
Circadian rhythmicity and reproduction in *Drosophila melanogaster*

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Abstract

Most species are adapted to rhythmic environments to improve survival and reproduction. In *Drosophila melanogaster*, rhythmicity and reproduction are well documented. I address how circadian rhythms affect reproduction and discuss the evolutionary relevance. Rhythms in locomotor activity, mating, courtship song and pheromone perception influence reproduction within *Drosophila melanogaster* but also between *Drosophila* species. Mutations in clock genes including *Period* lower reproductive fitness in males. *Period* encodes locomotor activity rhythms, but uncertain is whether this contributed to evolution by temporal reproductive isolation. Mating rhythms differ from locomotor activity and as hypothesized, males adapt activity rhythms in context of females responsible therefor, due to rhythmic female responses to courtship song and pheromone perception. Courtship song has species-specific properties allowing species recognition to prevent interspecies mating, thus possibly driving speciation. Separate clock mechanisms are present in olfactory systems accounting for rhythmic pheromone perception. Pheromones differ between species and indeed contribute to species recognition, but the role of circadian rhythmicity herein remains speculative. Species-specific rhythmicity in reproduction is likely due to both temporal and spatial reproductive isolation. Studies of environmental factors linked to rhythms in reproductive behavior are recommended as much remains unknown. Additionally, mostly laboratory schemes are utilized that are proven not to support all natural observations, addressing the need for more realistic research environments. The evolutionary background of interspecies differences in discussed rhythms remains mostly speculative. Thus, further research of interspecies differences could provide a better understanding of evolution of rhythmic reproductive behavior.

Introduction

Most species are adapted to rhythmic variations in the environment. Anticipating rhythmic environmental changes could improve survival and reproduction¹. Being able to predict environmental conditions allows avoiding dangerous situations, or foraging at optimal times of the day. This provides a benefit in comparison with those not well-adapted and thus more prone to situations endangering its survival and reproduction. The increase in fitness, a way of describing how well a species reproduces, can be the result of a clock mechanism adapted to environmental rhythms. The daily (circadian) light-dark cycle is a well-known example. The clock mechanism can wake up animals in anticipation of light. Animals which hunt during the night whereas their prey is active during the day have a disadvantage compared to those hunting during daytime. Another example is the adaptation to seasons². Trees lose their leaves during autumn in anticipation of cold conditions and bears prepare for hibernation. In the field of chronobiology, recently more focus is put on the interaction between clock genes and their influences on many traits. Given selection pressure by rhythmic environmental cues, variations in clock genes may cause temporal isolation of reproduction within a species and as a consequence, distinct groups of such species arise and evolve independently. Studying the interaction between circadian rhythms and reproduction may therefore provide insight on how organisms evolve with regards to rhythmic environments^{1,3,4}.

Mus musculus is a widely used rodent in chronobiological research. Interestingly, evidence for circadian influences on reproductive behavior in *Mus musculus* is controversial^{5,6,7}. Biologists believe circadian clocks promote fitness by adaptations to daily rhythmic conditions, whereas there is only clear evidence for this in cyanobacteria and plants⁸. A direct link between rhythmic genes and reproduction remains unclear^{5,6,7}. There is evidence for the influence of sex hormones

such as testosterone on when animals are active, suggesting at least interaction between reproductive and circadian systems⁵. Other studies, however, suggest no role for clock genes in reproduction and determined these do not alter spermatogenesis in a circadian fashion^{6,7}. Whereas in *Mus musculus* no solid evidence for influences of circadian rhythms on reproduction is found, the same does not apply to *Drosophila melanogaster*.

Drosophila melanogaster, the invertebrate fruit fly, is another species widely used in chronobiology, well known for its extensive research capacity. Its genome sequence is fully determined and thus very useful for gene research, besides from its ease of use in laboratory environments. Given that courtship behavior is a predominant activity in the species, it is well investigated⁹. In *Drosophila melanogaster*, much attention is paid to such gene-behavior analysis. In contrast with *Mus musculus*, there are studies documenting influences of circadian rhythmicity on reproduction in *Drosophila melanogaster*.

I aim to examine the influences of circadian rhythms on reproductive behavior, answering the following research question: How does the circadian rhythmicity of *Drosophila melanogaster* affect reproductive behavior?

Hypothesized is that circadian rhythms influence reproductive behavior through interactions with environment and clock genes. Mutations in specific clock genes could cause alterations in timing and stages of reproductive behavior, to such extent that variations in clock genes have caused evolutionary divergence between species of *Drosophila*^{3,4,10}. Furthermore, due to differences in behavior between males and females, possible differences in mating rhythms could be attenuated by social interactions. Given the choosy role of females, it is expected that males adapt to phases of females^{3,11}. Recognition of males and females would be due to specific pheromones under the influence of rhythmic mechanisms. Male pheromone emission and female pheromone reception may vary daily, creating optimal

times for reproductive behavior, and may provide a direct role in intra- and inter-specific variation in *Drosophila*¹².

General circadian rhythms in *Drosophila melanogaster*

Drosophila melanogaster undergoes a developmental path towards adulthood and there are differences between developmental stages of the fly regarding daily activity patterns¹³. The current section addresses the developmental path of the fly to then further discuss general circadian rhythms.

Larvae hatch (emerge) from their eggs about 12 to 15 hours after these are laid by adult females, tightly gated at dawn in wild-types. About 24 and 48 hours after hatching, the larvae undergo a process called ecdysis whereby their skin is shed. After 4 days the larvae transform in pupas and undergo metamorphosis lasting 4 days, after which adults appear from these pupae. This process is called eclosion. The flies can then diverge. These numbers apply under ideal conditions with a temperature of 25 °C. Deviation of this temperature results in prolonged or shortened development periods^{14,15,16}. While both emergence from eggs and eclosion are one-time events, these are under control of a peripheral clock system in the prothoracic gland (PG) which in turn is under control of the central clock system in Lateral Neurons (LNs) of the brain¹⁷. These clock systems gate timing of emergence and eclosion, whereby despite lack of multiple repeating events in an individual, it is considered a rhythm based on multiple events within a population. The period of this rhythm approximates 24 hours in wild-type flies^{14,17}. After eclosion, spermatogenesis takes approximately 2 days for *Drosophila melanogaster* males. Females require about less than 2 days for oogenesis to complete, entering physiological maturity. At this point, the flies can reproduce¹³.

Several of the main genes involved in clock systems are *Clock*, *Cycle*, *Timeless* and *Period*¹⁸. *Clock* defines the free-running period of locomotor activity (general movement) and eclosion rhythm, whereby mutants are known

for longer or shorter periods. Since both locomotor activity and eclosion rhythm were influenced, it was hypothesized that *Clock* acts on a central oscillator^{19,20}. The protein products of *Cycle* and *Clock* interact as a heterodimer on the *Timeless* and *Period* partner genes. Mutants of either have diminished circadian rhythmicity due to loss of

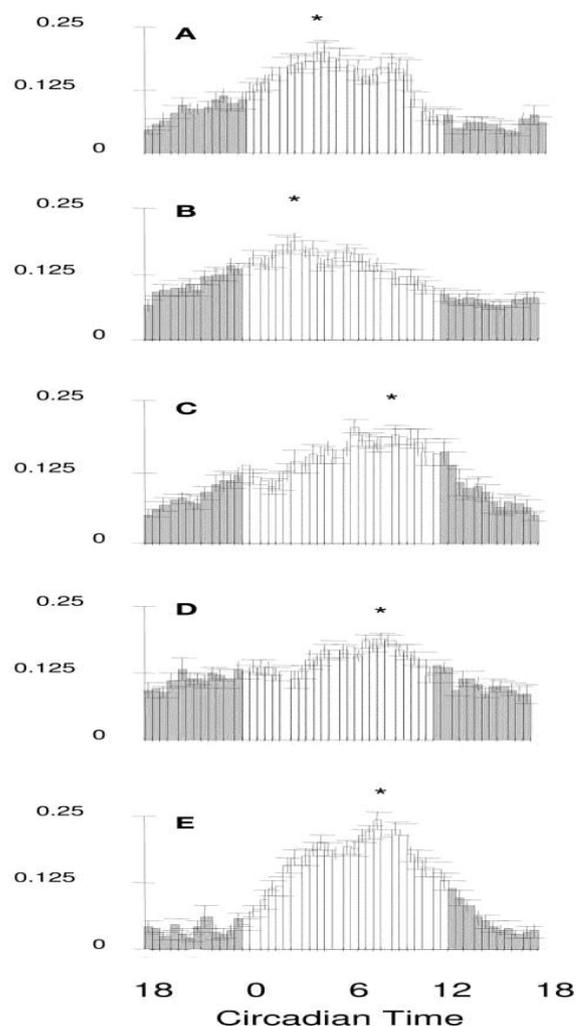


Figure 1 (edited citation: removed latter part): Free-Running Locomotor Profiles at 18 C of Males from *D. melanogaster*, *D. pseudobscura*, and Transformants Carrying *per* Transgenes from These Species. The x axis shows standardized circadian time. The y axis shows mean activity values (arcsin) and SEM. The dark area represents subjective night. An asterisk indicates the position of the activity peak as determined by a moving three-point average.

(A) *D. melanogaster* strain Canton-S, n24.

(B) *mel* transformant line 17a, n37.

(C) *D. pseudoobscura* strain AY, n19.

(D) *m_{ps}1* transformant line I-26, n18.

(E) Homozygous *m_{ps}1* insert (from line I-26) on a *per* background³.

Timeless and *Period* transcription²¹. *Timeless* and *Period* are at the core of the oscillation mechanism, whereby mutations in either alter or diminish the period of the circadian rhythm^{22,23}. *Timeless* and *Period* are essential components of this circadian rhythm whereby mutations disturb gating of the eclosion rhythm¹⁷. Furthermore, *Period* is essential for the emergence rhythm under laboratory environments whereas semi-natural environments only show weak rhythmicity¹⁴. Additionally, one study confirmed that mutations in either of the aforementioned genes result in lower reproductive fitness in male flies¹⁸. This study found a clock system in their reproductive system as well and earlier studies already confirmed the presence of separate clock systems in the antennae and excretion system^{17,18}.

Clock genes regulate both eclosion and locomotor activity rhythms^{19,20}. One key element in studies of circadian rhythms is the ability to synchronize endogenous rhythms to rhythmic environmental cues called Zeitgebers, such as light or temperature cycles, a process called entrainment. This shifts the rhythms forwards or backwards, depending when the Zeitgeber was presented. Generally, such Zeitgeber acts on sensors which relay information to central and/or peripheral clocks, which in turn induce transcription of clock genes with potential phase shifts as result²⁴. In laboratory environments, commonly a 12:12 light-dark cycle is used to study phase shifts. Locomotor activity is entrained by light-dark conditions, and temperature cycles as well^{24,25}. Under normal conditions, *Drosophila melanogaster* is diurnal, active mostly during the day^{3,26}. Panel A of Figure 1 shows locomotor activity rhythms of males at 18 °C, with significant peaks during the day and troughs during the night³. Figure 2 shows similar patterns for males (A), females (B) and male-female couples (C) under constant dark conditions, where circadian time represents the subjective time of the endogenous free-running (un-entrained) rhythm. There are differences between male and female rhythms

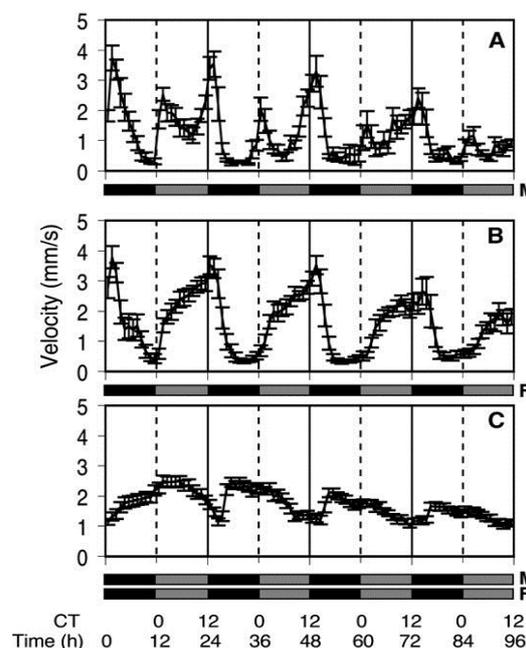


Figure 2 (edited figure: removed D-I; edited citation: removed descriptions of D-I): Locomotor Activity Is Different in Males and Females and Modified by Social Context. (A–C) Free-running circadian locomotor rhythm of individual flies (A & B) and FM combination (C) with a 2D arena. Locomotor-activity rhythms of single, sexually naive, and wild-type males (A) and females (B) are similar and show a minor and a major activity peak at subjective dawn and dusk, respectively, and rest during the subjective night. Note that males, but not females, exhibit reduced activity during the day. A novel activity rhythm is observed when a male and a female are present in the arena, with extended period of nightly activity (C). Black bars indicate the subjective night, and gray bars indicate the subjective day for males (M) or females (F), respectively²⁶.

but most intriguing is the male-female couple rhythm, discussed later²⁶.

Since *Drosophila melanogaster* is a cosmopolitan species found in around the world, one would consider the appearance of spatial and temporal isolation due to selection pressure. The different geographic habitats of the fly provide spatial isolation which in turn may lead to independent evolution of isolated populations. A similar phenomenon may occur due to variation in genes underlying timing of reproductive behavior, with temporal isolation as result. From a common ancestral *Drosophila* species, the *Drosophila melanogaster* and many other species of *Drosophila* may have risen and currently, new species of *Drosophila* may emerge from

existing ones. Hence – there are over 2000 species of *Drosophilids* currently known^{13,27}.

One study examined natural variation in *Period* and its relation to temperature²⁸. *Period* has a threonine-glycine Thr-Gly encoding repeat within its gene which is polymorphic in length. In natural populations of *Drosophila melanogaster*, 14 and 24 repeats occur most. In Europe and North Africa, (Thr-Gly)17 and (Thr-Gly)20 are the most common. These repeats influence the period of the molecular clock mechanism involved with *Period*, whereby temperature affects each variant differently. It was found, that (Thr-Gly)20 has the most efficient temperature compensation, where no significant difference between 18° (box) and 29°C (arrowhead) was found (Figure 3). The period was slightly below 24 hours, however. (Thr-Gly)17 displayed a period closer to 24 hours at 29°C but differed significantly from 18° with a shorter period. It was argued that these two variants are better adapted than others, considering cover up 90% of natural alleles. Since a (Thr-Gly)3 peptide forms a conformational monomer, “in-phase” series (Thr-Gly)14-17-20-23 show an approximately linear correlation between polymorphic length and temperature compensation. (Thr-Gly)14-17-20-23 series dominate, suggesting increments of 3 are most fit. This statement is reinforced by high occurrence of (Thr-Gly)17 in southern Mediterranean areas and (Thr-Gly)20 in northern Europe, polymorphisms apparently better adapted to their respective climates²⁸. Others speculate periods resonating closer with the environmental temperature cycle are better adapted, as less energy is spend in resetting the clock every day²⁹. The mechanisms behind temperature-correlated variation in this period were not detailed either, neither are there studies explaining this mechanism. Nevertheless, the study shows *Period* is prone to selection pressure caused by temperature, regarding the period of the clock mechanism²⁸. A different study confirms the phenotypic effect of varying repeat lengths is indeed directly related to efficiency of temperature compensation³⁰.

As previously detailed, *Drosophila melanogaster* is one of many kinds. Temporal and spatial isolation, along with great selection pressure, could cause new kinds of *Drosophila* to arise¹³. The following paragraphs will elaborate on differences in rhythmicity between *Drosophila* species and possible evolutionary explanations.

Unfortunately, not much research is done on interspecific differences in emergence and eclosion rhythms. There is a study of natural occurring *Period* rhythm mutants of *Drosophila melanogaster*²⁰. Longer-period, shorter-period and arrhythmic mutant *Period* strains revealed respective differences in both eclosion rhythm and

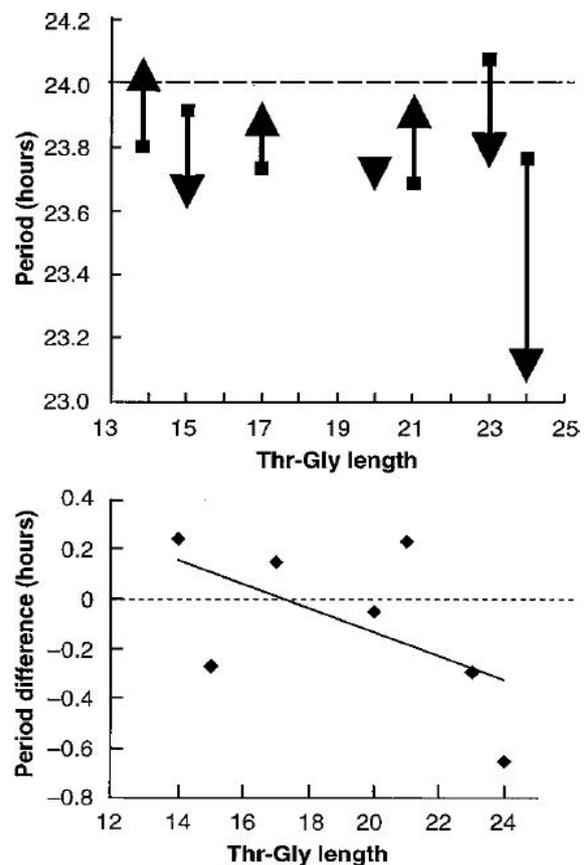


Figure 3 (Exact citation): (Top) Mean-free running periods at 18° (box) and 29°C (arrowhead) of males carrying different Thr-Gly length alleles. The means represent the pooled period averaged across the number of individuals within each Thr-Gly length (Table 1). The arrow reflects the change in direction of the period at 29°C. The (Thr-Gly)20 variants show almost identical periods at the two temperatures. (Bottom) The same data as (A) is used, but is plotted as the mean period obtained at 18°C subtracted from that obtained at 29°C. The regression line is plotted²⁸.

locomotor activity²⁰. No papers discussing interspecific emergence and eclosion rhythms were found. It can thus only be speculated that perhaps clock genes cause temporal isolation of developmental stages. Different eclosion rhythms may result in different hatching times. Divergence would then occur at different times, meaning specific mutants may be more probably to encounter conspecifics, but most likely there is still too much overlap for temporal isolation to occur. Interspecies differences in sexual maturation are documented, however. Sperm maturation in *Drosophila melanogaster* takes about 2 days, 9 days in *Drosophila hydei* and 3 weeks in *Drosophila bifurca*. Similarly, in females, mature oocytes appear several days after eclosion in *Drosophila melanogaster* while some Hawaiian *Drosophila* species may require 4 weeks¹³. With large differences, temporal isolation due to different development durations seems more plausible. Fast developing species may diverge before slow developing species eclose. Even then, overlap would still occur, as egg laying times vary. Thus, it remains unclear how emergence and eclosion rhythms of *Drosophila* are linked with evolution.

Figure 1 reveals the locomotor activity rhythm of *Drosophila pseudoobscura*³. While still clearly diurnal, the pattern differs from that of *Drosophila melanogaster*, mainly with its peak activity at circadian time ~8 hours, compared to circadian time ~3 hours of *Drosophila melanogaster*. These differences illustrate that circadian locomotor rhythms between species can be different, which possibly causes temporal isolation. Isolation may not be the proper term as there is still overlap. However, natural selection may require only small differences to drive speciation, so perhaps these differences in *Period*-dependent locomotor rhythms may have contributed to evolution of both species³.

The *Period* study involving polymorphisms in warmer and colder climates is an example of natural selection on spatially isolated populations²⁸. A gradient in environmental conditions rather than strict

isolation is present, but the term should suffice²⁸. As one would expect from intraspecific differences in a gene, there are differences in periods from homologue genes in other related species. Indeed, several studies have found species-specific variations of *Period*.

One study determined a different length of the Thr-Gly region in *Drosophila pseudoobscura*, having 200 residues (including non-(Thr-Gly)-peptides), compared to 50 of *Drosophila melanogaster*²⁹. For *Drosophila simulans* there are also variants with altered flanking haplotypes. However, a clear relation between environment and variants was not found. It can thus only be safely assumed the region is preserved among *Drosophila* species and selection may still play a role on this region in *Drosophila simulans*^{29,31}.

It should, too, considering functional studies discovered the region encodes species-specific circadian locomotor activity and rhythmicity in courtship songs of males of both species³². Not discussed previously is that the flanking regions coevolve with it, are species-specific, and essential for proper *PERIOD* functioning. Interspecific chimera studies have shown that flanking the region of *Drosophila melanogaster* with the *Drosophila simulans* haplotype, and vice versa, results in disrupted or abolished *Period* rhythms. Latter cases also displayed less stable forms of the *PERIOD* protein³¹. It is suggested, though without significant evidence, that variations in the repeat and flanking regions of *Drosophila simulans* are also due to differences in climate. It was further argued that specific variations occur mostly in specific climates due these variations being most energetically favorable as least metabolic cost is required for entrainment. This aspect of fitness was amplified with a study researching Cyanobacteria, where clock mutants with shorter and longer periods competed better in respectively shorter and longer periods of laboratory environments^{29,31}. It should be noted that the 'circadian' rhythms of Cyanobacteria may not be easily comparable to those of *Drosophila*, as Cyanobacteria originate from the astronomic period where

the rotation of the Earth around its axis lasted ~4 hours rather than the current ~24 hours. Regardless, the studies show a clear role of sexual selection in shaping species-specific rhythms²⁷.

Courtship behavior in male *Drosophila melanogaster*

Courtship defines male sexual behavior performed with the aim of producing offspring. The majority of behaviors related to reproduction is presented by males, though not exclusively. To avoid confusion, courtship defines the actions attributed to male sexual behavior. This section will explain male sexual behavior and its relation with rhythms.

Male courtship behavior involves a genetically defined set of actions, performed even if raised in social isolation. This activity defines most of their social life¹¹. One step of courtship is orienting towards the female and following her when she moves away. Here locomotor activity is an important visual cue to recognize a conspecific female. He then taps the back of the female with his foreleg. A species-specific courtship song is performed by vibrating one of his wings. He licks the genitalia at the female abdomen as well. Ultimately he attempts to copulate followed by actual copulation, by bending his abdominal genitals towards that of the female^{11,33}.

Mating activity of *Drosophila melanogaster* differs with age. No significant differences between day and night were found 3 days after eclosion, whereas significant differences appeared after 5 days³⁴. Both males and females show entrainment of mating rhythms from 5 days after eclosion in 12:12 light-dark cycles. The authors suggest a clock mechanism controls mating rhythms. *Period* and *Timeless* mutants were studied to confirm these genes affect the circadian mating rhythms, as knockout mutants show no significant rhythmicity in mating³⁴.

Another study also established locomotor activity is coupled to courtship, by comparing single sex, same-sex, and male-female setups measuring locomotor activity

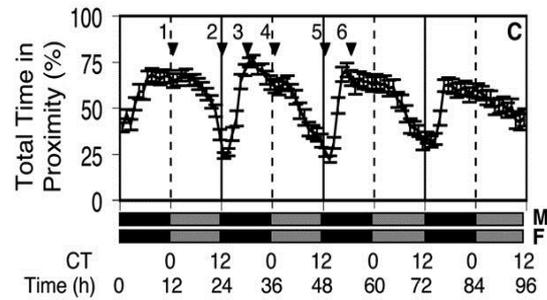


Figure 4 (edited figure: removed all but C; edited citation: removed all but description relevant to C): Rhythm of Close Proximity Reveals Circadian Nature of Male Sex Drive. FM couples (C) are in close proximity of each other for most of the night and large parts of the day, and they are interrupted for a few hours around subjective dusk. Black bars indicate the subjective night, and gray bars indicate the subjective day²⁶.

(Figure 2) and close proximity activity (ranging in centimeters, indicative of social interactions) (Figure 4)²⁶. The male-female close proximity rhythm is significantly rhythmic with peaks around subjective dawn and troughs at subjective dusk, as opposed to arrhythmicity found in same-sex couples. Comparison with single sex rhythms revealed phase shifts in the central clock mechanism to be male-specific, as this is independent of the female's phase. Additionally this so-called male sex drive is out of phase with locomotor activity rhythms (Figure 1), indicating males adapt in presence of females to display close proximity rhythm. Light-dark and constant dark conditions revealed no differences, suggesting locomotor activity and close proximity activity rhythms are due to social context. Courtship and mating rhythms were found to be highly synchronized. Observations imply locomotor activity and courtship to be linked, mainly due to females being chased by males as significant component of courtship²⁶. This might provide a means of temporal isolation, as it was found that different species of *Drosophila* have different courtship rhythms¹⁰. The researchers addressing these differences measured activity in sixteen species and found profound differences, but noted these might be partially due to pre-treatment of the flies. Regardless, it is

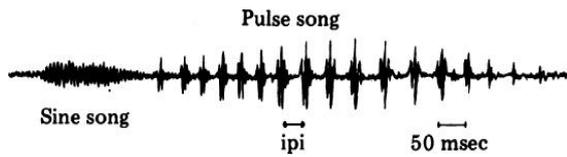


Figure 5 (novel citation): Courtship song of male *Drosophila melanogaster*. Song consists of Sine song and Pulse song with species-specific interpuls interval (IPI)³⁵.

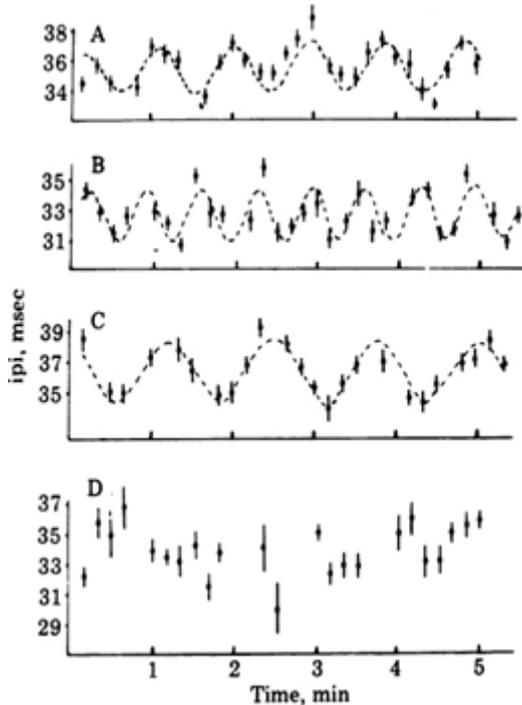


Figure 6 (exact citation): Courtship song profiles of the *per* mutants and a *per+* male (yellow on a Canton-S genetic background). (A) *per*⁺, $\bar{p} = 56$ sec; (B) *per*^s, $\bar{p} = 40$ sec; (C) *per*^l, $\bar{p} = 76$ sec; (D) *per*^o, $\bar{p} = ?$ ³⁵.

reasonable to assume that if species differ significantly in courtship and mating rhythms, temporal isolation may prevent interspecies copulation and thus enhances speciation¹⁰. One study did find significant differences in courtship and mating activity between *Drosophila melanogaster* and *Drosophila simulans*. During the day in LD cycles, both species showed high activity, whereas during the night, species were out of phase. In constant dark conditions, both species seem in anti-phase with each other. This further

suggests temporal isolation in mating rhythms³⁴.

One of the most important steps in courtship is courtship song³⁵. The vibration of a male wing produces an auditory signal for the female to recognize, with species-specific rhythmic properties. This signal is partially illustrated in Figure 5, whereby as part of the Pulse song, the interpuls interval (IPI) is displayed, the time in-between each pulse of wing vibration. For *Drosophila melanogaster*, this interval is approximately 34 ms. Occasionally, Pulse song is interrupted by a period of humming called Sine song. Not displayed is an overlaying rhythm fluctuating the IPI, with a period of about 1 minute in *Drosophila melanogaster*. Wing vibration is not essential for species recognition, but does contribute greatly to reproduction^{11,35}. *Period* is of great importance to courtship song, as it defines the fluctuation of IPI. It is yet unknown how *Period* affects a time scale of minutes compared to the circadian scale of 24 hours. Possibly, *Period* has non-circadian effects on transcription of factors related to courtship song³⁵.

The effect of natural polymorphism of *Period* on courtship song in relation to geographic habitat is doubtful²⁸. No significant differences in IPI regarding spatial location were discovered in a study examining *Drosophila melanogaster* populations taken world-wide³⁶. The researchers suggested a stabilizing selection acting on the species recognition system, whereby not environment drives selection but rather compatibility with other conspecifics, in order to maximize fitness. For this reason, genetic variation between populations would be minimal. This is an interesting result given that *Drosophila melanogaster* usually shows much variation among populations, seemingly with the exception of IPI. Other studies could not determine the evolutionary background of this phenomenon³⁶.

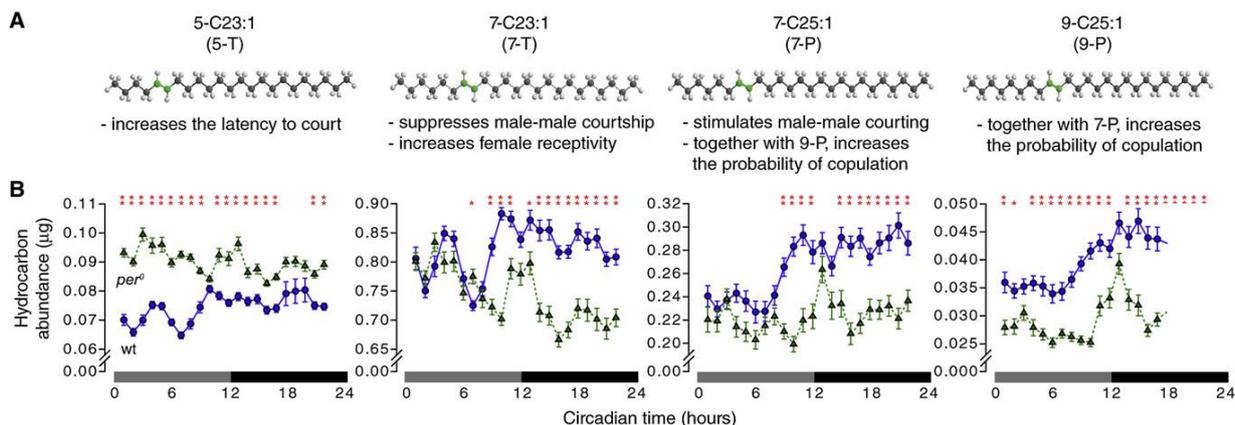


Figure 7 (edited figure: removed C; edited citation: removed latter part): Cuticular Hydrocarbon Accumulation Is Regulated by a *per*-Dependent Clock and Is Influenced by Exposure to Light. (A) Structural representations of hydrocarbon compounds 5-T, 7-T, 7-P, and 9-P. Each consist of a carbon chain (23C or 25C) with a single double-bond at position 5, 7, or 9 (green). (B) Comparisons in the temporal profile of the indicated hydrocarbon compound between wild-type and *per*⁰ males in DD (B)¹².

It was found that different *Period* mutants cause different variations in song rhythm and circadian locomotor activity rhythm³⁵. Longer-period (*per*^l) mutants show longer periods in both locomotor activity and IPI fluctuation. Shorter-period (*per*^s) mutants show shorter periods. Most interesting was *per*⁰ (arrhythmic) which, when placed with any other *per* allele, resulted in shortening of the song rhythm period (Figure 6). Consistent with the alterations in locomotor activity, respective results were found in the length of the courtship song period. No rhythm was found in the *per*⁰ allele. The study clearly shows a role for *Period* in both locomotor activity and courtship song³⁵.

Another important aspect of courtship is pheromone perception. Perception of pheromones occurs mostly in close proximity. Removal of olfactory antennal segments and Or83b knockouts (incapable of detecting most chemicals) revealed olfaction to be required for courtship²⁶. The antennae contain their own peripheral clock mechanism, suggesting a separate circadian rhythm in pheromone perception. Indeed, olfactory sensitivity is modulated by a clock mechanism (mostly *Period*-dependent) in olfactory neurons, independent of the clock in central brain neurons. Olfactory responses have highest sensitivity at night, an interesting finding given the high close proximity activity in male-female couplings at night²⁶. Emission is under control of a peripheral clock in oenocytes,

cells responsible for pheromone synthesis, also *Period*-dependent¹². Olfactory cues perceived by the antennae may cause phase shifts in other circadian rhythms such as locomotor activity and close proximity activity. It was found that olfactory cues are indeed required to maintain close proximity rhythmicity. Possibly, general locomotor activity differences in male-female coupling opposed to same-sex coupling are also due to pheromones. In *Drosophila melanogaster*, male sex pheromones are 5-C23:1 (5-T), 7-C23:1 (7-T), 7-25:1 (7-P) and 9-C25:1 (9-P), 7-T being most abundant. Their circadian expression profiles are displayed in Figure 7. 7-T appears to inhibit male-male courtship whereas it stimulates female receptivity, mostly at subjective night though a peak is also present mid-subjective day. Emission is greatly induced in close proximity encounters, independent of the conspecific sex^{11,12}. This suggests phase shifts in pheromonal emission occur in presence of conspecifics via olfaction of pheromones, emitted in basal quantities prior to courtship. Social interactions supposedly affect both central and peripheral clocks, given the influence on mating behavior and pheromone expression¹².

Unfortunately, phase shifts in pheromone rhythms were not further investigated and to my knowledge no papers currently address this issue. It thus remains speculative whether phase shifts occur and what the evolutionary relevance is. One could

argue that emission at times correlating with mating activity is preferential since these pheromones collectively induce reproductive behavior. Deviating from these times may provide a disadvantage for males as less reproductive behavior is induced in females at the appropriate times. Consequently, this may drive temporal isolation as conspecifics are adapted to species-specific emission and olfaction times. Differences between species could prevent interspecies mating. There are differences in mating rhythms between species due to differences in *Period*, suggesting similar differences in pheromone rhythms¹². Pheromone compounds also differ between species, mainly in molecular cuticular hydrocarbon (CH) structure and general expression profiles. This makes intraspecific research more difficult as comparisons between species are less easily made³⁷.

Sexual receptive behavior in female *Drosophila melanogaster*

Female sexual receptive behavior consists mostly of moving away from males present during social interactions, signaling males to recognize and follow the female to eventually court. The current section further addresses female rhythms and reproductive behavior.

Female locomotor activity is lowered in presence of a male, allowing the male to lick her genitalia¹¹. Females seem capable of determining mating rhythms by altering receptivity, under control of *Period* and *Timeless*^{11,34}. Courtship song and pheromones play important roles in female receptivity as demonstrated in studies comparing species of *Drosophila*^{12,26}. Presumably, these factors are cause for the change in locomotor activity and mating rhythms. Audition and olfaction are at least required for proper female receptivity. It was shown that courtship song and 7-T increase female receptivity^{11,26}. The study confirming differences in mating activity at different ages revealed a specific circadian mating rhythm in females³⁴. The researchers state that females show circadian rhythms in pheromone release and response to courtship

song, which may be causal for mating rhythms in male-female couplings. Since mating rhythms disappeared in *Timeless* mutant females crossed with regular males, and no rhythms were found in *Timeless* mutant males combined with regular or mutant females, it was argued that females determine circadian rhythmicity in mating. *Period* was found responsible for reduced mating activity at early subjective night and essential for the whole rhythm. The researchers were unfortunately not clear about the influence of courtship song or pheromones on rhythmicity³⁴. A different study addressing pheromones found that expression of main female pheromone 7,11-heptacosadiene (7,11-HD) differs in populations found worldwide, but no correlation was found with mating activity. No conclusions could be derived on an evolutionary perspective^{11,38}. Others have found a correlation between 7,11-HD and male courtship and concluded the pheromone induces courtship behavior and alters male preference, but this conclusion was wrongfully derived. The researchers eliminated all pheromones and focused on effects of 7,11-HD, neglecting the consequences of removal of other pheromones³⁷. Others found 7,11-HD to provide an inhibiting species barrier between different kinds of *Drosophila* rather than induce sexual behavior. Female *Drosophila melanogaster* flies lacking pheromones were even more preferred by males of varying kinds than wild-types³⁹.

With evolving male courtship songs, female preferences need to adjust accordingly – or vice versa⁴⁰. The most likely direction is that of males having to adapt to the current preference of females, as compatible courtship songs increase female receptivity whereas males appear to court indiscriminately^{11,34}. It was proposed that both signaling and preference systems share common genes. Hypothesized was that sexual selection of courtship song and female preference occurs either due to coupled genes or separate genes coevolving with each other. *Period* was selected as potential common gene. Wild-type (*per⁺*), longer-period (*per^l*),

shorter-period (*per^S*) and arrhythmic (*per⁰¹*) female flies were examined. They were presented with artificial songs matching those found in mutant male flies, in presence of wingless males. Given lack of mutant female preference for mutant songs, but choice for wild-type songs, mutant alleles are likely selected against in nature, thus supporting the coevolution hypothesis for the evolution of courtship song and female preference. Mutant females show similar preferences for courtship song as wild-type females. Further argued was that despite a small influence of *Period* on female receptivity, the proposed hypothesis of coupled genes could not be accepted. Rather, coevolution of separate genes seems a fitting explanation. For the shorter-period *Drosophila simulans*-like courtship song corresponding with the *per^S* male genotype, female receptivity seemed inhibited, thus implying species-recognition to prevent mating of *Drosophila melanogaster* females with *Drosophila simulans* males⁴⁰.

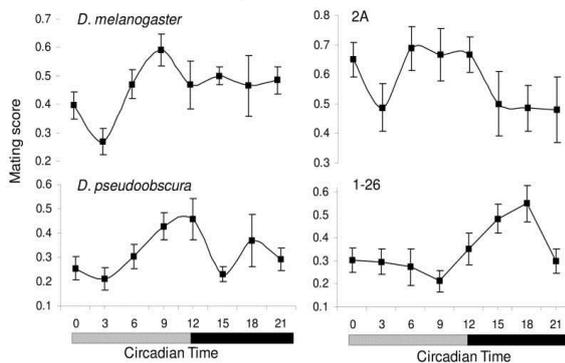


Figure 8 (edited citation: removed latter part): Free-Running Mating Rhythms at 18 °C

Top left panel, *D. melanogaster*; bottom left panel, *D. pseudoobscura*; top right panel, mel transformant line 2A; bottom right panel, mps1 transformant line 1-26 (per background). Each data point represents the mean proportion mating out of a possible ten (and SEM), from 10–15 experiments³.

This was confirmed in another study which added that the IPI has species-specific rhythmic fluctuation, with periods of approximately 1 and 0.5 minutes in respectively *Drosophila melanogaster* and

Drosophila simulans. The average IPIs themselves vary with approximately 34 ms respectively 48 ms. Further, not just these rhythmic parameters but also the general shape of the rhythms matter for species recognition^{35,41}. This adds to the theory that parameters of the courtship song are indicators of species for female receptivity³⁵. In terms of fitness, courtship song may indicate male genetic quality and subsequently that of offspring, thereby increasing fitness of females selecting such trait¹¹.

Other researchers challenged which genes encode female receptivity, for which hybrids between *Drosophila melanogaster* and *Drosophila simulans* were used⁴¹. It was hypothesized that certain sets of genes may encode both song rhythm and female preference. The hybrids share autosomal genes from both parent species, whereas the X-chromosomes are from 1 parental species. For each hybrid, song parameters were determined. It was concluded that differences in song rhythm are mainly due to X-linked genes whereas differences in IPI are due to autosomal genes. X-linked genes are however thought to still influence autosomal genes related to courtship song. Female song preference was examined with artificial songs in the presence of wingless deaf males. Preferences of hybrid females matched mostly the song parameters of their parents. Interesting results were obtained when presented with hybrid parameters. With a hybrid 45-second rhythm, showing lowest mating latency in all females, discrimination between IPIs was best pronounced whereby unique responses were obtained in hybrid IPIs. While the researchers were not clear on the genetics, it was shown that some form of sex-linked genetic coupling of song parameters and female preference was present⁴¹.

A transgenic study switching *Period* between *Drosophila melanogaster* and *Drosophila pseudoobscura* shows mating rhythms between species are different and controlled by *Period*³. There are significant differences in free-running locomotor activity

rhythms of both species, as mentioned earlier (Figure 1). Figure 8 shows a similar pattern for mating activity, complementing the correlation. Considering significant differences in mating peaks between the transgenic species, it was argued that *per* transgenes carry species-specific information about rhythmicity of mating and locomotor activity. It was further examined that flies prefer mating with flies carrying conspecific variants of *Period*. Males courted equally at any circadian time, implying females exert control over circadian mating phases³. This contradicts another study's finding that males determine mating rhythms rather than females²⁶. *Period* seems to define species-specificity in such a way that relatively small changes may already drive speciation, whereby differences in mating phase simply prevent interspecies mating, further enhancing temporal mating isolation³. This is in clear contrast with the view that differences in species occur due to accumulation of many small genetic changes⁴⁰. While females did prefer to mate with males carrying conspecific variants of *Period*, the genes responsible for female mating preference are unknown. The study does show that variation at only a single locus may drive speciation³.

Similar results were found in a study concerning different species of *Drosophila*. It should be noted that both *Drosophila melanogaster* and *Drosophila simulans* are cosmopolitan, whereas the other examined *Drosophila yakuba* is restricted to Africa. Isolation between the cosmopolitan species would, as argued before, be due to temporal isolation given variation in the *Period* gene governing circadian mating activity of both species^{3,10}. *Drosophila kikkawai* is another widely distributed species which, although covering similar geographic habitats, does not court with other species of *Drosophila*, given that courting occurs mostly at other times with respect to other species¹⁰.

Despite the apparent relative ease with which a new temporally isolated species can come in existence, a problem arises with the requirement for coevolution in the opposite sex, as it is currently understood that

male courtship and female preference are governed by separate sets of genes – or at least not by *Period*. Given that, mutations in *Period* altering courtship song are at a considerable disadvantage of not being preferred by female counterparts. In contrast with the most accepted model of gradual change, sudden changes are detrimental to the fitness of the individual and would thus not survive. As such, sexual selection requires that the mutation in *Period* (or any other sex-specific gene) is simultaneously accompanied by a mutation altering female preference in another individual – and for these individuals to court. Such coincidental event could have easily happened over millions of years of evolution, though the existence of easily variable and conspecifically accepted genes is also a likely explanation, borrowing at least some credit from the gradual evolution theory^{3,11,27}.

Discussion

Circadian rhythms affect reproductive behavior of *Drosophila melanogaster* by timing locomotor activity, mating rhythms, courtship song fluctuation and pheromone perception. The environment entrains locomotor activity by 24 hour light-dark and temperature cycles^{24,25}. *Period* and *Timeless* are here essential core genes of clock mechanisms. As the genes gate timing of emergence and eclosion, mutations alter these rhythms, in line with the hypothesis^{14,17}. Including *Cycle* and *Clock*, mutations lower fitness in males, underlining the importance of proper clock gene functioning¹⁸. *Period* encodes not only developmental rhythms but also locomotor activity and mating rhythms²⁶. Longer-, shorter- or arrhythmic-*Period* mutants show respective differences in both eclosion rhythm and locomotor activity²⁰. The hypothesis regarding evolutionary divergence by clock gene variation could not be proven however and remains speculative. Male-female social context alters male close proximity rhythms compared to single sex or same-sex locomotor activity. Phase shifts only occur in the male central clock, independent

of female phase²⁶. Females are as hypothesized indeed responsible for changing male behavior^{26,34}. This species-specific rhythm occurs by rhythmic female response to courtship song and pheromone receptivity, sustained by *Period* and *Timeless*^{3,11,34}. Courtship song and pheromones contribute to reproduction. The male wing vibrates with species-specific properties, most notably the *Period*-dependent rhythmic fluctuation of interpuls interval (IPI). Aforementioned *Period* mutants also show respective effects in the non-circadian period of this fluctuation³⁵. Species-specific courtship song parameters supposedly allow for species recognition to prevent interspecies mating, and thus to promote speciation⁴⁰. Separate clock systems are present in the olfactory antennae and pheromone excretion system, modulating circadian pheromone response and emission^{11,17,18,26}. Female receptivity is stimulated by male pheromone 7-C23:1 (7-T) which additionally inhibits male-male courtship. Close proximity encounters greatly induce emission, independent of sex^{11,12}. Female pheromone 7,11-heptacosadiene (7,11-HD) inhibits mostly interspecific male courtship behavior³⁹. Thus, as hypothesized, pheromones play a role in conspecific sex recognition. However, a role of circadian locomotor activity, mating activity and pheromone perception in intra- and interspecific variation among *Drosophila* remains speculative¹². The findings suggest that differences in aforementioned rhythms between species may be linked with temporal reproductive isolation. Unfortunately, much regarding the evolutionary background of interspecies differences in the discussed rhythms remains speculative and thus requires more research.

Reproductive isolation seems a plausible explanation for interspecies differences in reproductive rhythms. There must have been significant selection pressure to account for the more than 2000 species of *Drosophilids* currently known¹³. Given the wide geographical spread of many kinds, not just temporal but also spatial isolation could have occurred. Natural selection could act on

spatially isolated populations by driving adaptation to different habitats, though one should wonder how this can be accurately studied. *Drosophila melanogaster*, closely related species, and more distant related species could be studied in custom environments simulating their natural habitats. Alternatively, species other than *Drosophila* could be compared. Fitness should be measured in amount of offspring produced, relative to fitness in their natural environment. One might discover environmental factors that show clear correlations with reproduction, providing more insight in the evolutionary background.

Many studies utilize common laboratory schemes such as square (on-off) light-dark cycles or similar temperature cycles²⁵. These schemes, while proven useful, may not accurately reflect natural conditions. It is unclear how environmental factors influence rhythmic behaviors in natural conditions. Natural observations indeed do not support every key laboratory assumption of circadian behavior, such as anticipating light transitions or light dominating temperature⁴². Studies found that in contrast to laboratory conditions, afternoon locomotor activity peaks are present in semi-natural conditions^{25,42}. *Period* dependent emergence rhythmicity is attenuated in semi-natural conditions as well, compared to laboratory conditions¹⁴. Another example is the general hypothesis that *Drosophila melanogaster* is diurnal, yet no studies with natural conditions confirm this. Neither confirmed is whether male nocturnal sex drive occurs under natural conditions²⁶. Using realistic light and temperature cycles could provide more reliable results in studies on circadian rhythms⁴². Other ecological factors such as predators and food resources should be considered as well²⁶.

Another remark is that mainly *Period* was addressed. While essential for clock systems and well-studied, attention should also be paid to other clock genes¹⁷. As *Timeless* and *Period* are both at the core of clock systems, it can be assumed that natural variation in *Timeless* has implications for natural and sexual selection on rhythmic

traits³⁴. Other genes like *Clock* influence circadian rhythms as well^{19,20}. Studies on other clock genes and even their interactions with non-rhythmic genes could provide more insight on evolution of rhythmic reproductive systems.

As potential phase shifts in pheromone rhythms are not documented, further research is required. Social interactions are speculated to affect both central and peripheral clocks including the olfactory system^{11,12}. Females show rhythmic pheromone release, which might invoke mating rhythms in male-female couplings³⁴. Phase shifts might occur due to environmental factors or social context. Studies comparing pheromone release at different circadian times after administration of potential Zeitgebers, under which light and temperature alterations and social context, might uncover such phase shifts. Comparison of interspecies social context could reveal species recognition mechanisms.

Studying circadian rhythms and reproduction in *Drosophila melanogaster* could provide more knowledge about evolution of other species. In *Mus musculus* there is controversy regarding circadian influences on reproductive behaviors^{5,6,7}. However, female mice exhibit infradian estrous cycles, the mouse variant of menstrual cycles. These last ~4 days and consist of 4 main stages, including the estrous during ~10 hours, when females are most receptive⁴³. Receptive females prefer to mate conspecific males rather than *Mus domesticus* males, while in non-receptive state no preference is present⁴⁴. Male vocalization is also influenced by estrous state of the female. This illustrates endogenous rhythms influence reproductive behavior⁴⁵.

In both mice and humans, preovulatory pheromones advance the estrous/menstrual cycle while ovulatory pheromones create delays, thus affecting reproductive state^{46,47}. Possibly, olfactory reception and reproductive state varies daily for humans as well. A study comparing female preference for male sweated T-shirts, varying both wear and perception times, could

uncover such rhythm. Given the influence of seasons on animals, annual rhythms in reproductive state may also exist². Performing the proposed study at different times of the year would reveal this.

Studies on rhythmicity in reproductive behavior could provide more insights in the evolution of species with regards to temporal environmental variation. Much remains speculative and thus more research is required, where interspecies comparisons are important to trace back the relevant evolutionary forces. Ultimately such research provides a better understanding of how current life came to be and how future environments may steer evolution.

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