# Diet choice is insensitive to mating in male fruit flies

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Keywords: Animal nutrition, CAFE assay, diet choice, *Drosophila melanogaster,* macronutrients, multiple mating, P:C ratio.

*Data accessibility*

Data are provided in additional supplementary information for review.

*Authors contributions*

M.C.S. and J.C.P. conceived and designed the study; M.C.S. conducted the experiments with assistance from J.C.P.; M.C.S. performed data analysis and visualisation with advice and supervision from T.C. and J.C.P.; all authors wrote the original draft, and edited and approved the final draft for publication.

*Competing interests*

The authors declare no conflicts of interest.

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*Data availability*

All data used for this study are available at https://figshare.com/s/2b0375af6fda5bf5a49e.

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HIGHLIGHTS

- Female fruit flies show altered diet preference following mating.
- However, dietary responses to mating in males are largely unknown.
- We tested the impact of realistic mating rates on male fruit fly diet choice.
- Males do not alter food intake to recoup depleted ejaculate, despite fertility loss.
- These results highlight key sex differences in diet choice following mating.

# Diet choice is insensitive to mating in male fruit flies

Animals can adjust their consumption of different nutrients to adaptively match their current or expected physiological state. Changes in diet preference can arise from social and sexual experience. For example, in female *Drosophila melanogaster* fruit flies, a single mating triggers a behavioural switch in diet choice towards increased protein intake and total food consumption, which supports offspring production. In contrast, male diet choice appears to be unaffected by a single mating. However, one mating may not fully capture the impact of mating on male feeding behaviour. Males can often mate multiply in natural settings, and the costs of ejaculate production and energetic courtship may be cumulative, such that males might experience increased nutritional demands only after multiple matings. In this study we tested this prediction by measuring the effect of multiple matings on the diet choice of male *D. melanogaster* fruit flies. Males were assigned to one of three mating treatments – unmated, mated once or mated five times consecutively – and then allowed to feed freely on chemically-defined diets of protein and carbohydrate. In contrast to the prediction, we found that males that mated five times did not alter the amount of food, or the proportion of protein and carbohydrate consumed, when compared with unmated or once-mated males. This absence of a feeding response occurred despite substantial ejaculate depletion from multiple matings: males sired fewer offspring in each consecutive mating. These results reveal a lack of plasticity in male feeding behaviour according to mating status, despite substantial potential physiological costs, and highlight the remarkably distinct nutritional ecologies of males versus females.

## Key words

Animal nutrition, CAFE assay, diet choice, *Drosophila melanogaster*, macronutrients, multiple mating, P:C ratio

Animals have complex nutritional needs, with optimal diets varying with age, sex, metabolic rate and environment (Simpson and Raubenheimer, 2012). Previous studies have demonstrated that, in many species, individuals are able to sense their own physiological state and adjust feeding to match nutritional demand, by fine-tuning consumption of micronutrients, such as vitamins and minerals, and macronutrients, including carbohydrate, protein (amino acids) and lipids (Ribeiro and Dickson, 2010; Simpson et al., 2015). The balance of macronutrients eaten strongly affects an individual's fitness, with the balance of protein and carbohydrate (P:C ratio) being particularly important across animals of many different taxa. The impacts of P:C consumption have been studied in both vertebrate systems (e.g., in mice, *Mus musculus* (Solon-Biet et al., 2015) and rainbow trout, *Oncorhynchus mykiss* (Suárez et al., 2002)) and extensively in invertebrate systems (e.g., in locusts, *Locusta migratoria* (Raubenheimer and Simpson, 1993), field crickets, *Teleogryllus commodus* (Reifer et al., 2018), German cockroaches*, Blattella germanica,* (McPherson et al., 2021) and tephritid fruit flies, such as the Queensland fruit fly, *Bactrocera tryoni* (Prabhu et al., 2008)). Experiments carried out in *Drosophila melanogaster* fruit flies show that low P:C diets can maximise the longevity of both sexes, while optimal P:C diets for reproductive success differ between males and females (Lee et al*.*, 2008; Jensen et al*.*, 2015; Camus et al*.*, 2017; Carey et al*.*, 2022; but see Reddiex et al*.*, 2013). When males and female insects are able to choose, they generally favour diets that maximise reproductive success; for males this is generally a lower P:C diet than for females (Lee et al*.*, 2008; Maklakov et al*.*, 2008; Fanson et al*.*, 2009; but see Jensen et al*.*, 2015).

Mating, and the initiation of reproductive processes that stem from it, have important implications for nutrient demands in females. Females of many species are observed to increase food intake after mating, e.g. in the two-spot ladybird *Adalia bipunctata* (Perry, 2011), tephritid fruit flies (see Pérez-Staples and Abraham, 2023) and in *D. melanogaster* (Camus et al., 2018; Carvalho et al., 2006; Lee et al., 2013). Upon mating, female *D. melanogaster* also markedly increase the proportion of protein and yeast intake in their diet, in comparison with unmated females (Barnes et al., 2008; Camus et al., 2018; Corrales-Carvajal et al., 2016; Jensen et al., 2015; Lee et al., 2013; Newell et al., 2020; Ribeiro and Dickson, 2010). Macronutrient preference is similarly altered post-mating in the two-spotted cricket, *Gryllus bimaculatus* (Tsukamoto et al., 2014) and during pregnancy in *Rattus norvegicus domestica* rats (Leshner et al., 1972; Richter and Barelare, 1938; Simpson and Raubenheimer, 1997). In *D. melanogaster*, the increase in females' preference for protein after mating is thought to occur to meet the demands of elevated egg production (Bownes and Blair, 1986; Drummond-Barbosa and Spradling, 2001; Lee, Kim and Min, 2013; but see Ribeiro and Dickson, 2010).

In contrast, data on the effect of mating on male dietary preference are scant. The few studies to date have been conducted in species that produce resource-costly nuptial gifts such as the German cockroach *Blattella germanica* (Jensen and Silverman, 2018) and *A. bipunctata* (Perry and Tse, 2013). In both of these species, males exhibit some dietary compensation after their first mating. However, there is little evidence for a shift in male dietary preference after a single, first mating in non-nuptial gift-giving species. For example, in *D. melanogaster*, a single mating had no significant effect on male diet preference (P:C ratio) or the overall quantity of food consumed (Camus et al., 2018). This result is in accord with the view that male ejaculates may be relatively 'cheap' to produce (Bateman, 1948; Trivers, 1972) and hence that males might not need to increase protein intake to replenish reserves depleted by mating.

It is possible that males incur cumulative costs of ejaculate production, such that costs that are minimal after a single mating increase significantly with multiple matings. In fact, males of many species are able to mate multiply in quick succession. For example, males of the red flour beetle, *Tribolium castaneum*, can mate with up to seven females in 15 minutes (Lewis, 2004) and *D. melanogaster* males can mate up to 11 times in a day (Douglas et al., 2020). Moreover, contrary to the 'cheap' male ejaculate idea, it is now understood that the production of sperm and other ejaculate components can be costly and limiting for males (Dewsbury, 1982; Macartney et al., 2019; Olsson et al., 1997; Perry et al., 2013; Perry and Tse, 2013; Reinhardt et al., 2011; Simmons et al., 2022). For example, offspring production decreases with each consecutive mating by males of a parasitoid wasp, *Diaeretiella rapae* (Kant et al., 2012), *T. castaneum* (Lewis, 2004) and *D. melanogaster* (Douglas et al., 2020). Ejaculate components start to become depleted in male *D. melanogaster* after three sequential matings (Lefevre and Jonsson, 1962) and continue to decrease with additional matings (Hihara, 1981; Linklater et al., 2007; Loyau et al., 2012; Macartney et al., 2021). Males can be rendered infertile due to depletion of non-sperm ejaculate components (Hihara, 1981). Therefore, we predicted that a significant impact of mating on male diet preference would occur after multiple matings.

To test this prediction and improve understanding of how mating impacts male diet choice, we manipulated the mating rate of male *D. melanogaster* fruit flies and then measured male diet preferences and intake. We used *D. melanogaster* because protocols for measuring its dietary preferences are well-established, it is an important model for nutritional preference studies, and because males can mate multiply. Separate treatment groups of male flies were assigned at random to remain unmated, to mate once, or to mate five times consecutively. The unmated and single

mating treatments were included to investigate whether a feeding switch is triggered when males mate for the first time, as occurs in females. The five sequential mating treatment was chosen as this approaches the average maximal daily mating rate for this species (Douglas et al., 2020; Lefevre and Jonsson, 1962). We tracked the number of viable offspring produced from each mating to confirm that the highest sequential mating rate treatment did result in ejaculate depletion. Following the matings, male diet preference for all treatments was measured by offering male flies carbohydrate and protein solutions simultaneously using the Capillary Feeder (CAFE) assay (Ja et al., 2007). The CAFE method allows the quantification of food intake and macronutrient preference using synthetic diets with known nutritional content. We predicted that males from the highest sequential mating rate treatments would (1) suffer energy and ejaculate depletion, evident as cumulative reductions in their siring success with each mating bout; (2) increase total food consumption to recoup energy expenditure during courtship and mating; and (3) increase the proportion of protein eaten after mating to restore proteinaceous sperm and non-sperm components of the ejaculate.

## METHODS

### *Fly stocks*

Fly rearing and experiments were conducted in a 25°C humidified room under a 12-hour light-dark cycle. Experimental flies were collected from a large stock population of outbred wildtype Dahomey flies (Chapman, Trevitt and Partridge, 1994) maintained on a standard sugar-yeast-agar (SYA) diet (50g sucrose, 100g brewer's yeast, 15g agar, 30ml Nipagin (10% solution), 3ml propionic acid, 970ml water). To generate experimental flies, eggs from the stock population were collected on grape juice-agar plates with live yeast paste. First instar larvae were transferred into glass vials containing SYA medium at a controlled density of fifty larvae per vial. Experimental adults were collected as virgins using ice anaesthesia within 4-6 hours of eclosion. Flies were housed in single sex groups of 15 males or 10 females in glass vials containing 7ml SYA medium supplemented with live yeast granules for three days before mating assays.

Experiments were carried out in four experimental blocks. In blocks one and two an additional mating treatment of three sequential matings was included. Offspring data were collected from blocks one and two, dietary data were collected from blocks three and four, and mating data were collected from all four blocks (Appendix, Figure A1).

*Sequential mating treatments*

Experimental males were randomly assigned to one of three mating treatments: zero matings, one mating or five matings in a single day (*N*=528, all blocks). Males were gently aspirated into individual mating vials containing 7ml of 0.75% agar-water. This nutrient-lacking medium was used to remove any potentially confounding influence of a nutritional substrate on mating behaviour, while providing moisture. One virgin female was introduced to each vial for males assigned to the one and five mating treatments and latency to mate and mating duration were recorded by scan sampling across all the vials approximately every minute. Once mating was complete, the female was removed. For males assigned to the five matings treatment, this process was repeated until five consecutive matings were achieved. If no mating occurred within 60 minutes, the female was replaced with a new virgin female. Matings under five minutes were excluded (*N*=9) as short matings may not allow complete transfer of sperm and seminal fluids (Gilchrist and Partridge, 2000; Manier et al., 2010). Matings over 40 minutes were also excluded (*N*=4) because they appeared to represent cases in which males and females had become stuck during mating (Mason et al., 2016).

Males that were unable to complete their assigned number of sequential matings were dropped from the study (a total across all blocks of *N*=56 males, all from the five matings treatment, as expected given the cumulative effects of serial matings within a day). To test whether the exclusion of these individuals from the five matings treatment might have introduced bias, we compared like-for-like mating characteristics across mating treatments. We first analysed the duration of the first mating between individuals that completed five matings and individuals that did not. Blocks 1,2,3 showed no significant differences, while in block 4 non-maters (i.e., dropouts) had a somewhat longer first mating duration than maters retained in the final dataset ($P$= 0.03). We next compared the duration of the first matings between the successful maters across all treatment groups. Though this showed some differences in first mating duration among males from the different mating treatments, it was not consistent across all blocks (blocks 1 and 2 both $P$> 0.05; block 3 $P$=0.02, block 4 $P$=0.03). Overall, neither analysis provided evidence consistent with a systematic bias. We did not find differences in nutrient intake among males that mated different numbers of times. Thus, any slight differences between the males eventually retained in the different mating treatments, even if they were present, would be expected to have a conservative effect with respect to the main result we obtained. The no mating and one mating treatment vials were set up and handled in the same way as the five matings treatment vials. After the mating assay and prior to the CAFE assays of diet intake, experimental males were all housed singly in agar-water mating vials overnight.

*Diets and CAFE assay*

We used the CAFE assay (Ja et al. 2007) to measure the consumption of protein and carbohydrate liquid synthetic diets (Camus et al., 2018, 2017; Piper et al., 2014). Recipes are included in Appendix, Tables A1 and A2. Liquid diets included identical volumes of synthetic components (lipids, vitamins, and salts) to create a fully chemically defined diet. We added a supplement of 20% autoclaved yeast suspension to the protein diet following previous reports that *D. melanogaster* adults do not eat a pure protein solution (Camus et al., 2018, 2017). The resulting protein diet therefore contained 4% of carbohydrate due to the sugar content of the killed yeast suspension.

On the day following the mating assay, experimental males were transferred to fresh agar-water vials in groups of three, because preliminary experiments indicated that individual males ingest too little protein to detect through the CAFE assay. Coded labelling was used to ensure that the experimental treatments were anonymised relative to the observer. Vials were each provided with two 5µl microcapillary tubes (Ringcaps™; Hirschmann Instruments™) one containing the liquid protein diet and the other containing the liquid carbohydrate diet, held in place with a foam bung. Both microcapillary tubes were replaced every 24 hours for five days and the loss of liquid diet was measured from each tube at the meniscus using a digital calliper. We calculated total food consumption as the length of the vector from the origin to the intake values of protein and carbohydrate, in a protein and carbohydrate nutrient space (Appendix, Figure A2) (Camus et al., 2018). We calculated relative amounts of protein to carbohydrate ingested as the angle between this vector and the protein axis to give a value between 1-90°. Values of over 45° signify a greater proportion of carbohydrate consumed and values of less than 45° signify a greater proportion of protein consumed. During the experiment, vials were housed in a sealed 50 L lidded box containing a saturated salt solution to create a high humidity environment (77% on average) (Greenspan, 1977) to limit evaporation from the microcapillaries. Ten vials with protein- and carbohydrate-containing microcapillary tubes but without flies were interspersed amidst the experimental vials for each day of the measuring period to track evaporative loss. Mean evaporation was calculated from these vials for both protein and carbohydrate and subtracted from the feeding measures for each day of the measurement period. Instances where evaporation was greater than the measured diet consumption were excluded ($N$=18) because calculations of dietary preference (angle, see Appendix, Figure A2) require positive intake values. Vials where one or more males escaped or died during the assay were also excluded.

*Reproductive output*

Females that mated with experimental males (*N*=387, blocks one and two) were retained in individual SYA vials seeded with live yeast granules and allowed to lay eggs for approximately four weeks to allow us to profile offspring production over time from each mating. Females were moved to fresh vials every three to five days to ensure larvae would not be food limited. Vials were frozen 13 days after egg-laying to allow all offspring to eclose. The number of adult offspring was counted.

*Ethical note*

This research was conducted on fruit flies, which are not subject to any ethical restrictions in the United Kingdom.

*Statistical analysis*

Statistical analyses were carried out in R version 4.0.4 (The R Foundation for Statistical Computing, Vienna, Austria, http://www.r-project.org) and all statistical models can be found in Appendix, Table.A3.

*Dietary preference and mating duration*

We excluded outliers for diet consumption identified by Z-Score calculations (*N*=15 protein values, *N*=8 carbohydrate values). Outliers included tubes that had drained of liquid due to accidental contact with a substrate. To account for the effect of block on dietary and mating duration data, we first fitted generalised linear models with block assigned as a fixed factor for each of the response variables length of vector, angle, and duration of mating. Residuals from these models were analysed as response variables in generalised linear mixed models (GLMM) using the R package glmmTMB (Brooks et al., 2017) in which treatment and day were included as fixed effects for analysing length of vector and angle, and mate number for mating duration. Individual male id was included as a random effect. We also analysed differences between the treatment groups on day one only to untangle whether there was an effect of the mating treatment on feeding behaviour directly following the mating assay. Data from each block was additionally analysed separately for duration following tests for model fit. Additionally, we included angle and length of vector as response variables in a multivariate analysis of variance (MANOVA) to investigate the effects of treatment and day on the joint response of both variables (Pillai's trace). Angle and length of vector were centred around a mean of 0 for the MANOVA. Throughout, model fit was checked using the R package DHARMa (Hartig, 2022). Post-hoc

pairwise comparisons were carried out on estimated marginal means using the R package emmeans (Lenth et al., 2023).

*Reproductive output*

To investigate male sperm depletion with multiple mating, we tested the effect of a female's mate number (i.e., whether the female was the first, second, third, fourth or fifth mate of their male partner) on her offspring output over time (e.g., per vial). For each mated female, we calculated the slope of her adult offspring production regressed against time (i.e., each of the seven sequential 24-hour periods (vials)). Females without data for all seven timepoints were excluded ($N$=90, blocks one and two); such as those that died or escaped during the data collection period ($N$=76), as were females that produced no offspring, to exclude reproductive failure events ($N$=14). To account for effects of block, we first fitted a generalised linear model with block as a fixed factor and with individual slopes as the response variable. Residuals from the initial model were entered as the response variable in a linear model against mate number (whether the female was the first, second, third, fourth or fifth mate of their male partner).

*Latency to mate*

Latency to mate was analysed as a function of female mate number using the R package survival (Therneau, 2023; Therneau and Grambsch, 2000) and visualised using a Kaplan-Meier curve. Instances where the female partner was replaced were treated as censored values.

*Repeatability analysis*

Repeatability of male mating behaviour was analysed for both mating duration and latency for males that mated multiply (three- and five-times mating treatments), by using the R package rptR (Stoffel et al., 2019). Mate number was included as a fixed effect in both models to perform an analysis of enhanced agreement repeatability of male id. Instances of where the female partner was replaced were treated as censored values for repeatability of latency behaviour.

## RESULTS

*Multiple matings do not alter food intake or preference for protein and carbohydrate*

The combined total of protein and carbohydrate diet eaten by males (i.e., length of vector) over the 5 days was not significantly different between the 0, 1 or 5 mating treatment groups (Figure 1a) ($\chi^2_2$=2.12, $P$=0.35). There was no significant interaction between treatment and day ($\chi^2_8$=5.47, $P$=0.71)

suggesting little change in consumption over the 5 days of the assay. It is possible that an effect of mating on food intake might be strongest immediately after mating. However, the quantity of food consumed on day one was not significantly different among mating treatments ($\chi^2_2$=1.97, $P$=0.37). There was a significant effect of day on total food consumption ($\chi^2_4$=11.89, $P$<0.05) but no consistent effect of day between blocks.

The P:C diet composition (angle) that males ate was not significantly different between 0, 1- or 5-times mated males over the 5 days ($\chi^2_2$=3.18, $P$=0.20). There was no discernible effect of day ($\chi^2_4$=4.83, $P$=0.30) and no significant interaction between treatment and day ($\chi^2_8$=8.7, $P$=0.37) (Figure 1b). Males showed a consistent preference for carbohydrate over protein: all recorded P:C angles were over 45°, indicating a skew to carbohydrate (Appendix, Figure A2). We found no evidence for a stronger effect of mating on diet composition preference immediately after mating (no effect of treatment on day 1 diet composition; $\chi^2_2$=2.56, $P$=0.28). There was no treatment effect in the raw consumption data for either protein or carbohydrate (Appendix, Figure A3).

We tested whether treatment affected the joint feeding response of both total diet intake and diet composition using MANOVA. This showed no significant effect of treatment (Pillai test statistic $df_2$=0.0096, approx. $F_{4,1084}$=1.31, $P$=0.26) or interaction between treatment and day (Pillai test statistic $df_8$=0.0219, approx. $F_{16,\ 1084}$=0.75, $P$=0.74) in the multivariate analysis. The joint feeding response varied among days (Pillai test statistic $df_4$=0.0302, approx. $F_{8,1084}$=2.07, $P$<0.05). These results support the finding that there was no change in the nutrient intake for males as a function of their mating frequency.

*Males become ejaculate-limited after serial multiple matings*

As expected, the total number of offspring sired by a male increased when males were mated more than once in series ($F_{2,136}$=49.44, $P$<0.001) (Figure 2). Males that mated once produced 313 ±SE 16 ($N$=47) adult offspring on average, while an average of 642 ±SE 29 ($N$=46) and 681 ±SE 38 ($N$=46) offspring were produced by those that serially mated three and five times, respectively. The number of offspring produced by a male was significantly higher in males that mated serially three or five times compared to those that mated only once (post-hoc Tukey tests: one versus three matings: $t_{136}$=-8.08, $P$<0.001, one versus five matings: $t_{136}$=-9.04, $P$<0.001). However, reproductive output did not differ between the three and five mated groups ($t_{136}$=-0.96, $P$=0.61) despite an additional two matings with virgin females. This effect was evident in an analysis of the slope of decline in the numbers of offspring produced by each female (Figure 3). The slope of decline in female offspring production

279 depended on a female's position in the mating sequence ($\chi^2_4$=174.47, $P$<0.001). Post-hoc Tukey tests
280 showed that this slope of decline was significantly different when comparing across all mate numbers
281 ($P$<0.01 to $P$<0.001), with the exception of the fourth versus fifth females to mate with a male ($t_{290}$=-
282 1.3, $P$= 0.68). Mating latency and duration were not correlated with offspring production (Appendix,
283 Figure A4).

284 *Males took longer to mate if they had previously mated at least twice*

285 Latency to mate became significantly slower as males mated more times in sequence ($\chi^2_4$=108.65,
286 $P$<0.001) (Figure 4). The median time to mate was 8.0, 8.5, 11.0, 13.0 and 30.0 minutes for the first,
287 second, third, fourth and fifth matings, respectively. Latency between consecutive matings was not
288 significantly different, with the exception of the second versus third mating (post-hoc Tukey: $Z$=3.5,
289 $P$<0.01), while latency times between non-consecutive matings were significantly different from one
290 another (post-hoc Tukey*:* $P$<0.001 to $P$<0.01). There was significant variation in latency between
291 experimental blocks ($\chi^2_3$=21.31, $P$<0.001). Males also showed an increased reluctance to mate later in
292 the mating sequence, as evident in the increased requirement to add additional virgin females for
293 experimental matings to occur (Appendix, Figure A5). Latency to mate was not a repeatable individual
294 behaviour (repeatability score; $R$=0.02, $P$=0.19).

295 *Mating duration decreased after the first mating*

296 Mating duration was significantly altered by mate number ($\chi^2_4$=190.39, $P$<0.001, statistical model run
297 across data from all blocks) (Figure 5). However, significant deviation from the expected distribution
298 of residuals was detected during model fit tests. Therefore, blocks were also analysed separately.
299 These analyses showed that mate number had a significant effect on duration in all blocks ($P$<0.001 in
300 all cases). Pairwise comparisons showed that first matings were significantly longer than all
301 subsequent matings (post-hoc Tukey tests: $P$<0.001 to <0.05) with the exception of the fourth mating
302 in both block 1 ($t_{168}$=2.30, $P$=0.149) and block 2 ($t_{178}$=2.60, $P$=0.075). All matings after the first did not
303 differ from each other. There was significant but low repeatability in mating duration across the
304 multiple matings of an individual male, once accounting for the variation caused by mate number
305 (repeatability score; $R$=0.152, $P$<0.001).

306 ## DISCUSSION

307 Though it is often assumed that production of sperm incurs little cost to males, ejaculate production
308 costs as a whole can be significant (Dewsbury, 1982). Nutritional demands due to sperm depletion
309 may not be discernible after a single mating (Camus et al., 2018; Perry and Tse, 2013). However, they

are predicted to be evident after multiple matings, which males of many species experience in natural settings. To our knowledge, this broad prediction had not previously been tested in species lacking nuptial gifts. We tested whether mating multiply in series would trigger male fruit flies to alter their dietary preference to replenish ejaculate components. We tested 3 predictions. Consistent with prediction 1, we found that males in the high mating rate treatment were more ejaculate depleted. However, predictions 2 and 3 were not supported: we did not detect evidence for a change in macronutrient preference, nor did males eat more, in the high mating rate treatment compared to the unmated and low mating rate treatments.

*Male ejaculate depletion with consecutive matings*

We found that males were severely depleted in their ability to transfer ejaculates with fertilisation potential by their fifth mating. This was observed in the pattern of offspring production, with females that mated later in a male's mating sequence producing no, or markedly reduced, numbers of adult offspring compared with the first mate. Furthermore, the reduction in offspring produced showed a non-linear mate number-dependant decline. This is in accord with results on reproductive success in multiply mated males observed previously in several insect species (Abraham et al., 2020; Douglas et al., 2020; Hihara, 1981; Kant et al., 2012; Linklater et al., 2007; Macartney et al., 2021; Savalli and Fox, 1999). This effect is likely to result chiefly from seminal fluid rather than sperm depletion because it has been observed that males retain some sperm in their seminal vesicles, but lack fluid in the accessory gland, after four to five matings (Lefevre and Jonsson, 1962; Gillott, 2003; Macartney et al., 2021; see also Reinhardt, Naylor and Siva-Jothy, 2011). This effect occurs even though *D. melanogaster* males are reported to differentially allocate seminal fluid proteins (Wigby et al., 2009) suggesting that strategic allocation of ejaculates across many matings has some limits.
In the common bedbug, *Cimex lectularius*, reserves of seminal fluid are also reported to decline faster than sperm reserves in the male's reproductive organs - and seminal fluid depletion can even limit male remating (Reinhardt et al., 2011). Similarly, in the South American fruit fly, *Anastrepha fraterculus*, female fecundity was more strongly linked to male accessory gland size than to sperm transfer (Abraham et al., 2020).

An increased reluctance to mate was evident in males as they mated in sequence, males took longer to mate after the first two matings, later matings showed an increase in male refusal to mate, and fifth matings required more instances of swapping in new virgin females to get matings to occur. Duration of mating was also significantly shorter in all matings after a male's first. This could suggest reduced strategic investment in subsequent matings, or that males are becoming energetically

depleted. To distinguish these alternatives, it would be interesting to further study the adaptive allocation of behaviour, energy, and ejaculate resources across bouts of multiple matings.

*Ejaculate depletion does not alter male macronutrient intake or preference*

We predicted that following the depletion of ejaculate reserves after five matings, males would increase consumption of food, and especially protein, to facilitate efficient recharging of sperm and seminal peptides (Perry et al., 2013). Yeast availability in adult male diet, as a protein supplement, has been found to influence a male's ability to gain mates and sire offspring (Fricke et al., 2008). However, despite this, males remained on similar P:C trajectories with similar food intake rates regardless of their mating rate. Males were either unable to translate their physiological requirements into dietary preference or gained no benefit from doing so. Higher protein diets limited sperm viability in field crickets, *Teleogryllus oceanicus* (Ng et al., 2018). Dietary preference was consistent between singly mated and unmated male *D. melanogaster,* which is congruent with previous work (Camus et al., 2018) and with the observation that levels of seminal fluid are not significantly depleted after a single mating (Lefevre and Jonsson, 1962).

Males in this study favoured a low P:C ratio of 1:4 in their diet in all mating treatments. This is consistent with previous studies of male *D. melanogaster* (Camus et al., 2018; Jensen et al., 2015; Lee et al., 2013; Ribeiro and Dickson, 2010) and strong carbohydrate preference in other male insects (e.g., field crickets; *T. oceanicus* (Ng et al., 2019, 2018), cockroaches; *B. germanica* (Jensen and Silverman, 2018), and *Nauphoeta cinerea* (South et al., 2011)). Though both sexes of *D. melanogaster* prefer a carbohydrate-biased diet, male fruit flies exhibit an even greater preference for carbohydrate than females. This is thought to result from the demands of performing energetically costly courtship rituals, which are essential for male reproductive success (Bastock and Manning, 1955; Camus et al., 2018; Lee et al., 2013; Maklakov et al., 2008; von Schilcher, 1976). When placed with non-receptive females, males continue courting for hours (Bastock and Manning, 1955). Male attractiveness to mates in the speckled cockroach, *N. cinerea*, was also closely aligned with carbohydrate intake (South et al., 2011) and a carbohydrate-rich diet in juvenile male Jamaican field crickets, *Gryllus assimilis*, boosted their courting effort as adults (Reifer et al., 2018). It is possible that males might increase carbohydrate intake after multiple matings, to recoup nutritional resources lost to extra bouts of courtship. However, this prediction was not upheld, as we observed that carbohydrate intake remained unchanged across the treatment groups.

The results from this study suggest that male *D. melanogaster* choose a similar ratio of P:C and dietary intake rate regardless of sexual experience. This could indicate that diet intake in males is a non-plastic trait. Consistent with this, strict self-regulation of protein intake has been previously observed in this species (Rushby et al., 2023). No change in P:C ratio was also observed in male *T. oceanicus* in environments of varying sexual competition (Simmons and Chan, 2023). Some invariance in male diet preference is also suggested by a study of dietary preference following macronutrient deprivation (Ribeiro and Dickson, 2010). After maintenance on a sucrose-only diet, females strongly preferred yeast after only three days on sucrose, while males took 10 days to reach an equivalent yeast preference and lost this preference far more rapidly when returned to a yeast medium (Ribeiro and Dickson, 2010). That study provided some evidence that males are able to respond to a severe protein deficit but suggested that males do so more slowly than females. The absence of evidence in our study for increased food or protein ingestion by multiply mated males suggests that multiple matings do not induce such extreme protein limitation in males, even near the physiological limits for mating rate. In another study, five-times mated male *D. melanogaster* sired equivalent offspring to their first mating, provided that the fifth mating had taken place after a 24h respite period (Loyau et al., 2012). However, males in the Loyau et al. (2012) study remained on a solid cornmeal-agar-yeast diet, so it was unclear whether males had altered their food intake over the 24h respite period. The reproductive output of multiply mated males over 4h mating bouts on successive days has also been tested (Douglas et al., 2020). Males were reported to be able to remate multiply on consecutive days but did not necessarily sire more offspring, suggesting incomplete regeneration of ejaculate reserves between days (Douglas et al., 2020). The nutritional intake patterns of males that sustain a high mating rate over multiple days would be interesting to investigate further.

Sexual selection theory suggests that males typically maximise reproductive success by mating as many times as possible (Andersson, 1994; Arnqvist and Rowe, 2005). However, in natural settings, multiple factors may affect a male's ability to gain mating and remating opportunities, including access to females, female mate preferences and male-male competition. It may be that males have not evolved a mechanism to cope with sudden ejaculate depletion because the opportunity for consecutive, multiple rematings is rare in nature. There is also evidence that a mated male is less attractive as a mate to females (Douglas et al., 2020; Loyau et al., 2012; Savalli and Fox, 1999). Hence, in scenarios where access to females is unlimited, males may gain fitness by mating at maximal frequencies regardless of ejaculate depletion. Consistent with this idea, we observed that both multiply mated treatment groups had higher offspring numbers than those only mated once, while five times mated males were still able to sire offspring with their last mate and had a marginally

(though not significantly) higher reproductive output than three-times mated males. More data on male remating behaviour in natural contexts would be helpful for exploring these ideas.

Our results contrast markedly with findings in females, which respond to nutritional deficit and the initiation of reproduction and dynamically adjust their intake accordingly. Specifically, *D. melanogaster* females alter their diet preference from a low P:C diet as virgins (from a level that is similar to the observed P:C ratio of males) to a higher P:C ratio after mating (Barnes et al., 2008; Camus et al., 2018; Corrales-Carvajal et al., 2016; Lee et al., 2013; Ribeiro and Dickson, 2010). This sex difference is likely to ultimately result from contrasting reproductive strategies between the sexes, whereby females might gain most reproductive success from limiting their number of mates and have greater nutritional requirements to support offspring production. Proximate explanations for the sex differences in dietary responses to mating include gene expression differences in nutrient sensing pathways between the sexes (Bennett-Keki et al., 2023; Fowler et al., 2019) and the effects of the ejaculate sex peptide transferred to females during mating in the seminal fluid (Chapman et al., 2003; Hopkins and Perry, 2022; Liu and Kubli, 2003). The sex peptide has been identified as the key mediator of an increased preference for protein in females after mating (Carvalho et al., 2006; Hopkins and Perry, 2022; Ribeiro and Dickson, 2010).

In conclusion, we show that multiply mated *D. melanogaster* males do not, or cannot, adjust their intake of protein and carbohydrate diets compared with unmated or singly mated males. Surprisingly, this lack of dietary response occurs even despite significant ejaculate depletion. This study highlights a gap in knowledge regarding male nutrient homeostasis in response to sexual experience, in contrast to the data available on females. Given the importance of the relationship between the P:C ratio and mating in many insects, it would be useful to investigate the role of multiple mating in diet choice in additional invertebrate models. Males of many insect species adjust their ejaculate investment in response to the sexual environment (Gage and Barnard, 1996; Gage, 1991; Wigby et al., 2009). For example, males of the West Indian fruit fly, *Anastrepha obliqua*, are able to partition their sperm reserves when transferring ejaculate to multiple females and reserve sperm for possible future matings (Perez-Staples and Aluja, 2006). However, our data suggest that males of *D. melanogaster* don't support this shift in ejaculate allocation by responding to any nutrient debt it entails. In addition, since five-times-mated male *D. melanogaster* might still have motile sperm present in their vesicles (Gillott, 2003; Lefevre and Jonsson, 1962), perhaps *D. melanogaster* do not perceive a protein requirement until sperm reserves have been fully depleted. Overall, the results suggest that males

remain on a fixed feeding trajectory even when mating close to their daily functional maxima, and do not increase nutrient intake to recoup their reduced ability to transfer ejaculates to females.

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675 # APPENDIX

676 Table A1

677 Recipe for essential amino acid and non-essential amino acid stock solutions (Camus et al., 2018).

| Essential amino acid stock | |
|---|---|
| | (g/200 ml) |
| F (L-phenylalanine) | 3.03 |
| H (L-histidine | 2.24 |
| K (L-lysine) | 5.74 |
| M (L-methionine) | 1.12 |
| R (L-arginine) | 4.70 |
| T (L-threonine) | 4.28 |
| V (L-valine) | 4.42 |
| W (L-tryptophan) | 1.45 |
| Non-essential amino acid stock | |
| | (g/200 ml) |
| A (L-alanine) | 5.25 |
| D (L-aspartate) | 2.78 |
| G (glycine) | 3.58 |
| N (L-asparagine) | 2.78 |
| P (L-proline) | 1.86 |
| Q (L-glutamine) | 6.02 |
| S (L-serine) | 2.51 |

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Table A2

Recipe to make 200ml stocks of protein and carbohydrate diet solutions for *Drosophila* (Piper *et al.*, 2014; Camus *et al.*, 2017, 2018).

| Protein diet | | | Carbohydrate diet | |
|---|---|---|---|---|
| L-ile | Powder | 348mg | | |
| L-leu | Powder | 492mg | Sucrose | 6.5g |
| L-tyr | Powder | 252mg | | |
| All following components are identical for both diets | | | | |
| cholesterol | | 20mg/ml in EtOH | | 3ml |
| CaCl2 | | 1000x | | 200µl |
| MgSO4 | | 1000x | | 200µl |
| CuSO4 | | 1000x | | 200µl |
| FeSO4 | | 1000x | | 200µl |
| MnCl2 | | 1000x | | 200µl |
| ZnSO4 | | 1000x | | 200µl |
| H2O | | | | Up to 50ml |
| Autoclave resulting 50ml solutions at this stage | | | | |
| buffer | | 10x acetate buffer base | | 20ml |
| Nucleic acid/lipid solution | | 125x stock | | 1.6ml |
| Essential amino acid solution | | Stock (table S1) | | 18.154ml |
| Non-essential amino acid solution | | Stock (table S1) | | 18.154ml |
| Na glutamate solution | | 100mg/ml | | 5.464ml |
| Cys solution | | 50mg/ml | | 1.584ml |
| Vitamin mix | | 47.6x stock | | 4.2 ml |
| Folic acid | | 1000x stock | | 200µl |
| Propionic acid | | | | 1.2ml |
| Nipagin | | 110g/l stock in 95% EtOH | | 3ml |
| Make each to total volumes of 200ml with H2O and syringe filter into tubes for storage | | | | |
| Gently warm the protein diet to aid dissolution | | | | |
| Add 20% yeast solution to protein diet (at 20% concentration) before use | | | | |

Sucrose in carbohydrate diet is replaced 1:1 by amino acids in the protein diet. Ingredients for the vitamin mix can be found in Piper *et al.*, (2014).

692 Table A3

693 Statistical models used to analyse data in R version 4.0.4 (The R Foundation for Statistical Computing, Vienna,

694 Austria, http://www.r-project.org).

| Data | Model |
|---|---|
| | Intake data |
| Angle | glm(alpha ~ block, data=tidy.alpha) |
| | glmmTMB(resid ~ treatment*day + (1 \| id), data=tidy.alpha2) |
| Day 1 angle | glm(alpha ~ block, data=alpha1) |
| | glmmTMB(resid ~ treatment + (1\|id), data=alpha1) |
| Length | glm(distance ~ block, data=tidy.distances) |
| | lmer(resid ~ treatment*day + (1 \| id), tidy.distances2) |
| Day 1 length | glm(distance ~ block, distances1) |
| | glmmTMB(resid ~ treatment + (1\|id), data=distances1) |
| Raw protein | glm(P ~ block, data=tidyCP, family = Gamma()) |
| | glmmTMB(resid ~ treatment*day + (1 \| id), data=tidyCPP) |
| Raw carbohydrate | glm(C ~ block, data=tidyCP) |
| | glmmTMB(resid ~ treatment*day + (1 \| id), data=tidyCPC) |
| MANOVA | manova(cbind(alp, dis) ~ treatment*day, data = tidy.alphdis2) |
| | Offspring data |
| Male output | glm(totaloffspring ~ block, data=offspring2) |
| | glm(resid ~ male, data=offspring2) |
| Offspring slopes | c1 %>%group_by(id.b) %>% summarize(slope = coef(lm(adultperday ~ vial))[[2]], .groups = "drop") |
| | glm(slope ~ block, data=coefs) |
| | glmmTMB(resid ~ mate + ( 1 \| male), data=coefs) |
| | Mating data |
| Latency, survival | coxph(Surv(latency, lat.censor) ~ matenumber + block, data = Lmating.times) |
| | survfit(Surv(latency, lat.censor) ~ matenumber, data = Lmating.times) |
| Latency repeatability | rptGaussian(latency ~ matenumber + (1 \| id), grname = c("id", "Fixed"), data = REPmating.times, nboot = 1000, npermut = 0, adjusted = FALSE) |
| Duration | glm(duration ~ block, data=duration.times.2, family = poisson()) |
| | glmmTMB(resid ~ matenumber + (1 \| id), data=duration.times.2) |
| Duration repeatability | rptGaussian(duration ~ matenumber + (1 \| id), grname = c("id", "Fixed"), data = REPmating.times, nboot = 1000, npermut = 0, adjusted = FALSE) |
| Offspring x mating | glmmTMB(totaloffspring ~ scaledduration + scaledlatency + matenumber + (1\|id.b)) |

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Figure 1

The quantity (a) and composition (b) of protein and carbohydrate eaten over 24h periods for 5 days by experimental males that had mated 0, 1 or 5 times. (a) Combined consumption of protein and carbohydrate is represented by the length of vector, calculated as the distance of an intake value from the origin in P:C nutrient space (see Appendix, Figure A2). (b) Relative composition of protein to carbohydrate eaten (μl), represented by the angle between the length of vector and the protein axis to give a value between 0 – 90°. Angles over 45° indicate that more carbohydrate than protein was consumed, whereas angles under 45° indicate that more protein than carbohydrate was consumed. Boxes represent interquartile range (IQR), with medians as thick horizontal lines and means as large, filled circles. Whiskers represent 1.5 x IQR and small circles represent outliers.

Figure 2

Total numbers of adult offspring sired by males when mated once, three or five times sequentially. Data shown are from experimental blocks one and two where an additional three sequential matings treatment was included. Boxes represent interquartile range (IQR), with medians as thick horizontal lines and means as large, filled circles. Whiskers represent 1.5 x IQR and small circles are raw data points overlaid on their respective box. Letters above boxes denote significant differences among treatment groups as determined by post-hoc Tukey tests ($P$<0.001).

Figure 3

Total numbers of adult offspring produced over 24h by individual females that were a male's first, second, third, fourth or fifth mate ($N$=387). All females were initially virgins. Offspring were counted from vials in which females had been allowed to lay eggs for three to five days for a total of 21 days. These periods were normalised to 24h by dividing values by the number of days the female laid eggs in that vial. Shown in the figure is the total dataset for all females (including those that did and did not have data for all seven vials; data analysis was conducted on the dataset that included all seven vials only). Boxes represent interquartile range, with medians as thick horizontal lines and means as large, filled circles. Whiskers represent 1.5 x interquartile range and small circles are outliers. Data shown are from blocks one and two where a three times mated treatment was included.

Figure 4

Kaplan-Meier survival curves illustrating the proportion of experimental males initiating their first, second, third, fourth or fifth matings. Solid lines (with surrounding confidence intervals in matching colourways) represent each of the one to five sequential mating treatments, in which males were presented with a new virgin female. Data shown are taken from all four blocks.

Figure 5

Mating duration of individual males when mating for the first, second, third, fourth or fifth time with a virgin female in a single day. Boxes represent interquartile range (IQR), with median shown as the thick horizontal line and mean shown as the large, filled circle. Whiskers represent 1.5 x IQR and jittered raw data are overlaid on each box. Data shown are taken from all blocks.

Figure A1

Experimental set up of male mating treatments and variables collected across the four experimental blocks. Males were assigned at random to treatments of no mating, one, three or five sequential matings, each with a new virgin female, as shown. After the mating treatments, the dietary preferences of males in blocks three and four were tested using the CAFE assay. Mated females in blocks one and two were retained and offspring counted.

Figure A2

Graphical representation of the trigonometric conversion used to calculate angle and length of vector from raw feeding data. Protein and carbohydrate intake is represented by the large black circle. The length of vector for each intake is calculated using distance from the origin (0,0). Points further from the origin signify greater total consumption of macronutrients. The angle between the vector and the x-axis represents composition. Angle values less than 45° denote a greater proportion of protein (the blue space) and values greater than 45° denote a greater proportion of carbohydrate (the green space) eaten.

Figure A3

Mean (±SE) intake (µl) of (a) protein and (b) carbohydrate eaten over 24h periods by experimental males mated 0, 1 or 5 times. Experimental males were kept in vials in groups of three and presented with a choice of protein and carbohydrate synthetic liquid diets. Black vertical lines denote standard error around the mean (large, filled circles). (a) Raw protein intake was not significantly affected by treatment, day, or the interaction between treatment and day (treatment: $\chi^2_2$= 4.93, $P$=0.08; day: $\chi^2_4$= 6.25, $P$=0.18; treatment*day: $\chi^2_8$= 8.22, $P$=0.41). Treatment was not significant on day 1 only ($\chi^2_2$= 4.17, $P$=0.12). (b) Raw carbohydrate intake was not significantly affected by treatment or the interaction between treatment and day (treatment: $\chi^2_2$= 1.6, $P$=0.45; treatment*day: $\chi^2_8$=6.42, $P$=0.60). There was a significant effect of day ($\chi^2_4$=11.78, $P$<0.05). Treatment was not significant on day 1 only ($\chi^2_2$= 1.35, $P$=0.51).

Figure A4

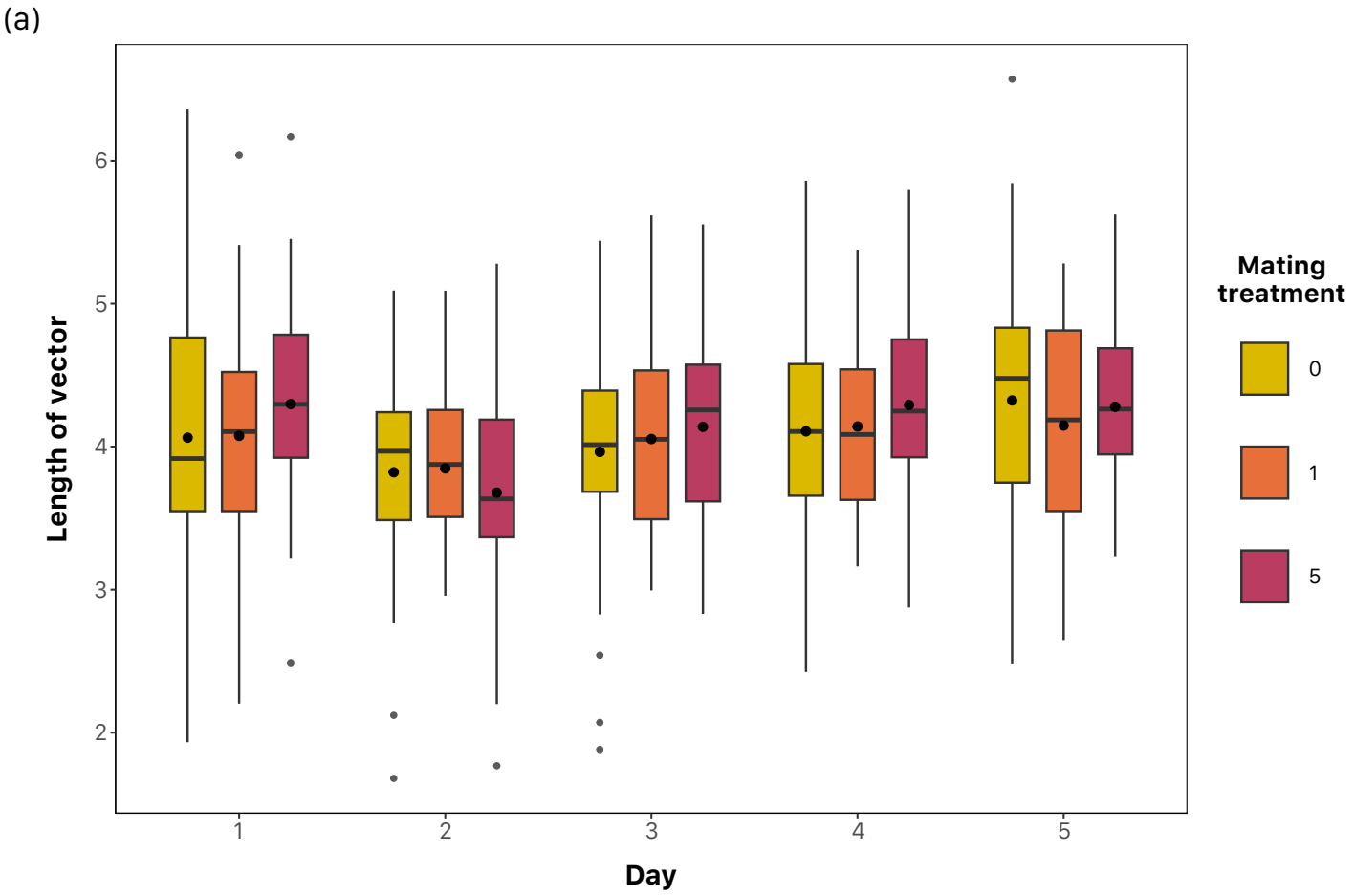
Relationship between mating traits and the offspring produced from a single mating. Data shows the offspring produced from a single mating between a male and female fly, where females were mated as virgins and were the first, second, third, fourth or fifth female to mate with a male partner, against the (a) latency to mate and (b) duration of each mating. Raw data points are shown as small circles and linear regression lines are overlaid in grey with coloured confidence intervals. Statistical analysis was carried out with transformed latency and duration data centred around a mean of 0. Preliminary modelling showed an insignificant effect of block, which was subsequently removed from the model. There was no significant interaction between duration, latency and mate number when included as a three-way interaction in a generalised linear mixed model (duration*latency*mate number; $P$=0.41). Model testing showed a reduced model to have the best fit, with mate number having a significant effect on offspring production ($\chi^2_4$=281.77, $P$<0.001), but not duration ($P$=0.07) or latency ($P$=0.96).

Figure A5

The number of virgin females presented to an experimental male before a successful first, second, third, fourth or fifth mating for that male took place. New, additional virgin females were added to mating arenas containing a single male when latency to mate with the previous female was >60 minutes. The “did not mate” category represents males that refused to mate within the period of the mating assay.

Figure 1





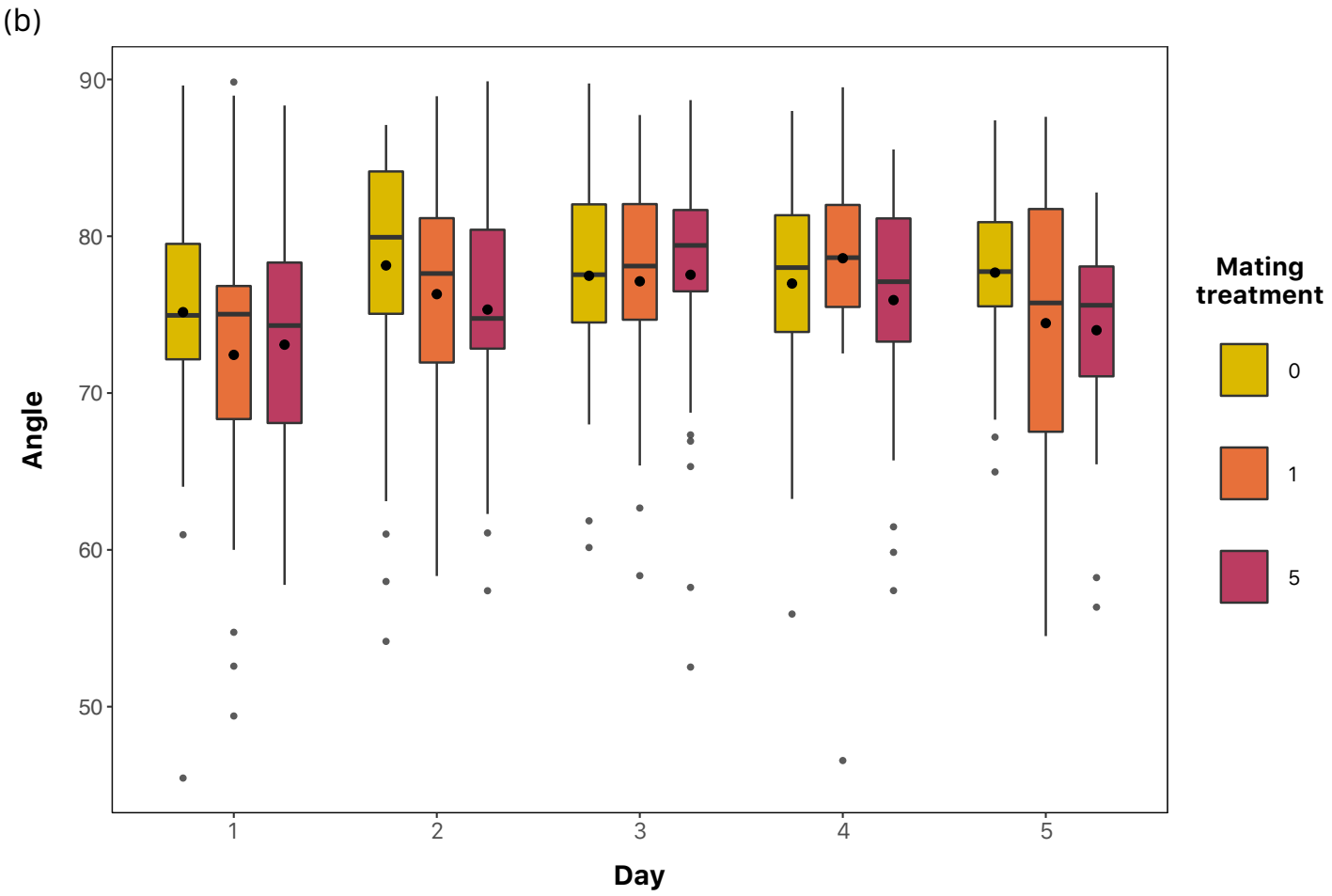



Figure 2

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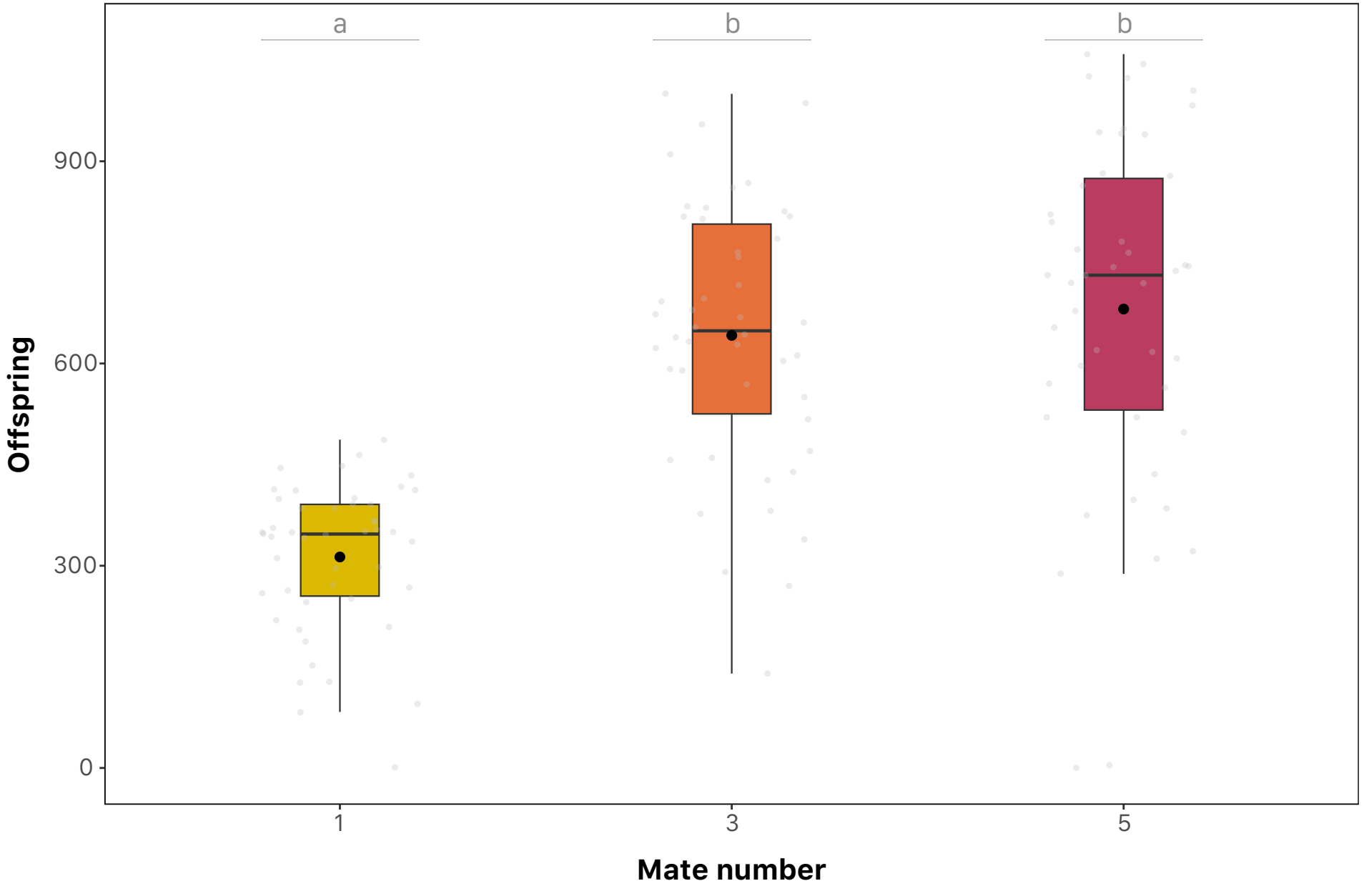

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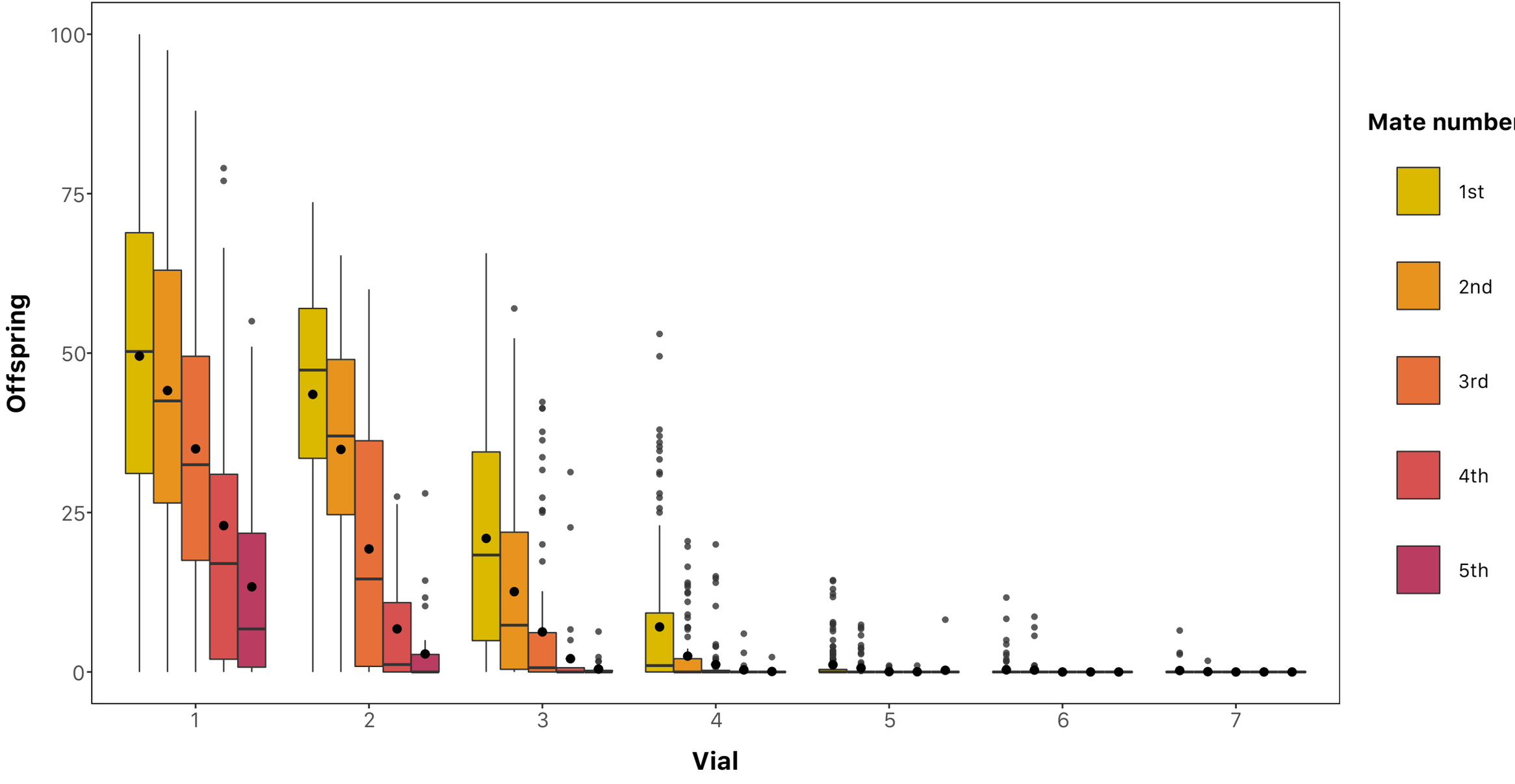

Figure 4

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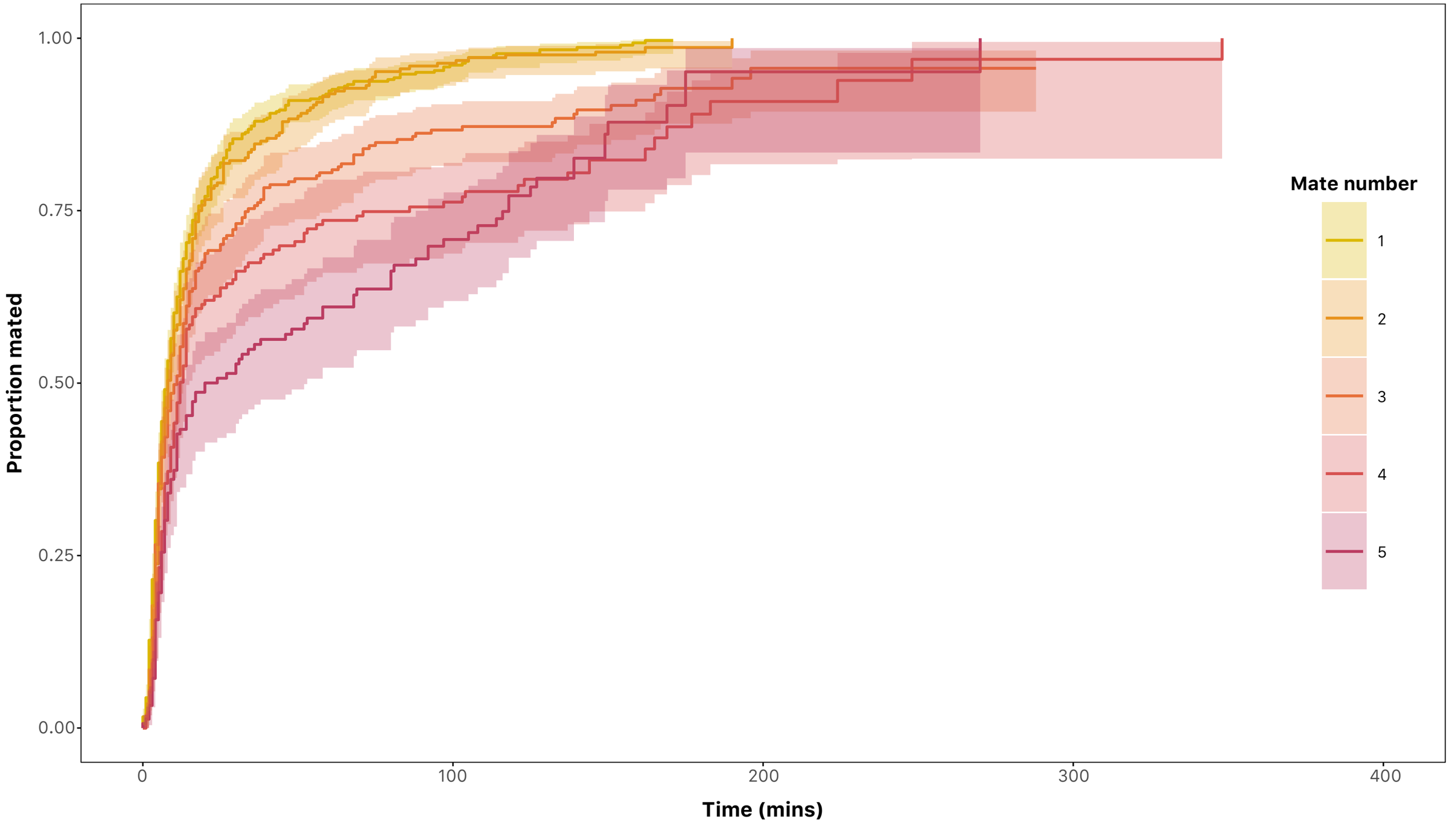

Figure 5

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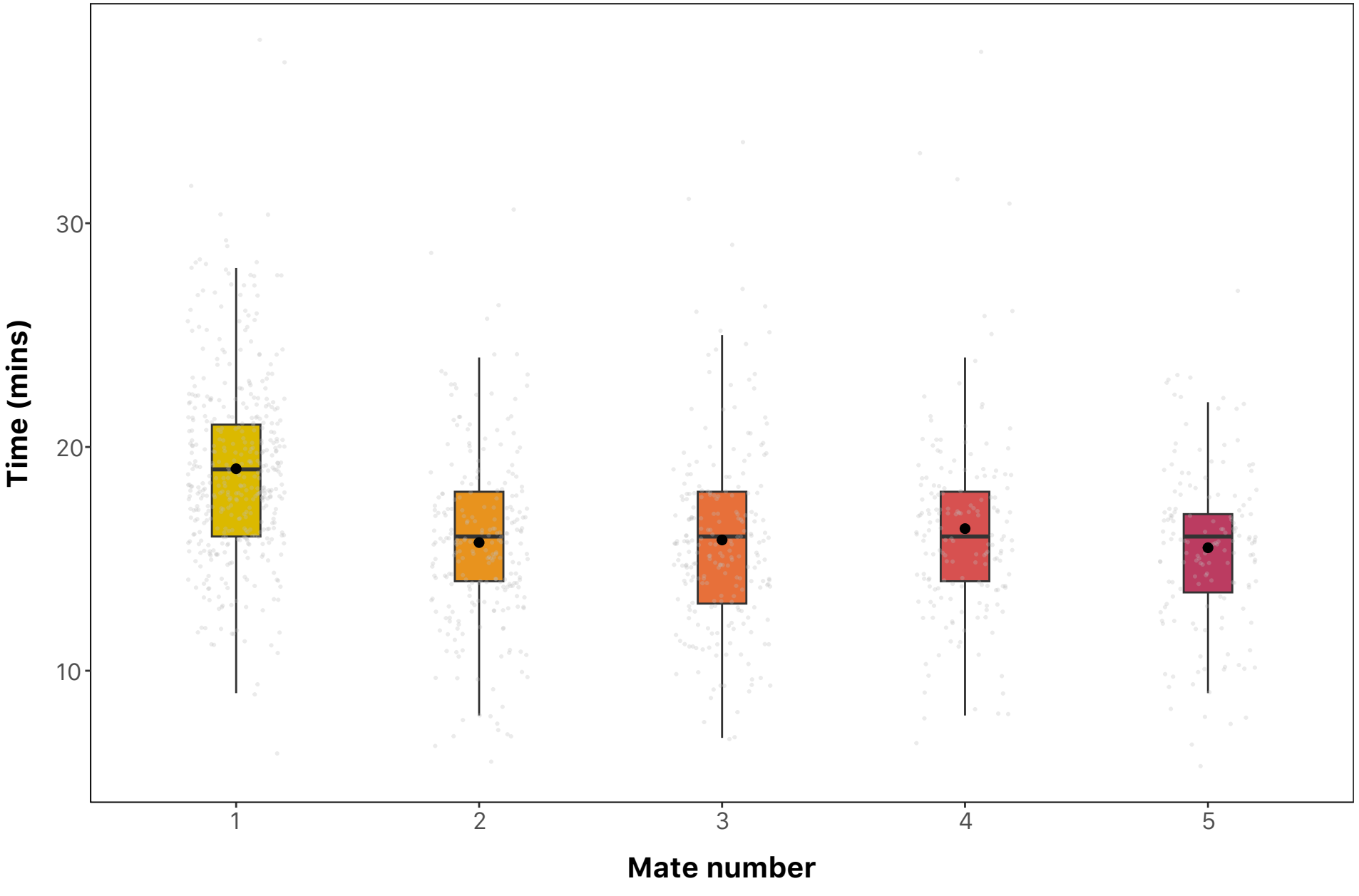

Figure A1



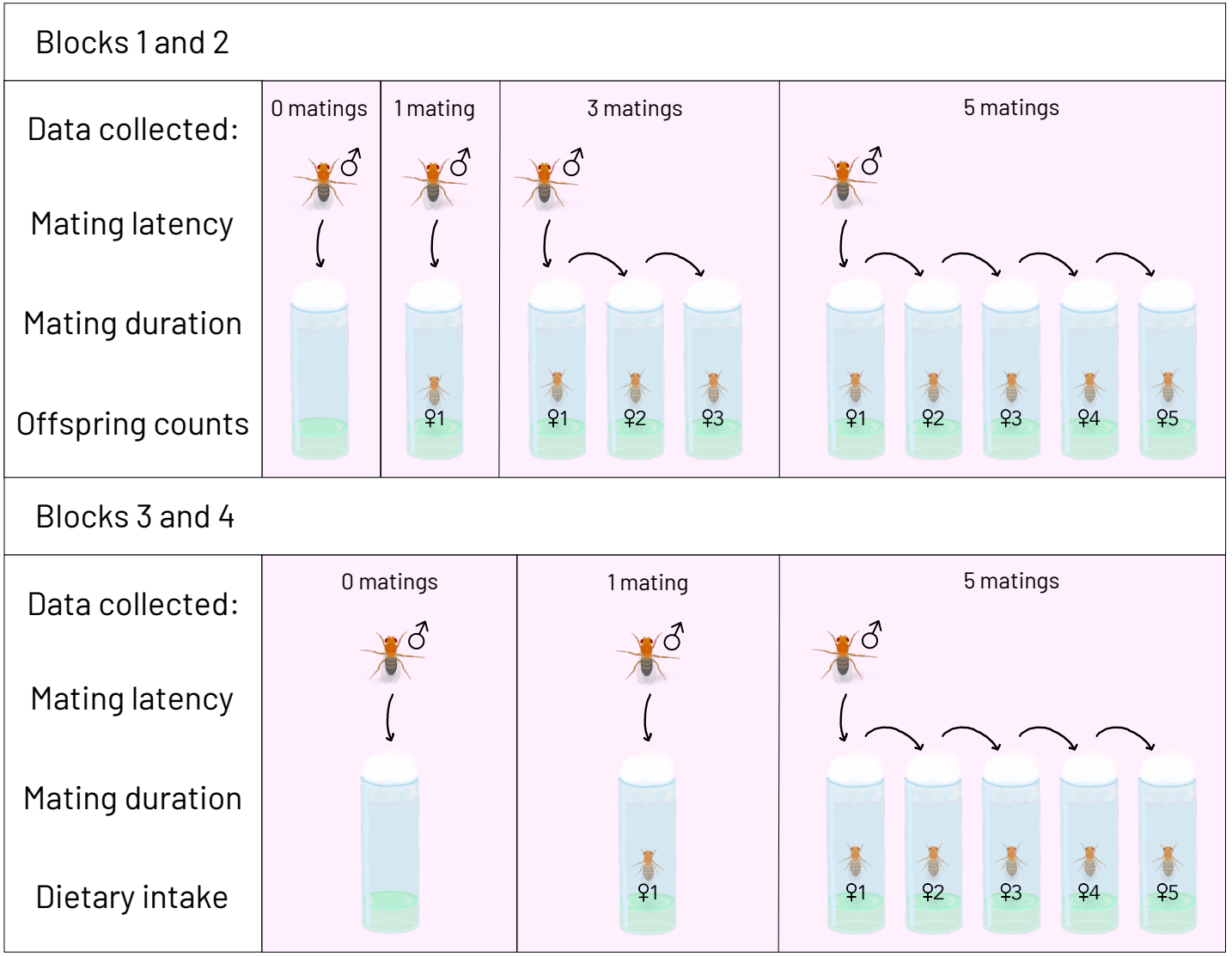

Figure A2

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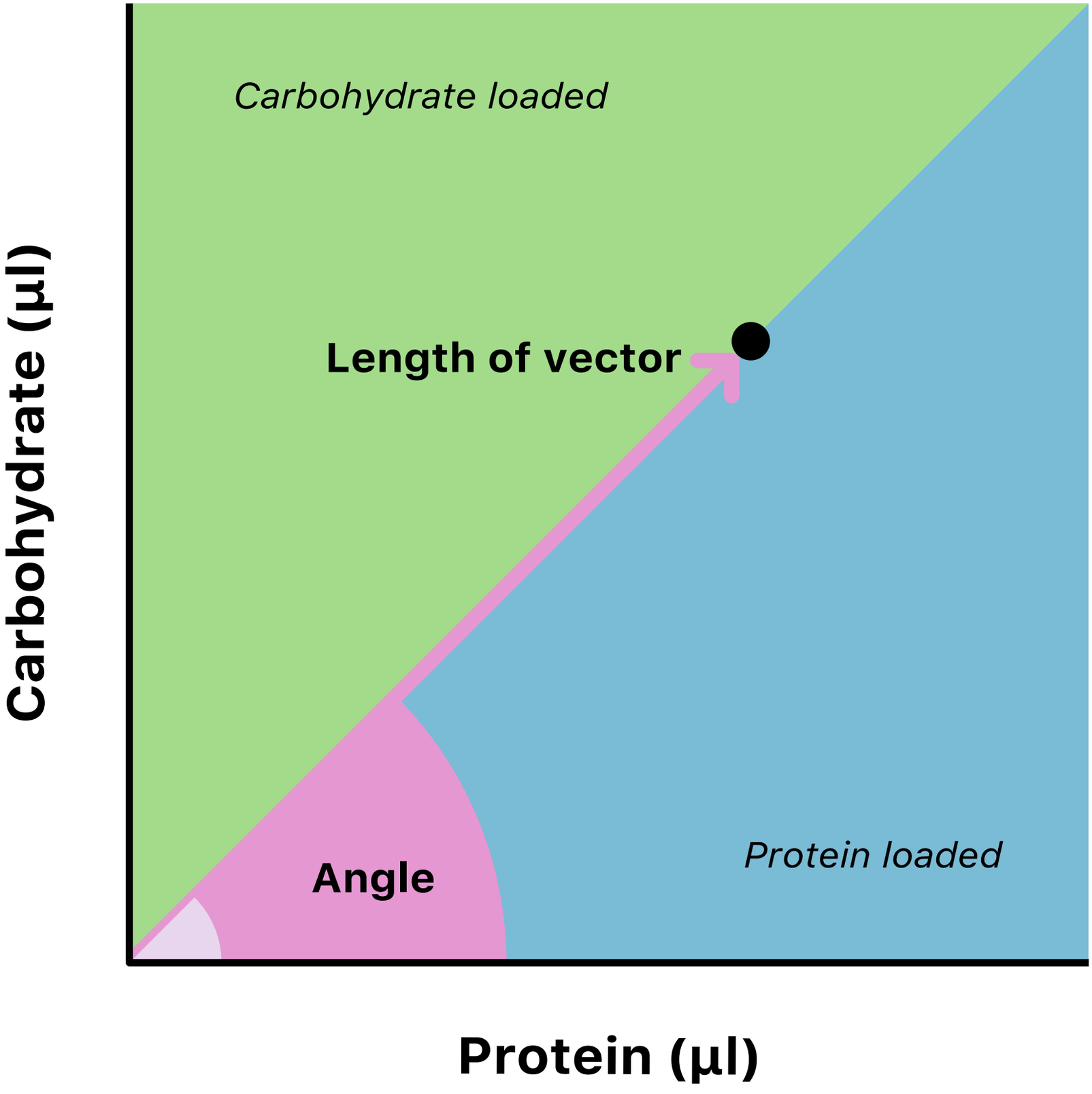

# Figure A3

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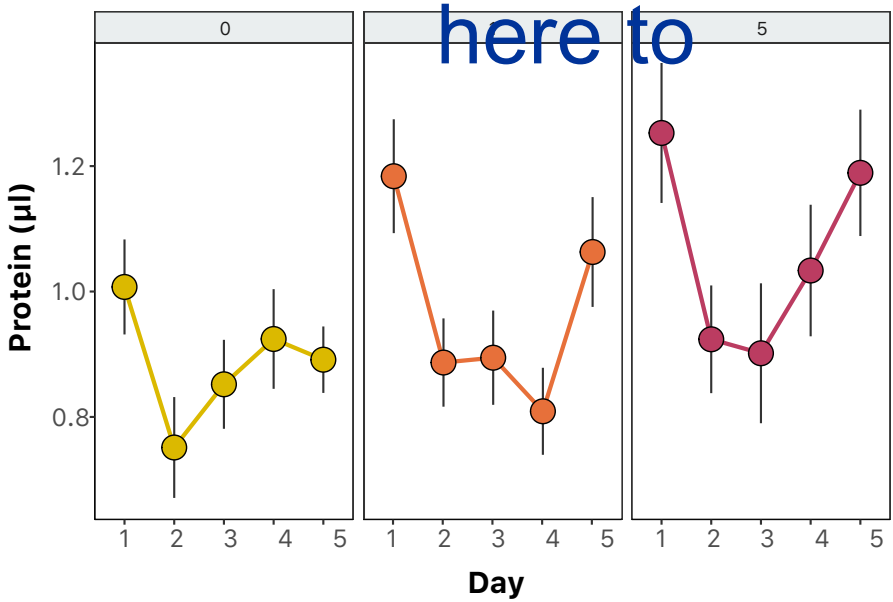


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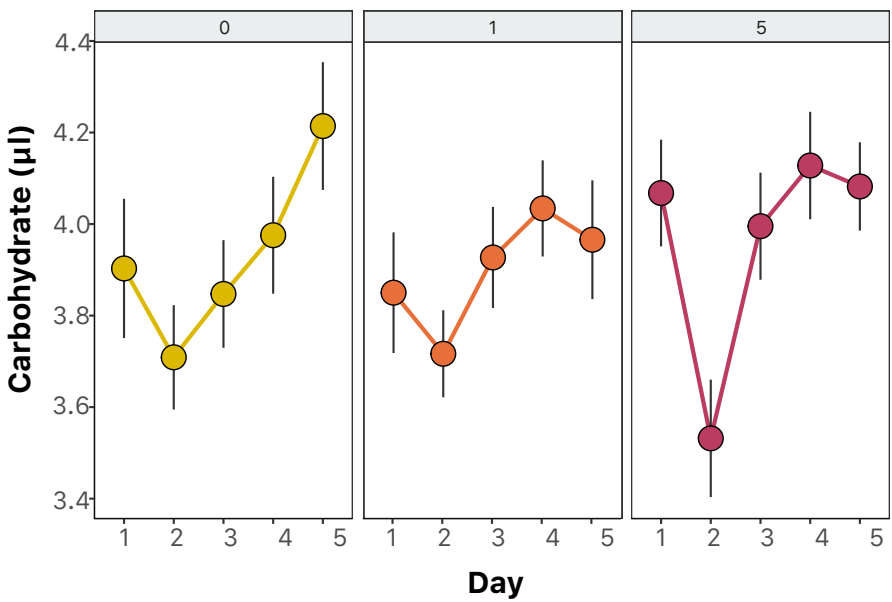

Figure A4

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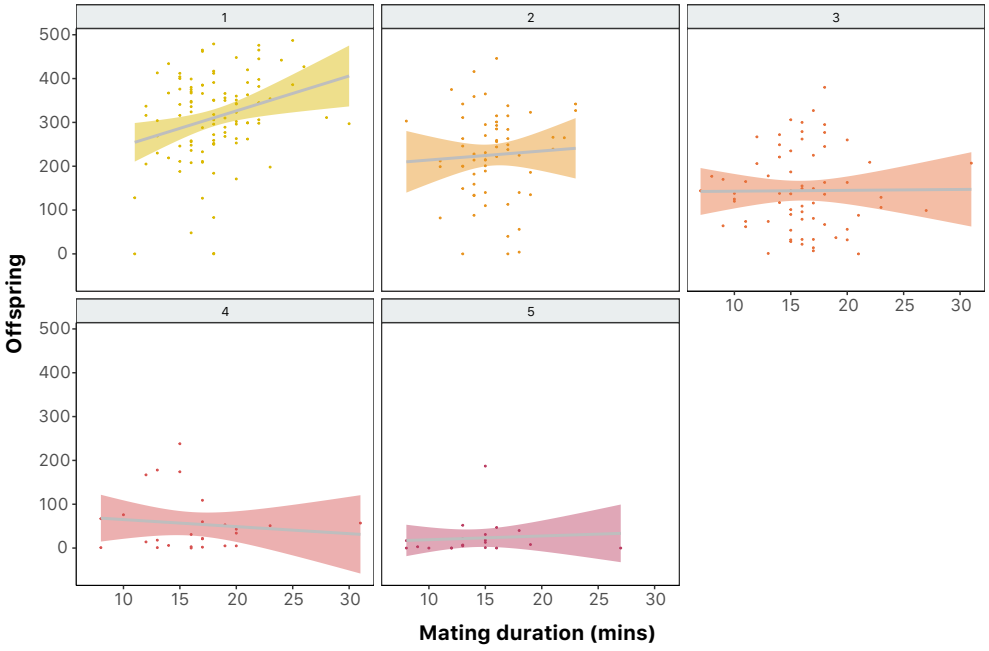
(a)

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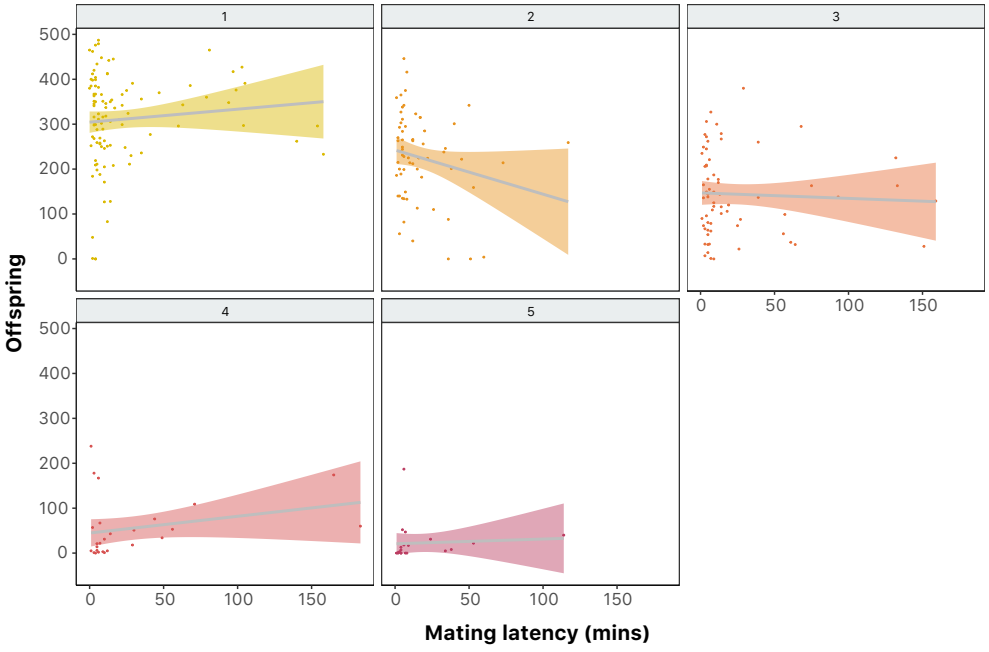

Figure A5

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