Decline in offspring quantity but not quality from successive matings in male rainforest *Drosophila,* with no evidence for genetic divergence in male mating behaviour along elevational and density gradients.

Andrew D. Saxon[1*], Eleanor K. O'Brien[1,2*], Natalie E. Jones[1] and Jon Bridle[1,3+]

[1] School of Biological Sciences, University of Bristol, Bristol, U.K.
[2] Centre for Precision Health, Edith Cowan University, WA, Australia
[3] Department of Genetics, Evolution and Environment, University College London, London, U.K.

* These authors contributed equally to this work

+To whom correspondence should be addressed

ACKNOWLEDGEMENTS

We are very grateful to Rhonda Snook for work assaying *D. birchii* sperm morphology and female reproductive tracts and to Tom Tregenza and two anonymous reviewers for helpful comments on an early draft of the manuscript. This work was supported by a University of Bristol PhD scholarship for ADS and a Natural Environment Research Council standard grant (grant number NE/G007039/1) to JRB.

ABSTRACT

Fertilisation success is a major component of male fitness, meaning males should capitalise on all opportunities for mating. However, other sources of variation in fitness mean that males often evolve life histories that limit their ability to mate frequently. We quantified mating latency, mating duration and offspring production in males of the tropical fly *Drosophila birchii* when presented with up to four females consecutively. Males were sourced from isofemale lines from the extremes of two elevational gradients (20 – 1100 m), that show substantial differences in population density, temperature and humidity. Total offspring sired increased with number of matings achieved, demonstrating substantial benefits of multiple mating. However, mean numbers of offspring declined with each successive mating, and mean mating durations increased, while mating latencies remained consistent. We saw no reduced fitness in male offspring from later matings, suggesting that declining offspring production is not associated with decreasing quality. Although differences between gradients were observed in total offspring production, reductions in offspring number were as great for males from high density sites as those from low density sites, despite expectations that males from high density sites would show higher mating investment. We also detected no divergence between high and low elevation sites for other traits, suggesting little adaptive divergence in mating strategies across this species' entire elevational range. The steep decline in offspring production over successive matings may reflect low encounter rates, or mating opportunities with females in natural populations of this species, even in high density environments, reducing relative investment in sperm or ejaculates.

Key words*:* Male mating, Bateman gradient, fitness, elevational gradient, offspring quality, adaptation, *Drosophila*

INTRODUCTION

Sexual selection theory assumes that the reproductive success of males is primarily determined by the number of matings they obtain (Arnqvist & Rowe, 2005, Kokko et al., 2006, Bateman, 1948, Janicke et al., 2015, 2016). The strength of this correlation between mating and reproductive success is often described as the “Bateman gradient”, and used to quantify the strength of sexual selection (Fromonteil et al 2023). However, there are often significant costs to mating for males, including the energetic costs of mate searching (Harvanek et al., 2017, Parker, 1978), courting and copulating with females (Cordts & Partridge, 1996, Brown et al., 2009, Martin & Hosken, 2004, Kotiaho & Simmons, 2003, Partridge & Farquhar, 1981) and competing with rival males (Bretman et al., 2013b, Gaskin et al., 2002). Ejaculate production itself can be energetically expensive (Edward & Chapman, 2011, Hayward & Gillooly, 2011, Dewsbury, 1982, Van Voorhies, 1992, Reinhardt et al., 2011, Perry et al., 2013), especially in species such as *Drosophila* that generate large sperm (Pitnick, 1996, Snook, 2005, Pitnick et al., 1995). Together, these costs may limit male mating rate and therefore the number of females that males can successfully inseminate within a given time period, driving a trade-off between current and future reproductive success. As a result, optimal investment in each mating will depend on opportunities for future matings within a genotype’s lifetime (Parker & Pizzari, 2010, Parker, 1982). If male mating opportunities are scarce, males will be expected to invest more in initial matings, whereas when mating opportunities are abundant, resources are likely to be strategically allocated among successive mates, particularly where the costs of mating are high (Wedell et al., 2002, Bonduriansky, 2001, Engqvist & Sauer, 2001).

Although several studies have characterised variation in male mating behaviour (e.g. number of rematings, copulation duration, courtship duration, time to remating) and reproductive allocation and success (number of offspring produced per remating, or egg-larvae survival) across successive mating opportunities and in various insect taxa (Douglas et al., 2020, Macartney et al., 2020, Linklater et al., 2007, Hopkins et al., 2019), there is a lack of data on the degree to which these key fitness traits vary in natural populations. Selection to prioritise current versus future mating will fluctuate across environments (Gromko & Markow, 1993, Harshman et al., 1988, Emlen & Oring, 1977), in response to factors such as population density, that affect the likelihood of acquiring mates by changing both the intensity of male-male competition and the chance of encountering conspecific females (Parker et al., 2013, Knell, 2009, Kokko & Rankin, 2006, Niemela et al., 2021). However, population density has complex effects on mating opportunities across many insect taxa including flour, soldier and fungus beetles, earwigs, and meal moths (McCullough et al, 2018; House et al. 2019; Winkler et al. 2023). Although male encounters with females may become more common at higher densities, inter-male competition may also increase, reducing mating efficiency and/or duration, or altering investment in mating versus in parental care. For example, male mating behaviour in *Drosophila melanogaster* evolves in response to greater exposure to rival males, resulting in increased mating latency and duration (Dore et al., 2021), while males also deplete ejaculates more rapidly where females are encountered more rarely (Linklater et al., 2007). Females may also show higher levels of sexual selection at higher densities, meaning that (for a given male) mating opportunities may be as infrequent at higher population densities as at lower densities, while also requiring higher investment in courtship and male-male competition (House et al. 2019). Female as well as male behaviour may also be affected in other ways, for example by reduced aggregation at oviposition sites at higher density, meaning increased population density may not necessarily increase male encounter rate (Churchill et al. 2020; Churchill et al 2021).

Changes to abiotic conditions can also alter resource allocation trade-offs between mating success and stress tolerance (Marshall & Sinclair, 2010, Gefen & Gibbs, 2009, Dolgin et al., 2006). Adaptation to the thermal environment (Dolgin et al., 2006, Katsuki & Miyatake, 2009, Sambucetti & Norry,

2015) and the quality and distribution of resources (Churchill et al., 2020, Blay & Yuval, 1997, Yuval et al., 1998, Aluja et al., 2009, Trajkovic et al., 2013, Fricke et al., 2008) have been shown to influence male reproductive potential in several fly species. Likewise, variation in male mating-related traits across latitude (Chahal et al., 2013) and season (Dev et al., 2013) in *D. melanogaster* suggests that ecological conditions place significant constraints on reproduction, especially where investment in mating is high, as is the case in many Drosophilidae (Markow & O'Grady, 2008). By comparing populations that vary in the likely frequency of mating opportunities due to differences in population density or other aspects of their ecology, genetic variation in male mating behaviour and reproductive success across multiple matings can be quantified. Such data can be used to test for existing adaptive divergence in reproductive traits as well as the potential for key male fitness traits to evolve.

Differences in male fitness may not result solely from the total quantity of offspring produced across successive matings, but also as a consequence of variation in offspring quality (Clutton-Brock, 1991). Although larger and more numerous ejaculates increase reproductive success when mating opportunities are common, ejaculate quality affects male fitness independently of quantity (Pattarini et al., 2006, Snook, 2005). Reductions in sperm and ejaculate quality can lead to lower quality offspring (Hosken et al., 2003, Siva-Jothy, 2000, Alavioon et al., 2017). Declining sperm quality (*e.g.* motility, viability) over rapid sequential ejaculations has been observed in rabbits (*Oryctolagus cuniculus*) (Ambriz et al., 2002), and in guppies (*Poecilia reticulata*), declines in sperm competitive ability are associated with the reduced reproductive success of sons (Gasparini et al., 2017). Variation in non-sperm seminal products has also been shown to affect key fitness traits in offspring (Crean et al., 2016, Crean & Bonduriansky, 2014). Although it has been demonstrated that multiple mating by females (polyandry) can confer fitness benefits via enhanced offspring quality in a number of species (Madsen et al., 1992, Ivy & Sakaluk, 2005, Gowaty et al., 2010, Jennions & Petrie, 2000), the effects of male multiple mating on offspring quality are, relatively unexplored; particularly in situations where ejaculates are likely to become depleted, affecting both quantity and quality of offspring (Crean et al., 2016, Macartney et al., 2018).

In this study, we test for variation in male reproductive investment strategies of the tropical fly *Drosophila birchii* between populations where the frequency of mating opportunities is likely to differ substantially. We measured key mating traits for individual *D. birchii* males from isofemale lines sourced from the high and low ends of two elevational transects (*i.e.* the entire elevational range of this species), that show significant differences in population density and abiotic factors such as mean, minimum and maximum temperature and humidity (Bridle et al., 2009, O'Brien et al., 2017). Traits measured were: (*i*) latency to achieve copulation, (*ii*) duration of copulation, (*iii*) number of offspring produced with each successive copulation, and (*iv*) total number of offspring sired, when initially virgin males were presented with four virgin females sequentially during a one-day period.

From previous work with *D. birchii*, we predicted that male reproductive success (mean number of offspring) would decline across successive matings. This is likely to be associated with changes in latency to mate, as males require refractory periods to replenish ejaculate reserves between mating bouts (Bissoondath & Wiklund, 1996, Radhakrishnan et al., 2009, Sirot et al., 2009, Macartney et al., 2021), and mating duration, as ejaculates become depleted over rapid consecutive copulations (Singh & Singh, 2000, Edvardsson & Canal, 2006, Linklater et al., 2007). However, we also hypothesised that the decline in offspring production would be lower for males from the higher density end of each elevation gradient than those from lower density sites, as higher population densities would select for higher male productivity across multiple matings. Finally, we tested the prediction that males produced from later matings (when ejaculates may be depleted) would be of lower quality and show reduced offspring productivity compared to those from earlier matings.

Predictions:

1. Sperm and/or other components of the ejaculate will decline with successive matings, which will result in:
    a. Decline in the number of offspring sired by male *D. birchii* across successive matings.
    b. Increased time spent courting and copulating with females, resulting in increased mating latency (time to commence mating) and duration (time from initiation to completion of copulation) across successive matings.
2. Males from high density populations, where mating opportunities may be more frequent, will partition investment across successive matings, resulting in a shallower decline in the number of offspring sired across matings than for males from lower density populations, and gain greater fitness benefits from multiple mating (in terms of total offspring sired).
3. Quality of offspring (siring success of sons) will decline across successive matings.

MATERIALS AND METHODS

Study species and populations

D. birchii is limited to tropical rainforest in north-eastern Australia and New Guinea, where it has an elevational range from ~0 – 1100 metres. *D. birchii* males display complex male courtship behaviour (Hoikkala et al., 2000), and show highly skewed siring success when presented with multiple females simultaneously (E O'Brien personal observation, 2010), suggesting limited capacity for remating.

Local abundance of *D. birchii* varies with elevation, with populations showing steep declines in abundance above 900 m and below 200 m (Bridle et al., 2009, O'Brien et al., 2017). We established *D. birchii* isofemale lines (see below) from populations at each elevational extreme of two elevational transects in north-east Queensland, Australia: Mount Lewis (Elevation: ~20 – 900 m, 16°35'S 145°17'E) and Mount Edith (Elevation: ~600 – 1100 m, 17°6'S, 145°38'E). Within these transects (each 1-5 km long) there is no significant differentiation in neutral genetic (ddRAD) markers, indicating high levels of contemporary gene flow across these distances. However, significant isolation by distance (explaining 7% of the variance across 600+ variable loci) is observed between these transects (Saxon, 2018). Both gradients show significant differences in mean daily temperature and humidity between high and low elevations (O'Brien et al., 2017; Table S1). Population density (measured as the number of male *D. birchii* captured per trap per day) varies with elevation along both of these gradients, but in opposing directions. At Mount Lewis, the density of the high elevation population is approximately three times greater than the low elevation population, while at Mount Edith, population density declines with elevation and is approximately eight times greater in the low than the high elevation populations sampled here (O'Brien et al., 2017).

(a) Establishment of isofemale lines

Drosophila birchii isofemale lines (called 'lines' hereafter) were founded from field-mated females collected from each population described above between March – May 2011. Flies were collected using banana baited buckets, sampled daily using fine sweep nets and sorted under a microscope using light CO_2 anaesthesia. Females were placed individually in vials to lay for 5 – 10 days. Each line was maintained across 3 – 4 40 ml vials containing 10 ml of *Drosophila* potato food medium (agar, instant mashed potato powder, raw sugar, inactive yeast, propionic acid, nipagin supplemented with live yeast) at ~100 individuals per generation for each line.

A ‘mass-bred’ stock was also established by combining 10 male and 10 female flies from each line, in order to provide a genetically mixed background population for test females to assess the mating behaviour and fitness of focal males. Mass-bred stocks were reared in 400 ml bottles with 100 ml of *Drosophila* medium and mixed across bottles each generation. All lines and the mass-bred population were maintained with non-overlapping generations. Mount Lewis experiments were conducted after ~25 laboratory generations, Mount Edith after ~50 laboratory generations.

(b) Experiment I: Assaying male mating traits across successive matings

Ten lines were used for the Mount Edith assay (five from each end of the gradient). Twenty lines were used for the Mount Lewis assay, with 10 originating from each population. Prior to the experiment, line stocks and the mass-bred population were reared at a constant 25 °C, 12:12 hour light:dark cycle for two generations to randomise and minimise any effects attributable to maternal condition. The experimental males and background females were reared for one generation at minimal density conditions to minimise larval competition, with five male and five female flies being allowed to mate and lay eggs in 10 ml of standard *Drosophila* medium for three days. Parental flies were then removed, and pupation card (75 x 30mm) inserted into the vial. On eclosion, flies were anaesthetised using CO_2, and sexed using a Leica (MZ9.5) microscope. 30 males from each of 10 lines (n= 300) from Mount Edith and 10 – 20 males from 20 lines (n= 285) from Mount Lewis were collected, along with mass-bred females, within 12 hours of emergence to ensure virgin status. All flies were held in single-sex vials for six days, at 25 °C at low density (maximum of 10 flies per vial) with fly food medium *ad libitum,* before the mating assay commenced.

Mating assay: The assay was conducted at a constant 25 °C and began within an hour of the daylight period, to coincide with time of peak activity in *Drosophila*. Each virgin male was placed in a vial with 8 ml of standard fly medium. All observations were made by Andy Saxon, assisted in vial and male and female preparation by the co-authors. A virgin female was placed with the focal male and the start time noted. If mating was initiated, the time was recorded to give time to copulation (latency), as was the end mating time (duration). Following copulation, the male was moved to a fresh vial and presented with a new female. This process continued with up to four matings allowed for each male within the one-day period, with males given up to two hours to mate with each female. If no mating occurred, the male was recorded as 'not mated' and the assay concluded for that male. The experiment was conducted over five days with ~60 male flies from each gradient tested per day, using equal numbers from each line. The order of males was randomised within each block to ensure that there was no systematic bias among lines due to diurnal effects, or the effect of successive matings. Blind ID codes were also used to avoid observer bias. Mated females were left in the vials for five days to ensure that they had laid all fertilised eggs. *D. birchii* females lack spermathecae, which means that sperm storage is minimal (R. Snook, personal communication, 2014). The female was then removed, and a pupation card added. At least 20 days after mating, the total productivity (number of offspring) of each successful mating for each male was recorded.

(c) Experiment II: Assaying the fitness of sons derived from successive paternal matings

Ten virgin males (sons) were collected from each of the paternal matings (first to fourth) for males from each isofemale line. These were randomly selected from a combination of all offspring of focal males (sires) from that line that had achieved the maximum four matings in Experiment I. Sons used were taken from seven of the ten Mount Edith isofemale lines (n= 280). At six days past emergence, each male was placed in a single vial of 8 ml of fly medium with a single, virgin mass-bred female of the same age. The pair were left for three days to mate under the same conditions as in Experiment

I. The male was removed, and each female was left to lay for a further five days. The female was then also removed, pupation card inserted, and all offspring left to emerge. The fitness of each son was assayed as the total number of offspring produced by this mating.

(d) Statistical analysis

All analyses were run in R (R Core Team, 2016) v 4.1.0.

Experiment I: The package *lme4* (v 1.1-27) (Bates et al., 2015) was used to fit linear mixed models to test whether male mating traits (latency to mate, duration of mating, number of offspring per mating) change across successive matings, and whether this varies among source populations. Separate models were fitted for each trait and elevational gradient. Mating number (as a continuous factor), elevation of origin (high or low) and their interaction were included as fixed factors. Mating duration and offspring per mating both showed non-linear relationships with mating number, so mating number was fitted as a second order polynomial using the *splines2* (v 0.4.4) package (Wang & Yan, 2021) in R. For latency, this did not improve model fit (based on a comparison of the log likelihood of models with and without the second order polynomial term), so only the linear relationship of this trait with mating number was modelled. Focal male and isofemale line were specified as random effects.

Generalised linear mixed models, with a Poisson distribution, were run using *lme4* to compare the mean number of matings obtained by males from high and low elevations at each gradient. Elevation of origin was included as a fixed effect and isofemale line as a random effect.

To evaluate the fitness benefits of multiple mating, we used linear mixed models to test whether the relationship between the number of matings achieved and the total number of offspring sired differed between males from high and low elevations from each gradient. Elevation, number of matings and their interaction were included as fixed effects. We included a second order polynomial term for number of matings (fitted using the *splines2* package) to test for a non-linear relationship of this factor with total offspring number. Isofemale line was included as a random factor.

Experiment II: To test whether there was a decline in the quality of sons produced from successive matings by Mt Edith males we fitted a linear mixed model of male fitness (number of offspring sired), where mating number was included as a fixed effect, and isofemale line as a random effect.

For each set of models, the significance of fixed effects was evaluated using F-tests, obtained using the *anova* function in R. The significance of random effects variance was evaluated using likelihood ratio tests to compare the full model with a model where that factor had been removed.

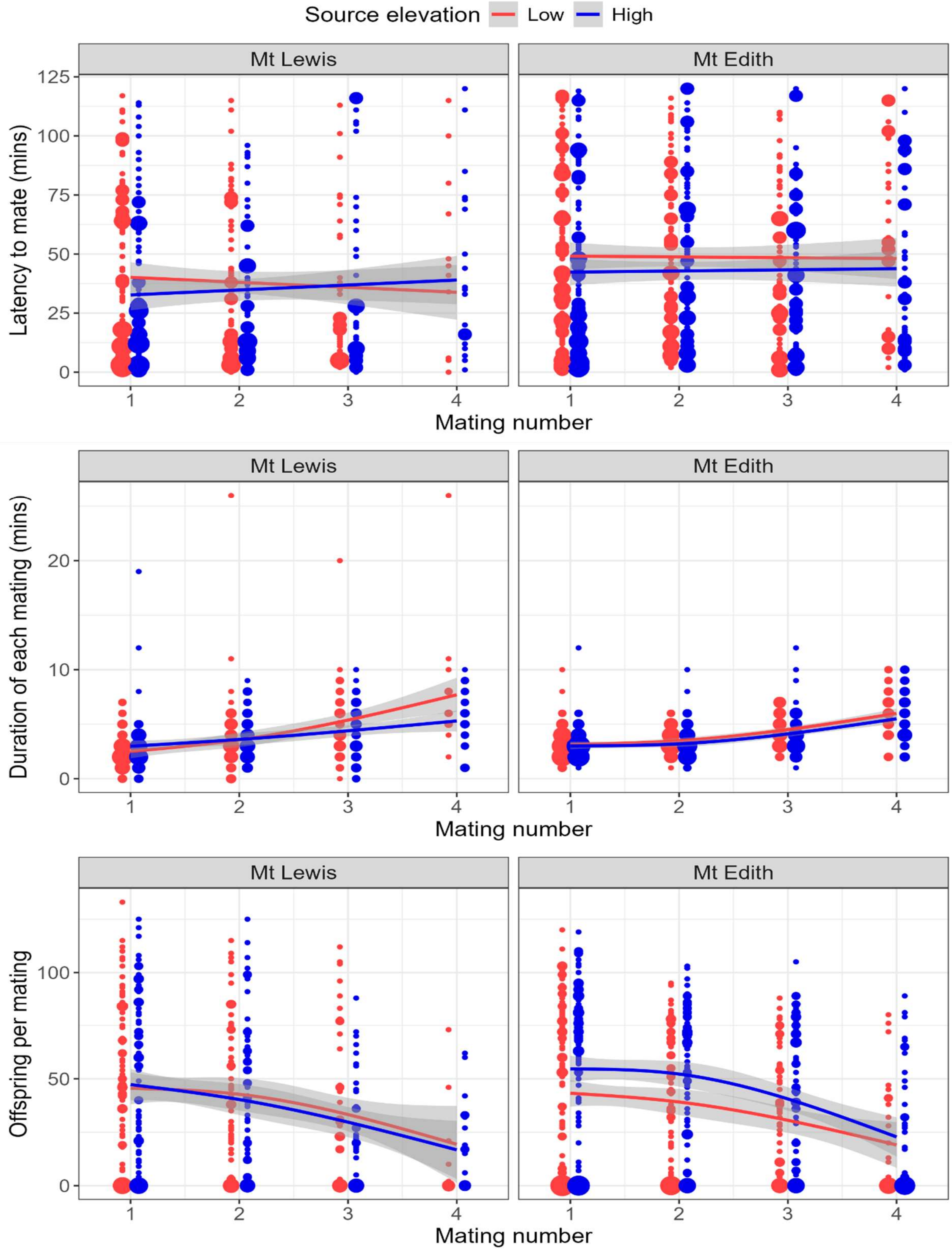

Figure 1. *Increased mating duration and reduced productivity with successive matings*. Plots showing mating latency, mating duration and number of offspring (productivity) from high elevation (blue dots) and low elevation (red dots) *D. birchii* isofemale lines from Mt Lewis (left) and Mt Edith (right) for each successive male mating (1 – 4). The size of each point is proportional to the number of males with a particular value for that mating number and trait (Number of matings observed: Mt Lewis 391 matings by 162 males from 20 lines; Mt Edith 589 matings by 221 males from 10 lines). Also plotted are regression lines from models fitted for each trait against mating number, using a

linear (mating latency) or quadratic (mating duration, number of offspring) function. The grey shaded regions indicate the 95% confidence interval around each fitted line.

Table 1. *Linear mixed effects analyses for male mating traits in Mount Edith and Mount Lewis lines*. Mating (1 – 4) and elevation of origin of isofemale line are fixed effects, with nested random effects of isofemale line and focal male. ANOVA tables of the fixed effects are shown for each trait at each gradient, and variance components for the random effects. *P*-values for random effects were obtained using likelihood ratio tests of the full model with the effect against a reduced model with the effect excluded. SS = sum of squares; MS = mean square.

Gradient	**Mating trait**	**Fixed effects**					**Random effects**			
		Predictor	**SS**	**MS**	$F_{(df1,\ df2)}$	***P***	**Variance component**	**Variance**	$\chi^2_{(1\ df)}$	***P***
Mt Lewis	Latency	Elevation	2554.3	2554.3	$2.47_{1,176}$	0.12	Line	14.18	0.13	0.72
		Mating	0.07	0.07	$1.00 \times 10^{-4}_{1,356}$	0.99	Male	19.44	0.13	0.71
							Residual	1035.4		
		Elevation x Mating	1634.5	1634.5	$1.58_{1,356}$	0.21				
	Duration	Elevation	7.81	7.81	$1.06_{1,72}$	0.31	Line	0.07	0.03	0.86
		$(\mathrm{Mating})^2$	483.04	241.52	$32.94_{2,384}$	6.29×10^{-14}	Male	0.00	0.00	1.00
							Residual	7.33		
		Elevation x $(\mathrm{Mating})^2$	67.20	33.60	$4.58_{2,384}$	0.01				
	Offspring per mating	Elevation	230	230	$0.22_{1,46}$	0.64	Line	53.81	2.74	0.10
		$(\mathrm{Mating})^2$	20939.8	10469.9	$10.09_{2,288}$	5.84×10^{-5}	Male	85.81	1.39	0.24
							Residual	1038.00		
		Elevation x $(\mathrm{Mating})^2$	704.2	352.1	$0.34_{2,288}$	0.71				
Mt Edith	Latency	Elevation	1535.0	1535.0	$1.34_{1,57}$	0.25	Line	21.73	1.14	0.29
		Mating	96.15	96.15	$0.08_{1,585}$	0.77	Male	0.00	0.00	1.00
							Residual	1147		
		Elevation x Mating	274.3	274.3	$0.24_{1,585}$	0.63				
	Duration	Elevation	0.19	0.19	$0.10_{1,12}$	0.76	Line	0.10	3.53	0.06
		$(\mathrm{Mating})^2$	446.7	223.3	$110.00_{2,485}$	4.05×10^{-40}	Male	0.23	7.83	0.01
							Residual	2.03		
		Elevation x $(\mathrm{Mating})^2$	0.83	0.41	$0.20_{2,485}$	0.82				
	Offspring per mating	Elevation	1183	1183	$1.56_{1,9}$	0.24	Line	224.7	35.71	2.29×10^{-9}
		$(\mathrm{Mating})^2$	62517	31259	$41.22_{2,422}$	4.47×10^{-17}	Male	221.0	21.07	4.43×10^{-6}
							Residual	758.4		
		Elevation x $(\mathrm{Mating})^2$	924	462	$0.61_{2,422}$	0.54				

RESULTS

(a) Experiment I: Variation in male mating traits across successive matings

Out of 589 observed matings of Mt Edith males, 166 (28.2%) did not produce any offspring, while for Mt Lewis males, 87 of 391 (22.3%) observed matings did not produce any offspring. We therefore ran analyses of mating traits with and without matings that produced 0 offspring. This made no qualitative difference to the results, therefore we present the results of analyses that include these matings. At Mount Edith, 73% of all focal males assayed attained a first mating (n= 220), with only 26% of all males reaching a fourth (n= 78). 18% mated only once. Mean productivity (number of offspring) per mating showed a 60% decline from mating 1 (mean offspring= 49.47) to mating 4 (= 19.71) for Mount Edith males (Fig. 1). At Mount Lewis, 57% of all focal males achieved a first mating (n= 162), while only 11% attained a fourth mating (n= 31). 14% of males mated only once. Mean productivity per mating showed a 62% decrease from mating 1 (mean offspring= 46.38) to mating 4 (= 17.74) for Mount Lewis males (Fig. 1). Latency to mating showed no change across successive matings at either gradient (Table 1). Mating duration, however, showed a significant increase across successive matings (Table 1), with durations increasing approximately twofold from the first to fourth mating at both gradients (Fig. 1).

For comparison, results of analyses of the relationship between mating number and mean number of offspring restricted only to males that mated four times are presented in the Supplementary Materials (Fig SM1 and Table SM1). As for the full data set, there was a significant decline in the number of offspring produced across successive matings (Fig SM1, Table SM1).

Although differences were observed between the geographically distinct gradients, there were no detectable differences between lines originating from high and low elevation sites, for any trait, at either gradient (Table 1). Generalised linear mixed models of the mean number of matings obtained by males at each gradient also revealed no significant difference between males from high and low elevations at either gradient (Table 2).

Table 2: ANOVA table of results of generalised mixed models testing for a difference between males from high and low elevation in the mean number of matings attained. This was modelled as a poisson distribution with isofemale line included as a random effect. MS = mean square.

Gradient	Predictor	MS	X^2_1	*P*
Mt Lewis	Elevation	0.46	0.44	0.51
Mt Edith	Elevation	0.24	0.24	0.63

There was a strong positive association between the number of matings obtained and the total number of offspring produced (Table 3, Fig 2), although the shape and slope of these Bateman gradients varied for males from the different transects. Mt Edith males that mated only once produced a mean of 43.02 (95% confidence interval (CI) = 32.6 – 53.4) offspring, while males that mated four times produce a mean of 171.88 (95% CI = 154.5 – 189.3) offspring across all matings (Fig. 2). Mt Lewis males that mated only once had a mean total of 59.18 (95% CI = 46.7 – 71.7) offspring, while males that mated four times produced a mean total of 104.39 (95% CI = 81.3 – 127.5) offspring (Fig. 2). The relationship between number of matings and total number of offspring did not vary with elevation at either gradient (Table 3).

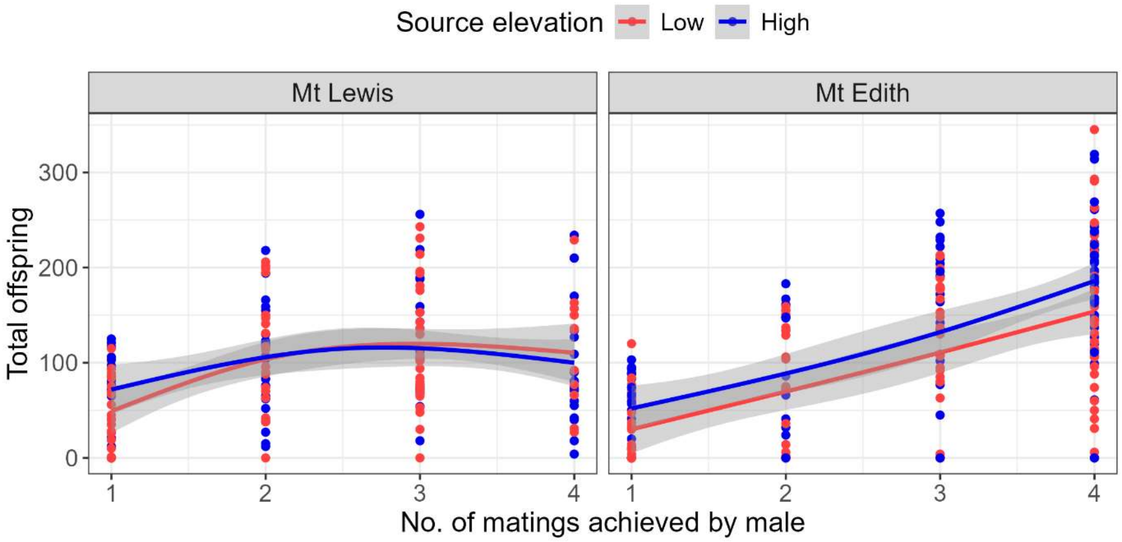

Figure 2. Plots showing the total number of offspring sired by males as a function of the total number of matings attained at each gradient (Number of matings observed: Mt Lewis 391 matings by 162 males from 20 lines; Mt Edith 589 matings by 221 males from 10 lines). Dots are individual data points and lines are fitted lines from linear mixed models for high elevation (blue) and low elevation (red) males. The grey shaded regions indicate the 95% confidence interval around each fitted line.

Table 3 ANOVA table showing results of linear mixed models testing whether the relationship between total number of matings attained and total number of offspring varies with elevation of origin at each gradient. Isofemale line was included as a random effect. A greater number of matings was associated with a significantly greater number of total offspring at each gradient (see also Figure 2), but this relationship did not differ with elevation of origin at either gradient. SS = sum of squares; MS = mean square.

Gradient	Predictor	SS	MS	$F_{(df1,\ df2)}$	*P*
Mt Lewis	Elevation	5656	5656	$2.02_{1,155.9}$	0.16
	(No of matings)^2	64594	32297	$11.54_{2,153.5}$	2.14×10^{-5}
	Elevation x (No of matings)^2	9021	4510	$1.61_{2,153.5}$	0.20
Mt Edith	Elevation	2560	2560	$0.75_{1,107.34}$	0.39
	(No of matings)^2	462451	231226	$67.55_{2,209.5}$	0.00
	Elevation x (No of matings)^2	1261	631	$0.18_{2,207.83}$	0.83

(b) Experiment II: Fitness of sons from successive paternal matings

The productivity of sons (only from Mt Edith males) was not explained by the paternal mating order ($1^{st} – 4^{th}$) that the sons were derived from. This was true whether mating number was treated as a continuous ($F_{1,272}$= 0.179, P = 0.673) or a categorical variable ($F_{3,270}$= 0.917, P = 0.433) (Figure 3).

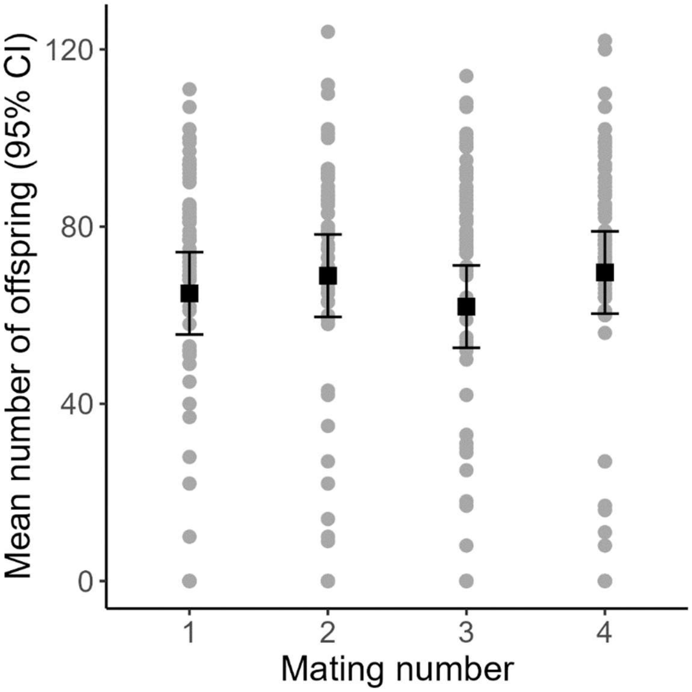

Figure 3. The number of offspring sired by 280 males from 7 lines from Mt Edith as a function of the mating ($1^{st} – 4^{th}$) from which they were produced. Grey dots show the number of offspring sired by individual males. Black squares and error bars are the estimated marginal means and 95% confidence intervals for each mating, obtained from linear mixed models that also included isofemale line as a random factor.

DISCUSSION

Productivity in D. birchii *males declines across subsequent matings*

Males typically maximise the number of offspring they sire by mating with multiple females (Bateman, 1948, Trivers, 1972). However, while mating is generally assumed to be more costly to females, it also often incurs costs for males, in the form of energetic and time demands, which are also likely to vary across environments (e.g. McCullough et al. 2018; Winkler et al. 2023). This means that across many taxa the capacity of males to mate frequently and continually produce offspring may be more limited than is often assumed, particularly where encounter rates with females are typically low, or where monopolisation of females by a few males is common.

We found that virgin males of the rainforest fruit fly *D. birchii*, when given up to four opportunities to mate over the period of one day, experienced steep and significant declines in productivity with each consecutive mating (Fig. 1). Similar patterns have been shown in *D. melanogaster* (Lefevre & Jonsson, 1962, Hopkins et al., 2019, Linklater et al., 2007), although both the mating rate and total productivity of *D. melanogaster* males exceed those of *D. birchii* in our study, making *D. birchii* much less capable of sustained remating that other *Drosophila* groups. *D. birchii* productivity is also substantially lower than in other members of the *serrata* species complex, *D. bunnanda* and *D. serrata* (O'Brien et al. 2017; O'Brien et al. 2022). Importantly, these reductions in productivity with each successive mating observed are not an artefact of the non-random subset of males for which estimates of fourth matings were possible (*i.e.* males that achieve four matings) because a comparable reduction in productivity across matings was seen for this subset of males too (Fig. SM1, Table SM1). This suggests rapid sperm or ejaculate depletion even in *D. birchii* males that successfully mate four times, which contrasts with results obtained from tropical tephritid flies (e.g. *Anastrepha obliqua*) where males allocated consistent sperm quantities over three successive matings (Perez-Staples & Aluja, 2006) and neriid flies (*Telostylinus angusticollis*) where frequently-mated males experienced no reduction in the quantity of offspring sired from subsequent mating opportunities, even when nutritionally stressed (Macartney et al., 2020).

Variation across matings and across lines in D. birchii male mating traits

Ejaculate components such as sperm (Wedell et al., 2002) or seminal fluids (Wigby et al., 2009) can limit male remating across insect taxa . Spermiogenesis in male *Drosophila melanogaster* takes five days (at 25 °C) (Fabian & Brill, 2012) and mated males with depleted seminal fluid proteins require three days of sexual inactivity before transferring equivalent quantities again (Sirot et al., 2009). Although sperm size in *D. birchii* is not known, long flagella and long ventral receptacles in females (R. Snook, personal communication, 2014) suggest that male investment in sperm is relatively high (Markow, 2015). If energetic costs of mating are also high for male *D. birchii*, successive mating events may increase male choosiness, as the relative cost of mating rises with male resource depletion (Byrne & Rice, 2006). In this study however, no variation was found in latency to copulation across matings (Fig. 1), suggesting no increase in male choosiness (i.e. inclination to remate) (Engqvist & Sauer, 2001) or reduction in male attractiveness to females (Taylor et al., 2007).

Edvardsson and Canal (2006) suggest that declines in copulation duration are associated with decreasing ejaculate transfer, with reductions in mating duration observed in males of several *Drosophila* species over consecutive matings (Linklater et al., 2007, Singh & Singh, 2000, Singh & Singh, 2013). By contrast, we found an approximate doubling (from approximately 3 to 6 minutes) of mean mating durations across four matings, even though the productivity of these matings declined (Fig. 1). Extending mating durations can also increase male fitness by increasing paternity (Bretman et al., 2013a, Mazzi et al., 2009). By contrast, in several other *Drosophila* species copulation durations decline over consecutive matings (Linklater et al., 2007, Singh & Singh, 2013, Singh & Singh, 2000), suggesting that duration decreases as male ejaculate becomes depleted (Edvardsson & Canal, 2006). In these experiments however, declining productivity with increasing duration indicates that extended copulation duration is not associated with increased sperm transfer.

No evidence for divergence in mating strategies across elevational, social or abiotic environments

We speculate that the apparent sperm or ejaculate limitation in *D. birchii* by low levels may be explained by a low encounter rate with females in natural populations, reducing the benefit of allocating more resources to sperm. Given the big differences in population density (and therefore encounter rate with females) along the elevational gradients studied, we might expect local adaptation to increase productivity on multiple matings at higher elevations, where densities are typically higher, and to evolve in response to the local ecological context (Abraham et al., 2020). This expectation is also supported by consistent evidence for higher productivity of matings for isofemale lines from high elevations in the lab (O'Brien et al 2017), and the fact that high and low elevation thermal environments (~7 °C difference in mean temperature) characterise the cold and warm ecological limits of this species, both latitudinally and with elevation.

Male allocation strategies across taxa are also likely to show adaptive responses to highly variable social, sexual, biotic and abiotic conditions in natural populations (Wigby et al., 2016, Wedell et al., 2002). For example, Pitnick and Markow (1994) propose that submaximal male ejaculate allocation over successive matings may act as bet-hedging for male *Drosophila*, particularly where environments are stressful, and variation in abiotic conditions also affects insect mating duration (Horton et al., 2002), sperm allocation and remating rate (Katsuki & Miyatake, 2009) within genotypes.

However, despite a significant advantage from being more productive in later matings (and males being of similar quality for 1st and last matings), we find no evidence for local adaptation with elevation at either transect, despite evidence for at least some genetic variation in these traits (Table 1). This may be because of trade offs with other costs, or because temporal variation in environment at each elevation may limit the power of selection to cause local adaptation across elevations, favouring investment in plasticity instead (O'Brien et al. 2022; Hoffmann and Bridle, 2022). Adaptive divergence in these mating traits may also not occur if male mating rates do not increase with population density, due to increased inter male competition, and monopolisation of females

(Winkler et al. 2023). Alternatively, adaptive divergence among high and low elevation sites may have existed in the field, but been lost in these lines during the 25-50 generations for which these isofemale lines have been in lab conditions.

Substantial variation in male mating strategies has been observed across *Drosophila* species (Gowaty et al., 2003, Singh & Singh, 2013), and this is likely to correlate with ecological parameters that relate to the risk of sperm competition, likelihood of repeated mating opportunities, and the energetic cost of mating relative to resistance to factors such as temperature stress and pathogen or parasitoid exposure (Rolff and Kraaijeveld, 2003) physiological stress, (Geffen and Gibbs, 2009). *Drosophila birchii* are often found at low densities in their rainforest habitat (Bridle et al., 2009, O'Brien et al., 2017; O'Brien et al. 2022), compared to other tropical drosophilids. Conspecific interactions are therefore likely to be relatively rare, which should shape resource allocation across matings (Aspi & Hoffmann, 1998, Willis et al., 2011, Shelly & Bailey, 1992), and this effect may vary depending on population density, provided the probability of multiple encounters (and matings) with females is increased. In particular, reduced risk of sperm competition may favour reductions in sperm quantity (Bjork et al., 2007), in favour of increased investment per gamete (Snook, 2005) and reduced allocation across multiple matings (Ingleby et al., 2010). Reductions in production of seminal fluid proteins also occur in less sexually competitive environments (Wigby et al., 2016). Such effects may be especially strong in *Drosophila,* given many species (including *D. birchii*) have relatively long sperm compared to most other insect taxa.

No evidence for reduced quality of males generated from later matings

The data reported here are surprising because they demonstrate that males produced from later matings in *D. birchii*, although fewer in number, are as high quality as those from earlier matings, and so would substantially increase fitness for males able to secure later matings. This is in contrast to Douglas *et al* (2020), who found that males who mate with the most females do not necessarily sire the most offspring in *Drosophila melanogaster*. Despite this they (and Davies *et al.* 2023) did find evidence for a positive relationship between mating success and reproductive success.

In addition, the presence of among line variance in productivity at Mount Edith suggests that (other things being equal) alleles that allow sustained productivity across matings should be favoured, provided mating opportunities are increased. The fact that sustained productivity is apparently not maximised supports the idea that encounter rate in the field may be much lower for *D. birchii* than for other montium *Drosophila,* even at high densities, or that encounters with females is less likely to lead to copulation or successful fertilisation.

Conclusions and future directions

We find no evidence for adaptive divergence in male mating traits, or an increase in ability to remate in *D. birchii* across these elevational transects, despite: (a) evidence for genetic variation in these traits within populations; (b) no decline in the fecundity of offspring from even forth rematings, meaning that selection should favour higher male remating rates where female encounter rates are higher. This lack of divergence in mating traits is surprising given these isofemale lines were sampled across elevational gradients that are equivalent to the changes in population density (up to a ten fold difference) and climate (a 7° C change in mean temperature, and a 20% reduction in humidity) that is equivalent to the entire ecological niche of this rainforest-limited species. Such a lack of adaptive divergence may suggest constraints on the evolution of these traits, possibly due to trade offs with other traits, especially those that allow better feeding, mobility or courtship at different climates. Alternatively, the lack of divergence observed may reflect the complex effects of changes in climate and (especially) population density on selection on mating traits. For example, although encounters (and potential matings) with females may be more frequent at higher population densities, competition or interference from other males may also increase, meaning that rematings remain just as rare (at least for most males) even at high population densities. If so, selection would continue to maximise output (and post copulatory success) from first matings, making divergence in male investment in subsequent matings less likely. Future work should focus on measuring and understanding trade offs across these traits, and the consequences of variable and changing environments on the mating and remating rates of males in response to different encounter rates with females, including when assayed in competition with males (see O'Brien et al 2022). Overall however, such conservatism in mating traits among populations exposed to significant changes in climate and population density may constrain ecological or evolutionary responses to rapid environmental change in the decades to come (Bridle and Hoffmann, 2022; Chevin and Bridle 2025).

SUPPLEMENTARY MATERIAL

Supplementary materials (Fig SM1 and Table SM1) are available.

DATA ACCESSIBILITY STATEMENT

Analyses reported in this article can be reproduced using the data provided by Saxon *et al.* (2021). The full data set for this study is available on Dryad: `10.5061/dryad.sqv9s4ngw`

REFERENCES

Abraham, S., Moyano, A., Dasso, S. M., Van Nieuwenhove, G., Ovruski, S. & Perez-Staples, D. 2020. Male accessory gland depletion in a tephritid fly affects female fecundity independently of sperm depletion. *Behavioral Ecology and Sociobiology* **74**.

Alavioon, G., Hotzy, C., Nakhro, K., Rudolf, S., Scofield, D. G., Zajitschek, S., Maklakov, A. A. & Immler, S. 2017. Haploid selection within a single ejaculate increases offspring fitness. *Proceedings of the National Academy of Sciences of the United States of America* **114**: 8053-8058.

Aluja, M., Rull, J., Sivinski, J., Trujillo, G. & Perez-Staples, D. 2009. Male and female condition influence mating performance and sexual receptivity in two tropical fruit flies (Diptera: Tephritidae) with contrasting life histories. *Journal of Insect Physiology* **55**: 1091-1098.

Ambriz, D., Rosales, A. M., Sotelo, R., Mora, J. A., Rosado, A. & Garcia, A. R. 2002. Changes in the quality of rabbit semen in 14 consecutive ejaculates obtained every 15 minutes. *Archives of Andrology* **48**: 389-395.

Arnqvist, A. & Rowe, L. 2005. *Sexual Conflict*. Princeton University Press, USA.

Aspi, J. & Hoffmann, A. A. 1998. Female encounter rates and fighting costs of males are associated with lek size in *Drosophila mycetophaga*. *Behavioral Ecology and Sociobiology* **42**: 163-169.

Bateman, A. J. 1948. Intra-sexual selection in *Drosophila*. *Heredity* **2**: 349-368.

Bates, D., Mächler, M., Bolker, B. & Walker, S. 2015. Fitting linear mixed-effects models using lme4. *Journal of Statistical Software* **67**: 1-48.

Bissoondath, C. J. & Wiklund, C. 1996. Effect of male mating history and body size on ejaculate size and quality in two polyandrous butterflies, Pieris napi and Pieris rapae (Lepidoptera: Pieridae). *Functional Ecology* **10**: 457-464.

Bjork, A., Dallai, R. & Pitnick, S. 2007. Adaptive modulation of sperm production rate in *Drosophila bifurca*, a species with giant sperm. *Biology Letters* **3**: 517-9.

Blay, S. & Yuval, B. 1997. Nutritional correlates of reproductive success of male Mediterranean fruit flies (Diptera: Tephritidae). *Animal Behaviour* **54**: 59-66.

Bonduriansky, R. 2001. The evolution of male mate choice in insects: a synthesis of ideas and evidence. *Biological Reviews* **76**: 305-339.

Bretman, A., Westmancoat, J. D. & Chapman, T. 2013a. Male control of mating duration following exposure to rivals in fruitflies. *Journal of Insect Physiology* **59**: 824-827.

Bretman, A., Westmancoat, J. D., Gage, M. J. G. & Chapman, T. 2013b. Costs and benefits of lifetime exposure to mating rivals in male *Drosophila melanogaster*. *Evolution* **67**: 2413-2422.

Bridle, J. R., Gavaz, S. & Kennington, W. J. 2009. Testing limits to adaptation along altitudinal gradients in rainforest *Drosophila*. *Proceedings of the Royal Society B-Biological Sciences* **276**: 1507-1515.

Bridle J and Hoffmann AA. 2022. Understanding the biology of species' ranges: when and how does evolution change the ecological rules of engagement? *Phil Trans Roy Soc Lond B.* 377: (1848).

Brown, E. A., Gay, L., Vasudev, R., Tregenza, T., Eady, P. E. & Hosken, D. J. 2009. Negative phenotypic and genetic associations between copulation duration and longevity in male seed beetles. *Heredity* **103**: 340-345.

Byrne, P. G. & Rice, W. R. 2006. Evidence for adaptive male mate choice in the fruit fly *Drosophila melanogaster*. *Proceedings of the Royal Society B-Biological Sciences* **273**: 917-922.

Chahal, J., Dev, K., Kataria, S. K. & Parkash, R. 2013. Opposite latitudinal clines for first mating and second mating (remating) in males of *Drosophila melanogaster*. *Ethology* **119**: 926-936.

Chevin LM and Bridle J. 2025. Impacts of local adaptation on population and community persistence in a changing environment. *Phil. Trans. Roy. Soc. Lond B.* **380** (1917).

Churchill, E. R., Bridle, J. R. & Thom, M. D. F. 2020. Spatially clustered resources increase male aggregation and mating duration in *Drosophila melanogaster*. *Animal Behaviour* **169**: 45-50.

Churchill, E. R., Dytham, C, Bridle, J. R. & Thom, M. D. F. 2021. Social and physical environment independently affect oviposition decision in *Drosophila melanogaster*. *Behavioural Ecology* **32**: 1391-1399.

Clutton-Brock, T. H. 1991. *The Evolution of Parental Care*. Princeton University Press, USA.

Cordts, R. & Partridge, L. 1996. Courtship reduces longevity of male *Drosophila melanogaster*. *Animal Behaviour* **52**: 269-278.

Crean, A. J., Adler, M. I. & Bonduriansky, R. 2016. Seminal Fluid and Mate Choice: New Predictions. *Trends Ecol Evol* **31**: 253-255.

Crean, A. J. & Bonduriansky, R. 2014. What is a paternal effect? *Trends Ecol Evol* **29**: 554-559.

Davies, N., Janicke, T. & Morrow, E.H. (2023). Evidence for stronger sexual selection in males than in females using an adapted method of Bateman's classic study of Drosophila melanogaster. Evolution, 77, 2420–2430.

Dev, K., Chahal, J. & Parkash, R. 2013. Seasonal variations in the mating-related traits of *Drosophila melanogaster*. *Journal of Ethology* **31**: 165-174.

Dewsbury, D. A. 1982. Ejaculate cost and male choice. *American Naturalist* **119**: 601-610.

Dolgin, E. S., Whitlock, M. C. & Agrawal, A. F. 2006. Male *Drosophila melanogaster* have higher mating success when adapted to their thermal environment. *Journal of Evolutionary Biology* **19**: 1894-1900.

Dore, A. A., Rostant, W. G., Bretman, A. & Chapman, T. 2021. Plastic male mating behavior evolves in response to the competitive environment. *Evolution* **75**: 101-115.

Douglas, T., Anderson, R. & Saltz, J. B. 2020. Limits to male reproductive potential across mating bouts in Drosophila melanogaster. *Animal Behaviour* **160**: 25-33.

Edvardsson, M. & Canal, D. 2006. The effects of copulation duration in the bruchid beetle *Callosobruchus maculatus*. *Behavioral Ecology* **17**: 430-434.

Edward, D. A. & Chapman, T. 2011. The evolution and significance of male mate choice. *Trends in Ecology & Evolution* **26**: 647-654.

Emlen, S. T. & Oring, L. W. 1977. Ecology, sexual selection, and evolution of mating systems. *Science* **197**: 215-223.

Engqvist, L. & Sauer, K. P. 2001. Strategic male mating effort and cryptic male choice in a scorpionfly. *Proceedings of the Royal Society B-Biological Sciences* **268**: 729-735.

Fabian, L. & Brill, J. A. 2012. *Drosophila* spermiogenesis: Big things come from little packages. *Spermatogenesis* **2**: 197-212.

Fricke, C., Bretman, A. & Chapman, T. 2008. ADULT MALE NUTRITION AND REPRODUCTIVE SUCCESS IN DROSOPHILA MELANOGASTER. *Evolution* **62**: 3170-3177.

Fromonteil, S., Marie-Orleach, L., Winkler, L. & Janicke, T. (2023). Sexual selection in females and the evolution of polyandry. PLoS Biol, 21, e3001916.

Gaskin, T., Futerman, P. & Chapman, T. 2002. Increased density and male-male interactions reduce male longevity in the medfly, Ceratitis capitata. *Animal Behaviour* **63**: 121-129.

Gasparini, C., Dosselli, R. & Evans, J. P. 2017. Sperm storage by males causes changes in sperm phenotype and influences the reproductive fitness of males and their sons. *Evolution Letters* **1**: 16-25.

Gefen, E. & Gibbs, A. G. 2009. INTERACTIONS BETWEEN ENVIRONMENTAL STRESS AND MALE MATING SUCCESS MAY ENHANCE EVOLUTIONARY DIVERGENCE OF STRESS-RESISTANT DROSOPHILA POPULATIONS. *Evolution* **63**: 1653-1659.

Gowaty, P. A., Kim, Y. K., Rawlings, J. & Anderson, W. W. 2010. Polyandry increases offspring viability and mother productivity but does not decrease mother survival in Drosophila pseudoobscura. *Proceedings of the National Academy of Sciences of the United States of America* **107**: 13771-13776.

Gowaty, P. A., Steinichen, R. & Anderson, W. W. 2003. Indiscriminate females and choosy males: Within- and between-species variation in *Drosophila*. *Evolution* **57**: 2037-2045.

Gromko, M. H. & Markow, T. A. 1993. Courtship and remating in field populations of *Drosophila*. *Animal Behaviour* **45**: 253-262.

Harshman, L. G., Hoffmann, A. A. & Prout, T. 1988. Environmental effects on remating in *Drosophila melanogaster*. *Evolution* **42**: 312-321.

Harvanek, Z. M., Lyu, Y., Gendron, C. M., Johnson, J. C., Kondo, S., Promislow, D. E. L. & Pletcher, S. D. 2017. Perceptive costs of reproduction drive ageing and physiology in male *Drosophila*. *Nature Ecology & Evolution* **1**: 152.

Hayward, A. & Gillooly, J. F. 2011. The cost of sex: Quantifying energetic investment in gamete production by males and females. *PLoS One* **6**: e16557.

Hoffmann AA and Bridle, J 2022. The dangers of irreversiblity in an age of increased uncertainty: revisiting plasticity in invertebrates. *Oikos* **4**: e08715.

Hoikkala, A., Crossley, S. & Castillo-Melendez, C. 2000. Copulatory courtship in *Drosophila birchii* and *D. serrata* - species recognition and sexual selection. *Journal of Insect Behavior* **13**: 361-373.

Hopkins, B. R., Sepil, I., Thezenas, M. L., Craig, J. F., Miller, T., Charles, P. D., Fischer, R., Kessler, B. M., Bretman, A., Pizzari, T. & Wigby, S. 2019. Divergent allocation of sperm and the seminal proteome along a competition gradient in *Drosophila melanogaster*. *Proc Natl Acad Sci U S A* **116**: 17925-17933.

Horton, D. R., Lewis, T. M. & Hinojosa, T. 2002. Copulation duration in three species of *Anthocoris* (Heteroptera : Anthocoridae) at different temperatures and effects on insemination and ovarian development. *Pan-Pacific Entomologist* **78**: 43-55.

House, C. M., Rapkin, J., Hunt, J., & Hosken, D. J. (2019). Operational sex ratio and density predict the potential for sexual selection in the broad-horned beetle. *Animal Behaviour*, 152, 63–69.

Hosken, D. J., Garner, T. W. J., Tregenza, T., Wedell, N. & Ward, P. I. 2003. Superior sperm competitors sire higher-quality young. *Proceedings of the Royal Society B-Biological Sciences* **270**: 1933-1938.

Ingleby, F. C., Lewis, Z. & Wedell, N. 2010. Level of sperm competition promotes evolution of male ejaculate allocation patterns in a moth. *Animal Behaviour* **80**: 37-43.

Ivy, T. M. & Sakaluk, S. K. 2005. Polyandry promotes enhanced offspring survival in decorated crickets. *Evolution* **59**: 152-159.

Janicke, T., David, P. & Chapuis, E. (2015). Environment-dependent sexual selection: Bateman's parameters under varying levels of food availability. The American Naturalist, 185, 756–768.

Janicke, T., Haderer, I. K., Lajeunesse, M. J. & Anthes, N. 2016. Darwinian sex roles confirmed across the animal kingdom. *Science Advances* **2**.

Jennions, M. D. & Petrie, M. 2000. Why do females mate multiply? A review of the genetic benefits. *Biological Reviews* **75**: 21-64.

Katsuki, M. & Miyatake, T. 2009. Effects of temperature on mating duration, sperm transfer and remating frequency in *Callosobruchus chinensis*. *Journal of Insect Physiology* **55**: 112-115.

Knell, R. J. 2009. Population density and the evolution of male aggression. *Journal of Zoology* **278**: 83-90.

Kokko, H., Jennions, M. D. & Brooks, R. 2006. Unifying and testing models of sexual selection. *Annual Review of Ecology Evolution and Systematics* **37**: 43-66.

Kokko, H. & Rankin, D. J. 2006. Lonely hearts or sex in the city? Density-dependent effects in mating systems. *Philosophical Transactions of the Royal Society B-Biological Sciences* **361**: 319-334.

Kotiaho, J. S. & Simmons, L. W. 2003. Longevity cost of reproduction for males but no longevity cost of mating or courtship for females in the male-dimorphic dung beetle Onthophagus binodis. *Journal of Insect Physiology* **49**: 817-822.

Lefevre, G. & Jonsson, U. B. 1962. Sperm transfer, storage, displacement, and utilization in *Drosophila melanogaster*. *Genetics* **47**: 1719-36.

Linklater, J. R., Wertheim, B., Wigby, S. & Chapman, T. 2007. Ejaculate depletion patterns evolve in response to experimental manipulation of sex ratio in *Drosophila melanogaster*. *Evolution* **61**: 2027-2034.

Macartney, E. L., Bonduriansky, R. & Crean, A. J. 2020. Frequent mating reduces male mating rate but not offspring quality or quantity in a neriid fly. *Evolutionary Ecology* **34**: 915-927.

Macartney, E. L., Crean, A. J. & Bonduriansky, R. 2018. Epigenetic paternal effects as costly, condition-dependent traits. *Heredity* **121**: 248-256.

Macartney, E. L., Zeender, V., Meena, A., De Nardo, A. N., Bonduriansky, R. & Lupold, S. 2021. Sperm depletion in relation to developmental nutrition and genotype in Drosophila melanogaster. *Evolution* **75**: 2830-2841.

Madsen, T., Shine, R., Loman, J. & Håkansson, T. 1992. Why do female adders copulate so frequently? *Nature* **355**: 440-441.

Markow, T. A. 2015. *Drosophila* reproduction: Molecules meet morphology. *Proceedings of the National Academy of Sciences of the United States of America* **112**: 8168-8169.

Markow, T. A. & O'Grady, P. 2008. Reproductive ecology of Drosophila. *Functional Ecology* **22**: 747-759.

Marshall, K. E. & Sinclair, B. J. 2010. Repeated stress exposure results in a survival-reproduction trade-off in *Drosophila melanogaster*. *Proceedings of the Royal Society B-Biological Sciences* **277**: 963-969.

Martin, O. Y. & Hosken, D. J. 2004. Copulation reduces male but not female longevity in Saltella sphondylli (Diptera : Sepsidae). *Journal of Evolutionary Biology* **17**: 357-362.

Mazzi, D., Kesaniemi, J., Hoikkala, A. & Klappert, K. 2009. Sexual conflict over the duration of copulation in *Drosophila montana*: Why is longer better? *Bmc Evolutionary Biology* **9**.

McCullough, E. L., Buzatto, B. A., & Simmons, L. W. (2018). Population density mediates the interaction between pre- and postmating sexual selection. *Evolution*, 72(4), 893–905.

Niemela, P. T., Tiso, S. & Dingemanse, N. J. 2021. Density-dependent individual variation in male attractiveness in a wild field cricket. *Behavioral Ecology* **32**: 707-716.

O'Brien, E. K., Higgie, M., Reynolds, A., Hoffmann, A. A. & Bridle, J. R. 2017. Testing for local adaptation and evolutionary potential along altitudinal gradients in rainforest *Drosophila*: beyond laboratory estimates. *Global Change Biology* **23**: 1847-1860.

O'Brien, E.K, Walter, GM, Bridle J (2022). Environmental variation and biotic interactions limit adaptation at ecological margins: lessons from rainforest *Drosophila* and European butterflies. *Phil. Trans. Roy. Soc. London. B:* **377:** 20210017.

Parker, G. A. 1978. EVOLUTION OF COMPETITIVE MATE SEARCHING. *Annual Review of Entomology* **23**: 173-196.

Parker, G. A. 1982. Why are there so many tiny sperm - Sperm competition and the maintenance of 2 sexes. *Journal of Theoretical Biology* **96**: 281-294.

Parker, G. A., Lessells, C. M. & Simmons, L. W. 2013. SPERM COMPETITION GAMES: A GENERAL MODEL FOR PRECOPULATORY MALE-MALE COMPETITION. *Evolution* **67**: 95-109.

Parker, G. A. & Pizzari, T. 2010. Sperm competition and ejaculate economics. *Biological Reviews* **85**: 897-934.

Partridge, L. & Farquhar, M. 1981. Sexual activity reduces lifespan of male fruitflies. *Nature* **294**: 580-582.

Pattarini, J. A., Starmer, W. T., Bjork, A. & Pitnick, S. 2006. Mechanisms underlying the sperm quality advantage in *Drosophila melanogaster*. *Evolution* **60**: 2064-2080.

Perez-Staples, D. & Aluja, M. 2006. Sperm allocation and cost of mating in a tropical tephritid fruit fly. *Journal of Insect Physiology* **52**: 839-845.

Perry, J. C., Sirot, L. & Wigby, S. 2013. The seminal symphony: How to compose an ejaculate. *Trends in Ecology & Evolution* **28**: 414-422.

Pitnick, S. 1996. Investment in testes and the cost of making long sperm in *Drosophila*. *American Naturalist* **148**: 57-80.

Pitnick, S. & Markow, T. A. 1994. Male gametic strategies - Sperm size, testes size, and the allocation of ejaculate among successive mates by the sperm-limited fly *Drosophila pachea* and its relatives. *American Naturalist* **143**: 785-819.

Pitnick, S., Markow, T. A. & Spicer, G. S. 1995. Delayed male maturity is a cost of producing large sperm in *Drosophila*. *Proceedings of the National Academy of Sciences of the United States of America* **92**: 10614-10618.

R Core Team (2016) R: A Language and Environment for Statistical Computing. pp. R Foundation for Statistical Computing., Vienna, Austria.

Radhakrishnan, P., Perez-Staples, D., Weldon, C. W. & Taylor, P. W. 2009. Multiple mating and sperm depletion in male Queensland fruit flies: effects on female remating behaviour. *Animal Behaviour* **78**: 839-846.

Reinhardt, K., Naylor, R. & Siva-Jothy, M. T. 2011. Male mating rate is constrained by seminal fluid availability in bedbugs, *Cimex lectularius*. *PLoS One* **6**: e22082.

Sambucetti, P. & Norry, F. M. 2015. Mating success at high temperature in highland- and lowland-derived populations as well as in heat knock-down selected Drosophila buzzatii. *Entomologia Experimentalis Et Applicata* **154**: 206-212.

Shelly, T. E. & Bailey, W. J. 1992. Experimental manipulation of mate choice by male katydids: The effect of female encounter rate. *Behavioral Ecology and Sociobiology* **30**: 277-282.

Singh, A. & Singh, B. N. 2013. Studies on remating behaviour in the *Drosophila bipectinata* species complex: Intra- and interspecific variations. *Behavioural Processes* **96**: 79-87.

Singh, S. R. & Singh, B. N. 2000. Male remating in *Drosophila ananassae*: Evidence for interstrain variation in remating time and shorter duration of copulation during second mating. *Zoological Science* **17**: 389-393.

Sirot, L. K., Buehner, N. A., Fiumera, A. C. & Wolfner, M. F. 2009. Seminal fluid protein depletion and replenishment in the fruit fly, *Drosophila melanogaster*: An ELISA-based method for tracking individual ejaculates. *Behavioral Ecology and Sociobiology* **63**: 1505-1513.

Siva-Jothy, M. T. 2000. The young sperm gambit. *Ecology Letters* **3**: 172-174.

Snook, R. R. 2005. Sperm in competition: Not playing by the numbers. *Trends in Ecology & Evolution* **20**: 46-53.

Taylor, M. L., Wedell, N. & Hosken, D. J. 2007. The heritability of attractiveness. *Current Biology* **17**: R959-R960.

Trajkovic, J., Pavkovic-Lucic, S. & Savic, T. 2013. Mating success and wing morphometry in Drosophila melanogaster after long-term rearing on different diets. *Behaviour* **150**: 1431-1448.

Trivers, R. L. (1972) Parental investment and sexual selection. In: *Sexual selection and the descent of man 1871-1971*, (Campbell, B., ed.). pp. 136-179. Aldine, Chicago.

Van Voorhies, W. A. 1992. Production of sperm reduces nematode lifespan. *Nature* **360**: 456-8.

Wang, W. & Yan, J. 2021. Shape-Restricted Regression Splines with R Package splines2. *Journal of Data Science* **19**: 498-517.

Wedell, N., Gage, M. J. G. & Parker, G. A. 2002. Sperm competition, male prudence and sperm-limited females. *Trends in Ecology & Evolution* **17**: 313-320.

Wigby, S., Perry, J. C., Kim, Y. H. & Sirot, L. K. 2016. Developmental environment mediates male seminal protein investment in *Drosophila melanogaster*. *Functional Ecology* **30**: 410-419.

Wigby, S., Sirot, L. K., Linklater, J. R., Buehner, N., Calboli, F. C., Bretman, A., Wolfner, M. F. & Chapman, T. 2009. Seminal fluid protein allocation and male reproductive success. *Current Biology* **19**: 751-7.

Willis, P. M., Ryan, M. J. & Rosenthal, G. G. 2011. Encounter rates with conspecific males influence female mate choice in a naturally hybridizing fish. *Behavioral Ecology* **22**: 1234-1240.

Winkler, L., Eilhardt, R. & Janicke, T. (2023). Population density affects sexual selection in an insect model, *Functional Ecology* **37:** 2734–2747.

Yuval, B., Kaspi, R., Shloush, S. & Warburg, M. S. 1998. Nutritional reserves regulate male participation in Mediterranean fruit fly leks. *Ecological Entomology* **23**: 211-215.