



Contents lists available at ScienceDirect

## Journal of Insect Physiology

journal homepage: [www.elsevier.com/locate/jinsphys](http://www.elsevier.com/locate/jinsphys)

## Effects of gender, age, and nutrition on circadian locomotor activity rhythms in the flesh fly *Sarcophaga crassipalpis*

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## ARTICLE INFO

## Keywords:

*Sarcophaga crassipalpis*  
Circadian rhythms  
Entrainment  
Locomotor activity  
Nutrition  
Anautogeny

## ABSTRACT

In many animal species, circadian rhythms of behavior are not constant throughout the lifetime of the individual but rather exhibit at least some degree of plasticity. In the present study, we have examined the potential influences of gender, age, and nutrition (presence or absence of liver) on the expression of circadian locomotor activity rhythms in the flesh fly *Sarcophaga crassipalpis*. We found no significant differences in endogenous circadian period under constant dark conditions with respect to gender, nutrition, or age for the duration of our experiments. On the other hand, both male and female flesh flies, as expected, were predominantly diurnal under light-dark cycles, but the pattern of entrainment differed between the sexes. Females also displayed higher activity levels than males. Also, in contrast with males, female activity levels increased with age. Moreover, females exhibited an extraordinary, but transient (one to three days), departure from diurnality which we characterize as “extended dark activity” (EDA). This phenomenon appeared as a continuous bout of locomotor activity that extended at least three hours into the early half of the dark phase at levels at least twice the median of the overall locomotor activity for the individual fly. EDA occurred as an age-dependent response to liver consumption, never appearing prior to day 4 post-eclosion but, thereafter, transpiring within one or two days after a 48-h exposure to liver. These results suggest a linkage between physiological events associated with egg provisioning and locomotor activity in the anautogenous flesh fly. Furthermore, our findings identify the existence of multiple influences on the expression of circadian clock-regulated behavior.

## 1. Introduction

Circadian clocks exert profound influences on physiological processes and behaviors in many organisms. Advantages associated with having endogenous circadian control of behavioral functions include enabling the organism to anticipate important events within its environment and scheduling behaviors at the most appropriate time of day or night despite seasonal changes in day and night durations. Key to anticipation and consistent scheduling is the ability of the circadian rhythm to entrain (maintain a fixed phase position) to various environmental oscillations (Pittendrigh and Daan, 1976). The best described environmental entraining signals (Zeitgebers) are light-dark (LD) cycles, temperature cycles, and daily occurrences of food (Aschoff, 1981; Mistlberger, 1994). Other factors, such as gender, age, and nutritional state may modify the manner in which the circadian rhythms entrain to environmental signals.

One of the preeminent model systems for understanding the molecular and neurogenetic bases of circadian behavior rhythms is *Drosophila melanogaster*. The phenotype of choice in the *D. melanogaster*

system is locomotor activity, due to the ease of measuring the behavior of individual animals under laboratory conditions and the fact that locomotor activity is considered to be a reflection of the animal's neural state of arousal (Brady, 1975; Martin, 2004). Support for this assumption is a recently discovered peptidergic neuronal circuit that links circadian clock neurons in the brain to motor circuits in the ventral nerve cord (King et al., 2017). Under laboratory LD cycles, *D. melanogaster* locomotor activity is diurnal and bimodal with a morning peak of activity at the lights-on (dawn) transition and an evening peak anticipating the lights-off (dusk) transition (Hamblen-Coyle et al., 1992; Helfrich-Förster, 2000). Under constant dark (DD) conditions, the activity free-runs with a period close to 24 h, often with both peaks free-running at the same period but occasionally splitting, thereby suggesting control from two different oscillators (Helfrich-Förster, 2000). In contrast, in *Drosophila pseudoobscura*, the evening peak free-runs in DD but the morning peak disappears (Engelmann and Mack, 1978), indicating exogenous control of the morning peak and endogenous clock control of the evening peak.

Gender-specific differences in circadian locomotor activity rhythms

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are common in insects. For example, in the cricket *Teleogryllus commodus*, males exhibit locomotor activity during the day (Loher, 1972) while the females show locomotor activity primarily during the night, when the males are singing (Loher, 1979). Although not as disparate as in crickets, *Nasonia* species exhibit male-female differences in locomotor activity rhythms with respect to the phasing of entrainment in LD and free-running periods in DD (Bertossa et al., 2013). In sandflies, *Lutzomyia longipalpis*, both sexes are active at dusk but males become active earlier and have a broader peak of activity than females (Rivas et al., 2008). In three different wild-type strains of *D. melanogaster*, the morning activity peak but not the evening peak of locomotor activity occurs significantly earlier in males than in females under LD cycles. Also, males have a shorter free-running period in DD than females and, in two of the three strains observed, females are more active overall than males (Helfrich-Förster, 2000). Furthermore, in *D. melanogaster*, males exhibit a marked reduction in activity (i.e., sleep) at mid-photophase (the mid-day “siesta”) that is not present in females (Andrejic and Shaw, 2005; Ho and Sehgal, 2005). In stark contrast, male and female hoverflies *Eristalis tenax* show no significant differences in locomotor activity (Thyselius and Nordström, 2016).

Weakened rhythmic behaviors and fragmented sleep episodes increase with age in mammals (Kolker et al., 2004; Zhdanova et al., 2011), including humans (Duffy and Czeisler, 2002; Cajochen et al., 2006). Similar age-related changes occur in *D. melanogaster*, including activity fragmentation and an increase in activity in the dark (Koh et al., 2006; Rakshit et al., 2012) as well as reductions in rhythm strength and longer free-running periods (Rakshit et al., 2012). Other circadian behavioral changes occurring with aging in insects include earlier (Kanno, 1979) or later (Castrovilho and Cardé, 1979) onsets in the time of calling in female moths and the transition from behavioral arrhythmicity to rhythmicity in adult worker honey bees (Moore et al., 1998; Toma et al., 2000; Shemesh et al., 2010; Eban-Rothschild et al., 2012).

Another factor known to influence the expression of behavioral circadian rhythms is nutritional state, for which there exists a diversity of responses. For example, in *D. melanogaster*, increases in dietary sucrose concentration, but not total caloric consumption, increases activity bout length and activity intensity in both sexes, and a yeast supplement reduces sleep in males and shortens bout length in females (Catterson et al., 2010). In contrast, female sandflies, *L. longipalpis*, following a blood meal, exhibit reduced locomotor activity as well as reduced expression of the canonical clock genes *period* and *timeless* (Meireles-Filho et al., 2006). Nutritional stress, such as starvation, may lead to increased locomotor activity, as observed in *D. melanogaster* (Knoppien et al., 2000; Lee & Park, 2004; Meunier et al., 2007; Sarov-Blat et al., 2000), increased spontaneous flight activity, as shown in tsetse flies (Crump and Brady, 1979), or have no effect on locomotor activity levels, as in the hoverfly *E. tenax* (Thyselius and Nordström, 2016).

In the present study, we examine the effects of gender, age, and nutrition on the circadian rhythm of locomotor activity in an autogenous insect, the flesh fly *Sarcophaga crassipalpis* Macquart (Diptera: Sarcophagidae). Joplin and Moore (1999) determined that the locomotor activity of adult *S. crassipalpis* is predominantly diurnal under LD cycles and under circadian clock control, as demonstrated by free-running periods close to 24 h under constant dark, constant temperature conditions (Joplin and Moore, 1999). However, the recordings were not differentiated with respect to sex, leaving open the possibility of sexual differences in both entrainment patterns and endogenous free-running periods. The potential for sexual differences in locomotor behavior in *S. crassipalpis* is suggested by sex-specific differences in spatial behavior (Paquette et al., 2008) and aggression (Moore et al., 2014; Shropshire et al., 2015).

In addition to the prospect of gender-specific differences in locomotor rhythms in *S. crassipalpis*, there is the potential for interactions among gender, diet, and age. Adult females have a nutritional

requirement (a protein meal necessary for egg maturation) that increases with age but is not present in males (Hahn et al., 2008b). In this study, our overarching objective is to determine if nutritional state (i.e., availability of liver) in this autogenous flesh fly has age- and/or gender-specific effects on locomotor activity rhythms, reflecting gender-specific differences in behavior observed under natural conditions. Specifically, the following hypotheses are proposed: (1) overall activity levels are lower in males than females, reflecting the sit-and-wait strategy males use to capture females for mating, (2) in the absence of a liver source, female activity escalates with age, paralleling the dissipation of larval protein reserves (Hahn et al., 2008a), (3) after a liver meal, female activity decreases, similar to the effect of a blood meal in sandflies (Meireles-Filho et al., 2006), and (4) the addition of liver to the male diet boosts overall arousal, leading to greater locomotor activity levels in both photophase and scotophase but not changing the fundamental pattern of entrainment, similar to the effects of dietary supplementation in *D. melanogaster* (Catterson et al., 2010). Our results reveal a number of significant interactions among gender, age, and nutritional factors influencing locomotor activity rhythms in *S. crassipalpis*. Additionally, we describe a previously unreported phenomenon: a radical but transient change (lasting one to three days) in locomotor activity pattern in female flesh flies, involving intense nighttime activity in this otherwise diurnal organism. Furthermore, this “extended dark activity” (EDA) is related to the timing (age) at which liver is available.

## 2. Materials and methods

### 2.1. Maintenance of flesh flies

The flesh flies *S. crassipalpis* used for all of the experiments described here (except for the constant dark experiments) were reared in a colony (colony 1) started at East Tennessee State University (ETSU) in 1994, derived from a colony established at The Ohio State University in 1972 (Denlinger et al., 1972). A second colony at ETSU (colony 2) was initiated in 2014: this colony also originated from the same Ohio State colony from 1972. The constant dark experiments (see below) used flies from colony 2. Colonies were maintained in non-diapause conditions of LD 15:9 h at  $25 \pm 0.5$  °C (Denlinger, 1985). Beginning two days after larviposition, cohorts of flies for the present study were switched to LD 12:12 h at  $25 \pm 0.5$  °C, maintaining non-diapause development (Denlinger, 1985; Flannagan et al., 1998) as well as establishing the entrainment conditions for adults (Joplin and Moore, 1999). Beef liver and sugar cubes provided the nutrition colony maintenance.

Despite being maintained in a continuous lab culture for many years, the flesh fly *S. crassipalpis* has continued to show robust (80–90%) diapause under diapause-inducing conditions (K.H. Joplin, personal communication), diurnal circadian rhythms of locomotor activity (Joplin and Moore, 1999), and an extensive behavioral repertoire including male-to-male aggression (Moore et al., 2014), reproductive behavior (Shropshire et al., 2015), and gender differences in inter-individual spacing (Paquette et al., 2008). We, therefore, consider our laboratory cultured *S. crassipalpis* to be a valuable model system for investigating fundamental mechanisms underlying behavior.

### 2.2. Measuring locomotor activity

An infrared (IR) beam interruption-based activity monitor (LAM25, TriKinetics Inc., Waltham, MA) was used to measure locomotor activity. Individual flies were placed in translucent plastic tubes (25 mm diameter, 12.5 cm in length) with a screen on one end and a water supply on the other. The activity tube contained a transparent strip (1 cm wide) at its center, permitting IR transmission and detection. The tube was placed in the monitor so that an array of three evenly spaced IR emitter/detector pairs was positioned over the transparent center strip. Interruptions of the IR beams were recorded at 1-min intervals.

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