TENERAL MATINGS AND SITUATIONAL CAFFEINE TOLERANCE IN *DROSOPHILA MELANOGASTER*

AN INVESTIGATION OF TENERAL MATINGS, MALE COERCION, AND FEMALE RESPONSE. A SECOND INVESTIGATION OF CAFFEINE TOLERANCE IN *DROSOPHILA MELANOGASTER.*

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Abstract

Chapter 1-4 focuses on investigating whether forced copulations occur in teneral females, and how the female responds. There has only been one paper to report mating in newly eclosed (teneral) female matings in fruit flies (*Drosophila melanogaster)*, and it was suggested to be forced. The focus of this thesis is to determine whether teneral matings are forced and how this may affect the future remating and reproductive success of females. Within the thesis, chapter 1 and 4 results suggest that teneral matings occur in both Canton-S and wild caught females, and occur in females less than 30 minutes post eclosion. Chapter 3 compared the male/female interaction of teneral females vs. immature females that successfully reject male mating attempts. Males were more aggressive with teneral females, and females displayed more rejection behaviours during courtship and mating. Chapter 4, was aimed at investigating what the reproductive consequences are, and results suggest that a teneral mating yields less progeny than a mature mating, and 68% of tenerally mated females remate at maturity.

Chapter 5 and 6 focuses on investigating whether situational caffeine tolerance can be developed in fruit flies. Chapter 5 results indicate that caffeine causes a rest disruption, and a general tolerance to the rest disrupting effects can be gained over 6 days of repeated administration. The experiments in chapter 6 used various protocols to investigate whether a situational tolerance will develop, using odours and colours as associative cues. No conclusive results were found

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Sexual Conflict

The evolutionary interests of males and females often conflict, making sexual conflict a general feature in the evolution of both sexes (Arnqvist & Rowe, 2005; Clutton-Brock, 1991; Andersson, 1994). In the majority of non-monogamous species, the variance in reproductive success is much higher in males than in females. The general idea is that because of anisogamy (gametes of different size), male and females have different investment in offspring, and have evolved different sex roles and reproductive strategies. Trivers (1972) argued that the sex with more parental investment (often female) will be more selective. The differences in parental investment, cause different physical and behavioural strategies between males and females, which result in sexual conflict and sexually antagonistic coevolution (coevolutionary arms race) between the sexes (Bulmer & Parker, 2002; Parker, 1972).

Females often resist mating and have a bias towards certain male phenotypes, and as a consequence, males have evolved many alternative reproductive strategies to increase mating success. Some strategies include mating plugs (Contreras-Garduno et al., 2006; Duvoisin et al., 1999), adaptations to restrain resistant females during mating (Arnqvist & Rowe, 1997; Sakaluk et al., 1995), mate guarding behaviour (Rondeau & Sainte-Marie, 2001; Zeiss et al., 1999), infanticide (Hrdy, 1979), and transfer of anti-aphrodisiacs (Andersson et al., 2000), seminal toxins (Harshman & Prout, 1994) and infertile sperm that delay female remating (Cook & Wedell, 1999) in male ejaculate. Female counter adaptations to these strategies include removal of mating plugs, increased female aggression, and breakdown or decreased sensitivity to ejaculatory compounds. Female resistance can cause increased fitness for the males with coercive phenotypes, and a coevolutionary "arms race" in antagonistic traits in females (Arnqvist & Rowe, 2005).

Forced mating

There are some species where females show active precopulatory resistance and males have strategies or traits to overcome resistance (Eberhard, 1996). Rice (1996) noted that it is important to differentiate selective resistance, an attempt by females to select the healthiest and dominant males as mates, from forced copulation, which is when females are unsuccessful at resisting an unwanted male mating attempt. In species with selective resistance, every mating is resisted, aggression usually only occurs prior to mating and higher quality males are the males who achieve mating (Allen & Simmons, 1996; Lalumiere et al., 2005). There is yet to be a clear definition of forced mating, but it is usually differentiated from selective resistance in numerous ways. Forced mating often only occurs in certain situations (i.e. courtship attempts are rejected, males are competitively disadvantaged, unequal operational sex ratio). These matings involve increased male aggression and female resistance compared to a typical mating in that species (Lalumiere et al., 2005). Mating may occur in an atypical position, and female struggling may occur throughout the mating (Lalumiere et al., 2005). The following examples have been generally accepted as cases of forced copulation and are not considered a case of selective female resistance.

Forced mating in vertebrates

In orangutans, smaller males choose a forced mating strategy (Mitani, 1985). There are two male sizes, large and small. The large males have more sexually dimorphic traits, and are pursued by females. The smaller males do not often get to mate with females, and if they do, a larger male usually interrupts it and mates with the female himself. Males and females display different mating behaviours when small males are mating compared to large males. Small males have more aggressive behaviours than large males, including grabbing and biting, and females

show more precopultory resistance with small males, which continues throughout the mating (Mitani, 1985). The Lake Eyre dragon (*Ctenophorus maculosus*), a small Australian lizard, has aggressive courtship, which includes grabbing the female, but only when a male is courting an unreceptive female (Olsson, 1995). Aggressive courtship attempts are typically made by males that have not formed pair bonds.

Forced mating has also been reported in many species of waterfowl (Cunningham, 2003; McKinney et al., 1983). Mallards dive after females and aggressively grab feathers and attempt to mount. Females try to escape but sometimes males (often as groups) successfully mount and mate with females which often causes an injury. Forced mating can be distinguished because of lack of typical courting attempts, increased female resistance, and lack of typical mating posture of females during mating. Ducks also have a male biased operational sex ratio, and there is usually a shortage of females to mate with (McKinney et al., 1983).

Forced mating in invertebrates

A very direct mating method, is found when males compete for access to female pupae, and mate with them before pupae emergence. The mosquito (*Opifex fuscus*) (Slooten & Lambert, 1983) and butterfly (*Heliconius hewitsoni*) (Dienert et al., 1994) mate with females when they are in the pupal stage, giving females no opportunity for female choice.

In scorpionflies (*Panorpa japonica*) a nuptial gift is given to females for mating acceptance. In situations where males are unable to obtain food for a nuptial gift, they rely on their notal organ, a clamp like structure on the abdomen, which grasps the forewings of resisting females prior to and during mating (Thornhill 1980; Thornhill & Sauer, 1991; Thornhill & Palmer, 2000). When the notal organ is covered in wax and males do not have a nuptial gift, mating attempts will fail, where if a nuptial gift or salivary mass is given, matings are still

achieved without the use of their notal organ. When given a choice, males will choose the more consensual mating, as forced matings are 50% less successful at producing progeny. Males who typically choose the forced mating strategy are those who do not have access to a nuptial gift, are smaller and are less symmetrical (Thornhill 1987; Thornhill, 1992).

A less studied example of situational forced mating strategies involves grasshoppers (*Melanoplus sanguinipes*). The male provides females with a spermatophore, and females typically choose the best foragers as mates as they provide the largest spermatophore. The better foragers show typical courtship behaviours, orienting towards the female and vibrating the femora. The weaker foragers had a different strategy, called "stalking", where they follow close behind the female slowly and then leap on her back and begin mating (Belovsky et al., 1996).

Markow (2000) reported field observations of what appeared to be forced copulation in newly eclosed fruit flies (*Drosophila melanogaster*). A laboratory test found that mature males will mate with very young, newly eclosed females (teneral). Markow implied it was forced, however, there is yet to be data investigating this phenomenon. It seems likely that fruit flies are a species in which forced copulation occurs (see below), and the purpose of my thesis is to further investigate this phenomenon.

Sexual Conflict in *Drosophila melanogaster*

The fruit fly has been a very popular model to study sexual conflict. Bateman (1948) discovered that male reproductive success is highly variable compared to females. Chromosomally marked strains of male and female flies were measured for reproductive success. All males attempted to mate, however, some males had no reproductive success and others had a much higher success than the most successful female. All females were courted, and 96% accepted a male and only mated once. So, some males mated with numerous females and others

failed to mate even once. Male reproductive success is highly influenced by their ability to attract a mate and is biased toward certain male phenotypes.

A typical male-female interaction in fruit flies involves a courtship display which females either accepts or rejects (Hall, 1994). Females benefit from being choosy when accepting a male, as egg production is energetically costly (Lamb, 1964; Partridge et al., 1987). Mating itself can be costly, as males have evolved phenotypes to help increase offspring production, which have negative consequences on the female. The accessory gland secretions in the seminal fluid are designed to increase the females rate of egg-laying (Rice, 1996), reduce receptivity towards other males (Rice, 1996), and kill sperm from any previous matings (Harshman & Prout, 1994). Male toxins increase male reproductive success, but are detrimental to female health and associated with reduced female longevity (Chapman et al., 1995). It is, therefore, in the female's best interest to mate with a high quality male, which should outweigh the costs the mating incurs.

Females do exercise mate choice, and show preference for larger males or males who have high nutrition and environmental enrichment during post eclosion adult development (Dukas & Mooers, 2003; Dukas, 2005; Fricke et al., 2008; Partridge & Farquhar, 1983; Pitnick 1991). Larger males have a higher mating success, shorter mating duration, and are more likely to win a fight against a smaller male (Partridge & Farquhar, 1983). Markow et al. (1978) showed that females favour virgin males over genetically similar males with a reduced fertility caused by previous mating. If a female has a suboptimal mating, they will remate, and the last male's sperm takes precedence over the first males (Prout & Bundgaard, 1977). Remating can depend on the amount and quality of ejaculate from the first mating (Gromko & Markow, 1993; Van-Vianen & Bijlsma, 1993). Females who mate with already mated males or males of lower quality of semen

are quicker to remate, and choose higher quality males to remate with (Van-Vianen & Bijlsma, 1993).

Females are generally slow to remate; sperm stores are usually depleted by the 4th or 5th day (Van-Vianen & Bijlsma, 1993) but may last up to 11 days. There is an unequal operational sex ratio, and more males are ready to mate than females at a given time (Hutter et al., 2007). Multiple matings by females and a biased operational sex ratio exposes males to sperm competition and results in male adaptations (seminal compounds) that reduce sperm competition or increase the competitiveness of sperm. These adaptations have been found to have negative physical consequences on females. Rice (1996) reported that females evolve resistance to these male tactics, and demonstrated a nice example of antagonistic coevolution in the fruit fly. In the study, males were allowed to evolve, and not females. Males evolved seminal compounds that stimulated female egg laying, reduced receptivity to other males, and killed rival sperm from a previous mating. When males were tested with females that were not allowed to evolve, the males were better at mating with unreceptive females and the female death rate was higher compared to the evolved control females. The females that evolved alongside the males, were better at rejecting unwanted mating attempts, which suggests they evolved behavioural adaptations to resist males.

It is suggested that females have post copulatory sexual selection techniques and bias sperm use and oviposition rates to favour certain males (Devries, 1964; Eberhard, 1996; Zimmering & Fowler, 1968). Females typically use sperm from their own strain more efficiently than sperm from another strain, and it is unrelated to the amount of sperm stored (Devries, 1964; Zimmering and Fowler, 1968; Zimmering et al., 1970). The amount of sperm in the females main storage organ is dependent on the strain of the female (Devries, 1964). Female fruit flies

have different storage organs, the main storage organ (central spermathecae) and the lateral spermathecae. Sperm stored in the main storage organ is used more, and it is suggested that the lateral organs are used as a 'back-up' (Fowler, 1973).

In addition to the physiological adaptations described above, female fruit flies also have behavioural adaptations for resisting male mating strategies. Females typically are able to physically reject male mating attempts. Typical rejection behaviours include increased locomotion, wing flicking, decamping, kicking, elevation of abdomen tip and ovipositor extrusion (Kim & Ehrman, 1998; Lasbleiz et al., 2006; Markow & Hanson 1981). The majority of the literature reports that females do not mate until sexually mature, which varies, but is usually around 2 days old (Manning, 1967).

Markow (2000) reported mating in newly eclosed females, and reported it as forced implying that females are unable to reject male mating attempts. The state following eclosion is referred to as teneral, which is described by Markow as a two hour period posteclosion, when the wings are folded, the body is soft and movement is slow. It has been described in the whitefly (*Aleyrodes brassicae)* as the period of time before flight begins (Iheagwam, 2004).

As far as I know, no further data has been published to investigate this phenomenon and it is not reported in the lab. It is still unclear whether teneral matings are forced, and if so,what the effects are on female choice. To answer these questions, I conducted a series of experiments and behavioural observations, asking (1) do teneral matings occur, (2) is there a time window in the teneral stage that matings are more likely to occur, (3) what is the male-female interaction, and how does it differ from an interaction between males and immature females that successfully reject male mating attempts, (4) do female rejection attempts occur throughout mating, (5) do tenerally mated females exercise mate choice and remate when they reach sexual maturity, (5)

what is the rate of progeny production of tenerally mated females that do not remate, compared to mature mating, and lastly (6) do teneral matings occur in a wild caught population.

General Methods.

I used Canton-Special (C-S) flies kept in two population cages containing a few thousand individuals inside an environmental chamber at 25°C with 60% RH. Flies were kept on a 12:12 light: dark cycle with lights on at 1000h. Each cage contained two 200-ml standard fly food bottles. The flies used in the experiments developed from bottles containing approximately 300 larvae. Flies were collected within 8 hours from eclosion, anaesthetized with CO_2, sexed and placed in groups of 20 in single sex vials with food containing corn meal, yeast, glucose, sucrose, agar and methyl paraben. All males were four days old virgins when introduced to females, and isolated 20 hours prior to data collection to increase courtship behaviour. All flies were taken out of the incubator at least one hour prior to data collection, to ensure they were adjusted to daylight. Flies were housed in the same environmental conditions as the population cages. All data collection and behavioural coding was conducted by observers which were blind to the nature of the experiment. Mating durations and latencies were recorded, mating duration was defined as any period with which mounting exceeded 2 minutes and mating latency was defined as the length of time for a mounting to begin, after females were placed in a vial with males.

1) Teneral Matings

1.1.) Experiment 1: Proportion of teneral matings

Many studies assume that female *Drosophila melanogaster* have choice over which males they mate with, and that this choice occurs when they are sexually receptive (2 days old) (Manning, 1967). Field research by Markow (2000) reported observations of mature males mating with teneral females. Markow (2000) also conducted a laboratory test put recently

eclosed *D. melanogaster* females in a vial with either two conspecifics or two *D. simulans* males and observed copulation durations for one hour. Matings were reported, and in over half the cases, mating occurred in the absence of courtship. If courtship occurred it lasted under 10 seconds. Surprisingly, no further work on this topic has been done. The purpose of this study is to test whether teneral females will mate with sexually mature males.

Methods

Observations were carried out for one hour beginning at 0800. There were two treatments, teneral females, which were collected immediately upon eclosion, and 4 day old virgin females. Females were placed in a vial with two males, mating latencies and durations were recorded. 20-30 observations were made per age group, for 3 consecutive days with new subjects each day. In total 70 observations were made per group. Mating latencies for the control group were recorded for the last 2 days of the experiment, latency and duration could not be calculated for one mature fly because latency was not properly recorded. I then examined the proportion of matings in teneral and mature females using a logistical regression and conducted ANOVAs to compare mating latency and duration between female age groups.

Results

About 18.6% (13/70) of the teneral females and a significantly higher proportion of mature females mated (Wald test: $\chi 2=41.1$, $P=0.007$; Fig. 1a). The teneral females had longer mating latencies ($F_{1,49}=175.4$, $P<0.01$; Fig. 2a) and shorter mating durations ($F_{1,49}=12.0$, $P=0.01$; Fig. 3a) than the mature females.

Discussion

Experiment 1 suggested that teneral matings do occur. Of females that were collected in a teneral state, 20% mated with mature males (Fig 1a). Compared to mature females the mating

latency was longer (Fig 2a) and had a shorter duration (Fig 3a). These results are similar to what was reported by Markow (2000), however, I found a much longer mating latency.

1.2.) Experiment 2: Teneral mating window

The purpose of experiment 2 was to determine the window of time that teneral females are most likely to mate. Markow (2000) implied a 2 hour time window of teneral matings, but our results suggest a shorter window. It seemed to be that females which were collected immediately following eclosion were mating, compared to females near the end of the teneral stage. So, the purpose of experiment 2 was to investigate whether likelihood could be predicted by the time since eclosion.

Methods

The experiment had 3 treatments, females which were 0, 30 and 60 minutes old. To eliminate time of day and time of eclosion as potential confounds we had two conditions. Condition 1 controlled for time of day (time tested), flies were observed for one hour beginning at 0800 and 0900. To ensure that females were at the proper age, newly eclosed flies were collected at 0700, 0730 and 0800. At 0800, 10 females that were 0, 30 and 60 minutes old were put singly into vials each containing two males, and observed for one hour. The same procedure was repeated at 0900, with females collected at 0800, 0830, and 0900. This procedure was carried out for 3 consecutive days. A total of 60 flies per condition were observed, half beginning at 0800 and half beginning at 0900.

The second condition controlled for the time of collection (time collected), newly eclosed flies were collected at 0800 and tested for one hour beginning at 0800, 0830, or 0900. 10 observations were made per group, for 3 consecutive days. The second eclosion time was 0900, and observations began at 0900, 0930 and 1000. 10 observations per group, for 3 consecutive

days. A total of 60 flies per group were observed, half which were collected at 0800, and half which were collected at 0900.

In total 120 flies per group were observed and mating latency and durations were recorded. Two logistic regression analyses were run using time of day (0800 and 0900) and time of eclosion (0800 and 0900) as predictor variables, with mating as dependent variable. We predicted that females collected and tested immediately upon eclosion would be most likely to mate, followed by 30 minutes old, and lastly, females over 60 minutes old would not mate. I conducted an ANOVA to compare mating latency at 0800 and 0900 in time of day, and again in time collected group. I also compared mating durations in time of day and time collected group using an ANOVA.

Results

Females in the 0 minute old treatment mated more frequently than both females in the 30 and 60 minute old treatment. This was found in both time tested (Wald test: $\chi 2=11.9$, $P=0.003$; Fig. 4) and time collected (Wald test: $\chi 2=7.5$, $P=0.02$; Fig. 4) conditions. The average mating latency of the 0 min old teneral female was 29.8 (± 2.3 mins), and duration was 11.9 (± 0.6 mins), which are consistent with experiment 1 results. The two matings in the 30 minute group had latencies of 12 and 17 minutes and durations 12 and 17 minutes.

In the time tested group, matings occurred faster for flies at 0900 (28.3 ± 3.5 mins) than they did for flies at 0800 (41.1 ± 4.9 mins), $F_{1,21}= 4.6$, $P= 0.045$. No time of day differences were found in the time collected condition ($F_{1,14}=0.2$, $P=0.9$). The mating durations were similar in the time tested and time collected group (11.9± 0.8 mins and 12.1±1.1 respectively; $F_{1,33}= 0.03$, $P=0.87$).

Discussion

Experiment 2 results suggest that the teneral mating window is shorter than what was suggested by Markow (2000). The majority of matings (95%) occurred in females who were tested within minutes of eclosion (Fig 4). There were only a couple matings in the 30 minute old condition, and no matings in the 60 minute old females.

2.) Behavioural observations: Male coercion and female response

2.1.) Experiment 3A: Male aggression during courtship and female response

Next, I decided to closely observe postcopulatory male aggression and female rejection behaviours, comparing the interaction between mature males with teneral females vs. immature females, which are a few hours old and typically reject male mating attempts. I measured male aggression , including proportion of time spent courting, and number of touches, grabs, and mounting attempts, and female rejection, including activity and kicking. Decamping and wingflicking are also common female rejection behaviours (Lasbleiz et al., 2006), however, it is unlikely teneral females use these behaviours as their wings are still folded, and the vial we used is too small for decamping. I predicted that males would have increased aggression towards teneral females, and it was unknown whether females would show active rejection behaviours in response.

Method

All interactions were video recorded from 0800-1000. A single male and female were aspirated into a vial (10mm diameter and 5mm height) and placed under a microscope with 10 x magnification, which was imaged and recorded from a laptop screen using a videorecorder. Teneral females were collected immediately upon eclosion, and immature females were between 2-10 hours old. Video recordings were 15 minutes long, and alternated between teneral and

immature females, to make a total of 10 recordings per group. All vials were washed with ethanol and water between uses.

Behavioural coding

Behaviours were analyzed with Observer 5.0 computer software (Noldus Information Technology, Netherlands). Male behaviour was coded into two states, courtship and noncourtship, which were mutually exclusive. Courtship included any chasing, wing vibration, and orientation while vibrating wings. Any other behaviours were coded as 'non courtship' (i.e. preening, immobility, wing vibration without orientation, and orientation without wing vibration). Male behavioural events included touching, grabbing and mounting attempts. Touching included any front leg contact the male made with the female body, which did not include contact with wings, as only immature females had wings. Grabbing was strictly defined as when a male used his front legs to hold the body of the female, with enough strength to move her. A mounting attempt was coded as any mount which did not end in mating.

The amount of time females spent active, kicking and immobile was coded as behavioural states, and were mutually exclusive. There were a few instances where the females kicked and moved at the same time, and kicking took priority. For male and female behaviours the proportion of time spent in each state was calculated as well as number of events per minute. A rate of movement value was calculated by counting the number of times a female crossed the midline of the vial in the first two minutes of each trial. Group differences were compared using a nonparametric Mann Whitney U test, for each dependent variable.

Results

Teneral females experienced more grabbing ($U = 0$, $P< 0.001$; Fig. 5a) and mounting attempts from males (U=17.5, P=.008; Fig. 5b) than the immature females. Teneral and

immature females were courted for an identical proportion of time (0.96 (± .01) and 0.96 (±.01); U = 43, $P = 0.6$) and were touched by males a similar number of times (38.8 (± 9.5) and 44.4 (± 18.9); U=46.5, $P = 0.8$).

Teneral females spent a larger proportion of time kicking (U=17.5, P=0.013; Fig. 5c) than the immature females. Both groups of females spent most of their time active (0.96 (±.01) and 0.92 (±.03); U=43, P=0.6), however, the teneral females had a slower rate of activity than the immature females. Teneral females crossed the midline 14.8 ± 1.2 times per minute, and immature females crossed it 24.4 ± 1.8 times per minute (U=8, P=0.001).

Discussion

Results from experiment 3A found teneral females had a slower rate of activity, spent more time kicking (Fig 5c) and were grabbed (Fig 5a) and mounted (Fig 5b) more compared to immature females. Both groups were equally courted and active, which suggests that despite of increased kicking, teneral females were less successful at rejecting male mating attempts.

2.2 Experiment 3B: Female rejection behaviours during copulation

The purpose of experiment 3b was to observe if female rejection behaviours occur during a teneral mating. As described above, forced copulation is differentiated from selective resistance because female rejection attempts continue throughout mating, and only occur in particular situations (Lalumiere et al., 2005). It was expected that teneral females would exhibit more rejection behaviours during mating compared to mature females, providing evidence to support forced copulation.

Method

The same recording set up and procedure was used that was previously described in experiment 3a. Instead of immature females we used 4 day old virgin females, and recorded mating activity rather than courtship. We recorded 10 teneral and 10 mature matings.

Behavioural coding

The same female behavioural states from experiment 3a were coded during mating. Kicking, moving and immobility were mutually exclusive events and the proportion of time in each event was calculated. I examined the effect of female age on each behavioural state using a nonparametric Mann Whitney U test.

Results

While mating, the teneral females spent more time kicking (U=11, P=.003; Fig. 6a), and more time active (U=12, P=.004; Fig. 6b) than the mature females did. Mating latencies were shorter for teneral females (582.1 ± 55.2 secs) than they were for mature females (917.7 ± 56.5 secs) ($F_{1,18} = 18.1$, $P < 0.001$).

Discussion

Results from experiment 3b found more kicking and activity during mating in teneral females compared to mature females (Fig 6a,b). The mating duration was shorter for teneral females as well. These results, provide strong evidence to support the hypothesis that matings are forced by males.

3) Fitness effects of teneral matings on females

3.1) Experiment 4A: Effects of forced copulation on remating

Experiments 1-3 indicated that newly eclosed teneral females do not appear to have control on their mate choice. Such choice of males is crucial for the operation of sexual selection.

What appears to be forced copulation of teneral females would interfere with female choice unless tenerally mated females remate soon after reaching reproductive maturity and use the latter sperm for their eggs. I conducted another experiment in which I investigated whether females that mated while teneral respond by remating at sexual maturity.

Methods

The experiment consisted of three treatments, an experimental group and two controls. The experimental treatment consisted of females that mated when teneral and were tested for remating when 3 days old. The second treatment was a control for duration between mating (duration control group), females mated when 3 days old and tested at 6 days old. The third treatment was a control for age (age control group), females mated at 2 days old and were tested at 3 days old. Beginning at 0800 on day 1, I collected newly eclosed teneral females and placed one in a vial with 2 males. While setting up teneral females, I alternated, and aspirated 3 day old females into a vial with 2 males, which was the "duration control group". On day 3, the age control group was treated the same, using 2 day old females. I tried to record 20 matings per group per day. For the two controls treatments, most females mated, and we used about 20-22 vials. For the teneral females we set up 60-120 vials per day, to get approximately 20 matings. All females that mated were aspirated into a single food vial containing live yeast and kept until the test day. A randomly selected subset of the females was aspirated onto new food vials daily, which will be discussed in Experiment 4b. On the test day (day 4), all females that had previously mated were tested for remating by aspirating them into a vial with 2 males. The test began at 0800, and flies from each treatment were set up in an alternating fashion. This procedure was repeated 8 times, to get a total of approximately 160 females to test for remating

per group. In total, 9 teneral and 12 mature females were injured during handling and could not be used during the remating test. Observation periods were two hours.

I used 3 logistical regressions to compare group differences in the proportion of initial matings, rematings, and deaths prior to remating test. ANOVAs were used to compare the latency and duration of rematings of females in each group.

Results

Initial Mating

On day one, an average of 31% of teneral females mated and almost all mature females mated (Wald test: $\chi2=162.5$, $P<0.001$; Fig 1b). Before the remating test, a nonsignificantly higher proportion of teneral females (n=41) died, than did the duration and age control groups (n = 10) (Wald test: $\chi2= 5.8$, $P=0.056$). The teneral females had longer mating latencies ($F_{1,422}=403.6$, $P<0.01$; Tukey post hoc $P<0.001$; Fig. 2b) and shorter mating durations ($F_{1, 433}=85.2$, $P<0.001$; Tukey post hoc $P<0.001$; Fig 3b) than both mature groups. In total, I tested 141 females from treatment 1, 148 females from treatment 2, and 147 females from treatment 3 for remating.

Remating

Tenerally mated females had a higher proportion of rematings than the mature mated females ($\chi2= 56.4$, $P<0.001$; Fig. 7). There were no group differences in remating latency ($F_{1,113} = .17$, $P = .85$; Fig 2b) or duration ($F_{1,113}=.92$, $P=.41$; Fig 3b). A further analysis of teneral females, found that the tenerally mated females that remated had shorter initial mating durations than those that did not remate ($F_{1, 140}=10.42$, $P=0.002$; Fig. 8).

Discussion

In experiment 4a, the majority (64%) of tenerally mated females remated when mature, and there were more matings compared to both control groups (Fig 7). The flies that remated had a shorter teneral mating duration than the flies that did not remate (Fig 8). This suggests that females with a short mating duration may have had less sperm transfer, and were remating to replenish sperm stores.

3.2) Experiment 4B: Daily rate of progeny production from teneral and mature matings

Results from experiment 4a found that 36% of tenerally mated flies did not remate, and had a longer teneral mating duration than those that did remate. It is unclear as to why some females did not remate, but the purpose of experiment 4b was to measure progeny production of females in this group. If a teneral female has fewer progeny, as expected, this could be detrimental to reproductive fitness.

Methods

Experiment 4b was conducted during experiment 4a. In three of the 8 rounds, female flies were transferred to a fresh food vial daily, which began immediately following mating. All teneral flies that did not remate continued to be transferred daily, until egg production slowed down and they were then transferred on day 16, 18 and 21. A matched number of controls that did not remate were kept and transferred as well. Vials were left for two weeks after egg laying and all offspring were anaesthetized and counted. A preliminary analysis indicated a similar pattern of progeny production rate in the two mature female groups (Repeated measures ANOVA, $F_{1,27} = 1.3$, $P = 0.26$) so we compared the teneral females to the mature females.

Results

The tenerally mated females produced approximately one third the progeny than females which mated when mature (Repeated measures ANOVA, $F_{1,38}$=31.1, P<0.001; Fig. 9), with much of the difference seen in the first 6 days following mating.

Discussion

Experiment 4b results were as predicted, and tenerally mated females had fewer daily progeny than mature females (Fig 9). The majority of the differences were seen in the first six days following mating. It is still unclear as to why these females did not remate, it is possible that the teneral mating caused damage. It would have been interesting to interrupt rematings, and compare the progeny production of females that were tenerally mated and did remate, to see how their progeny rate compares.

4) Teneral matings in wild caught fruit flies

4.1) Experiment 5: Proportion of teneral matings in caught fruit flies

Experiment 2 found that teneral females which are less than 30 minutes old mate with mature males. Our behavioural observations from experiment 3, show strong evidence to suggest that teneral matings are forced. Experiment 4 found that females that mated while teneral are more likely to remate, compared to females that mated while mature. Our final experiment is to test whether teneral matings occur in wild caught flies.

Method

The same procedure was used as experiment 1, but using wild caught flies instead of C-S flies. Each day 30 flies in each treatment were tested for 3 consecutive days. All flies were second generation flies that were caught in Hamilton. To confirm the flies were *Drosophila melanogaster,* 10 wild caught males were put into a vial with a Canton S female, all 10 mated

and produced male and female progeny. There is no other local species that would produce offspring with *Drosophila melanogaster*. I first conducted a logistical regression to compare the proportion of rematings in teneral and mature females, followed by ANOVAs to compare mating latency and duration.

Results

About 15% of the teneral females and 92% of the mature females mated (Wald test: $\chi 2=$ 73.9, $P<0.001$; Fig. 1b). The teneral females had longer mating latencies ($F_{1,94}=32.3$, $P< 0.01$; Fig. 2c) and shorter mating durations ($F_{1,94}=12.9$, $P=0.01$; Fig. 3c) than the mature females.

Discussion

Experiment 5 replicated the finding in experiment 1, about 15% of teneral females mated (Figure 1c). Teneral females had a longer mating latency (Fig 2c) and shorter mating duration (Fig 3c) than mature females. The latency for teneral matings appeared longer in wild caught flies compare to what was found in C-S. Males in general seemed less aggressive, but no other behavioural observations were recorded so it is unknown what the male-female interaction was.

General Discussion

Major findings

The results here provide the most comprehensive evidence, to date, that male fruit flies use forced copulation as a mating strategy. Teneral females had more rejection behaviours during mating than a mature female (Fig 6a,b) and matings were consistently shorter (Fig 3a,b,c) and produced less offspring than a consensual mating (Fig. 9). Based on the courtship observations, it appeared that teneral females were less successful at rejecting male mating attempts. Males courted teneral and immature females equally, but teneral females moved at a lower rate. Teneral females spent more time kicking (Fig 5c), but were still mounted (Fig 5b) and grabbed

(Fig 5a) more often by males. Experiment 2 also showed that it was teneral females that were introduced to males immediately following eclosion that mated, rather than females over 30 minutes old, but still in a teneral state (Fig 4). The above results suggest that females were unable to reject male attempts during the first 30 minutes of the teneral stage.

Experiment 4a tested whether tenerally mated females will remate when sexually mature. Females that mated while teneral and retested 3 days later were more likely to remate than both the duration control and age control groups (Fig 7). The females that remated had a shorter teneral mating duration than those that did not remate (Fig 8), which suggests that remating occurred because the first mating was not optimal and sperm stores were low. This seems likely because females appeared to vigorously reject males while mating, and mating durations were shorter than an average mating involving a mature female. The duration of the rematings were longer than the original teneral mating duration (Fig 3b), and occurred much faster (3a), which suggests the remating was by choice. Of the flies that did not remate, the daily progeny production rate was much lower than in females that mated while mature (Fig 9). This suggests that forced mating may have negative fitness consequences on the female, as they produced a suboptimal amount of offspring.

Lastly, we tested whether teneral matings occur in wild caught *Drosophila* . We found mating in 15% of females, which was similar to what was found in experiment one (Fig 1b). The average mating latency was longer in wild caught fruit flies (Fig 2c) than in the Canton-S (Fig 3c) which suggests the teneral mating time window might be longer for wild caught flies.

Adaptive Significance

These results could open up a brand new area of research aimed at understanding sexual conflict in fruit flies, and may have implications for how sexual selection occurs in the wild. It is

already known that mating for females is costly, and mature females show strong preference toward specific male phenotypes (Van-Vianen & Bijlsma, 1993). This makes male mating success highly variable (Bateman, 1948). Males have evolved phenotypes to increase reproductive success (Harshman & Prout, 1994; Rice, 1996). Our results suggest that females may have less choice than was once thought and that sexual selection may, in part, be driven by male choice. The majority of females that had forced teneral mating, remated at maturity. It is not conclusive as to why some females remated and not others, our results suggest it may be due to decreased sperm transfer, due to a short mating duration. Future research should measure how much sperm is transferred during a teneral mating in both females that remate and do not remate. Much more can be done to fully understand what the affect of forced teneral matings are on a females future mate choice, and overall reproductive fitness.

Remating can be adaptively significant, as it renews an exhausted sperm supply (Gromko & Pyle, 1978) and also may help female upgrade from previous mating due to last male sperm precedence (Aspi, 1992). However, mating is very costly to females in terms of survival (Lamb, 1964; Chapman et al., 1995) so forced copulations may still have detrimental fitness effects, even though a female has exercised mate choice at maturity. Remating at maturity may not be as successful as a female that successfully rejects males while teneral and only mates while mature. It was suggested by Markow (2000) that teneral matings cause injury to females. Our results did find a higher death rate among teneral compared to mature females which was close to significance. A future experiment could investigate whether females that mate while teneral have a higher death rate than females that successfully reject males while teneral. Some tenerally mated females did not remate when mature. These flies had fewer progeny than the females that only mated while mature, which might suggest that it can be detrimental to fitness.

It is already known that accessory gland proteins stimulate egg production and ovulation and cause a change in the physiology of the reproductive tract to a mated state (Heifetz & Wolfner, 2004). Accessory proteins in male sperm cause the reproductive tract to operate at its highest efficiency. Future research could investigate whether forced mating increases maturation and egg laying rate for teneral females. If females lay eggs at a younger age, and remate at maturity, it is possible that forced mating is beneficial for reproductive fitness and progeny production of female.

Future Studies

There are many potential possibilities for future research. As mentioned above, future research could focus on what affects a teneral mating has on the female, such as decreased longevity, either from male aggression, increased energy expenditure, or the toxic effects of the sperm. For the females that do remate, they may not be as successful at producing offspring as a mature mating. Progeny production of the second mating could be measured and compared to a female that only mated while mature. Fruit flies would be a great model to use as an economic approach, to understand the cost and benefits of forced copulation are for males, females and the population as a whole.

There was only a small proportion of teneral matings, and a lot of day to day variability. It is unclear what causes this variability. In many species, forced copulations are situationally specific, and are used as an alternative reproductive strategy (Belovsky et al., 1996; Thornhill, 1987; Olsson, 1995). Males will force copulate with females when mating opportunities are low. In scorpionflies, males will only use their notal organ when nuptial gifts are unavailable (Thornhill, 1980). The dance fly (*Empis borealis)* is a species of fly that uses nuptial gifts to obtain matings (Svensson et al., 2008), forced teneral mating could be investigated in this species

as an alternative male strategy. In grasshoppers, seals, and orangutans forced mating only occurs in males of lower quality or status (Belovsky et al., 1996; Mitani, 1985). A lot of this research is from observation, rather than quantifiable data. Future studies could experimentally manipulate male qualities based on what is already known about female mate preferences and test whether forced mating occurs in the less desirable male phenotypes.

Markow (2000) also suggested that it is dependent on the number of mating opportunities. Markow did not find teneral matings in *Drosophila mojavensis*, which has a much higher remating rate than *D. melanogaster* and a less male biased operational sex ratio than what is seen in *D. melanogaster*. More in depth comparative studies could be done, to investigate whether forced teneral matings is a function of male mating opportunities. This is the first study to show forced mating in fruit flies, and this phenomenon can be investigated in other species of fruit flies.

This is also the first study, to my knowledge, that has conclusively demonstrated forced copulation during a teneral stage. Other invertebrates have mating strategies while they mate with pupae, such as the funnel web spider, mosquito and butterfly (Deinert, 1994; Singer et al., 2000; Slooten & Lambert, 1983). I know of no study that has found mating during the teneral period. It is likely that this has evolved in other species and has been overlooked, as the teneral period is often very short. *Drosophila* females store sperm, so mating while teneral will still produce progeny. Future studies should investigate this phenomenon in other species where females have sperm storage as well.

I previously discussed the idea that forced teneral mating may be situational specific. In my experiment, all males were virgin and had never been in contact with a female, so the situation was controlled for, however, males may have phenotypic differences that contribute to

mating success and explain the variability in male success. Male fruit flies show a lot of genetic variation in aggression (Edwards et al., 2009). There could be a persistence or aggressiveness trait evolved in response to female resistance that is particularly successful when courting teneral females that are weak. A follow up experiment could test whether males that are successful at mating with teneral female have more aggressive courtship than those that are unsuccessful. Some of the males simply may have been more aggressive than others. There could also be physical differences that help increase male success, such as size, speed, agility, or sex comb length. Forced copulation could explain sexual selection and why there is variation in male sex traits in *Drosophila.* Ahuja and Singh (2008) study the evolution of male sex combs in *Drosophila* and there is a large variety of males which could be tested on teneral females. I mentioned agility because of personal observation, males that force copulated appeared to change their body position quickly, to jump and mount females. Males often mounted from an abnormal position, which is often found in forced copulation. It makes sense that certain phenotypes that overcome female resistance have evolved. If mating is consensual, than these coercive phenotypes would not be necessary.

Forced mating may also be aimed towards females with specific phenotypes (smalle size, slow speed). It may be that male mate choice is used to select the weakest females, or that all males are equally aggressive, and only the weakest females are those with which matings are achieved. Thornhill (1987) reported that the smallest females are more likely to be force copulated with. In *Drosophila* smaller females have a lower fecundity than larger females (Lefranc & Bundgaard, 2000). So, understanding if male choice is involved, or if certain female phenotypes are at risk, will help understand the implications for forced mating on female phenotypic selection, which could explain the low reproductive success reported. Future

research could use selection techniques to understand how forced mating evolved, or measure phenotypes in females that mated vs rejected males. If forced mating is common in nature as it is in our study, sexual selection and the evolution of male and female traits may need to be re-evaluated.

Other pertinent questions revolve around how common forced copulation is in fruit flies in the wild. Given that it was initially observed in the wild, and is found in wild caught flies, it seems clear that it does occur. Females in the wild, may not exercise mate choice to the degree that is evident in all laboratory experiments. Markow (2000) suggested that forced mating may occur more often in a natural context compared to laboratory, as males may patrol emergence sites, waiting for teneral females to eclose. If I had more time to work on this project, I would create a naturalistic laboratory setting and measure the proportion of teneral matings that occur. If teneral matings occur in a more naturalistic setting, the operational sex ratio of the environment could then be manipulated to test if certain environments elicit forced mating.

Conclusion

This thesis presents strong evidence that forced matings occur in teneral *Drosophila melanogaster*. This has strong implications for understanding the nature of sexual conflict in fruit flies. In the wild, female choice may be very different from what is typically seen in the laboratory. I believe that fruit flies would be a great model for future research on forced copulation.

Chapters 5 & 6

Learned anticipatory drug responses

A form of learning that fruit flies exhibit is classical (Pavlovian) conditioning. In classical conditioning, a stimulus which precedes an event becomes associated with the event, and with repeated pairing, the stimulus elicits the same response that the event elicited. Pavlovian conditioning have been shown to play an important role in drug tolerance development (Siegel et al., 2000) Siegel et al. (2000) proposed a model of situational specific tolerance, where environmental cues previously associated with drug administration (conditioned cues) elicit a physiological response to counteract the drug effects. Learned anticipatory responses respond to an anticipated perturbation to homeostasis to regulate physiological homeostasis, which is disrupted by drug use. Stimuli which were previously associated with drug use can elicit a response to balance the drug effects and establish homeostasis, resulting in a learned drug tolerance. So, tolerance is more pronounced in the presence of drug associated cues that were previously paired with drug administration.

Situational specific tolerance has been shown in many drugs, including morphine (Siegel, 1988), nicotine (Cepeda-Benito et al., 1998), and ethanol (Seeley et al., 1996), to name a few. Morphine tolerance in humans can be situationally specific, where self administration is a conditioned cue and when injected intravenously results in a significantly higher drug effect (Ehrman et al., 1992). Students who consume alcohol in an unfamiliar drink (blue peppermint mixture) have a significantly decreased performance on cognitive and motor tasks, and rate themselves as more intoxicated than students who consume an equal amount of alcohol in a familiar way (beer) (Remington et al., 1997).

Situational specific caffeine tolerance

The drug of particular interest in this paper is caffeine. Caffeine is widely used, as it increases attention, working memory, and mood while decreasing daytime sleepiness (Kopplestaetter et al., 2008). Research in humans has found some support for a conditioned tolerance development. The eye blink reflex slows down when habitual coffee drinkers are given decaffeinated coffee (anticipatory response) and remains stable when given caffeinated coffee (Andrews et al., 1998). The decaffeinated coffee may be cueing a compensatory response by slowing central nervous system in anticipation that the body is receiving caffeine. Human research can be difficult, as most participants are already habitual coffee drinkers, so there is less experimental control and more mixed results (Flaten & Blumenthal, 1999). A model organism which would be a great model for studying caffeine tolerance, as it can be easily controlled and manipulated, is the fruit fly (*Drosophila melanogaster).*

Fruit flies as a model organism for situational caffeine tolerance

Recent evidence shows that fruit flies engage in sleep related behaviours that are common amongst animals (Hendricks et al., 2000; Shaw et al., 2000). Like humans, flies wake up in the early morning, have an afternoon siesta, and are inactive at night (Shaw et al., 2000). They have a species specific posture, increased arousal threshold which increases with time resting, and a dose dependent rest rebound in response to rest deprivation. Fruit flies have dose dependent rest-disrupting effects when administered caffeine, however, caffeine tolerance has yet to be investigated in the fruit fly.

Fruit flies are a model organism for neurogenetics. It has been used with alcohol tolerance; Cowmeadow et al. (2005) found that tolerance does not occur without expression of a

specific calcium activated potassium channel gene (*slowpoke*). The fruit fly would be a good species to measure caffeine tolerance at a neuronal level. At a neuronal level, caffeine blocks adenosine receptors, and increases dopamine. Repeated caffeine administrations cause neuromodulatory changes and the down regulation of dopamine in the mammalian brain (Fredholm, 1995). Using fruit flies as a model, Andretic et al. (2008) found caffeine administration leads to down regulation of dopamine, specifically in the mushroom body, a part of the brain responsible for learning and memory. If flies exhibit a situational tolerance to caffeine, in similar ways other mammals do, they would provide a great model to investigate the neuromodulatory and neurogenetci processes involved.

Positive effects of associative learning on fitness have been found in the fruit fly (Berger et al., 2008; Dukas, 2005; Mery & Kawecki, 2002). Anticipatory responses that are mediated by learning have could have a positive fitness advantage. It seems likely that fruit flies can develop anticipatory responses to drug effects by using associative cues.

Caffeine is poisonous to insects, and coping with toxic agents is important for fitness and survival so it is important to understand what mediates this ability (Hollingsworth et al., 2003). Caffeine has been proposed as a possible pesticide, as it is recognized as safe by the US food and Drug Administration. So understanding the mechanism behind learned tolerance could in the future help with crop production and pest control in an environmentally healthy way.

As far as I know, no data has investigated if fruit flies have situational drug tolerance. To answer this question, I conducted a series of experiments, asking (1) does caffeine cause rest disruption in the fruit fly, (2) can a tolerance be gained to the rest disrupting effects of caffeine and (3) can tolerance be situational specific, and do fruit flies only gain a tolerance to the rest disrupting effects of caffeine when it is paired with associative cues from a previous pairing.

General Methods

I used Canton-Special (C-S) flies from University of Toronto. The stock was kept in two population cages containing several thousand individuals inside an environmental chamber at 25°C with 70% RH. Flies were kept on a 12:12 light: dark cycle with lights on at 1000h. Each cage contained two 200-ml standard fly food bottles. The flies used in the experiments developed from bottles containing approximately 300 larvae. Female flies were collected within 8 hours from eclosion, anaesthetized with CO_2, sexed and placed in groups of 10 single sex vials with food containing corn meal, yeast, glucose, sucrose, and agar. All data collection was done using five day old virgin females. Flies were housed in the same environmental conditions as the population cages.

Flies were isolated into empty vials 8 hours prior to caffeine administration, unless stated otherwise. Approximately 1mL of caffeine solutions was dropped onto a kim wipes in a petri dishes to completely saturate it. Flies were individually aspirated into petri dishes and left unperturbed to drink for 30 minutes. All solutions contained 3 drops of food colouring and were 10% sucrose. After 30 minutes, flies with visibly coloured abdomens were aspirated into activity monitor tubes for night time recording. Lights out was 2200 and data was recorded until 1000 the following day.

Behavioural measurements.

All activity data was recorded using a 16 tube activity monitor, which records activity signals using an infrared (935 nm) transmitter. The infrared beam is invisible to fruit flies, whose vision extends only to 650nm (Bertholf, 1932). When the fruit fly is at rest the output of the monitor is equal to zero volts, and movement registers as a positive voltage. Signal detection theory was used to find activity threshold values; a value < 0.015 indicates inactivity. I generated

activity values for 2-sec bins and behavioural inactivity ≥ 5 minutes long was considered sleep, a value previously determined by Shaw et al. (2000). Flies had access to a piece of wet cotton while in the activity monitor. The first hour of recording (22:00-23:00) was not used in the analysis. Seven hours of recording were analyzed (23:00-06:00).

5) Rest disrupting effects of caffeine and general caffeine tolerance in the fruit fly

5.1) Experiment 1: Rest disrupting effects of caffeine

Flies in previous literature show a dose dependent decrease in night time rest following caffeine administration (Hendricks et al., 2000; Shaw et al., 2000). Hendricks et al. (2000) used 1-day-old flies, sex not specified and Shaw et al. (2000) used 5-day-old virgin females. Here, I will attempt to replicate these results, using 5-day-old virgin females, as sleep patterns are found to stabilize at day 3 (Shaw et al., 2000). It was predicted that caffeine administration one hour before activity recording will significantly decrease sleep duration in a dose-dependent fashion.

Methods

Following eclosion, 40 virgin females were placed in vials and left unperturbed for five days. At 1300 on day 1 of the experiment, flies were isolated, and then given a sucrose solution (control), a 2.5mg/ml caffeinated solution or 5mg/ml caffeinated solution (treatment groups) before activity recording. This study had 5 replicates, with 13 flies recorded for each replicate. The first hour of recording (22:00-23:00) was not used in the analysis. Seven hours of recording were analyzed (23:00-06:00). I compared rest duration of controls and the two treatment groups using an ANOVA. One fly in the 5mg/ml group died during data recording and was not used in analysis.

Results

There was a significant main effect of caffeine ($F_{2,61} = 4.2$, $P = 0.02$; Fig. 10). Tukey HSD post hoc results reveal that flies given 5mg/ml of caffeine spent fewer minutes per hour resting than caffeine free control flies, $P=0.03$. A more liberal, LSD post hoc found flies with no caffeine had more night time rest than those given 2.5g/l of caffeine ($P=0.03$) and those given 5 mg/ml ($P=0.1$; Fig. 5.1), but this was not found using Tukey HSD post hoc ($P>0.05$). No significant differences were found between flies given 2.5 mg/ml and 5mg/ml of caffeine ($P>0.05$).

Discussion

In experiment 1, caffeine reduced night time rest in flies. Flies that had 5mg/mL of caffeine rested significantly less than flies give no caffeine (Figure 10). There was no clear dose dependent effect of caffeine, as flies given 2.5mg/mL and 5mg/mL rested a similar amount.

5.2) Experiment 2: Caffeine tolerance after 7 days of caffeine administration

The purpose of experiment two was to test whether tolerance can develop after repeated caffeine administration. Fruit flies have been shown to gain alcohol tolerance (Berger et al., 2004). Previous studies have found evidence suggesting fruit flies gain tolerance to the rest disrupting effects of caffeine after 4 days of repeated administration (L. Prevot, unpublished data), but such results are yet to be published. Our study attempted to replicate this finding, but had 6 days of repeated administration. It was predicted that flies will show a rest disruption following caffeine administration, but return to a normal amount of rest after repeated administrations. It was also predicted that control flies given caffeine only on the final night of administration will show rest disruption. Flies given no caffeine, but an equally aversive substance should have no rest reduction.

Methods

Activity measures were tested on the first and seventh (test) night of a tolerance development period, on night two through seven flies were put into individual vials and housed in an incubator. Three treatment groups were used. Experiment 1 results found that 5mg/mL was enough to elicit a caffeine response, however, previous research suggests it may be too much for continued use (Prevot, unpublished) so I used 4mg/mL. All flies were given a solution for 6 consecutive nights, and were tested on the 7th night. Two control groups received sucrose solution during 6 days, and either a 1% quinine solution (sucrose/quinine) or 4 mg/ml caffeinated solution (sucrose/caffeine) on test night. The quinine is used as a control for the bitterness of caffeine (Prevot, unpublished data). The tolerance group was be given 4 mg/ml of caffeine on all seven nights (caffeine/caffeine)

Night time rest duration was compared, with Day as within in subject factor and treatment (caffeine/caffeine, sucrose/caffeine, and sucrose/quinine) as between subject factor. There was a 32% loss in flies from handling over the 7 days of data collection.

Results

Flies in the tolerance group rested more on night seven compared to night one ($F_{1,10}$ = 15.77, $P < 0.01$; Fig 11). The sucrose/caffeine flies rested less on night seven compared to night one, the difference was close to significance ($F_{1,6}$ = 5.61, P= 0.056; Fig. 11), however, there were only seven flies in this condition, so increasing the sample size may yield more promising results. The sucrose/quinine flies rested similar amounts on night one and night seven ($F_{1,8}$ = 1.52, P= 0.25; Fig 11).

A between groups analysis found significant group differences on test night ($F_{2,28}$ = 3.9, P=0.03; Fig. 11). A Tukey HSD post hoc comparison found the sucrose/quinine had significantly

more rest than sucrose/caffeine, P=0.05, suggesting the reduction in rest is caused by caffeine and not by effects of a novel substance. The caffeine/caffeine treated flies rested more on test night than the sucrose/caffeine flies, P=0.05, suggesting a developed tolerance to caffeine.

Discussion

Flies that were given caffeine for 6 consecutive days rested more on night 7 than on night 1 (Fig 11). Flies given sucrose for six nights showed a rest reduction only when give caffeine on night 7 compare to quinine (Fig 11). These results gave evidence to support a tolerance to caffeine can develop in fruit flies over a six day tolerance period.

6.) Situational specific tolerance to caffeine

6.1) Experiment 3: First test for situational caffeine tolerance

Results from experiment 2 suggest a general caffeine tolerance. The purpose of experiment 3 was to test whether tolerance to caffeine can be situational specific. Previous studies have found evidence to suggest wild caught fruit flies can gain a situational tolerance, however results are not conclusive (L. Prevot, unpublished data). I attempted to replicate these results using Canton S flies, as they are typically found to be associative learners. We predicted that flies receiving a caffeine odour pairing different than the pairing they experienced during a tolerance phase would have a larger rest reduction rest, compared to flies given the same caffeine odour.

Methods

Amyl acetate (AA) and benzaldehyde (BA) solutions were paired with solutions and used as associative odour cues. Half AA solutions were caffeinated 4mg/ml (AA+) and half were 1% quinine control solutions (AA-). The BA had 4mg/mL of caffeine (BA+) or 1% quinine (BA-) as well. For 6 consecutive days flies were either given AA+ or BA- (Treatment 1), and BA+ or AA-

(Treatment 2). Each solution was given 3 of the 6 nights with the order randomized in two day blocks. On test night flies half of the flies in each treatment received the same caffeine odour solution as during tolerance acquisition, and half received caffeine with the odour that had been previously paired with quinine and night time rest was recorded. The study had five replicates and recorded 12 flies per replicate. Rest activity was only recorded on the first and last night. An ANOVA compared the amount of rest per hour on test night in flies given the "same" and "new" caffeinated odour pairing. A total of 54 flies were used in analysis, 6 flies were lost during handling throughout data collection.

Results

On test night, flies in the "same" and "new" odour condition rested a similar amount ($F_{1,54}$= 1.0, P=0.3; Fig. 12). I then ran separate ANOVAs to compare "same" vs. "new" in each treatment. In treatment 1 (AA+/BA-) flies given the same (AA+) and new (BA+) caffeinated solution rested similar amounts ($F_{1,30}$ = 0.6, P=0.5; Fig 13). However, in treatment 2 (BA+/AA-) flies given the same odour pairing (BA+) rested more compared to a new odour pairing (AA+) ($F_{1,24}$ = 4.8, P=0.04; Fig 13).

On night one, caffeinated flies had a rest reduction compared to quinine flies (47.2 ± 3.6 and 30.8 ± 3. and mines; $F_{1,68}$ = 13.5, P< 0.01). A within subject ANOVA found that flies given the same conditioned caffeine-odour pairing on night one and night seven rested a similar amount (28.2 ± 3.8 and 26.5 ± 3.7 mins; $F_{1,19}$ = .52, P=0.6).

Discussion

Results from experiment 3 did not show strong evidence to support situational specific tolerance development. Flies given caffeine with the "same" odour did not have reduced rest compared to flies given caffeine with a "new" odour (Fig 12). Results from treatment 2 give

evidence to support a situational specific tolerance to caffeine, however, this result was not found in treatment 1 (Fig 13). Results do not give evidence to support a general tolerance, as flies given the "same" caffeinated solution did not have an increase in rest on night 7 compared to night 1. Rest per hour on night 1 and night 7 were comparable to rest per hour for caffeinated flies on night one in experiment 2 (24.0 ± 2.8). However, in experiment 2, rest per hour increased on night 7 (39.7 ± 3.2), which was not found in this study.

6.2) Exp 4: Caffeine tolerance after 3 days of administration. A test for protocol 1.

In experiment 2, flies were administered caffeine for 6 consecutive days, and results support a general tolerance development. The experiment 3 protocol had only 3 days of caffeine administration, which were over 6 days, and randomized with 3 opponent odour quinine pairs. Three trials over six days may not be enough for tolerance to develop, conditioning trials may need to be closer together and more frequent. The purpose of this study was to test whether a general tolerance can develop after 3 nights of caffeine administration. Results from this experiment will help develop a stronger protocol to test for situational specific tolerance. The same predictions were expected as experiment two.

Methods.

The same protocol was used as experiment 2, except there was 3 conditioning trial. Rest activity was recorded on night 1 and night 3. The experiment had 3 replicates and 14 flies were recorded per replicate. Eight flies were lost during the data collection.

Results

On night one, flies in the caffeine tolerance group rested a similar amount as control flies ($F_{1,34} = 0.3$, $P > 0.05$; Fig. 14). On test night there was a significant effect of group, $F_{2,34} = 4.9$, $P = 0.02$. A Tukey HSD post hoc found the caffeine/caffeine rested more than sucrose/caffeine

($P< 0.02$; Fig. 14). The sucrose/quinine group rested longer than the sucrose/caffeine flies, but the difference was not significant ($P=0.06$; Fig. 14).

Discussion

Results from experiment 4 did not show clear tolerance development over 3 days. There was no rest reduction in caffeine/caffeine flies on night one, and they rested a similar amount to the controls on both night 1 and night 7 (Fig. 14). On night 7 the sucrose/caffeine had a strong reduction in rest compared to night 1, and compared night 7 of the caffeine/caffeine flies. This may suggest the caffeine/caffeine flies gained a tolerance, but it cannot be concluded, as there was no initial rest reduction on day 1 in the caffeine/caffeine flies (Fig. 14).

6.3) Exp 5: Protocol 2: A second test for situational caffeine tolerance

Results from experiment 4 did not show strong evidence to support a 3 day tolerance development to rest disrupting effects of caffeine. The main difference from experiment 2, was that there was no caffeine effect on night one. The sample size was low and there were severe thunderstorms for all the nights of data collection. Changes in barometric pressure can affect activity patterns in fruit flies (Raboutet, 1959), so it was possible that this was the reason flies were inactive. In my previous experiments (1,2,3) I had strong evidence supporting the rest disrupting effects of caffeine, so I decided to continue with a new protocol to test for situational tolerance. The protocol for experiment 5 had 6 days of consecutive administration rather than 3. I also introduced colour as as well as odours to the solutions to provide flies with visual and olfactory cues, which may increase likely hood of associations being made between caffeine administration and environmental cues.

Methods

The same solutions were made as in experiment 3, however, green food colouring was added to AA solutions and blue food colouring was added to BA solutions. There were 4 treatments, two of which were given the same caffeinated solutions for 6 consecutive days (either AA+ or BA+), and the other two were given same quinine solution for 6 consecutive days (either AA- or BA-). On night 7, both caffeinated treatments received caffeine, either with the "same" or "new" odour/colour, and both quinine treatments received quinine, with the "same" or "new" odour/colour. The experiment had 6 replicates and recorded 14 flies per replicate. A total of 9 flies were lost during collection.

It was expected on night 7, that flies given a "new" caffeine solution would have reduced rest compared to flies give the "same" caffeine solution. I expected flies given a "new" quinine solution would have similar rest to flies given the "same" quinine solution, which would indicate that changing the odour/colour of a solution does not influence consumption or rest activity.

Results

On test night, all group rested an equal amount ($F_{1,42}$=1.8, P=0.16). There was no reduction in rest in flies given a new caffeine solution compared to those given the same solution (40.7 ± 4 mins vs. 38.6 ± 3.5 mins). There were no group differences in rest activity in AA conditioned group ($F_{1,20}$=0.4, P=0.8) or BA conditioned group ($F_{1,18}$=3.2, P=0.07).

On night one, there was a main effect of treatment ($F_{1,71}$ = 4.7, P=0.047). A Tukey HSD post hocs test found AA + rested significantly less than AA- (P< 0.01) and no other group differences were found (Fig 15).

Discussion

There were no data to support a situational tolerance development. Flies given a new caffeinated solution did not have reduced rest compared to flies given the same caffeinated solution. On night one, there only appears to be a caffeine response in AA+ group (Fig 15).

6.4) Exp 6: Protocol two revisited. A third test for situational caffeine tolerance

The flies failed to show a caffeine response in experiment 4 and 5. Before starting a new experiment I wanted to test different food deprivation lengths to see if this would increase caffeine consumption resulting in more of a caffeine effect. I did some tests (in total 5) to test different feeding protocols. I wanted to test for optimal starvation time, to hopefully increase the amount of solution consumption. I also wanted to test for optimal consumption amount. The current criteria was to use flies that had coloured abdomens, I had noticed a lot of variation. I wanted to develop more accurate criteria to visually quantify the amount of solution ingested. From these results, I developed a final protocol which I tested in experiment 6.

Preliminary Tests

For each test I used 20 flies. First, I investigated what happened when flies were isolated from food for different amounts of time. Test 1 began by isolating flies for 24 hours and rested only 9.2 mins her hour. Test two isolated flies for 12 hours and flies only rested 22 hours per night. Flies were kept isolated from food completely. In both tests 1 and 2, a large amount of solution was consumed, however, all flies died by day 2. It appeared as though they drank too much solution, their abdomens were very colourful, and they had twitchy behaviours after consumption, some which ended in death instantly. For test 3, I isolated flies for 8 hours, and gave them an agar water solution overnight to help decrease dehydration. Flies rested for 30 mins per hour, but only lived 3 days on this protocol. In test 4 and 5, flies were give an agar/sucrose

solution overnight and were isolated 10 and 7 hours respectively. They showed a slight decrease in rest with 10 hours of isolation (23 mins/hour), but no effect of caffeine with 7 hours of isolation (37.3 mins/hour). The flies lived longer than the flies in test 1-3, but only lived for about 6 days. Based on these results, we decided to give flies full food overnight and isolate them for 10 hours instead of 8, to help increase solution consumption.

My selection criteria for experiment 1-5 was that flies had abdomen colour, so before continuing on to experiment 6 I wanted to test whether I could visually quantify the variation in abdomen colour I was seeing. I wanted a more strict criteria to eliminate flies a small amount of solution and were less likely to show a caffeine effect. I came up with a visual criterion, and measured rest activity in flies that appeared to have a small, medium or large amount of colour in their abdomen (Fig. 16). The number was too small to run stats, but based on these results, I only used flies in experiment 6 that appeared to have a medium or large amount of colour in their abdomen as they appeared to have a stronger caffeine effect.

Methods

The experiment five protocol was replicated, the only difference is that flies were isolated for 10 hours instead of 8, and I only used flies that drank a medium amount of solution. The experiment had 12 replicates with 10 flies per replicate. A total of 26 flies were lost during the data collection.

Results

On test night, there were no group differences in rest activity, and flies given a "new" caffeinated solution rested a similar amount than flies given the "same" caffeinated solution and both quinine groups ($F_{1,92}$= 1.8, P=0.2). A within subject ANOVA did not give evidence to support situational tolerance development. All groups had less rest on test night than on night one $P< 0.05$. On night one, a between group ANOVA found no group differences and flies

administered caffeine did not have a rest reduction compared to flies given quinine $F_{1, 92}=1.5$, $P=0.3$.

Discussion

Experiment 6 results did not show evidence for situational caffeine tolerance, and flies given caffeine in a new colour/odour solution rested a similar amount to flies given the same caffeine odour solution. There was no evidence to suggest a general tolerance development, the caffeine/caffeine flies in the "same" condition did not rest more on night 7 compared to night 1. All groups had less rest on night 7. There was also no caffeine effect found on night 1, and caffeinated flies rested a similar proportion compared to control flies.

General Discussion

Major findings

The results here do not support evidence for situational drug tolerance in Drosophila. In experiment 1 and 2, I was able to show that caffeine induced rest decrease (Fig 10,11), which replicated previous results that caffeine disrupts night time rest in Drosophila (Hendricks et al., 2000; Shaw et al., 2000). In experiment 2, I was able to show a 6 day general tolerance development (Fig 11), but was unable to show a 3 day tolerance development in experiment 4 (Fig 14). In experiment 3, 5, and 6 I tested 3 different protocols to test whether tolerance to rest disrupting effects of caffeine is situational specific. In experiment 3, flies were given 3 days of caffeine administration and 3 days of quinine, in a randomized order. They did not show evidence of situational caffeine tolerance, or a general tolerance development (Fig 12,13), but did show an effect of caffeine on night 1. In experiment 5, I administered caffeine for 6 consecutive day and introduced colours as well as odours for associative cues. A caffeine effect was only found with one odour (AA) and not BA (Fig 15). AA and BA were equally consumed,

and a further test found a more quantifiable way measure the amount of solution drank by flies. Experiment five was re ran as experiment 6, and flies were isolated from food for an extra two hours each day, to encourage more solution consumption, and only flies that drank a 'medium' amount of solution were used. Results found no initial caffeine effect, and no evidence of general or situational tolerance development.

Future research

The next step would be to develop a method to accurately administer caffeine. I only found an initial caffeine effect in experiment 1,2,3 and 5 (AA group only). It is unclear to me why a caffeine effect was not found in experiment 4 and 6. There was no noticeable difference in abdomen colour compared to previous experiments and the same protocols were used. One major drawback to my protocol what that I did not have direct control over the amount of caffeine ingested. If situational tolerance was to be found, we would have had to measure the amount of solution ingested daily to ensure the results were not due to a change in eating habits or preference. If I was to continue on this project, I would develop a more accurate method of administration.

The most common method of caffeine administration is through a food medium (Xu, 1998), but it seems to be mostly effective as a method that requires only one time administration (Xu, 1998), or effects of long term exposure (Andretic et al., 2008; Carillo & Gibson, 2002; Zimmering, 1977). Most of these studies measure neuronal changes in the fly brain after prolonged drug exposure and did not control for amount of caffeine consumption. So, the method of ingestion that I used, has drawbacks for my research, as I wanted to administer daily doses. I did not have control over the amount each fly drank, which made it difficult to get a caffeine

effect. This confound made it difficult to measure tolerance development, as it is hard to ensure equal drug amounts are administered daily.

Another popular method of drug administration is volatilization, it is yet to be used for caffeine administration in fruit flies, but has been successfully used for ethanol, nicotine, and cocaine (Manev et al., 2000). It is possible to volatilize caffeine, but there is no clear protocol to show it is effective for administration to mammals or invertebrates. Using this method it is also hard to determine the amount consumed, however, it seems like a more accurate way to administer equal doses than through a food medium.

A more accurate, and more invasive method is injection (Manev et al., 2000) and direct application to the nerve cells of a severed neck (Andretic & Hirsh, 2000; Torres & Horowitz, 1998). These methods are typically only used for drugs that cannot be ingested or vaporized and would not be useful for the question I am asking.

If I was to do this experiment again, I would try topical administration. This method is less invasive than injection and more accurate than food or volatilization. Caffeine has yet to be delivered to flies topically, however recent literature has shown it can be used for cocaine administration. Lease and Hirsh (2005) topically applied cocaine to anaesthetized flies and found it elicited the same behaviours that are seen from volatilization. Based on these results they developed a method of spraying ("airbrushing") flies with the topical drug spray. The topical administration and airbrushing method elicited the same behavioural response as cocaine volatilization with less variation which suggests a more uniform dosing. Topical caffeine administration to flies quite possibly has not been tried yet, as it is a fairly new protocol being tested in invertebrates. Recent research has applied caffeine topically to bees (Barron et al.,

2007; Malechuk, 2009; Kucharski & Maleszka, 2005; Si et al., 2005), where most research in the past administered caffeine through a food source (Kucharski & Maleska, 2002).

So, some future ideas could be to try and see if a topical administration protocol can be set up. Lease and Hirsh (2005) anaesthetized and applied cocaine to the fly. Si et al. (2005) had bees tethered and performed a proboscis extension reflex (PER) assay to test the effects of caffeine on associative learning. The conditioned cues were odours, and the bees were given exposure to them as they were tethered. PER assays have been performed on fruit flies, so it could be possible to use a similar protocol to tether the flies and administer the caffeine topically while giving them exposure to a paired odour.

So, an important future step is to develop a method that accurately administers caffeine. If a strong protocol is developed, future studies could be done to investigate whether situational specific tolerance can develop. There is a large body of literature on associative learning and anticipatory responses, which show that it increases fitness and survival (Hollis, 1997; Ricco et al., 1991). It seems likely that this ability has evolved, and more research should be done to investigate it. The fruit fly has already been used as a model for the neurogenetics of drug tolerance (Cowmeadow et al., 2005) and understanding how the environment mediates tolerance on a neuronal level would be a great avenue for future researchers interested in the topic.

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Figure Legends

Figure 1. (a) Experiment 1: Proportion of matings in Canton S teneral (n=70) and mature (n=70) females during a one hour observation period. (b) Experiment five: Proportion of matings in wild caught population teneral (n=90) and mature (n=90) females during a one hour observation period.

Figure 2. (a) Experiment 1a: The average (± SE) mating latency (secs) of teneral (n=13) and mature (n=38) females during a one hour observation period. (b) Experiment 4a: The average (± SE) mating latency (secs) divided by group, based on age (day) of initial mating and retest. Mating latency values are given for day one, 0 and 3 (n = 141), 3 and 6 (n=148) and 2 and 3 (n= 147) and test day, 0 and 3 (n = 89), 3 and 6 (n = 18) and 2 and 3 (n = 9) on test day. (c) Experiment 5: The average (± SE) mating latency (secs) of wild caught teneral (n=13) and mature (n=83) females during a one hour observation period.

Figure 3. (a) Experiment 1a: The average (± SE) mating duration (secs) of teneral (n=13) and mature (n=38) females. (b) Experiment 4a: The average (± SE) mating duration (secs) divided by group, based on age (day) of initial mating and retest. Mating duration values are given for day one, 0 and 3 (n = 141), 3 and 6 (n=148) and 2 and 3 (n= 147) and test day, 0 and 3 (n = 89), 3 and 6 (n = 18) and 2 and 3 (n = 9) on test day. (c) Experiment 5: The average (± SE) mating duration (mins) of wild caught teneral (n=13) and mature (n=83) females during a one hour observation period.

Figure 4. Experiment 2: The proportion of females that mated, separated by time of day and time tested as predictor variables. Females were paired with two males at 0 (n=60), 30 (n=60) or 60 (n=60) minutes old and vials were observed for one hour.

Figure 5. Experiment 3a: (a) Average (± SE) number of times the male grabbed either teneral (n=10) or immature (n=10) females during 15 minute observation. (b) Average (± SE) number of times the male mounts teneral (n=10) or immature (n=10) females during 15 minute observation. (c) Average (± SE) proportion of time teneral (n=10) and immature (n=10) females spent kicking males during 15 minutes of observation.

Figure 6. Experiment 3b: (a) The average (± SE) proportion of time the teneral (n=10) and mature (n=10) females spent kicking during mating. (b) The average (±SE) proportion of time teneral (n=10) and mature (n=10) females spent active while mating.

Figure 7. Experiment 4a: The proportion of rematings of females that mated while teneral (n=141), 3 days old (n=148) and 2 days old (n=147). The '3 and 6' group had a 3-day gap between mating and the remating test like the tenerally mated females, while the '2 and 3' group was tested at the same age as the tenerally mated females.

Figure 8. Experiment 4b: The average teneral mating duration (± SE) of females that either remated (n=90) or did not remate (n=51).

Figure 9. Experiment 5: The average (± SE) number of progeny produced from females mated when teneral (n= 11), 3 days old (n= 17), and 2 days old (n=12).

Figure 10. Experiment 1: Average (± SE) rest per hour. Bars represent the average from 2300 – 0600 for 0mg/ml caffeine (n = 24), 2.5mg/l caffeine (n = 21), and 5 mg/l caffeine (n= 19).

Figure 11. Experiment 2: Average (± SE) rest per hour during the period 2300-0600. Flies were given caffeine/caffeine (n = 12) , sucrose/caffeine (n = 7) and sucrose/quinine (n = 9) on the first 6 days, and last day of administration.

Figure 12. Experiment 3: Average (± SE) rest duration (mins) from 2300-0600 on test night. Flies administered the same 4mg/ml caffeine odour pairing (n = 27) or a novel caffeine odour pairing (n = 27) which was not administered during conditioning phase.

Figure 13. Experiment 3: Average (± SE) rest duration (mins) from 2300 – 0600 on test night, separated by group. The AA conditioned flies were given the "same" AA 4 mg/ml caffeinated solution (n = 15) that was administered during the conditioning phase, or a "new" BA 4 mg/ml caffeinated solution (n = 15). The BA conditioned flies were given the "same" BA 4mg/ml caffeinated solution (n = 12) or a "new" AA caffeinated solution (n = 12).

Figure 14. Experiment 4: Average (± SE) rest duration (mins) from 23:00 – 07:00 on night one and test night, separatred by group, caffeine /caffeine (n = 11), the sucrose/quinine (n = 12) and sucrose/caffeine (n = 11).

Figure 15. Experiment 5: Average (± SE) minutes resting per hour on night one, separated by group, AA+ (n=17) , AA- (n=20), BA+ (n=20), and BA- (n=18).

Figure 16. Test for experiment 6 protocol: Average (± SE) minutes resting per hours for flies that appeared to have a small (n=5), medium (n=8) and large (n=1) amount of colour in their abdomen. A small amount was what appeared to be less than 50% of their abdomen, medium including 50% or more, and large included a full abdomen.

Fig. 1

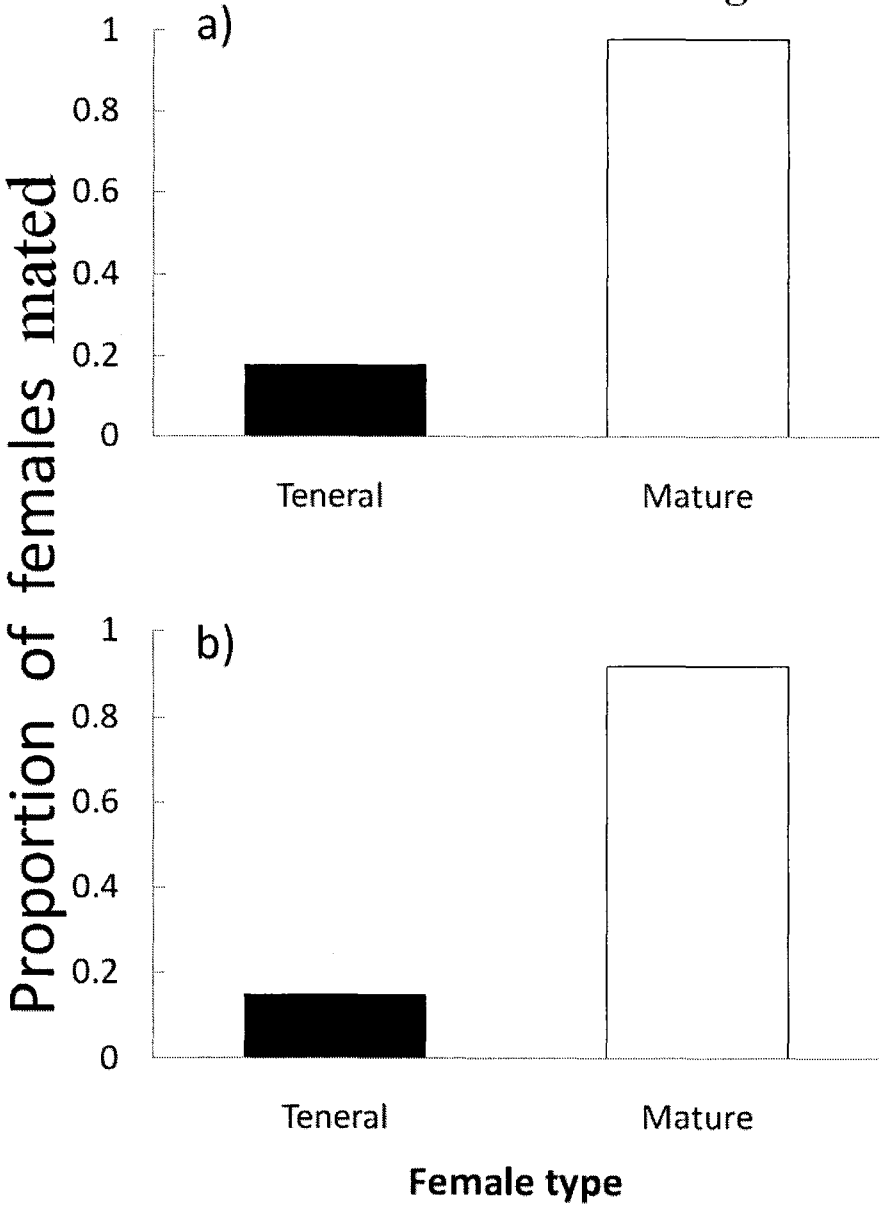

a)
b)
1
0.8
0.6
0.4
0.2
0
Proportion of females mated
Teneral
Mature
Female type

Fig. 2

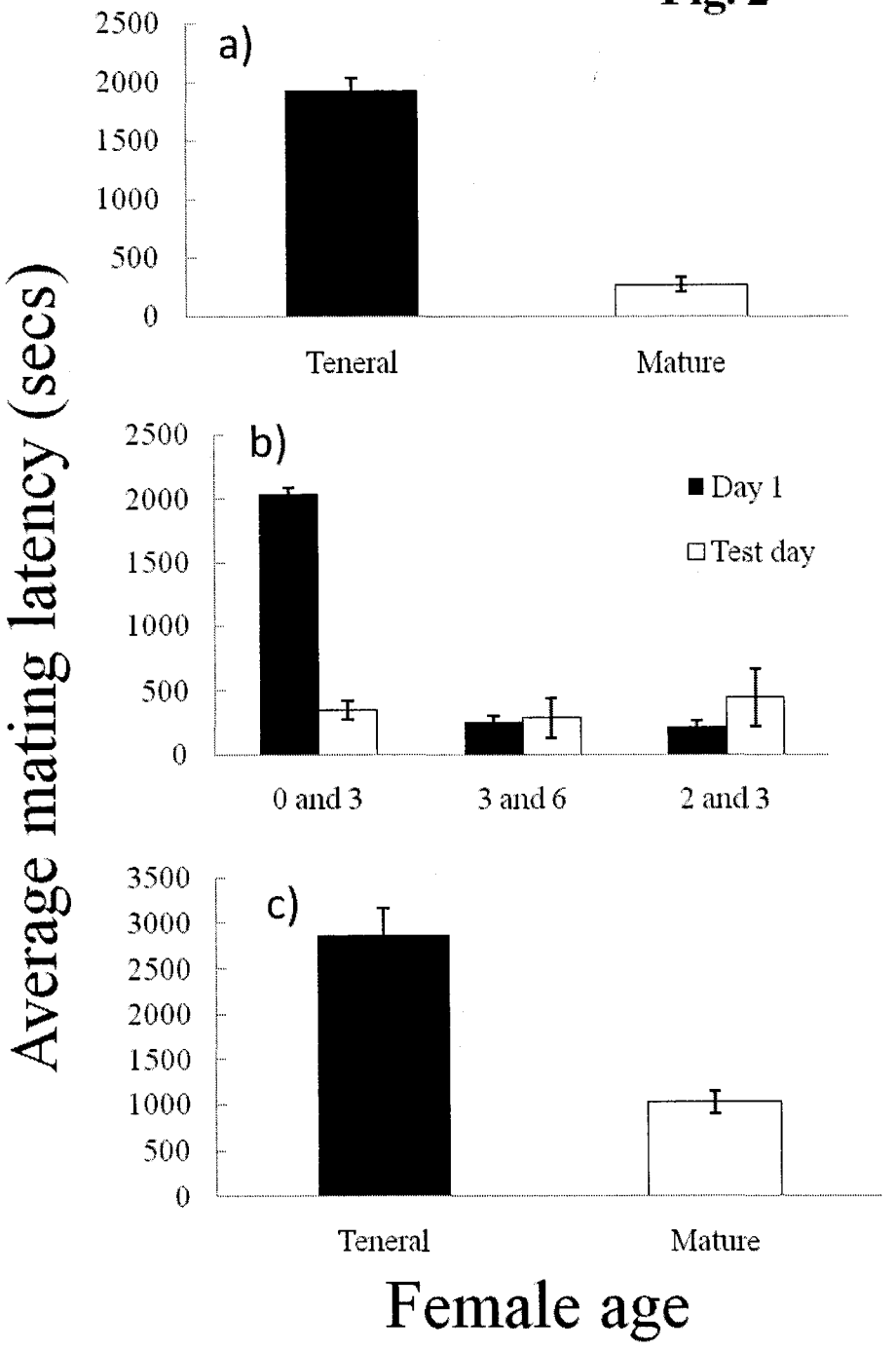

a)
2500
2000
1500
1000
500
0
Teneral
Mature
b)
Day 1
Test day
0 and 3
3 and 6
2 and 3
c)
3500
3000
2500
2000
1500
1000
500
0
Teneral
Mature
Average mating latency (secs)
Female age

Fig. 3

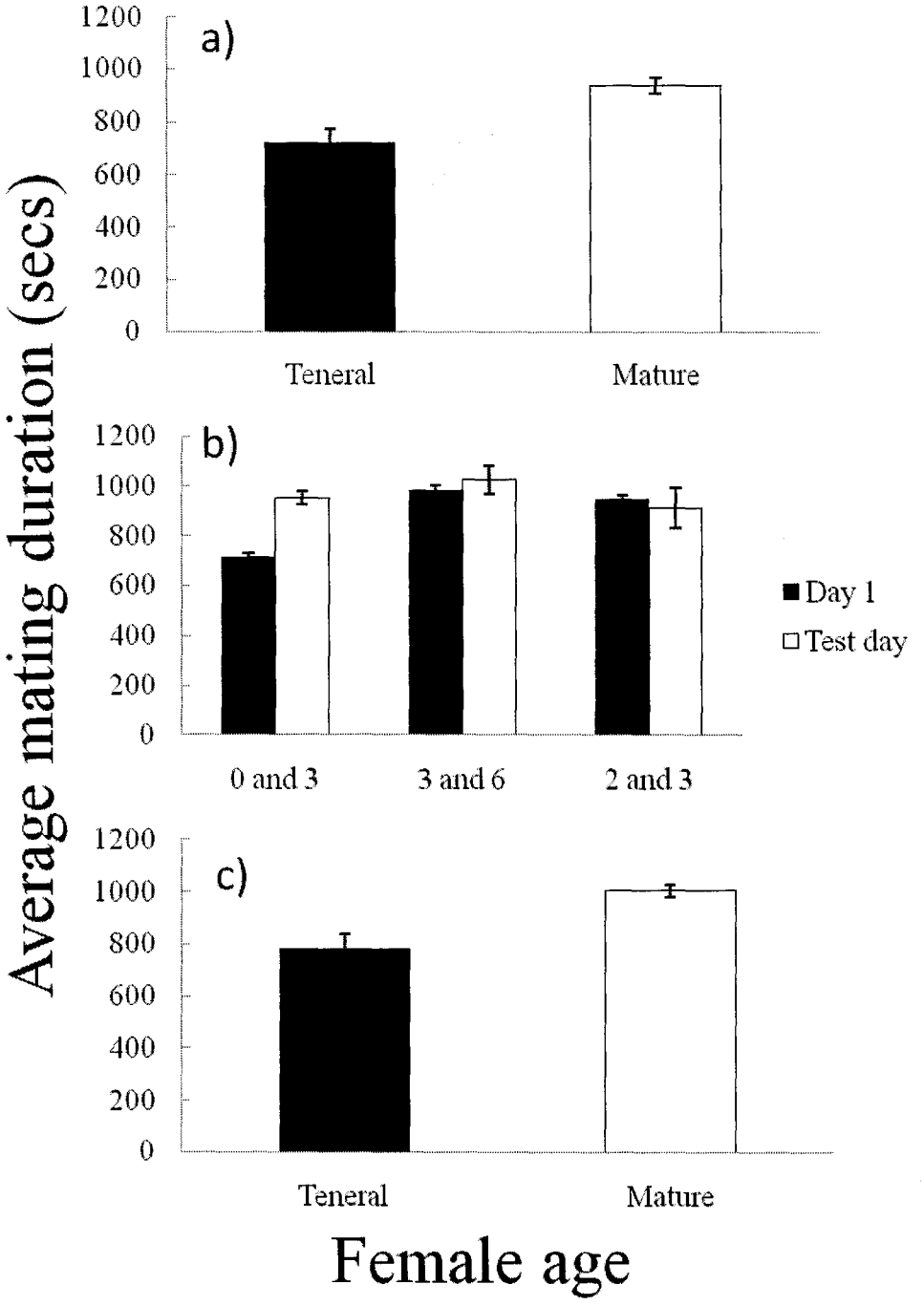
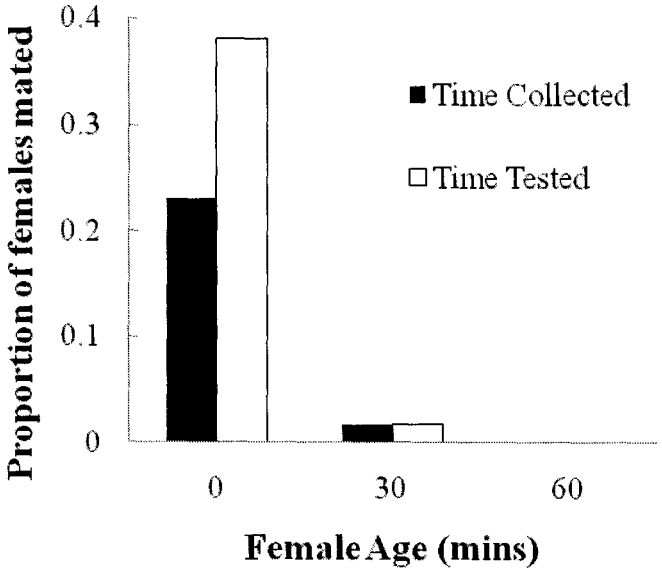

a)
b)
c)
1200
1000
800
600
400
200
0
Teneral
Mature
0 and 3
3 and 6
2 and 3
Day 1
Test day
Average mating duration (secs)
Female age

Fig. 4


0.4
0.3
0.2
0.1
0
Proportion of females mated
Time Collected
Time Tested
0
30
60
Female Age (mins)

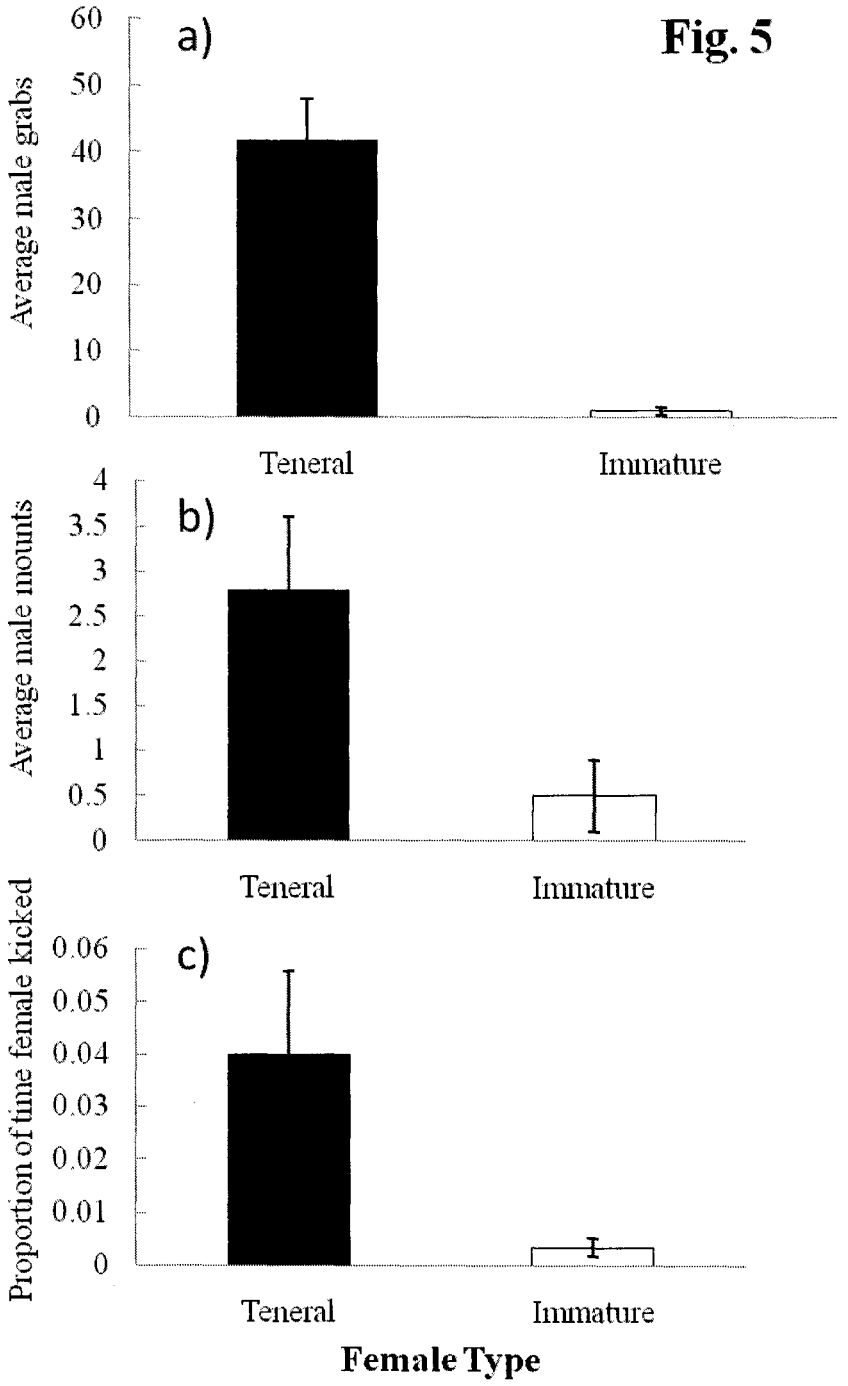

a)
Average male grabs
60
50
40
30
20
10
0
Teneral
Immature
b)
Average male mounts
4
3.5
3
2.5
2
1.5
1
0.5
0
Teneral
Immature
c)
Proportion of time female kicked
0.06
0.05
0.04
0.03
0.02
0.01
0
Teneral
Immature
Female Type

Fig. 5

Fig. 6

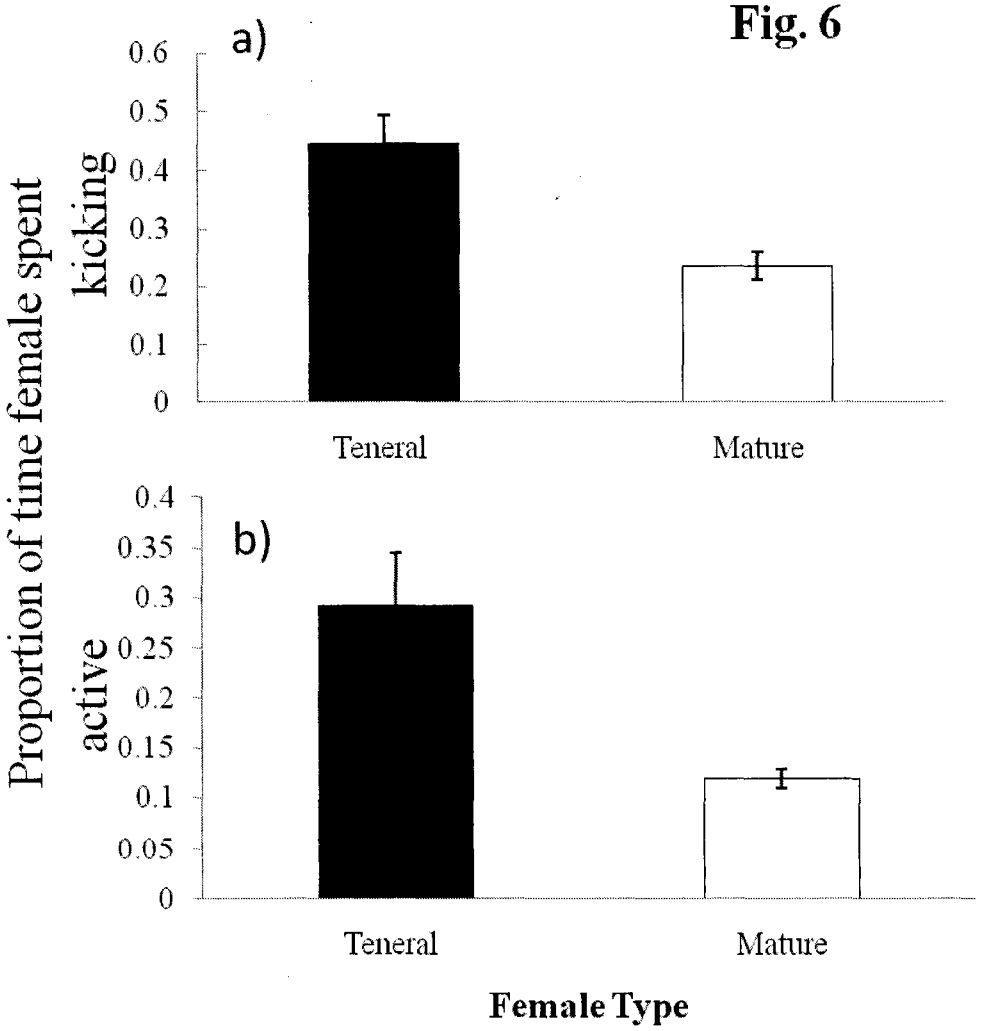

a)
0.6
0.5
0.4
0.3
0.2
0.1
0
Teneral
Mature
b)
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Teneral
Mature
Proportion of time female spent kicking
active
Female Type

Fig. 7

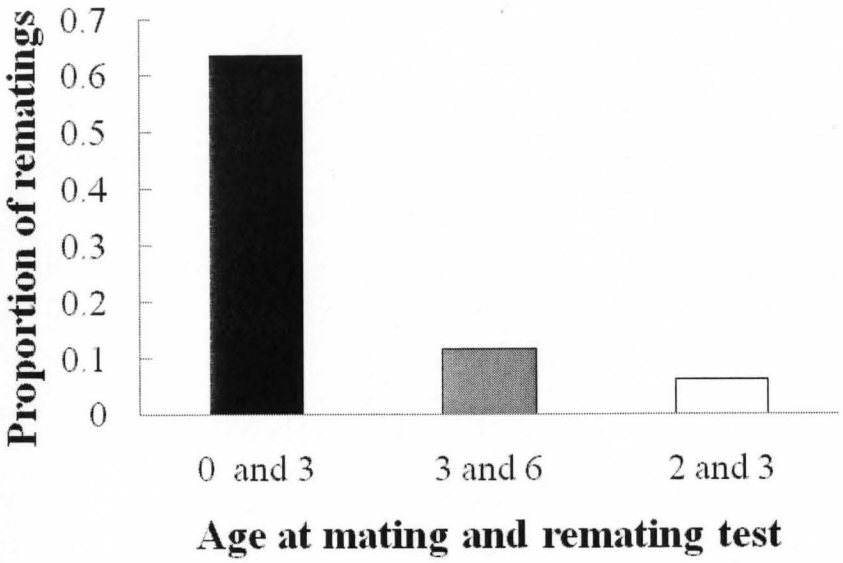

Proportion of rematings
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0 and 3
3 and 6
2 and 3
Age at mating and remating test

Fig. 8

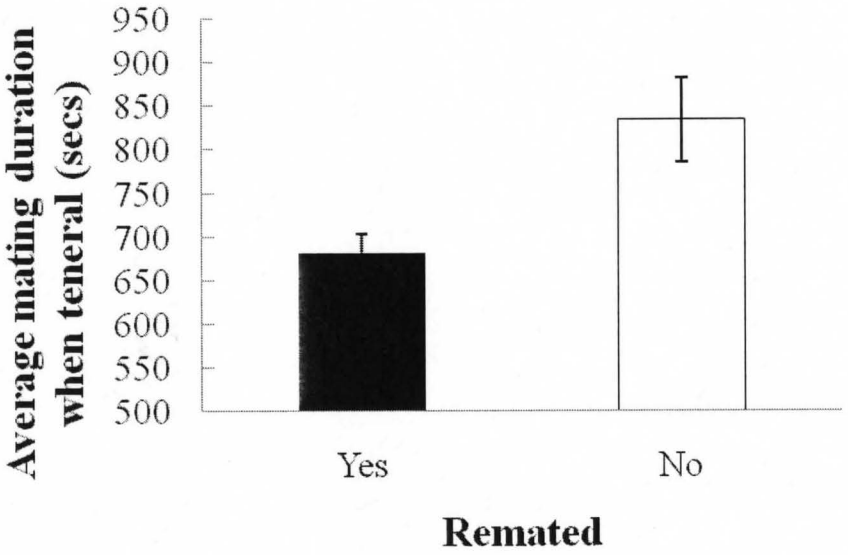

Average mating duration when teneral (secs)
950
900
850
800
750
700
650
600
550
500
Yes
No
Remated

Fig. 9

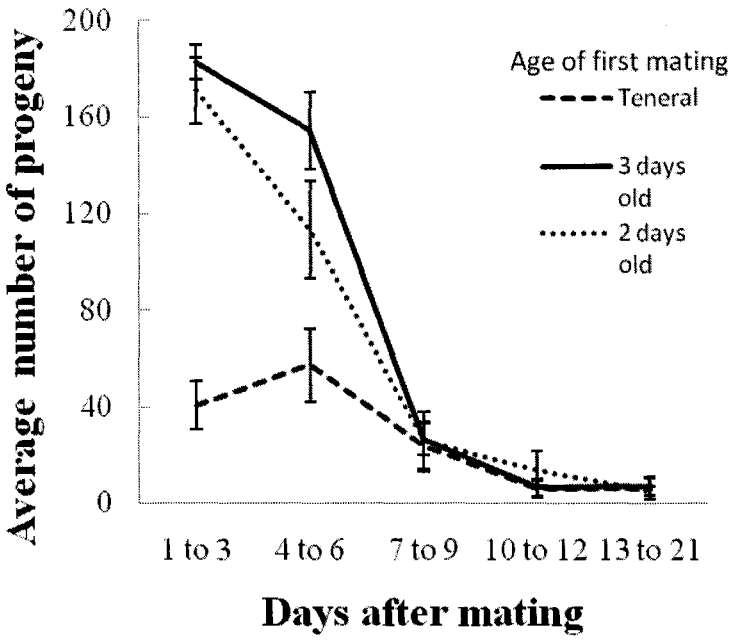

Average number of progeny
200
160
120
80
40
0
Age of first mating
Teneral
3 days old
2 days old
1 to 3
4 to 6
7 to 9
10 to 12
13 to 21
Days after mating

Fig. 10

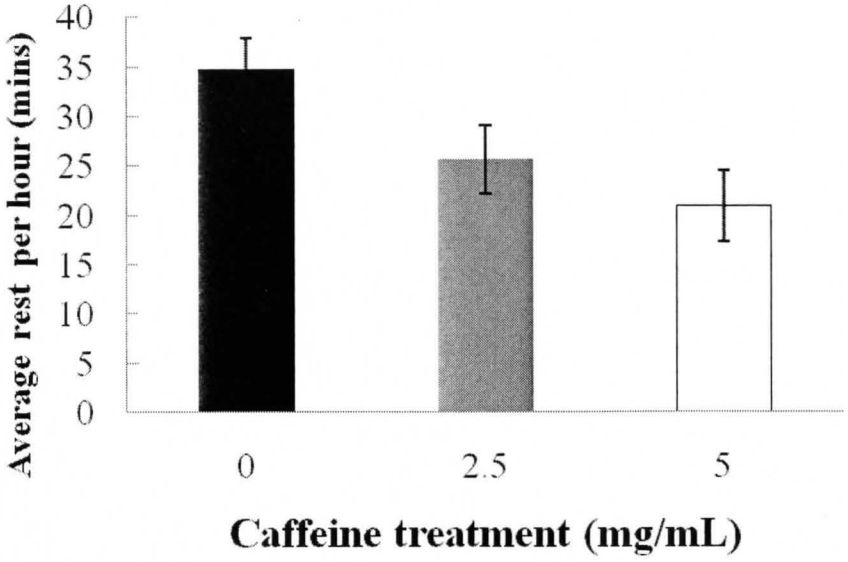

Average rest per hour (mins)
40
35
30
25
20
15
10
5
0
0
2.5
5
Caffeine treatment (mg/mL)

Fig. 11

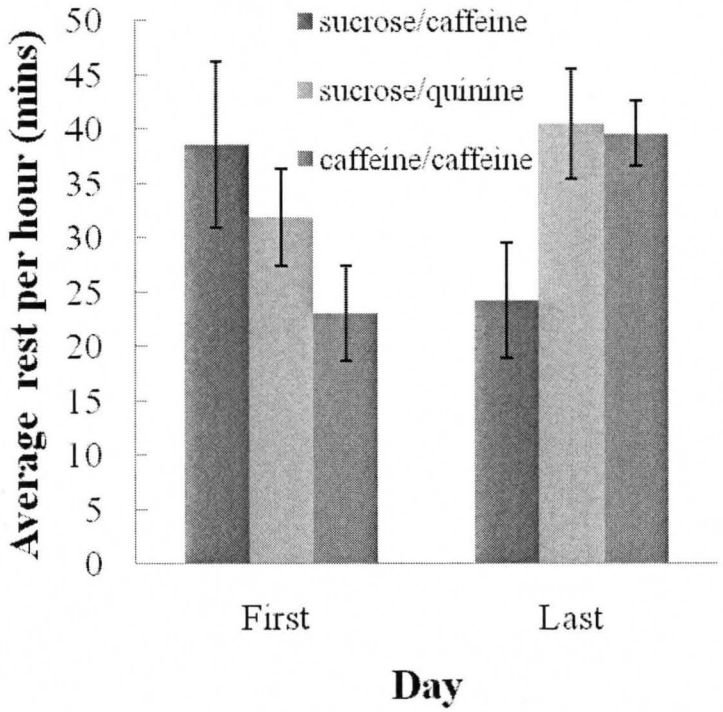

Average rest per hour (mins)
50
45
40
35
30
25
20
15
10
5
0
sucrose/caffeine
sucrose/quinine
caffeine/caffeine
First
Last
Day

Fig. 12

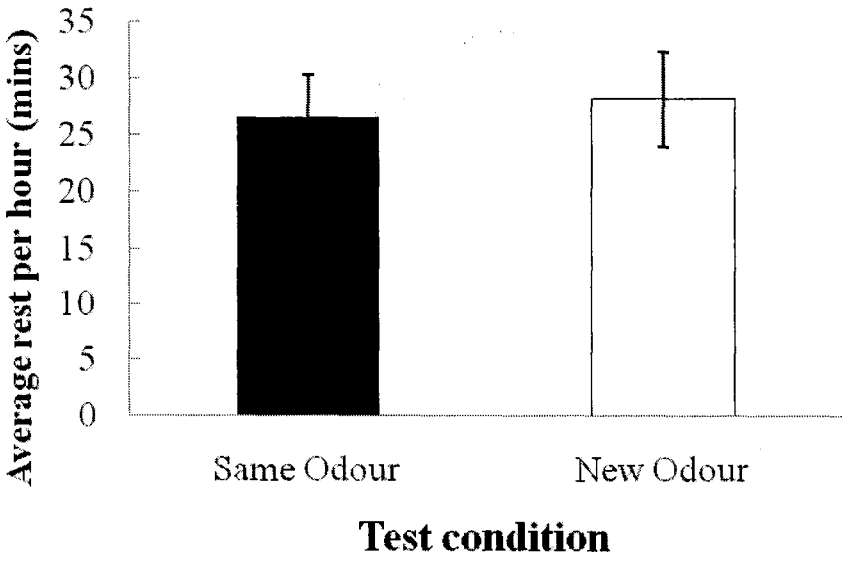

Average rest per hour (mins)
35
30
25
20
15
10
5
0
Same Odour
New Odour
Test condition

Fig. 13

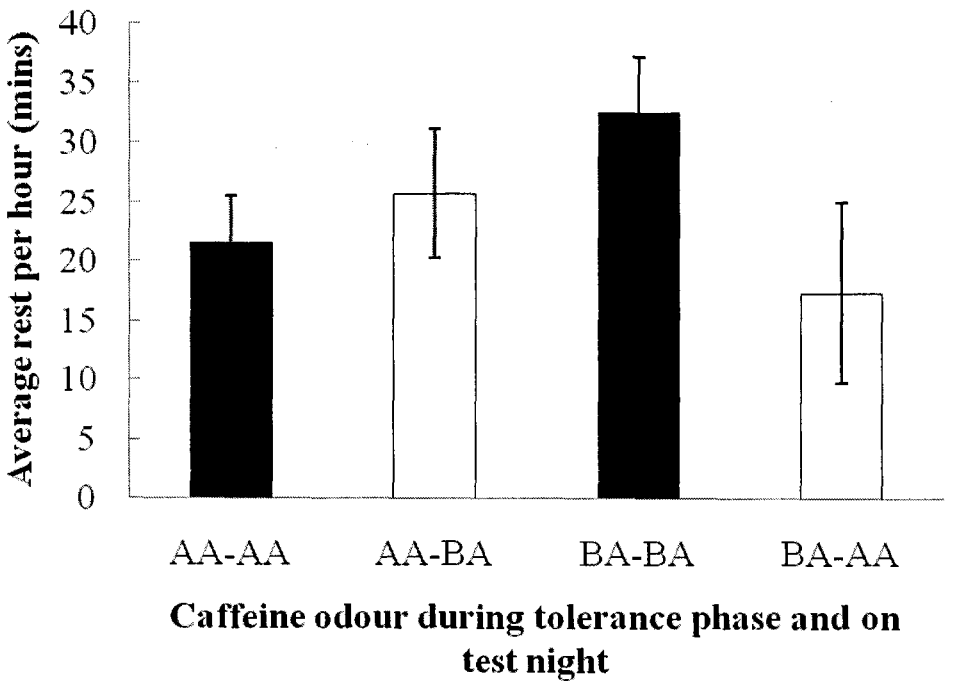

Average rest per hour (mins)
40
35
30
25
20
15
10
5
0
AA-AA
AA-BA
BA-BA
BA-AA
Caffeine odour during tolerance phase and on test night

Fig. 14

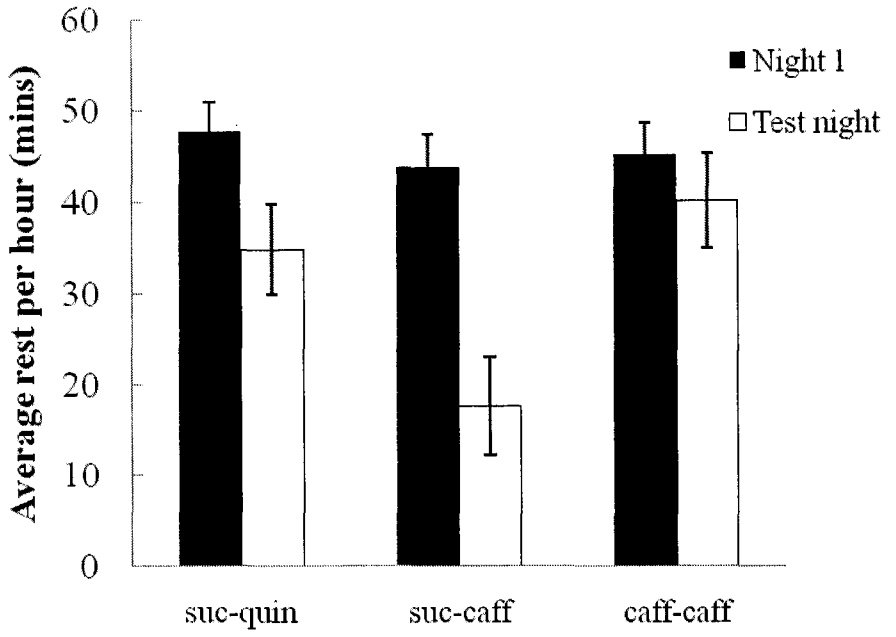

60
50
40
30
20
10
0
Average rest per hour (mins)
Night 1
Test night
suc-quin
suc-caff
caff-caff
Condtion during tolerance phase and on test night

Fig. 15

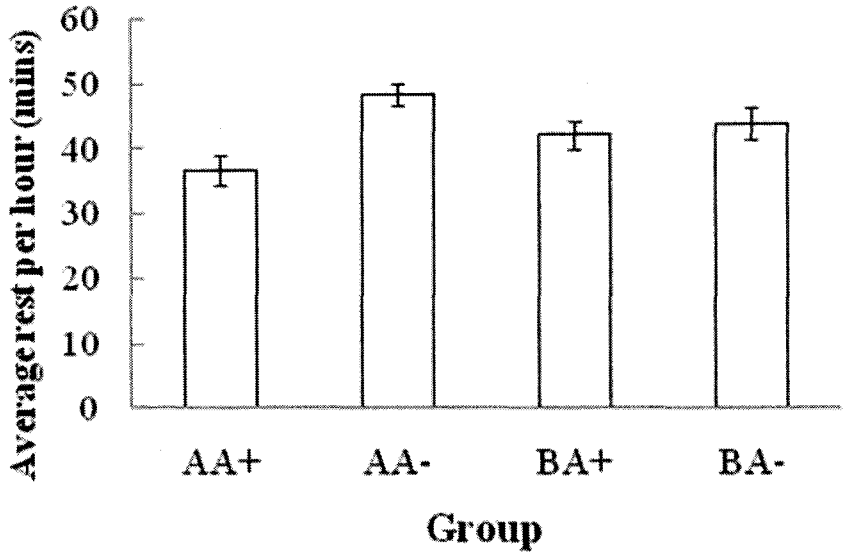
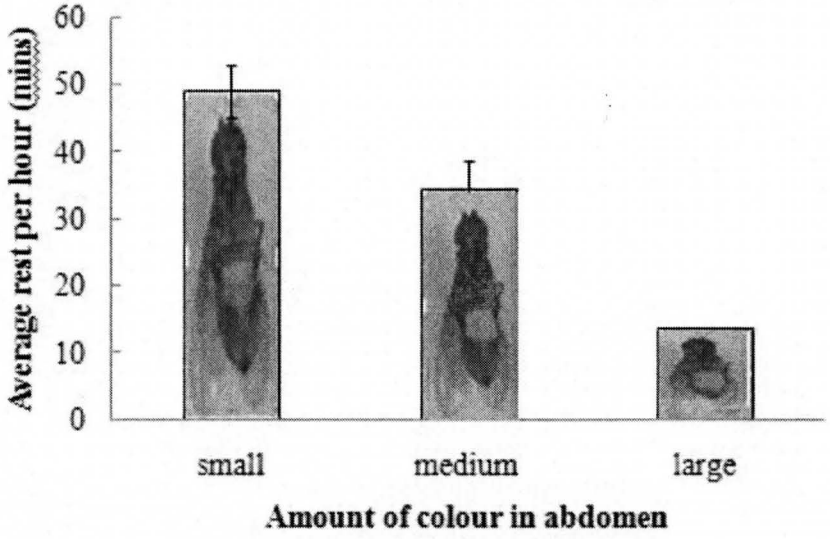

60
50
40
30
20
10
0
Average rest per hour (mins)
AA+
AA-
BA+
BA-
Group

Fig. 16


Average rest per hour (mins)
60
50
40
30
20
10
0
small
medium
large
Amount of colour in abdomen