus* larvae, with the most rearing success on *A. augustifolia*. Larvae were not as aggressive as in *D. gilippus*, but were raised with a maximum of 2 fifth instar larvae per cup as a precaution. Individuals that were reared on *Asclepias subulata* had significantly shorter adult lifespans and a darker coloration.

E. chalcedona larvae were collected at Round Valley on Sycamore Creek in the Tonto National Forest and were fed *Keckiella antirrhinoides*. Larvae were not aggressive. Around 10% were internally parasitized by tachinid fly larvae and did not successfully complete pupation.

J. coenia larvae were collected at Mesquite Wash and were reared on *Mimulus guttatus*. Larvae were non-aggressive; no instances of cannibalism were noted.

V. cardui larvae were obtained either in the field at Mesquite Wash or from Carolina® Biological Supply. Wild-caught larvae were reared on *Cirsium vulgare*; captive-bred larvae were reared on the artificial diet supplied with the animals. Artificial diet dried out quickly in the standard larval cups, resulting in negligible consumption and high early instar death rate. Captive-born larvae were individually raised in 35mm film canisters to decrease moisture loss to the artificial diet. This second method had a 100% survival-to-pupation rate.

B. philenor eggs and larvae were collected at Mesquite Wash and were reared on *Aristolochia watsonii*. They were housed with a maximum of two fifth-instar larvae per larval cup to prevent cannibalism.

Upon pupation, animals were placed in a naturally-lit greenhouse on the Tempe campus of Arizona State University. Pupae were video recorded beginning 1-2 days before eclosion; the day of eclosion was predicted using a pupal candling method, developed in previous experiments (Sencio et al, 2015). At night, red light was used to illuminate pupae for video viewing. As supported by Watari and Tanaka's (2014) experiment on the onion fly, *Delia antiqua*, infrared light does not affect insect eclosion behavior. Eclosion time was defined as the first movement of the animal as it exited the chrysalis.

Statistical analyses were done using the R 'circular' statistics package (Agostinelli & Lund, 2013). The R program was used to display the data on a circular plot format (R Core Team, 2016). The study focused on the eclosion of nymphalid butterflies in relation

to sunrise, a reliable temporal reference (Cornwall et al., 2017). Display of species data on a 24-hour, clock-like plot allowed easy interpretation of results.

Eclosion times were plotted on circular, clock-like graphs that display three vectors. The black vector represents the species data set, blue represents males, and purple represents females. The length of the vector corresponds to the data's rho value, with the circle being 1.00. P-values are displayed near the vectors. The vector points to the mean eclosion value of the data set. Rayleigh tests were performed on each species and each sex within species to investigate the presence of non-uniformly distributed eclosion. Waston U^2 tests were performed to test for differences in data among species and sexes within species.

RESULTS

Battus philenor

Figure 2 shows the time of eclosion relative to the time of sunrise for both males (blue) and females (purple). Population eclosion, those for males, and females alone were significantly non-uniformly distributed and clustered around 88 min after sunrise. The difference in eclosion times for males and females was not significant ($U^2 = 0.0625$, $p > 0.05$).

Nymphalidae

Figure 3 shows the time of eclosions relative to the time of sunrise for both males (blue) and females (purple).

Danaus gilippus

Population eclosions were significantly non-uniformly distributed. The population was clustered around 69 min after sunrise. Male and female eclosions both had non-clustered distributions. The difference in eclosion times for males and females was not significant ($U^2 = 0.0858$, $p > 0.05$).

Danaus plexippus

Population, male, and female eclosions were not significantly clustered in distribution.

Agraulis vanillae

Population eclosion, those for males, and females alone were significantly non-uniformly distributed and clustered around 144 min after sunrise. The difference in eclosion times for males and females was not significant ($U^2 = 0.0775$, $p > 0.05$).

Vanessa cardui

Population, male, and female eclosions were not clustered in distributions. The difference in eclosion times for males and females was not significant ($U^2 = 0.1477$, $p > 0.05$).

Euphydryas chalcedona

Population, male, and female eclosions were significantly non-uniformly distributed and clustered around 201 min after sunrise. The difference in eclosion times for males and females was not significant ($U^2 = 0.0947$, $p > 0.05$).

Junonia coenia

Population, male, and female eclosions were significantly non-uniformly distributed and clustered around 73 min after sunrise. The difference in eclosion times for males and females was not significant ($U^2 = 0.0496$, $p > 0.05$).

Table 2 shows a summary of individuals collected within each species, population, male, and female average eclosion times, and the results of Watson's U^2 Test on male-female population pairs within species. None of the pairs showed a significant difference and can be considered homogeneous populations. Table 3 shows the results of Watson's U^2 Test, comparing each species to one another as pairs. All nymphalid species were significantly different and can be considered heterogeneous populations.

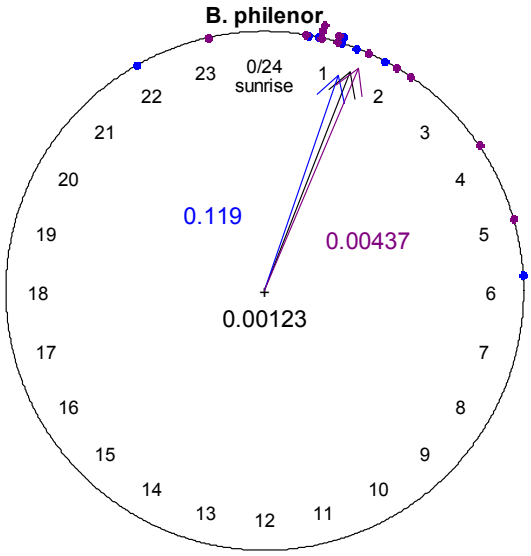


Figure 2: Eclosion times for *B. philenor* females (purple), males (blue), and all individuals (black arrow) relative to the time of sunrise.

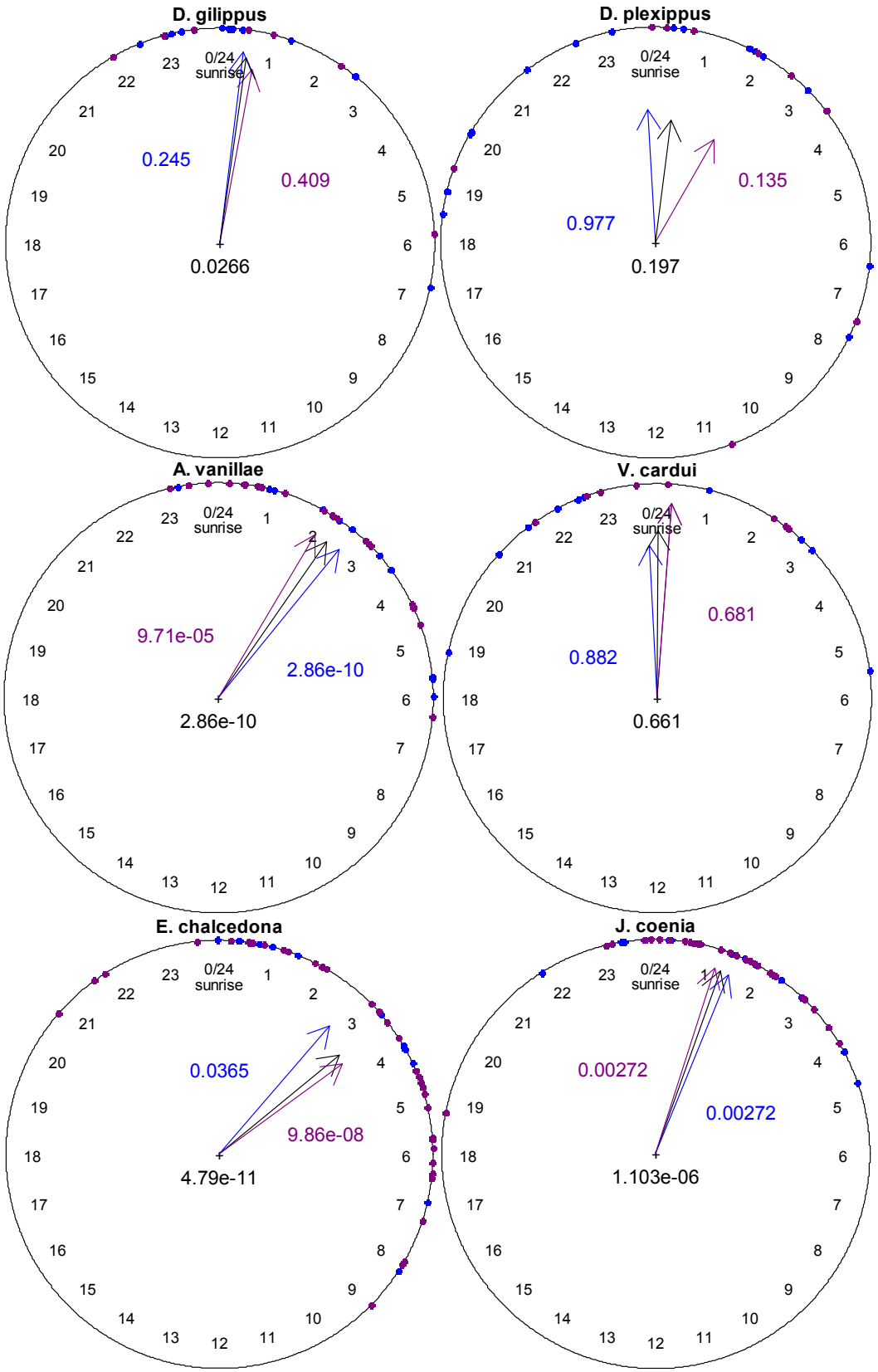


Figure 3. Eclosion times for the six species of nymphalid butterflies.

Table 2: Data used in Figures 2 and 3. Watson's Tests of Homogeneity were conducted to find statistically significant differences between sexes within species. All male-female comparisons did not reject the null hypothesis and can be considered homogeneous.

Watson's U^2 Tests on Pairwise Male-Female Populations within Species			
Data Set	Count	Mean Eclosion Time after Sunrise	Watson U^2 Test
B. philenor	20	88.3 min	
B. philenor Males	8	80.5 min	$U^2 = 0.0625$
B. philenor Females	12	93.5 min	$p > 0.05$
D. gilippus	26	68.81 min	
D. gilippus Males	15	37.2 min	$U^2 = 0.0858$
D. gilippus Females	7	52.14 min	$p > 0.05$
D. plexippus	26	64.58 min	
D. plexippus Males	16	1.68 min	Non-clustered
D. plexippus Females	9	147.67 min	distribution
A. vanillae	43	143.81 min	
A. vanillae Males	19	158.95 min	$U^2 = 0.0775$
A. vanillae Females	21	124.10 min	$p > 0.05$
V. cardui	25	15.76 min	
V. cardui Males	13	-8.00 min	Non-clustered
V. cardui Females	8	17.13 min	distribution
E. chalcedona	53	201.08 min	
E. chalcedona Males	12	172.33 min	$U^2 = 0.0947$
E. chalcedona Females	36	208.22 min	$p > 0.05$
J. coenia	54	73.44	
J. coenia Males	18	88.17	$U^2 = 0.0496$
J. coenia Females	28	65.93	$p > 0.05$

Table 3: Each species was tested for homogeneity amongst each other species. Grayed cells indicate that the pair cannot be considered statistically different.

Watson's U^2 Tests on Pairwise Species					
	B. philenor	*D. gilippus*	*A. vanillae*	*E. chalcedona*	*J. coenia*
B. philenor	-	$U^2 = 0.3005$ $p < 0.01$	$U^2 = 0.2401$ $p < 0.05$	$U^2 = 0.3438$ $p < 0.01$	$U^2 = 0.3403$ $p > 0.1$
D. gilippus	-	-	$U^2 = 0.3764$ $p < 0.01$	$U^2 = 0.3618$ $p < 0.01$	$U^2 = 0.2778$ $p < 0.01$
A. vanillae	-	-	-	$U^2 = 0.2874$ $p < 0.01$	$U^2 = 0.21$ $p < 0.05$
E. chalcedona	-	-	-	-	$U^2 = 0.56$ $p < 0.001$
J. coenia	-	-	-	-	-

DISCUSSION

Eclosion times in *B. philenor*

In the greenhouse used in this study, *B. philenor* showed an extremely similar eclosion pattern similar to that reported in a previous study where individuals eclosed in an artificial light environment (Sencio et al., 2015). Natural light environment eclosions clustered around 88.3 minutes and artificial light eclosions clustered around 76 minutes after sunrise and were not significantly different (Watson's U^2 Test, $U^2 = 0.7904$, $p > 0.001$). This confirms the assumption that artificial environment in an incubator gives a reliable representation of the eclosion behavior of this species in the field.

Nymphalid eclosion patterns: general patterns

Four of the nymphalid species show a significantly restricted eclosion time, with clustering close to sunrise. Only *D. plexippus* and *V. cardui* displayed eclosion times that were not clustered. In pairwise comparisons, the mean eclosion times were statistically different for all species pairs.

Within species with clustered eclosion patterns, there were no sex differences in eclosion times. Many nymphalids and species from other butterfly families (e.g. heliconiids; Walters and Harrison, 2008) display protandry, in which males eclose a few days before females. This is thought to promote male mating success by allowing males to be ready to mate as females emerge (Degen et al., 2015). The results indicate that protandry does not occur on the order of hours within the selected group of study species.

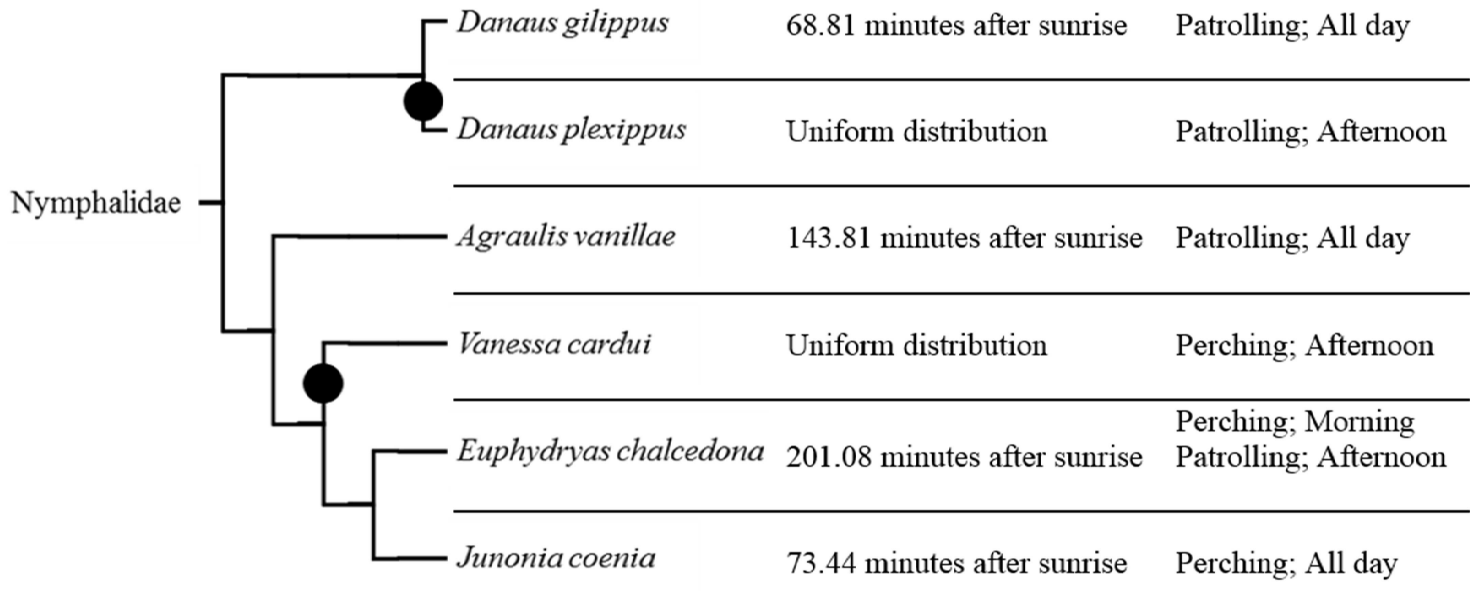
Eclosion patterns and mating systems

The results provide support a for the predicted relationship between mate-finding and eclosion timing. *D. gilippus, E. chalcedona, A. vanilla,* and *J. coenia* supported morning eclosion predictions, all emerging within 201 minutes of sunrise. Male-male competition would lend greater benefit to those individuals eclosing during early morning in these species because mate-finding begins in the morning and continues throughout the day. Relaxed eclosion pressures may be present in the afternoon mate-finding species; *V. cardui* and *D. plexippus* show non-restricted eclosion distributions.

Unlike in heliconid females, perhaps the nymphalid species do not mate on the day of eclosion. Upon eclosion, animals often position themselves towards direct sunlight to speed wing expansion. Four of the experimental species' mean eclosion times occurred within hours of sunrise. If mating does not take place the same day of eclosion, morning emergence would be most advantageous for diurnal Lepidoptera because it allows for nectaring and, in some species, territorial establishment.

Phylogenetic relationships in these six species were used to explore correlation between mate-finding times and eclosions times. Mapping mate-finding times onto the phylogeny reveals two nodes at which mate-finding behavioral timing diverged (Table 4). Eclosion patterns also diverged at these nodes and in the predicted direction, supporting the hypothesis.

Table 4: Comparison of mate-finding behavior and average population eclosion times among species. The circles show nodes at which divergence in mate-finding timing occurred.



Eclosion and mate-finding appear to be temporally linked behaviors in a small group of nymphalid butterflies. This experiment found that four species had mean eclosion times during morning, with the remaining two species displaying a non-restricted distribution of eclosion. Although these species provide support for a relationship between eclosion and mate-finding, sexual selection pressure may not be a singular factor in Nymphalid eclosion timing. Other selective pressures, including predation and food availability, may have a stronger influence on eclosion timing and are worth pursuing.

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