Low but genetically variable male mating ability in a tropical *Drosophila* despite fitness benefits of remating

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Abstract

Male mating success is a key source of variation in fitness, particularly in organisms where males can mate multiply and there is potential for high variance in reproductive success. Males should therefore produce large numbers of gametes to capitalise on all opportunities for mating. In addition, strong selection on male mating success should reduce genetic variation in male mating traits relative to other traits. Despite this, males of the tropical Australian fruitfly *Drosophila birchii* show significant variation in their remating potential and the resulting number of offspring. We quantified mating latency, mating duration and productivity in *D. birchii* males, in 30 isofemale lines collected from across two elevational gradients (20 – 1100 m), when they were given opportunities to mate with up to four females consecutively. Male

remating rates were low compared to other *Drosophila* species (only 14 – 27% of males achieved a fourth mating in ~1 day). Mean mating duration also approximately doubled across successive copulations. However, although successive remating produced progressively fewer offspring than a male's first mating, it consistently increased overall male reproductive success. Critically, we found no reduction in the productivity of the (male) offspring derived from these later matings, indicating a sustained cumulative fitness benefit to remating. Heritable variation was observed for all traits (H^2 = 0.035 – 0.292) except mating latency, although no evidence was found for divergence of trait means across elevation. The surprisingly restricted remating ability of male *D. birchii* may be explained by a low female encounter rate due to the species' low densities in the field, possibly combined with a high cost of sperm (or ejaculate) production.

1. Introduction

Sexual activity reduces male lifespan in *Drosophila melanogaster* (Partridge & Farquhar, 1981) and a range of energy, time and risk-related costs are associated with variation in mating success in males (Hayward & Gillooly, 2011; Dowling & Simmons, 2012; Bretman et al., 2013b). These include the cost of searching and competing for females (Dewsbury, 1982), perceiving female signals (Harvanek et al., 2017) and of complex courtship displays (Connolly et al., 1969; Cordts & Partridge, 1996). Prolonged copulation duration and extended mate guarding to maximise paternity share also incur significant costs (Mazzi et al., 2009; Bretman et al., 2013a). Critically, while male gametes were once assumed to be effectively unlimited (Bateman, 1948), sperm production can also be energetically expensive (Dewsbury, 1982; Van Voorhies, 1992; Paukku & Kotiaho, 2005; Hayward & Gillooly, 2011), especially in *Drosophila* species that generate large sperm (Pitnick et al., 1995). As investment in sperm size or length increases, sperm are therefore likely to be increasingly limited in number (Pitnick, 1996), meaning sperm depletion may limit male reproductive success if mating opportunities are frequent (Pitnick & Markow, 1994; Preston et al., 2001; Wedell et al., 2002; Hines et al., 2003).

The evolution of males to be able to mate repeatedly will be constrained by a trade-off between current and future mating success (Parker, 1982). Males should invest more in initial copulations if female mating frequency and/or encounter rate is low (Reinhold et al., 2002). However, if sperm is costly and the male mating rate is high, strategic allocation of resources

over successive matings is expected (Byrne & Rice, 2006). Several studies have established that males do not maximally inseminate during initial copulations, but partition sperm across consecutive females (Pitnick & Markow, 1994; Galvani & Johnstone, 1998; Perez-Staples & Aluja, 2006). Understanding the evolution of male mating traits therefore necessitates measurement of the size of fitness benefits that males accrue from obtaining multiple matings. This involves determining (*i*) the total number of offspring produced by remating and (*ii*) whether the offspring generated in later matings increase paternal fitness as much as those from first matings (Wedell & Tregenza, 1999).

Sperm quantity, in the form of larger and more numerous ejaculates, is associated with higher fitness (Simmons, 2001). However, sperm quality affects male fitness independently of sperm quantity (Snook, 2005; Pattarini et al., 2006). Declines in sperm quality (e.g. motility, viability) have been observed over rapid sequential ejaculations in mammals, especially as ejaculates become depleted (Ambriz et al., 2002). Variation in sperm quality among males has also been found to result in differences in the quality of offspring (Siva-Jothy, 2000; Hosken et al., 2003; Alavioon et al., 2017). For example, in male guppies (*Poecilia reticulata*), declines in sperm competitive ability are associated with reduced reproductive success of sons (Gasparini et al., 2017). These results suggest that offspring produced later in frequent successive matings may have reduced fitness than those from initial matings.

If opportunities for remating are common in nature, rapid ejaculate replenishment should be under strong directional selection (Trivers, 1972). In *Drosophila*, sperm are transferred in a complex seminal fluid comprising numerous costly components (Dewsbury, 1982; Perry et al., 2013). These components include accessory gland proteins (Acps), which increase male reproductive success by stimulating female egg-laying and reducing female receptivity to further matings (Gillott, 2003). However, the production of such seminal components may also limit the number and size of ejaculates males can produce (Lefevre & Jonsson, 1962; Linklater et al., 2007; Sirot et al., 2009; Wigby et al., 2009; Reinhardt et al., 2011), with the lag time for replenishment of seminal fluids (or maturation of sperm) correlated with the refractory period observed before male remating occurs (Partridge, 1988; Lehmann & Lehmann, 2007). Edvardsson and Canal (2006) suggest that declines in copulation duration are associated with decreasing ejaculate transfer. Accordingly, reduction in mating duration over successive matings is observed in males of several *Drosophila* species (Singh & Singh, 2000; Linklater et al., 2007; Singh & Singh, 2013).

Selection to prioritise current versus future mating may also vary across environments, and with population density. Genetic variation in the partitioning of male reproductive resources should therefore be observed in heterogeneous environments (Engqvist & Sauer, 2001). In *Drosophila*, males preferentially court larger females (Byrne & Rice, 2006) and variation in ejaculate size correlates with variation in female fecundity, mating status and age (Wedell et al., 2002; Lupold et al., 2011), all of which can vary across habitats. Similarly, males adjust copulation duration and ejaculate quantity in the presence of rival males, presumably associated with the likelihood of sperm competition (Gage & Baker, 1991). Ecological conditions also affect mating patterns (Gromko & Markow, 1993), due to trade-offs between stress tolerance and reproduction (Harshman & Zera, 2007; Marshall & Sinclair, 2010). For example, low resource availability may affect male mating rates (Blay & Yuval, 1997) and their ability to produce sperm (Gage & Cook, 1994). Latitudinal clines in male mating traits indicate that environmental variation can determine such allocation patterns (Parkash et al., 2011; Chahal et al., 2013).

Sexual conflict, 'good genes' and 'sexy sons' models of sexual selection all predict that males pass on genetic benefits to offspring (Wedell & Tregenza, 1999; Kokko, 2001; Taylor et al., 2013). However, mating success traits should have lower heritabilities than morphological or physiological traits because of their close association with fitness. Alternatively, low heritability estimates in mating traits could result from their higher sensitivity to environmental variation (Price & Schluter, 1991). Some studies have shown that traits associated with increased male mating success, such as territoriality (Hoffmann, 1991) or mating latency and duration (Hoffmann, 1999; Taylor et al., 2007) have intermediate heritabilities. However, other studies have found little evidence of genetic variation in latency, duration (Taylor et al., 2013) or copulatory success (Whittier & Kaneshiro, 1995). In either case, it is important to estimate genetic variation in mating traits to understand their evolutionary potential and the ecological factors that are likely to limit their evolution in natural populations (Bretman et al., 2014).

Studies of male mating traits in *Drosophila* typically focus on cosmopolitan species that are easy to rear in the laboratory and occur at high density throughout their ecological range (Gowaty, 2012). However, tropical species typically have narrower abiotic and biotic tolerances which are likely to have direct and indirect effects on reproduction (Markow & O'Grady, 2008). In this study, we assayed mating behaviour and reproductive success for males of the tropical fly *Drosophila birchii*, to investigate the low male remating rate previously observed (E.K. O'Brien, personal communication, 2014). This species, limited to wet

rainforest in Australasia, varies substantially in density across its narrow elevational range (Bridle et al., 2009), shows complex male courtship behaviour (Hoikkala et al., 2000) and high levels of variation in male mating success under different thermal regimes (Saxon et al., 2018). We assessed genetic variation across the elevational distribution of this species (using isofemale lines collected from sites along two elevational gradients in northern Queensland) for the following mating and reproductive traits: (*i*) latency to achieve copulation, (*ii*) duration of copulation, (*iii*) number of matings and (*iv*) number of offspring produced with each successive copulation, when virgin males were presented with four virgin females sequentially. We then tested how this variation in male mating traits affects male reproductive success, both in terms of offspring and grand-offspring. Surprisingly, the remating ability of was found to be remarkably low across all habitats, despite (*a*) higher remating rates consistently increasing fitness and (*b*) significant levels of genetic variation suggesting evolutionary potential in key male mating traits.

2. Materials and Methods

(a) Establishment of isofemale lines

Drosophila birchii isofemale lines (called 'lines' hereafter) were founded from field-mated females collected from two low or high elevation sites along two gradients (eight in total), Mount Edith (Elevation: ~600 – 1100 m, 17°6'S, 145°38'E) and Mount Lewis (Elevation: ~20 – 900 m, 16°35′S 145°17′E), in Queensland, Australia in 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 established by combining 10 male and 10 female flies from each line, as a genetically mixed background population to provide a source 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 between generations. All lines and the mass-bred population were maintained with non-overlapping generations at 19 °C on a 12:12-h light:dark cycle at 60% relative humidity prior to the experiments. Mount Lewis experiments were conducted after ~25 laboratory generations, Mount Edith ~50 generations.

(b) Experiment I: Assaying male remating and productivity

Ten lines were used for the Mount Edith assay, with five lines originating from two high elevation sites and five from two lower elevation sites. Twenty lines were used for the Mount Lewis assay, with 10 originating from two high and two low sites. 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 transgenerational effects attributable to maternal condition. The experimental males and background females were reared at minimal density conditions to minimise larval competition. Five male and five female flies mated and laid 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 under CO_2 and sexed using a Leica (MZ9.5) microscope. 30 males for each of 10 lines (N = 300) from Mount Edith and 20 males from 20 lines (N= 400) 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 in low density vials (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* (De et al., 2013). Each male was placed in a vial with 8 mL of standard fly medium. 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, with males given up to two hours to mate with each female. If no mating occurred, then the male was recorded as ‘not mated’ and the assay concluded for that male. The assay took place over five days with 60 (for Mount Edith) and 80 (for Mount Lewis) male flies assayed 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. Blind ID codes were used to avoid observer bias.

Mated females were left in the vials for five days, which ensured that they had laid all fertilised eggs, given that the lack of spermathecae in *D. birchii* females 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 first to fourth (1 – 4) paternal matings of focal males (sires) in Experiment I, that had achieved the maximum four matings. Sons were derived from seven of the ten Mount Edith isofemale lines (N = ~280). Each six-day old male was placed in a single vial of 8 ml of fly medium with a single, virgin mass-bred female of the same age (± 12 hours). The pair were left for three days to mate under the same control conditions as Experiment I. The male was then removed and the female was left to lay for a further five days. The female was then removed and pupation card inserted. After all offspring had emerged, the mean productivity of matings from these sons was assayed with respect to the order of mating and their paternal line.

(d) Statistical analysis

The R (R Core Team, 2016) package *lme4* was used to fit linear mixed models to test for effects of successive matings on male mating traits (latency to mate, duration of mating, number of offspring). Separate models were fitted for each trait and gradient. All data were untransformed because trait data were normally distributed. Mating number and elevation of origin of line (high or low) were included as fixed factors and focal male nested within isofemale line were specified as random effects. The significance of each factor in the model was determined using likelihood ratio tests to compare the full model with a model where that factor had been removed (Experiment I). P-values were corrected for multiple comparisons following the False Discovery Rate method of Benjamini and Hochberg (1995), allowing an FDR of 0.05.

Models were also run for total number of matings gained per male and total offspring per male, using only elevation of origin of line as a fixed factor and line as a random effect. Linear mixed models were also run to analyse overall differences in the mean proportion of males per line that attained each successive mating and the mean cumulative number of offspring per line at each mating. Mating number was used as a fixed factor with line as a random effect. For Experiment II, a linear mixed model with mating as a fixed factor and line as a random effect

was used to analyse the productivity of sons derived from successive paternal mating events when mating with a single, virgin female from the mass-bred stock.

To estimate broad-sense heritability (H^2) for mating traits, between line variances from the linear mixed models were used (Experiment I), using within (V_w) and between (V_b) line variance components derived from models fitted using REML. Inbreeding coefficients (F_t) (with the assumption of full-sibship) for the number of generations and population size (~100) at which lines had been held at in the laboratory were calculated using the method of Falconer and Mackay (1996). H^2 was then calculated following Hoffmann and Parsons (1988)(see SM). The significance of the H^2 was evaluated from the significance of the between-line variance component in the linear mixed models.

3. Results

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

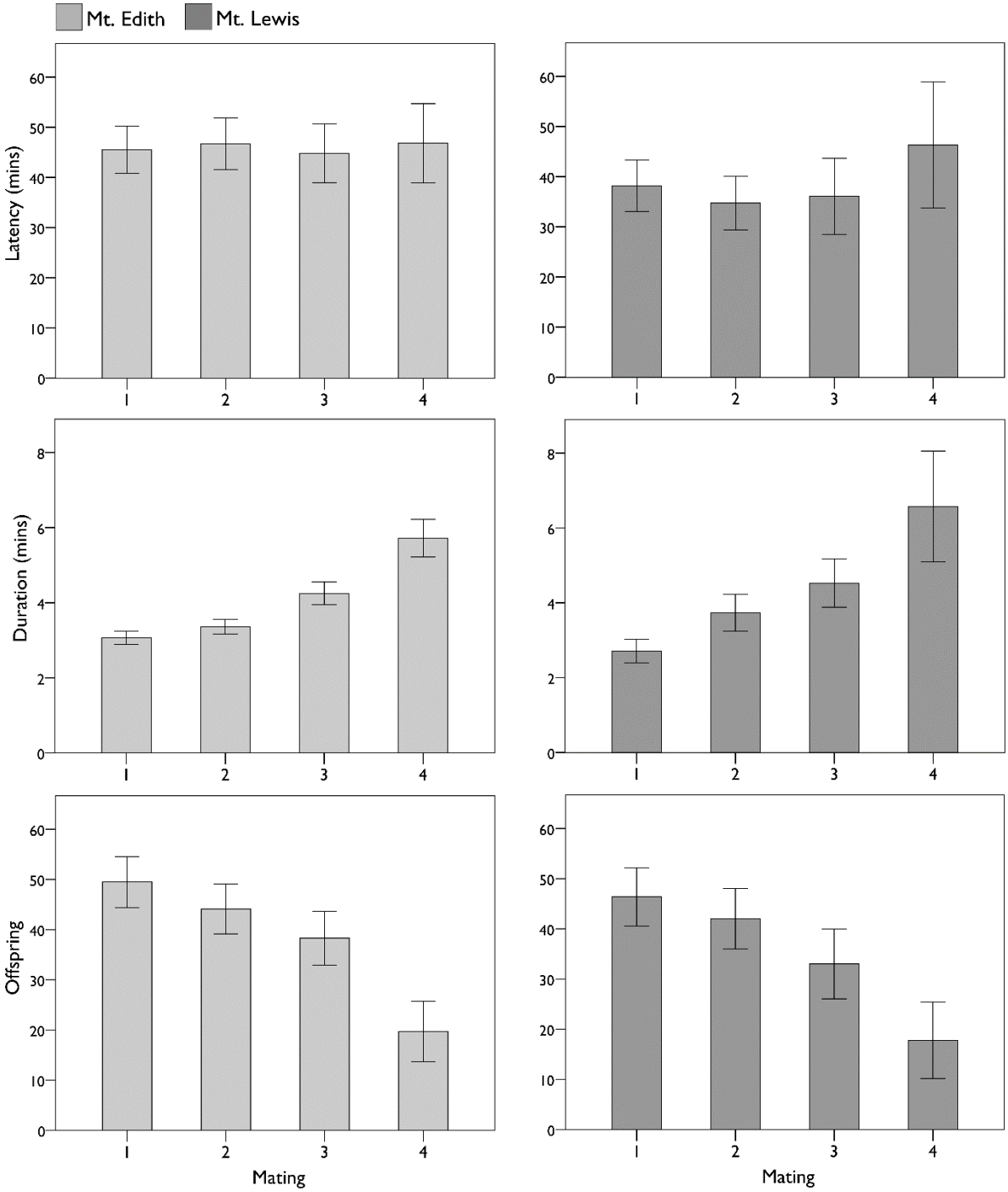

Figure 1. *Increased mating duration and reduced productivity with successive matings*: Mean mating latency, mean mating duration and mean number of offspring (productivity) for all isofemale lines for each successive male mating (1 – 4). Data from Mount Edith lines are shown in light grey (left), Mount Lewis lines shown in dark grey (right). Error bars indicate 95% confidence intervals. For associated statistic output see Table 1.

Table 1. Linear mixed effects analyses for male mating traits in Mount Edith and Mount Lewis lines. Mating number and elevation of origin of isofemale line are fixed effects, with nested random effects of isofemale line and focal male. *P*-values were obtained by likelihood ratio tests of the full model with the effect against the model with the effect excluded (*ns*= not significant, *adjusted $P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$).

Mating trait	***Gradient***	***Fixed effect***				***Random effects***		
		Predictor	***χ^2***	***df***	***P***	***Variance component***	***Variance***	***P***
Latency	Mt. Edith	Mating	0.5111	3	*ns*	Isofemale line	28.61	*ns*
						Male	9.803×10^{-13}	*ns*
		Elevation	2.097	1	*ns*	Residual	1162	-
	Mt. Lewis	Mating	4.0038	3	*ns*	Isofemale line	10.15	*ns*
						Male	39.95	*ns*
		Elevation	0.7601	1	*ns*	Residual	1031.17	-
Duration	Mt. Edith	Mating	184.84	3	***	Isofemale line	0.08798	*
						Male	0.04592	*ns*
		Elevation	0.653	1	*ns*	Residual	3.00321	-
	Mt. Lewis	Mating	64.142	3	***	Isofemale line	0.7751	*ns*
						Male	6.507×10^{-14}	*ns*
		Elevation	0.5598	1	*ns*	Residual	8.535	-
Offspring per mating	Mt. Edith	Mating	76.697	3	***	Isofemale line	221.5	***
						Male	106.3	*ns*
		Elevation	1.9363	1	*ns*	Residual	958.3	-
	Mt. Lewis	Mating	18.959	3	***	Isofemale line	48.47	*
						Male	184.27	*
		Elevation	0.051	1	*ns*	Residual	1012.86	-
Total matings	Mt. Edith	Mating						
		Elevation	0.3199	1	*ns*	Isofemale Line	0.1525	**
						Residual	1.2997	-
	Mt. Lewis	Mating						
		Elevation	1.6943	1	*ns*	Isofemale Line	0.06595	*ns*
						Residual	1.08995	-
Total offspring	Mt. Edith	Mating						
		Elevation	1.8045	1	*ns*	Isofemale Line	1831	***
						Residual	5809	-
	Mt. Lewis	Mating						
		Elevation	0.094	1	*ns*	Isofemale Line	365.1	*ns*
						Residual	3148.5	-

Table 2. Broad-sense heritability (H^2) estimates for mating traits for Mount Edith and Mount Lewis lines. Calculated from between (V_b) and within (V_w) line variance components and inbreeding coefficient (F_t) for number of laboratory generations. Significant H^2 estimates are in **bold.**

Mating trait	*Gradient*	V_b	V_w	F_t	H^2
Latency	Mt. Edith	28.61	1162	0.4104	0.029
	Mt. Lewis	10.15	1031.17	0.3317	0.015
Duration	Mt. Edith	0.08798	3.00321	0.4104	**0.035**
	Mt. Lewis	0.7751	8.535	0.3317	0.126
Offspring per mating	Mt. Edith	221.5	958.3	0.4104	**0.229**
	Mt. Lewis	48.47	1012.86	0.3317	**0.069**
Total matings	Mt. Edith	0.1525	1.2997	0.4104	**0.128**
	Mt. Lewis	0.06595	1.08995	0.3317	0.086
Total offspring	Mt. Edith	1831	5809	0.4104	**0.292**
	Mt. Lewis	365.1	3148.5	0.3317	0.157

In Experiment I, 76% of males at Mount Edith attained a first mating (N = 220 males), with only 27% reaching a fourth. 19% only mated once. 63% of males at Mount Lewis attained a first mating (N = 166 males), while only 14% attained a fourth mating, with 14% of males mating only once (Figure 2). Mean cumulative productivity, while significantly increasing across successive matings, slowed its rate of increase with each copulation (Fig. 3). For Mount Edith lines, mean productivity per mating (for all males) showed a 64% decrease from mating 1 to mating 4. Focal males that mated only once had mean total offspring of 43.02 (standard error ± 5.176), while males that mated four times had a mean total cumulative productivity of 145.07 (± 18.305). For Mount Lewis lines, mean productivity per mating declined by 72% from mating 1 to mating 4. Males that mated only once had mean total offspring of 63.97 (± 6.010), while males reaching a fourth mating had a mean total productivity of 133.31 (± 7.041). The subset of males that reached a fourth mating showed a similar pattern of declining productivity to the pattern observed for all males (Figure SM1).

Latency to mating did not vary with successive matings for line from either gradient. Between-line variance in this trait was also not found at either gradient. However, both mating duration and productivity showed a highly significant change across successive matings for both gradients. Mating duration increased approximately twofold and productivity more than halved over the four successive matings (Figure 1; Table 1). Line also accounted for a significant proportion of variance in these traits (with the exception of mating duration in the Mount Lewis

lines), although there was no systematic difference between lines originating from high and low elevation sites for any of these traits. Broad-sense heritabilities (H^2) for variance components in the Mount Edith and Mount Lewis (Table 2) lines gave low to moderate estimates. Mating duration gave low H^2 values (0.035 – 0.126), although did not significantly differ from zero at Mount Lewis. H^2 estimates were significantly different from zero for number of offspring produced per mating, ranging from 0.069 – 0.229. Total number of matings attained and total productivity per male exhibited significant heritability at Mount Edith (0.128 and 0.292 respectively), but not at Mount Lewis.

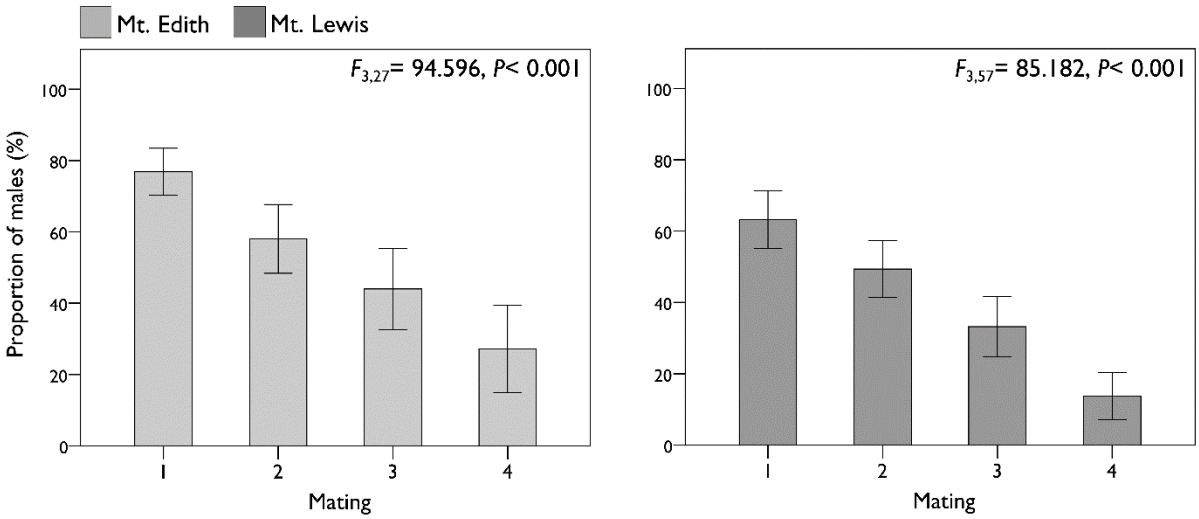

Figure 2. *Mean proportion (%) of males per line reaching each mating*. Mount Edith (left) and Mount Lewis (right) with 95% confidence intervals.

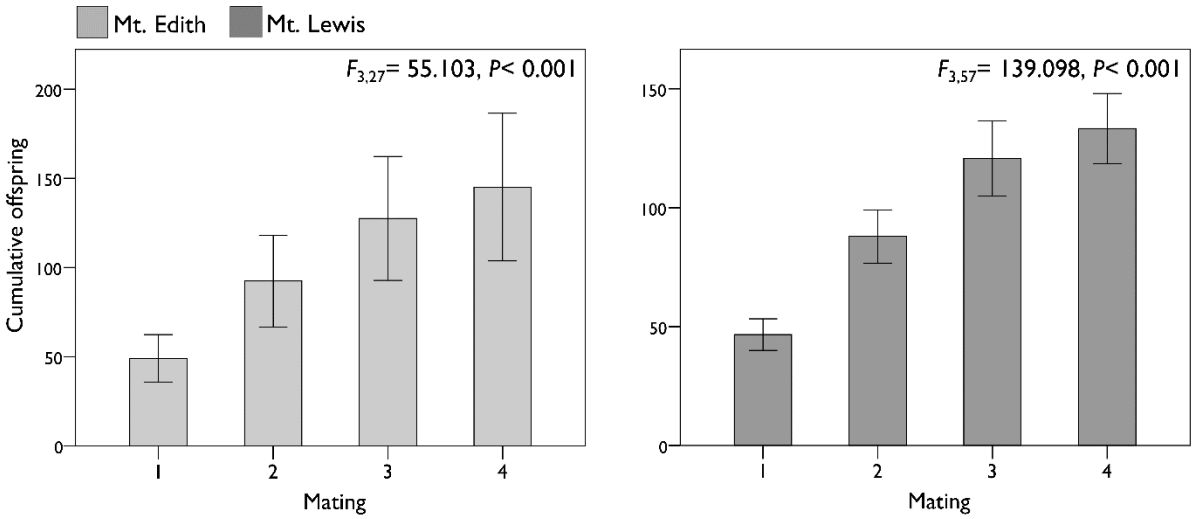

Figure 3. *Cumulative number of offspring produced per line with successive matings*. Mount Edith (left) and Mount Lewis (right) with 95% confidence intervals.

(b) Experiment II: Offspring produced by sons from successive paternal matings

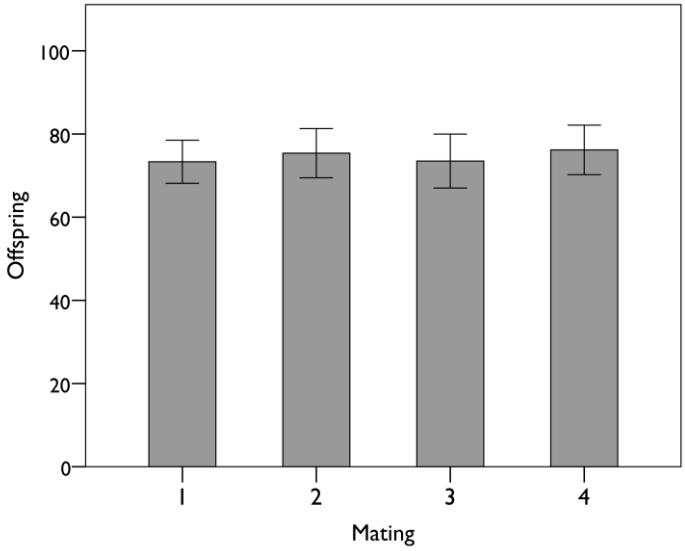

Figure 4. *No decline in reproductive success of male offspring from later matings*. Number of offspring produced by sons derived from successive paternal matings (1 – 4) when mating with a single, virgin female (with 95% confidence intervals). For Mount Edith lines only.

Table 3. *No difference in productivity between sons derived from successive sire matings or different isofemale lines*. ANOVA with number of offspring as dependent variable. Male (son) derived from sire mating number (1-4) was used by as a fixed factor, isofemale line as a random factor and interaction between terms. *F*-ratio, degrees of freedom (*df*) and associated probability (*P*).

	F	*df*	*P*
Mating number	0.519	3, 221	0.675
Line	1.344	6, 221	0.288
Mating no. * Line	0.647	18, 221	0.860

There were no significant differences in the productivity of sons derived from successive paternal matings in Experiment II (Fig. 4). Mean number of offspring produced from mating with a single, virgin female was similar for sons obtained from sire mating 1 – 4: Mating 1= 73.3 (SE ± 2.59), Mating 2= 75.4 (± 2.96), Mating 3= 73.5 (± 3.24), Mating 4= 76.2 (± 2.98). There was also no significant variation in productivity among sons attributable to isofemale line used (Table 3).

4. Discussion

There is substantial interspecific variation in male mating strategies in *Drosophila* (Gowaty et al., 2003; Singh & Singh, 2013). This variation 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 local abiotic and biotic factors such as temperature stress and pathogen or parasitoid exposure respectively. Here, we demonstrate that males of the rainforest specialist *Drosophila birchii* exhibit a relatively low mating rate in comparison with cosmopolitan species previously assayed. For example, *D. melanogaster* males will copulate with 10 females per day given the opportunity (Mossige, 1955; Gromko, 1992) or *D. hydei*, which can mate up to 10 times in two hours (Markow, 1985). By contrast, we observed that only 27% and 14% of *D. birchii* males from Mount Edith and Mount Lewis lines respectively, attained a fourth mating over the one-day experimental period, with only 58 and 49% mating more than once (Fig. 2). In addition, there was a substantial increase in the total number of offspring produced from males that mated four times compared to those that mated only once (Fig. 3), even though multiple mating was associated with a reduction in the per mating number of offspring produced in later mating events (Fig. 1). This is also observed in *D. melanogaster* (Lefevre & Jonsson, 1962), despite the substantially lower absolute productivity recorded per mating in *D. birchii*. Importantly, these patterns were not to be an artefact of the non-random subset of males for which estimates of fourth matings are possible (i.e. those males that successfully copulate four times) because a comparable reduction in productivity across matings was seen for this subset of males too (Fig. SM1). This suggests rapid ejaculate depletion even in males that successfully mated the maximum number of times, and contrasts with studies in tropical tephritid flies that found males do not deplete sperm stores, allocating similar quantities over three consecutive matings (Perez-Staples & Aluja, 2006).

Drosophila birchii are often observed at low densities in their rainforest habitat (Bridle et al., 2009; O'Brien et al., 2017), compared to other drosophilids. This means the female encounter rate for males may be low, along with the density and distribution of males themselves, which will shape resource allocation across matings (Gage, 1991; Shelly & Bailey, 1992; Aspi & Hoffmann, 1998; Willis et al., 2011). In particular, sperm competition risk favours increased sperm quantity in other *Drosophila* (Bjork et al., 2007), and leads to increased allocation across multiple matings (Ingleby et al., 2010), although sperm numbers may trade-off against other characteristics of sperm including size, viability or longevity, at different levels of sperm

competition (Snook, 2005). Increases in production of seminal fluid proteins can also occur in sexually competitive environments (Crudgington et al., 2009; Wigby et al., 2016).

Ejaculate components such as sperm (Wedell et al., 2002) or seminal fluids (Wigby et al., 2009) can limit male remating. Spermiogenesis in male *Drosophila melanogaster* takes five days (at 25 °C) (Fabian & Brill, 2012) and mated males with depleted seminal fluid proteins can require three days of sexual inactivity before they can transfer initial quantities again (Sirot et al., 2009). Species with larger sperm (i.e. higher costs at each mating) are also more likely to partition ejaculates (Pitnick & Markow, 1994). Although sperm size in *D. birchii* has not been assayed here, long flagella and long ventral receptacles in females (R. Snook, personal communication, 2014), indicate that male investment in sperm is likely to be high (Markow, 2015). If energetic costs of mating are high for male *D. birchii*, successive mating events may be accompanied by increased male mate discrimination, as the relative cost of mating rises with male resource depletion (Byrne & Rice, 2006). In this study however, no overall variation was found in latency to copulation across matings (Fig. 1), suggesting no detectable effect of multiple mating on male choosiness (i.e. inclination to remate) (Engqvist & Sauer, 2001) or male attractiveness to females (Taylor et al., 2007). However, our experiments used 'no choice' mating assays (i.e. the focal male was offered a single virgin female sequentially), which makes male choosiness difficult to measure directly, as sequential (as opposed to simultaneous) mate choice means that males cannot assess comparative female quality or identify if further mating opportunities are likely to occur (Barry & Kokko, 2010).

Extending mating durations can increase male fitness by increasing paternity (Mazzi et al., 2009; Bretman et al., 2013a). In *D. birchii*, a consistent increase in mean mating duration was observed with each successive copulation, from ~3 to 6 minutes over four matings (Fig. 1). Conversely, in several other *Drosophila* species copulation durations decline over consecutive matings (Singh & Singh, 2000; Linklater et al., 2007; Singh & Singh, 2013), suggesting that, in these cases, duration decreased as male ejaculate becomes depleted (Edvardsson & Canal, 2006). In these experiments however, declining productivity with increasing duration over successive matings indicate that extended copulation duration is not associated with sperm transfer. Gilchrist and Partridge (2000) found that sperm transfer occurs rapidly after mating is initiated in *D. melanogaster* and that the function of extended copulations is to delay female remating by transmitting seminal fluids that boost fecundity and reduce receptivity in females. Observed increases of mating duration with repeated male copulations in the housefly (*Musca domestica*), were likely due to the depletion of such accessory secretions, with males only

terminating copulations when the levels transferred were sufficient to stimulate an inhibitory response to further mating in females (Leopold et al., 1971).

Alternatively, prolonging copulation might represent a mate guarding strategy by males, particularly as sperm becomes limited (Simmons, 2001). Sexual conflict over mating duration is likely, with male optima generally exceeding that of females (Blanckenhorn et al., 2007). While mating duration is largely controlled by males in several *Drosophila* (Parsons & Kaul, 1966; MacBean & Parsons, 1967; Patty, 1975; Singh & Singh, 2000; Jagadeeshan & Singh, 2006), studies with *D. birchii* have shown that females can dislodge males (Hoikkala & Crossley, 2000). Similarly, females have an influence on copulation duration in *D. mojavensis* (Krebs, 1991), *D. elegans* (Hirai et al., 1999) and *D. montana* (Mazzi et al., 2009). Such species-specific differences in male ability to control mating are likely to generate variation in mating duration optima across successive matings (Hoikkala et al., 2000; Edvardsson & Canal, 2006). However, the relatively small absolute increases in mean copulation duration in this study make such hypothesised mate guarding unlikely. Further research is necessary to clarify female fitness effects of prolonging copulations, particularly given that virgin females experienced only a single mating in this assay, and female *D. birchii* will rarely remate within the same day (E.K. O'Brien, personal communication, 2014). Furthermore, unlike many *Drosophila* species, female *D. birchii* cannot store sperm due to the absence of spermathecae (R. Snook, personal communication, 2014). This may alter mating strategies for males (Parker, 1984; Pitnick et al., 1999), because of the strong last male precedence in paternity for *D. birchii* when females remate (E.K. O'Brien, personal communication, 2017).

Males were assayed under standardised (constant 25 °C) laboratory conditions. However, male allocation strategies are not fixed for a given genotype and are likely to show adaptive responses to the more variable conditions experienced in natural populations (Wedell et al., 2002; Wigby et al., 2016). Pitnick and Markow (1994) propose that submaximal male ejaculate allocation over successive matings may constitute bet-hedging for male *Drosophila*, particularly where environments are stressful. Variation in abiotic conditions also affects mating duration (Horton et al., 2002), sperm allocation and remating rate (Katsuki & Miyatake, 2009) within genotypes in insects. Latitudinal variation in male remating traits within two cosmopolitan *Drosophila* species has also been established (although only two consecutive matings were assessed) (Singh & Singh, 2000; Chahal et al., 2013). Bouletreaumerle et al. (1982) found that female fecundity was reduced in tropical versus temperate populations of *D. melanogaster*, suggesting that males of tropical species may invest more in sperm or seminal

components or in overall resistance to biotic factors (e.g. pathogens) associated with the relatively stable environments they inhabit.

This study showed no sign of genetic divergence in any mating trait across either elevational gradient (Table 1), even though the differences in thermal conditions at high and low elevation are considerable (~7 °C difference in mean temperature) and characterise the ecological limits of the species (influencing both population abundance in the field and productivity in the laboratory). There was significant genetic variation between isofemale lines in number of offspring (productivity) over successive matings at both gradients, with between line variance representing ~17% of total variance in Mount Edith lines and ~4% at Mount Lewis. Similar differences were observed between lines for mating duration at Mount Edith although not at Mount Lewis. Broad-sense heritability (H^2) estimates varied substantially between gradients with Mount Edith showing consistently lower levels of genetic variance. For instance, offspring per mating gave values of 0.229 at Mount Edith but 0.069 at Mount Lewis. However, latency to mate showed no between-line variation and mating duration only exhibited significant H^2 at Mount Edith. Taylor et al. (2013) also found no evidence for significant heritability in male latency and duration estimates in *D. melanogaster*. These results can be compared to the wide-range of estimates obtained in *D. melanogaster*, for male courtship traits (0.033 – 0.094) (Gaertner et al., 2015), mating latency (0.01), duration (0.007) (Gromko, 1987) and mating success (0.25) (Tucic et al., 1988).

If ejaculate quantity decreases over successive matings in males, there may be accompanying declines in sperm quality that affect the fitness of offspring from later matings. Experiment II demonstrated that the paternal (sire) mating sequence had no effect on the reproductive success of sons from fourth matings compared to those from first mating, when they were mated to a single female (Fig. 4; Table 3). This means that no deterioration in sperm/ejaculate quality is observed at least until males' fourth remating, which is associated with a 237% relative increase in mean total productivity at Mount Edith and 111% increase at Mount Lewis compared to a male that mates only once. This variation in male remating rate within *D. birchii* populations therefore remains surprising, given the high opportunity for mating in these laboratory populations and the apparent unequivocal fitness advantage it provides.

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