ROVER/SITTER POLYMORPHISM & SEXUAL SELECTION IN FRUIT FLIES

MAINTENANCE OF THE ROVER SITTER POLYMORPHISM AND THE EFFECT OF MALE-MALE COMPETITION ON MATING SUCCESS IN FRUIT FLIES

By JOSEPH MENTLIK, H. BSc.

A Thesis Submitted to the School of Graduate Studies in Partial Fulfillment of the Requirements for the Degree Master of Science

MASTER OF SCIENCE (2016) McMaster University
Psychology, Neuroscience, & Behaviour Hamilton, Ontario

TITLE: Maintenance of the rover sitter polymorphism and the effect of male-male competition on mating success in fruit flies

AUTHOR: Joseph Mentlik H. B.Sc. (McMaster University)

SUPERVISOR: Reuven Dukas, Ph.D.

NUMBER OF PAGES: vii, 71

Abstract

The fruit fly, *Drosophila melanogaster* is an exceptional model species for understanding both proximate and ultimate causes of variation in behaviour. Here, we have examined two behaviors: foraging and mating. We discuss these research projects in turn. Almost 40 years ago, the classic foraging behaviour polymorphism rover/sitter was first discovered. Recently, evidence has suggested that the polymorphism is maintained by negative frequency-dependent selection at the larval stage. In two separate experiments we failed to replicate negative frequency-dependence in the survival of rover and sitter larvae. However, we empirically demonstrated differences in burrowing behaviour between the rover and sitter morphs which may be a possible mechanism causing the negative frequency-dependent relationship between the morphs. Future replication of negative frequency-dependence is necessary if we are to understand the maintenance of this polymorphism in nature. We examined mating behaviour from the perspective of two mechanisms of sexual selection: female choice and male-male competition. The study of sexual selection requires careful isolation of these mechanisms. Owing to difficulties in experimental design, studies have yet to properly separate and quantify the individual effects of female choice and male-male competition in fruit flies. Here, we designed a novel arena to assess true female choice. We then used this arena to test the effect of male courtship interference on mating outcomes. However, due to an unforeseen amount of male harassment of females in the arena, we withhold any strong conclusions about the effect of male-male interference behaviour. We also attempt to demonstrate the potential for sexual selection on traits associated with interference behaviour in males.

Acknowledgments

I would like to thank Reuven Dukas for his guidance. I believe I have become a more critical thinker because of him. I would like to thank my committee members Ian Dworkin and Paul Andrews for their input. They have helped shape these projects. Additionally, I thank Ian for his help in the genotyping. To my lab mates: Carling Baxter, Andrew Scott and Blake Anderson. You were constantly helpful, and fun to hang out with every day in the office or the lab. I am grateful to the undergraduates Mohamed Djebli, Shehani Wickramasinghe and Ieta Shams for their hard work and to Kevin Abbott for many insightful conversations. I would also like to thank Miroslav Cika for contributing immensely to the design of the female choice arena. Lastly, I thank Emily Stacy. I couldn't have done it without her.

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CHAPTER 1: MAINTENANCE OF THE ROVER SITTER POLYMORPHISM

INTRODUCTION

The now classic difference between the two larval foraging strategies of the fruit fly, *Drosophila melanogaster*, was first described by Sokolowski (1980). The two phenotypic variants of this polymorphism are termed rover and sitter. The rover/sitter dichotomy is most detectable in 3rd instar larvae (Graf & Sokolowski, 1989) in a behavioural assay where rovers typically traverse a large area while foraging on a paste of live yeast, as opposed to sitters which traverse a smaller area. The difference in locomotion between the morphs disappears in the absence of food and is therefore thought to be foraging related (Graf & Sokolowski, 1989; Sokolowski, Kent, & Wong, 1984). Moreover, the difference in locomotion is not due to differential muscular ability between the morphs (Sokolowski & Hansell, 1992). Although the yeast paste behavioural assay described above is relatively simple, the behaviour in itself is a complex phenotype that can be the result of interactions between many genes and the surrounding environment. The resulting variation in foraging behaviour due to genetic and environmental influences, can be important in successful exploitation and exploration of food and as a result, competitive ability (Bakker, 1962). Indeed, it has been shown that the foraging behaviour of *D. melanogaster* larvae has a measurable effect on egg-to-adult viability (Ohnishi, 1979). It follows that understanding the cause of this variation in behaviour as well as its effect on an individual's fitness is of interest.

Much work has been done on establishing the genetic control of foraging behaviour in larvae. Initially, using isogenic lab stocks homozygous for the second and third chromosomes, Sokolowski (1980) showed that a substantial component of the phenotypic variation in foraging behaviour could be attributed to the influence of alleles present on the second chromosome. A hypothesis for a single gene two allele model was put forth and support has been garnered from several experiments. The first supporting evidence for a simple Mendelian model was shown using isofemale lines which are strains established from individual females collected in the field (Bauer & Sokolowski,

1984). Isofemale lines are useful because within lines they have little to no genetic variance, but between lines they display genetic variance as would be expected in a natural population. Bauer and Sokolowski (1984) tested larvae of 15 isofemale lines for foraging behaviour on yeast paste, after which flies of the two phenotypically extreme lines were crossed. The resulting phenotypes expressed in the reciprocal crosses and backcrosses followed a pattern of Mendelian ratios with rover being completely dominant over sitter and no sex-linked or maternal effects. Again, this variation was attributed to the second chromosome (Bauer & Sokolowski, 1985). Subsequently, a full complement of 16 reciprocal crosses between the isogenic sitter line from Sokolowski (1980) and isogenic rover line from Bauer & Sokolowski (1984), also supported a single major gene, segregating in a Mendelian ratio (de Belle & Sokolowski, 1987). Novel techniques using gamma irradiation allowed for the mapping of the rover sitter trait to the left arm of chromosome 2 (de Belle & Sokolowski, 1989); and further, generated 5 chromosomes of lethal tagged sitter mutants on rover genetic backgrounds (de Belle, Hilliker, & Sokolowski, 1989). Non-complementation among pairwise tests of 3 of these mutants identified the gene which was named *foraging* (*for*). Perhaps the best evidence for the role of *for* in foraging behaviour was shown by Osborne et al. (1997). Using restriction fragment length polymorphism analysis, they mapped *for* to the gene *dg2*, a cGMP-dependent protein kinase (PKG) gene. Over expressing *dg2* in sitter larvae induced rover like behaviour in a sitter genetic background showing that PKG is responsible for variation in rover-sitter foraging behaviour.

While the molecular and genetic evidence supporting a role of *dg2* in rover/sitter like variation in lab strains is strong, correlating evidence for discontinuous phenotypic variation in natural populations is weak and has several lines of evidence that do not support it. Under the hypothesis that variation in the phenotype is largely discontinuous and segregates in a manner consistent with a single locus with 2 alleles of large effect, foraging behaviour of larvae from natural populations of *D. melanogaster* should follow a bimodal distribution. There are several cases of natural populations that conform to a bimodal distribution, but contrastingly, there is also support for the phenotype following a

continuous distribution. The first report of the distribution of phenotypes in a natural population was described by Sokolowski (1980) comprising 70% rovers and 30% sitters. This ratio remained consistent over a sampling period of several weeks. However, the actual distribution of path lengths measured is not made known, and so does not provide evidence for bimodality. Soon after this, a similar sampling was undertaken of larvae from an orchard (Sokolowski, 1982). Here, a distribution of path lengths was reported showing distinct bimodality. However, there are several potential problems with this distribution. First, larvae were collected from the field and then tested and thus their behaviour may have had a large environmental component and is not necessarily the result of *for* (Turner, Giauque, Schrider, & Kern, 2014). Larvae were tested within a 24 hour window after collection from the field, but it is not specified whether they were tested all at once. So, rather than the environmental variation adding random noise to the distribution, larval behaviour may have changed over the course of testing in response to the lab environment. Especially if testing was partially done immediately following collection and then finished the following day. Second, the sitter peak in this distribution is largely larvae that crawled less than 1 cm, and the distribution of path lengths is otherwise relatively continuous. Because the data are binned, it is not known if this sitter peak is a result of larvae simply not crawling. A more clear bimodal distribution that lacks potential flaws of the above distribution (Sokolowski, 1982) was generated from larvae descended from a wild population caught a year prior to the behavioural test (Sokolowski, Pereira, & Hughes, 1997). The results of a complementation analysis using this wild population and the isogenic sitter from Sokolowski (1980) suggest that the variation exhibited in the bimodal distribution was a result of *for*.

On the other hand, there are several lines of evidence both from within the Sokolowski lab and from other labs that do not support the existence of a bimodal distribution in nature. There are several groups of isofemale lines that have been collected by the Sokolowski lab at different times that do not exhibit a bimodal distribution, but rather a continuous one (Bauer & Sokolowski, 1984; Carton & Sokolowski, 1992). Data from other labs also fails to support bimodality. Among a panel of 22 isofemale lines,

there was a continuous distribution of path lengths (Reed et al., 2010); and in fact, the rover strain (Bauer & Sokolowski, 1985) appears to be an outlier among this distribution. In addition, there are two distributions from larvae of the *Drosophila* Genetic Reference Panel (DGRP), a collection of 208 genetically identical inbred lines which display natural genetic variation between lines (Mackay et al., 2012). In our lab, a continuous distribution has been shown in the rate of 2nd instar larval movement over a ten minute period among 29 DGRP lines (Anderson, Scott, & Dukas, 2015). A similarly continuous distribution was shown by Turner et al. (2014) among 36 DGRP lines as well as the rover and sitter lines for reference. However, this distribution should be interpreted with caution. Their behavioural assay may have been biased as they only collected 3rd instar larvae that were burrowed in the food for testing. It is likely that they did this in order to avoid larvae in the wandering stage. Wandering larvae search for a pupation sites and so their locomotion is not foraging related. The Sokolowski lab likewise avoids wandering larvae, but only avoids larvae on the wall and ceiling of the larval rearing dish, and otherwise randomly samples larvae either in or on the food. As a possible consequence of the sampling bias, the path lengths of the Turner et al. (2014) distribution are much shorter than those from any other study. Indeed, the mean rover path was only 2.66 cm. Collectively, there are multiple distributions that argue against bimodality and thus against the hypothesis that there are alleles of large effect at the *foraging* locus in natural populations.

However, assuming that there really are alleles of large effect at the *foraging* locus existing in nature, the question of how the rover and sitter alleles could be maintained has only been partially explained. It was originally hypothesized that the heterogeneity of the environment caused disruptive selection on the rover and sitter phenotypes with rover being advantageous in a discontinuous environment and sitter in a continuous one (Sokolowski, 1980). Other mechanisms of maintenance have been suggested including wet and dry pupal microhabitats (Sokolowski, 1985), apostatic selection due to parasitoids (Carton & Sokolowski, 1992), and density dependent selection (Sokolowski et al., 1997). The most convincing hypothesis was recently tested empirically by Fitzpatrick, Feder, Rowe, and Sokolowski (2007). In this experiment they showed that variation at *for*

could be maintained through negative frequency-dependent selection acting on rovers and sitters during bouts of resource competition. In doing so, they also show that the rover and sitter alleles can be maintained in the absence of any of the above mentioned mechanisms.

Fitzpatrick et al. (2007) replicated negative frequency-dependence in three separate ways. All three experiments were performed using an 85% reduction in nutrition quality from the standard lab food to increase competition among larvae. Vials contained 32 rover and sitter larvae in three ratios 24:8, 16:16 or 8:24 of rover to sitter. In experiment 1, green fluorescent protein (GFP) was used to mark either rovers or sitters so that their survival could be assessed visually. To produce GFP strains, an *Ubiquitin* promotor fused with GFP was inserted on chromosome 3 and then this chromosome was substituted into either the for^R or for^s strains. Regardless of which strain was GFP marked, both rovers and sitters had their highest survival to pupation when they were least frequent in the population. In experiment 2, it was shown that the difference in survival was solely the effect of the *for* gene. They did this by using a wild type for^R marked with GFP and an unmarked mutant sitter $for^{s(2)}$. The mutant sitter $for^{s(2)}$ behaves as a sitter, but aside from a mutation at *for*, shares the rover genetic background. Again, negative frequency-dependent survival of both rovers and sitters was observed demonstrating the effect of *for*. In experiment 3, it was shown that the difference in survival was not an effect of GFP nor an epistatic effect from the mutation process in generating the mutant sitter $for^{s(2)}$. To show this, unmarked rover and sitters were used. Under the same conditions as experiment 1 and 2, larvae surviving to pupation were genotyped using polymerase chain reaction and a restriction digest in order to differentiate the for^R and for^s strains. The relative survival of each morph was again found to be frequency dependent. Overall, it was suggested that maintenance of both alleles in the population was due to the fact that variation for survival was driven by greater intra-morph competition than inter-morph. However, how this competition was generated and how it drove selection was not clear.

A possible mechanism to allow for greater intra- than inter-morph competition for food would be niche separation between the morphs (Barker, 1971; Parsons, 1975). Larvae segregate on the surface of food in two dimensions as seen in the classic foraging assay. In addition, there is some evidence that rover and sitter larvae also segregate in three dimensions by burrowing to different depths within the food. Based on indirect methods, Sokolowski (1982) suggested more digging behaviour by rover than sitter larvae. Conversely, B. Anderson (personal communication, 2015) had observed that sitters were more likely to remain burrowed during late development compared to rovers. Lastly, in homozygous larval rearing dishes of rovers and sitters, we personally observed different magnitudes of digging and dispersal. By chance we observed that 60 hours post-hatch 3rd instar rovers had dug less and dispersed to the edges of the larval rearing dish, whereas sitters dug more and were distributed evenly throughout the dish.

Overall orthologs of the *dg2* gene have been found to affect food-related behaviours and contributes to phenotypic plasticity in several species including *C. elegans*, *D. melanogaster*, *A. mellifera*, *P. barbatus*and *P. pallidula* (Ben-Shahar, 2002; Fujiwara, Sengupta, & McIntire, 2002; Ingram, Oefner, & Gordon, 2005; Lucas & Sokolowski, 2009). We are working towards understanding how polymorphism at the *foraging* locus is maintained in *D. melanogaster* and assessing its evolutionary significance as orthologs of the *dg2* gene may be phylogenetically widespread.

GENERAL METHODS

We obtained *Drosophila melanogaster* strains of rover and sitter from Marla Sokolowski at the University of Toronto (see table 1). We kept flies in vials at a density of ~30 flies per vial, each containing 5 mL of standard food (900 mL water, 90 g sucrose, 32 g yeast, 75 g cornmeal, 15.5 g agar, and 2 g of methyl paraben dissolved in 20 ml of 95% ethanol). We placed adults into new vials with fresh food every 6-7 days. We kept all flies in an incubator at 25°C and 60% relative humidity, on a 12:12 h light cycle with lights on at 01:00 h.

The following experiments required age matched larvae. In order to achieve this, egg collection consisted of two stages of egg laying, one right after the other. The initial stage of egg laying aimed to minimize hatching asynchronies between larvae as females can retain developing embryos while searching for oviposition sites. At 13:00 h, we placed rover and sitter adults separately into vials containing surface scraped, standard food and added live yeast to stimulate egg laying. Females were able to lay eggs for an hour. After the initial stage, adults were transferred to new vials containing only surface scraped, standard food. Here, we left females to lay eggs for an hour and a half.

All data were analyzed using R version 3.1.3 (R Development Core Team, 2015). The lme4 library was used for generalized linear mixed models (Bates et al., 2015) and the survival library was used for Cox regression (Therneau, 2015).

Table 1. Description of rover and sitter strains obtained from the Sokolowski lab.

Date Obtained	Phenotype	Strain Description	Associated Publication
Fall 2013	Rover	B15B15: derived from isofemale population, isogenic for chromosomes 2 and 3	Bauer & Sokolowski, 1985
	Sitter	E2E3: derived from ebony mutant, isogenic for chromosomes 2 and 3	Sokolowski, 1980
Spring 2015	Rover/sitter	Same as above	Same as above
Summer 2015	Rover/sitter	Same as above	Same as above
Summer 2015	Rover	Chromosome 2: B15 derived from isofemale population, Chromosome 3: *Ubiquitin* promotor fused with GFP	Fitzpatrick, 2007
	Sitter	Chromosome 2: E2 derived from ebony mutant, Chromosome 3: *Ubiquitin* promotor fused with GFP	Fitzpatrick, 2007

Experiment 1: Rover and sitter movement behaviour verification tests

RATIONALE

In order to do experiments on the rover and sitter variants, their behavioural traits had to be confirmed in a preliminary test which should align with those found originally by Sokolowski (1980). We tested four pairs of rover and sitter lines (Table 1). We hypothesized that in all pairs of rover and sitter strains, rovers would have longer path lengths than sitters.

METHODS

To obtain synchronous 2nd and 3rd instar larvae, at 16:00 h the day following egg collection as described above, we transferred the subsequent 1st instar larvae using a moist paintbrush to 8.5 cm diameter petri dishes containing 60 mL standard food at a density of 50 larvae per dish where we left them for 45 or 93 hours to reach 2nd and 3rd instar respectively.

At 10:00 h on the day of testing, we filled 8.5 cm test petri dishes with 20 mL of agar solution (450 ml water, 7.75 g agar). After the agar had solidified, we coated it with a thin homogeneous layer of aqueous yeast suspension (distilled water and Fleischmann's bakers' yeast in a 4:1 ratio by weight) applied by pouring liberally and then removing the excess. After coating the test dishes, we used a paintbrush to carefully separate the food medium of the rearing petri dishes and randomly chose larvae from each strain to be tested. We avoided using larvae on the dish ceiling. All manipulations of larvae and testing were done under red light so as to not affect the movement behaviour of the larvae (Godoy-Herrera, 1977). We ran trials four at a time, at 25°C and 80 ± 10% relative humidity. Larvae were able to forage for a duration of 5 minutes. At the end of the trial, we removed the larva from the test dish and took a photo of its path in the yeast paste. We measured path length using ImageJ 1.48v software using the measure tool. We compared mean path lengths with two sample t-tests. Welch two sample t-tests were used when variances between groups were unequal, tested for using a Bartlett test of homogeneity of

variances. We also observed digging behaviour among 3rd instars of the fall 2013 and spring 2015 lines. We analyzed this with generalized linear models with a binomial distribution and logit link. We modeled digging as a response variable predicted by morph.

In our first test of the fall 2013 lines, we tested 2nd instars. We assayed late 2nd instar larva as behavioural differences in movement between rover and sitter larvae are not significant at the 1st instar stage (Graf & Sokolowski, 1989) and we suspected that the increased digging behaviour in the 3rd instar stage would bias movement patterns. However, because 2nd instars showed little difference, tests 2-4 were performed on 3rd instars only.

RESULTS

For rovers and sitters of the fall 2013 lines, 2nd instar rovers did not crawl a significantly greater distance than 2nd instar sitters ($t = 0.92$; d.f. = 17; $p = 0.19$; Fig. 1a). However, 3rd instar rovers did crawl a significantly greater distance than 3rd instar sitters ($t = 6.14$; d.f. = 16.75; $p < 0.0001$; Fig. 1b). 42% of 3rd instar sitters dug down into the agar during the trial duration, whereas none of the rovers did so (Wald $\chi^2_1 = 8.2629$; $P < 0.01$).

For rovers and sitters of the spring 2015 lines, 3rd instar rover larvae crawled a marginally significant greater distance than 3rd instar sitter larvae ($t = 1.72$; d.f. = 26; $p = 0.048$; Fig. 1c). 10% of spring 2015 rovers dug into the agar whereas no sitters did so (Wald $\chi^2_1 = 1.4065$; $P = 0.2356$). Differences in path length between rovers and sitters of the fall 2013 population were replicable. When the fall 2013 lines were tested a second time, 3rd instar rovers crawled significantly greater distances than 3rd instar sitters ($t = 7.23$; d.f. = 37; $p < 0.0001$; Fig. 1c). 35% of fall 2013 sitters dug into the agar whereas no rovers did (Wald $\chi^2_1 = 10.81$; $P = 0.001$).

For rovers and sitters of the summer 2015 lines, unmarked 3rd instar rovers crawled a significantly greater distance than unmarked 3rd instar sitters ($t = 1.91$; d.f. = 85; $p = 0.03$; Fig. 1d). However, GFP marked 3rd instar rovers did not crawl a

significantly greater distance than GFP marked 3rd instar sitters ($t = 0.94$; d.f. = 76.79; $p = 0.18$; Fig. 1d).

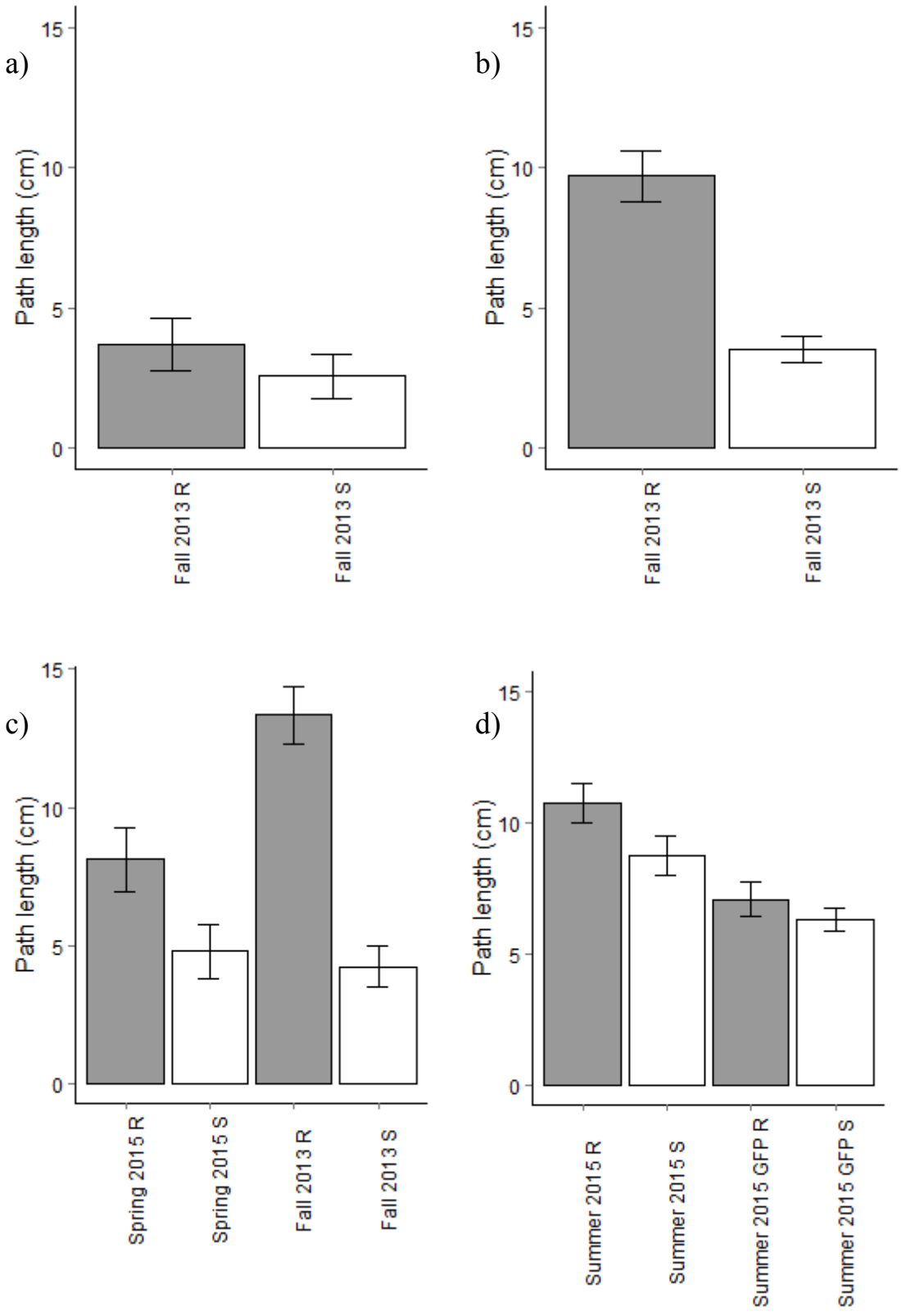
a)
b)
c)
d)
Path length (cm)
0
5
10
15
Fall 2013 R
Fall 2013 S
Spring 2015 R
Spring 2015 S
Summer 2015 R
Summer 2015 S
Summer 2015 GFP R
Summer 2015 GFP S

Fig. 1: Mean path length ± SE of rover and sitter larvae after 5 minutes on a paste of yeast. Data within each graph were collected in a single morning. a) 2nd instar rovers (N = 10) and sitters (N = 9) of the fall 2013 populations. b) 3rd instar rovers (N = 12) and sitters (N = 12) of the fall 2013 populations. c) 3rd instar spring 2015 rovers (N = 20) and sitters (N = 8) and fall 2013 rovers (N = 19) and sitters (N = 20). d) 3rd instar summer 2015 rovers (N = 44) and sitters (N = 43) and green fluorescent protein marked 3rd instar rovers (N = 45) and sitters (N = 46).

Experiment 2: Rover and sitter 3D spatial location in the food substrate

RATIONALE

Before proceeding with this experiment, we wished to first replicate the negative frequency-dependence as shown in Fitzpatrick et al. (2007). However, due to issues of timing in receiving GFP larvae, we went ahead with the spatial location experiment before the replication.

The behaviour verification tests of 3rd instars performed on the fall 2013 population (Fig. 1b, 1c) confirmed that the phenotypes were as expected. Paths lengths were comparable to those found in the literature using the same strains e.g. mean path length ± SE of rovers and sitters found in de Belle & Sokolowski (1987) were respectively 13.74 ± 0.84 and 3.71 ± 0.39. Therefore, it is unlikely that these lines had changed behaviourally through any contamination or substantial amount of genetic drift. However, the lines we received in the spring of 2015 displayed only a marginally significant difference in path length and so we rejected them in favour of the fall 2013 lines which, showed a replicable significant difference in path length (Fig. 1b, 1c). Consequently, we used the fall 2013 lines for both experiments 2 and 3.

Here, we wished to test if a difference in digging behaviour, whether it be spatially or temporally, allowed the two morphs to exploit separate niches. To test this hypothesis, we placed individual larvae in vials and observed their position in three dimensions over time. Based on preliminary personal observations, we predicted that sitters would spend a greater proportion of time burrowed in the food in the late 2nd and 3rd instar stages than rovers. And we predicted that sitters would be found in the center of the vial whereas rovers would be found at the edge of the vial.

METHODS

At 15:00 h the day following egg collection as described in the general methods, we collected synchronous rover and sitter larvae from our fall 2013 population. Using a moist paint brush we carefully transferred newly hatched larvae individually into vials

(N=50 vials per morph). For the duration of the experiment, we kept these larvae in the incubator at 25°C and 60% relative humidity, on a 12:12 h light cycle with lights on at 01:00 h with the exception of brief periods of observation.

To mimic the conditions used by Fitzpatrick et al. (2007) when frequency-dependant selection was found, each vial contained 5 mL of low nutrition food (900 mL water, 13.5 g sucrose, 4.8 g yeast, 15.5 g agar, and 2 g of methyl paraben dissolved in 20 ml of 95% ethanol).

We recorded all observations blind to larval genotype beginning the day after transferring newly hatched larvae to vials. We recorded observations at 8:00 h, 12:00 h, and 16:00 h for seven days. After that, we continued to record observations once a day at 8:00 h until larvae reached pupation or twelve days post hatching. We scored digging behaviour as a binary response: yes or no with digging defined as larval mouthparts below the surface of the food and an absence of crawling (Durisko, Kemp, Mubasher, & Dukas, 2014). We also scored the location of the larvae as a binary response: centre or edge with edge defined as being within 1 mm from wall of the vial regardless of whether the larva was on the surface of or within the food substrate. We scored pupation once the larva stopped wriggling and turned brown in colour (Bainbridge & Bownes, 1981). All observations were done under red light so as to not affect the digging or movement behaviour of the larvae (Godoy-Herrera, 1977).

We analyzed the first seven days of digging behaviour and location as more than half of the larvae had pupated by 16:00 h on day seven. We used a generalized linear mixed model with a binomial distribution and logit link function in a repeated measure design. In our full model, binomial response variables digging (Table 2) or location (Table 3) were predicted by the fixed effects time, a quadratic transformation of time, genotype and both of the time by genotype interactions. A random effect was included allowing the intercept as well as time to vary by individual larvae. To test for significance of the fixed effects, we used the Anova function from the Car package (Fox & Weisberg, 2011) testing for type III sums of squares. In a separate analysis, we analyzed survival to pupation

using a Cox regression from the survival package (Therneau, 2015). Here, genotype was the only fixed effect, tested for again using the Anova function.

RESULTS

Overall, sitters spent a greater proportion of their time burrowed in the substrate than rovers (Wald $\chi^2_1 = 9.78$, $P < 0.002$; Fig. 2.1). However, between the two morphs, we found only weak support for a difference in the rate of change in digging behaviour throughout development (Genotype by quadratic time interaction: Wald $\chi^2_1 = 3.33$; $P = 0.06$; Fig. 2.1).

Neither morph displayed an overall preference for the edge (Wald $\chi^2_1 = 1.82$, $P = 0.178$; Fig. 2.2). However, the proportion of larvae found at the edge of the vial for each genotype did change slightly over time with rovers spending more time at the edge later in development (Genotype by time interaction: Wald $\chi^2_1 = 7.17$, $P = 0.007$; Fig. 2.2).

There was no significant difference in pupation latency between the rovers and sitters (Cox regression: Wald $\chi^2_1 = 0.05$, $N = 93$, $P = 0.8$; Fig. 2.3).

Table 2. Fixed effects of the generalized linear mixed model for larval digging behaviour.

Fixed effect	Chi-square value	DF	P-value
Intercept	54.69	1	<0.001
Genotype	9.78	1	0.002
Time	94.17	1	<0.001
Time^2	56.77	1	<0.001
Genotype x Time	0.21	1	0.650
Genotype x Time^2	3.33	1	0.068

Table 3. Fixed effects of the generalized linear mixed model for larvae location.

Fixed effect	Chi-square value	DF	P-value
Intercept	19.82	1	<0.001
Genotype	1.82	1	0.178
Time	6.56	1	0.010
Time^2	6.63	1	0.010
Genotype x Time	7.17	1	0.007
Genotype x Time^2	0.42	1	0.518

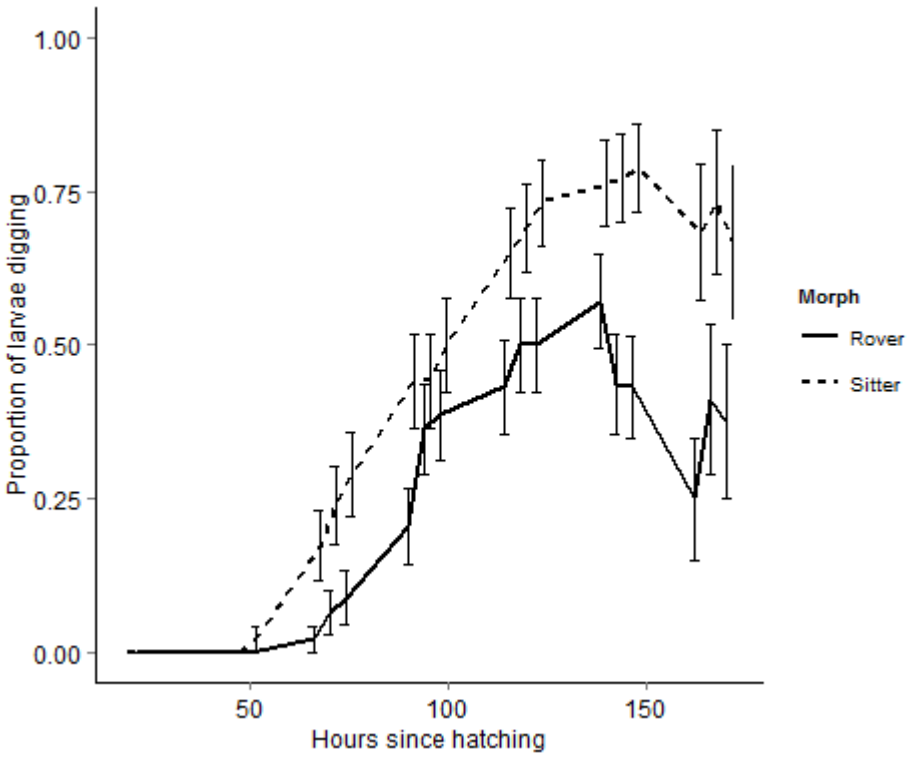

Fig. 2.1: The proportion of rover (N=47) and sitter (N=46) larvae burrowed into the substrate throughout development.

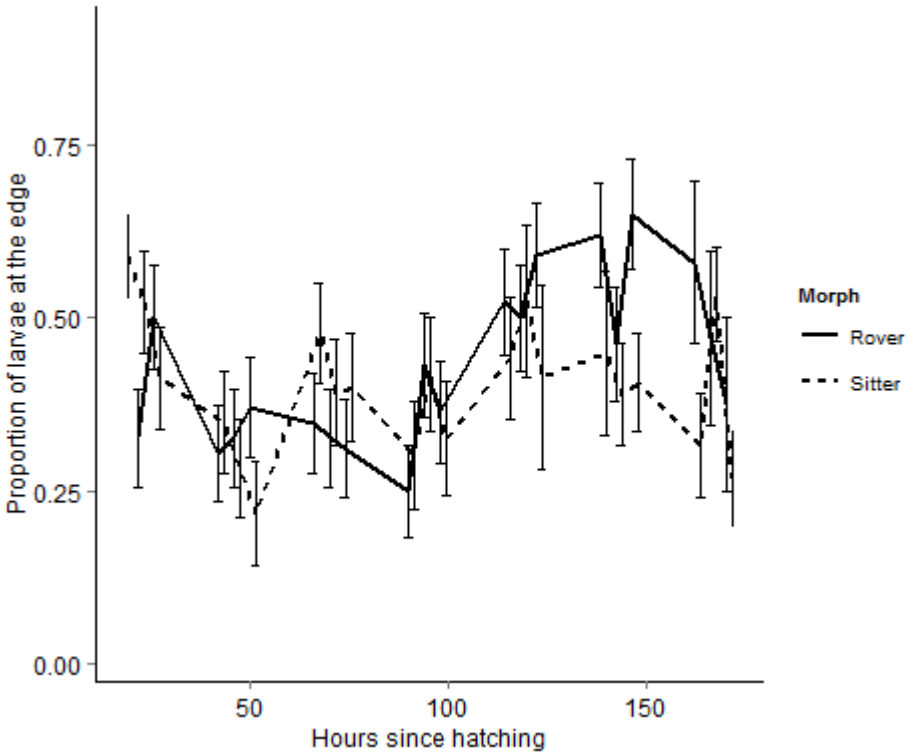

Fig. 2.2: The proportion of rover (N=47) and sitter (N=46) larvae positioned at the edge of the vial throughout development.

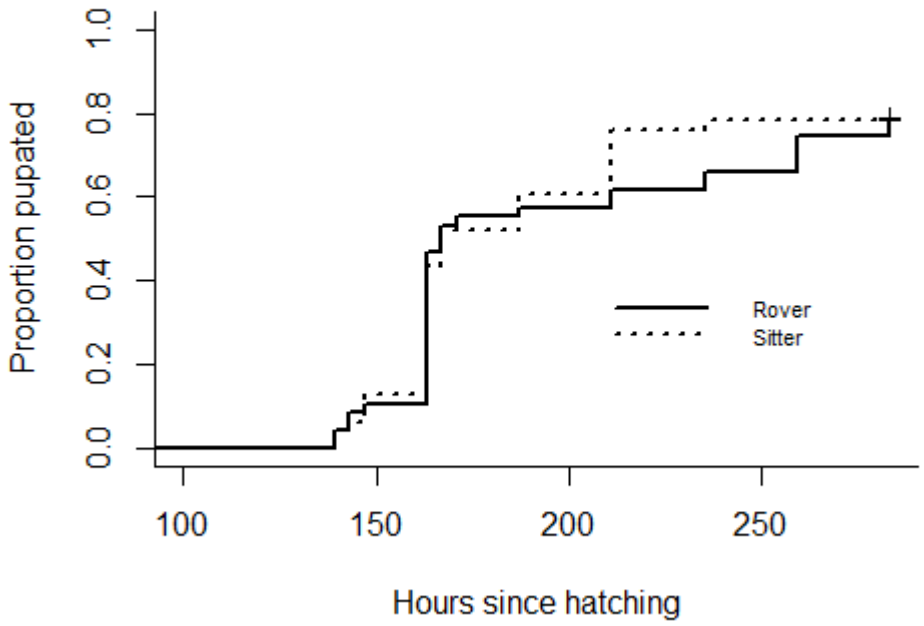

Fig. 2.3: The cumulative proportion of rover (N=47) and sitter (N=46) larvae pupating over time.

Experiment 3: Pilot of frequency dependent survival in rovers and sitters

RATIONALE

Before obtaining the GFP rovers and sitters to replicate Fitzpatrick et al. (2007), we ran a smaller scale pilot assessing frequency dependence. The pilot design comprised three frequencies of the morphs: only rover, only sitter or equal parts rover and sitter. Under the hypothesis of negative frequency-dependence, we predicted that in the treatment with an equal number of rovers and sitters, total larval survival should be higher than in either treatment containing a single morph.

METHODS

We seeded 15% nutrition food vials (N=33) with 32 synchronous 1st instar larvae in one of three frequencies (1:0, 1:1 and 0:1) of rover to sitter. In addition, vials contained one of three levels agar altering food hardness relative to our standard food (50%, 100% and 150%). We measured survival to pupation as an approximation of fitness (Rodriguez, Sokolowski, & Carton, 1991) and observed pupation for 13 days post hatch, counting and removing pupa from vials each day at 10:00 h. We observed for 13 days as development is delayed from standard because vials contain poor nutrition food.

We analyzed the data using generalized linear mixed effects model with a binomial distribution and a logit link function. We modeled the proportion of larvae pupating as the dependent variable and we modeled as fixed effects: the frequency of morphs and time. To account for repeated measures, we included a random effect allowing the intercept as well as time to vary by vial. We tested for the significance of fixed effects using the Anova function.

RESULTS

The mean proportion of larvae pupating in the sitter alone treatment was 8% greater than in the rover + sitter treatment and 9% greater than in the rover alone

treatment. However, the main effect of frequency was not significant. (Wald $\chi^2_2 = 5.51$, P = 0.06; Fig. 3).

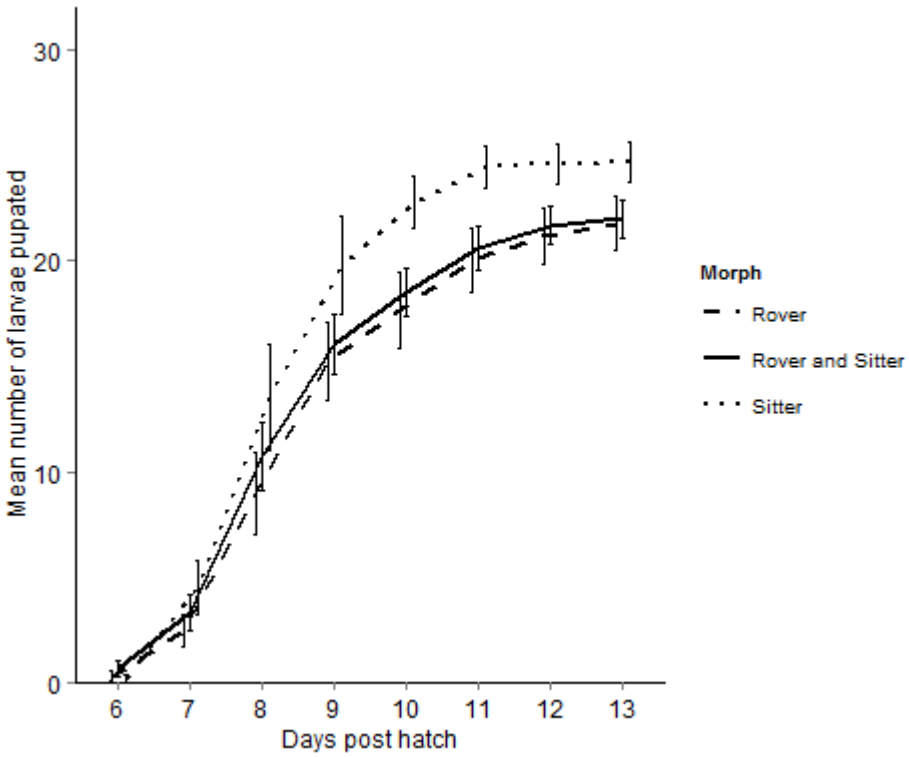

Fig. 3: Mean cumulative larval pupation ± SE up to 13 days post hatch. Vials contained 32 larvae in frequencies of either all rover, all sitter or 16 larvae of each rover and sitter in the same vial. Development is delayed from standard as vials contain poor nutrition food.

Experiment 4: Replication of negative frequency-dependent selection in rover and sitter larvae

RATIONALE

If we were able to replicate the result of negative frequency-dependence, it would allow us to conclude that the polymorphism is at least partially maintained by some larval interaction between the rovers and sitters on poor quality food. Whether or not frequency dependence is caused by competition for food is ambiguous. In nature the opportunity for food deprivation and thus competition is high due to overcrowding at food patches (Atkinson, 1979). Larval competition is typically described as scramble competition, where the best competitor is the one who can consume the food at the fastest rate (Joshi & Mueller, 1988). However, the experimental design of Fitzpatrick et al. (2007) utilized an ample amount of poor quality food to generate competition. In this design, the larvae do not deplete the food enough to allow for scramble competition. Instead, there is a situation of chronic malnutrition (Vijendravarma, Narasimha, & Kawecki, 2012) where there may be some form of interference competition, possibly though the buildup of harmful ammonia waste product (Borash, Gibbs, Joshi, & Mueller, 1998). In this experiment, we predict that as in Fitzpatrick et al. (2007), rovers and sitters will have their highest survival when they are the less frequent morph in each vial.

The green fluorescent protein marked lines that were to be used in this experiment showed no difference in path length and surprisingly, did not fluoresce under UV light. As a result, we used the fall 2013 lines and genotyped surviving adults to assess rover and sitter survival.

METHODS

Using a moist paintbrush, we placed rover and sitter 1st instar larvae (± 1h in age) into vials. Into each vial, we either placed 24 rovers and 8 sitters or 24 sitters and 8 rovers (N = 20 vials per frequency). This was done over four successive days with N=5 vials per frequency per day. Each vial contained 6 mL of low nutrition food (as described in

Experiment 2). We placed vials in a vial rack in the incubator at 25°C and 60% relative humidity, on a 12:12 h light cycle with lights on at 1:00 h. We randomized their position within the rack daily to eliminate any asymmetric abiotic effects of the incubator.

We measured fitness as the proportion of larvae that eclosed from their pupal case. Once a day at 10:00 h, from days 5 to 18 post hatch, we removed adults from each vial and then stored them individually in 95% ethanol at -20ºC.

To genotype the adults, we first completed single fly DNA extractions. To do this, we placed each fly into a microcentrifuge tube with 49 µL squish buffer (80 µL [0.5 M] EDTA, 200 µL [5 M] NaCl, 400 µL [1 M] TrisHCl pH 8.2, 39.32 mL ddH_2O) and 1 µL [10mg/mL] proteinase K and then used a pestle to grind them until homogenous. We pipetted 50 µL of each sample into 96 well plates and then placed them in a thermocycler for 2 hours at 37ºC and then at 98°C for 2 minutes to denature the proteinase K.

We then ran polymerase chain reactions (PCR) using a cocktail comprised of 6.25 µL ddH_2O, 0.2 µL $MgCl_2$ [final concentration 2.5 mM], 1 µL 10x reaction buffer (FroggaBio), 0.4 µL primers (95 µL ddH_2O, 2.5µL [100 µM] forward primer TTGATGACTATCCTCCCGATCCT (Integrated DNA Technologies), 2.5µL [100 µM] reverse primer AAGGCAACCCGATTTGTATGC (Integrated DNA Technologies)), 1 µL deoxynucleotides (FroggaBio), 0.15 µL [5U/µL] Taq polymerase (FroggaBio) and 1 µL of DNA. Conditions were as follows: 94ºC for 2 minutes; 35 cycles of 94ºC for 15 seconds, 58ºC for 25 seconds, 72ºC for 60 seconds, followed by a final step of 72ºC for 1 minute.

We used the ie4/iB55 RFLP and the restriction enzyme Pst1 to digest the PCR product. The amplification product is 578 base pairs and the subsequent restriction results in two fragments (123bp and 455bp) if sitter and one fragment (578bp) if rover. Digests contained 5.8 µL ddH_2O, 1 µL 10x CutSmart® Buffer (New England Biolabs), 0.2 µL PstI-HF® (New England Biolabs), and 3 µL PCR amplified product. We ran digests for 8 hours at 37ºC. We then visualized the digested product using a 1.3% agarose gel run at 160 mV for 30 minutes.

To test for any imbalances in the data due to day effects, we assessed the proportion of eclosion in each vial by day, frequency and their interaction using an ANOVA. We were unable to genotype 12.9% of the surviving adults missing 1.9%, 1.7%, 7.1%, and 4.8% adults per day. We analyzed the genotyped adults using a generalized linear model with a binomial distribution and a logit link function. The response variable was the proportion of larvae that eclosed. Day, frequency of sitters in the vial, adult genotype and their interaction were modeled as fixed effects.

RESULTS

There was no pattern of negative frequency-dependence as would be indicated by a frequency × morph interaction (Wald $\chi^2_1 = 0.0017$, $P > 0.9$; Fig. 4). Although the slope of the rover morph appears to be negatively correlated with rover frequency, it is not significant (Wald $Z = 0.202$, $P > 0.8$; Fig. 4). There was a significant linear day effect on the proportion of adults eclosing from each vial ($F_{1, 72} = 6.799$, $P=0.01$) as days 3 and 4 had respectively 14% and 23% less eclosion than day 1. However, there was no significant day by frequency interaction ($F_{3, 72} = 2.14$, $P =0.1$).

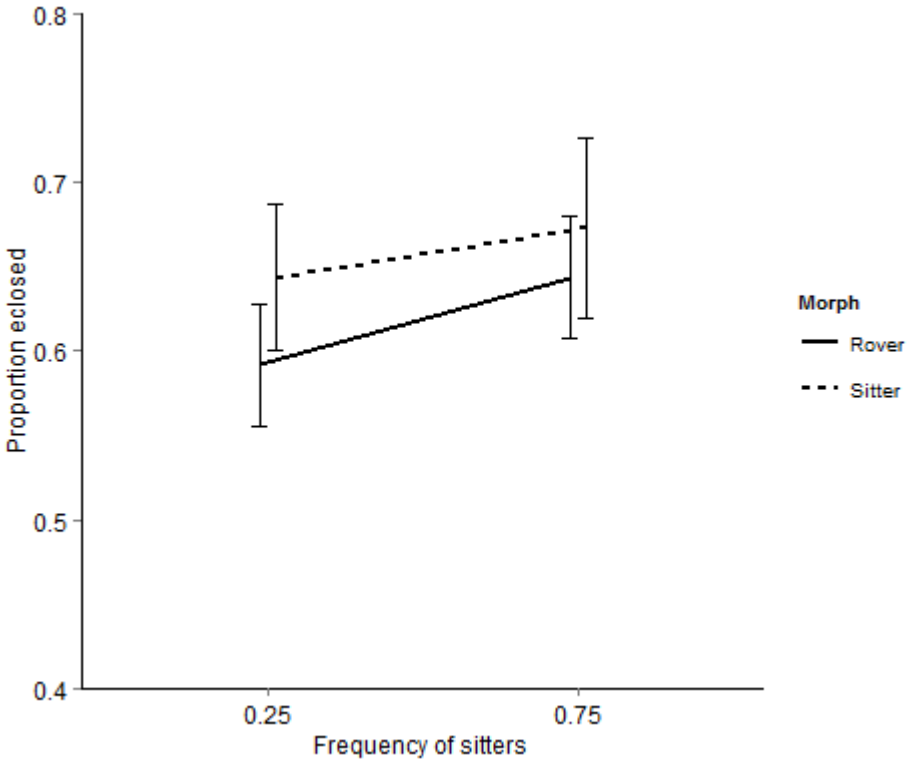

Fig. 4: The effect of rover and sitter frequency on rover and sitter fitness measured as the mean ± SE proportion of adults that eclosed. Morphs were reared together in either a 24:8 or 8:24 rover to sitter ratio (N = 20 per treatment).

DISCUSSION

The 3D spatial location experiment indicated that sitters spend a greater amount of time burrowed in the substrate than do rovers. Although this result conflicts with the results of Sokolowski (1982), they are not fully incompatible. Sokolowski used indirect observational methods by adding charcoal to the bottom 2/3^{rds} of the food and checking larvae for charcoal stained digestive tracts. Consequently, it was found that in single genotype groups of ten larvae, a greater proportion of rovers dug into the substrate than did sitters. These two sets of results are contrasting, but it is possible that while sitters spend more time burrowed in the substrate, they only dig to shallower depths. Both results indicate a difference in the behavioural phenotype for digging and may allow for some form of microdispersal. This dichotomy of burrowing behaviour parallels that of *D. melanogaster* and *D. simulans* larval burrowing patterns. *D. simulans* larvae burrow deeper into the food than *D. melanogaster* allowing for niche separation between the species and coexistence on food patches (Barker, 1971; Parsons, 1975). Similar resource partitioning as a result of divergent feeding behaviours has reduced intraspecific competition in the polymorphic cichlids, *Herichthys minckleyi* (Swanson, Gibb, Marks, & Hendrickson, 2003) and *Perissodus microlepis* (Hori, 1993). Niche separation among rovers and sitters in the vertical plane may be the cause of the negative frequency-dependant selection and the maintenance of both the rover and sitter alleles. Such a system may have evolved from an ancestral generalist experiencing disruptive selection, until the fitness of each phenotypic variant reached an adaptive peak.

In addition to vertical displacement, we also tested for segregation in the horizontal plane. We found a small difference in horizontal displacement between the morphs. We predicted that rovers would spend more time at the edge of the vial, but this was based largely on the distribution of rover burrowing sites in a larger petri dish. The small surface area of the vial may have been too constrained to see a large effect of 2D dispersal. Regardless of this, in the 3rd instar stage, rovers spent more time at the edge of the vial than did sitters. We propose two possible explanations for this behaviour. First, late 3rd instar larvae may be entering the wandering stage, where they seek out a suitable

location to pupate. In vials, rovers typically pupate higher on the vial wall than do sitters (Sokolowski, 1985). Moreover, in nature, rovers tend to pupate in the soil or underneath the fruit whereas sitters pupate on the fruit. This tendency to leave the food during the wandering stage may be causing a bias in the amount of time rovers are observed at the edge during the late 3rd instar stage. Second, over time as the food dries, it comes away from the wall of the vial. The increased crawling effort and decreased digging of rover larvae suggests that they may be searching at the edge for alternative entry points into the food that require less energy to excavate. Searching behaviour for trenches that allow for easier excavation of the food has been demonstrated previously as larvae aggregate around areas where the surface of the food is broken (Durisko, Kemp, Mubasher, & Dukas, 2014).

In nature there are multiple potential benefits to burrowing in the substrate. First, burrowing may reduce attack rates of parasitoid wasps (Carton & David, 1985), which are a large source of larval mortality (Fleury et al., 2004). Second, burrowing may help to maintain homeostasis in larvae (Reaume & Sokolowski, 2006) as there may be less fluctuation in temperature and humidity within a fruit than on its surface. Finally, groups of larvae are able to suppress the growth of harmful mould (Rohlfs, 2005) and further, enhance the growth of beneficial yeast species (Stamps, Yang, Morales, & Boundy-Mills, 2012) possibly due to the churning of the food as a result of burrowing. However, under lab conditions and specifically the single larva design of experiment 2, it is unlikely that these benefits are realised. And in agreement with this, we observed no significant difference in the rate at which individual rovers and sitters reach pupation (Fig 2.3).

Alternatively, in groups of both rovers and sitters, we should expect differences in survival under the hypothesis of negative frequency-dependence selection. The results of experiment 3 did not suggest negative frequency-dependence as the proportion of larvae pupating in the mixed rover and sitter vials was not any greater than the pupation in the rover alone vials and was much less than the sitter alone vials (Fig. 3). It appears as though the sitter morph may be better adapted to our specific lab conditions. There is an energetic cost of crawling (Berrigan & Lighton, 1993) which, for the rovers, may not pay

off in a homogenous lab environment (Sokolowski, 1980). Evidence for greater survivorship for either morph under conditions of poor nutrition is conflicting. Kaun et al. (2007) demonstrated greater survival of rovers than sitters. The opposite was found by Vijendravarma et al.(2012) using food with less yeast (3.1 versus 7.5 g per litre) than Kaun et al. (2007).

In accordance with experiment 3, the replication of Fitzpatrick et al. (2007) did not suggest a pattern of frequency dependent selection. However, Fitzpatrick et al. (2007) found no evidence for frequency dependence in conditions using food with marginally better nutrition (75% vs 85% reduction in yeast and sugar from standard). It is possible that our food recipe was not similar enough to that used by Fitzpatrick et al. (2007) (85% nutrition reduction of Dukas lab recipe: 1,000 ml H2O, 15 g sucrose, 7.5 g Fleischmann's yeast, 15.5 g agar, 20 ml methylparaben solution; 85% nutrition reduction of Sokolowski lab recipe: 1,000 ml H2O, 15 g sucrose, 7.5 g Fleischmann's yeast, 16 g agar, 8 g $C_4H_4KNaO_6$, 1g KH_2PO_4, 0.5g NaCl,0.5 g $MgCl_2$, 0.5g $CaCl_2$, 0.5g $Fe_2(SO_4)_3$). Differences in rover and sitter survival between Vijendravarma et al. (2012) and Kaun et al. (2007) certainly exhibit the nuances of the lab diet and experimental conditions. To add to the difficulty in interpreting these results, it has been shown that foraging behaviour is plastic and that food scarcity can alter the expression of *for*. In response to food scarcity rovers express reduced PKG levels and increase their food uptake to match that of sitters (Kaun et al., 2007).

With these experiments, it is still not clear under what conditions frequency dependent selection operates and what the exact mechanism is that is maintaining this polymorphism. A possible testable mechanism is that sitters and rovers are respectively producers and scroungers. This hypothesis is based on the greater burrowing behaviour of sitters and the greater searching behaviour of the rovers. If rovers pay an energy cost of locomotion, but save energy by making use of previously excavated tunnels, then they will have the highest fitness when the population has a high frequency of burrow producing sitters, whereas they will have the lowest fitness when the population has a

high frequency of scrounging rovers. It should be noted that our replication of Fitzpatrick et al. (2007) does not support this as we did not find negative frequency-dependence.

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CHAPTER 2: THE EFFECT OF MALE-MALE COMPETITION ON MATING SUCCESS IN FRUIT FLIES

INTRODUCTION

Sexual selection is the differential reproduction of individuals due to variation in traits affecting their ability to obtain mates and fertilizations (Andersson, 1994). First described by Darwin (1871), the two mechanisms that drive the sexual selection of traits are intersexual-choice and intrasexual-competition. Due to the disparity in gamete production with sperm being less costly relative to eggs, male's reproductive success tends to be limited by the number of matings achieved. On the other hand, females are limited by their egg production and so it is typically more optimal to obtain fewer mates of higher calibre. Consequently, females are often choosey while males compete with other males for access to females. Although there are exceptions to this, I focus on it for simplicity. More often than not, these two mechanisms, female choice and male-male competition do not operate independently (Berglund, Bisazza, & Pilastro, 1996; Qvarnström & Forsgren, 1998; Wong & Candolin, 2005). In some cases, a single trait may be selected for by both male competition and female choice. For example, in the field cricket, *Gryllus bimaculatus*, male body size is selected for by both male contest for burrows as well as female choice (Simmons, 1986a, 1986b). In this respect, competition and choice act in the same direction and reinforce each other (Hunt, Breuker, Sadowski, & Moore, 2009). Alternatively, the two mechanisms may be acting in opposition, where male competition and female choice act in different directions on the same trait or select for different traits entirely. So, although, mating success is often skewed in favour of dominant or large males, it is not always clear whether this is a result of male-male competition, female choice or their interaction (Wong & Candolin, 2005). In such cases as this, it is important to disentangle the effects of each mechanism.

However, separating the effects of choice and competition can prove to be very difficult depending on the mating system and natural history of the species and requires careful use of experimental protocols. For example, in the mating system of the scarlet-tufted malachite sunbird, *Nectarinia johnstoni*, field observations combined with

experiments have shown that females prefer to mate with males that have longer tail feathers, but a male's ability to hold territory is instead, based on the size of his pectoral tufts (Evans & Hatchwell, 1992a, 1992b). The difference in selection for either tail feather length or pectoral tuft size is likely due to the natural history of this species. The elongated tail feather is moulted each year and is relatively costly to regrow compared to pectoral tufts we can be replaced more easily. The low cost of maintaining the tufts allows males to defend a territory year round, whereas the costly tail feather is needed only briefly during the breeding season as an indicator to females of male quality. Compared to the field cricket, where male body size is selected for by both male contest and female choice, it is clear in the case of the sunbird, that distinct traits affect male mating success and so need to be studied by separating choice from competition.

In the past, female choice has been assessed using two experimental designs which I discuss in turn. The first design is a choice test, where each female is given a choice between two (or more) males or stimuli presented simultaneously (Dougherty & Shuker, 2015; Wagner, 1998). Although it has long been recognized that controlling for male competition is important in identifying female choice (Halliday, 1983), the type of interactions between males and females in each mating system can make quantifying the individual effect of female choice challenging when using a choice test design. In species where mating interactions are primarily visual or auditory, choice tests can be used to demonstrate isolated female choice while controlling for male competition. In visual systems, choice tests are often implemented by separating males behind clear partitions and assessing female choice via her proximity to either male or her engagement in courtship behaviour. This is common practice in fish where male pairs differ in traits such as ornament size or coloration. Similarly, in systems that primarily have auditory courtship interactions such as frogs or crickets, two loud speakers playing separate male calling signals can be used, where female choice can be tested for differences in repetition rate or duration of male calls. Conversely, in species where mating interactions include physical contact, controlling for male competition can be much more difficult. One method to prevent males from competing while allowing a female to interact with them is

to tether them to separate areas (Galef, Lim, & Gilbert, 2008; Nilsson & Nilsson, 2000). However, this is not feasible for many species due to either physical constraints such as small size or due to abnormal behaviour of males when tethered. As a result of this, male-male competition in fruit flies is often ignored or assumed to be negligible when using choice tests involving physical contact (Gershman, Delcourt, & Rundle, 2014). Overall, it is important to consider the natural conditions under which courtship and mating occur. Differences in experimental design such as tethering versus partitioning of males alter the set of cues a female receives and thus can alter her preferences (Nilsson & Nilsson, 2000).

The alternative design to a choice test, which alleviates some of the above difficulties, is a no-choice or forced-choice test, where a female is presented with a single male or stimulus. This can also be done as a sequential choice test where the female is repeatedly presented new males if the previous male is rejected. Because a no-choice test has the benefit of eliminating male competition, it is commonly used to assess female preference based on mating latencies (Gowaty, Steinichen, & Anderson, 2002). However, this design has the drawback that females do not have multiple concurrent males to choose between. This means that depending on the number of previous male encounters and consequently, the expectation of future mating opportunities, the cost of rejection may be high relative to a choice design where if one male is rejected there is still another mating option. Therefore, in the no-choice design, one should expect a positive relationship between the cost of rejection and the randomness of a female's mating decisions with respect to a male trait of interest (Dougherty & Shuker, 2015). For example, a female may choose to mate with a low quality male if the expectation of future male encounters is low based on the past male encounter rate. In many experimental protocols, the female subject tested is isolated from males so that she remains virgin. As this duration of male deprivation increases, so does the cost of rejection and thus randomness of mating outcomes.

We believe that a more refined experimental design is required to accurately assess female choice in the fruit fly, *Drosophila melanogaster*, a prominent model species

used to study sexual selection. Prompting this were recent and relatively novel observations in our lab of courtship interference behaviour, whereby males exhibit aggressive competition during the courtship of females. Courtship interference behaviour has been studied in several species including sticklebacks (Nilsson & Nilsson, 2000), salamanders (Howard, Moorman, & Whiteman, 1997), and gobies (Kangas & Lindström, 2001). However, courtship interference among male fruit flies has only been briefly noted in the past in nature (Partridge, Hoffmann, & Jones, 1987), as well as more recently in the lab (Debelle, Ritchie, & Snook, 2016) and its effect on mating outcomes is yet to be quantified. We postulate that courtship interference in fruit flies is not just a lab artifact as, in nature, there is ample chance for multiple males to compete for a female as the operational sex ratio in fruit flies is strongly male biased (Bateman, 1948; Spieth, 1974). Furthermore, flies congregate on fallen fruit, producing high densities which allow for intense competition. As such, we suspect that the effect of male-male competition in *D. melanogaster* has been underestimated and should not be ignored when assessing female choice.

Although the classic assumption has been that choice and competition act in unison on the same traits and that female preference for larger, dominant males is dependent on male competition as a signal of quality (Darwin, 1871; Berglund et al., 1996), this assumption has been challenged (Qvarnström & Forsgren, 1998). Females may incur costs mating with larger or dominant males. Females of *D. melanogaster* die sooner and lay fewer eggs when mated to larger males (Friberg & Arnqvist, 2003; Pitnick & García-González, 2002). Certainly, male courtship interference behaviour can prevent females from mating with their preferred mate if traits other than male-male competitive ability are desired i.e. if the outcome of male-male competition is not optimal with respect to the female's fitness. Moreover, there is a growing body of work showing a lack of correlation between male fighting ability and male attractiveness to females in frogs (Morrison, Hero, & Smith, 2001), lizards (López, Muñoz, & Martín, 2002), birds (Andersson et al., 2002; Evans & Hatchwell, 1992a, 1992b), fish (Wong, 2004), and insects (Moore & Moore, 1999; Moore et al., 2001). In light of courtship interference

behaviour among male fruit flies, it is likely that female choice and male competition are working in opposition or are even selecting for different traits. Because of this, we wanted to first ask if we could quantify male courtship interference behaviour separately from female choice and whether this behaviour affected a male's ability to gain matings. Second, we wanted to ask whether there is potential for sexual selection on traits associated with courtship interference behaviour.

Previously in the lab, we examined the extent of male courtship interference behaviour as it varies with age, size and genetic background, with both larger and older males being better competitors (Baxter et al., 2015c, unpublished raw data). We have observed two components to courtship interference in fruit flies: takeover and blocking. A takeover occurs when a male aggressor moves between a courting male and a female, often pushing the other male out of the way, and then proceeds to court the female himself. A takeover is defined then if a male successfully stops another male's courtship while initiating or continuing courtship himself. Conversely, a block occurs when a courting male successfully defends against a takeover by positioning himself between the female and the male aggressor. In the following experiments we focused on takeover behaviour as it is clear and quantifiable, whereas a courting male's intent to block a male aggressor is not always clear.

In order to quantify the effect of male courtship interference behaviour separately from female choice we designed an arena which separated males to prevent male competition while allowing a female to physically interact with either male and then choose a mate. This choice design arena addresses the problem of the need for physical contact between males and females during courtship while avoiding problems associated with no-choice tests.

To assess whether there is potential for sexual selection on traits associated with courtship interference behaviour, we screened for genetic variation in takeover behaviour and then wished to critically test whether male takeover behaviour results in a mating and subsequent fitness advantage.

EXPERIMENT 5: Quantifying the effect of male-male competition on male mating success using a novel mating arena

RATIONALE

In order to quantify the effect of male courtship interference behaviour we used males that would differ in competitive ability by using males of different ages. In general, older males typically have a mating advantage compared to younger males (Long, Markow & Yaeger, 1980). Previously in the lab, it had been shown that 4 day old males more frequently takeover the courtship of 1 day old males when competing for a female (Baxter et al., 2015c, unpublished raw data). The resulting effect of male age is apparent in the courtship and mating frequency of competing males. When a sexually immature, virgin female, who rejects males yet is attractive to them, was placed in a vial with both a 4 day old and 1 day old male simultaneously, the 4 day old male spent significantly more time courting the female than did the 1 day old, even though, when males of each age were placed alone with a sexually immature, virgin female, both courted the female for equal durations (Baxter et al., 2015b). From this, we hypothesize that the increased takeover behaviour of 4 day old males allows them to dominate the courtship of females when in competition with a 1 day old male. Furthermore, the mating frequency follows a pattern parallel to the courtship observations. When 4 day old or 1 day old males were placed alone with a sexually mature, virgin female, the mating frequencies were equal, but when both males competed for a single female, the mating frequency of the 4 day old male was more than three times greater than 1 day old males (Dukas & Baxter, 2014). From this, we hypothesize that in addition to dominating the courtship, the greater takeover frequency of 4 day old males allows them to gain a greater proportion of matings. However, the experiments mentioned above were done without controlling for male competition, so it is still unknown whether these older males enjoy a mating advantage over the young because of their increased competitive ability or because of female preference. In the following experiment, we predict that when observing initial matings, 4 day old males will be more likely to mate than 1 day old males when in direct

competition than when the mating outcome is dictated by female choice alone. Additionally, we predict more re-mating from 4 day old males than 1 day old males when in direction competition compared to female choice.

METHODS

We used flies from our wild population, descended from wild-caught *D. melanogaster* collected in southern Ontario in August 2014. We kept cages of several hundred flies in an environmental chamber at 25°C and 60% relative humidity, on a 12:12 h light cycle with lights on at 10:00 h.

To generate true female mate choice and compare it to the standard set up of concurrent choice and competition, we constructed two types of arenas. The true choice arena is a novel arena of our design. It has two adjacent rectangular compartments that isolate the males from each other. Each compartment is 1 x 1 x 4 cm in size. In the shared wall of the two compartments are 24 holes approximately 0.95 mm in diameter. The holes are small enough to prevent large males from passing between compartments, but at the same time large enough that small female is able to pass though and visit both males. The second arena, which allows for simultaneous choice and competition, we will term for simplicity, the competition arena. It is only a single compartment of dimensions 1 x 1 x 6.5 cm. Both arenas are filled with standard food to the 1.5 cm mark from one end and sealed with a foam plug at the other. After adding food, the space in the competition arena is twice the length of the true choice arena so that the total space the female can explore in both arenas is equivalent.

To develop small females, we allowed females to lay eggs on a small amount of standard food with a live yeast suspension. The resulting vials contained approximately 250 eggs per 1 ml of food (Byrne, Rice, & Rice, 2008; Baxter et al., 2015b). Although small females are less attractive than large females as shown by less courtship from males, both 4 day old and 1 day old males court small females with equal intensity (Baxter et al., 2015b). To develop large males, we allowed females to lay eggs in bottles

and removed excess eggs to reach a density of 100 eggs per bottle containing 50 ml of standard food.

To collect mature males, three days prior to the trial, we cleared bottles at 7:00 h and live sexed flies by aspiration at 11:00 h. We housed these males individually in vials with standard food and kept them in the chamber. Young males and females were collected the day of the trial. Again, clearing at 7:00 h, live sexing at 11:00 h and housing flies individually. By sexing flies within four hours we ensured that flies are virgin and lack mating experience.

At 14:00 h, we coloured males either pink or blue so that we could identify them in the competition arena (Crumpacker, 1974). We coloured all males regardless of which type of arena they would be placed in and counter balanced colour between the two male ages. After colouring, males were left in cages in the chamber with access to food for 7 hours so that they could preen off the excess colouring powder.

At 21:00 h ± 1 hour, we gently aspirated coloured males, either 3 day old or newly eclosed males into their respective compartments in each arena. A newly eclosed female was aspirated simultaneously with one of the males in the true choice arena or with both of the males in the competition arena. The initial position of females in the true choice arena was counter balanced in both the physical side of the arena as well as with which aged male she started the trial with.

The immaturity of the female allowed her a period of approximately 24 hours to explore the arena and learn about both males. During this time the males were able to court her. Both young and mature males find immature females attractive and will court the female with equal intensity (Dukas & Baxter, 2014). However, immature females reject male advances (Dukas & Dukas, 2012). Once females became sexually mature, they were able to make informed choices of whether to mate with either a 4 or 1 day old male. We ran trials in humidified bins at 25°C and 80 ± 10% relative humidity with bright ambient light. Trials lasted 48 hours beginning when we placed flies into the arenas.

We recorded behaviour with iPods using the time-lapse application, OhSnap!. Each iPod recorded twelve arenas capturing a single frame every three minutes. From the

resulting time-lapse, we scored matings if for 3-5 consecutive frames the same male was mounting the female. We believe this was an adequate measure as matings in *D. melanogaster* typically last for about 15 minutes (Ashburner, 1989). All observations were completed blind to male age.

A priori, we excluded from the data, arenas where females did not receive courtship by both males before mating as this did not constitute a choice by the female. We also excluded arenas where females did not mate during the trial. All data analysis for the following experiments was done using R version 3.1.3 (R Development Core Team, 2015). We analyzed the data in two steps. First, we tested whether or not male age and level of competition affected the latency and proportion of the initial matings. To do so, we used a Cox proportional-hazards regression model where mating latency and proportion of mating were predicted by male age, the level of competition and their interaction (Table 4). We also assessed for the interaction of the effects of each covariate with time by testing the proportional-hazards assumption which tests the correlation between the scaled Schoenfeld residuals and time. Second, we tested if the level of competition affected the number of matings obtained by young and mature males (i.e. initial mating plus all subsequent remating). For this, we used a generalized linear mixed model with a Poisson distribution and log link function (Table 5). The model comprised the number of matings as the dependent variable and the predictor variables: male age, level of competition and their interaction. In both of these analyses, we were interested in the age by competition interaction as it informs us whether or not competition allows mature males to gain more matings than would be expected from female choice alone.

RESULTS

Observing only the initial matings in each arena, we found a non-significant interaction between male age and competition level (Interaction: Wald $\chi^2_1 = 2.947$; P = 0.086; Fig. 5.1). Mature males were more likely to mate than young (Main effect: Wald $\chi^2_1 = 6.568$; P = 0.010; Fig. 5.1). However, there was no overall difference between the two competition levels (Main effect: Wald $\chi^2_1 = 2.366$; P = 0.124; Fig. 5.1). This was the

result of an interaction of competition level with time (Test of proportional-hazards assumption: Wald $\chi^2_1 = 5.202$; P =0.023; Fig. 5.1), where the effect of competition level disappeared around the 21 hour mark.

When we included re-matings, we found no significant interaction in the number of matings between male age and competition level (Interaction: Wald $\chi^2_1 = 1.591$; P = 0.207; Fig. 5.2). Females mated significantly more times with mature males than young (Main effect: Wald $\chi^2_1 = 6.875$; P < 0.01; Fig. 5.2). There were more matings in the competition arena than the choice arena (Main effect: Wald $\chi^2_1 = 5.730$; P = 0.017; Fig. 5.2).

Table 4. Fixed effects of the Cox proportional-hazards regression model of mating latency.

Fixed effect	Chi-square value	DF	P-value
Male	6.5678	1	0.010
Competition	2.3656	1	0.124
Male x Competition	2.9466	1	0.086

Table 5. Fixed effects of the generalized linear mixed model of the number of matings.

Fixed effect	Chi-square value	DF	P-value
Intercept	7.404	1	0.007
Male	6.875	1	0.009
Competition	5.730	1	0.017
Male x Competition	1.591	1	0.207

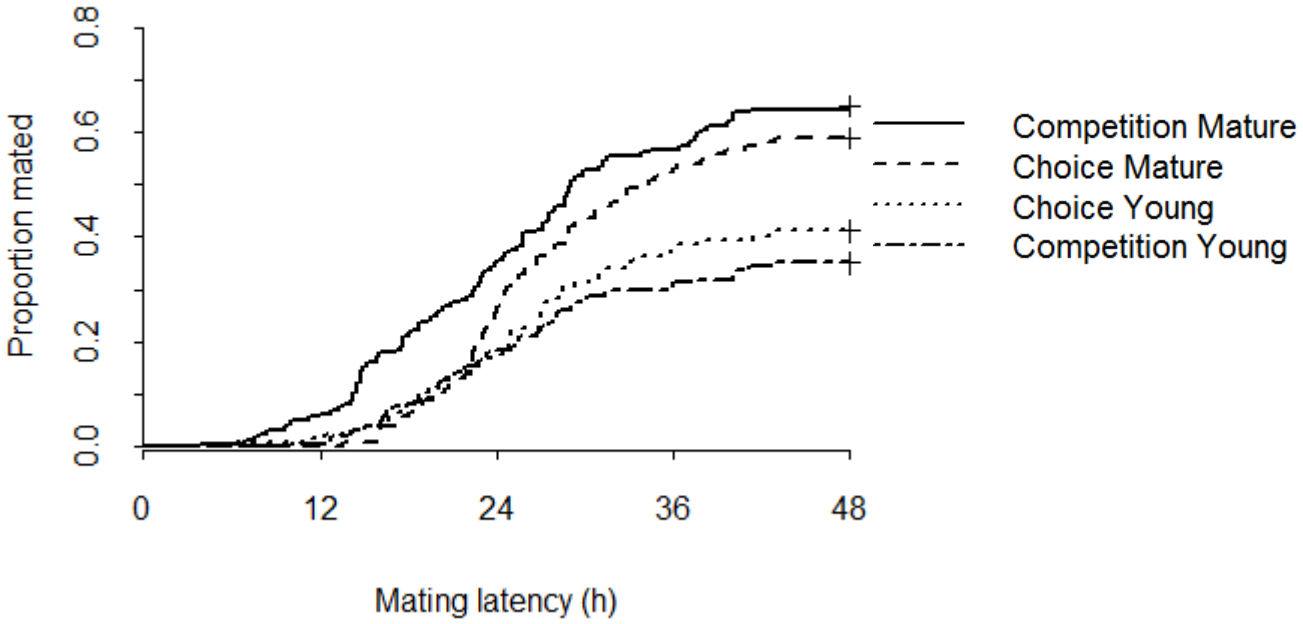

Fig 5.1: Proportion of initial matings between young and mature males over time in either the true female choice treatment (N = 124) or the competition treatment (N = 157). After the full trial duration of 48 hours, the final mating proportion for mature and young males was respectively 0.59 and 0.41 in the choice arenas and 0.65 and 0.35 in the competition arenas. The onset of female sexual maturity occurs at about the 9 hour mark. However, this is based on the sexual maturity of normal sized females and sexual maturity may be more variant and delayed in small females. The mean mating latency ± SE for mature and young males was respectively 26.2 ± 0.8 and 25.0 ± 1.1 hours in the choice arenas and 23.3 ± 0.9 and 24.5 ± 1.1 hours in the competition arenas.

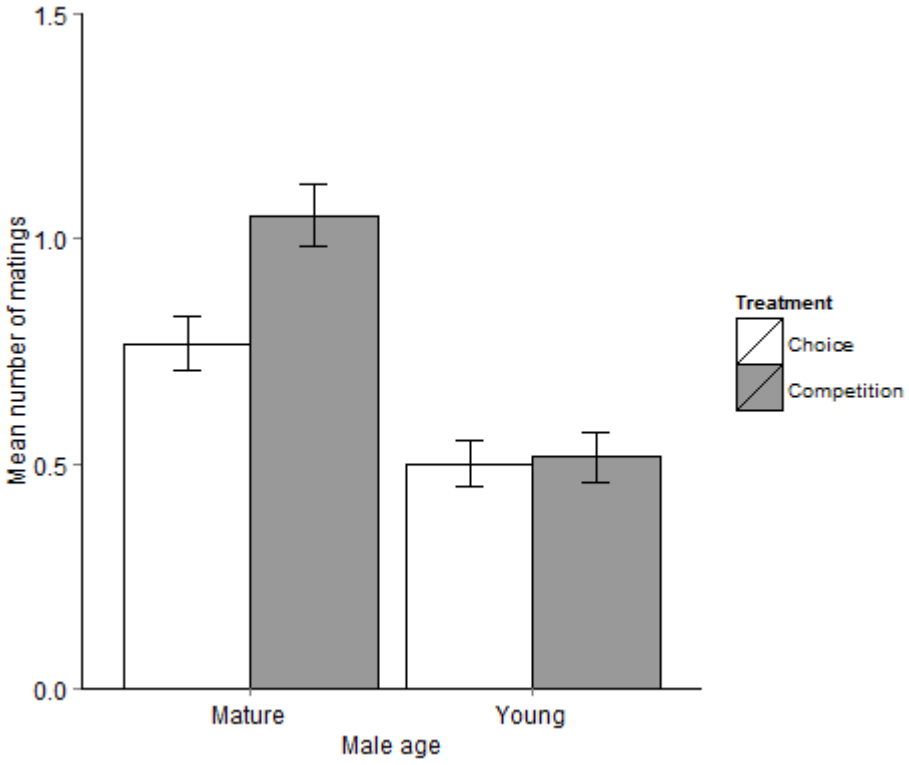

Fig. 5.2: Mean ± SE number of matings of mature and young males paired in either a choice (N = 124) or competition treatment (N = 157).

EXPERIMENT 6: Assessing genetic variation in courtship takeover behaviour

RATIONALE

Although genetic variation in courtship takeover behaviour had been demonstrated previously in our lab, recent efforts to show a resultant mating advantage of aggressive males have failed. This was likely due to the use of inbred lines. In order to correct this, we screened for variation in takeover behaviour among hybridized lines of the *Drosophila* Genetic Reference Panel (DGRP). The DGRP are a collection of 208 genetically identical inbred lines which display natural genetic variation between lines (Mackay et al., 2012). Hybrid lines allowed us to screen for natural variation in behaviour while avoiding the effects of inbreeding depression.

METHODS

We kept DGRP lines in vials of approximately 30 flies with standard food. We kept a reference DGRP line, line 83 in a cage of several hundred flies. All flies were kept in an environmental chamber at 25°C and 60% relative humidity, on a 12:12 h light cycle with lights on at 10:00 h.

We chose 10 DGRP lines of moderate activity levels (Anderson, Scott, & Dukas, 2015). Each line was then hybridized with the single reference DGRP line, line 83. We mated virgin females of line 83 with males of the 10 focal DGRP lines to create hybrid F_1 offspring.

To screen for courtship takeover behaviour, we competed 4 day old males of each hybrid DGRP line with 4 day old reference males from our wild population (see experiment 5 methods for description of wild population). We chose to use a wild reference line due to the potential for high relatedness between a DGRP reference line and our focal DGRP, as males may reduce courtship effort in the presence of kin (Martin & Long, 2015). We allowed both males to simultaneously court a sexually immature, wild female for 15 minutes. Although immature females reject the courtship of males,

males find them to be very attractive (Dukas & Dukas, 2012). Therefore, using immature females allowed us to observe takeover behaviour for the entire trial duration.

To collect focal, hybrid DGRP males and reference, wild competitor males, we cleared at 8:00 h and live sexed by aspiration at 12:00 h. To collect wild females, we cleared at 19:00 h and live sexed at 7:30 h. All flies were housed individually in vials with standard food after sexing.

We ran trials in 3.5 cm petri dish arenas. We did not place food in the arena to avoid behaviour associated with resource defence polygyny (Baxter et al., 2015a). To deter flies from walking on the ceiling or walls of the dish, we coated them with a slippery liquid, Surfasil (Sigma Aldrich, Oakville, ON, Canada), and placed a circle of filter paper on the floor of the arena. This allowed us to video record behaviour in a shallow depth of focus for maximal resolution. To distinguish wild males from focal males, we coloured the wild males pink (Crumpacker, 1974). After trials were completed, we measured the wing length of male pairs from a subset of arenas (Gilchrist & Partridge, 1999). Wing length is highly correlated with body size which has a large influence on competitive mating success (Partridge & Farquhar, 1983).

We recorded behaviour with iPods using the application, Filmic Pro. We began recording immediately after aspirating flies into arenas. After, we observed videos using Noldus (Noldus Information Technology, Wageningen, The Netherlands). Observers, blind to focal male line, recorded for both the focal and wild male, the courtship duration and number of courtship takeovers. Takeover frequency was measured as the number of courtship takeovers performed by each male divided by the courtship duration of his competitor.

Prior to analysis, we eliminated trials without courtship from either male. We compared wing lengths of DGRP and wild males using a Welch two sample t-test which accounts for unequal variance between groups. Because we know that male body size affects takeover frequency (Baxter et al., 2015c, unpublished raw data), we assessed whether wing difference, which was calculated as DGRP wing length minus wild wing length for each competing pair, varied by line using an ANOVA.

We then assessed whether there was significant variation between DGRP lines for takeover behaviour. Because the distribution of takeover frequencies was zero inflated, we used a Tweedie generalized linear model from the tweedie package (Dunn, 2014) with a Compound Poisson response distribution and a log-link which is used for non-negative continuous data with a mass at exactly zero. The model comprised DGRP takeover frequency as the dependent variable as predicted by DGRP line and the covariate wing difference.

We also assessed variation in the difference in takeover frequency between DGRP lines. Takeover frequency differences were calculated as DGRP takeover frequency minus wild takeover frequency. In part, this accounts for blocking behaviour which we did not score due to its cryptic nature. For example, DGRP males can gain a greater difference in takeover frequency from wild males by either performing more takeovers or alternatively by blocking wild males and preventing them from taking over courtships. Thus, the takeover frequency difference score is a proxy for overall male competitive behaviour. As in the above mentioned distribution of DGRP takeover frequency, the difference in takeover frequency scores was also zero inflated. However, because some wild males outcompeted their DGRP counterparts, some takeover frequency difference scores were negative and are hence not suitable for a Tweedie distribution. The zero inflated takeover frequency difference data was non-normal as tested with a Shapiro-Wilk normality test. Therefore, we used a Kruskal-Wallis test to compare takeover differences by DGRP line.

RESULTS

We found no significant difference between DGRP lines in takeover behaviour (Main effect: Wald $\chi^2_9 = 5.743$; P = 0.765; Fig. 6.1a). We also found no significant effect of differences in wing size on takeover frequency (Main effect: Wald $\chi^2_1 = 1.967$; P = 0.0.161). Similarly, we did not find significant variation in competitive ability (Kruskal-Wallis rank sum test: Wald $\chi^2_9 = 9.283$; P = 0.4116; Fig. 6.1b). Due to rearing densities, DGRP males were significantly larger than wild (t = 18.9; d.f. = 126; P < 0.001; Fig. 6.2a,

b). Additionally, the size difference between focal and reference male pairs varied by line ($F_{9, 67}$ =2.9; P < 0.01; Fig. 6.2a, b).

Table 6. Fixed effects of the Tweedie generalized linear model of DGRP takeover frequency.

Fixed effect	Chi-square value	DF	P-value
Wing size difference	1.967	1	0.161
Line	5.743	9	0.765

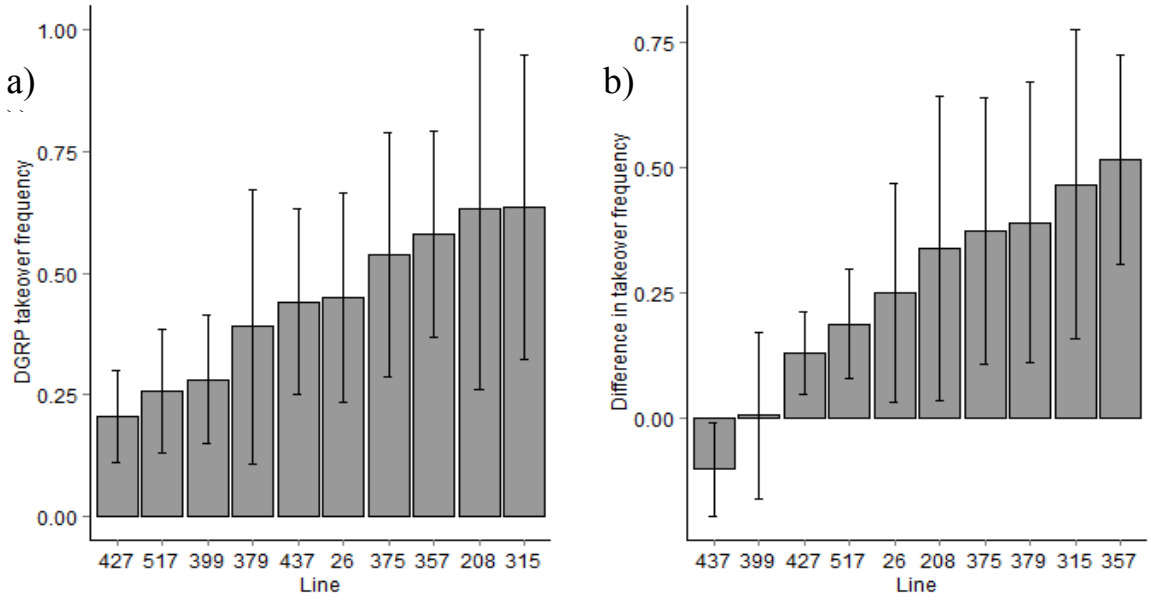

Fig. 6.1: a) Mean ± SE takeover frequency of hybrid DGRP lines (N = 9 to 14 replicates per treatment). b) Mean ± SE difference in takeover frequency calculated as DGRP takeover frequency minus wild takeover frequency (N = 9 to 14 replicates per treatment). Difference in takeover frequency is a proxy for overall competitiveness.

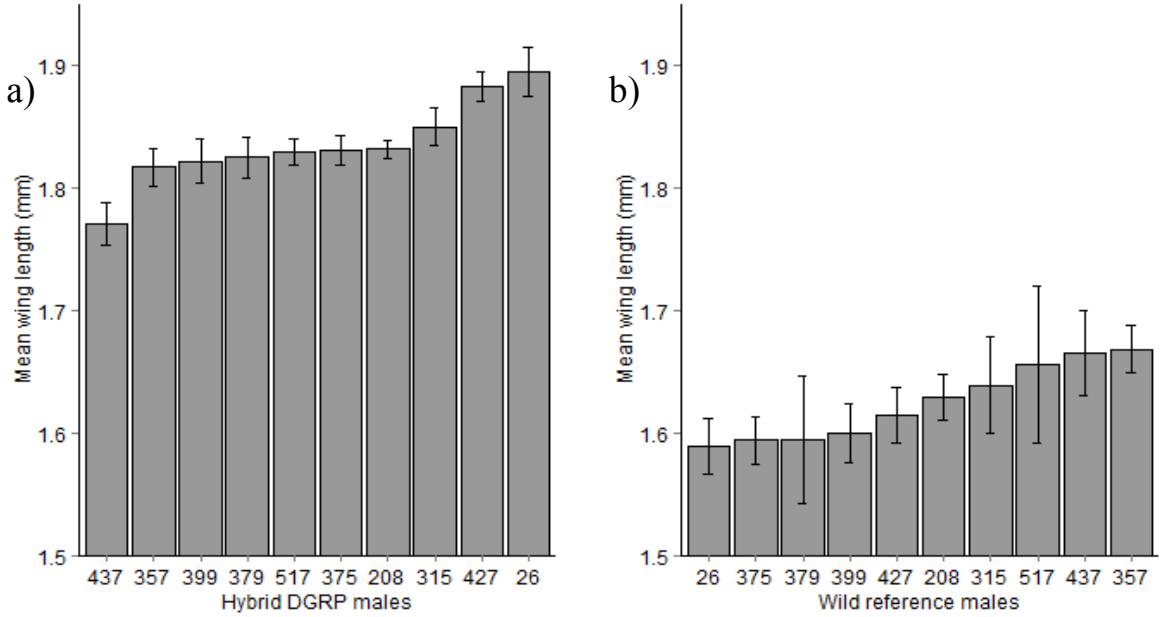

Fig. 6.2: Mean ± SE wing length (mm) (N = 5 to 11 per treatment). a) Hybrid DGRP males by line. b) Wild reference males grouped by their DGRP competitor.

EXPERIMENT 7: Replication of courtship interference behaviour among dominant and submissive lines

RATIONALE

Admittedly, the sample sizes in the above screen of genetic variation in takeover behaviour were small and there was large variation in takeover behaviour within each line. However, it was only to serve as a preliminary screen. From the screen, we chose three lines 208, 315, and 357 that performed the greatest frequency of takeover behaviour, hereby termed dominant (Fig. 6.2a). Our choice of submissive lines took into account differences in takeover frequency, proportion of courtship and size effects. We did not choose lines 427 and 399 even though both were in the bottom three lines for takeover frequency because both spent much more time courting than did their wild competitor (data not shown). Lines 517 and 437 both performed a low frequency of takeover behaviour (Fig. 6.2a), and were taken over more often by wild males (Fig. 6.2b). Additionally, we chose as a third submissive line, line 26, as it had both the largest mean body size and faced the smallest wild reference males, yet was in the bottom five lines for competitive ability (Fig. 6.2b).

In this experiment, we aimed to replicate the tendency for takeover behaviour in each line with a larger sample size. Moreover, we checked that the takeover behaviour observed in the initial screen was not transient in that the three dominant lines that we chose, in addition to out-competing wild males in experiment 6, also successfully out-compete the three submissive lines that we chose. In addition to this, we wished to choose the 2 most dominant and 2 most submissive lines to test the effect of courtship interference on mating success.

METHODS

Methods are as described in experiment 6 with the exception that we coloured both dominant and submissive males either pink or blue in a counterbalanced fashion to discriminate them.

We assessed differences between dominant and submissive pairs using a general linear mixed effects model. Takeover frequency was predicted by a single fixed effect level of competitiveness i.e. dominant or submissive. We also included a random effect allowing both the intercept and the level of competitiveness to vary by treatment i.e. there are 9 individual pairings of dominant and submissive males. The main effect of competitiveness tells us whether or not the dominant and submissive behaviour of each line is replicable and not transient. We tested for this using the Anova function with type III sums of squares. The slope of the random effect tells us whether or not there is a difference between treatments in the degree of dominance to submissiveness. We tested for this by parametric bootstrapping with 1000 iterations using the pbkrtest package (Halekoh & Højsgaard 2014).

RESULTS

Across all treatments, dominant lines had a greater takeover frequency than submissive (Main effect: Wald $\chi^2_1 = 9.660$; P = 0.002; Fig. 7). However, there was a significant difference among treatments in the difference in takeover frequency between dominant and submissive lines (PBtest: P = 0.014; Fig. 7)

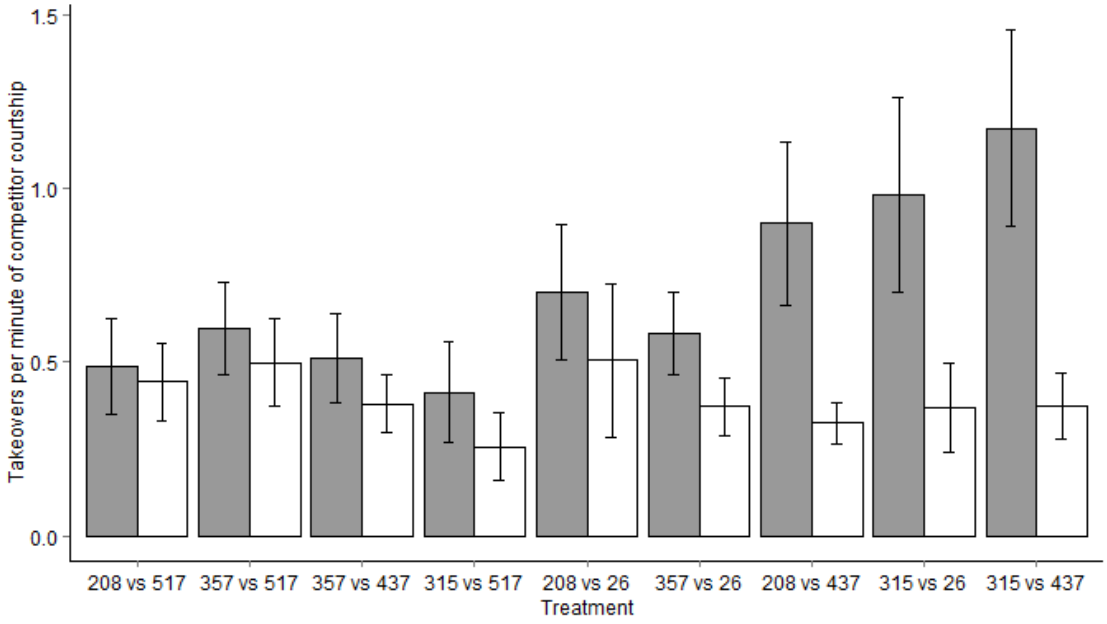

Fig. 7: Mean ± SE takeover frequency of dominant (grey) and submissive (white) DGRP lines (N = 15-21 replicates per treatment). Ordered from left to right by the increasing difference in takeover frequency between the dominant and submissive line.

EXPERIMENT 8: Testing the effect of genetic variation in takeover behaviour on mating frequency

RATIONALE

We wished to critically test whether the differences in takeover behaviour result in differences in competitive mating success. From the results of experiment 7, we chose to test the two most dominant lines, 315 and 208 and the two most submissive lines, 437 and 26 (see right-most bars in Fig. 7). We predicted that the dominant lines would have a greater proportion of matings than submissive in the competition treatment than when alone.

METHODS

We used two treatment groups, each with four levels. In the first group, we placed into each arena, a 4 day old male from one of the four lines 315, 208, 437 and 26 alone with a 4 day old sexually receptive, virgin female. In the second group, we placed into each arena, dominant and submissive male pairs 315 vs. 437, 315 vs. 26, 208 vs. 437 and 208 vs. 26 with a 4 day old sexually receptive, virgin female.

We used arenas as described in experiment 6 and coloured males as described in experiment 7. For each arena, we recorded, blind to male line, the mating latency and the colour of mated males. If no mating had occurred by 30 minutes, we replaced the female in the arena and observed for an additional 30 minutes. If no mating had occurred after the combined hour duration, we scored males as not mating. After trials, we measured the wing length in a subset of males.

RESULTS

Due to a complication in the maternal parent line that was not apparent during the execution of experiments 6 and 7, upwards of 90% of hybrid F_1 males had wing deformities and thus were not suitable for testing. As a result, we were only able to run 3 treatments: the dominant line 208 alone, the submissive line 26 alone and 208 vs. 26 in

competition. The sample sizes were respectively, N = 13, 10, and 28. As there is typically large variation in mating frequencies, the standard sample size in our lab for testing this is N = 100 per treatment. Hence, we believe the data collected do not merit statistical analysis.

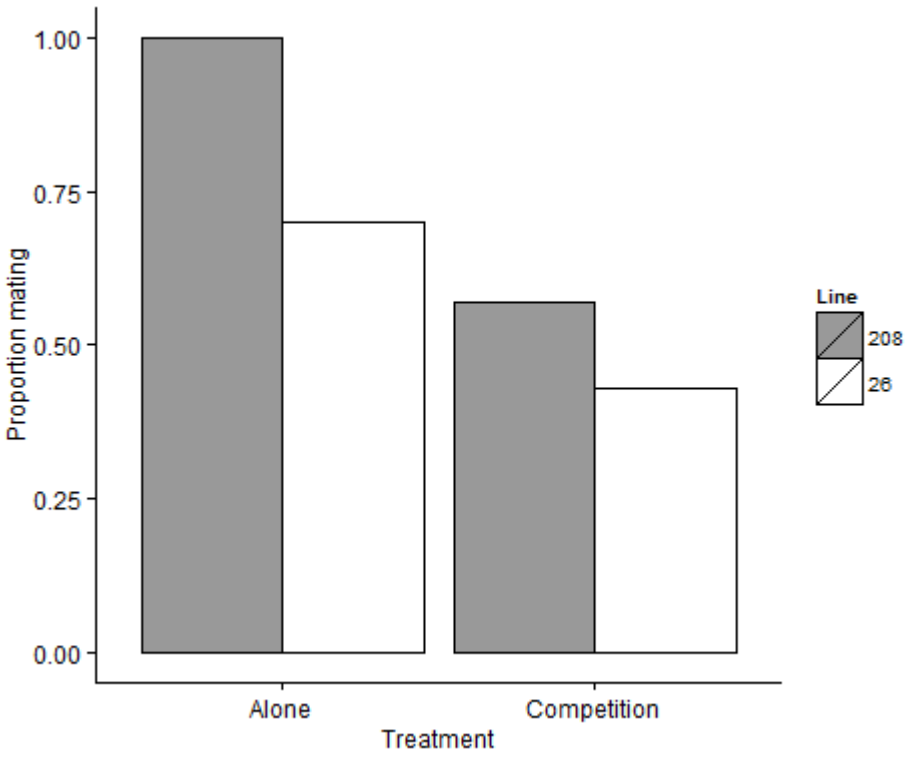

Fig. 8: Proportion of dominant (grey) and submissive (white) males mating (N = respectively 13, 10, and 28 for treatments 208 alone, 26 alone and 208 vs 26 in competition).

DISCUSSION: True female choice vs. competition

We did not find strong evidence for an effect of male competition on mating success in the choice vs. competition arenas. Here, we discuss possible reasons why and propose future experiments that address concerns with the experimental design.

We predicted that mature males would have a greater advantage in securing initial matings as well as re-matings in the competition arena compared to the choice arena. This prediction was based on the mature males' ability to prevent the young males from access to females through courtship takeovers. To some degree, it could be argued that this is a weak prediction because in fruit flies, females are able to reject males' advances and as a result, female choice may override male competition. Similarly, in the field cricket, male competition results in competitive males holding burrows and therefore the opportunity to mate (Simmons, 1986b). But ultimately, because the female must mount the male, female choice may dictate a male's reproductive success (Simmons, 1986a). In fruit flies, the female is typically larger than the male and thus may have the final say on the length of the courtship period and which male mates (Merrell, 1949).

However, by definition, female mate choice requires the rejection of some males and because of intersexual conflict, this can be costly to the female. There is evidence to suggest that in *D. melanogaster*, persistent male harassment through courtship reduces the lifespan of females (Friberg & Arnqvist, 2003; Partridge & Fowler, 1990). Moreover, in an experimental monogamous population in which sexual selection was removed, male courtship intensity was reduced (Holland & Rice, 1998). Furthermore, females of the monogamous population died sooner than females of a control polyandrous population when both were housed with males from the polyandrous population. Taken together, persistent harassment through male courtship can have a negative effect on females' fitness, which females incur by rejecting said males. In polyandrous mating systems such as *D. melanogaster*, there is generally positive directional selection for males to mate more (Friberg & Arnqvist, 2003). Therefore, males may remain persistent in their courtship even in the face of female rejection signals (see: Dukas & Scott, 2015). Furthermore, male persistence may increase with age if older males discount the future to

a greater degree than young or if past experience suggests that there are few potential mates.

Overall, this means that female choice is not equivalent to female preference, but rather, is a cost benefit decision. For females, each subsequent mating has diminishing returns in terms of viable offspring production, but may retain costs such as suboptimal mating conditions in the timing or place of mating. The cost of mating has been shown empirically as a reduction in lifespan in females (Fowler & Partridge, 1989), with a suggested mechanism of toxic male seminal fluid products (Chapman et al., 1995). Therefore, because there are both benefits and costs to mating, females should have some optimal intermediate mating frequency (Friberg & Arnqvist, 2003) where the cost of rejection is balanced against both costs and benefits of mating with a more attractive male.

As expected from previous studies assessing the mating rates of mature vs. young males in competition (Dukas & Baxter, 2014; Long et al., 1980), mature males in the competition arenas were both more likely to mate first and had a greater number of total matings than their younger competitors. However, we observed a similar pattern of mating in the female choice arenas. After viewing female behaviour in the choice arena, we cannot say with confidence that we accurately tested for female preference by reducing the costs of rejection. Female movement in the choice arenas was largely driven by evasion of male harassment. It was typical of females to spend more time with the less aggressive young males during the first half of trials. This is apparent in the interaction of competition level and time. Early on, restricted mature males in the choice arena have fewer matings than unrestricted mature males in the competition arena (Fig. 5.1). One could argue that as a result of constant female evasion of male harassment later in the trial duration, females may have eventually given up and allowed a male to mate regardless of preference. This follows the logic of convenience polyandry where the cost of rejection outweighs the cost of mating (Arnqvist & Rowe, 2005). Such coercive mating is more likely to occur under conditions of male biased sex ratios where males are persistent in courtship and mating attempts (Wigby & Chapman, 2004). Recent work in our lab has

shown that mature males are more likely than young males to be aggressive towards females resulting in more coercive matings (Baxter & Dukas, 2016). Therefore, the extent to which mature males mated in the female choice arena may reflect greater coercion by mature males rather than female preference.

In a small sample of preliminary trials, we tested a female choice arena with three compartments instead of two. The center compartment allowed the female a refuge from males which reduces the opportunity for males to harass females. A spatial refuge may allow females to mate at a rate that is closer to their optimum rather than the optimum of males (Byrne et al., 2008), and thus more accurately tests for female preference. However, we noted that females visited both male compartments at a lower rate than our current no refuge arena. It could be argued that females can make a choice without receiving courtship from both males based on olfactory (Arienti et al., 2010) or visual cues, but we did not wish to make this assumption in assessing preference. Therefore, if using the refuge arena, we would have had to eliminate a greater number of trials where no choice occurred, causing potential bias in the data.

The inability of the female in either the competition or the choice arena to escape male harassment is an obvious flaw as it does not reflect natural conditions. A possible solution to this would be a sequential choice test where a female is repeatedly presented a new male if the previous male is rejected. To reduce the effect of male harassment we would provide females with ample space by using a cage and allowing them to signal male rejection by decamping, a behaviour where a female flies away from a male. Immediately following decamping, females would be transferred to a new male and cage where she again may make a choice that involves less potential cost of male harassment. A potential critique of this design is whether or not females of *D. melanogaster* have the cognitive capability to compare males as the latency between male encounters increases. Often in nature females arriving at a food patch will be courted by an average of five males (Markow & Sawka, 1992). Whether or not a sequential choice design is representative of female choice in natural conditions merits further exploration.

DISCUSSION: Genetic variation in takeover behaviour

To assess for the potential of sexual selection on traits associated with courtship interference behaviour, we screened for genetic variation in takeover behaviour. In the initial screen, we did not find significant variation among the ten lines tested in either DGRP takeover frequency or their general competitive ability (Fig. 6.2 a, b). This could in part be attributed to the small sample size of trials as well as large variance within each line which was largely driven by zero inflation. Takeover frequencies of zero did sometimes occur for lack of intense competition between males, but in many trials intense competition was observed yet lacked takeovers. When a female remained somewhat immobile against a wall, pairs of males would simultaneously court a female with intensity, but were less likely overtake each other. On the other hand, takeovers occurred more readily during bouts of chasing. There was a significant difference in the takeover frequency scored by each observer. However, this can be difficult to avoid in behavioural observations as observers may differ in their level of conservativeness when scoring.

In the follow up experiment, the dominant line in all treatments tended to takeover more than the submissive. Although it is clear that any difference between the dominant and submissive lines was minimal. In the future, it is likely that to find clearly dominant and submissive lines, a greater number of lines need to be screened.

In the final experiment, between the weak differences among the dominant and submissive lines screened and the lack of sample size it would be naïve at this point to draw any conclusions about the potential for sexual selection on traits associated with courtship interference behaviour. It is possible to re-run the final experiment after re-establishing the maternal parent line, as it is a relatively short experiment compared to the initial screens. Although, the lack of variation in the screens reduces the chance that dominant males will be able to secure more matings than submissive males under competition than when alone making any effect likely conservative. Because of this, it may be best to run this experiment on a larger scale and screen upwards of 60 DGRP lines as opposed to 10 and make use to computer vision to increase the efficiency of observations.

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