CAFFEINE TOLERANCE IN DROSOPHILA

CAFFEINE TOLERANCE IN THE FLY *DROSOPHILA MELANOGASTER*

By

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ABSTRACT

Responding to cues that precede and signal important biological events (anticipatory learning) may have important fitness consequences. Anticipatory learned responses have high adaptive value when intervene in the regulation of physiological states, and one widely studied example of learning-mediated homeostatic regulation is tolerance to drugs. Physiological responses that offset drugs' effects, like many other autonomic responses, can become classically conditioned to environmental cues that are repeatedly paired with drug intake. Caffeine is a widely used model substance for studying mechanisms related to drug intake, tolerance and addiction. In this report I provided evidence of tolerance development to some caffeine effects in the fruit fly, *Drosophila melanogaster*, which is a novel finding. I then showed that the tolerance is mediated by classical conditioning. A surprising result was that tolerance was completely and not, as more generally found, only partially mediated by conditioning. Together with other drugs, caffeine appears to be a promising substance to be used in the fly to study the pharmacology of substances with addictive potential. Also, these results indicate that this system may be an optimal model for the study of the adaptive value of learning in an insect species.

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Introduction

Learning capabilities allow organisms to adjust to changing environmental conditions, and are likely to have positive fitness consequences when changes occur in the short term, that is, within the organisms' lifespan.

The adaptive value of learning has received growing attention in recent years, and evidence for it has been steadily accumulating. Fitness benefits of learning are suggested in the literature related to a variety of organisms, from relatively simple ones like insects (e.g. Dukas, 2005; Papaj and Prokopy, 1989) to mammals, and its widespread distribution in the animal kingdom may be thought of an indication of its adaptive significance.

One of the most basic forms of learning is classical (Pavlovian) conditioning. In classical conditioning, an organism learns to associate a stimulus with a biologically significant event, generally with the stimulus closely preceding the event.

Responding to cues that precede and signal important biological events may have important fitness consequences. For example, in the Japanese quail conditioning is implicated in increased success in male-male competition for access to a reproductive female (Gutierrez and Domjan, 1996). Also, conditioning was shown to increase the probability that insemination would results in fertilization, and therefore reproductive success (Domjan et al., 2003; Adkins-Regan and MacKillop, 2003). Hollis and colleagues have used the blue gouramis (*Trichogaster trichopterus*) as model system to show other facets of sexual conditioning. For example, they showed that Pavlovian signalling of female accessibility attenuated males' aggressive behaviour and increased their reproductive success (Hollis et al., 1997).

Anticipatory learned responses have high adaptive value when intervene in the regulation of physiological states, or homeostasis; in the words of B. Dworkin, "learning is one of the physiological mechanisms that give the body its wisdom" (Dworkin, 1993, p.185). One widely studied example of learning-mediated homeostatic regulation is tolerance to drugs. Drugs can profoundly disrupt organismal balance, but appropriate control mechanisms are in place that sense the alteration, intervene to offset the drugs' effects and eventually re-establish the normal state. It is now widely accepted that the physiological responses that offset drugs' effects, like many other autonomic responses (Dworkin, 1993) can become classically conditioned to environmental cues that are repeatedly paired with drug intake. A number of theories have been proposed that explain the role of conditioning in drug tolerance (Poulos and Cappell, 1991). One of the most successful models was proposed by Siegel (Siegel, 1976), and is known as situational specificity of tolerance. In this framework, a drug administration event can easily be thought of as a conditioning trial. The drug's effects are the unconditioned stimuli, as they elicit homeostatic (compensatory) responses prior to any learning, i.e., unconditionally. These homeostatic responses are compensatory in that they are opposite to the drug's effects and therefore act to reduce or offset them and reinstate homeostasis. With repeated pairing of the drugging procedure with the drug's effects, environmental and subjective cues become associated with those effects (i.e., become conditioned stimuli). Thus, administration of the drug *in presence of the conditioned stimuli* causes

the organism to activate in advance the compensatory responses to the drugs' effects, which results in tolerance (e.g., Siegel et al., 2000).

Such kind of anticipatory conditioned responses to drugs have been shown for a variety of substances, in many species, and indeed the modern definition of tolerance to drugs incorporates them as a prominent factor (Kalant, 1996; Poulos and Cappell, 1991).

Fast, plastic responses to noxious substances may play a particularly relevant role in the ecology of organisms that face a high risk of poisoning and intoxication. In the case of phytophagous insects, for example, the challenge of noxious compounds – secondary plant compounds – is especially serious. Almost all plant species produce secondary compounds (also, allochemicals). Some allochemicals are both unpalatable and toxic, some are only toxic, and others are unpalatable but otherwise harmless; allochemicals in this last category may cause behavioural aversive responses and therefore erroneous rejection of valuable nutritious resources (e.g. Glendinning, 1996, 2002).

The existence of these different classes of allochemicals poses a difficult decision-making problem, which, together with the general problem of a nutritionally poor diet (Slancky, 1993) compounds the complexity of foraging for herbivorous insects. Accordingly, they have evolved an impressively vast and diverse array of mechanisms that allow a great deal of flexibility in dealing with plant allochemicals. Among others, preingestive (Schoohnven and van Loon, 2002) and postingestive (Glendinning, 1996) detection mechanisms, that lead to rejection of toxicants, comprehending aversion learning (e.g., Bernays and Chapman, 2000); various aversive responses overriding mechanisms that allow utilization of unpalatable but otherwise harmless compound (Shield and Mitchell, 1995; Glendinning et al., 2001a); and both constitutive and acquired resistance (tolerance) (Karban and Agrawal, 2002; Glendinning, 2002; Scott 1999; Stevens et al., 2000).

Both constitutive and inducible tolerance to noxious compounds is well characterized in insects. Constitutive tolerance is typical of insect species that specialize on particular host plants, and is mostly mediated by specific enzymatic systems. For example, Heliconius caterpillars employ an unusual suite of enzymes that allows them to convert cyanogenic glycosides into thiols, thus bypassing the release of highly toxic cyanide gas (Engler et al., 2000).

Resistance to allochemicals however is more often an inducible response (Terriere, 1984), that is, facultatively activated by exposure to plant metabolites. This is the case for example of most of the alloforms of the major detoxifying enzyme system, the cytochrome P450 system (Feyereisen, 1999).

Whereas most of what is known about tolerance in insects is based on work on enzymatic activity, in mammals at least two non-mutually exclusive physiological mechanisms of acquired tolerance are well characterized. First, metabolic (or pharmacokinetic) tolerance refers to a diminished effect due to changes in the absorption, distribution or degradation of a substance (Tabakoff et al., 1986). Enzymatic changes belong to this group. A second form of tolerance is functional (pharmacodynamic) tolerance, which is generally mediated by adaptations at neural level, for example, up or down regulation of receptors in the CNS (e.g, Fadda and Rossetti, 1998).

Theoretically, learning could play a role in both metabolic and functional tolerance, that is, both enzymatic and neuroadaptive changes could become conditioned to environmental cues, and thus elicited by the appropriate conditioned stimuli. This possibility was proposed early on, for example by Tabakoff et al. (1986). In support, they cited two studies that seemed to suggest conditioned metabolic tolerance to two drugs in mice (the two papers are Melchior and Tabakoff (1985), with alcohol, and Roffman and Lal (1974), with hexobarbitral). However, to my knowledge these have remained the only two reports of conditioned metabolic tolerance since, and the evidence was provocative but not conclusive. On the other hand, evidence for conditionally mediated functional tolerance is supported by a huge literature, and a wealth of data are available for various vertebrate species and for a number of chemicals. One of the most studied is ethanol. A widespread toxic compound in nature, ethanol is of particular interest here because it is the only one for which functional tolerance has been so far showed in an insect, the fruit fly (*Drosophila melanogaster*). The fruit fly has recently been introduced as model system in alcohol research as a powerful tool to gain access to the molecular bases of tolerance (Berger et al., 2004). Two different forms of tolerance, rapid and chronic, have been described in the fly, which closely resemble two of the three forms known in mammals, and with which show remarkably similarities. In mammals, tolerance can be *acute*, which occurs within the administration of a single, prolonged infusion of alcohol. A second form is *rapid*, which refers to tolerance to a second dose given between 8 hours and 3 days after the effects of a first dose have been completely extinguished. Lastly, tolerance can be *chronic*, which develops after a certain number of repeated administrations (Kalant, 996).

In the fruit fly, Scholtz et al. (2000) and Dzitoyeva et al. (2003) have reported on tolerance development to alcohol motor impairing effects after a single (rapid tolerance) or multiple exposures (chronic tolerance). Berger et al. (2004) have demonstrated for the first time a form of chronic ethanol tolerance that seems to be dissociable genetically and pharmacologically from rapid tolerance. Both forms of tolerance appear to be functional in nature, that is, involving neural adaptations. Furthermore, development of tolerance was impaired in a number of neurally impaired mutant strains. Likewise, functional biosynthesis of octopamine, a neuromodulator and neurotransmitter analogous to mammalian norepinephrine (Roeder, 1999; 2005), was required for tolerance to develop.

Another widely used toxic substance that appears especially suitable for studying functional tolerance is caffeine (Rozin et al., 1984). A number of reasons guided the decision to use caffeine in this project. First, caffeine is a toxic plant secondary compound (alkaloid), and occurs naturally in the leaves, seeds or fruit of more than fifty species (Carrillo, 2004), the most well known being coffee, tea and cocoa. Second, caffeine is a toxic agent that shares some of the alcohol's characteristics. It has been long known as a poisonous agent in insects (Nathanson, 1984; Mathavan et al., 1985) where its toxic action has been characterized (e.g., Slansky and Wheeler, 1992). At the same time, caffeine is well known for its neuromodulatory effects in mammals. In fact, caffeine is the most used neuromodulatory substance in humans, who profit from its positive effects like sleep removal, enhanced attention and mood improvement (reviewed e.g. in Smith, 2002). This opens the interesting possibility that caffeine, like alcohol, may have a

dual pharmacological action in insects. Third, in mammals tolerance to some of caffeine's effects is well documented. Importantly, there is some evidence that tolerance to some effects of caffeine is mediated by conditioning (e.g, Andrews et al., 1998; Corti et al., 2002; Rozin et al., 1984). Finally, the fruit fly has been recently shown to respond to caffeine in much the same way mammals do. Shaw et al., (2000) and Hendricks et al. (2000) were interested in circadian activity regulation in the fruit fly, and produced two detailed studies of its nighttime rest behavior, which they referred to as a sleep-like state. In fact, night rest in the fly shares many of the features that characterize sleep in mammals. One of these is that it is similarly affected by two psychoactive substances, caffeine and hydroxyzine, that modulate waking and sleep in mammals. Specifically, caffeine was shown to dose-dependently decrease nighttime rest, and hydroxyzine to decrease sleep latency. Taken together, these results suggest that caffeine may acts on neural substrates in the fly, as it does in mammals.

In sum, coping with toxic chemicals is critical to insect survival; tolerance mechanisms play a relevant role in it, and conditioning is known to play a major role in tolerance expression, at least in mammals. Functional tolerance requires neuroadaptive changes, and recent evidence was provided of neuromodulatory actions underlying tolerance to a toxic agent in the fruit fly. All in all, there seem to be the preconditions to advance the hypothesis that conditioning-mediated, anticipatory responses may also have evolved in insects, a further mechanism among many, to cope with ubiquitously occurring noxious compounds.

The methods section of this manuscript is divided into fours parts. The goal of the first experiment, in the first section, was to reproduce the published results on the circadian organization of rest and activity during a 12:12, dark:light cycle in individual flies. In the second section, I describe an experiment that addressed the question of caffeine effects on fly's nighttime rest, and tried to replicate its published rest-disrupting effect. The experiment followed a protocol similar to Hendricks et al. (2000); I gave flies a caffeinated solution just before dark, and recorded their activity for the following 12 hours long night. I predicted that flies given caffeine just before night would rest less than control flies receiving a placebo. After showing rest-disrupting effects of caffeine, the third section reports on an experiment aimed at testing development of tolerance to those effects. In the experiment, I gave flies caffeinated solution before night for several consecutive nights to elicit development of tolerance, and then recorded their nighttime rest on the final, test night. I hypothesized that flies given caffeine for a period of several consecutive nights would rest more after a final challenge with caffeine – that is, would become tolerant – than flies similarly challenged after receiving for the same period a placebo. As hypothesized, I found evidence of development of tolerance to the rest-disrupting effects of the drug. The fourth section of this manuscript reports on an experiment that tested the possibility that the acquired tolerance is partly mediated by learning (classical conditioning). I administered flies either a caffeine solution flavoured with either one of two flavours, pineapple or orange, or a non-caffeinated solution flavoured with the other flavour on alternate nights, for 6 nights; this was the conditioned tolerance acquisition phase. On the 7^{th} night (test night), half the flies received a pineapple-flavoured caffeinated solution, the other half an orange-flavoured caffeinated

solution. I predicted that flies receiving, on test night, a flavour-caffeine pairing different from the pairing experienced during their conditioned tolerance acquisition phase would exhibit a significant loss of tolerance to caffeine.

General methods

I used a stock of *D. melanogaster* flies initiated from wild flies collected in Hamilton, Ontario. The flies were kept in a population cage inside an environmental chamber, at constant temperature of 25°C and 70% RH, on a 12:12 dark:light cycle, with light on at 0600h. The population cage contains a few thousands flies and two bottles of standard fly food. The flies used in the experiments developed at low density from bottles containing ca. 300 larvae. I collected and sexed flies within 8 hours from eclosion and placed them in 40-ml vials containing 5 ml of standard medium, 10 flies per vial, if not otherwise specified in the methods subsections below. I kept the flies inside the environmental chamber until they reached the appropriate age for each experiment. In all experiments I used virgin females. In the following methods subsections, *test day* refers to the day when activity recording was performed.

In experiments 2 to 6, I gave flies different types of solutions to drink before night. Plain sucrose solution was used as control in experiments where the other(s) treatment(s) received caffeinated or quininated solutions. Irrespective of type however, all the solutions contained 100 mg/ml of sucrose (10% w/v). Drinking behaviour varied among flies. Therefore, the solutions provided were blue colored with food color, allowing approximate quantification of flies' drinking with close inspection of the flies' abdomen color.

1. Circadian rest-activity patterns

Experiment 1

Rationale

In two recent papers (Shaw et al., 2000; Hendricks et al., 2000) two separate laboratories provided evidence supporting the notion that night time rest in the fruit fly may be a sleep-like state, since it presents most of the features that define sleep in mammals. Flies' developmental rest pattern parallels the mammalian's, is homeostetically regulated and shows the typical rebound effect after deprivation. Also, they were able to show that caffeine, hydroxyzine, and cyclohexyladenosine, three agents that modulate sleep and waking in mammals (see below, experiment 1), have comparable effects on flies; caffeine increased motor activity and waking, whereas hydroxyzine and cyclohexyladenosine increased rest.

In the series of experiments reported in this manuscript, I used night time rest as a behavioural measure of caffeine effects. Therefore, as a first step, I needed to reproduce the published result and show the circadian organization of rest during the 24 hours cycle. I expected that flies would show higher activity during the light period and that rest would be concentrated during the dark period.

Further details regarding the activity monitors' technical parameters and details on raw data treatment are reported in appendix B.

Methods

I used 5 days old females. I kept flies in groups of ca.10 in 40 ml vials with standard food until recording; vials were substituted every two days. I constrained the space in the vials with a cotton plug to habituate the flies to the dimensions of the recording tubes (see Appendix B). At 1800h of test day, I placed the flies into the activity monitor and their activity was recorded for the ensuing 24 hours, of which 12 hours of dark (night) and 12 hours of light (day). For this study, only one activity monitor was available, therefore the study had 4 replicates, each conducted on a different day, with 7 flies recorded each replicate. I used a repeated measures ANOVA (period as within and replicate as between sbj. factor) to compare the two periods. The reported sample size of 26 instead of 28 flies was due to loss of two flies during handling.

Results and discussion

Flies subjected to 12 hour:12 hour light:dark cycles exhibited sustained periods of activity and quiescence, with most of quiescence occurring during the dark period (Fig. 1). In figure 1 it is clearly visible the high activity displayed upon introduction in the

activity chamber that lasted for approximately one hour. Examples of other activity patterns throughout the 24 hours for individual flies are reported in Appendix A. Flies rested more during the night than during the day ($F_{1,25} = 60.5$, $p < 0.001$; Fig.2). Time (± SE) spent resting was 50.6 ± 1.3 min and 33.4 ± 1.9 min for night and day, respectively; flies showed a 44% reduction in rest during the day.

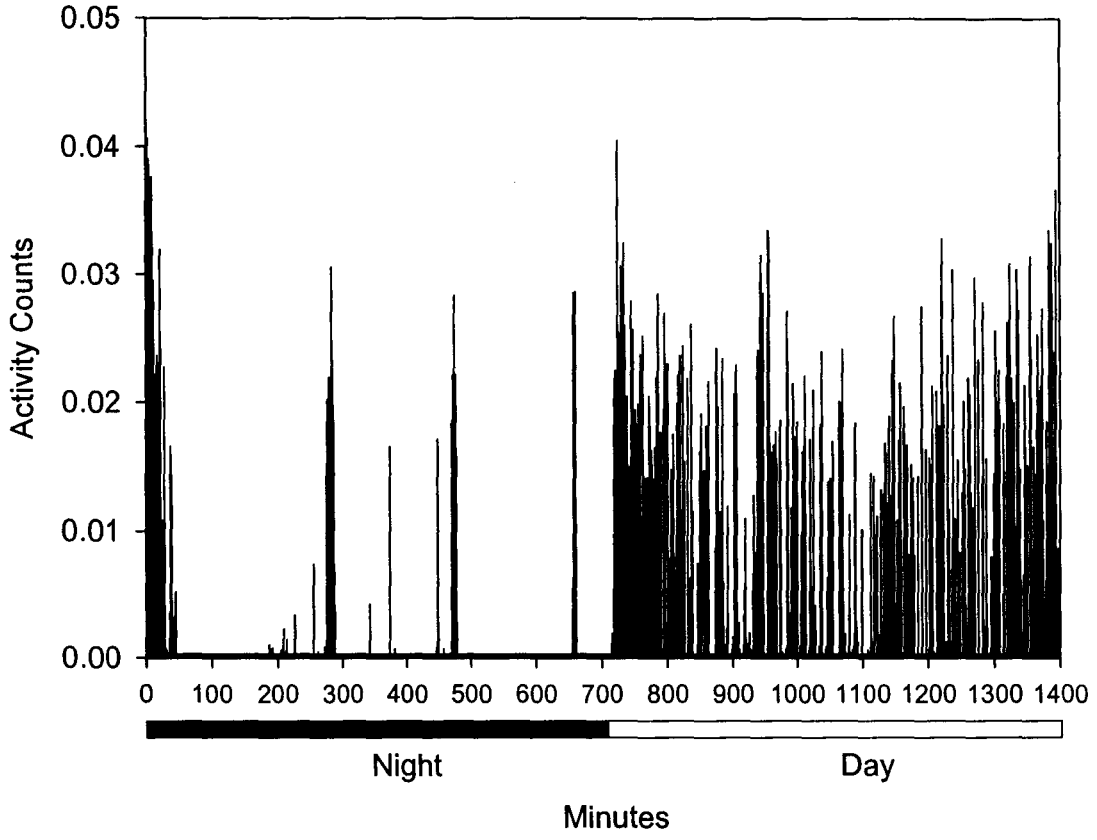

Figure 1. Activity record of a sample fly maintained on a 12 hour/12 hour light (horizontal open bar) /dark (horizontal solid bar) cycle. Activity counts indicate the number of perturbations of the infrared beam detected over 1-min bins.

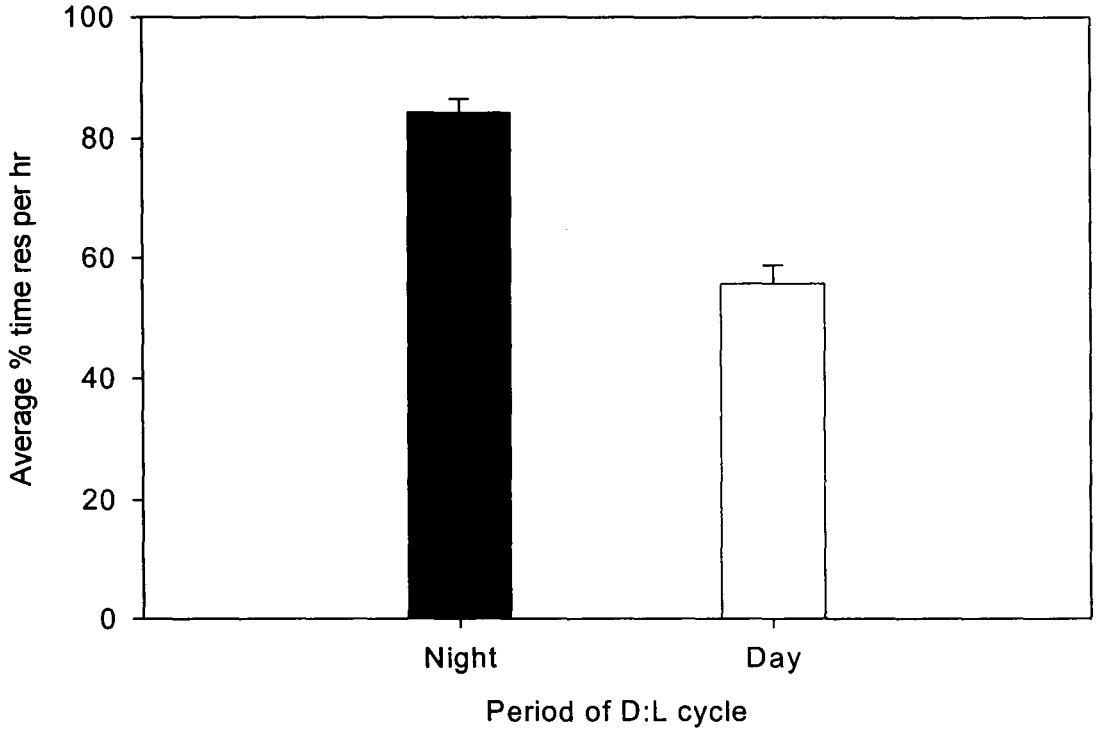

Figure 2. Average (± SE) % rest per hour spent resting during the night and the day (ANOVA, $p<0.001$, $n = 26$ flies).

2. Effects of caffeine on night time rest

Experiment 2

Rationale

Flies in experiment 1 showed a robust circadian organization of activity and rest, with rest concentrated during the night hours. The two published studies referred to above (Shaw et al., 2000; Hendricks et al., 2000) showed that two agents that neuromodulate arousal in mammals produce the same effects in the fly. Caffeine was shown to dose-dependently decrease night rest. Concentrations of 2.5 mg/ml and 5 mg/ml of caffeine in sucrose solution reduced sleep of about 20% and 45% respectively. While the results are intriguing, and may suggest that caffeine acts on conserved neural mechanisms, a simpler explanation is that in fact the reduction in rest is due to stress or a non-specific effect of the substance. To examine this possibility, flies were also treated with agents known to increase sleep and sleep latency in mammals. In one study, the highly selective A1 adenosine receptor agonist cyclohexyladenosine caused a significant increase in rest (Hendricks et al., 2000). In the second study, the H1 histamine receptor antagonist hydroxyzine was shown to increase rest as well as to reduce its latency (Shaw et al., 2000).

In the following experiment I replicated the cited caffeine effects. I tested the prediction that a caffeinated solution at two different concentrations of 4 mg/ml and 5 mg/ml would reduce a fly's night rest, when ingested just before onset of the night.

Methods

I used 5 days old females. On the morning of the test day, I transferred the flies into empty vials in groups of 10, and starved them for 8 hours at 25°C and 30-40% RH, from 0930 to 1730 h. After starvation, I gave control flies a drop of plain sucrose solution (see general methods), and treatment flies a drop of sucrose solution containing either 4 mg/ml or 5 mg/ml caffeine, allowing drinking for 5 min. To increase motivation to drink I placed the vials for additional 10 min into a chamber kept at 30°C. I then transferred 7 control and 7 treatment flies to the activity monitor and at 1800h I turned the lights off and activity recording began, for the ensuing subjective night (12 hours).

Analysis

To compare tolerant with control flies I used repeated measures ANOVA for the 4 mg/ml caffeine concentration. For caffeine 5 mg/ml I used Mann-Whitney *U* tests since normality assumptions were not met and I could find no suitable transformation to normalize them. I discarded the first hour of recording because all flies, irrespective of

treatment, showed the same high level of activity upon placement in the small tubes in the activity monitor, (for both caffeine 4 and 5 mg/ml, *ps* > 0.05, for the average time rest during the first hour in control and caffeine flies respectively).

Results

Caffeine 5 mg/ml decreased the average time spent resting per hour during both the 1900-2200 hours interval (Mann-Whitney *U* test: U=218.5, N_1=30, N_2=34; P<0.001; Fig.3), and the 1900-0100 interval (Mann-Whitney *U* test: U=307, N_1=30, N_2=34; P=0.006). The mean (± SE) minutes rest per hour was 43.5±2.2 min (72% average time rest per hr) for control, and 28.2±2.1 min (42% average time rest per hr) for treatment flies; caffeine-treated flies showed a 36% reduction in rest. Caffeine 4 mg/ml also decreased the average time spent resting per hour during 1900-0100 hours ($F_{1,24}$=4.7, P=0.04; Fig. 3), although for the interval 1900-2200 hours the difference was only marginally significant (p=0.1).

As figure 4 shows for the 5 mg/ml caffeine concentration, the two treatments showed significantly differences in rest for the first 4 hours (F=25, p<0.001; F=12, p=0.001; F=11, p=0.001; F=7, p=0.01 respectively), and in the caffeine-treated group rest tended to return to normal values with time, most likely due to a decline in caffeine's action. Although no data are available in insects, the half-life of caffeine for doses comparable to ones used here is 0.7-1.2 h in rodents and 2.5-4.5 hours in humans (Nehlig, 1999). Based on these considerations, in the successive experiments I expressed the results as average of the hours in the 1900-2200 h interval.

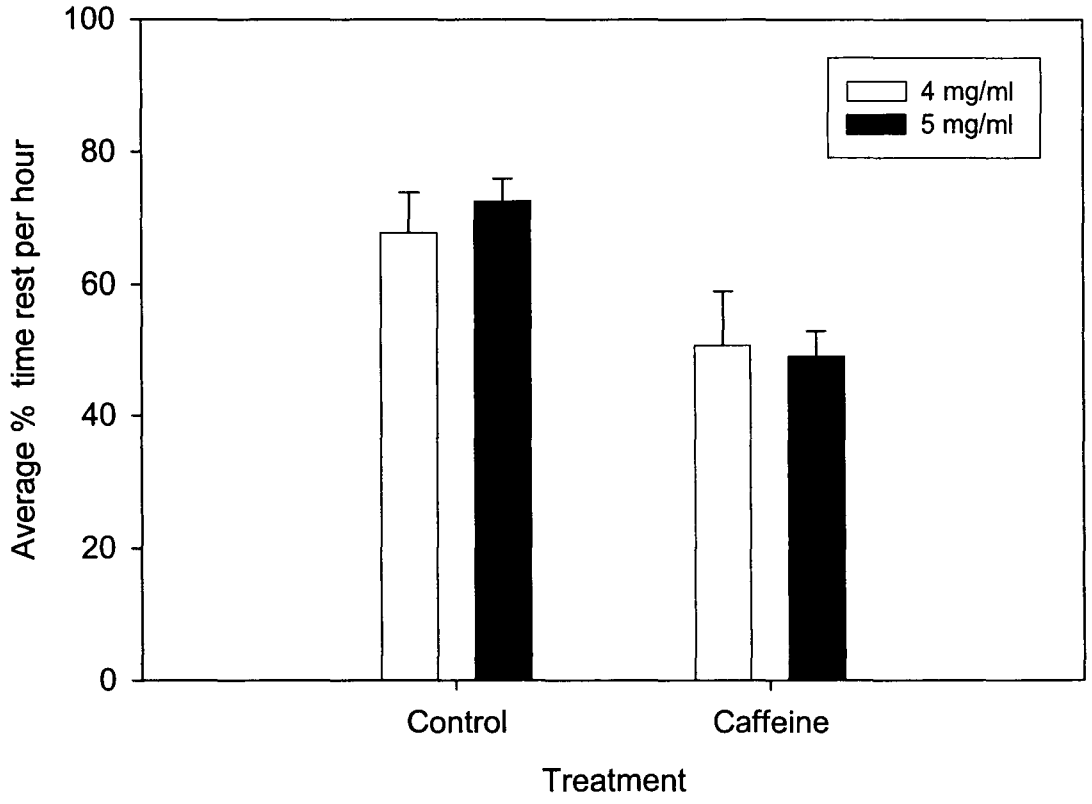

Figure 3. Mean (±SE) percent time rest per hour. Bars represent averages for the period 1900 PM-0100 AM for caffeine 4 mg/ml (ANOVA, $p=0.04$), and 1900 PM-2200 PM for caffeine 5 mg/ml (Mann-Whitney *U* test, $p<0.001$).

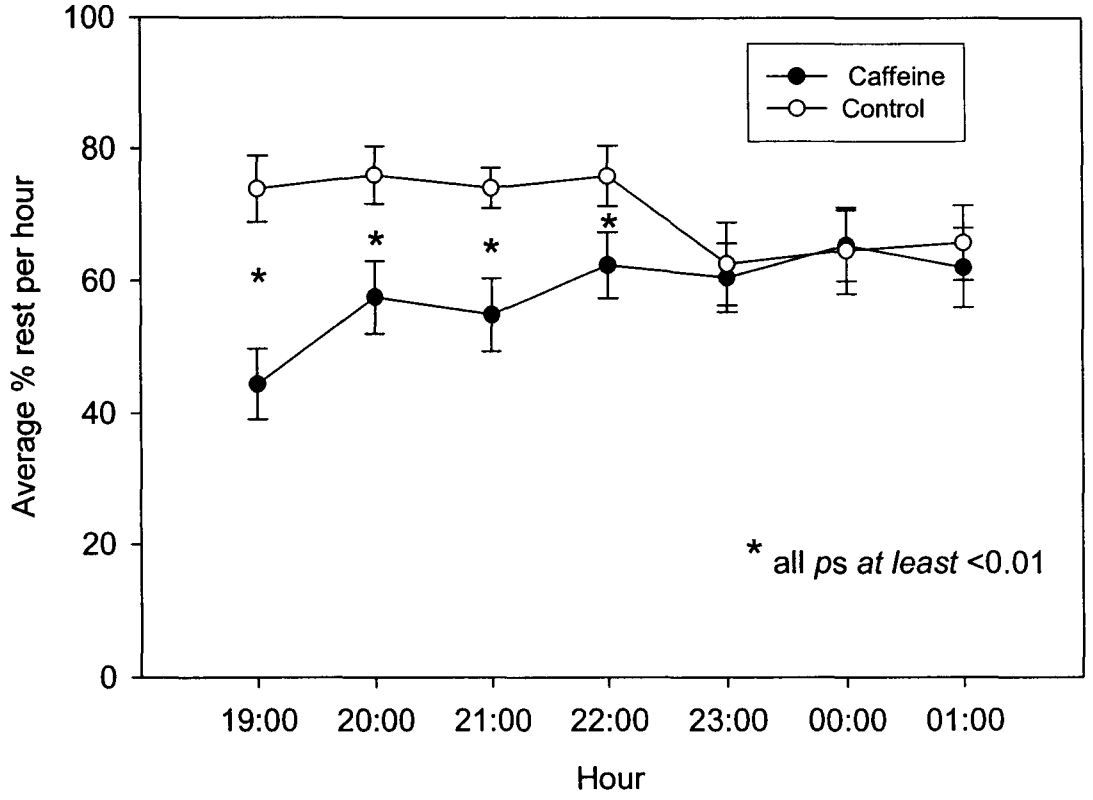

Figure 3. Mean (± SE) % rest per hour, during the period 1900 PM-0100 AM, for flies that did and did not drink caffeine (5 mg/ml).

3. Caffeine tolerance development

3.1 – Experiment 3

Rationale

In experiment 2, caffeine was shown to reduce night time rest in the flies. With the following experiment I tested the prediction that flies would develop tolerance to caffeine rest-disrupting effects over the course of repeated administrations.

Evidence of caffeine tolerance is varied. Studies have been mostly carried out in rodents and humans, but in the two the focus is placed on different caffeine effects.

In humans, caffeine has a variety of pharmacological effects as a psycho- and motor-stimulant, cardiotonic and diuretic (for review see e.g. Benowitz, 1990; and especially Freedholm et al., 1999). Evidence of tolerance is strong for some and mixed for other effects. For example, in the most recent review of pressor effects, Nurminen et al. (1999) concluded that a wide interindividual variability doesn't allow general statements to be made.

The literature on tolerance to cognitive effects also provides mixed results, but this is mostly due to the fact that until very recently many studies were systematically flawed by the use of withdrawn subjects. It is well established that humans develop addiction to caffeine and upon discontinuation suffer from withdrawal symptoms, for up to 7-14 days. Therefore, only studies where either the subjects maintain their normal caffeine consumption or are tested after a washout at least 1 week long provide meaningful data. It has been just relatively recently that this notion was assimilated, and as a result demonstration of tolerance to psychostimulant and mood and cognitive-enhancing effects of caffeine remains unclear.

Of the few studies that investigated tolerance to sleep-disrupting effects of caffeine in humans strong evidence was provided by Zwyghuizen-Doorenbos et al. (1990). Their protocol utilized a mixed model design with day as within subject factor (3 days) and a caffeine and placebo groups as levels of the between subject factor. Subjects in the two treatments received either 500 mg of caffeine in 2 cups of coffee during the day (the equivalent of 4 to 5, 150 ml cups of percolated coffee), or decaffeinated coffee. Among other tests, a multiple latency test (MSLT) was performed 4 times a day to assess the latency to sleep during brief (20 min) naps. It was shown that whereas latency remained low and constant for the placebo group, caffeine group showed a significantly higher and linearly declining latency to sleep over the course of the 3 days, that is, caffeine disrupted sleep, but tolerance to that effect developed over the three days.

In rodents, most of the work has concentrated on the increase in locomotory activity, tolerance to it, and neurological mechanisms underlying this tolerance. Motor stimulation is due to a synergistic blockade of adenosine A1 and A_{2A} receptors (Karcz-Kubicha et al., 2003; Halldner et al, 2004). It is now established that tolerance to motor-activation effects of caffeine occurs in rodents chronically treated with caffeine concentrations similar to the typical concentrations experienced by habitual coffee

drinkers, and the most likely underlying mechanism involves mainly upregulation of A_1 and downregulation of A_{2A} receptors, whereas metabolic changes seem not to play a role (Dassesse et al., 2001; Svenningsson et al., 1999). In sum, there is good evidence that in mammals tolerance develops to the type of effects that are the subject of the present investigation.

In this experiment, flies were tested for nighttime rest on the first and the last day of the tolerance development period, in a within-subject design. On the first night, all the flies received a caffeinated solution; the first night's recording thus provided for each naïve fly a measure of caffeine rest-disrupting effects. I then treated flies in the Daily Caffeine group with caffeine during a tolerance acquisition period of several consecutive days, whereas flies in the Control group underwent a similar manipulation, but received a non-caffeinated solution, containing only sucrose, for the same period. I predicted that flies in the tolerance group, when receiving a caffeinated solution before dark on the last night, at the end of the tolerance development period, would show more sleep in the subsequent night than control flies receiving the same caffeinated solution.

Methods

Tolerance development phase

I used 1.5 days old (<36 hrs) flies. All flies were kept individually inside wells in 12-wells tissue cell plates (Falcon 353043) covered with perforated lids. On the morning of day 1, I starved the flies for 10 hours at 26°C and ~40% RH, from 0730-1730 hours. After starvation, I gave flies in both the Control and Daily Caffeine groups a drop of caffeinated (5 mg/ml) sucrose solution. To increase motivation to drink I placed the flies for additional 10 min into a chamber kept at 30°C. I then transferred them into the activity monitor for nighttime recording. The next morning, and for the successive 7 days, I starved the flies for the same 10 hour period in empty cell plates. Just before night I then gave Control flies sucrose solution and Daily Caffeine flies caffeinated solution, and after 10 min in the 30°C chamber I transferred the flies into another cell plate with fresh standard food for the night.

Last day test phase

On day 8, I repeated the above procedure until starvation, but I then gave both Control and Daily Caffeine group flies the caffeinated solution. I placed the flies into the 30°C and then introduced them into the activity monitor; starting at 1800h, their activity was recorded for the ensuing subjective night (12 hrs).

Analysis

I used a mixed model repeated measures ANOVA to compare Daily Caffeine with Control flies, with Day as within subject factor and Treatment as the between subject factor. I discarded the first hour of recording because all flies, irrespective of treatment, showed the same high level of activity upon placement in the small tubes in the activity monitor. In the first hour, mean (± SE) rest was 18.2 ± 3.4 min and 15.1 ± 3.3 min for control and tolerant flies on day 1, and 21.1 ± 2.4 min and 17.1 ± 2.8 min on day 8, and they didn't differ (both *p*s>0.1).

Results and discussion

Figure 5 shows that flies in both tolerance (n=18) and control (n=14) group rested more at the end of the tolerance development period. The main effect of the within subject factor, Day, was highly significant ($F(1,26)=30$, $p<0.001$), the interaction was non significant.

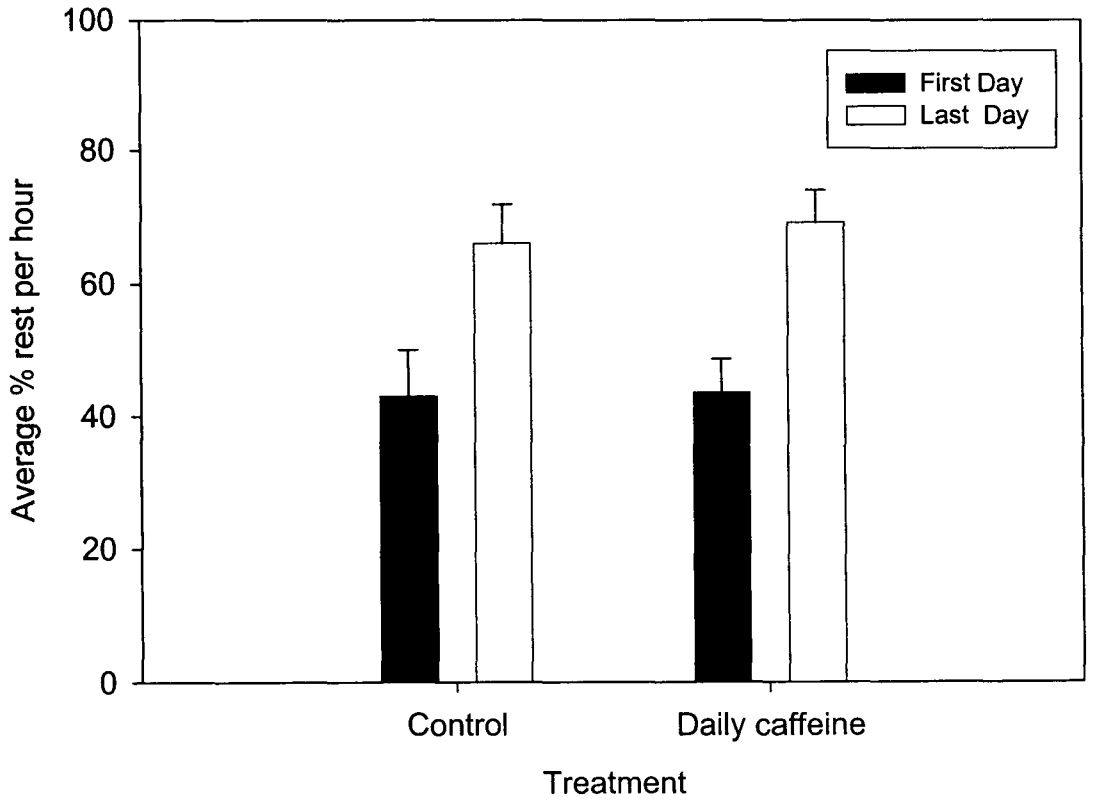

Figure 4. Mean (± SE) % rest per hour, during the period 1900-2100 PM. Control group, n=14; tolerant group, n=18.

These results appear puzzling. One the one hand, flies in the Daily Caffeine group rested more on the last night, which is consistent with the development of tolerance to caffeine. On the other hand, the Control group showed the exact same pattern, even though they didn't undergo a tolerance-inducing period. There are at least two possible explanations for these results. First, it must be considered that the Control flies in fact were given caffeine on the first day, and so potentially may have developed tolerance after just that one administration. As discussed in the introduction, this would constitute an instance of rapid tolerance, which in flies has been repeatedly reported occurring with alcohol (Berger et al., 2004; Dzitoyeva et al., 2003; Scholz et al., 2000). Second, the experiment lasted 8 days and manipulations of the flies may have been stressful. Therefore fatigue or distress may partly account for lack of responsiveness to caffeine in both treatments on the final test day. In order to address these concerns, I conducted two other tolerance experiments, which are discussed below.

3.2 – Experiment 4

Rationale

The main issue I wanted to address was avoiding a possible development of rapid tolerance as is suggested in experiment 3, which requires that Control flies don't receive caffeine on the first night. In such a protocol, one would expect Control flies to show normal sleep during the first night, and reduced rest on the last, when given caffeine; and that Daily Caffeine flies to show reduced rest (due to caffeine) during the first night but normal rest (due to tolerance) during the last night. However, this design introduces a further problem, i.e., that the control group would then experience caffeine for the first time on the test night. Therefore, an alternative explanation for the predicted results would be a novelty effect, that is, that are not the neuromodulatory properties of caffeine per se, but the experience of a novel, unexpected, and bitter substance that causes rest reduction in the control flies on the last night.

To control for novelty effects, in these experiments I therefore introduced other control groups: on the last test night, I divided both Control and Daily Caffeine flies in two subgroups, one of which received the caffeinated solution, the other a solution containing a bitter substance, quinine. In the remainder, I will refer to these groups as control-caffeine group, control-quinine group, caffeine-caffeine and caffeine-quinine group.

I predicted that (1) at the end of the tolerance development phase, when given caffeine before night, flies in the control-caffeine group would display less rest than caffeine-caffeine flies, whereas (2) flies in the control-quinine group would display similar amount of rest as caffeine-quinine flies.

Methods

Quinine concentration was chosen such as to mimic the bitterness of the caffeinated solution. In preliminary egg laying choice experiments, I found that 5 days-old, mated females didn't show a preference between a food 0.1 mg/ml in quinine and a food 5 mg/ml in caffeine (paired t-test, t_{19}= 1.4, p= 0.17); a higher quinine concentration of 0.4 mg/ml was on the contrary more aversive than the same caffeine solution (paired t-test, t_{19}= 2.3, p= 0.03).

Tolerance development phase

I used 1.5 days old (<36 hrs) flies, kept in groups of 15 in standard 40 ml polypropylene vials. On the morning of day 1 of the tolerance phase, I starved flies for 10 hours at 26°C and ~40% RH, from 0730-1730 hours in empty vials. After starvation, I gave control groups sucrose solution and tolerance groups a drop of caffeinated (5 mg/ml) sucrose solution. I then transferred the flies into vials with fresh food for the night. I repeated this procedure for 7 days.

Last day test phase

On day 8, I repeated the above procedure until starvation, but I then gave the control-quinine and caffeine -quinine flies a novel solution, containing 10% sucrose and 0.01% quinine, and the control-caffeine and caffeine -caffeine the caffeinated solution. I placed the flies into the 30°C chamber, then I introduced them into the activity monitor and, starting at 1800h, their activity was recorded for the ensuing subjective night (12 hrs). For each pair of replicates (2 consecutive nights), I used a counterbalanced number of flies for each treatment; that is, on the first night, I used 3 quinine-treated (for both control and caffeine groups) and 4 caffeine-treated flies (for both control and caffeine), whereas on the second night, I used 4 quinine-treated and 3 caffeine-treated flies, for a total of 7 flies in each of the four groups. Unequal sample sizes were due to mortality.

Analysis

I used a one-way ANOVA with a priori Bonferroni-corrected contrasts. As in experiment 3, I discarded the first hour of recording because flies showed the same high activity level irrespective of treatment ($F<1, p>0.1$).

Results and discussion

As shown in Figure 6, flies in the control-caffeine group rested significantly less than caffeine-caffeine flies (ANOVA, linear contrast, t_{27}=2.4, p=0.026), whereas rest by control-quinine flies was not significantly different from either the caffeine-caffeine or control-caffeine group (ANOVA, linear contrasts, p=0.2 and p=0.6 respectively).

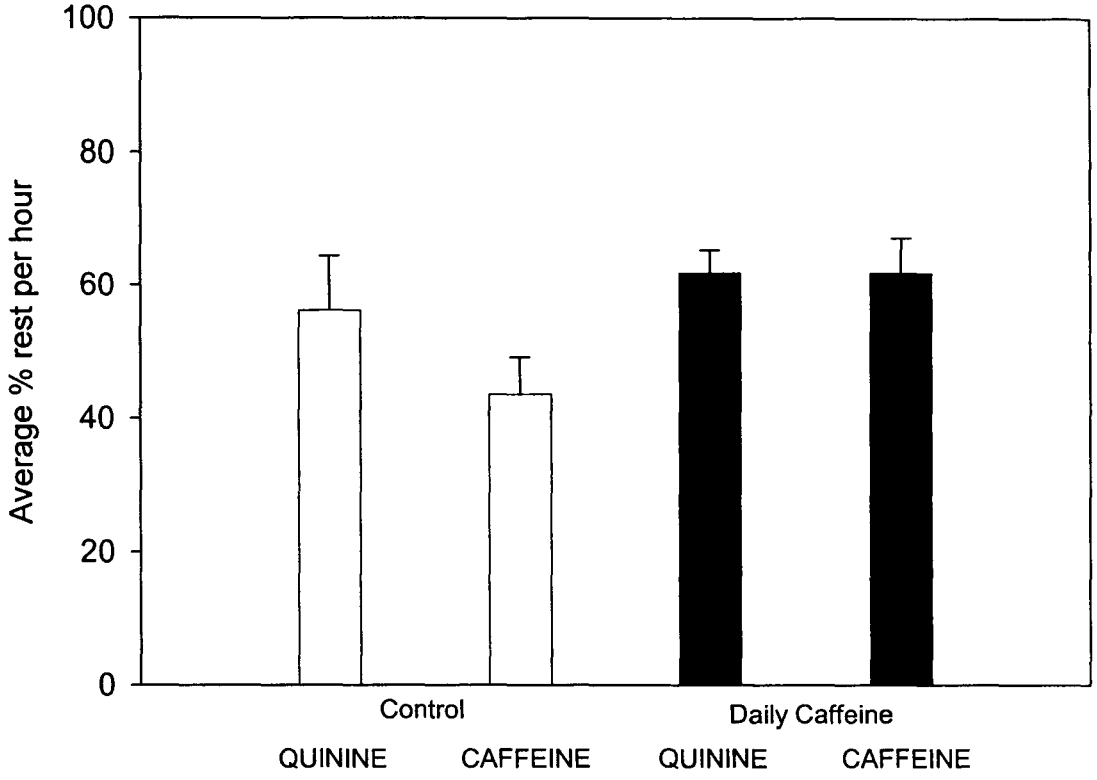

Figure 6. Mean (± SE) % rest per hour, during the period 1900-2100 PM. Control-caffeine flies rested significantly less than caffeine-caffeine flies

Flies in the control groups received caffeine only on the last day, thus cannot have developed rapid tolerance. Although they did show a tendency to rest more, the behaviour of control flies given quinine remains unclear, and therefore these data cannot rule out the possibility of a novelty effect. However, the somewhat big standard error for that particular group, the low sample size and the between subject design are all factors that reduced the power to detect differences between treatments. Moreover, the experiment lasted 8 days, as experiment 3, due to schedule overlapping for the use of the activity monitor. With the following experiment, I thus addressed these issues by using a within-subject protocol and reducing the tolerance phase to 4 days.

3.3 – Experiment 5

Rationale

This experiment's design was similar to that of experiment 3. Flies were tested for nighttime rest on the first and the last day of a tolerance development period, in a within-subject design. There were three, and not four, treatments: a control-caffeine group, a control-quinine group and a daily caffeine-caffeine group, that is, the caffeine-quinine group was eliminated from the design. As in experiment 4, to avoid the possibility of rapid tolerance development, control flies were given sucrose solution on the first night.

I predicted that (1) control-caffeine flies would show more rest during the first than the last night; (2) caffeine-caffeine flies would show an increase in rest between the two nights, whereas (3) control-quinine flies would show no difference in rest between the two nights.

Methods

Tolerance development phase

I used 1.5 days old (<36 hrs) females. Flies were kept individually into wells in 12-wells tissue cell plates (Falcon 353043) covered with a perforated lid. On the morning of day 2, I starved flies inside empty vials for 10 hours at 26°C and ~40% RH, from 0730-1730 hours. After starvation, I gave control flies a drop sucrose solution, and treatment flies a drop of 5 mg/ml caffeinated solution. To increase motivation to drink I placed the vials for additional 10 min into a chamber kept at 30°C. I then transferred the flies into the activity monitor for the first night's activity recording. The next morning, and for the successive 4 days, I transferred the flies into empty tissue cell plates for day-long starvation. I then gave control flies sucrose solution, whereas flies in the tolerance group received caffeinated solution. After 10 min in the 30°C chamber, at 1800h I transferred the flies into another plate with standard food for the night.

Last day test phase

On day 5, I repeated the above procedure until starvation. Then, I further divided the control flies in two groups, as mentioned above. After starvation, I gave the control-quinine a novel solution 0.1 mg/ml in quinine, whereas control-caffeine and caffeine-caffeine flies received the 5 mg/ml caffeinated solution. After 10 min in the 30°C chamber I introduced them into the activity monitor and, starting from 1800h, their activity was recorded for the ensuing subjective night (12 hrs). The number of flies in each treatment was counterbalanced across replicates in order to obtain approximately equal Ns. The experiment had 5 replicates, each conducted on a different day. Final sample size differences among groups were due to mortality, which was negligible (<10%).

Analysis

I used a mixed model repeated measures ANOVA to compare tolerant with control flies, with Day as within subject factor and Treatment as the between subject factor. As above, the first hour of recording was discarded because all flies, irrespective of treatment, showed the same high level of activity upon placement in the small tubes in the activity monitor ($ps>0.05$ on both first and last day)

Results and discussion

The main analysis revealed a not significant main effect of Day ($p=0.3$), and significant Day×Treatment interaction ($F(2,45)=8.9$, $p=0.001$). As shown in Figure 7, flies in the daily caffeine group developed tolerance to the caffeine rest-disrupting effects over the course of 5 days (simple main effect of Day, $F(1,45)=14$, $p=0.001$), and flies in the control-caffeine group showed the predicted reduction in rest (simple main effect of Day, $F(1,45)=4.8$, $p=0.034$). Flies in the control-quinine group showed no difference in rest (simple main effect of Day, $p=0.8$). This pattern of results therefore supports the idea that reduction in rest is caused by caffeine and not by non-specific effects of a novel substance.

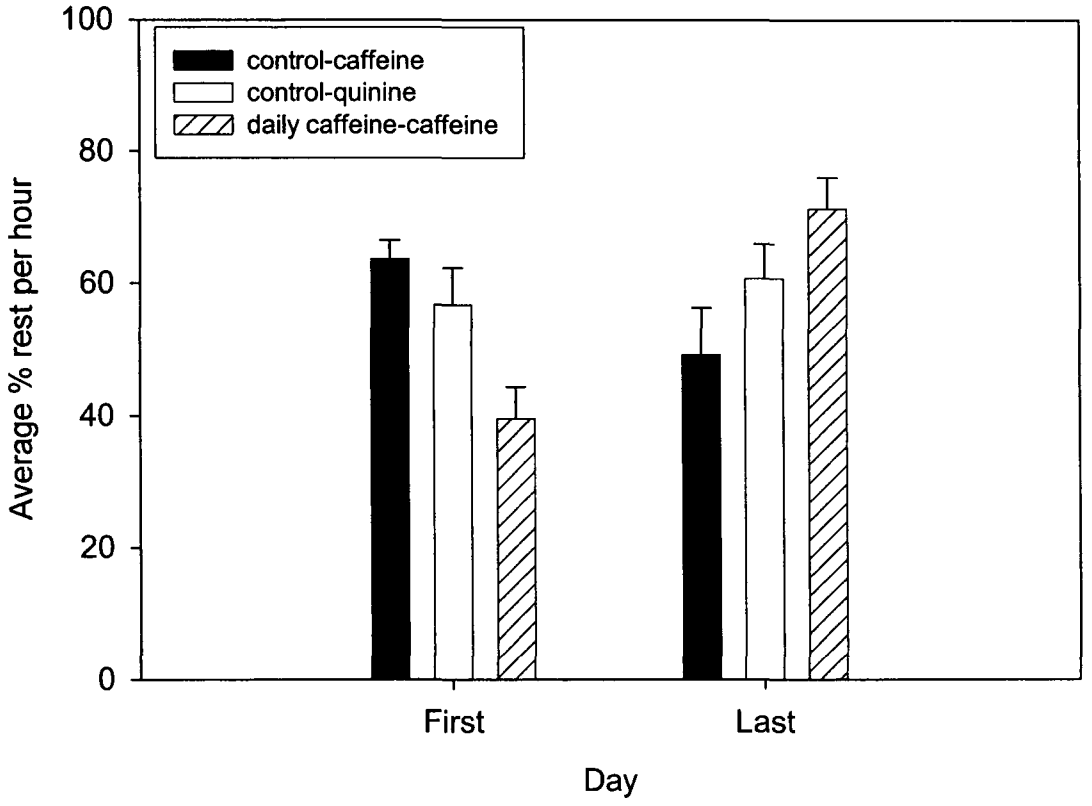

Figure 7. Mean (± SE) % rest per hour during the period 1900-2200 PM. Control-caffeine group showed a significant decrease in rest (ANOVA, p=0.03, n=18); control-quinine showed no variation (ANOVA, p=0.8, n=21); and caffeine-caffeine group showed a significant increase (ANOVA, p=0.001, n=21), over the course of 5 days.

4. Conditioned tolerance to caffeine

Experiment 6

Rationale

As stated in the introduction, in the literature there is some evidence to support the idea of conditioned tolerance to caffeine. First, early on Rozin and colleagues (1984) provided evidence that tolerance showed by regular coffee drinkers to salivation-inducing effects of caffeine was mediated by conditioning. Especially interesting was the fact that decaffeinated coffee caused reduced salivation, which can be seen as indication of compensatory conditioned responses (CCRs) to decaffeinated coffee. Another interesting report of CCRs to an autonomic response to caffeine is the study of Andrews et al. (1998) on startle eyeblink reflex in humans, where they showed that onset latency of the startle was longer in subjects given decaffeinated compared to caffeinated coffee. Recently, Corti et al. (2002) reported on a study on caffeine effects on blood pressure and muscle nerve sympathetic activity. The effects of the same plasma-level concentration of caffeine administered via intravenous bolus and caffeinated coffee was studied in habitual and nonhabitual coffee drinkers. Whereas intravenous caffeine increased systolic pressure in both groups, caffeinated coffee produced the same effect only in nonhabitual coffee drinkers. Siegel and al. (2003) suggested that this effect may be interpreted as an instance of situational specificity of tolerance to caffeine, because coffee, but not intravenous administration (IV), provides the CS associated with caffeine effects, and therefore no compensatory responses were produced to contrast the caffeine IV.

With the following experiment I directly tested whether conditioning is involved in caffeine tolerance. This experiment's design was similar to that of experiments 3 and 5. Flies were tested for nighttime rest on the first and the last day of a tolerance development period, in a within-subject design. I predicted that flies receiving a flavour-caffeine pairing different from the pairing that they experienced during their conditioned tolerance acquisition phase would exhibit a significant loss of tolerance to caffeine.

Methods

Conditioning phase

The conditioning protocol consisted of administration of either a 0.1 mg/ml sucrose, 5 mg/ml caffeine solution (denoted as +) flavoured with either one of two flavours, pineapple (P) or orange (O), or a non-caffeinated, 0.1 mg/ml sucrose solution (denoted as –) flavoured with the other flavour. Flavour was provided by adding enough concentrated fruit juices (No Name brand) such as to obtain a dilution of 1:4 and the correct sucrose concentration. Each night, flies received one of the two solutions; and each solution was given for the same number of nights, with the order of presentation

randomized in 2-days blocks. To control for flavour effects, 2 treatments experienced one caffeine-flavour pairing (P+ and O –), and 2 treatments the reverse pairing (O+ and P –). In preliminary experiments flies showed no preference for either flavour in the concentration used.

I used 1.5 days old (<36 hrs) flies. Flies were kept individually inside wells in 12-wells tissue cell plates (Falcon 353043) covered with perforated lids. On the morning of the first day of the conditioning phase, I put flies to starve at 26°C and ~40% RH, from 0730-1730 hours. After starvation, I administered the scheduled solution, which in all cases was the caffeinated one. After 10 min into the 30°C chamber, I transferred the flies into the activity monitor for the first night's activity recording.

The next morning, and for the successive 5 days I repeated the following procedure: I transferred the flies into empty tissue cell plates for day-long starvation in the morning, administered the scheduled solution before night, and transferred the flies back to a cell plate with fresh food at 1800h for the night.

Test phase

On day 7, I repeated the above procedure identical until starvation. Then, I divided the flies in 2 groups which both received a caffeinated solution, one flavoured with pineapple and one with orange. Therefore, half the flies received the previously conditioned caffeine-flavour pairing (conditioned group) and half received caffeine paired with the flavour that during training was not associated with caffeine (not-conditioned group). After 10 min in the 30°C chamber, I introduced the flies into the activity monitor and, starting at 1800 h, their activity was recorded for the ensuing subjective night (12 hrs). In total, the sample size was 21 and 24 flies for the conditioned and non-conditioned group. Mortality was negligible (~12%).

Analysis

I used a mixed model repeated measures ANOVA to compare tolerant with control flies, with Day as within subject factor and Treatment as the between subject factor. As above, the first hour of recording was discarded because all flies, irrespective of treatment, showed the same high level of activity upon placement in the small tubes in the activity monitor (ps>0.1 on both first and last day).

Results

The main analysis produced a significant main effect of Day ($F(1,37)=10.4$, $p=0.003$), and a significant Day×Treatment interaction ($F(1,37)=7.6$, $p=0.009$).

In Figure 8, *Learned Pairing* treatment refers to those flies that on the last night received the same flavour-caffeine pairing they had experienced during the training; as predicted, they rested more on the last than on the first night (simple main effect of Day, $F(1.37)=19.5$, $p<0.001$); that is, they showed tolerance to caffeine. *Diff. Pairing* treatment refers to the flies that received a different flavour-caffeine pairing than that they were

trained with, and showed no tolerance (simple main effect of Day, $F(1,37)=0.1$, $p=0.7$), which can be interpreted as a loss of tolerance due to the missing proper flavour CS.

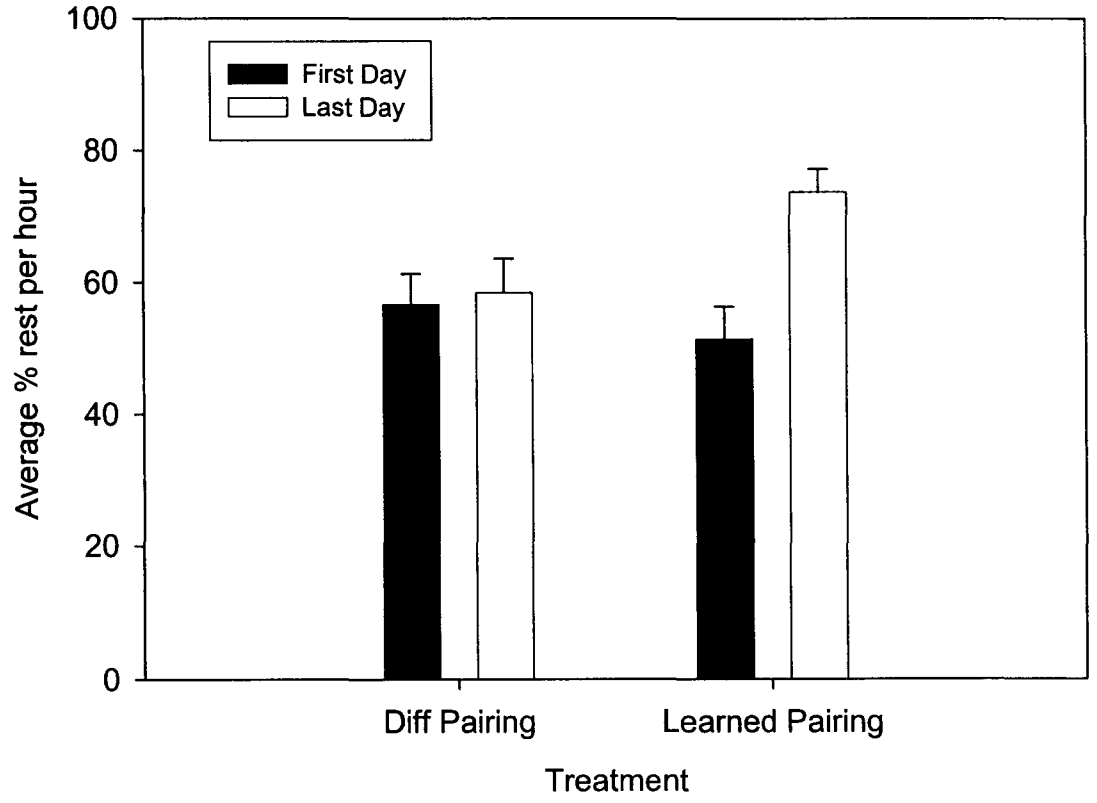

Figure 8. Mean (± SE) % rest per hour during the period 1900-2200 PM. Diff. Pairing group, $n=21$, Learned Pairing group, $n=24$.

General discussion

The results reported here reproduce and extend previous findings. First, I showed that flies display a higher amount of behavioural quiescence during the night than during the day. Following two previous reports (Hendrick et al., 2000; Shaw et al., 2000), I defined this behavioural quiescence *rest*. Hendrick et al. (2000) and Shaw et al. (2000) have investigated the features of nighttime rest in the fruit fly and found suggestive parallels with those of sleep in mammals. One finding was that, as I report here, flies showed a higher amount of behavioural quiescence during the subjective night than the day. In the cited papers, a number of other features of fly's nocturnal quiescence were shown to mimic those of mammalian sleep. First, flies, as mammals, had an increased arousal threshold while resting. For example, vibratory stimuli of greater intensity (6 g (acceleration)) were necessary to elicit a response in flies that were resting than those (0.05g and 0.1 g) necessary in flies that had been behaviourally awake. Second, flies showed the phenomenon of rest rebound, that is, flies deprived of rest for 12 hours during the night exhibited a large increase in rest in the next 12 light hours compared to baseline. This suggests that in flies, as in mammals, nighttime rest is homeostatically regulated (Edgar et al., 1993). A third finding was that two well known substances that exert a neuromodulatory action in mammals produced the same effects in the fly: caffeine was shown to dose-dependently decrease nighttime rest, and hydroxyzine to decrease sleep latency. On the basis of these parallels, I was thus encouraged to use rest as a measure of caffeine effects.

Before proceeding with the illustration of further results, a word of caution is needed in relation to the definition of rest in the fly. The idea of nighttime rest as a sleep-like state in the fly is a novel construct and its definition remains somewhat arbitrary. Shaw et al. (2000) decided to define rest any bout of uninterrupted behavioural quiescence lasting at least 5 minutes, whereas Hendrick and colleagues (2000) considered rest bouts longer than 1 minute. In my analysis I initially adopted this second definition in the evaluation of differences in rest and tolerance. A more careful observation of the data (not shown) however suggested that, differently from what reported by Hendricks et al. (2000, p.129), rest bouts lasting 1 min are quite common and are typically interspersed within normal daytime activity bouts. In other words, at least judging from my data, behavioural quiescence up to 5 minutes appears to be a normal feature of daytime activity. All the data presented here are therefore based on the 5 minutes definition of rest, notwithstanding that it is not less arbitrary. However, at this stage, for the purpose of investigating caffeine effects and tolerance to them, it is not strictly necessary that the behavioural quiescence is indeed a form of sleep. In fact, the overall effect of caffeine in mammals is a general increase in arousal, which in rodents is always measured as locomotory activity. Indeed, tolerance to caffeine has been shown most clearly to these effects in rodents. After all, the *operational* definition of rest used here is in fact decreased locomotory activity, occurring during the night.

As a second step in my investigation, I showed that caffeine decreases nighttime rest, and there is an indication of a dose-dependent action, since caffeine 4 mg/ml

decreased rest for the period 1900-0100 hours, although the difference was not significant for the sole first 3 hours of the night (1900-2200 hours).

The third step in my investigation was to test for development of tolerance to caffeine rest-disrupting effects. In previous work, using alcohol as toxicant, two different form of tolerance were reported in the fly. Scholtz et al. (2000) and Berger et al. (2004) showed rapid tolerance, which developed after a single intoxicating dose of alcohol, and the latter showed chronic tolerance, which developed over the course of 4 to 48 hours. Here I report on development of tolerance to caffeine over the course of 5 days. In experiments 4 and 5, the flies that received caffeine for 5 consecutive nights became tolerant compared to naïve flies that received placebo solution for the same period and a caffeine challenge on the last night. In experiment 3 I used a different protocol, in which flies received caffeine on the first night in order to measure baseline caffeine effects. Then, control and tolerance group were treated as in experiments 4 and 5 and tolerance was assessed on the 8th night. The results were unexpected, because all the flies, in both groups, showed the same increase in rest, thus apparently developing tolerance. Stress related phenomena might account for the lack of responsivity to caffeine due to the length of the experiment. However, based on the data presented here, it is not clear whether in fact manipulation for a period as long as 8 days can be considered an adverse factor. In experiment 3 mortality was 20%, a value somewhat higher than that in the other experiments; however, experiment 6 was almost as long (7 days) and mortality was only 10%.

Another possible explanation is that flies developed rapid tolerance following the exposure to caffeine on the first night. Rapid tolerance is a well-established phenomenon that appears to mirror most of the features of chronic tolerance and responds in the same way as chronic tolerance to most pharmacological manipulations (see Kalant, 1996, for references). In most alcohol experiments, it typically requires two days to be instated, which is also the type of protocol in experiment 3. According to Khanna et al., (1992), there is evidence that rapid tolerance, at least in mammals and in alcohol studies, is functional in nature and does not include a dispositional (metabolic) component. Recent data on rapid tolerance to alcohol in the fly provide a direct support to those finding. Berger et al. (2004) have explored features of rapid and chronic tolerance to alcohol in the fly. First, they showed that neither forms involve changes in ethanol pharmacokinetics, which strongly suggests a functional nature. Second, the rapid but not the chronic form was impaired in mutant TβH flies lacking octopamine, a norepinephrine-analog. As discussed below when considering in more details the connection between tolerance and learning, this point may be particularly interesting. Octopamine in the fly is required in some forms of classical conditioning, and a possibility is that it is thus required for the development of conditioned tolerance; in such case, rapid tolerance development may prove to be a better paradigm than chronic. Finally, I must draw the attention to one of the findings in the two studies reporting rapid tolerance in the fly, which may undermine rapid tolerance as an explanation of the results in experiment 3. Berger et al. (2004) found that chronic tolerance lasted longer than rapid tolerance, the two being completely dissipated after 24 and 48 hours respectively. Scholz et al. (2000) showed that the rapid form lasted at least 24 hours. Clearly, if a parallel can

legitimate be drawn from alcohol to caffeine, then rapid tolerance cannot explain my results since I tested tolerance 7 days after the 1st exposure, a period after which rapid tolerance should have been long dissipated.

Experiment 6 was a first step towards exploring the role of classical conditioning in tolerance to caffeine in the fruit fly. My results supported the hypothesis that tolerance is mediated by learning; in fact, the magnitude of tolerance loss in flies that experienced the non-conditioned caffeine-flavour pairing was surprisingly large. Tolerant flies displayed a 41 % increase in rest, whereas flies in the other group displayed almost no tolerance showing only a 3.2 %.

These preliminary results are suggestive, but don't address the question of which mechanism is involved. The next logical step to follow up the present investigation would then be to first differentiate between metabolic or neuroadaptive changes, which is rather straightforward, because of the general features of metabolic and functional tolerance. As a general pharmacological principle (Fadda and Rossetti, 1998), if tolerance is metabolic in nature, a tolerant individual needs the same circulating concentration of a drug as a non-tolerant individual to display the same effects, but a longer acute exposition to the drug is needed for that concentration to be reached, because absorption is reduced and/or toxicant's clearance is faster. Conversely, the definition of functional tolerance implies that a tolerant individual will display the same degree of impairment as a naïve individual only at significantly higher circulating concentrations of drug. Therefore, as has been done in alcohol studies, it would be easy to determine the content of circulating caffeine in naïve and tolerant flies (Berger et al., 2004) after a challenge with the drug. Lack of difference between the two groups would support the idea that no changes in absorption or metabolism occour. However, caution should be used, since this approach may be complicated by the fact that, at least in mammals, caffeine is metabolized to other active metilxanthines. Theophylline, theobromine and some other paraxanthines act on the same receptors and have the same or even higher activity than caffeine (Svenningssonn et al. 1999), and their pharmacokinetics and distribution are unknown in the fly.

A second point that could be addressed is the nature of the conditioned response that was showed in experiment 6. The modern definition of tolerance incorporates learning processes as a prominent factor (Kalant, 1996). As briefly described in the introduction, according to Siegel's model of conditioned tolerance, tolerance results from compensatory responses (CRs) that become conditioned to the situational-specific stimuli (CS) after repeated drug administations and that are opposite in direction to the drug's effects. One of the implications is that when the conditioned stimuli are presented *in the absence of the drug*, the CRs cause a perturbation of homeostasis, but that is opposite in direction to that unconditionally produced by the drug. In experiment 6, I showed tolerance and the loss of tolerance caused by the elimination of the conditioned stimulus (flavour) that supposedly elicited the CRs. A further direct prediction of the model is that a third group that received the caffeine-paired flavour without caffeine may evidence the sole effect of the CRs, that is, increased rest. It would be easy to introduce such third group in a next experiment. Negative results however would not necessarily indicate lack of compensatory responses, since, for example, they may involve anticipatory

physiological adjustments that however don't result in increase in rest, like decoupling at the receptors' level. Therefore, any further investigation of the compensatory model would need careful thinking in order to take into account such alternative mechanisms.

Further investigation of role of learning in tolerance to caffeine should capitalize on the recent findings in both learning and tolerance studies in the fly. In the fruit fly classical conditioning has been extensively studied in paradigms typically using electric shocks (Roman, 2001) and odours (e.g., Mery and Kaweky, 2002) as conditioned stimuli. The fly has been shown to be able to associate these CS with food, egg laying sites, mates. The cellular basis of associative learning in the fly is currently successfully investigated using mutants strains with various impaired neural functions (e.g., Schwaerzel et al., 2003 and references therein). Schwaerzel and colleagues (2003) showed that two different neurotransmitters are selectively involved in the acquisition of olfactory memory in the fly. Octopamine, a neuromodulator and neurotransmitter analogous to mammalian norepinephrine (Roeder, 1999; 2005) was required for the acquisition of an olfactory memory with sugar as US (a positive reinforcer). Dopamine was involved in acquisition with electric shock US (a negative reinforcer). Lack of either one neurotransmitter selectively impaired the formation of one or the other type of memory. What is extremely interesting is the fact that dopamine and octopamine are involved in tolerance processes where learning is though to play a role. First, as mentioned above, Scoltz et al. (2000) reported evidence that TβH mutants flies, lacking the key octopamine-synthesizing enzyme showed impaired rapid and tolerance. In other words, a neurotransmitter that is necessary for conditioning acquisition also impaired acquisition functional tolerance to alchol, suggesting a possible role of conditioning in the process.

Second, in mammals dopamine is involved in acquisition of tolerance to caffeine. It is firmly established that caffeine causes increase in activitiy by modulating the function of adenosine receptors in the brain, more specifically, synergic blockade of A_1 and A_{2A} receptors is needed. But also, caffeine effects are produced by indirectly modulating the dopaminergic system, with a reciprocal antagonist interaction of A2A and D2 receptors (Fuxe et al., 2003; Cauli and Morelli, 2005). It is also clear that tolerance involves neuroadaptive responses within these neurotransmitting systems (Svenninngsson et al., 1999 for adenosine; Powell et al., 2001 for dopamine). Thus, a neurotransmitter that is involved in tolerance to caffeine, at least in mammals, is also a key element of acquisition of conditioning in the fly.

A typical strategy in tolerance research is to choose a specific neural system or neuromodulator that one suspects is involved in the development of tolerance, selectively alter its function and then verify the results on tolerance acquisition (Kalant, 1996). In light of the promising parallels discussed above, the wealth of knowledge on conditioning in the fly, paired with the readily available neurally impaired mutant strains make seem such approach a very powerful tool in the context of caffeine tolerance research.

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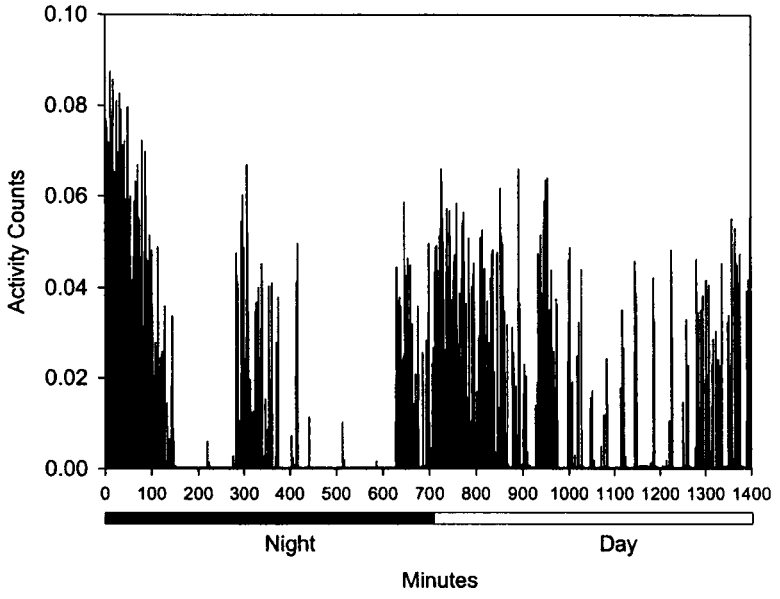
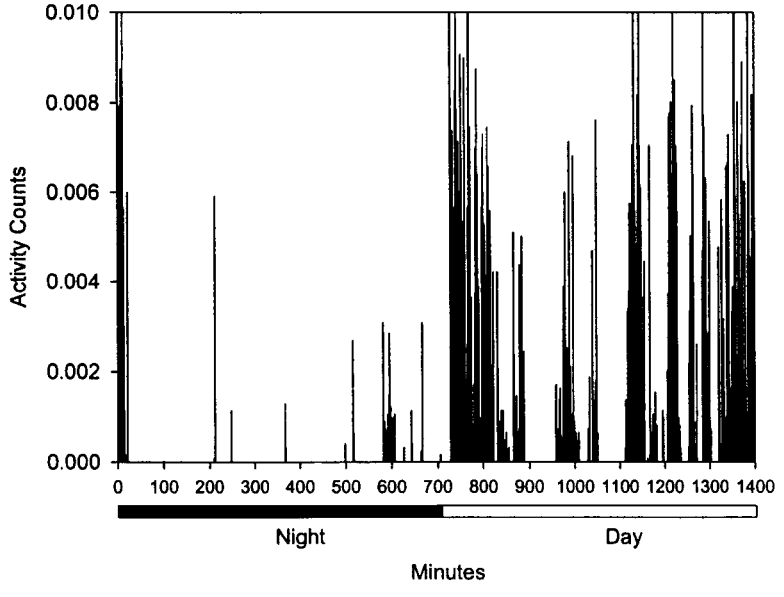
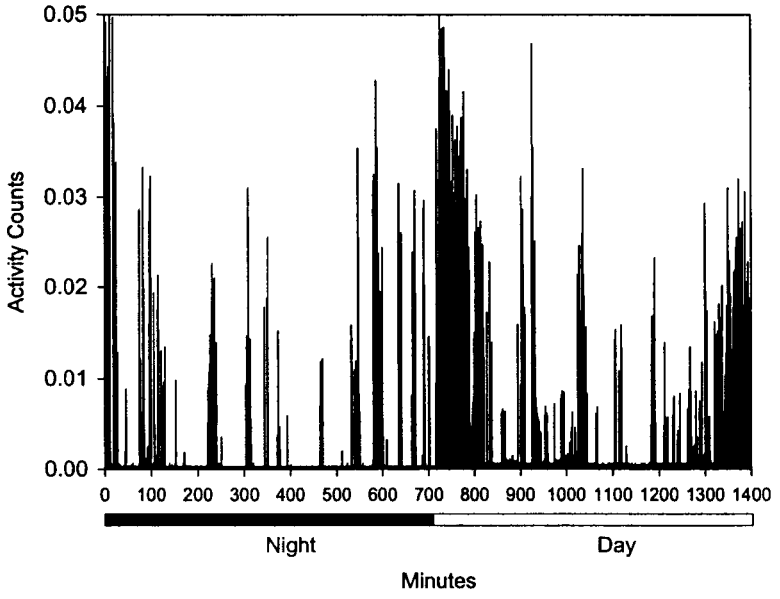
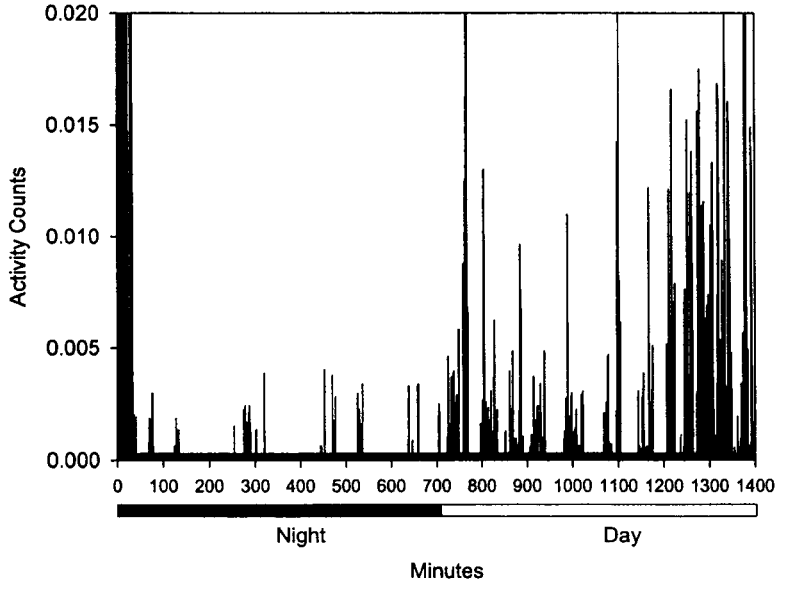
Appendix A.

Activity recordings of 4 different flies during a 12:12 h, dark:light cycle, represented in 1-sec bins. Notwithstanding a clear circadian organization, figures show a certain amount of interindividual variation in activity and rest patterns both during the day and the night.



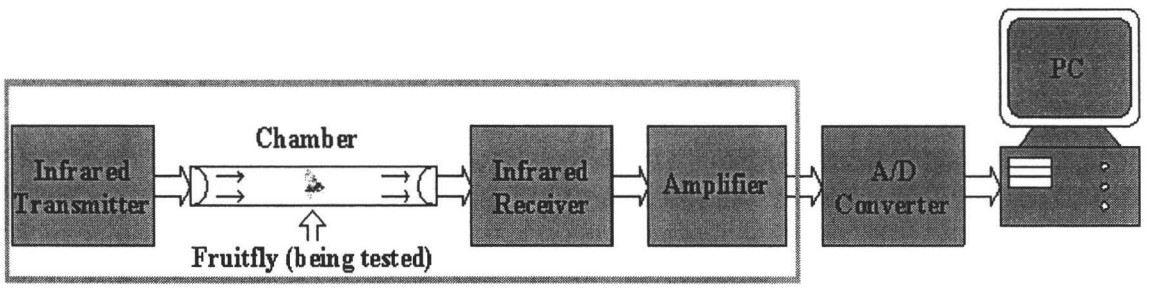



0.05
0.04
0.03
0.02
0.01
0.00
Activity Counts
0 100 200 300 400 500 600 700 800 900 1000 1100 1200 1300 1400
Night
Day
Minutes


0.020
0.015
0.010
0.005
0.000
Activity Counts
0 100 200 300 400 500 600 700 800 900 1000 1100 1200 1300 1400
Night
Day
Minutes

Appendix B. Activity monitor specifics and data considerations.

The activity monitor records activity signals using an infrared (935 nm) transmitter-receiver mounted on small plastic, transparent test tubes (length, 60 mm, ∅ 5 mm). The IR beam crosses lengthwise the tube, encompassing the whole tubes' internal area, and is modulated by movements of the fruitfly. Infrared light is invisible to fruitflies, whose vision extends only up to 650 nm. (Bertholf, 1932). The output signal is an AC voltage waveform. When the animal is at rest, output is equal to zero volts, and movements cause output voltage variations proportional to their amplitude. The device is insensitive to variations in external light, sound, temperature, electromagnetic interference, humidity. During the night recordings however, the tubes are kept in complete darkness, at constant temperature and RH. The tubes are perforated for air circulation and humidification; a small opening in the tube is filled with standard food.



Activity monitor schematic.

Each of the 8 tubes output to an analogue/digital (A/D) converter's separate channel (16-bit accuracy). The output is logged on a PC with the Logger VI software (National Instruments). Signal is sampled at 50 Hz. One tube is always empty and provides a baseline noise signal. The signal from each other tube, where a fly is placed, is then subtracted from the empty tube signal to generate the raw data. I wrote a program that does successive data processing. I currently generate activity values for 1-sec bins (that is, a median absolute deviation (m.a.d.) values of activity per each sec). Median values for each minute are then calculated from the m.a.d. values.

Sleep-like states in insects lack an agreed upon definition, that is, what is the minimum duration of an immobility bout that meaningfully defines an instance of sleep. For example, isolated, 1 min long events of quiescence that occur among bursts of activity are more unlikely to represent sleep than longer, sustained periods of immobility. Nevertheless, following Shaw et al. (2000), I defined 'rest' as a period of uninterrupted behavioural quiescence $\geq$ 5 minutes long. In order to obtain the data analyzed and reported in the results sections above, I calculated frequencies of rest bouts thus categorized: 1 min, 2-4 min, 5-9 min, 10-19 min, 20-29 min, $\geq$30 min long, and unless otherwise specified, I used only rest bouts $\geq$ 5 minutes long.