Neurogenetic Studies of the Egg-Laying Rhythm of *Drosophila melanogaster*

A Thesis

Submitted for the Degree of

Master of Science

by

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For Amma

Who waited long enough for the clouds to pass

DECLARATION

I hereby declare that the thesis entitled "**Neurogenetic Studies of the Egg-Laying Rhythm of *Drosophila melanogaster***" submitted towards the fulfilment of the Integrated PhD degree is the result of investigations carried out by me at the Evolutionary and Organismal Biology Unit of the Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, India, under the supervision of Professor Vijay Kumar Sharma. The work incorporated in this thesis did not form the subject matter of any other thesis submitted by me for any other degree elsewhere.

Due care has been taken to acknowledge the work and findings of other investigators in the light of the present study, keeping in view the practice of reporting scientific observations. Any omission that may have occurred due to misjudgement or oversight is deeply regretted.

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CERTIFICATE

This is to certify that the work described in this thesis entitled "Neurogenetic Studies of the Egg-Laying Rhythm of *Drosophila melanogaster*" is the result of studies carried out by Ms. Shambhavi Chidambaram in the Chronobiology Laboratory, Evolutionary & Organismal Biology Unit of Jawaharlal Nehru Centre for Advanced Scientific Research, Jakkur, Bangalore 560 064, under my supervision, and that the results discussed in the thesis have not previously formed the basis for award of any other diploma, degree or fellowship.

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CHAPTER 1

THE OVIPOSITION RHYTHM OF *DROSOPHILA MELANOGASTER*: AN INTRODUCTION

INTRODUCTION

Nearly all organisms on Earth are subject, in one way or the other, to the effects of the planet's daily rotation about its axis and need to adjust their physiology and behaviour to the daily cycling of environmental conditions. The observation that living things exhibit rhythms in their physiology is not a recent one: as far back as the early 18th century, Jacques d'Ortous de Mairan noted that the common "touch-me-not" plant of the Fabaceae family opened and closed its leaves once every 24 hours, even when placed in the dark without any light stimulus (de Mairan, 1729), indicating that something within the plant was producing the rhythmic phenomenon, rather than it being merely a reaction to rhythmic stimuli. The clinching evidence for an endogenous source of daily rhythms came nearly two centuries later however, when Erwin Bünning demonstrated that navy bean plant variants differed in their endogenous periodicities from 24 h (Bünning, 1935). He correctly deduced that this meant the plants were not following a rhythmic environmental cue that repeated itself everyday, as this would produce exactly 24 h rhythms – instead, something from within the plant was producing a rhythm with a near-24 h period.

Internal time-keeping mechanisms or biological clocks have been found in organisms spanning a diverse range of taxonomic groups: from cyanobacteria (Stal and Krumbein, 1985; Mitsui et al., 1986), to fungi like *Neurospora* (Feldman and Hoyle, 1973), plants (reviewed in Somers, 1999), solitary insects like *Drosophila* (see Sheeba, 2008; Peschel and Helfrich-Förster, 2011 for reviews) eusocial insects like honeybees (Moore and Rankin, 1985), birds (Aschoff et al., 1962) and mammals like mice (Panda et al., 2002). A few of these organisms have been adopted as laboratory systems to investigate the genetics, neurobiology, physiology and behaviour of the output of the circadian clock, of which the greatest success has been seen in mice and *Drosophila.*

The fruit-fly *Drosophila melanogaster* has been described as the "workhorse" of experimental genetics (Jennings, 2011) due to the availability of a large number of genetic tools and methodologies that allow very precise manipulations to the fly. This permits a detailed understanding of the neurological and genetic basis of the fly's behaviour, including circadian behaviour. Numerous aspects of the fly's physiology have been conclusively shown to have a circadian rhythm (covered in more detail below): activity and rest (see Peschel and Helfrich-Förster, 2011 for review); adult emergence (see Myers, 2003 for review); olfactory

physiology (Krishnan et al., 1999; Tanoue et al., 2004); gustatory physiology (Chatterjee et al., 2010); feeding behaviour (Xu et al., 2008); cuticle deposition (Ito et al., 2008); the ability to learn (Quinn et al., 1974; Sakai et al., 2004); mating activity (Sakai and Ishida, 2001) and oviposition (see Howlader and Sharma, 2006 and Manjunatha et al., 2008 for reviews).

Of these the activity/rest rhythm is probably the most extensively studied, with the underlying genetics, molecular biology and neurobiology extensively worked out. Some, such as the olfactory and gustatory rhythms and the rhythm in mating, have been observed to share at least some of their clock genes with the locomotor activity rhythm (Krishnan et al., 1999; Tanoue et al., 2004), with the clock itself being an autonomous peripheral oscillator in the case of olfaction and gustation. Others, such as the 'fat-body clock' which is thought to drive the rhythm in feeding behaviour, is known to exist, but the core clock genes involved are not known in any detail (Xu et al., 2008).

The oviposition rhythm is quite an outlier in this case, with virtually nothing known about which genes are involved in it, or even where the clock lies. There is no doubt, however that there *is* an endogenous oscillator that produces this rhythm: it free-runs under constant conditions of light and dark (LL and DD respectively) with periodicities that fall within the circadian range (David and Fouillet, 1973; Allemand, 1977; Fluegel, 1978; Sheeba et al., 2001), and is both temperature- and nutrition-compensated (Howlader et al., 2006).

There are large gaps in our understanding of the oviposition rhythm, beginning with the fact that it is not entirely clear why there should be such a rhythm in the first place. In *D. melanogaster*, for example, the rhythm is unimodal in nature. In a 12:12 hour light/dark (LD) cycle the peak of the rhythm occurs close to dusk, i.e., the transition between light and dark, rather than being evenly spread out (see Figure 1).

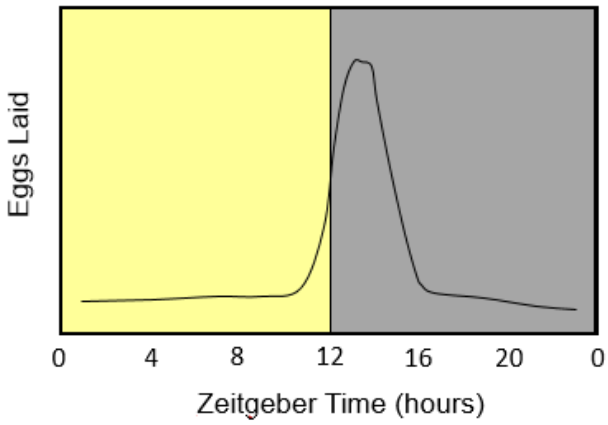


Figure 1: A representation of a typical oviposition profile under 12:12 h LD cycles

possible explanation for this timing of the peak is that eggs laid at dusk would be protected from desiccation due to high temperatures (Howlader and Sharma, 2006) and low humidity during the day, and as the larvae hatch 12-18 h later, they can burrow into the oviposition substrate before peak daytime temperatures the next day. This hypothesis is yet to be rigorously tested: what is certainly true however is that there are egg-laying rhythms in many insect species. Other than this, the details of the genetic and neurological control of the rhythm are almost completely unknown, and a great deal of the behavioural characterization of the rhythm still remains to be worked out.

This review aims to highlight the following: the fact that multiple oscillators exist within the fruit fly; that these oscillators can show a large degree of autonomy; to show that rhythms in reproduction exist in several insects species; to elucidate our current understanding of the egg-laying rhythm in *D. melanogaster* and emphasize several unique aspects of it that contradict what we might expect based on the far more extensively studied activity/rest rhythm; and finally, to point out directions for future research by providing some hypotheses and models for testing.

A MULTI-OSCILLATORY SYSTEM

A system of multiple oscillators

The clock residing in the ventral lateral neurons (the small ventral lateral neurons or s-LNvs) of the brain and dictating the fly's activity and sleep is one among many autonomous pacemakers in the body of the fly (see Giebultowicz, 1999; Peschel and Helfrich-Förster, 2011 reviewed in Tomioka et al., 2012 for reviews), which control different behavioural rhythms (Sheeba et al., 2001). An autonomous pacemaker is considered as such when it fulfils the following criteria (Giebultowicz, 1999): circadian oscillations that are sustained in vitro and direct entrainment by environmental signals. While the s-LN_v clock is thought of as a 'master' pacemaker because of its ability to drive the rhythm in locomotor activity, the extent to which it dominates the other pacemakers in the fly's body is not yet entirely clear. This is unlike the circadian organization of the mammalian body, which involves a strict hierarchy. Without the constant input of the Supra-Chiasmatic Nucleus (SCN), the 'master' pacemaker, peripheral oscillators lose their rhythmicity within four or five days (Yamazaki et al., 2000).

Certainly the time of day at which a fly is awake or asleep will decide whether it feeds, oviposits, smells or tastes at that particular time, so there is at least a partial behavioural dependence of 'peripheral' rhythms on the 'central' rhythm. This is not to say, however, that clocks in parts of the fly's body other than the s-LN_vs cannot tick along without input from the latter. Using real-time luciferase reporting of *per* transcription in living adult flies (Brandes et al., 1996; see also Plautz et al., 1997), with *per*-driven Green Fluorescent Protein as a visible spatial marker, Plautz et al. (1997) observed a circadian rhythm in bioluminescence in different body tissues maintained in vitro: antenna, proboscis, leg, wing, Malphigian tubule, testis and eye. Notably, the ovary did not show rhythmic PER expression, an oddity which we will examine more closely in the context of the egg-laying rhythm later in this review. These rhythms could entrain to new LD cycles and gradually decreased in amplitude when switched to DD.

The autonomous nature of peripheral oscillators is further highlighted by the excretory organs of *Drosophila* (the hindgut, Malphigian tubules and rectum). The Malphigian tubules alone were shown to be an autonomous pacemaker by their rhythmic PER expression in decapitated

flies (Hege et al., 1997) and when transplanted into flies that had been entrained to a different LD cycle the *timeless* (TIM) protein in the host tubules continued to cycle out of phase with that in the donor tubules for at least two days post-operation (Giebultowicz et al., 2000).

While the same molecular mechanism appears to underlie both the central and peripheral oscillators, some of the less crucial clock components seem to be tissue-specific, as the example of *cryptochrome* (CRY) illustrates. This blue light-sensitive protein is one of the principle light input pathways to the central circadian clock (Emery et al., 1997): associating with the F-box dependent protein JETLAG in a light-dependent manner, CRY interacts with TIM to induce its degradation and reset the circadian clock (Peschel et al., 2009). The CRY-TIM pathway appears to be the mechanism behind the entrainment of the clock in both the Malphigian tubules as well as the brain, but unlike the central clock where CRY is not an intrinsic component of the core molecular mechanism, *cry*-depleted mutants (cry^b) showed no cycling of TIM in this particular peripheral clock under constant darkness (Ivanchenko et al., 2001). CRY appears to an equally integral part of the antennal oscillator and seems to serve a function other than as a light input molecule: cry^b show severely dampened electroantennogram rhythms as well as in the cycling of PER and TIM in the antennae, and are unable to entrain to temperature cycles (Krishnan et al., 2001).

One example of a non-autonomous peripheral rhythm is the rhythm in adult-emergence. The peripheral clock in question lies in the pro-thoracic gland, where both PER and TIM show circadian oscillation (Emery et al., 1997). As in other peripheral oscillators, CRY appears to be an integral part of the mechanism, as cry^b mutants are arrhythmic for eclosion (Myers et al., 2003). Both the central clock as well as the oscillator in the pro-thoracic gland are required to produce the rhythm in adult-emergence: disrupting the former by ablating the Lateral Neurons or disrupting the latter by the over-expression of TIM both lead to a loss of eclosion gating (Myers et al., 2003). Moreover, Pigment Dispersing Factor (PDF) appears to be the means of communication between the Lateral Neurons and the pro-thoracic gland.

Altogether it would appear that the central and peripheral oscillators of *Drosophila,* while perhaps not a set of completely independent circadian oscillators (each entraining individually to external environmental cycles), are far less hierarchical in their interaction than one might imagine.

It is likely that a fine balance between the oscillators is required for the health and reproductive fitness of the organism. It has been shown, for example, that a de-synchrony between the fat body clock (Xu et al., 2008) and the brain clock, induced by restricting the flies' access to food to CT9-15 h when feeding is normally low, causes a reduction in the flies' egg output (Xu et al., 2011).

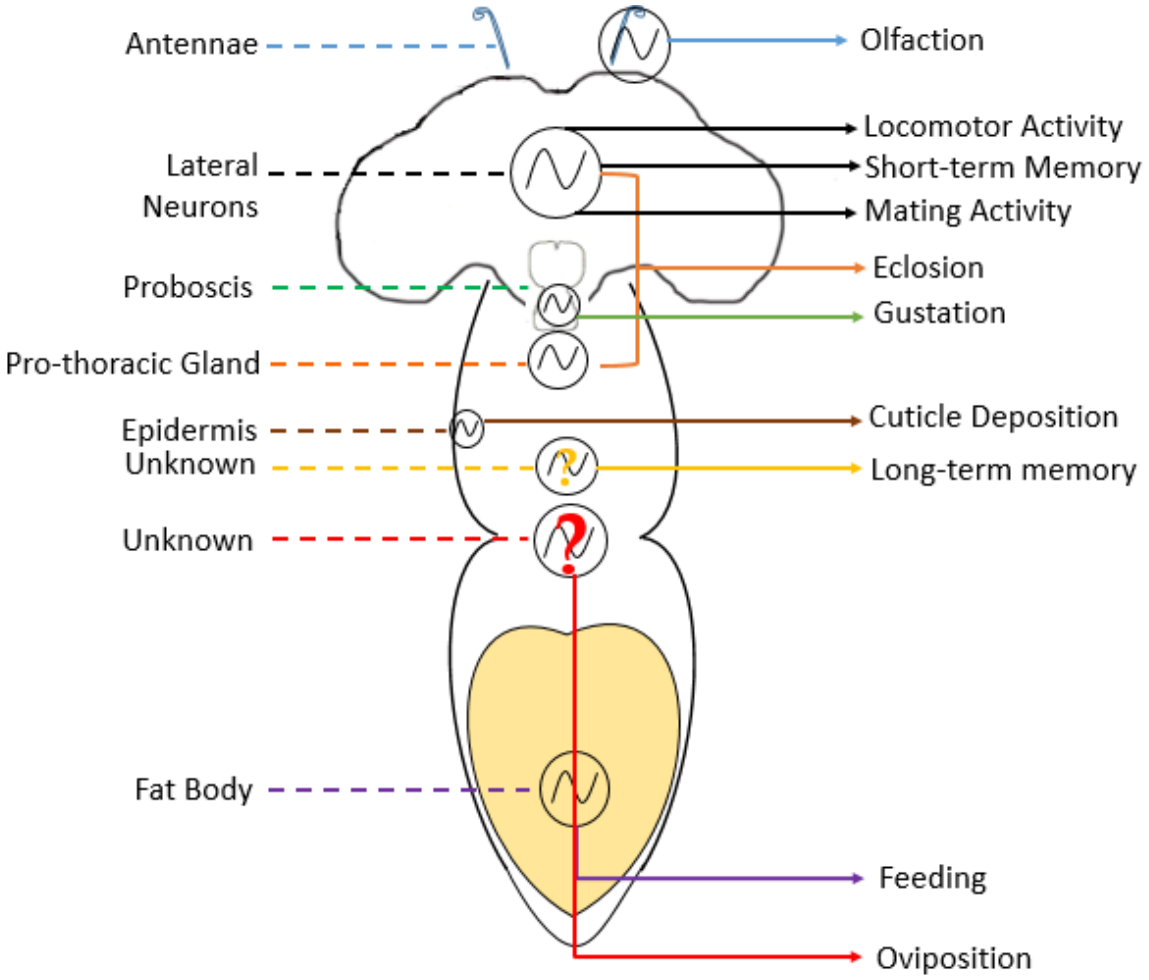


Figure 2: A schematic of those central and peripheral clocks, along with their location, whose behavioural or physiological output is known

Synchrony is particularly important in the case of single clocks with multiple components that must work together to produce a single behavioural rhythm. An excellent case in point of this is the central clock itself.

Oscillators within an oscillator

Two endogenously-generated bouts of activity every day, one in the morning and one in the evening are to be observed in a diverse range of organisms: birds, various insects (Aschoff, 1966), tens of different mammal species (reviewed in Aschoff, 1962) including the lab model systems mice (Jagota et al., 2000) and hamsters (the SCN itself has a remarkably heterogeneous organization: see Lee et al., 2003 for review), and even fish such as the Baltic herring (Bityukov, 1959). It is not surprising, therefore, that the "dual oscillator theory" dates back to Pittendrigh and Daan's proposal in 1976 (Pittendrigh and Daan, 1976): the widespread dual peak activity rhythm is due to the existence of two different oscillators. One oscillator tracks the onset of the light phase (dawn) and accelerates in response to light; the other tracks the offset of the light phase (dusk) and decelerates in response to light. In this manner, the circadian clock is capable of plastic responses to changes in photoperiod, allowing organisms to adjust their activity to the shifting seasons.

D. melanogaster also exhibits a morning and an evening peak of activity, and the study of the fruit-fly's bimodal activity rhythm gave the dual oscillator theory a cogent neurological mechanism. Expressing PER in specific subsets of neurons in a per^{01} genetic background revealed that the morning peak of activity of the fly is determined by the s-LN_vs and the evening peak by both the s-LN_vs and the dorsal Lateral Neurons (LN_ds), and the former alone seems to be adequate for driving the system (Grima et al., 1994; Stoleru et al., 1994). The 'morning (M)' and 'evening (E)' oscillators are functionally coupled to each other (Stoleru et al., 2004) with PDF-positive neurites projecting from the M-cells to the E-cells and CRY-positive, PDF-negative projections from the E-cells to the M-cells.

Intensive studies on the fruit fly have updated and revised the dual oscillator theory (reviewed fully in Yoshii et al., 2012). Importantly, another aspect of the original theory was held up: light does in fact speed up the M-oscillator and slow down the E-oscillator (It is of note that temperature has a similar effect – Majercak et al., 1999). Using mutant flies that lack the normal levels of CRY protein (the presence of functional CRY causes arrhythmicity under LL – see below under The Oviposition Rhythm of *Drosophila melanogaster*: What Do We Know So Far?), it has been demonstrated that under high light intensity, the activity rhythm begins to split into short- and long-period components, and the levels of PER in the M-cells fluctuated in synchrony with the short-period oscillator and that of the E-cells fluctuated with the long-period oscillator (Yoshii et al., 2004; Dolezelova et al., 2006). Furthermore, both the

M and E-cells appeared to underlie the evening activity: after the short-period M activity began to free-run, a second short-period component detached itself from the E activity peak, indicating that the M-oscillator might be the lead oscillator (Rieger et al., 2006), consistent with the original findings of Stoleru et al. (2004), and Grima et al. (2004).

A system of oscillators within oscillators cannot function without interaction between the rhythmic components. The neurotransmitter PDF is an insect homologue of the Pigment Dispersing Hormone, which was first characterized in fiddler crabs (Rao et al., 1985). The first indication that PDF might have a role to play in the *Drosophila* circadian clock came a few years later (Helfrich-Förster and Homberg, 1993): staining the fly brain with β-PDH antiserum revealed an arborisation pattern of PDF-positive neurons that closely overlapped with those neurons expressing PER. Co-staining with PER and the PDH antiserum made the link clearer: out of the five pairs of small ventral lateral neurons, four expressed PDF and the LN_ds did not (Helfrich-Förster, 1995). PDF is released in a circadian fashion in the region of the brain where the pacemaker is located, the dorsal protocerebrum (Park et al., 2000), a lack of PDF in the pacemaker neurons results in severe abnormalities in the locomotor activity rhythms (Renn et al., 1999) as did the ectopic overexpression of PDF in neurons projecting to the dorsal cerebrum close to the terminals of the LNv neurons, although such overexpression in PER and TIM-expressing neurons did not (Helfrich-Förster et al., 2000). All of this put together led to the following understanding of the fly's dual oscillator pacemaker: the master oscillator, the M-cells consisting of the 4 PDF-positive s-LNvs produced PDF in a rhythmic fashion, which provides input to the E-cells, comprising the PDF-negative fifth s-LNv and the CRY-positive PDF-negative LNds (see Figure 3a).

This classical model has been updated since then based on more recent investigations to incorporate the fact that the clock is more heterogeneous than the model depicts. Firstly, PDF was found not to be the fly's only circadian neurotransmitter. Neuropeptide F (NPF), which was previously known to modulate ethanol sensitivity (Wen et al., 2005) was found to be expressed in the LN_ds of male flies alone, and *npf* mRNA was undetectable in these cells in the *Clock* (*Clk*^{Jrk}) and *cycle* (*cyc*^{01}) mutants (Lee et al., 2006). Given that the LN_ds form part of the E-oscillator, it is not surprising then that ablating NPF-expressing clock neurons causes an advance of evening activity (Hermann et al., 2011).

sNPF is the other major neurotransmitter whose role in the circadian system has been partially characterized. It is known to be expressed in the four PDF-positive s-LN_vs of the M-

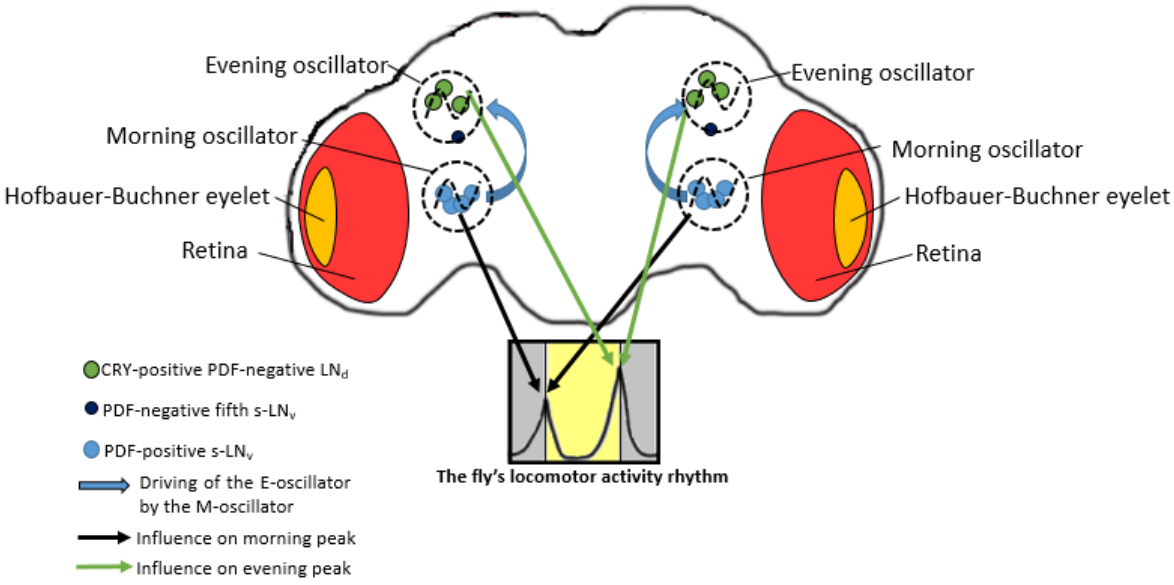
oscillator, and two out of the three CRY-positive LN_ds (Johard et al., 2009), with a role in the regulation of sleep levels and maintaining sleep homeostasis (Chen et al., 2013; Shang et al., 2013).

An investigation of the effect of PDF-release by the M-cells on the E-cells showed that the PDF-positive neurons do not govern the molecular clocks in all the PDF-negative neurons (Yao and Shafer, 2014), and further examination of the PDF-negative neurons revealed distinct oscillators that differed in their functionality and neurochemistry. More recent electrophysiological studies of the PDF-neurons (Guo et al., 2015) indicate that, while the M and E-cells can independently generate activity rhythms, the PDF-neurons themselves receive light information from the environment and transmit it to the E-cells via the rhythmic release of PDF.

The current model of the dual oscillator system is something as follows, as Yao and Shafer (2015) have put it. The E-oscillator can be divided into three neurophysiologically distinct oscillators: PDF-negative LN_ds, sNPF-positive LN_ds and the ITP-positive LN_d and fifth s-LN_v. Of these, the latter two groups are strongly coupled to the M-oscillator (the four pairs of PDF-positive s-LN_vs) and the first is only weakly coupled to it. The M-oscillator is responsible for the morning activity peak, as was the case with the classical model, but the updated E-oscillator now consists of the latter two groups of non-PDF-expressing groups of neurons, excluding the PDF-negative LN_ds, responsible for the evening activity peak.

The wealth of newly-discovered detail about the central pacemaker of the fruit-fly shows that even a single clock responsible for a single behavioural output can be composed of a complex hierarchy of multiple oscillators coupled together with varying degrees of strength. It remains to be seen whether the other rhythms of the fly prove to be equally complicated in their neuronal organization. It is not an unreasonable guess that the oviposition rhythm, fundamentally tied with the vital Darwinian task of reproduction really will be so, with many redundant components and compensatory circuits.

(a)



(b)

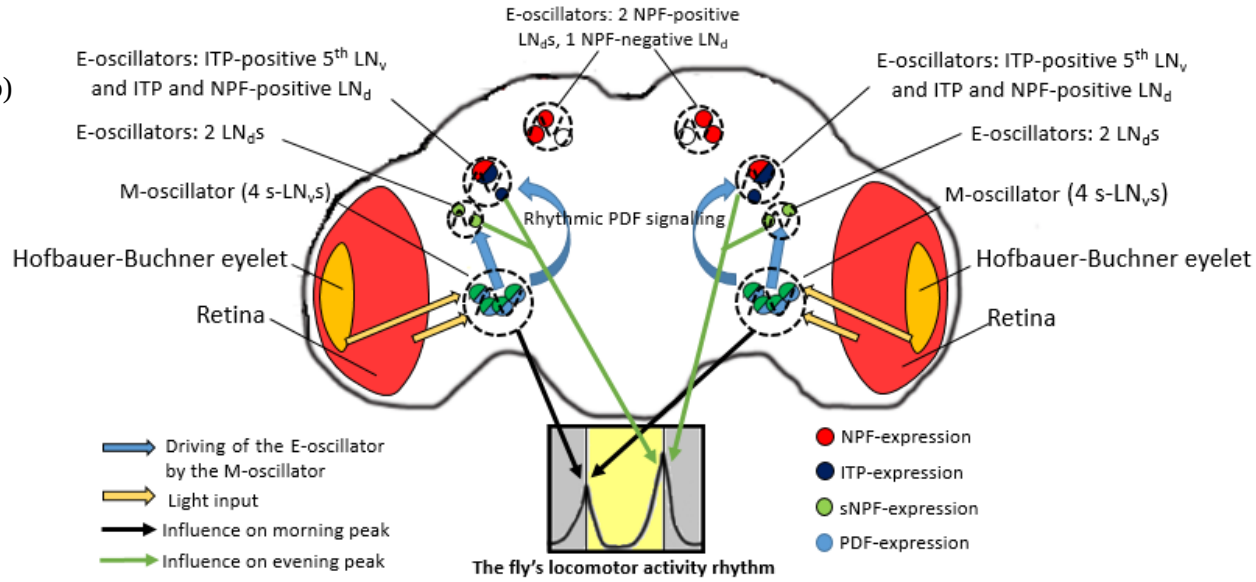


Figure 3: Oscillators within an oscillator – the central pacemaker of the fly which dictates the locomotor activity rhythms (see text for explanation)

a) The classical dual oscillator model
b) The updated dual oscillator

EGG-LAYING RHYTHMS IN INSECTS

Reproductive rhythms: a widespread and wide-ranging phenomenon

Reproductive physiology, as we have briefly seen so far, is also subject to the influence of the circadian clock in *D. melanogaster*. Rhythmicity in reproduction is a concept familiar to us, as mammals, in the monthly estrous cycle. This too, however is subject to circadian rhythmicity. The levels of Luteinizing Hormone and Follicle Stimulating Hormone have a circadian rhythm in rodents such as rats (Goldman and Mahesh, 1968) and Syrian hamsters (Goldman and Mahesh, 1969) and this rhythm is under control of both the ovaries (Goldman et al., 1971) as well as the mammalian master circadian pacemaker, the Supra-Chiasmatic Nucleus, through its entrainment of the pineal melatonin rhythm (Moore, 1995). Seasonal rhythms in reproduction are very common in mammals; due to the vast amount of energy and resources required to gestate and raise newly-born progeny, these rhythms appear to serve the purpose of optimally timing birth to occur when food resources are most abundant. For this sort of seasonal rhythm, mammals track changing photoperiods (see Goldman, 1999 and references therein). Light is a zeitgeber and when it occurs at a particular phase of the circadian cycle, the clock responds by behaving as though affected by long, summer photoperiods. This is known as the external coincidence model based on Erwin Bünning's original proposition that light could induce long photoperiod responses when present at particular times during the circadian cycle (Bünning, 1936; 1960).

Reproductive rhythms in non-mammalian species range from circadian (Howlader and Sharma, 2006) to circatidal and circalunar in aquatic animals (reviewed in Raible et al., 2011) and circannual (Duston and Bromage, 1988; Bailey, 1981; Gwinner, 1996). Insects, with their terrestrial lifestyles and life cycles on the order of a few days to a few months generally display only circadian rhythms in their reproductive physiology, and this has mainly been studied from the point of view of rhythmic sperm release and rhythmic egg-deposition.

Studies on the egg-laying rhythms of insects have tended to focus on those species that have some impact on human beings, whether they are agricultural pests such as the turnip moth (*Agrotis segetum* – Byers, 1986) and the European corn borer (*Ostrinia nubilalis* – Schurr and Holdaway, 1966), disease vectors such as mosquitoes (*Aedes aegypti* – Wong et al., 2011) and kissing bugs (*Triatoma infestans* and *Triatoma phyllosoma* – Constantinou, 1984),

or even insects implicated in the forensic sciences (*Calliphora vicina* – George et al., 2015). Oviposition rhythms have also been in studied in several Drosophilids, including *D. melanogaster* (Sheeba et al., 2001), *D. ananassae* and *D. malerkotliana* (Priya, 2014). As we shall see in the next section, despite its suitability to laboratory maintenance and study, vast gaps remain in our understanding of the egg-laying rhythm of *D. melanogaster*.

There is no doubt that many insect species display a daily rhythm of period approximately 24 h in their egg-deposition (see below). Many of these rhythms are unimodal with a single, daily peak of egg-output, and demonstrate entrainment of the rhythm to changing LD cycles. I have presented a brief review below of these rhythms in various disease vectors, agricultural pests and some species closely related to *D. melanogaster*.

Disease vectors

Insects that act as disease vectors in humans and other mammals need to time their daily activities in accordance with the external environment, but also with the physiology and activity of their respective hosts to better their chances of successful infection or infestation. Ideally, egg-deposition should be maximized when the host is likely to be sleeping, resting or otherwise stationary, which is why many parasitic or haematophagous insects show peaks in their daily oviposition rhythm around or after sunset. The cat flea *Ctenocephalides felis*, for example, show a peak of egg deposition between midnight and 3 AM when its host is likely to be resting; the eggs are then clustered in cat resting areas to increase the chances of being picked up by another host (Kern et al., 1992).

Mosquitoes are still some of the most dangerous insect vectors of human disease in the world, and several species have been examined to pick up rhythms in their reproductive physiology, the better to control their populations. The malaria-spreading *Anopheles aquasalis* (Chadee and Mohammed, 1996) and *Anopheles kruzii* (Chahad-Ehlers et al., 2007) both show something akin to a daily rhythm though the evidence as to its endogenous nature seems to be very unconvincing. *Anopheles gambiae,* known for spreading the most dangerous malaria parasite of all (*Plasmodium falciparum*) shows two slightly different rhythms depending on where they were reared: wild-caught mosquitoes showed two different peaks, one about an hour after sunset and another peak a couple of hours after that, while green-house reared mosquitoes only showed the first peak (Sumba et al., 2004). It must be borne in mind, however, that these 'rhythms' have only been observed under natural LD cycles and the presence of a free-running rhythm has yet to be demonstrated under constant conditions of

light or dark. *Aedes aegypti*, another mosquito disease vector responsible for spreading such diseases as yellow fever, dengue fever and chikungunya, shows a bimodal rhythm in its oviposition. Its peaks appear to be diurnal, however, with a smaller one occurring between 0600 and 0800 h and the larger one between 1600 and 2000 h in a 0800 to 2000 h LD cycle (Wong et al., 2011). This species, like the *Anopheles* species has yet to be tested under constant conditions.

One genus of disease-carrying mosquitoes that *has* been tested under constant conditions is *Culex. Culex pipiens* appears to have a crepuscular rhythm, with a peak at sunrise and another peak at sunset in its egg-laying (Savage et al., 2006); *Culex tritaeniorhynchus* on the other hand appears to be more nocturnal: the peak occurs in the first half of the dark phase when placed in 12:12 h LD cycles, and this persists even when the light regime is changed and made anti-phasic (Aslam et al., 1977). However, it seems fairly clear that these rhythms are not endogenous in origin: *C. pipiens* shows a loss of its morning peak under shorter photoperiods and the rhythm of *C. tritaeniorhynchus* is greatly diminished under constant light.

Unlike mosquitoes, 'kissing bugs', which most famously transmit diseases like Chagas disease have been unequivocally shown to have circadian rhythms (Constantinou, 1984). Members of the genus *Triatoma* are blood-sucking human parasites and two species (*T. infestans* and *T. phyllostoma*) have been shown to have a nocturnal unimodal peak, beginning about 3 h after lights-off in 12:12 h LD cycle (Constantinou, 1984). Under increasing photoperiod, the peak of egg-laying shifts closer to the transition between light and dark and even when the photoperiod is 20 h long, egg-laying continues to be nocturnal, though it now becomes biphasic, with a peak at each end of the dark phase. Under 2:22 h LD cycle, the rhythms were shown to free run with an endogenous period of about 23 h. Another closely related species *Panstrongylus megistus* shows a similar peak of oviposition shortly after lights-off, and free-runs under DD with a period of 23 h. *Rhodnius prolixis,* which is the principal vector of Chagas disease in many parts of Latin America, is similar to *D. melanogaster* in many aspects of its oviposition rhythm: it too displays a unimodal rhythm under 12:12 h LD cycle with a peak around the transition between light and dark, though this peak is much more narrowly gated than in the fruit-fly; its free-running rhythm has a period of about 25 h; and the virgin insects also lay eggs, though fewer than mated females do (Ampleford and Davey, 1988).

Agricultural pests

There is a fairly obvious reason why the study of the rhythms in the reproductive behaviour of pests is of interest. Recognizing the times of day when oviposition and/or mating behaviours peak will enable us to target the insects more effectively by concentrating our efforts at this time, minimizing the collateral damage caused by the indiscriminate use of such pest-control measures as synthetic pesticides and insecticides (see, for example, Miyatake, 2011). Unlike disease vectors who must target their hosts when they are most vulnerable, environmental conditions appear to be the primary factor that determine the timing of oviposition peaks in pest insects.

Glenea cantor, the longhorn beetle and potential invasive pest in several parts of Asia, has peaks of both oviposition and mating that occurs almost exclusively in the photophase of a 14:10 h LD cycle. The oviposition rhythm appears to have two peaks, one 2-5 h after the start of the photophase and another one 7-11 h after the start of the photophase, while mating has a unimodal rhythm with the peak occurring 10-14 h after the start of the photophase (Lu et al., 2013). The bean bug *Riptortus clavatus* shows a diurnal peak in its oviposition rhythm, occurring 8-12 h after lights-on in 12:8 h LD cycle. Free-running rhythms in both constant light and constant dark have been demonstrated, testifying to the endogenous nature of this rhythm with free-running periods of 20-21 h in DD and 22-25 h in LL (Numata and Matsui, 1988). The milkweed parasite *Oncopeltus fasciatus* has a similarly diurnal rhythm of oviposition, with the peak of egg-laying occurring between 4 and 10 hours after the start of the photophase (Rankin et al., 1972). This holds true under a range of photoperiods, and under constant light, although the insects continue to be rhythmic, their free-running periods are shown to drift and get longer over several days (Caldwell and Dingle, 1967).

The importance of ecological factors in determining the oviposition rhythm of plant pests is evidenced by the rhythm of *Ostrinia nubinalis*, commonly known as the European corn borer. *O. Nubinalis* appears to require moist conditions for egg-laying as rainfall seems to induce oviposition, and a sharp increase in humidity could act as an important zeitgeber for this rhythm (Schurr and Holdaway, 1966). Under natural LD cycles the peak occurs precisely at 10 PM. Under DD the rhythm free-ran with a period of about 20 h, though it became far less precise (Skopik and Takeda, 1980) and under LL the rhythm was suppressed.

Other agricultural pests have also been shown to have what appears to be a rhythm in their daily egg-output, but whether these rhythms are truly endogenous in nature have yet to be

determined by assaying them under constant conditions. The oviposition rhythms of such insects can show peaks in either light or dark. The Spruce Budworm *Choristoneura fumiferana* shows a diurnal peak in oviposition between 1200 and 1800 h (Sanders and Lucuik, 1975); the Turnip Moth (*Agrotis segetum*) and the Indian mealmoth (*Plodia interpunctella*) are nocturnal in their activity, and show peaks of oviposition at or shortly after the start of the scotophase (Byers, 1987; Lum and Flaherty, 1969).

Carrion flies

The presence of blowfly larvae on corpses are often used to determine the time of death of a corpse, and thus the oviposition rhythms of the genus Calliphoridae are of special relevance to forensic science. The circadian biology of *Calliphora vicina* is particularly well-studied. Its locomotor rhythm shows a fairly constant level of flight activity during the photophase and the fly becomes relatively inactive during the dark phase (Saunders and Hong, 2000). Its oviposition activity appears to be primarily diurnal as well, and when the LD cycle is reversed, oviposition shifts to track the light phase (George et al., 2015). This is one of the few non-Drosophilid species in which the mRNA rhythms of clock genes *per* and *tim* were tracked: PER and TIM mRNA show cycling in both the head as well as the ovaries with a peak in the middle of the night at around 0200 h, which provides further evidence of the endogenous nature of both rhythms, and the conserved role of these clock genes, which have been so extensively studied in *Drosophila melanogaster*.

The locomotor activity rhythm of at least two other Calliphorid species have been examined: *Lucilia cuprina* (Smith, 1983) and *Phormia terraenovae* (Aschoff and von Saint Paul, 1982; Hamasaka et al., 2001) both of which have similar rhythms to *Calliphora vicina*: diurnal activity and nocturnal inactivity. However, there seems to be some amount of controversy regarding whether oviposition is nocturnal or diurnal in blow flies. While some studies report no evidence of nocturnal oviposition activity in blowflies (Tessmer et al., 1995; George et al., 2015; Amendt et al., 2008) others clearly report that it does happen (Greenberg, 1990; Singh and Bharti, 2001).

Drosophilids

Drosophila pseudoobscura

The oviposition rhythm of *Drosophila pseudoobscura* shows enormous variability from day to day even when placed in rhythmic light-dark conditions, sometimes even skipping days and with a great deal of inter-individual variation as well (Fluegel, 1983). This could just as well mean that the oviposition rhythm is more complex than that of other species, and requires more sophisticated protocols to study and if one is developed it is just possible that a novel circadian mechanism is awaiting discovery.

Drosophila melanogaster

Our necessarily succinct overview of egg-laying rhythm in various species of insects gives us a few tentative conclusions to draw, which we may meaningfully apply to the study of this rhythm in *D. melanogaster*.

It seems to be that the life cycle and ecology of the organism play a significant role in determining where the peak of oviposition lies. For example, parasites that target their hosts when the latter are asleep or resting might show a nocturnal peak in oviposition. In understanding the adaptive significance of the phasing of the *D. melanogaster,* a better understanding of its ecology and life cycle would help formulating and testing hypotheses. A number of different factors can shape the amplitude, phasing, period and light-sensitivity of the oviposition rhythm. Studying the rhythm under several different light conditions and examining strains from different geographical locations and altitudes seem indicated to understand the effect of these factors. Finally, it is possible that the oviposition clock could operate by a very different mechanism than the other better-studied oscillators in the fly system. There may be a great many redundancies and compensatory circuits in place as the act of egg-laying is crucial to a fly's Darwinian fitness and this could contribute to the complexity of the oscillator.

THE OVIPOSITION RHYTHM OF *DROSOPHILA MELANOGASTER*: WHAT DO WE KNOW SO FAR?

The established canon of fly circadian biology

The locomotor activity rhythm of *Drosophila* is the best studied of all its rhythmic behaviours and what we know about it rather represents the established canon of fly circadian biology. The fruit fly is a crepuscular organism and show two peaks of activity – one each in the morning and evening – and a period of rest during midday. The neural circuitry or clock responsible for generating this bimodal rhythm has been localized to about 150 'pacemaker' neurons in each brain hemisphere, comprising seven different anatomical subgroups (Sheeba, 2008).

At the molecular level, the circadian clock is composed of two tightly interlocked transcription-translation feedback loops, the clock genes *per*, *tim*, *clk* and *cyc* being the core components (see Hall, 2003, and Peschel and Helfrich-Förster, 2011). In brief, the molecular circadian clock functions as follows (see Figure 4 for a representative schematic). The proteins CLOCK (CLK) and CYCLE (CYC) form a heterodimer that binds to the E-box enhancer element of *per*. This activates the transcription of *per* (Rutila et al., 1998; Darlington et al., 1998; Allada et al., 1998). The transcription of the gene *tim* is also activated by CLK and CYC and as PER and TIM proteins form, they are translocated to the cytoplasm. PER is phosphorylated and degraded by the kinase *doubletime* (*dbt*) (Price et al., 1998) unless protected by TIM; TIM itself is phosphorylated by SHAGGY (SGG). PER and TIM bind together to form a heterodimer along with DBT, and the PER-TIM-DBT complex is translocated into the nucleus at least partly through the effect of SGG (Martinek et al., 2001). Inside the nucleus, this complex binds to the CLK-CYC heterodimer through the interaction of PER and CLK (Lee et al., 1999). This hyperphosphorylates CLK thereby stops the CLK-CYC heterodimers from initiating *per* and *tim* transcription by preventing their binding to the DNA (Yu et al., 2006). As light degrades TIM by means of the blue-light photoreceptor *cryptochrome* (CRY) (Stanewsky et al., 1998; Peschel et al., 2009), the suppression of CLK and CYC is relieved and the circadian rhythm starts anew.

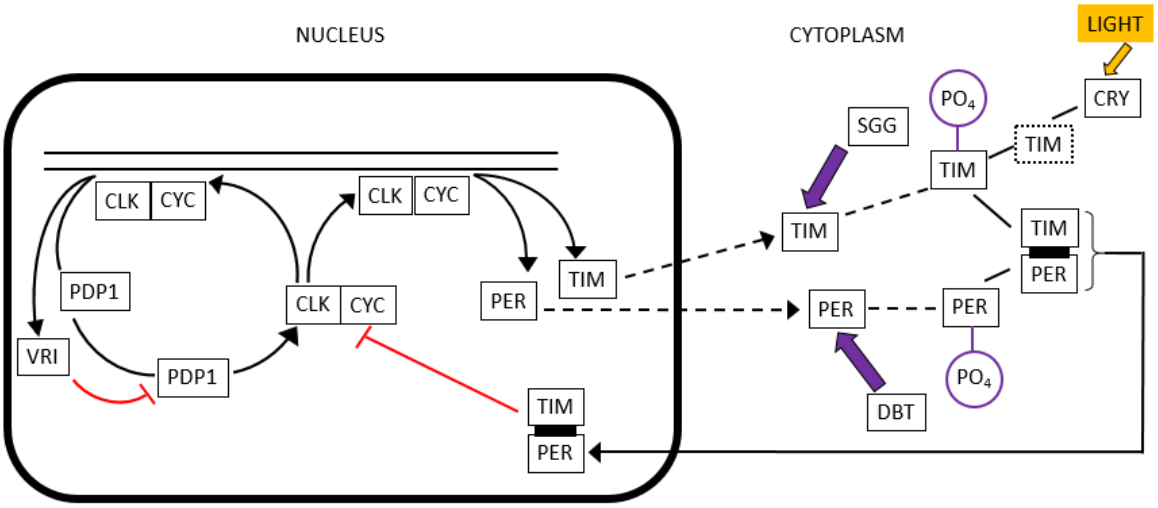


Figure 4: A schematic of the transcription-translation feedback loop underlying the *D. melanogaster* circadian clock

The Egg-laying and activity/rest rhythms: a comparison

However, a great deal of what we know of the functionality of the egg-laying rhythm is at odds with the well-worked out molecular and neurobiology of the activity/rest rhythm.

Firstly, the egg-laying rhythm persists under conditions of constant light of high intensity (Sheeba et al., 2001; Menon et al., 2014), whereas the activity/rest rhythm only persists under LL of low intensity (Bachleitner et al., 2007). As mentioned above, light acts on TIM through CRY: light induces CRY to bind to TIM causing the protein JETLAG (JET) to preferentially degrade TIM rather than CRY (Peschel et al., 2009). This indicates that the JET-TIM-CRY pathway is probably not the means by which the egg-laying rhythm gains its light input.

Secondly, the period of the egg-laying rhythm is extraordinarily variable for a circadian rhythm under LL and DD. Flies with one significant period yielded values ranging from 18 to 28 h in constant as well as periodic LD conditions (Sheeba et al., 2001), unlike rhythms in locomotor activity and eclosion where the period values cluster tightly around 24 h (Saunders, 2002). This does not seem to be due to maintenance in an aperiodic environment, as the authors of Sheeba et al. (2001) originally proposed, as we see a similarly broad range in period values even in flies maintained for many generations in a periodic environment (*Radhika Shindey, unpublished data*). This suggests that, while oviposition rhythm *per se* may be essential to the fly's evolutionary fitness, a strict adherence to a near 24 h period is not.

Thirdly, the oviposition rhythm's limits of entrainment are different than those of the activity/rest rhythm (16 to 32 h – Wheeler et al., 1993) and similar to the eclosion rhythm (20 to 28 h – Paranjpe et al., 2004).

Fourthly, the LN_vs of the fly brain and the neuropeptide PDF that are known to orchestrate the activity/rest and eclosion rhythms (Myers et al., 2003) appear to be completely uninvolved in the egg-laying rhythm. Flies with a null mutation for the gene *pdf* and flies whose LN_v neurons had been ablated using the GAL4-UAS system were consistently rhythmic in their egg-output (Howlader et al., 2006), demonstrating that the oviposition clock neither resides in the pacemaker neurons nor makes use of the rhythmic production of PDF.

Fifthly, many of the known clock genes – *per, tim, clk, cyc, cry* – do not appear to be responsible for generating the oviposition rhythm; in other words, the molecular clockwork described above seems not to be involved in the oviposition rhythm.

The daily oscillation of the protein and mRNA levels of the *per* gene, the heart of many autonomous and peripheral oscillators in the fly body (Brandes et al., 1996; Plautz et al., 1997a, b), is absent in the ovary, as is the oscillation of *tim* mRNA and protein (Liu et al., 1988; Saez and Young, 1988; Liu et al., 1992; Hardin, 1994; Beaver et al., 2003). While null mutations of *per (*per^{01}*)* does not cause a loss of rhythmicity, these flies show much shorter and more variable periods than wild-type flies (McCabe and Birley, 1998). The alleles of *per* that induce a shorter period (per^S) and longer period (per^L) respectively in the activity/rest rhythm have a similar effect on the period under DD, and there is a significant phase advance under LD in per^S flies as compared to per^L flies (McCabe and Birley, 1998; *Anuj Menon, unpublished data*). The exact nature of the contribution of *per* to the rhythm in egg-laying is currently unknown and an open question. Chapter Three will attempt to address it.

Sixthly, the effect of socio-sexual interactions on these rhythms produces very different outcomes. Female Canton-S (CS) flies paired with males showed an overall reduction in their activity levels but remained persistently rhythmic (Lone and Sharma, 2011). Female CS flies paired with males showed fecundity higher than that of virgin females and females that had been exposed to males only for one day (Menon et al., 2014). Under 12:12 h LD cycles in continuous male presence, however, the robustness of the oviposition rhythm is greatly reduced: the number of eggs laid during the peak of oviposition is lower, leading to nearly half the flies becoming arrhythmic in their egg-output.

Almost the only important point of similarity between the oviposition and locomotor activity rhythms is that they can entrain to both light and temperature cycles and these zeitgebers exert an associative effect on both the rhythms. Under periodic LD cycles, an increase in temperature from 25 to 30 °C causes a more nocturnal shift in activity and flies entrain to thermophase-cryophase (TC) cycles under LL (which would otherwise induce arrhythmicity) and DD (Tomioka et al., 1997). They further found that when temperature and light cycles were administered together but with a 6-h phase advance in the temperature cycle, the evening peak began just after cryophase, consisting of a burst of activity, but maintaining the same phase of termination at the onset of the dark phase well into the cryophase. Consistent with this finding, different sets of clock neurons were shown to be responsible for light and temperature entrainment respectively, the Lateral Neurons (LNs) responsive to light input and the Dorsal Neurons (DNs) and Posterior Lateral Neurons (LPNs) responsive to temperature input (Miyasako et al., 2007).

Kannan et al. (2012) demonstrated a similar associativity between light and temperature cycles in their entrainment of the oviposition rhythm: temperature cycles entrained the rhythm under LL and DD, and when the two cycles were given six hours out of phase with each other the flies displayed two prominent peaks, one at the onset of the dark phase (similar to the peak shown under LD cycles) and one at the onset of the cryophase (similar to the peak shown under TC cycles). Whether this too means that separate groups of neurons mediate the entrainment of the egg-laying rhythm to light and temperature cycles must await investigation perhaps until the discovery of the oviposition clock itself.

It is evident that there is fresh, fertile ground to be broken in the investigation of this unusual rhythm. If what we know so far is any indication, a better understanding of even its most basic aspects – which nervous tissue generates it, what genes are involved, what are its properties – could shed an entirely new light on the circadian biology of the fly.

THE WAY FORWARD

The adaptive significance of the oviposition rhythm

There is clearly a great deal to be discovered yet about the mechanism of generation of the oviposition rhythm, as well as many of its properties and indeed its adaptive significance. As evidence from other insects indicate, the ecology and life history of the organism probably have something to do with the phasing of the rhythm and the time of day at which the peak or peaks occur. *D. melanogaster* is neither a parasite nor an agricultural pest. Its main oviposition substrate is fallen fruit, which stay on the ground until consumed or crushed by a larger animal, which may or may not happen at any time of the day. Therefore, the timing of the oviposition peak to coincide with dusk may not have anything to do with the circadian availability of the oviposition substrate in the way pest species must aim to infect their hosts when they are stationary. One possibility is that the peak has evolved to exploit the lower temperatures and higher humidity of dusk compared to the middle of the day. Humidity is highest early in the morning shortly before sunrise but the peak at dusk would not only help avoid desiccation of the eggs as they are laid, but the bulk of the time before the first instar larvae hatch 12-18 h later would be at night. As the larvae hatch in the middle of the day, they can then burrow into the food and escape the heat of mid-day.

If this is indeed the case it would be possible to test the hypothesis by examining the egg-laying rhythm under temperature and humidity cycles in DD. Kannan et al., (2012) showed that, under temperature cycles, the peak of egg-laying does indeed occur close to the onset of the low-temperature phase. The effect of humidity cycles on the egg-laying rhythm has not been examined thus far. This could be done in two ways: 12 h periods of alternating high and low humidity or a ramping up of humidity levels to mimic natural conditions, with 8 h each of low, moderate and high humidity. If the desiccation hypothesis is correct one would expect to see the oviposition peak shift to coincide with the beginning of the period of high humidity in the first case, and the beginning or middle of the period of moderate humidity in the second case.

Socio-sexual interactions and the oviposition rhythm: the effect of male presence

Unusually enough, as we have seen, the continuous presence of male flies appears to induce rhythm attenuation for egg-laying and this phenomenon only occurs under LD cycles. There

are several points that can be tentatively inferred from this. Firstly, light duration may have something to do with the disturbance to the female, as this effect is not seen in DD or LL. Secondly, both male and female must display rhythms of a similar period, as under constant conditions when both male and female free-run, the rhythms must necessarily diverge from each other, and we see the phenomenon of arrhythmic egg-laying disappear. Thirdly, if a behavioural rhythm of the male interferes with the egg-laying rhythm of the female, it is likely to be the activity rhythm of the male in question that serves to disturb the female. As we know, flies display two peaks of activity under 12:12 h LD cycle, one close to lights-on and the other close to lights-off. The timing of the evening peak of the males overlaps with that of the egg-laying peak of the female.

Thus we can construct the following scenario to explain the effect of male presence. The evening activity peak of the male coincides with the oviposition peak of the female; the male either directs this activity towards the female by attempting to mate with her or merely serves to disturb her without active attempts to mate, both of which result in the female being unable to execute the oviposition motor program (Yang et al., 2008) and oviposit on the substrate. As a result, the oviposition peak disappears, and the female is forced to retain the eggs in her uterus/ovaries through the night and during the rest of the day when she is unable to retain them any longer, and oviposition is rendered arrhythmic. This hypothesis still requires to be tested systematically.

The canonical clock genes and the oviposition rhythm

The genes that form the molecular transcription-translation feedback loop that lies at the heart of the locomotor activity rhythm, i.e., the canonical clock genes, do not appear to be responsible for generating the egg-laying rhythm. As several studies have demonstrated, expression of *per* and *tim* genes do not cycle in the ovary (either their mRNA or protein levels) and they are expressed constantly in the cytoplasm, not the nucleus (Plautz et al., 1997; Hardin, 1994; Saez and Young, 1988; Liu et al., 1988; Kaneko and Hall, 2000). This is made all the more strange by the fact that *per* does cycle in the testes (Plautz et al., 1997) and this functions as a reproductive clock in males by regulating rhythmic sperm release (Beaver et al., 2002).

Per and *tim* appear to have non-circadian roles to play in both male and female reproduction. Per^{01} and tim^{01} males show a greater time in copula, and this phenomenon does not act via the known mechanism of the clock, as mutations in other clock genes do not seem to affect

copulation duration (Beaver and Giebultowicz, 2004). In fact, these clock mutants show a decrease in sperm quantity and thus compromised reproductive fitness (Beaver et al., 2002). In female flies, PER and TIM are similarly linked to fecundity, as null mutants show a decline in surviving progeny and a similar reduction in their production of mature oocytes. This does not seem to act via the clock either, as disrupting clock function alone while PER and TIM are present does not exert these deleterious effects on female fertility (Beaver et al., 2003).

The *per* gene itself presents a most unusual and intriguing case. McCabe and Birley (1998) showed that the null-mutant per^{01}, while not arrhythmic, shows shorter and far more variable periods than the wild-type. They also demonstrated that per^{S} and per^{L} females show shorter and longer oviposition periods respectively. This is consistent with preliminary results from our lab (*Anuj Menon and Shambhavi Chidambaram, unpublished data*), though why the null mutation should not cause arrhythmicity, but the two alleles should, is very much an open question. One possibility is that the per^{S} and per^{L} alleles exert their effect on the oviposition rhythm by acting through the activity/rest rhythm. An interaction between the two oscillators would have the effect of slowing down or speeding up the oviposition clock in concert with slowing down or speeding up the central pacemaker. If *per* is not part of whatever molecular mechanisms drive the oviposition clock, a lack of wild type *per*, which renders activity arrhythmic, would have no effect whatsoever on egg-laying rhythm.

The use of these two alleles of *per* to shed light on the location of the neural circuitry governing the clock is covered in Chapter Three. As far as the investigation of the neurogenetic basis of the oviposition rhythm goes, it is important to establish that the clock itself is neuronal in origin. Manipulations on such a large scale in the nervous system, however, would greatly compromise the health and reproductive physiology of the fly, up to the point where she could no longer oviposit. This has been observed, for example, when parts of the Mushroom Body have been either depolarized or hyperpolarized (*Shambhavi Chidambaram, unpublished data*). Expressing per^{S} and per^{L} in the nervous system would have such effects, and would allow manipulation of the entire central nervous system. Thus, the pan-neuronal driver *elav*GAL4 was used to drive the expression of PER^{S} and PER^{L} in all the neurons of the adult fly, and another GAL4 driver was used to express these proteins primarily in the thoracic-abdominal ganglion. If indeed the neurons of the nervous system are involved in governing the oviposition rhyhm, such an approach should result in the shortening (with PER^{S}) and lengthening (with PER^{L}) of the period.

This hypothesis would also help to explain why several other clock mutants show perfectly normal egg-laying rhythms (*Anuj Menon, unpublished data*). One way to test the coupled oscillator hypothesis would be to interfere with the central pacemaker in such a way that its function is not compromised, but so as to produce a noticeable change in its output, and examine the corresponding change, if any, to the oviposition clock. Overexpressing TIM and PER in the lateral neurons only through the use of a *pdf*GAL4 driver would be one possible approach.

Another important question that has no answer yet is the identity of the light input pathway to the clock. The oviposition rhythm entrains to light, as we know (Kannan et al., 2012), but given that the TIM-CRY pathway does not appear to be the means by which light information is conveyed to the clock, an unknown mechanism seems to be at work here.

The neural basis of the oviposition rhythm: the oviposition clock

Howlader et al. (2006) conclusively demonstrated that the PDF-expressing Lateral Neurons play no role in the generation of the oviposition rhythm, leaving the question of the location of the oviposition clock ripe for exploration. The process of oviposition involves several inter-connected steps, so it seems logical that the clock could be located in one of the tissues involved in reproductive output and primarily exerts its influence at one or more steps of the process of egg-production.

Figure 6 summarizes the steps involved in the production of a fertilized egg, from courtship through to the act of inserting an egg into a substrate. Courtship and mating and the neural circuitry that control a female's responsiveness to these can effectively be ruled out as the originators of the rhythm, as unmated females are also demonstrably rhythmic in their egg-output. The gene *take-out* is a clock-controlled gene that helps regulate feeding behaviour in flies (So et al., 2000; Sarov-Blat et al., 2007) and the protein is chemically similar to Juvenile Hormone (JH) (Touhara and Prestwich, 1992; Touhara et al., 1993). JH is released from the Corpora Allata in the brain (Saunders, 2002) and this hormone is thought to be an important regulator of insect reproduction (Koeppe et al., 1985).

As the oocytes pass through the oviducts from the ovaries, ovulation takes place. The muscles surrounding the ovaries, oviduct and uterus contract with an ultradian rhythm of a period on the order of minutes or seconds (Middleton et al., 2006), but there is known to be a circadian rhythm in the production of mature and immature oocytes under 12:12 h LD (*Anuj Menon, unpublished data*). The neurotransmitter Octopamine plays an important role in modulating

the contraction of the oviduct, necessary for ovulation and a network of octopamine-expressing neurons permeates the reproductive tract (Rodriguez-Valentin et al., 2006).

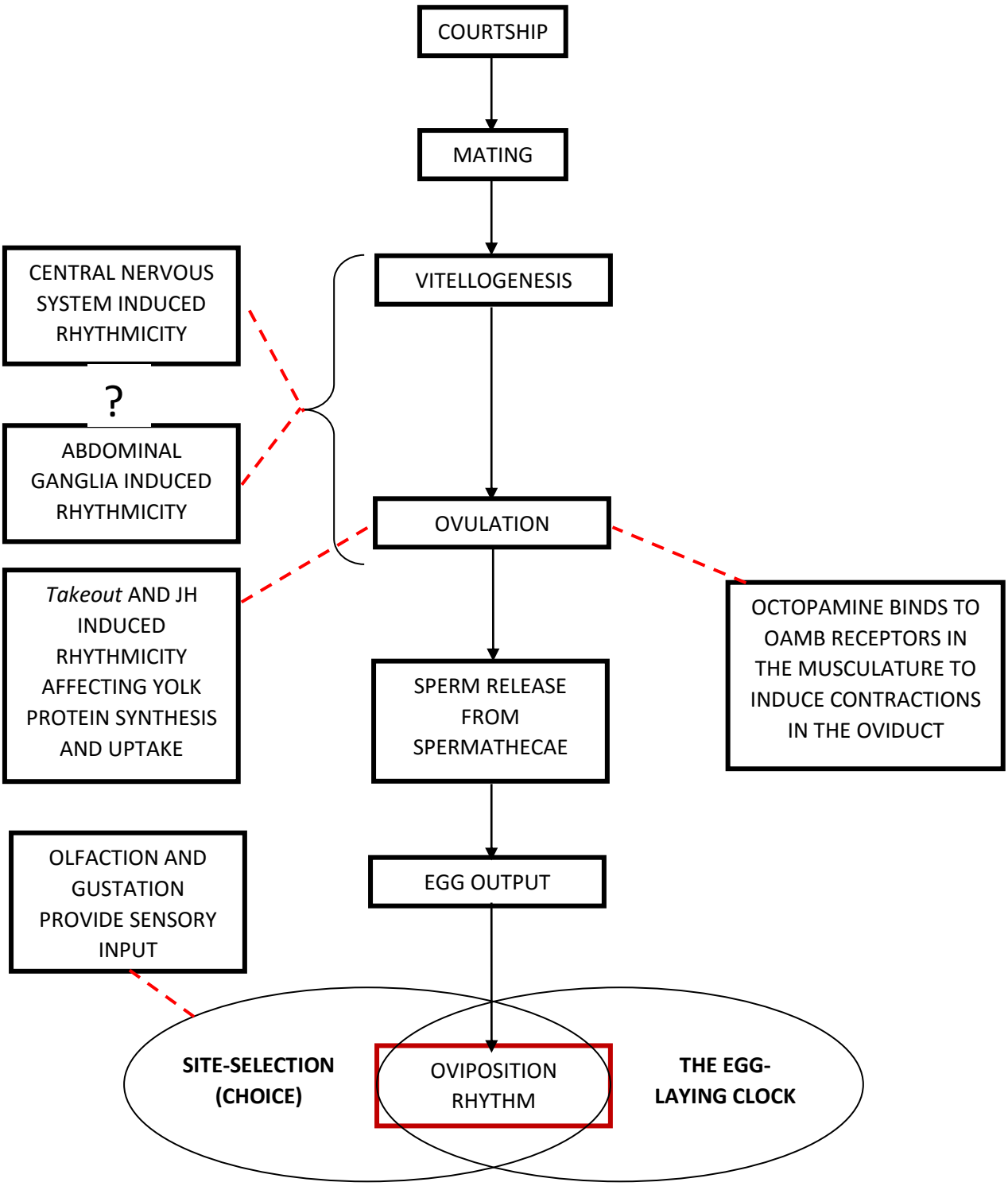


Figure 5: The steps in the rhythmic production of fertilized eggs

The receptor OAMB (Octopamine receptor in the Mushroom Body) is present in the walls of the musculature of the reproductive tract, and the binding of Octopamine to these receptors induces the contraction of the oviduct (Lee et al., 2003; Lee et al., 2009). Octopaminergic neurons, particularly the ones innervating the oviduct and uterus, and the OAMB receptor are therefore prime candidates for the neural control of oviposition. Whether octopamine is released rhythmically, or OAMB shows a rhythm in its sensitivity to Octopamine is currently not known, and this line of inquiry might yield further indications as to the role of Octopamine in the oviposition rhythm. The female fly exercises a certain amount of choice in selecting oviposition sites, and evaluates her choices through olfaction and gustation. In Chapter Two, the female fly's preferences, the neural circuitry involved in these sensory modalities and their potential roles in the egg-laying rhythm are more fully discussed and experimentally investigated. Olfaction and gustation are involved in the process of selection of suitable oviposition substrates (Yang et al., 2007; Joseph et al., 2009; Azanchi et al., 2013) and both show a circadian rhythm in their neurophysiology as well as behaviour (Krishan et al., 1999; Chatterjee et al., 2010). The hypothesis was therefore formulated as follows: a rhythm in olfactory and gustatory sensitivity to potential oviposition substrates could lead to a rhythm in ovipositional preference for a substrate, which could in turn influence the oviposition rhythm itself. Using the GAL4-UAS system, it was possible to manipulate the olfactory and gustatory circuits of the fly so as to render them either arrhythmic or hyperpolarized (so neurophysiological activity is diminished) and thus examine their role in the oviposition rhythm. This is the first step in working out the role of sensory modalities in the egg-laying rhythm, and the way in which sensory information is conveyed to the oviposition clock.

The fact that the oviposition rhythm seems not to follow any of the known rules or employ any of the known mechanisms or circuits poses an exciting challenge. The rhythm is intimately involved with the activity most crucial to the fly's Darwinian fitness, and it is possible that being connected with something so vital has resulted in the formation of multiple back-up circuits and redundant mechanisms, which could explain why rendering the fly arrhythmic is so difficult. It could also be that the rhythm is the final output of multiple factors, including an endogenous clock, all co-operating synergistically to produce a final output, which could explain the tremendous variability in period and the general noisiness of the rhythm. In this scenario, it is the task of the chronobiologist to identify the influence of the individual factors and remove them to expose the clock at the heart of it all. If there is any

rhythm in the fly that stands the greatest chance of exposing fresh and hitherto unknown principles of chronobiological organization, the oviposition rhythm is a better candidate than all others, and this by itself is reason enough to subject it to rigorous investigation through as many methods as possible.

CHAPTER 2

OLFACTION, GUSTATION AND THE OVIPOSITION RHYTHM

INTRODUCTION

The act of oviposition, unlike locomotor activity, is critically dependent on the presence of a suitable external environment, i.e., a substrate to oviposit on. Precise oviposition site preference is characteristic of Drosophilids (Richmond and Gerking, 1979) and in the absence of such a substrate, female *Drosophila melanogaster* will withhold their eggs until one becomes available (Allemand and Bouletreau-Merle, 1989; *Anuj Menon, unpublished data*). Ripe, rotting fruit is the preferred oviposition site of *D. melanogaster* (Ashburner, 1998; Becher et al., 2012) and flies are consequently attracted to medium containing ethanol (positionally attractive) and acetic acid (positionally repellent) (Eisses, 1997; Dudley, 2002; Joseph et al., 2009; Azanchi et al., 2013).

Various fruit volatiles have different levels of ovipositional attraction to flies, though there is some evidence that such volatile compounds play a secondary role to the more attractive smell of fermenting yeast (Becher et al., 2012). Sucrose-containing, or sweet-tasting, medium by contrast seem to be ovipositionally repellent to female flies as when presented with a choice between sucrose-containing and lobeline-containing (bitter-tasting) medium, they avoid sweet-tasting media consistently (Yang et al., 2007; Wu et al., 2015).

It is imperative that a female lay her eggs on a substrate that will maximize the survival of her progeny to adulthood, and the attraction and repulsion produced by various concentrations of ethanol, acetic acid, volatile fruit odourants, fermenting yeast and such suggests that a female is capable of evaluating and discriminating between the choices available to her.

Gustation and olfaction appear to play a central role in this evaluation process. Olfaction is the primary sensory modality used to detect volatile compounds such as ethanol from a distance (Dudley, 2000; 2002). Before a female lays an egg, she walks over the substrate, and actively inserts her proboscis and ovipositor into it in an attempt to evaluate its suitability (Yang et al., 2007), and all of these body parts - legs, proboscis and ovipositor – contain gustatory receptors (Scott et al., 2001; reviewed in Montell, 2003; Chyb, 2004). Ethanol-containing mediums are characterised as preferred ovipositional sites, and the lack of gustatory and olfactory ability results in an ovipositional aversion to ethanol containing medium (Azanchi et al., 2013), just as hyperpolarizing gustatory neurons sensitive to sweet tastes abolishes the fly's innate ovipositional repulsion to sucrose-containing medium (Yang

et al., 2007). When the fly's ability to taste is compromised overall, the gustatory attraction to acetic acid-containing medium is diminished significantly, and the positional repulsion to it is enhanced (Joseph et al., 2009).

The circadian clocks of the fruit fly govern the daily pattern of much of the animal's behaviour and physiology. Activity/rest rhythm of fruit flies is governed by core circadian pacemakers located in its brain (reviewed in Nitabach and Taghert, 2008; Sheeba, 2008; Peschel and Helfrich-Förster, 2011), which also serves to coordinate the activity of several peripheral oscillators distributed throughout the fly body (Giebultowicz, 1999; reviewed in Glossop and Hardin, 2002). Many of these tissue-specific peripheral oscillators are capable of functioning in a completely autonomous manner, independent of the central brain clock, and such independently ticking timers have been reported in the Malphigian tubules, wings, testes, legs (Brandes et al., 1996; Plautz et al., 1997), ring gland (Emery et al., 1997), and, relevant to the above discussion, the antennae and proboscis as well.

The fly's sensitivity to olfactory stimulants, measured by the response of the electroantennogram (EAG) to odourants is also found to follow circadian rhythm (Krishnan et al., 1999). This constitutes a truly autonomous circadian pacemaker, as these rhythms persist in *in vitro* organ culture (Plautz et al., 1997), and even when the core pacemaker neurons are ablated, though they are abolished in some of the core clock gene null mutants. The antennal circadian pacemaker is both necessary and sufficient for the olfactory rhythm to persist in *Drosophila* (Tanoue et al., 2004). As opposed to the activity/rest rhythm, but similar to the rhythm in oviposition, the olfactory rhythm is unimodal with a single peak occurring at ZT16 in a 12:12 h LD cycle, shortly after the single ovipositional peak occurs around the time of transition from light to dark (Krishnan et al., 1999).

Similarly, the gustatory rhythm in *Drosophila* is regulated by autonomous peripheral oscillators: labellar gustatory receptor neurons show circadian rhythms in their spiking amplitude, frequency and duration in response to gustatory stimuli (Chatterjee et al., 2010). This is also the neural basis for an important gustatory behaviour, i.e., the Proboscis Extension Reflex (PER). The rhythm in gustatory neuron activity is both necessary and sufficient for the rhythm in the proboscis extension reflex, and like the rhythm in olfaction, both are abolished in clock gene null mutants.

Given the involvement of gustation and olfaction in the evaluation and selection of an ovipositional substrate, the rhythm in olfaction and gustation could be playing a similar role

in the regulation of oviposition rhythm. Essentially, a daily and rhythmic fluctuation in olfaction and gustation could be driving the daily and rhythmic fluctuation in egg-output by influencing the fly's ability to evaluate the substrate that she is exposed to. Egg-output is rhythmic even when the fly is provided with the same food medium throughout the day, eliminating any influence of conscious choice between different potential ovipositional sites, so if the rhythm in smell and taste is to influence the rhythm in egg-laying behaviour, it must act via altering the fly's sensitivity to the constant food medium, thus altering her evaluation of it as a suitable ovipositional substrate.

Two different neurogenetic approaches to manipulate the female's olfactory and gustatory systems were adopted, using the GAL4-UAS bipartite method:

Firstly, the olfactory and gustatory behaviours were rendered arrhythmic by expressing a dominant negative allele of the gene *Clock* in antennal (*Or83b*) neurons and in the gustatory receptor neurons (*Gr5a*) responsible for the rhythm in the PER.

Secondly, the flies' overall ability to smell and taste were compromised by hyperpolarizing the *Or83b* and *Gr5a* neurons by expressing a Kir2.1 potassium channel. This would have the effect of ablating rhythmicity in membrane potential. Since the *Gr5a*-expressing gustatory receptor neurons comprise only a subset of the 67 classes of gustatory receptor neurons (Scott et al., 2001), two further manipulations were done.

We also assayed the oviposition rhythm of flies expressing a mutant allele of *poxneuro*. This gene mediates a developmental switch between mechanosensory and chemosensory bristles on the fly's body, and the mutant allele causes much of the latter to develop into the former (Awasaki and Kimura, 1996, 2001).

Finally, CLKΔ (Clock-Dominant Negative) and Kir2.1 were expressed in the sub-oesophageal ganglion, a neurological region behind the brain that functions as the primary gustatory association area (Vosshall and Stocker, 2007).

MATERIALS AND METHODS

***Fly maintenance*:** All fly strains were maintained in glass vials (95 mm height and 10 mm diameter) in approximately 10 mL of a culture medium primarily composed of ripe banana and unrefined cane sugar (jaggery). The external conditions were a light/dark regime of 12 hours each (LD12:12). The flies were maintained at 25°C and ~75% humidity, and the adults were kept at a density of 60 to 70 flies per vial. Before each assay, the flies were collected within six hours of eclosion from their pupal cases, sexed and maintained separately in same-sex groups as virgins for 1-2 days under a 12:12 h LD at 25 °C.

Fly strains: Most of the fly genotypes used in these experiments were obtained from the lab of Dr. Sheeba Vasu, JNCASR. In order to manipulate olfactory neurons, the GAL4 driver yw; *Or83b*GAL4 was used, along with the UAS lines w[*]; UAS-Kir2.1 and w[*]; UAS-ClkΔto create the experimental genotypes *Or83b*GAL4-UASKir2.1 and *Or83b*GAL4-UASClkΔ. These UAS lines were also used in the manipulation of sweet gustatory receptor neurons, using the GAL4 driver w[*]; *Gr5a*GAL4 to create *Gr5a*GAL4-UASKir2.1 and *Gr5a*GAL4-UASClkΔ and in the manipulation of the sub-oesophageal ganglion (GAL4 line: w^{1118};P{GawB}1471, which I shall from now on refer to as the SOGGAL4 line for the sake of clarity) to create SOGGAL4-UASKir2.1. The cross between SOGGAL4 and UASClkΔ yielded no viable progeny as the flies perished in the first instar larval stage. The poxneuro allele $poxn^{70}$ was obtained as a gift from the lab of Dr. Ken-Ichi Kimura of the Hokkaido University of Education, Iwamizawa, Hokkaido, Japan.

The control genotypes for each of these assays consisted of the GAL4 and UAS parental lines crossed to the strains of their respective genetic background. The control for the $poxn^{70}$ flies was *CS*, as this line had been crossed to the *CS* background several times in the lab of Dr. Kimura to remove other lethal mutations from the strain (Kimura, personal communication).

Egg-laying Assays: The egg-laying behaviour of the flies was assayed under constant darkness (DD) at the same temperature and humidity as the flies were maintained in during their development. Virgin females and *CS* males, aged 1-3 days, were anaesthetized with CO_2 and transferred into glass vials as single opposite-sex pairs with low levels of banana-jaggery food, approximately 3 mL. Continuous male presence was required for the assays in order to boost females' fecundity, as preliminary assays (data not shown) had indicated that solitary

females mated only for 24 h showed a drop in daily egg output which made the detection of a rhythm difficult.

Individual pairs were maintained in assay conditions for 24 h to allow for recovery from the anaesthetization, to acclimatize to the assay conditions and to avoid confounding the data with statistical artefacts caused by the burst of oviposition seen immediately after virgins mate for the first time. Each assay was initiated with 20 females of each genotype, so that, accounting for deaths and escaped flies, at least 12–15 flies were present by the end of seven 24 h cycles. The male flies were not removed, and the egg-laying behaviour was assayed in continuous presence of males. This, as has been demonstrated before (Menon et al., 2014) does not disrupt the egg-laying rhythm in any way under either DD or constant light (LL). Males lost during the course of the assay were replaced with virgin males maintained individually in the assay conditions.

All of the assays were carried out manually. Every two hours, except on the first day of the assay, the male-female pairs were gently transferred to fresh vials without any anaesthesia with the help of a dim red lamp, light of wavelength greater than 650 nm. The vials were then examined under a light microscope (Leica, Germany) to estimate the number of eggs laid by the females over the course of the previous two hours. Each assay was conducted for eight days in total, and data was obtained from all the days except the first.

Verification of the GAL4 lines: The expression pattern of the GAL4 constructs were verified by immunocytochemistry. The flies were crossed to the line UAS-GFP and brains or the brains and ventral nerve cord of the 0-2 day old progeny were dissected after rearing them under 12:12 h LD cycle at 25 °C. Flies were anaesthetized in ice and then dissected under phosphate buffer saline (PBS), fixed in 4% formaldehyde and washed three times with 0.5% PBT (0.5% Triton-X in PBS). They were then blocked in a solution of 10% horse serum and 0.5% PBT for an hour, again washed three times with 0.5% PBT and incubated overnight at 4 °C with primary antibody - anti-GFP (chicken, 1:1000). Six more washes with 0.5% PBT under a black covered container followed, and Alexa Fluor dye (anti-chicken, 1:10, 488 nm) was used as secondary antibody. The brains were then cleaned again and mounted in 3:7 1X PBS:Glycerol mounting medium. These data are not shown here.

Verification of the UAS lines: The flies were reared under 12:12 h LD cycle at 25 °C. 4-5 day old virgin male flies were loaded into locomotor activity tubes and their activity/rest

profiles were recorded separately in Drosophila Activity Monitors (DAM, Trikinetics, Waltham, MA, USA) under DD for seven days (see Figure 21).

***Statistical Analysis*:** The time-series data obtained from the egg-laying assays was examined to determine the rhythmicity of the individual female using Chi-squared periodogram, done on the raw data and available in the software CLOCKLAB (Actimetrics, Evanston, IL). Females that did not oviposit for 48 h or more or that were lost (died or escaped) before the end of the 7 day long assay were not included for the analysis.

It is known that the oviposition rhythm of *Drosophila* has a far more variable period than the activity/rest rhythm, ranging between about 18 to 30 h (Sheeba et al., 2001). Those females which showed significant rhythmicity at a *p* value less than 0.05 within this range were considered for further analysis.

The percentage of rhythmic flies per genotype was calculated as the ratio between the number of flies that were rhythmic and the total number of flies that remained at the end of each assay. The "centre of gravity" indicates where the weighted mean of the circular distribution lies. This point in time was then calculated for each day, and the phase value of each day except the first was subtracted from that of the previous day so on to give a daily period.

Precision of the rhythm of individuals was calculated as the inverse of the standard deviation of the daily period values, considering the start of the assay as phase 0 and end as 24, using an original MATLAB code developed in the Chronobiology Laboratory by Anuj Menon (unpublished), using the CircStat2012a MATLAB toolbox (Berens, 2009). Robustness was calculated as the difference between the maximum amplitude and the cut-off amplitude set by the *p* value. To examine the effect of genotype on the period, fecundity or robustness of the rhythm, ANOVAs were performed using tools available in STATISTICA v5.0.

RESULTS

Manipulating the olfactory and gustatory neuronal circuits does not induce arrhythmic egg-laying behaviour

GENOTYPE	NUMBER OF FLIES	% RHYTHMICITY
*Or83b*GAL4-UASClkΔ	10/13	76.92
*Or83b*GAL4-UASKir2.1	9/13	69.23
*Or83b*GAL4/+	7/15	46.67
UASClkΔ/+	6/10	60
UASKir2.1/+	10/23	43.48

Table 1: Summary of the percentage of rhythmic flies among the lines whose olfactory neural circuits were manipulated

GENOTYPE	NUMBER OF FLIES	% RHYTHMICITY
*Gr5a*GAL4-UASClkΔ	13/16	81.25
*Gr5a*GAL4-UASKir2.1	8/14	57.14
*Gr5a*GAL4/+	8/16	50
UASClkΔ/+	6/10	60
UASKir2.1	10/23	43.48

Table 2: Summary of the percentage of rhythmic flies among the lines whose gustatory neural circuits were manipulated

GENOTYPE	NUMBER OF FLIES	% RHYTHMICITY
SOGGAL4-UASKir2.1	7/14	50
SOGGAL4/+	10/13	76.92
UASKir2.1/+	10/23	43.48

Table 3: Summary of the percentage of rhythmic flies among the lines whose sub-oesophageal ganglion was manipulated

GENOTYPE	NUMBER OF FLIES	% RHYTHMICITY
poxneuro^{70} /CyO	11/16	68.75
Canton-S	5/10	50

Table 4: Summary of the percentage of rhythmic flies – the developmental mutant

The percentage of rhythmic flies out of the entire sample, excluding those individuals which had been lost or excluded, is an indicator of whether oviposition rhythm persists or not: when fewer than 50% of the flies turn out to be arrhythmic, there is reason to believe than the rhythm has been greatly attenuated. None of the experimental manipulations described above produced arrhythmic oviposition rhythm, and the rhythm persisted in the progeny of the GAL4 and UAS parents at much higher than 50% (data summarized in Tables 1 to 4).

Unexpectedly, only 50% of the two of the control genotypes (*Or83b*GAL4/+ and UASKir2.1/+) displayed rhythmic behaviour (Figures 1 to 3). It is unclear at this point why this should be so, but it is possible that in the case of the latter, leaky expression of the genetic construct, leading to the hyperpolarization of some neurons could result in this phenotype. Another possibility is that this line has been inbred for many generations, resulting in low average fecundity. What is clear, however, is that hyperpolarizing the neurons involved in the gustatory and olfactory circuits does not produce arrhythmic egg-laying behaviour.

The rhythms of the experimental genotypes are just as robust and precise as those of the control genotypes

The neural manipulations did not produce a significant change in either the robustness or the precision of the period of the egg-laying rhythm (Figures 7 to 12), and the length of the period itself was similar to that of the non-manipulated flies (Figures 4 to 6).

Variations in fecundity, while significant, do not contribute to arrhythmicity, nor does it explain the variation in percentage rhythmicity

There were statistically significant differences in the average daily fecundity between the various genotypes (Figures 13 to 15).

In the case of the flies whose olfactory neurons were manipulated, there was no difference in its fecundity from that of the control genotypes. The lower percentage rhythmicity of the control *Or83b*GAL4/+ could not be explained away as being due to reduced daily fecundity. While a lower egg output can make it more difficult to detect a circadian rhythm and thus register more individuals as being arrhythmic, *Or83b*GAL4/+ had a higher fecundity than *Or83b*GAL4-UASClkΔ as well as the other two parental lines, UASKir2.1/+ and UASClkΔ/+.

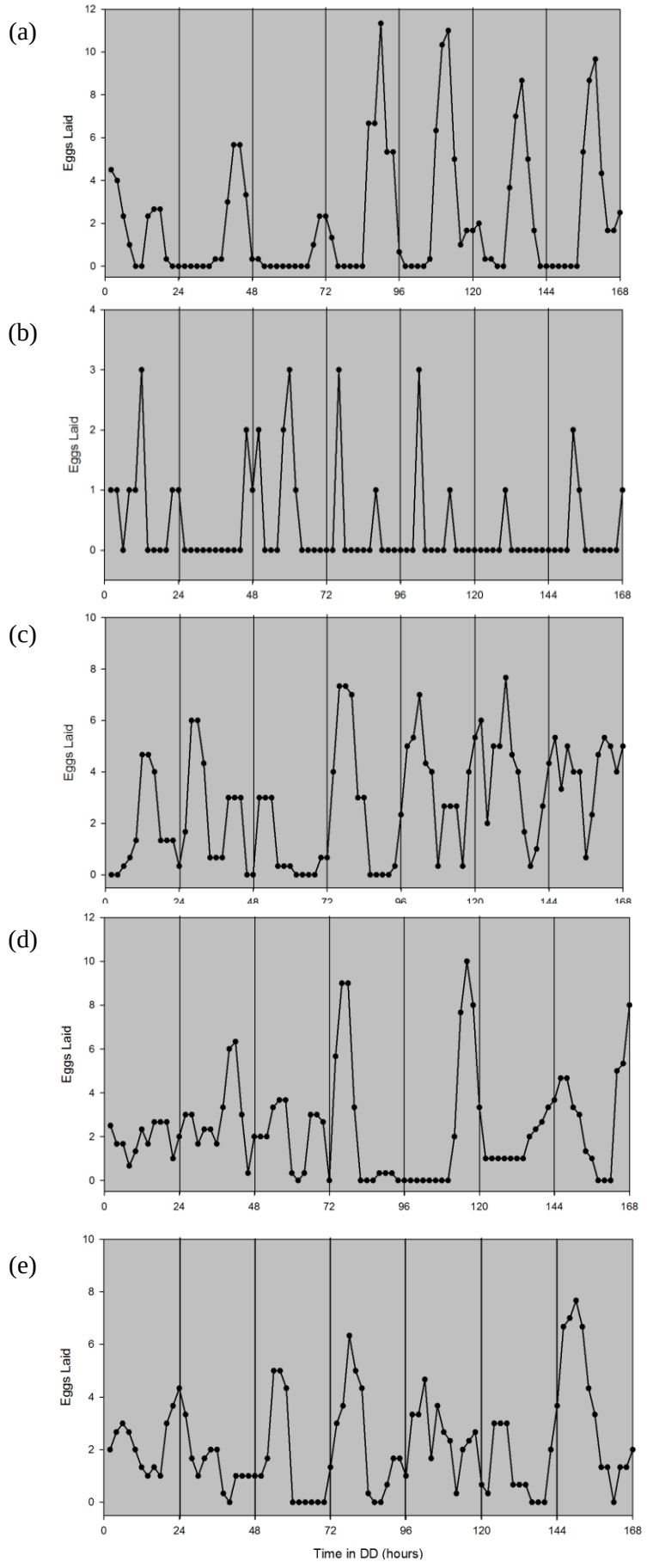


Figure 1: Cycle by cycle profile of a single fly across all seven days of the assay of lines with olfactory circuits manipulated

a) *Or83b*GAL4-UASClkΔ(N=13)
b) *Or83b*GAL4-UASKir2.1 (N=13)
c) *Or83b*GAL4/+ (N=15)
d) UASClkΔ/+ (N=10)
e) UASKir2.1/+ (N=23)

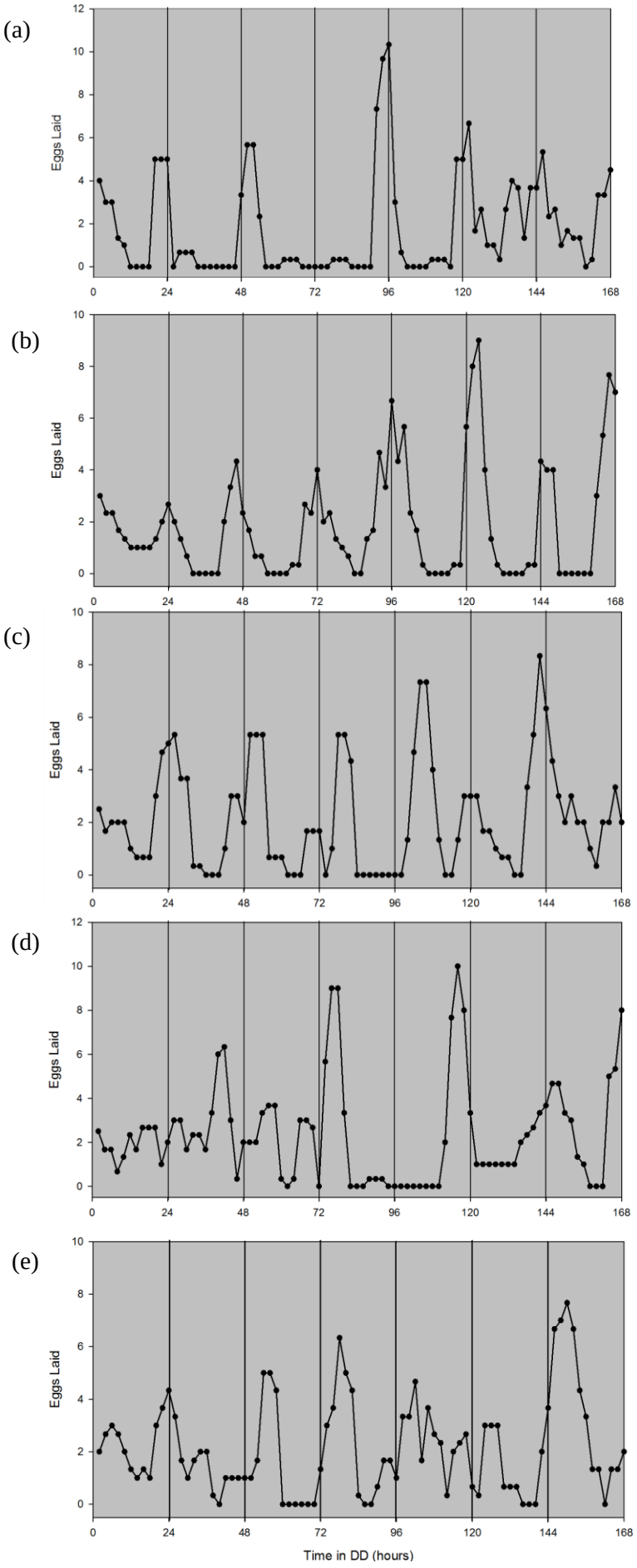


Figure 2: Cycle by cycle profile of a single fly across all seven days of the assay of lines with olfactory circuits manipulated

a) *Gr5a*GAL4-UASClkΔ (N=16)
b) *Gr5a*GAL4-UASKir2.1 (N=14)
c) *Gr5a*GAL4/+ (N=16)
d) UASClkΔ/+ (N=10)
e) UASKir2.1/+ (N=23)

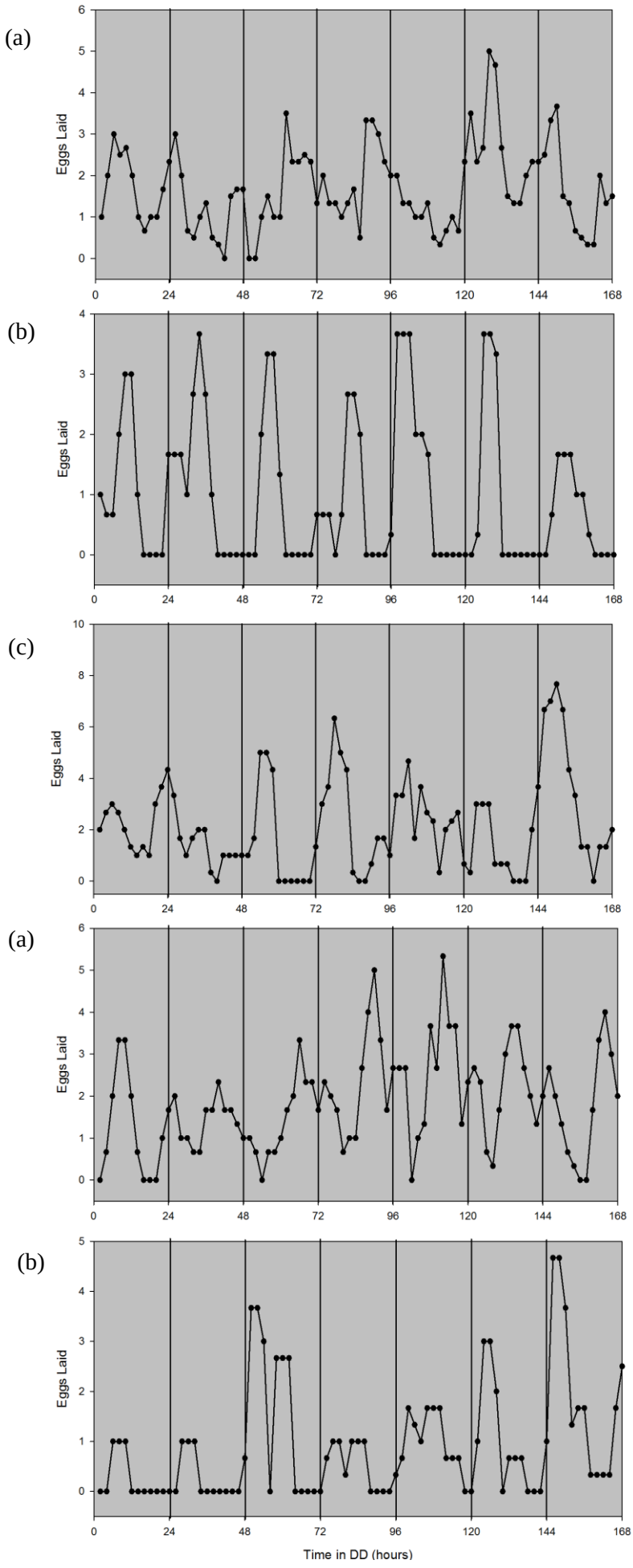


Figure 3: Cycle by cycle profile of a single fly across all seven days of the assay of lines with olfactory circuits manipulated

a) SOGGAL4-UASKir2.1 (N=14)
b) SOGGAL4/+ (N=13)
c) UASKir2.1/+ (N=23)

Figure 4: Cycle by cycle profile of a single fly across all seven days of the assay of lines with olfactory circuits manipulated

a) *poxneuro*^{70} (N=16)
b) Canton-S (N=10)

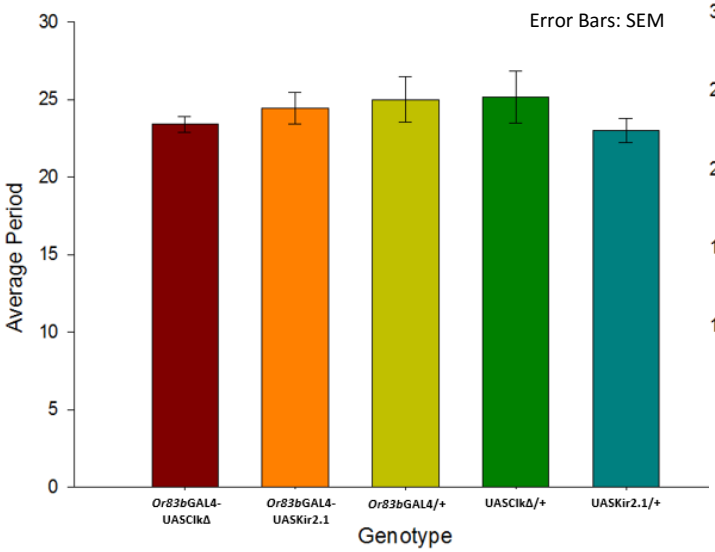


Figure 5: Period length averaged across days of lines with olfactory circuits manipulated

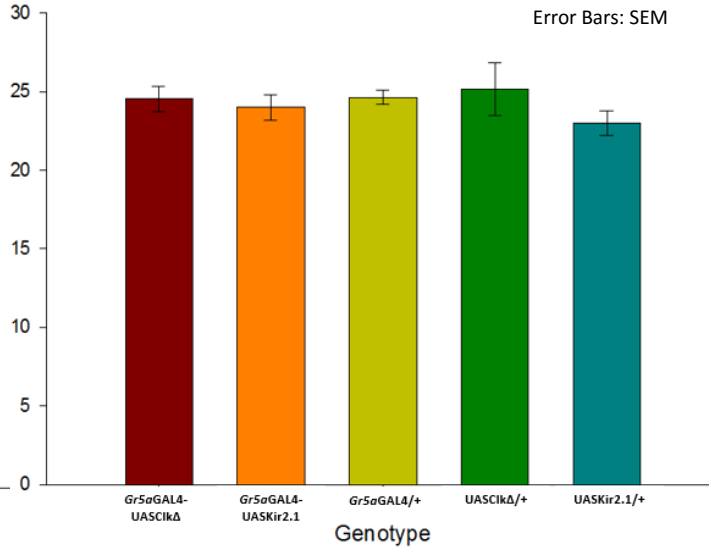


Figure 6: Period length averaged across days of lines with gustatory circuits manipulated

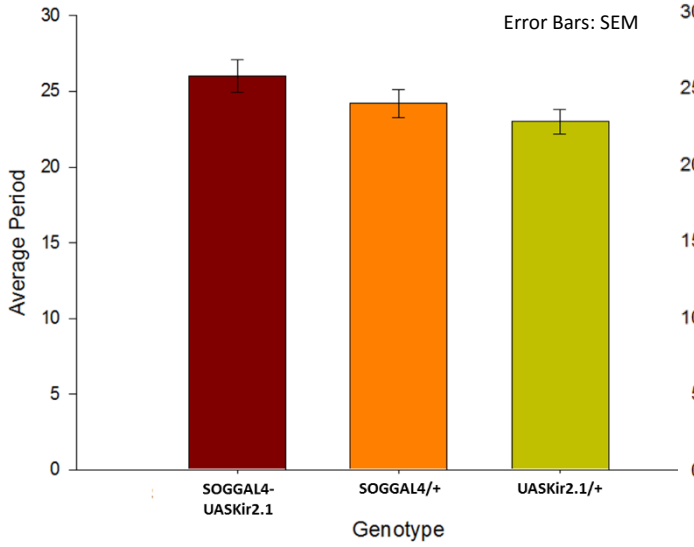


Figure 7: Period length averaged across days of lines with the sub-oesophageal ganglion manipulated

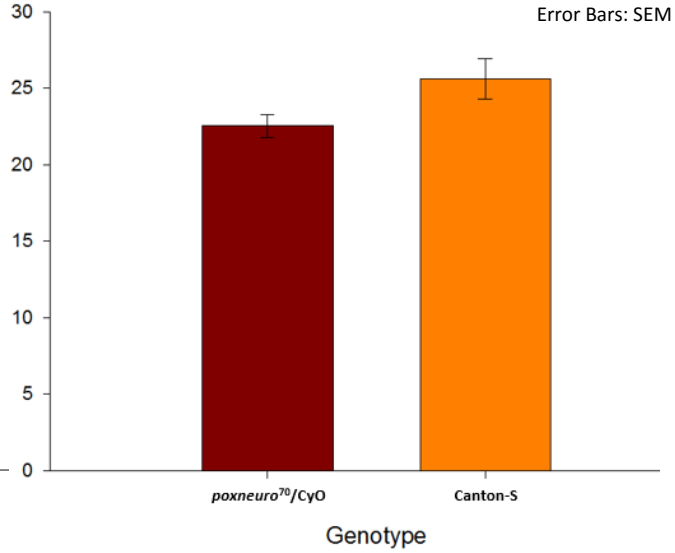


Figure 8: Period length averaged across days of *poxneuro*^{70} and its control

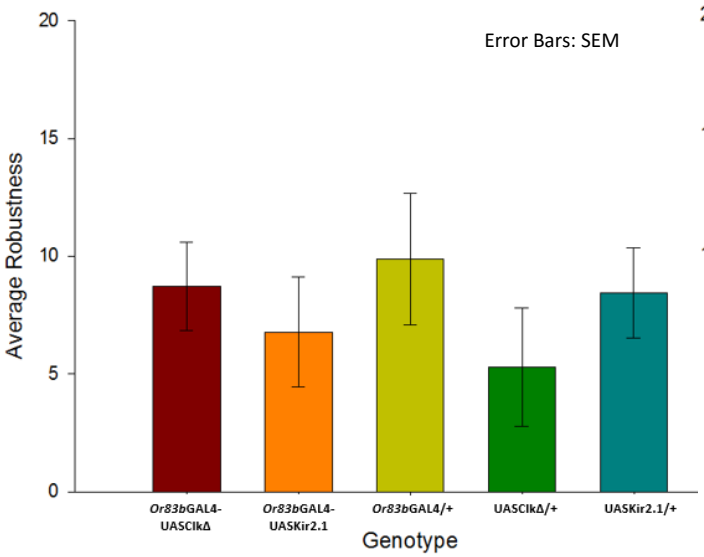


Figure 9: Robustness averaged across days of lines with the olfactory circuits manipulated

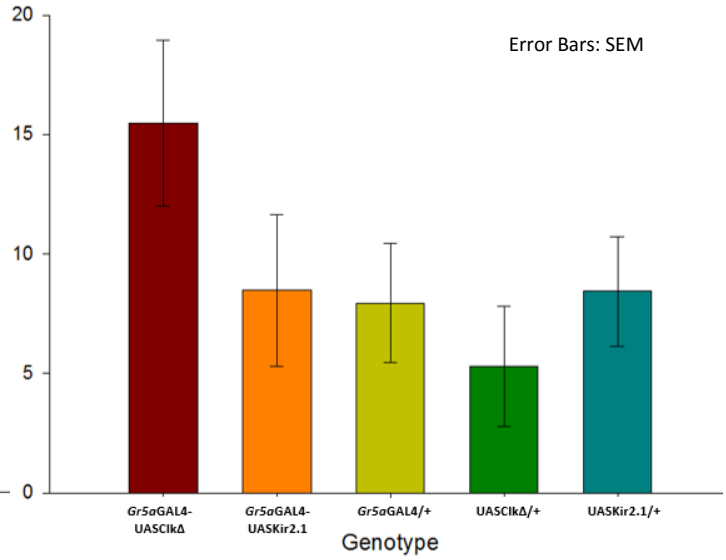


Figure 10: Robustness averaged across days of lines with the gustatory circuits manipulated

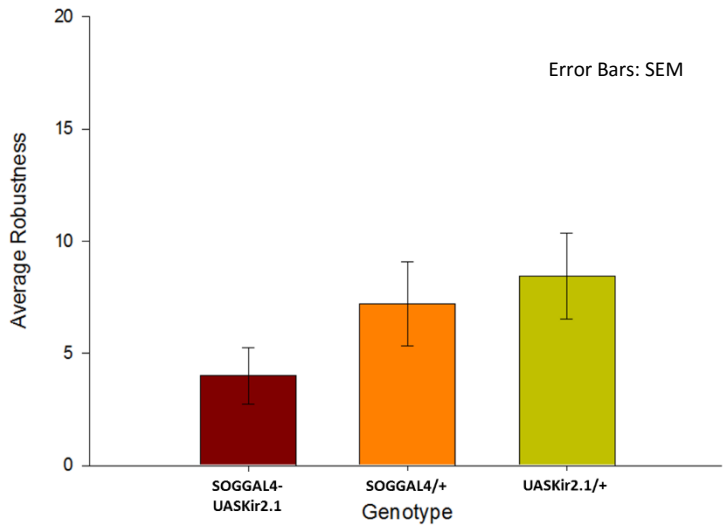


Figure 11: Robustness averaged across days of lines with the sub-oesophageal ganglion manipulated

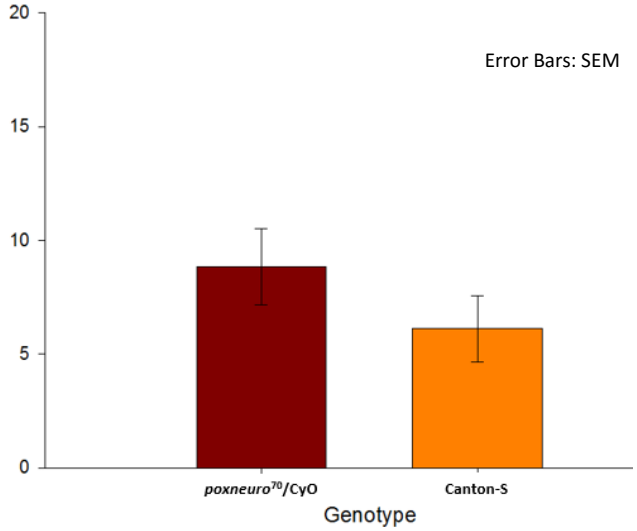


Figure 12: Robustness averaged across days of *poxneuro*^{70} and its control

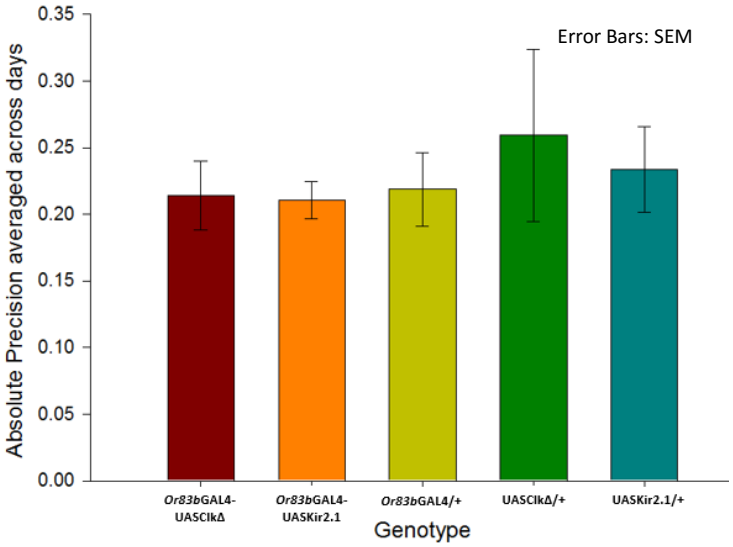


Figure 13: Precision averaged across days of lines with the olfactory circuits manipulated

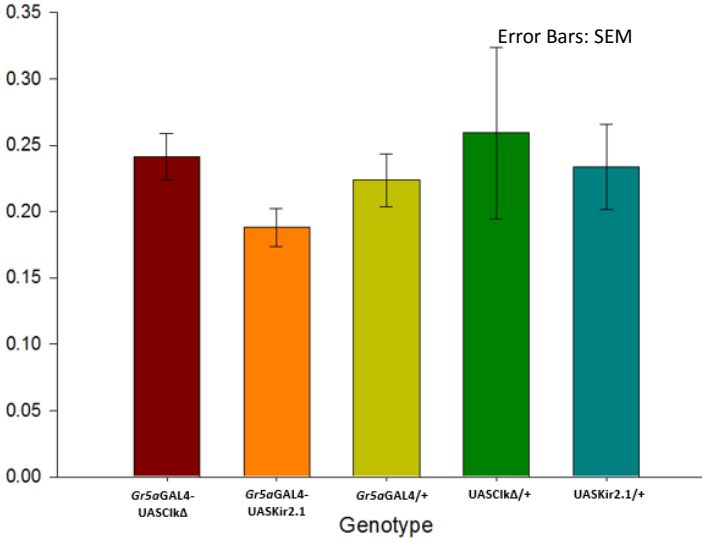


Figure 14: Precision averaged across days of lines with the olfactory circuits manipulated

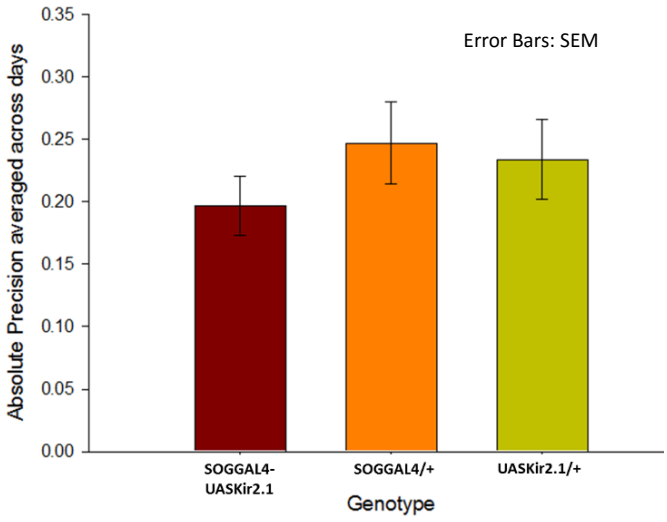


Figure 15: Precision averaged across days of lines with the sub-oesophageal ganglion manipulated

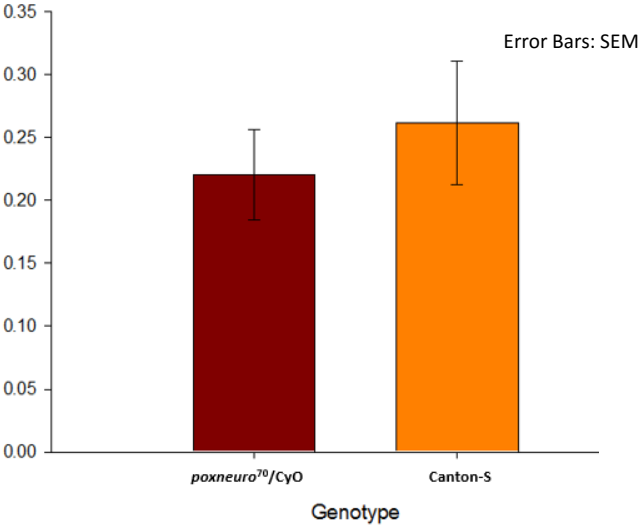


Figure 16: Precision averaged across days of *poxneuro*^{70} and its control

The manipulation of the *Gr5a* gustatory receptor neurons yielded a consistent difference in the fecundity between the experimental and control lines ($F = 13.431$, $p < 0.001$) with the parental lines being more fecund than the experimentally-manipulated flies. There was a similar difference in fecundity between the flies with the hyperpolarized sub-oesophageal ganglion neurons and their respective controls.

The *poxneuro*^{70} mutation, renders the fly incapable of tasting from an overall, developmental point of view, as most of the chemosensory bristles on the fly's body are switched to developing into mechanosensory bristles (Awasaki and Kimura, 1997; Awasaki and Kimura, 2001) was surprisingly no different from its control in terms of fecundity.

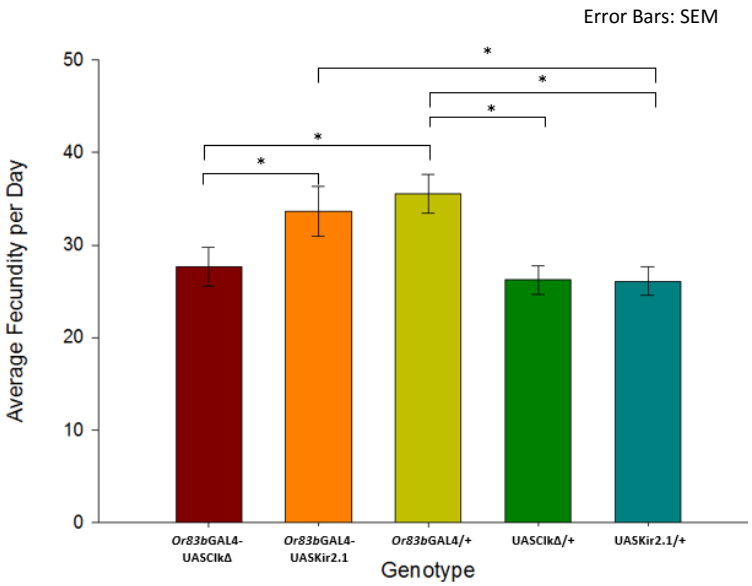


Figure 17: Fecundity averaged across days of lines with the olfactory circuits manipulated

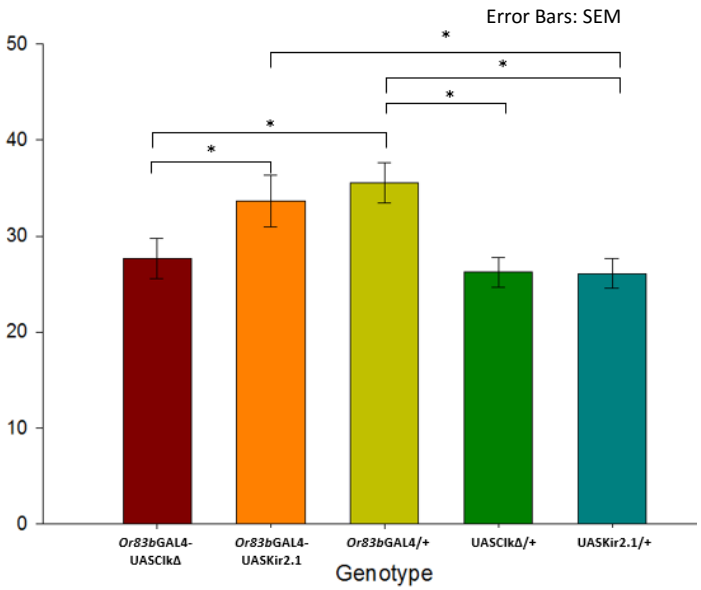


Figure 18: Fecundity averaged across days of lines with the gustatory circuits manipulated

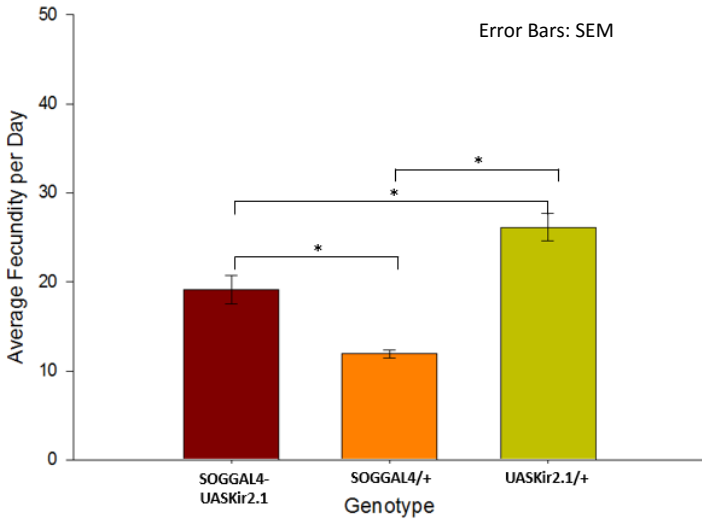


Figure 19: Fecundity averaged across days of lines with the sub-oesophageal ganglion manipulated

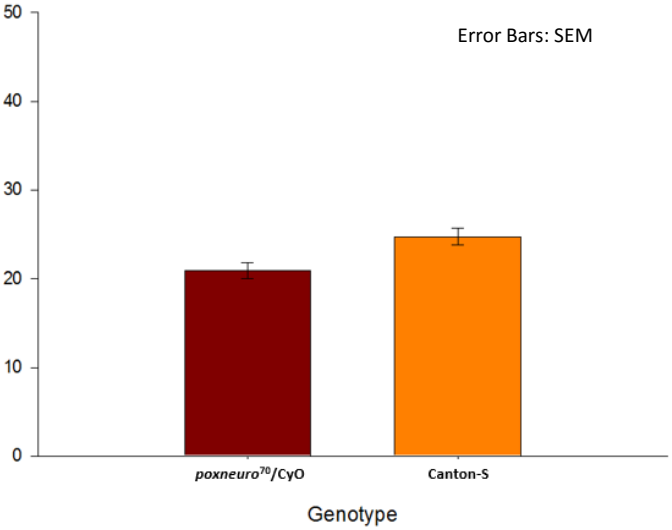
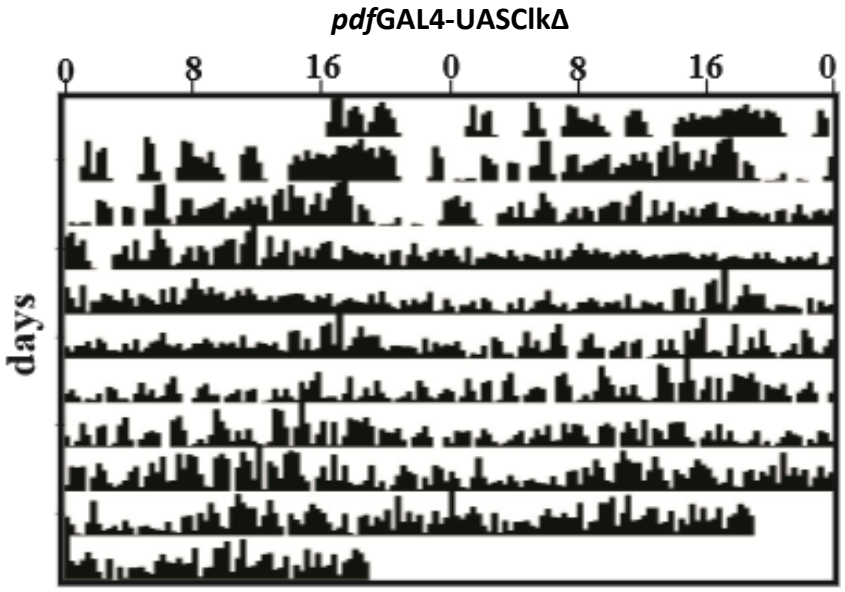


Figure 20: Fecundity averaged across days of *poxneuro*^{70} and its control

(a)



(b)

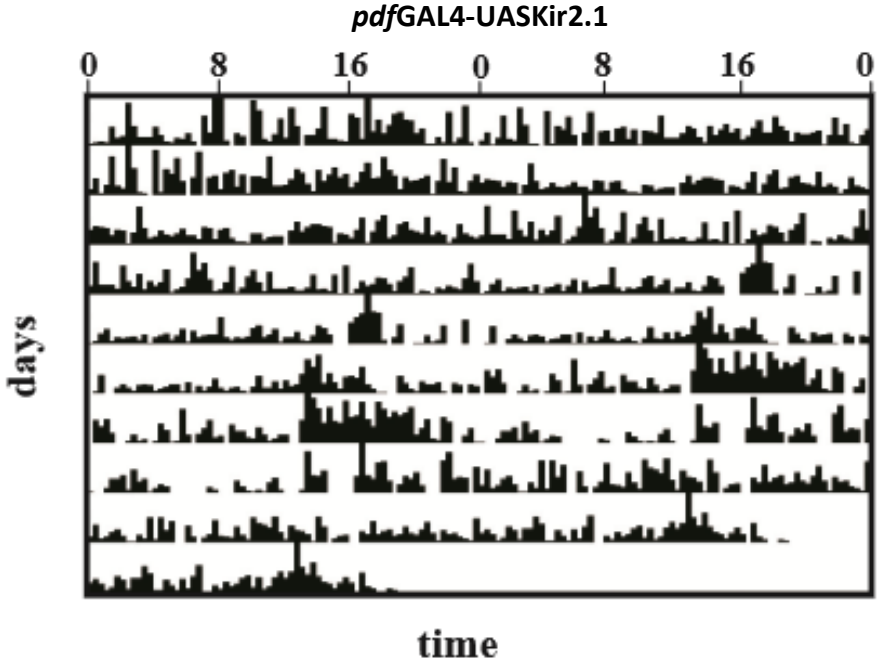


Figure 9: Representative double-plotted actograms of virgin male flies, conducted at 25°C in constant darkness. Time of day is plotted on the abscissa (X-axis) and the number of days is plotted on the ordinate (Y-axis). Genotype is indicated by the labels above the

DISCUSSION

The fact that none of the fly lines displayed arhythmicity is a clear indication that the olfactory and gustatory circuits do not contribute in any way to the oviposition rhythm. The periods of the manipulated lines were as robust and precise as the control lines, so the oviposition clock seems to be unaffected in any way.

We began with the hypothesis that the rhythm in smell and taste translated to a rhythm in food evaluation as a suitable substrate, leading, at least in part, to the oviposition rhythm. If this is not the case, then the rhythms in smell and taste do not lead to a rhythm in food evaluation for oviposition. Instead, it is possible that the rhythmicity in egg-laying arises mainly from the oviposition clock itself, located elsewhere in the central nervous system, and that the choice to oviposit based on olfactory and gustatory information is secondary to the clock's output.

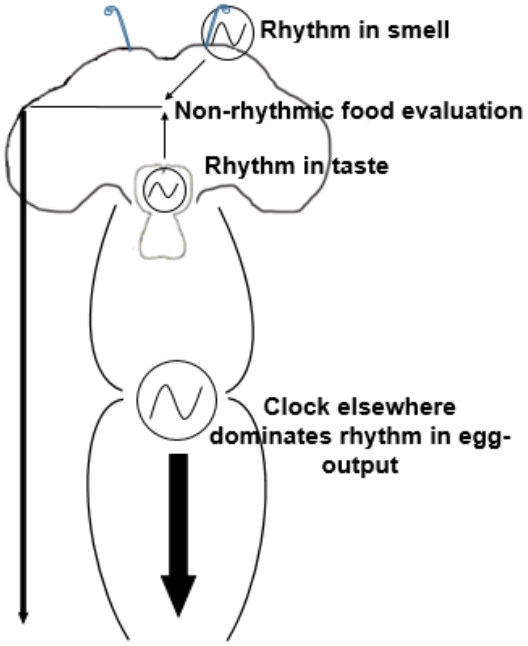


Figure 21: The proposed model: rhythmic sensory input leads to non-rhythmic food evaluation, with the oviposition clock located elsewhere in the central nervous system producing the rhythm in egg-output

One possibility is that there is a hierarchical process involved in rhythmic egg-laying, and the clock lies at the top of the hierarchy, dictating the timing of oviposition. Choice mediated through sensory modalities could be lower down in the hierarchy, and rhythmic information from the clock could interact with it. This might produce weaker rhythms on substrates that the fly judges to be unsuitable for egg-laying, but they remain nevertheless.

Another possibility would be that the clock and sensory modality-mediated choice could interact continuously with each other, a phenomenon that would not be evident when the flies are provided with only one type food medium throughout the day, as was done in all the assays described above. Whether this is the case would best be elucidated by providing the flies with different types of food at different time of the day. Flies with intact olfactory and

gustatory circuits could be presented with suitable and unsuitable oviposition substrates at different times of day, to examine whether the drive to oviposit at the right time of day is stronger than the drive to oviposit only on, say, bitter-tasting media.

Another way of examining the relationship between the clock and oviposition substrate choice would be to examine the rhythm on different types of food media. It remains to be seen whether, in the set of physiological steps required to oviposit, the clock is dominant over smell and taste-mediated choice, as might be indicated by rhythmicity even on wholly unsuitable substrates.

Compromising olfactory physiology appears to result in a general trend of higher fecundity and compromising gustatory physiology appears to result in a trend towards lower fecundity. There is no clear indication at this point as to why many of the control genotypes were more fecund than their respective experimental genotypes. It is likely that this is due to genetic background effects – *poxneuro*^{70} for example, is on a *CS* background, and when compared directly to *CS* is not significantly different in its fecundity. The *Or83b*GAL4 line, for example, is on a *yw* background and the UAS lines with which it was mated are on a w^{1118} background. Genetic interaction effects between these backgrounds could possibly have contributed to the differences in fecundity between them.

CHAPTER 3

PERIOD ALLELES AND THE OVIPOSITION RHYTHM

INTRODUCTION

The experimental approach to manipulating olfactory and gustatory neurons was to either render them arrhythmic through the expression of ClkΔ, or to hyperpolarize the neurons by expressing additional Potassium channels. These neurons, while good candidates for the oviposition clock, comprise only a small subset of the fly's entire nervous system. Therefore, the extreme effect of these manipulations on the neurons did not result in either the death of the fly or in their physiology being compromised to the extent that they were unable to oviposit. Such effects are seen when UASKir2.1 and UASClkΔ are expressed broadly in the brain or in the Mushroom Body (*Shambhavi Chidambaram, unpublished data*). If the oviposition clock is indeed in the nervous system, a systematic process of elimination and narrowing-down is required to locate the nervous tissue involved in the oviposition rhythm. We know, for example, that the PDF-expressing ventral-lateral neurons do not contribute to the oviposition rhythm (Howlader et al., 2006), but there is no indication as to which tissue *is* doing so. Therefore, any investigation of the nervous system must begin from scratch, and any manipulation of the nervous system must avoid compromising the physiology of the reproductive system to the extent that the fly cannot oviposit.

The aims in the set of experiments covered in this chapter were as follows: to determine if the neurons of the nervous system are involved at all in the oviposition rhythm, and, if they are, whether the clock control arises from the brain or the ventral nerve cord.

The alleles of the gene *period,* namely per^S and per^L, were chosen in order to achieve the desired manipulations. McCabe and Birley (1998) showed that per^{01}, per^S and per^L flies were all rhythmic in their oviposition under three light regimes: 12:12 h light/dark (LD) cycle, constant light (LL) and constant darkness (DD). Under LD cycle, the flies showed rhythmic egg-laying with an average period of 21.3 $\pm$ 1.8 h.

Under LL and DD, all four genotypes showed free-running rhythms. Wild-type flies showed an average period value close to 24 h, per^{01} and per^S flies showed a rather shorter period (20.8 $\pm$ 1.1 h under DD; 20.9 $\pm$ 0.9 h under LL for per^S flies), especially the former (19.2 $\pm$ 3.0 h under DD and 14.0 $\pm$ 7 h under LL), and the per^L flies showed a longer period than 24 h (25.8 $\pm$ 7.0 h under LL and 28.0 $\pm$ 0.5 h under DD).

McCabe and Birley's data were produced using groups of flies, rather than individual flies. However, their results are replicable with our protocol. This is evidenced by unpublished data from both Gitanjali Howlader and Anuj Menon. Howlader showed that individual per^{01} flies are rhythmic with an average period of about 21 h. Menon showed that per^{S} and per^{L} flies display shorter and longer oviposition rhythms respectively

The *elav* gene plays an important role in both neuronal differentiation as well as maintenance (Yao et al., 1992; Robinow and White, 1988) and is expressed in all neurons in the adult fly. The *elav*GAL4 driver is a broadly expressed, pan-neuronal driver, which makes it a good starting point to determine if the neurons of the nervous system were involved in the oviposition rhythm at all. per^{S} and per^{L} were expressed using the *elav*GAL4 driver, and to avoid the potential confounding effects of wild-type PER protein in the system, the oviposition rhythm of *elav*GAL4-UASper^{S} and *elav*GAL4-UASper^{L} were examined on both a wild-type *per* background as well as a per^{01} background.

In order to further localize the neuronal input to the rhythm, the same set of manipulations were performed on another GAL4 line of the following genotype: w^{1118};P{GMR42H11-GAL4}attP2. This line shows weak to no expression in the brain, but ubiquitous expression in the parts of the ventral nerve cord: the abdominal ganglion, accessory mesothoracic ganglion, mesothoracic ganglion, metathoracic ganglion, prothoracic ganglion and tectulum (Pfeiffer et al., 2008). Nerves from the abdominal ganglion innervate the reproductive tract (Monastirioti, 2003; Hasemeyer et al., 2009), making the thoracic-abdominal ganglion a good candidate for the oviposition clock. This GAL4 driver shall be referred to as the TAG-GAL4 line for short from here on.

MATERIALS AND METHODS

***Fly maintenance*:** The fly strains used in this experiment were maintained in the same way as those used in the experiments manipulating olfactory and gustatory circuits (see Chapter 2).

Fly strains

The *elav*GAL4 line was obtained from the lab of Dr. Sheeba Vasu of the Neurosciences Unit, JNCASR. The TAGGAL4 line was obtained from the Bloomington Stock Centre (Stock ID: 41255).

The UASper^{S} and UASper^{L} lines were obtained as a gift the lab of Dr. Michael Rosbash of Brandeis University, Waltham, Massachusetts, USA. The Stock Centre of the National Centre for Biological Sciences provided me with the per^{01} strain (per^{01} 506 *iiry*) and the quadruple balancer lines required to put the GAL4 and UAS lines on the per^{01} background.

The experimental lines on the wild-type *per* background consisted of *elav*GAL4-UASper^{S}, *elav*GAL4-UASper^{L}, TAGGAL4-UASper^{S} and TAGGAL4-UASper^{L}, with their corresponding controls being *elav*GAL4/+ and TAGGAL4/+.

The above experimental genotypes were created on a per^{01} background with the help of quadruple balancer strains. *elav*GAL4 and UASper^{L} are genetic constructs on the second chromosome and TAGGAL4 and UASper^{S} are on the third chromosome; the *per* gene is located on the X-chromosome. Therefore, the quadruple balancers that were used are as follows: FM7/FM7; Tft/CyO; + (barred eyes, tufted hair on the thorax and curled wings were the phenotypic markers) and FM7/FM7/; +; Tb/Sb (barred eyes, shortened larvae and pupae and stubbled hair on the adult body were the phenotypic markers). A series of five crosses were performed to create the following experimental genotypes:

- per^{01}; *elav*GAL4/+; UASper^{S}/+
- per^{01}; *elav*GAL4/ UASper^{L}; +
- per^{01}; +; TAGGAL4/UASper^{S}
- per^{01}; UASper^{L}/+; TAGGAL4/+

A similar set of crosses were performed to create the corresponding set of control genotypes

- *per*^{01}; *elav*GAL4/+; +
- *per*^{01}; +; TAGGAL4/+
- *per*^{01}; +; UAS*per*^{S}/+
- *per*^{01}; UAS*per*^{L}/+; +

Egg-laying Assays: Oviposition assays were done in DD at 25 °C, after the flies were reared under 12:12 h LD cycle at the same temperature. Adult flies were collected within 6-8 h of their emergence from the pupae, sexed and separated. They were maintained this way for 1-2 days until a sufficient number of female flies had emerged to begin the assay. The females were then paired with *Canton-S* (*CS*) males and placed into glass vials (identical to the vials they were reared in) which contained approximately 3-4 mL of standard banana-jaggery fly medium after anaesthesia with CO_2. After the heterosexual pairs had had 24 h to recover from the transfer, handling and anaesthetization, and the initial burst of oviposition that follows the mating of virgin females was over, data were collected from the flies.

At every two hour interval for seven days (after the first 24 h during which the flies recovered) the flies were manually transferred into fresh vials and the eggs laid in the previous 2 h period were counted under a light microscope (Leica, Germany).

Verification of the GAL4 lines: The GAL4 lines' expression pattern was verified in much the same way as the lines described in Chapter 2 were verified. The data are not represented here.

Verification of the UAS lines:

The male flies of the experimental genotypes that emerged at the end of the crosses were assayed to examine their activity/rest rhythms under DD at 25 °C, and the data from two of these lines (*per*^{01}; *elav*GAL4/+; UAS*per*^{S}/+ and *per*^{01}; *elav*GAL4/ UAS*per*^{L}; +) are represented here in Figure 9.

***Statistical Analysis*:** The same statistical analyses that were performed on the experimental data in Chapter 2 were performed on these experimental data, using the same tools.

RESULTS

The % rhythmicity of lines expressing per^{S} and per^{L} in the nervous system of the fly on the wild-type per background and on a per^{01} background

Tables 1 and 2 summarize the % rhythmicity of lines expressing the *per* alleles in the fly's nervous system. Curiously, one of the control lines (*per*^{01}; *elav*GAL4/+) showed a reduction in rhythmicity to about 20%, while none of the other lines on the *per*^{01} did so.

GENOTYPE	NUMBER OF FLIES	% RHYTHMICITY
*elav*GAL4-UAS*per*^{S}	6/12	50
*elav*GAL4-UAS*per*^{L}	10/13	76.92
elavGAL4/+	7/14	50

GENOTYPE	NUMBER OF FLIES	% RHYTHMICITY
TAGGAL4-UAS*per*^{S}	13/15	86.67
TAGGAL4-UAS*per*^{L}	13/13	100
TAGGAL4/+	13/17	78.95

Table 1: A summary of percentage rhythmicity of the experimental and control lines on a wild-type *per* (*per*$^{+}$) background

GENOTYPE	NUMBER OF FLIES	% RHYTHMICITY
per^{01}; *elav*GAL4/+; UAS*per*^{S}/+	7/10	70
per^{01}; *elav*GAL4/ UAS*per*^{L}; +	4/9	44.4
per^{01}; *elav*GAL4/+; +	3/15	20
per^{01}; +; UAS*per*^{S}/+	9/12	75
per^{01}; UAS*per*^{L}/+; +	9/11	81.82

GENOTYPE	NUMBER OF FLIES	% RHYTHMICITY
per^{01}; +; TAGGAL4/UAS*per*^{S}	6/9	85.71
per^{01}; UAS*per*^{L}/+; TAGGAL4/+	6/9	85.71
per^{01}; +; TAGGAL4/+	12/15	75
per^{01}; +; UAS*per*^{S}/+	9/12	75
per^{01}; UAS*per*^{L}/+; +	9/11	81.82

Table 2: A summary of percentage rhythmicity of the experimental and control lines on a *period*-null (*per*^{01}) background

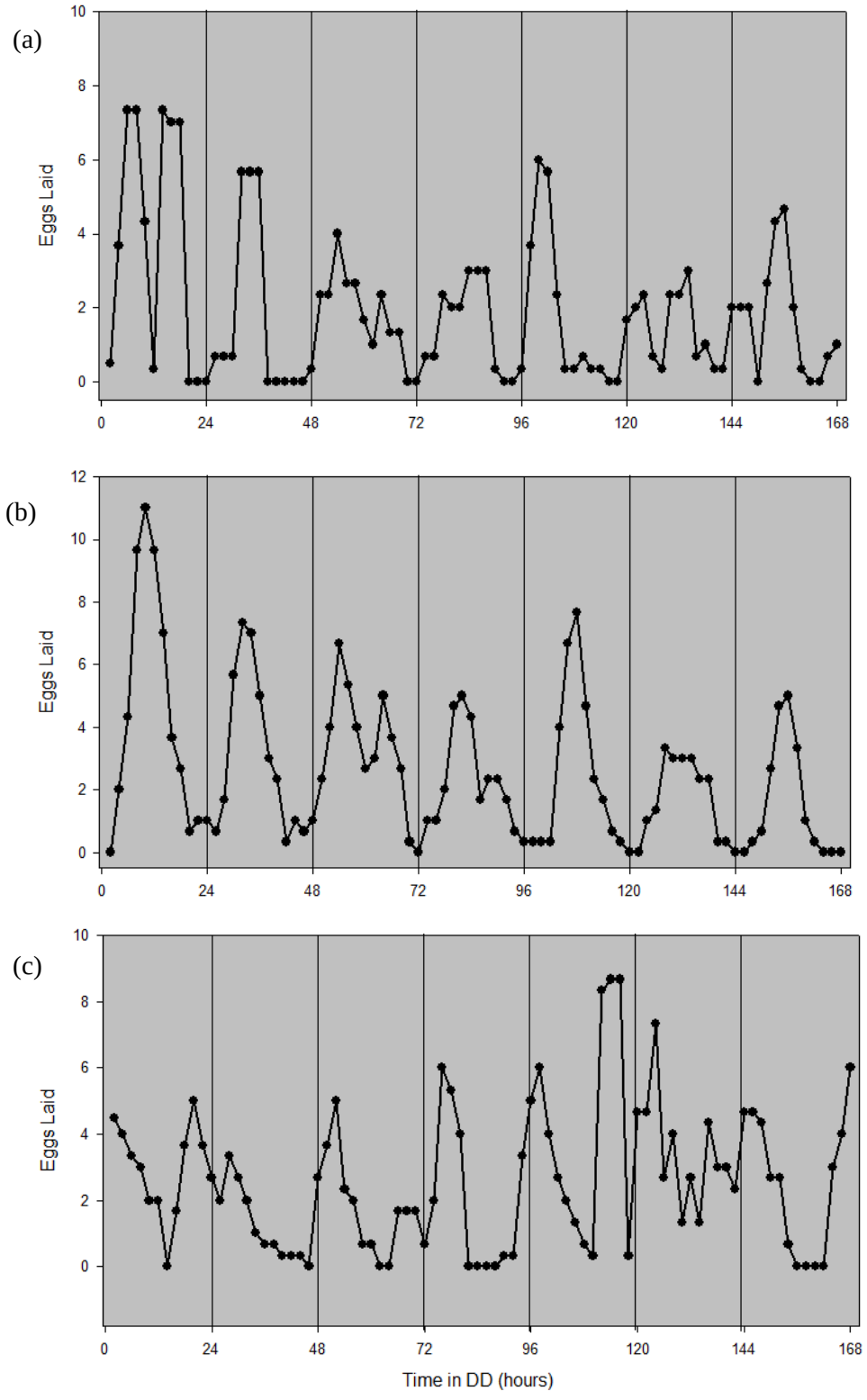


Figure 1: Cycle by cycle profile of a single fly across all seven days of the assay with lines wherein per^S and per^L are expressed pan-neuronally on a wild-type per background – a) *elav*GAL4-UASper^S (N=12) b) *elav*GAL4-UASper^L (N=13) c) *elav*GAL4/+ (N=14)

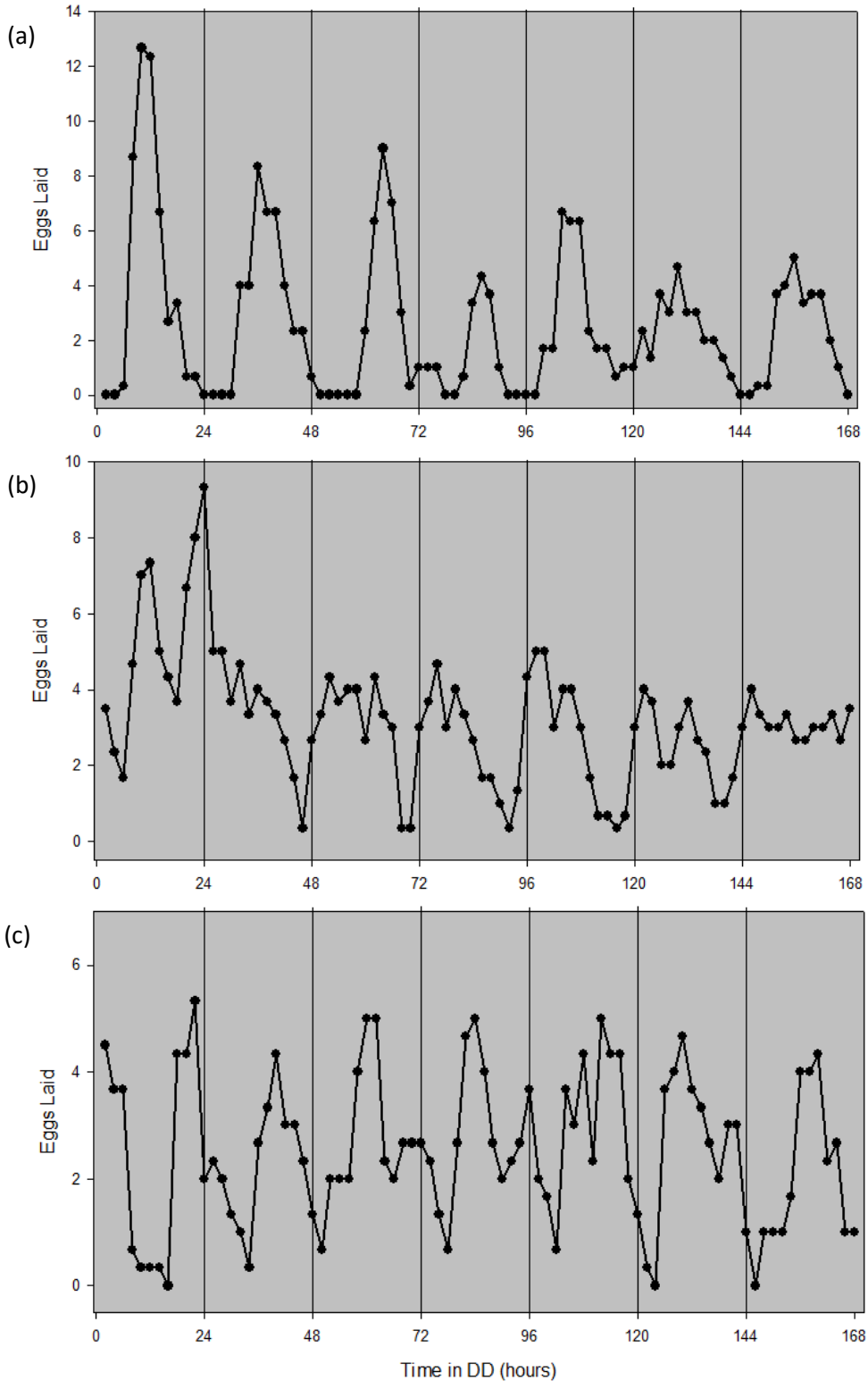


Figure 2: Cycle by cycle profile of a single fly across all seven days of the assay with lines wherein *per*^{S} and *per*^{L} are expressed primarily in the thoracic-abdominal ganglion on a wild-type per background – a) TAGGAL4-UAS*per*^{S} (N=15) b) TAGGAL4-UAS*per*^{L} (N =13) c) TAGGAL4/+ (N=19)

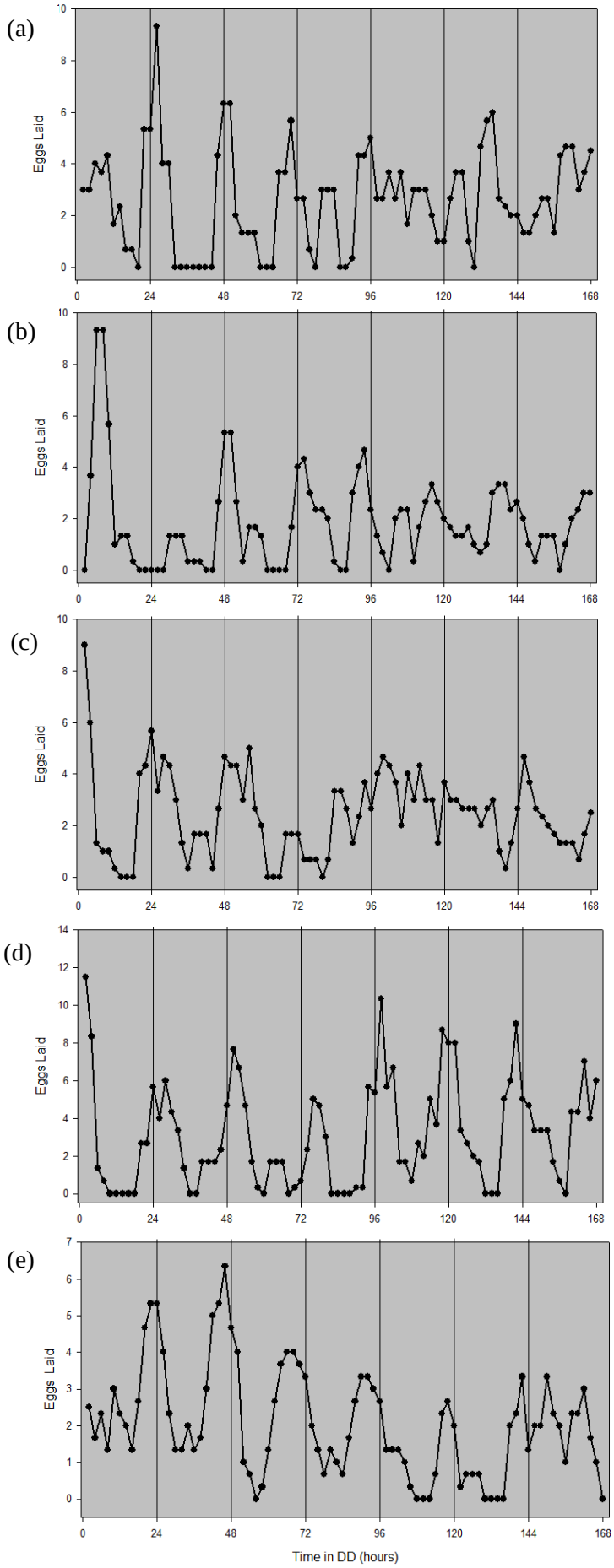


Figure 3: Cycle by cycle profile of a single fly across all seven days of the assay with lines wherein *per*^{S} and *per*^{L} are expressed pan-neuronally on a *per*^{01} background – a) *per*^{01}; *elav*GAL4/+; UAS*per*^{S}/+ (N=10) b) *per*^{01}; *elav*GAL4/ UAS*per*^{L}; + (N=8) c) *per*^{01}; *elav*GAL4/+; + (N=15) d) *per*^{01}; +; UAS*per*^{S}/+ (N=12) e) *per*^{01}; UAS*per*^{L}/+; + (N=11)

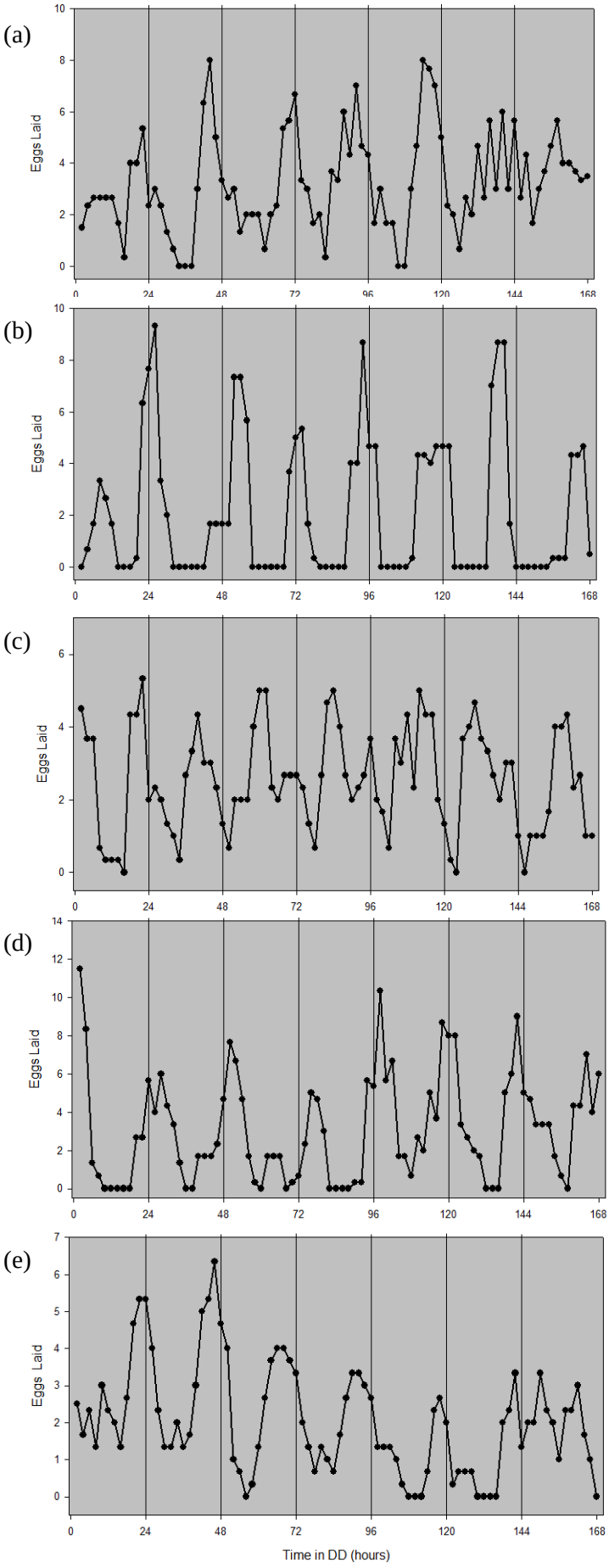


Figure 4: Cycle by cycle profile of a single fly across all seven days of the assay with lines wherein *per*^{S} and *per*^{L} are expressed pan-neuronally on a *per*^{01} background – a) *per*^{01}; +; TAGGAL4/UAS*per*^{S} (N=7) b) *per*^{01}; UAS*per*^{L}/+; TAGGAL4/+ (N=7) c) *per*^{01}; +; TAGGAL4/+ (N=12) d) *per*^{01}; UAS*per*^{S}/+ (N=12) e) *per*^{01}; UAS*per*^{L}/+ (N=11)

The expression of per^S and per^L in the nervous system of the fly did not effect a change in the average period value

Surprisingly, the expression of the PER^S and PER^L proteins in the nervous system of the fly did not bring about the expected significant change in the period value of the flies, whether on a *per*^{01} background or not (see Figures 4 and 5). It is of note, however, that the average period of the line *per*^{01}; *elav*GAL4-UAS*per*^{L} is 26.5 $\pm$ 2.13 h (SEM) and the average period of the line *per*^{01}; *elav*GAL4-UAS*per*^{S} is only 22.9 $\pm$ 0.7 h (SEM). There appears to be a trend towards a longer period in the former and towards a shorter period in the latter. This difference is not significant, and the possible explanations of this phenomenon are discussed below.

This trend was non-existent in the lines wherein only the thoracic-abdominal ganglion expressed PER^S and PER^L, as *per*^{01}; TAGGAL4-UAS*per*^{L} had an average period of 25 $\pm$ 0.75 h (SEM) and *per*^{01}; TAGGAL4-UAS*per*^{S} had an average period of 24.5 $\pm$ 1.45 h (SEM).

Significant differences in fecundity are seen

While there are few significant differences in fecundity across the lines, there is no clearly evident pattern to the differences. On the wild-type *per* background, for example, TAGGAL4/+ had a significantly lower average fecundity than the two experimental lines (see Figure 6), but this difference between control and experimental lines is not consistent. On a *per*^{01}background, the three control lines are significantly different from each other, but *per*^{01}; TAGGAL4/+ is not significantly less fecund than either one of the two experimental lines.

It has been reported that the null mutants of *period* and *timeless* have a role to play in the fecundity of the female fly (Beaver et al., 2003) as well as in the reproductive fitness of the male fly (Beaver et al., 2002): *per*^{01} and *tim*^{01} flies are less fecund than their wild-type counterparts. However, such a comparison of fecundity across flies carrying the null mutation for *period* and those carrying the wild-type gene (between the controls *elav*GAL4/+ and *per*^{01}; *elav*GAL4/+, for instance) would be confounded by the effects of different genetic backgrounds, and the crossing of the such backgrounds could lead to hybrid vigour that obscures the effect of the lack of PERIOD on the fecundity of the female fly.

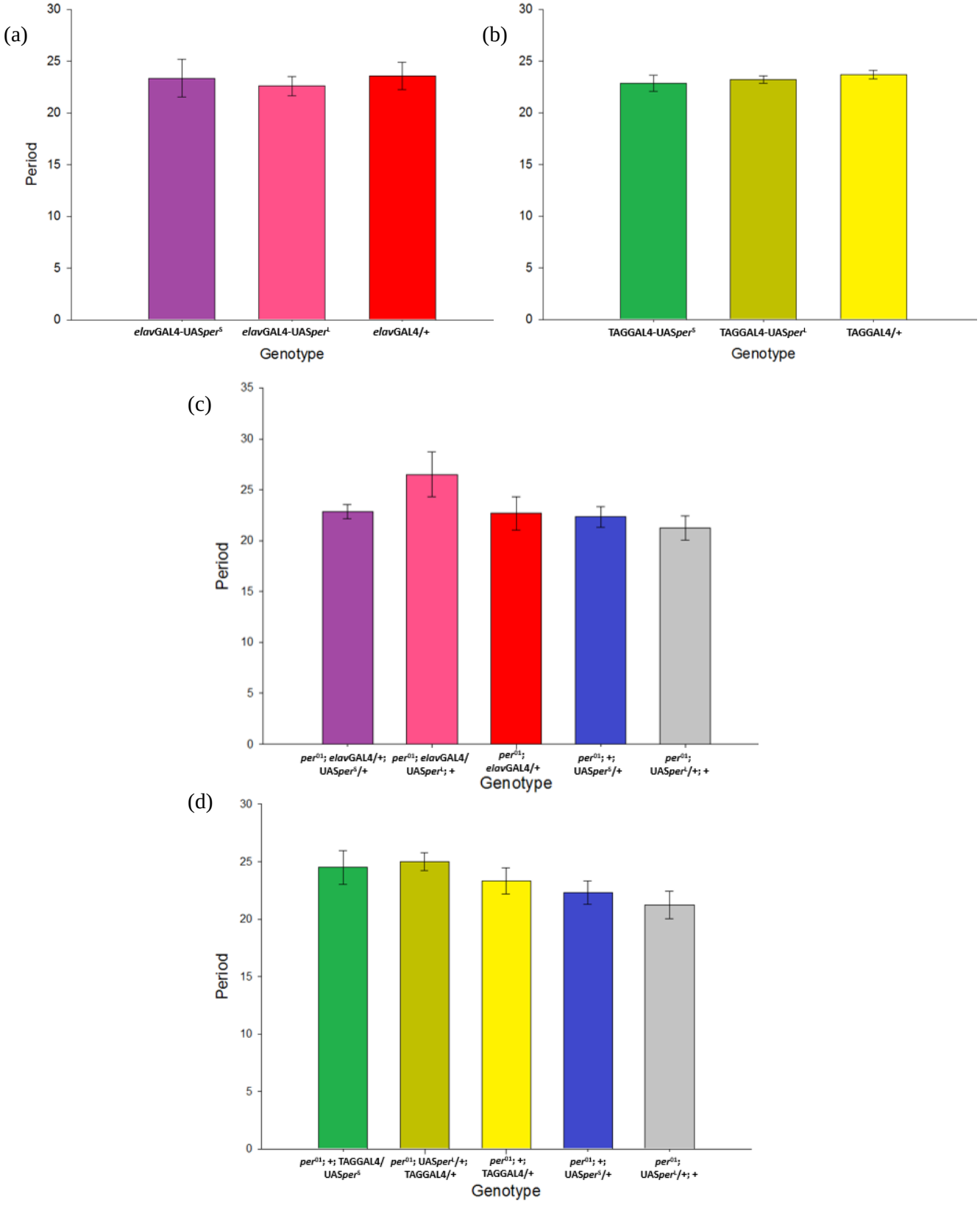


Figure 5: Period length averaged across days for pan-neuronal expression of PER^S and PER^L on a per^+ background (a) and per^{01} background (c) and expression in the thoracic-abdominal ganglion on a per^+ background (b) and per^{01} background (d). Error bars are SEM

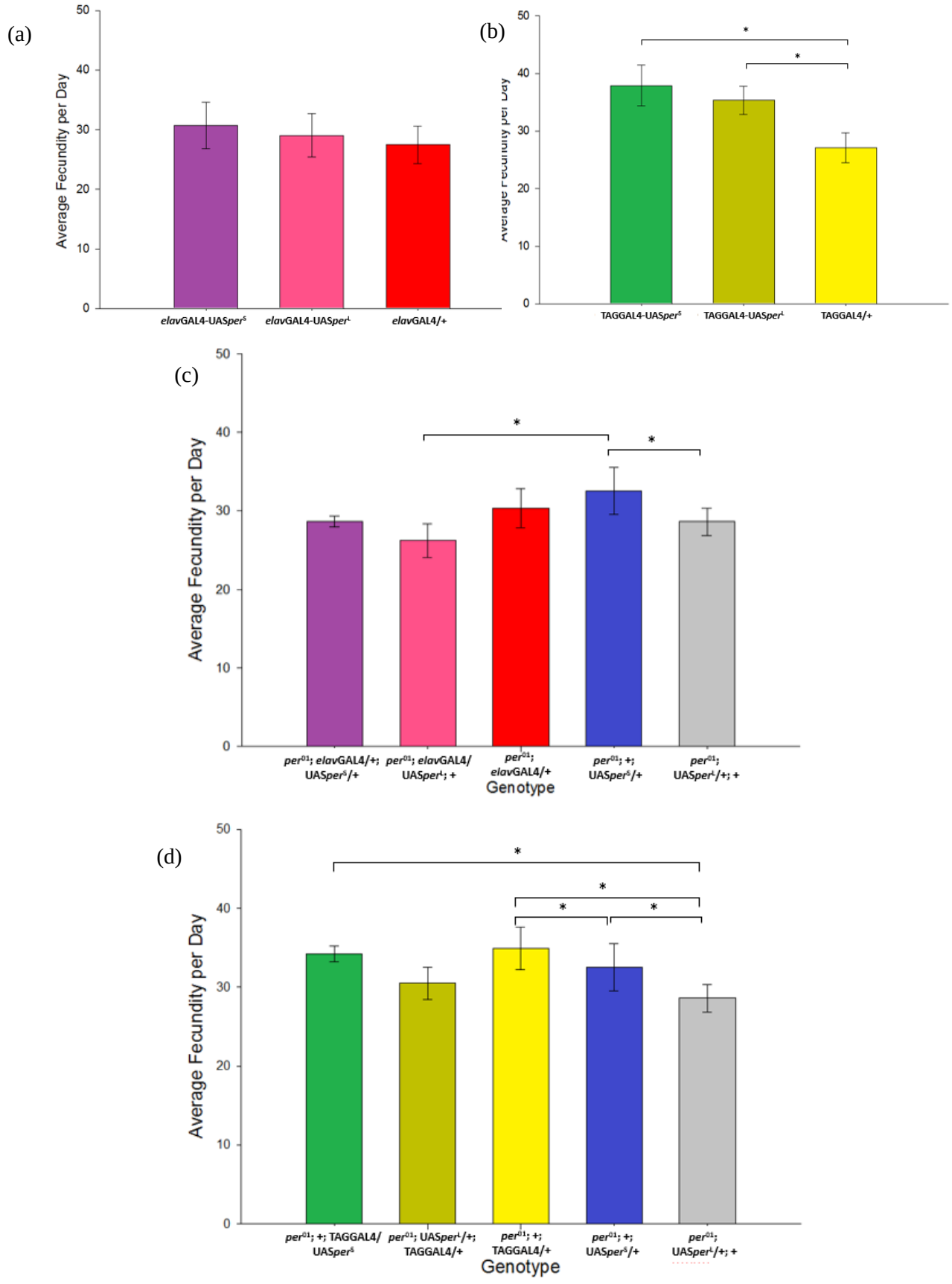


Figure 6: Average fecundity per day averaged across individuals for pan-neuronal expression of PER^S and PER^L on a wild-type *per* background (a) and per^{01} background (c) and expression in the thoracic-abdominal ganglion on a wild-type *per* background (b) and per^{01} background (d). Error bars are SEM

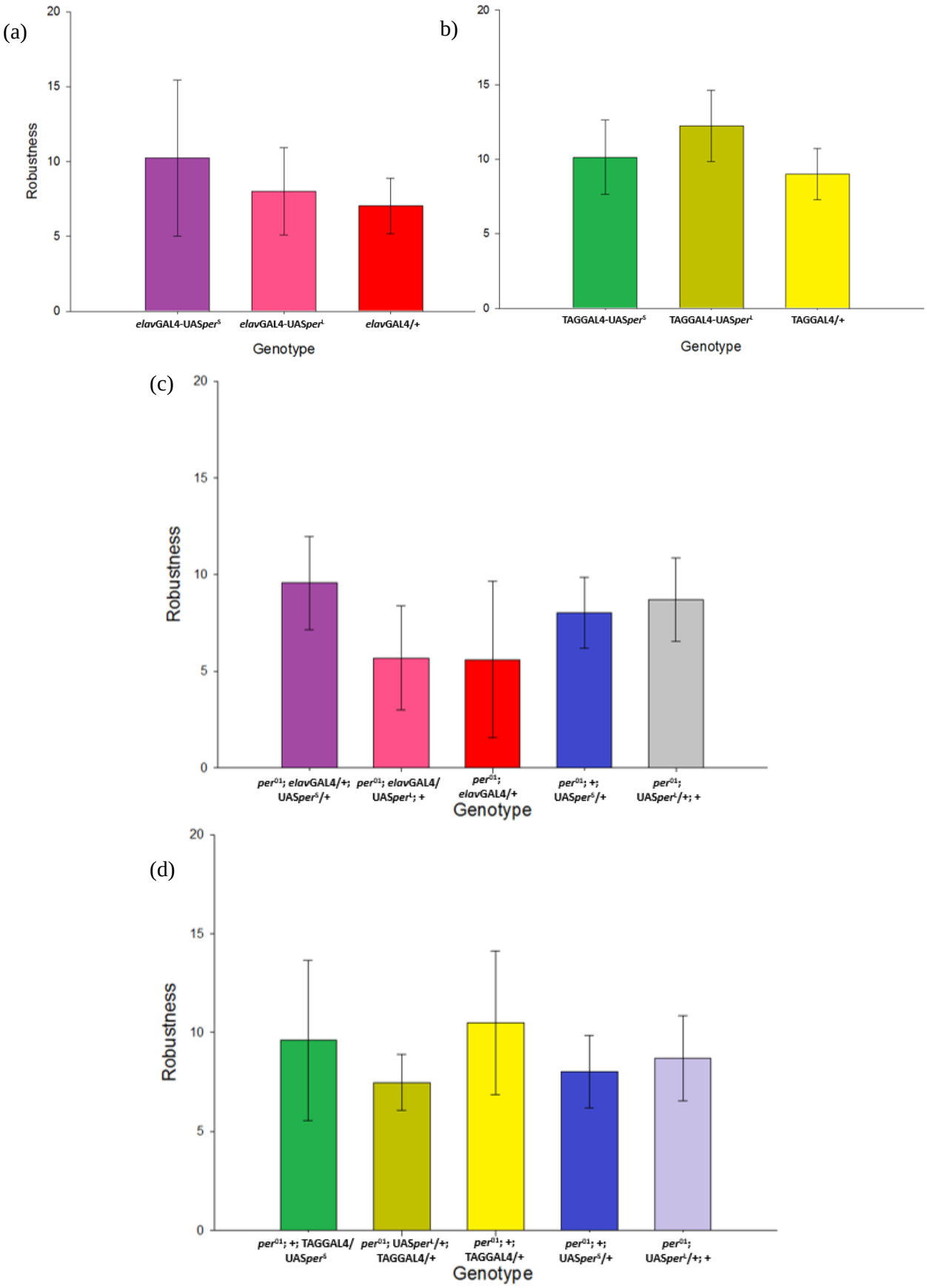


Figure 7: Robustness averaged across days for pan-neuronal expression of PER^S and PER^L on a wild-type *per* background (a) and per^{01} background (c) and expression in the thoracic-abdominal ganglion on a wild-type *per* background (b) and per^{01} background (d). Error bars are SEM

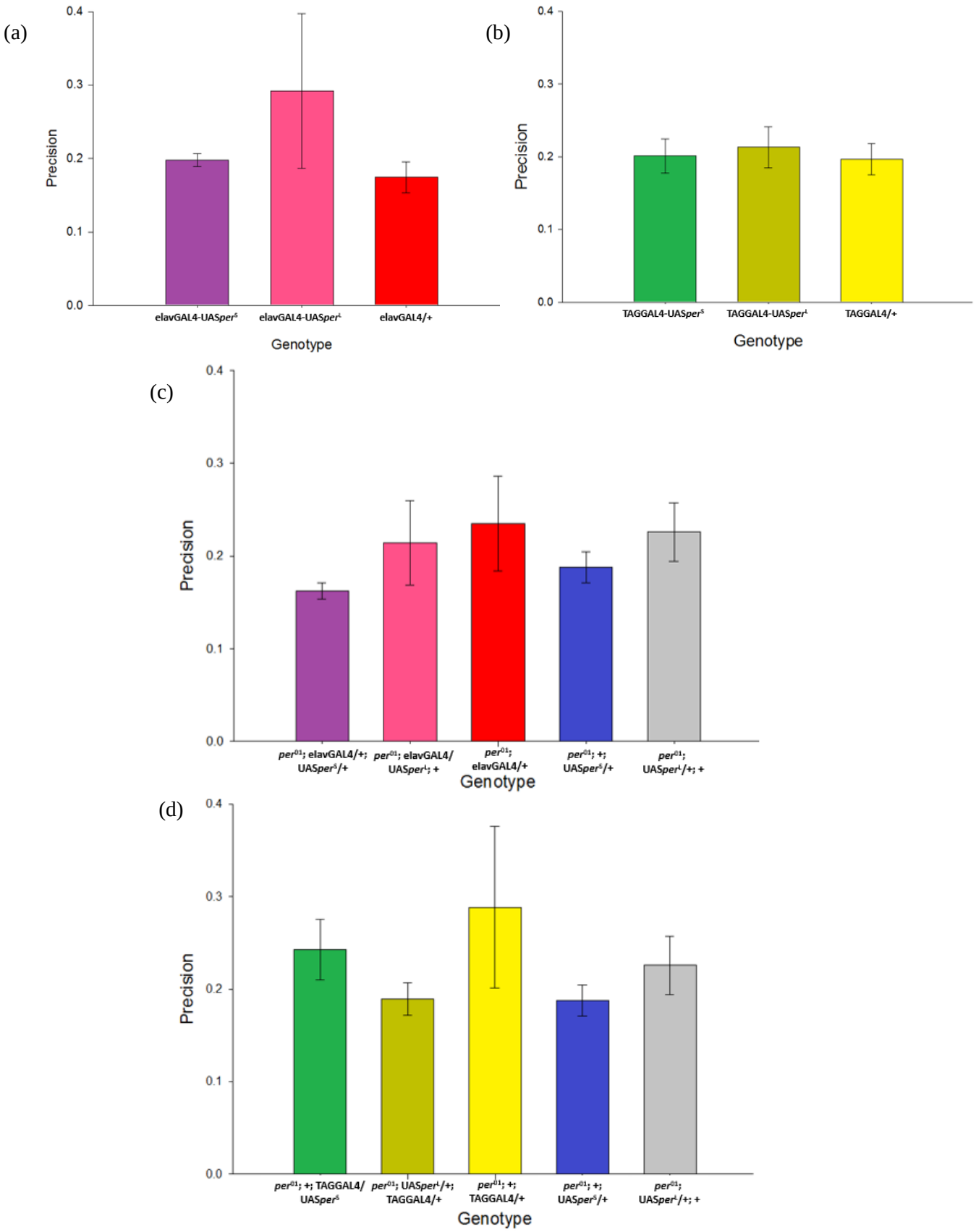


Figure 8: Robustness averaged across days for pan-neuronal expression of PER^S and PER^L on a wild-type *per* background (a) and per^{01} background (c) and expression in the thoracic-abdominal ganglion on a wild-type *per* background (b) and per^{01} background (d). Error bars are SEM

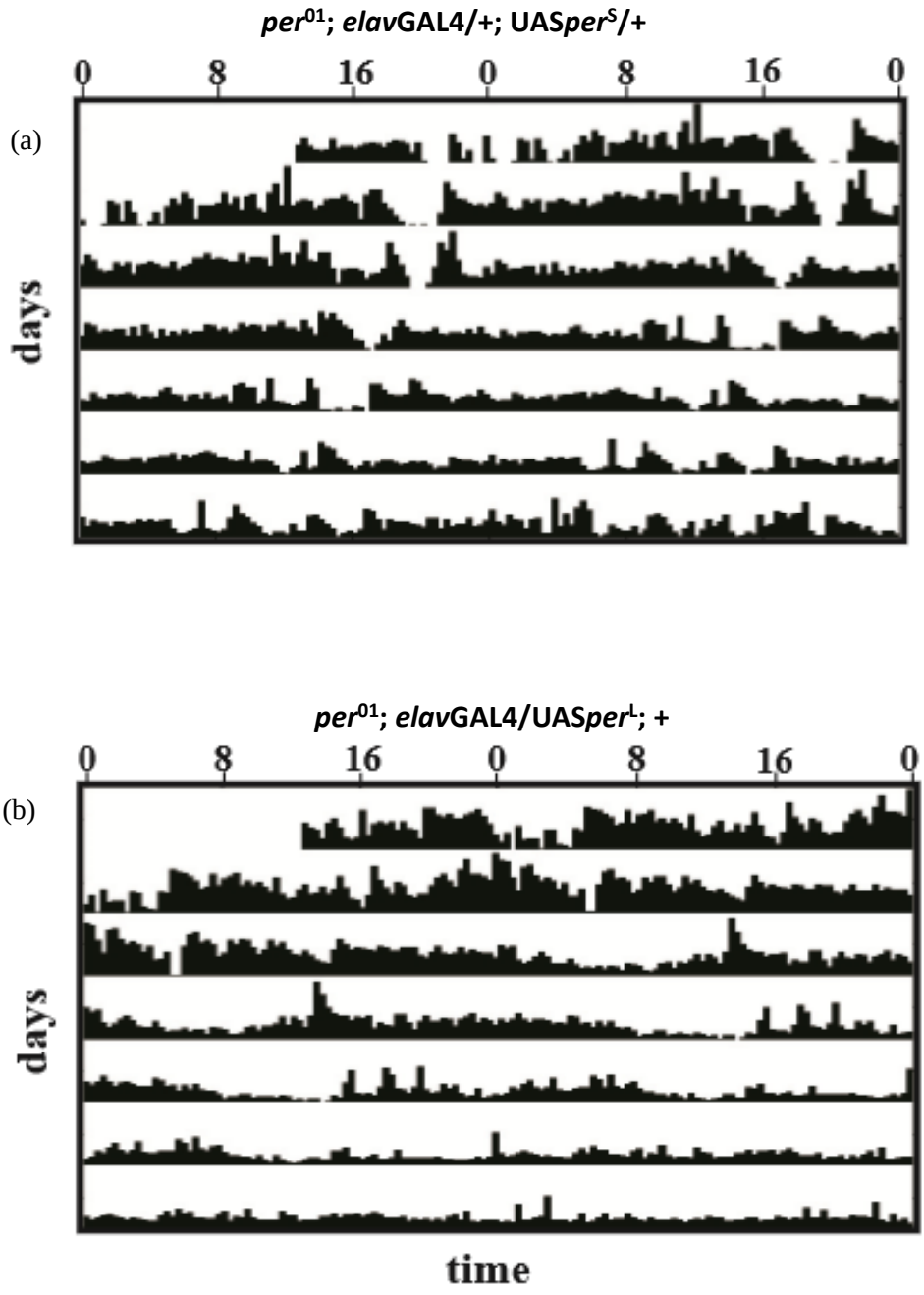


Figure 9: Representative double-plotted actograms of virgin male flies, conducted at 25 °C in DD. Time of day is plotted on the abscissa (X-axis) and the number of days is plotted on the ordinate (Y-axis). Genotype is indicated by the labels above the plots

DISCUSSION

The average period values of *per*^{01}; *elav*GAL4/+; UAS*per*^{S}/+ and *per*^{01}; *elav*GAL4/UAS*per*^{L}; + are not significantly different from each other, possibly due to the high variance of the latter's period values. However, the trend is unmistakeable: *per*^{01}; *elav*GAL4/UAS*per*^{L} has a higher average period value than *per*^{01}; *elav*GAL4/+; UAS*per*^{S}/+. This can be interpreted in one of or a combination of different ways.

Methodological constraints

The manual transfer of flies into fresh vials every two hours introduces a certain amount of noise in addition to the noise inherent to the oviposition rhythm, due to the disturbance to the flies. This disturbance could result in the flies taking additional time to return to their normal behaviour. Furthermore, the length of the time-series data may not have been sufficient to pick up the shifts in period, especially as the resolution of the data points is as high as 2 h. These two factors could interact to obscure the true difference between the two different manipulations of the nervous system, and result in a non-significant trend. While the control lines also have low period values quite comparable to *per*^{01}; *elav*GAL4/+; UAS*per*^{S}/+, this is consistent with the findings of McCabe and Birley (1998), who showed that the oviposition rhythms of *per*^{01} flies have highly variable but low period values, close to those of *per*^{S} flies.

A repeat of this experiment to produce data of a higher resolution or a longer time-series would seem indicated.

Another factor that might contribute to the non-significant trend is the fact that the final sample size of the experimental lines was rather low, on the order of 7-10 flies. This was due to low survivorship, the loss of flies during the manual transfers and the necessity to exclude flies that did not oviposit for 48 h continuously.

A repeat of this experiment to produce data with a larger sample size of flies would seem indicated.

The oviposition clock is non-neuronal

Should the effects of sample size, low resolution and extraneous noise be ruled out, and the difference still come up as non-significant, it would lead us to the conclusion that there is a non-neuronal clock responsible for the egg-laying rhythm.

One candidate for a non-neuronal clock in the nervous system itself is the neuroglial system. If the rhythm emerges from the fly's glia, this could be verified using the *repo*GAL4 driver, whose expression pattern encompasses all the glia in the fly's nervous system (Xiong et al., 1994), but not the neurons themselves.

If the glia too are ruled out, the clock probably lies outside the nervous system, though it may possibly convey rhythmic information to the reproductive system through the nervous system. As reproduction is closely regulated by hormones, the endocrine system of the fly might provide non-nervous candidates for the oviposition clock. Juvenile Hormone regulates reproduction in many insects (Koeppe et al., 1985) and in *Drosophila melanogaster* it is produced by the Corpora Allata (Saunders, 2002). This, and the ring gland would be worth investigating.

Oogenesis is a rhythmic process (Allemand, 1976), and is probably tied to fat metabolism as yolk proteins are synthesised. The fat body clock (Xu et al., 2008) would also be worth investigating for its role in oviposition rhythm.

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