Anxiety-like behaviour is regulated independently from sex, mating status and the sex peptide receptor in *Drosophila melanogaster*

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A major goal in behaviour research is to understand why and how sex differences in cognitive-emotional states like anxiety arise and are regulated throughout life. Anxiety allows individuals to detect and respond to threats. Mating is a candidate regulator for anxiety because threats are likely to change, often in sex-specific ways, when individuals shift to a postmating reproductive state. However, we know little about how mating mediates anxiety-related behaviour in males and females, or about how males might influence female anxiety via seminal proteins transferred during mating. To address this gap, we examined anxiety-related behaviour in the fruit fly *Drosophila melanogaster*, an emerging model animal for anxiety, with respect to sex, mating and sex peptide, a seminal protein known to modulate a host of female postmating responses in fruit flies. We assayed anxiety-like behaviour using the open-field assay to assess individual avoidance of the interior of an arena (‘wall-following’ behaviour). We found sex differences in activity level, but no evidence for sex differences in wall-following behaviour. We found no effects of mating in either sex, or of the presence of the sex peptide receptor in females, on wall following. Our results suggest that anxiety is not one of the cognitive-emotional states regulated by mating and sex peptide in fruit flies, and that researchers need an alternative model for sex differences in anxiety.

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finches, Taeniopygia guttata (Kullberg, Jakobsson, Kaby, & Lind, 2005) and green-veined white butterflies, Pieris napi (Almbro & Kullberg, 2012). Because mating reshapes behaviour and physiology in females much more than in males, mating might mediate sex differences in anxiety.

Furthermore, males in some species appear to manipulate female postmating responses for their own benefit (Arnqvist & Rowe, 2005). Males might benefit from manipulating female anxiety; for example, a female's optimal level of anxiety manages risks to her lifetime reproductive success, whereas a male does best if his mates maximize immediate offspring output before remating, setting up the potential for conflict. Female animals often do show profound changes in diverse behaviours after mating (Hopkins, Avila, & Wolfer, 2018), including behaviour related to cognitive-emotional state. In some insects, mating affects female behaviour: it impacts long-term memory retention (Scheunemann, Lampin-Saint-Amaux, Schor, & Preat, 2019), reduces sleep (Isaac, Li, Leedale, & Shirs, 2009), increases locomotion (Brutscher, Baer, & Niño, 2019) and alters aggression towards conspecifics (Bath et al., 2017; Chamorro-Florescano, Favila, & Macias-Ordoñez, 2017; Papadopoulos, Carey, Liedo, Müller, & Sentürk, 2009). In other insects, mating influences male behaviour: for example, male parasitoid wasps show altered locomotion after mating (King & Owen, 2012). Furthermore, studies in vertebrates and insects have reported mating-induced changes in neural tissue in both sexes (e.g. Alvarado-Martinez; Paredes, 2018; Ellis & Carney, 2010; Imonmen, Sayadi, Bayram, & Arnqvist, 2017; Xu et al., 2019), suggesting that mating-induced changes in cognition and emotion might be more widespread than currently appreciated. However, whether mating induces shifts in anxiety-related behaviour in males and females remains unknown.

We addressed this knowledge gap by investigating anxiety-like behaviour in the fruit fly Drosophila melanogaster. Fruit flies are a promising model for anxiety (Iliadi, 2009; Kaur, Simon, Chauhan, & Chauhan, 2015; Perry & Baciadonna, 2017). They display anxiety-like behaviour that shares environmental sensitivity, and neuro-chemical and genetic regulation, with vertebrates (Besson & Martin, 2005; de la Flor et al., 2017; Iliadi, 2009; Lebretón & Martin, 2009; Mohammad et al., 2016). Fly anxiety-like behaviour is often measured in open field tests where individuals can choose between exposed and sheltered areas (Doria, Morand-Ferron, & Bertram, 2019; Finn, Rutledge-Gorman, & Crabbe, 2003; Perals, Griffin, Bartomeus, & Sol, 2017). Like rodents and humans, fruit flies prefer to stay close to the arena perimeter, and more anxious individuals spend more time close to it (‘wall following’; Kallai et al., 2007; Lebretón & Martin, 2009; Mohammad et al., 2016; Simon, Dupuis, & Costentin, 1994).

However, whether fruit flies exhibit sex differences in anxiety, and their magnitude, remains unclear. Male and female fruit flies differ in habitat use (Taylor & Keck, 1988) and in body size, which might generate sex-specific risk and vulnerability, and thereby sex differences in anxiety. Some studies report sex differences in the wall-following anxiety-like behaviour of fruit flies, but these differences are often small and inconsistent. Some studies report higher wall following by females (Besson & Martin, 2005; Liu, Davis, & Roman, 2007), but others report no sex differences (Lebretón & Martin, 2009; Martin, 2004) or differences that vary with nutritional state (Argue & Neckameyer, 2013). These discrepancies in reported sex differences might relate to variation in arena design, with more recent studies using round arenas instead of the square ones from earlier work (Besson & Martin, 2005; Lebretón & Martin, 2009; Martin, 2004) because fruit flies prefer to spend time in the darkened corners of square arenas (Soibam et al., 2012), which might disrupt measures of wall following. Moreover, previously observed sex differences in anxiety-like behaviour might have been caused by sex differences in responses to stress (e.g. Harris, D’Eath, & Healy, 2008), with previous assays conducted in stressful conditions (arenas devoid of food or moisture).

It is also unclear whether mating impacts anxiety-related behaviour in fruit flies. Mating induces a shift in both habitat use (Prokopy & Roitberg, 1984) and egg load (Sirot, Wong, Chapman, & Wolfer, 2015), which might influence risk and vulnerability. Many postmating changes in female behaviour have been detected in fruit flies (Sirot et al., 2009), with many changes regulated by sex peptide, a male accessory gland protein transferred during copulation (Sirot et al., 2015). Mating in general and sex peptide in particular are therefore promising candidate regulators for sex differences in anxiety-like behaviour in fruit flies. If fruit fly anxiety is indeed regulated by sex or by mating, flies offer significant experimental advantages for uncovering the genetic, developmental and neurobiological bases for sex-specific anxiety, including high-throughput behavioural phenotyping and advanced molecular and genetic tools (Anholt & Mackay, 2004; Neville & Goodwin, 2012; Sokolowski, 2001).

Here, we investigated sex differences in wall-following anxiety-like behaviour and how they are mediated by mating and sex peptide in D. melanogaster. To do this, we assayed wall following in two experiments. In the first, we compared the effect of mating on wall following in males and females. In the second, we compared the effect of mating on wall following in females that were deficient in the sex peptide receptor gene (hereafter, SPR- females) or in genetically matched control females. SPR- females do not bind sex peptide transferred from males and hence show disrupted post-mating responses, as well as a reduction in sleep independently from mating (Oh et al., 2014). We measured total locomotion so that we could relate any differences in wall following to overall activity level.

METHODS

Fly Stocks and Culture

We used flies from an outbred, laboratory-adapted Dahomey genetic background (Partridge & Farquhar, 1983). To obtain adult flies for each experiment, we collected eggs from population cages and raised larvae at a standardized density on standard fly food medium (Clancy & Kennington, 2001). Emerging adults were collected as virgins within 8 h of eclosion using ice anaesthesia and housed in same-sex vials containing food media in groups of 10. Flies were maintained and experiments conducted at 25 °C on a 12:12 h dark:light cycle.

SPR- flies bore the genetic deficiency Df(1)Eelix6234, a deletion that covers the sex peptide receptor gene and four adjacent genes of unknown function (Yapici, Kim, Ribeiro, & Dickson, 2008). We used an SPR- stock backcrossed into a Dahomey genetic background, into which the w1118 allele (conferring white eye colour) had been backcrossed. The Df(1)Eelix6234 carries a white+ transgene that partially rescues the w1118 phenotype, such that SPR- flies had red eyes. We used the genetically matched, white-eyed w1118 Dahomey flies as controls to allow us to easily distinguish SPR- flies from controls (see also Dean, Perry, Pizzari, Mank, & Wigby, 2012; Perry et al., 2016). White-eyed flies have impaired vision, which can affect locomotion and other behaviours (Krstic, Boll, & Noill, 2013; Reed & Reed, 1950).

Experimental Design

In each experiment, we assigned flies to a mating or nonmating treatment and then assayed their anxiety-like behaviour in 10 min filmed trials, following protocols in flies where this is sufficient...
time to detect treatment differences (e.g. Argue & Neckameyer, 2013; Burnet, Burnet, Connolly, & Williamson, 1988; de la Flor et al., 2017; Liu et al., 2007; Mohammad et al., 2016; Soibam et al., 2012). Previous research in chipmunks, Tamias striatus, and mice, Mus musculus, indicates that results from shorter trials are consistent with those from longer ones (Montiglio, Garant, Thomas, & Réale, 2010). We conducted the experiment over successive days.

Experiment 1: sex and mating effects

to test for sex differences in wall following, and whether sex differences depend on mating, we assayed behaviour in mated and unmated virgin males and females in a fully factorial design. For the mating treatment, pairs of 2-day-old male and female flies were transferred via gentle aspiration into vials containing food media, and observed until mating occurred. We recorded the latency to and duration of mating. Pairs that did not mate within 5 h were discarded. Following mating, flies were separated into individual vials containing food media. Virgin flies were handled in an identical manner, being transferred to new vials containing food media twice throughout the same period as mated flies. All flies in this experiment were derived from the Dahomey stock population and had red eyes. Sample sizes were 37 virgin males, 28 mated males, 40 virgin females and 31 mated females.

Experiment 2: role of the sex peptide receptor

to test whether SPR mediates female wall following, we compared the behaviour of SPR- and control females that were experimentally assigned to a mating or nonmating treatment. The mating treatment was conducted as described above. We used male flies from the control Dahomey background. Sample sizes were 37 virgin control females, 32 mated control females, 31 virgin SPR-females and 41 mated SPR-females.

Behavioural Assays

We assayed wall following in 3-day-old flies, 1 day following mating. We followed an open-field protocol to measure the proportion of time flies spent near the perimeter or centre of an arena (Besson & Martin, 2005; Iliadi, 2009; Mohammad et al., 2016). To do this, each fly was placed in a petri dish (inner diameter 54 mm) that was partially filled with agar medium to leave 5 mm between the agar surface and lid. Circular arenas elicit more activity than square arenas, as animals tend to spend more time without moving in the corners of square arenas (Liu et al., 2007; Soibam et al., 2012), such that it is unclear whether preference for wall following or preference for corners varies across treatments. We placed filter paper that had a marked central inner zone (diameter 36 mm) onto the agar to form a dip surface. Preliminary trials indicated that this division of space was effective in capturing fly movement along the perimeter versus into the centre. We used a fresh arena and filter paper for each trial. Flies were transferred into arenas by gentle aspiration and, after a 5 min acclimation period, were recorded for 10 min using digital cameras (Toshiba Camileo X400). All trials were conducted between 1 and 7 h Zeitgeber time.

A single observer scored videos using JWatcher (v 1.0; Blumstein & Daniel, 2007). Scores were recorded blind to the mating treatment, but sex and eye colour were visible. We recorded movement and location and calculated (1) the proportion of each 10 min trial spent in the outer zone; (2) the proportion of total moving time spent in the outer zone, and (3) the total time spent moving.

Analyses

We used two measures of anxiety-like behaviour: the proportion of time spent in the outer zone (of the 10 min trial) and the proportion of time spent moving in the outer zone (of the total time spent moving). The former is proportional to another commonly used measure, the number of crosses flies make into the centre zone (Martin, 2004). For experiment 1, we tested for effects of sex, mating and their interaction (as fixed factors) on these measures using quasibinomial generalized linear models (using the R package ‘lme4’; Bates, Maechler, Bolker, & Walker, 2015). We weighted the proportion of time spent moving in the outer zone by the total time spent moving. We included time of day as a polynomial covariate and its interaction with sex because males and females have distinct activity patterns across the day (Isaac, 2019; Isaac et al., 2009). We initially included trial date as a random factor, but it explained little variance in behaviour (0.000% for the proportion of time spent moving in the outer zone), so we removed it from final models. We tested for effects of these same factors on total movement time using a generalized linear model with a gamma distribution. For experiment 2, we tested for effects of SPR genotype, mating and their interaction on measures of anxiety and total movement time, using analogous models. We again included time of day as a polynomial covariate. We used the ‘outlierTest’ function in the ‘car’ package in R to identify outliers for each model using a Bonferroni outlier test (Fox & Weisberg, 2019). We identified several outliers (experiment 1: N = 2 for wall-following behaviours, N = 4 for total time moving; experiment 2: N = 1 for wall-following behaviours, N = 4 for total time moving) and winsorized these outliers using the Winsorize function in the ‘DescTools’ package (Signorelli et al., 2019). Analyses were conducted in R (version 3.6.1; R Core Team, 2016).

RESULTS

Experiment 1: Sex and Mating Effects

Wall-following behaviour

We found no evidence for differences in wall following between the sexes or between virgin and mated individuals, nor evidence for an interaction between sex and mating status, for our two measures of wall following (proportion of time spent in the outer zone: Fig. 1a: sex: χ²(1,129) = 1.04, P = 0.31; mating status: χ²(1,129) = 0.02, P = 0.89; interaction: χ²(1,129) = 0.001, P = 0.97; proportion of moving time spent in the outer zone: Fig. 1b: sex: χ²(1,129) = 1.21, P = 0.27; mating status: χ²(1,129) = 1.35, P = 0.21; interaction: χ²(1,129) = 0.91, P = 0.34). When we restricted the analyses to individuals that spent at least half of the trial moving, the results were qualitatively similar, suggesting that inactive individuals did not influence this result.

We found some evidence that the extent of wall following varied nonlinearly throughout the day, with an initial increase followed by a decrease and then a smaller increase (time of day: cubic): proportion of time spent in the outer zone: χ²(3,129) = 11.67, P = 0.008; proportion of moving time spent in the outer zone: χ²(3,129) = 9.16, P = 0.03). As with wall following, we found similar results when we restricted the analyses to individuals that spent at least half of the trial moving.

Time spent moving

Males spent more time in motion than did females (χ²(1,129) = 8.33, P = 0.004; Fig. 1c). There was no evidence for an effect of mating on moving time (χ²(1,129) = 0.48, P = 0.49), nor evidence for an interaction between sex and mating (χ²(1,129) = 0.20, P = 0.65). There was no detectable effect of time of day on the total time spent moving (χ²(3,129) = 2.73, P = 0.43). We found qualitatively similar results when restricting our analysis to individuals that spent at least half the trial in motion.
Experiment 2: Role of the Sex Peptide Receptor

Wall-following behaviour

We predicted that if wall-following behaviour is mediated by sex peptide transferred during copulation, then we would observe an interaction between mating and SPR-genotype. We found no evidence for an interaction for either measure of wall following (proportion of time spent in the outer zone: Fig. 2a: \( \chi^2_{1,134} = 0.45, P = 0.50 \); proportion of moving time spent in the outer zone: Fig. 2b: \( \chi^2_{1,134} = 0.68, P = 0.41 \)). However, SPR-females engaged in more wall following than did control females regardless of mating, spending relatively more time in the outer zone than control females (proportion of time spent in the outer zone: Fig. 2a: \( \chi^2_{1,134} = 18.15, P < 0.001 \); proportion of moving time spent in the outer zone: Fig. 2b: \( \chi^2_{1,134} = 27.14, P < 0.001 \)). We found no evidence for an effect of mating on wall following (proportion of time spent in the outer zone: Fig. 2a: \( \chi^2_{1,134} = 0.00, P = 0.97 \); proportion of moving time spent in the outer zone: Fig. 2b: \( \chi^2_{1,134} = 0.21, P = 0.65 \)), consistent with experiment 1. We found no evidence for time of day effects (proportion of time spent in the outer zone: \( \chi^2_{3,134} = 4.83, P = 0.18 \); proportion of moving time spent in the outer zone: \( \chi^2_{3,134} = 6.36, P = 0.10 \)), although the pattern appeared similar to that of experiment 1.

Time spent moving

There was no evidence for effects on overall activity of mating (\( \chi^2_{1,134} = 1.25, P = 0.26 \); Fig. 2c), SPR-genotype (\( \chi^2_{1,134} = 0.64, P = 0.42 \)), their interaction (\( \chi^2_{1,134} = 0.05, P = 0.82 \)) or time of day (\( \chi^2_{3,134} = 0.35, P = 0.95 \)).
DISCUSSION

We investigated sex differences in wall following, a commonly used measure of anxiety-like behaviour (Finn et al., 2003), and its regulation by mating through the sex peptide pathway. We found no evidence for sex-specific wall following, and no evidence for regulation of wall following by mating in males or females, or by the sex peptide receptor in females. However, we found that control females, which had impaired vision, showed reduced wall following, consistent with a previous finding of reduced wall following by blind females (Besson & Martin, 2005). This result, together with our finding of differences in overall activity between the sexes, suggests that insufficient replication does not explain the absence of sex or mating effects. Our results suggest that anxiety is not one of the cognitive-emotional states regulated by mating and sex peptide in fruit flies.

We found no evidence for sex differences in wall-following behaviour. These results are consistent with the idea that male and female fruit flies display similar anxiety-like behaviour because they experience similar risks and vulnerability, factors predicted to shape adaptive anxiety (Bateson et al., 2011). Many studies of wall following in fruit flies, and in most animals, ignore sex. Evidence from studies of fruit flies that have looked for sex differences has been mixed, with some studies reporting more wall following by females (Besson & Martin, 2005; Liu et al., 2007), but others finding little support for sex differences (Lebreton & Martin, 2009; Martin, 2004). Another study found more wall following by females when flies had been deprived of food, but no sex differences when flies were satiated (Argue & Neckameyer, 2013). The variation among studies might be explained by unknown idiosyncratic differences in conditions between laboratories, or by genetic variation among fly populations. All studies used flies with a Canton-S background, but
some substrains of Canton-S show divergent behaviours (Columb & Brems, 2015). Alternatively, the outcomes from some studies might have been influenced by the square arenas used for observations (Besson & Martin, 2005; Lebretón & Martin, 2009; Martin, 2004) because flies prefer the darkened corners of square arenas (Soibam et al., 2012). Furthermore, these previous studies of sex differences used observation arenas that lacked food or moist medium. These stressful conditions should have enhanced sex differences (e.g. Argue & Neckameyer, 2013; Harris et al., 2008) and make it difficult to attribute previous observations of sex differences to an innate difference versus a sex-specific stress response. Future studies of sex differences in other behaviours will help to shed light on whether male and female fruit flies show broadly similar cognitive-emotional states.

We did not find evidence that wall following is regulated by mating in males (experiment 1) or females (experiments 1 and 2). Our results are consistent with a previous study that reported no difference in wall following between virgin and mated female D. melanogaster (Martin, 2004), but that study had limited power to detect differences because it assessed only four flies for each treatment and used square arenas. Many other behaviours are influenced by mating in female fruit flies (Siot et al., 2005), including both activity level (Isaac et al., 2006; Martin, 2004) and behavioural differences in long-term memory (van Alphen & Swinderen, 2013). Mating-induced changes in female behaviour are sometimes hypothesized to stem from sexual conflict and male manipulation of females (Arnvist & Rowe, 2005); the absence of mating effects observed here suggests that males have little scope to influence female anxiety-like behaviour in fruit flies. An interesting direction for future study would be to test the hypotheses that male fruit flies do not influence female cognitive-emotional states because males would not benefit from doing so, or because females have evolved resistance to male influence on their emotions, such that no influence is detectable.

We detected decreased wall following by control females, which had reduced vision, compared with SPR- females. The decrease was similar in virgin and mated females, and hence not dependent on receipt of sex peptide, consistent with our observation that mating itself did not influence wall following. The difference between SPR- and control females was probably caused by the reduced visual ability of control females, consistent with the decreased wall-following behaviour of blind females (Besson & Martin, 2005). An alternative hypothesis is that wall following is regulated by SPR or any of the other four genes covered by the SPR deletion. An effect of SPR that is independent of mating is plausible: some female behaviours are regulated by the SPR pathway independently from mating (e.g. sleep; Oh et al., 2014). Disrupted sleep is associated with reduced cognitive function and behavioural disorders in flies and humans (van Alphen & Swinderen, 2013), suggesting that SPR might function as a link between sleep and anxiety-like behaviour in D. melanogaster.

We found that males spent more time in motion compared with females. Sex differences in movement in D. melanogaster vary with genetic background. Females tend to move more than males in Canton-S (Bégacem & Martin, 2006; Helfrich-Förster, 2000; Martin, 2004; see Martin, Ernst, & Heisenberg, 1999) and in a wild-derived population (Burnet et al., 1988). Mated females move more than males in Oregon-R flies, whereas virgin females and males show similar movement levels (Helfrich-Förster, 2000; Isaac et al., 2009). No sex differences in movement were detected in the Berlin strain (Helfrich-Förster, 2000) or in several inbred lines of unspecified genetic background (Fernández, Grant, Tulli, Karkowski, & McClearn, 1999). Male flies moved more than females in another inbred line (Fernández et al., 1999). Together, these results highlight the pronounced variation in behaviour across strains within D. melanogaster. Our study is the first, to our knowledge, to examine sex differences in overall movement in D. melanogaster with the Dahomey background. Our finding that mating had no effect on movement is inconsistent with the pattern in Canton-S flies (Helfrich-Förster, 2000; Isaac et al., 2009), but consistent with results in the Oregon-R and Berlin strains (Helfrich-Förster, 2000; see also Isaac’s (2019) study of patterns of female group movement following mating).

Overall, our work suggests that sex differences in anxiety are minimal in fruit flies, that there is little evidence for a postmating change in anxiety-like behaviour as part of the shift to a reproductive state and that there is little scope for male manipulation of female anxiety-related behaviour in fruit flies. It will be of interest to test how general these findings are in other species. Understanding sex differences in anxiety has the potential for applied value because human populations show sex differences in the frequency and severity of anxiety symptoms (Feingold, 1994), with strong sex differences in how mental health disorders influence health and longevity (Murray & Lopez, 1997). Fruit flies are a useful animal model for psychiatric conditions generally (van Alphen & Swinderen, 2013) and an emerging model for anxiety specifically (e.g. Mohammad et al., 2016). However, our results suggest that alternative animal models are needed to recapitulate the sex differences in anxiety observed in humans. Further study is needed to determine whether anxiety-related behaviour in D. melanogaster is modulated by aspects of the social environment apart from mating.

**Data Availability**

The data for all experiments in this paper are publicly available on the Oxford University Research Archive (doi: https://doi.org/10.5287/bodleian:pdz8dD60x).

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