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# Adult sex ratios affect mating behaviour in the common housefly *Musca domestica* L. (Diptera; Muscidae)

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## Abstract

Adult sex ratio determines the level of mate availability and intrasexual competition for each sex. Sex ratio biases have been proposed to enhance the productivity of animal rearing procedures. However, behaviour may change in response to sex ratio manipulations that may counteract potential benefits. We investigated how sex ratios affected mating behaviour of the housefly *Musca domestica*, a species used in the animal feed industry. We hypothesized a reduced courtship effort and mating latency and increased ejaculate allocation (copulation duration) under male-biased sex ratios, whereas female-biased sex ratios would lead to the opposite effects. However, courtship effort was reduced in female-biased groups, implying reduced male harassment. Mating latency was lower and copulation lasted longer in female-biased groups, which may reduce reproduction time and increase female fecundity and lifespan. Our results indicate that in houseflies, female-biased sex ratios cause behavioural changes in both sexes that could positively contribute to reproductive output.

## Keywords

courtship behaviour, mating latency, sex ratio bias, insect breeding, sexual behaviour, male–male competition, female mating behaviour.

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## 1. Introduction

Sexual selection is a major factor in the evolution of mating systems and in shaping mating behaviour (Shuster, 2009). Males produce smaller gametes than females (Parker et al., 1972; Parker & Pizzari, 2010) and therefore invest less in individual offspring in terms of allocating resources to each gamete (Arnqvist & Nilsson, 2000). This could allow males to have considerably more offspring than females, provided that both sexes dedicate the same amount of energy to gamete production (Bateman, 1948). Consequently, males and females can evolve different reproductive strategies, where males typically compete with each other for access to females and females usually display mate choice (Lindsay et al., 2019). Both sexes can also invest in other factors related to offspring production. Males typically invest into securing mating and fertilization (Arnqvist & Nilsson, 2000) via mate searching, competition for mates and courtship performance (Leftwich et al., 2012), as well as producing large quantities of sperm in response to the risk of sperm competition, which reduces the likelihood of successful fertilization (Parker & Pizzari, 2010). Females invest in offspring production and parental care (Arnqvist & Nilsson, 2000) and female choice is costly through delayed commencement of reproduction, and the risk of remaining unmated (Andersson & Simmons, 2006; Lindsay et al., 2019; Kohlmeier et al., 2021). Investment in specific aspects of reproduction is usually plastic and their payoff in terms of costs and benefits depends on conditions such as the social environment, level of competition and mate availability (Billeter et al., 2012; Leftwich et al., 2012; Dore et al., 2020).

The plasticity of reproductive behaviour, and the influence of controllable factors thereon, provides both opportunities and threats in the context of applications in which reproductive capacity is of major importance. Examples of this include the mass rearing of insects, e.g. for feed production and as a more sustainable alternative to traditional livestock (van Huis & Ooninx, 2017), or for biological control and sterile insect technique (SIT) programmes (Sørensen et al., 2012). One controllable factor that influences reproductive behaviour is manipulation of the sex ratio, as biased sex ratios alter the level of intrasexual competition and availability of mates. Sex ratio manipulation could be used to select for desirable reproductive behaviour that contributes to optimization of mass rearing of insects (Fitz-Earle & Barclay, 1989; Desa et al., 2018; González-López et al., 2019; Gou et al., 2019),

and it is therefore important to study how population sex ratios affect mating behaviour.

Different sex ratio types have been defined, including the primary sex ratio (number of male and female offspring; Székely et al., 2014), adult sex ratio (number of sexually mature males and females; Székely et al., 2014) and operational sex ratio (ratio of males and females available for mating/reproduction; Emlen & Oring, 1977). Although the adult and operational sex ratio can be similar and are sometimes used interchangeably, it is important to distinguish between the two (Kokko & Jennions, 2008). The adult sex ratio determines the initial operational sex ratio but is not dependent on reproductive behaviour, whereas the operational sex ratio is altered whenever an individual's mating status changes, which can cause the adult and operational sex ratios to diverge over time. Adult and operational sex ratios are therefore not necessarily closely linked and can depict different temporal patterns (Carmona-Isunza et al., 2017) and are predicted to differently affect the evolution of sex roles and mating behaviour (Kokko & Jennions, 2008; Székely et al., 2014; Jennions & Fromhage, 2017). As such it may be important to consider which sex ratio to manipulate.

Sex ratio biases may have different effects on female and male behaviour depending on the sex that is overrepresented. Under male-biased sex ratios, males experience higher levels of intrasexual competition, and may therefore invest in pre-copulatory traits that enhance their mating success (Leftwich et al., 2012). Males increased courtship performance in the presence of other males in the fruit fly *Ceratitis capitata* and housefly *Musca domestica* (Carrillo et al., 2012; Leftwich et al., 2012). The presence of males prior to copulation can also decrease the mating latency (Bretman et al., 2009; Abraham et al., 2015; Dore et al., 2020), possibly speeding up reproduction time. Moreover, female remating exposes both sexes to post-copulatory sexual selection, e.g. through sperm competition and (cryptic) female choice (Birkhead & Pizzari, 2002). Males can increase sperm and seminal fluid protein production (Bretman et al., 2009; Fedorka et al., 2011) and allocation (Wigby et al., 2009; Garbaczewska et al., 2013; Abraham et al., 2015) under increased risk and intensity of sperm competition, which may influence female fecundity. For example, female houseflies *M. domestica* had larger first clutch size and higher offspring survival under male-biased sex ratios, presumably resulting from increased accessory gland protein transmission to females by males

(Carrillo et al., 2012). Male-biased sex ratios could therefore positively affect productivity, depending on the species.

Female-biased sex ratios are however more commonly expected to enhance reproductive output compared to equal or male-biased sex ratios (Fitz-Earle & Barclay, 1989). This is because only females lay eggs and owing to their vastly larger output in terms of gametes, one male ought to suffice to inseminate many females. Resources can then be used more efficiently as fewer resources are expended on maintaining a surplus of males, effectively bypassing the twofold cost of sex (Maynard Smith, 1971, 1978). Indeed, female fecundity was increased in female-biased sex ratios in the Tsetse flies *Glossina fuscipes fuscipes* and *Glossina pallidipes* (Desa et al., 2018) and the Mexican fruit fly *Anastrepha ludens* (González-López et al., 2019). Moreover, female *Drosophila melanogaster* initiate oviposition sooner when in a group than when alone, and sooner in higher densities (Bailly et al., 2021), which may increase production in the short term but lower it in the long term. However, female-biased sex ratios may only be profitable to a certain extent, as large biases can lead to a shortage of males and unmated females (Desa et al., 2018). Moreover, males may alter their reproductive behaviour as well, which may confound the expected effect of a female-biased sex ratio on productivity. Given that both male- and female-biased sex ratios can alter reproductive behaviour in a manner that may benefit the reproductive output, it is important to study how exactly biased sex ratios influence the mating behaviour in the species of interest.

We investigated the effect of both male- and female-biased adult sex ratios on mating behaviour in the common housefly *M. domestica*. Houseflies are increasingly used for feed production, are easily reared, and the ability of its larvae to grow on waste products makes them a useful resource in establishing a more circular economy (Francuski et al., 2020). Female egg production is a limiting factor in rearing efficiency of houseflies (Pastor et al., 2015). If reproductive behaviour is influenced by the adult sex ratio, biasing the sex ratio could improve the efficiency of rearing, e.g. through shorter courtship display or reduced mating latencies. Moreover, male seminal fluid proteins are associated with increased female fecundity and lifespan (Arnqvist & Andrés, 2006). Adult sex ratios may vary in natural housefly populations (Feldman-Muhsam, 1944) and interpopulation crosses can cause strong sex ratio biases as populations differ in the number and composition of male and

female sex determiners (Feldmeyer et al., 2008; Kozielska et al., 2008; Pastor et al., 2014; Hamm et al., 2015; Li et al., 2022). Houseflies also display protandry, the faster development and earlier emergence of males compared to females (L. Francuski, data not shown). Houseflies may therefore experience biased sex ratios spatially and temporally (Carrillo et al., 2012) and be able to adjust their mating behaviour accordingly.

Here, we test the effects of adult sex ratio bias on the courtship and mating behaviour of houseflies. Male-biased sex ratios are generally expected to lead to increased male intra-sexual competition and therefore increased courtship effort, for example in the butterfly *Bicyclus anynana* (Holveck et al., 2015) and med fly *Ceratitis capitata* (Leftwich et al., 2012). However, male-biased sex ratios can also lead to a lower courtship rate through increased courtship interruptions or lower female encounters (Weir et al., 2011). Male houseflies spent less effort on courtship performance in high compared to low density groups (Hicks, 2004). Moreover male *D. melanogaster* evolving under male-biased sex ratios adapted reduced and different courtship effort (Dore et al., 2020). Therefore, we first tested the hypothesis that males will reduce courtship effort in male-biased groups but increase their effort in female-biased groups. Females depicted reduced mating latencies in male-biased groups in the fruit fly *Anastrepha fraterculus* (Abraham et al., 2015) and *D. melanogaster* (Bretman et al., 2009), but *D. melanogaster* females evolving in female-biased sex ratios exhibited the opposite in another study (Dore et al., 2020). Second, we hypothesized that houseflies will exhibit lower mating latencies in male-biased sex ratios, and increased mating latencies in female-biased sex ratios.

Housefly matings last about an hour, during which sperm is transferred in the first approx. 15 min, whereafter only seminal fluid proteins are transferred (Riemann et al., 1967; Leopold et al., 1971a). Male seminal fluid proteins generally inhibit female remating at least until oviposition, and the inhibitory effect is dependent on the quantity of seminal fluids transferred, as indicated by increased female remating frequencies when copulations are interrupted. (Riemann et al., 1967). Seminal fluid proteins are costly to produce (Chapman, 2008) and males are depleted of their inhibitory seminal fluid proteins after multiple (3–4) matings (Riemann et al., 1967; Leopold et al., 1971a). Therefore, males are hypothesized to allocate seminal fluid proteins prudently to mitigate the costs of sperm competition. Using copulation duration as a proxy for resource allocation we hypothesized males to increase

the copulation duration in male-biased groups to avoid sperm competition and decrease allocation in female-biased groups where sperm competition risk is low. Copulation duration is positively correlated with increased body size difference between sexes in houseflies (Baldwin & Bryant, 1981). Body size is generally correlated with fitness in insects (Beukeboom, 2018). Considering that male houseflies prefer larger females (Shin et al., 2003), we hypothesized that copulations last longer between mating partners with increased body size differences as males may invest more ejaculate. As males are expected to alter mating behaviour such as courtship performance and ejaculate investment in response to changes in sex ratio, female productivity is likely also influenced under mass-rearing conditions.

## **2. Material and methods**

### *2.1. Strain collection and housing culturing*

The laboratory strain SPA4-2020 (collected in 2020 in Barcelona, Spain, 41°30'19.2"N, 2°05'46.8"E) was used in all mating experiments. Adult flies had been kept in plastic cages (3250 ml; Semadeni; Ostermundigen, Switzerland) in a climate room for 11 generations before use in experiments. Flies were kept at 25°C and a L12:D12 cycle, also during experiments. Adult flies were provided with water, sucrose solution (20%, w/v) and milk powder. After 4–6 days, egg substrate (wet food) was provided in 2 cups (35 ml each). Wet food was prepared by mixing 200 g dry food (1000 g wheat bran, 150 g flour, 120 g milk powder, 50 g inactivated yeast), 4 ml nipagin solution (10 g nipagin powder/100 ml ethanol 96%; Spruijt-Hillen; IJsselstein, The Netherlands), and 250 ml tap water in a beaker. After 3–5 days, egg-laying cups were removed from the cages (and if necessary, new cups were provided), the contents of the egg-laying cups were emptied into beakers (750 ml) and mixed with additional wet food. Beakers were closed off using lids with nylon meshes. After 7–10 days, emerging (virgin) flies were anaesthetized using CO<sub>2</sub> (g), separated by sex and transferred to plastic cups (280 ml) with milk powder and 1 vial of sucrose solution (20%). Flies were kept in groups of 10 to prevent any potential differentiation in sperm/seminal fluid protein synthesis in males due to variation in perceived competition levels. Dead flies were removed and replaced using an aspirator to maintain equal group sizes. Flies used in the experiment were from the 11th to the 14th generation after initial collection.

## 2.2. *Experimental setup*

To study the effect of the adult sex ratio on mating behaviour, males and females were grouped in different adult sex ratios (male:female): M5:F1, M3:F1, M1:F1, M1:F3, M1:F5 and M3:F3. Adult sex ratio is the relative number of sexually mature males and females in a group. The M1:F1 and M3:F3 equal sex ratios served as control for the biased sex ratios, as well as for an effect of density. Flies were 4–8 days old when used in experiments. They were transferred from the storage cups to observation arenas with an aspirator. Each observation arena consisted of a 100 mm Petri dish. An observation area with a particular sex ratio was considered a replicate. A hole (approx.  $1 \times 0.5$  cm) was created in the side of Petri dish using a soldering iron to allow for the addition of flies via an aspirator. Afterwards, tape was added to cover the hole to prevent fly escape. A 35 mm Petri dish with egg-laying substrate was attached to the centre of the observation arena with tape. Finally, a cotton plug dosed in sugar water (20%, w/v) was added to the side of the arena. Flies were handled briefly and with care to minimize handling stress. The only individual of the sex present in the arena of each sex ratio was added last to minimize unrecorded interaction between sexes, but for the M1:F1 and M3:F3 control males were added first, then females. Two replicates of each sex ratio (i.e., 12 observation arenas) were assessed per day. If one or more flies died during recording the replicate was excluded from data analysis.

Flies were colour coded under light CO<sub>2</sub> anaesthesia at least 72 h prior to mating assays to minimize any potential influence of anaesthesia on mating behaviour. Where possible, dyes were applied simultaneously with sex separation to avoid repeatedly anaesthetizing flies. Dyes consisted of acrylic model paint and were applied with a paint brush to the fly's notum. Flies were dyed with white and green or red acrylic paint for identification under red light.

Observation arenas were placed on a white background in an incubator, resulting in a set-up of 12 arenas per rack. Two cameras (c920 HD pro webcam, Logitech, Lausanne, Switzerland) were placed above the arenas. Flies were recorded using the recording software 'Security Monitor Pro' (Deskshare, Plainview, NY, USA). As flies were kept at a L12:D12, and have low sensitivity for red light (Goldsmith, 1965), a red LED light strip of 630 nm (Buyleystrip; Geldrop, The Netherlands) was attached to the roof of the incubator to allow for continued observation during the dark phase. Flies were given time to accommodate to the new environment; hence, the



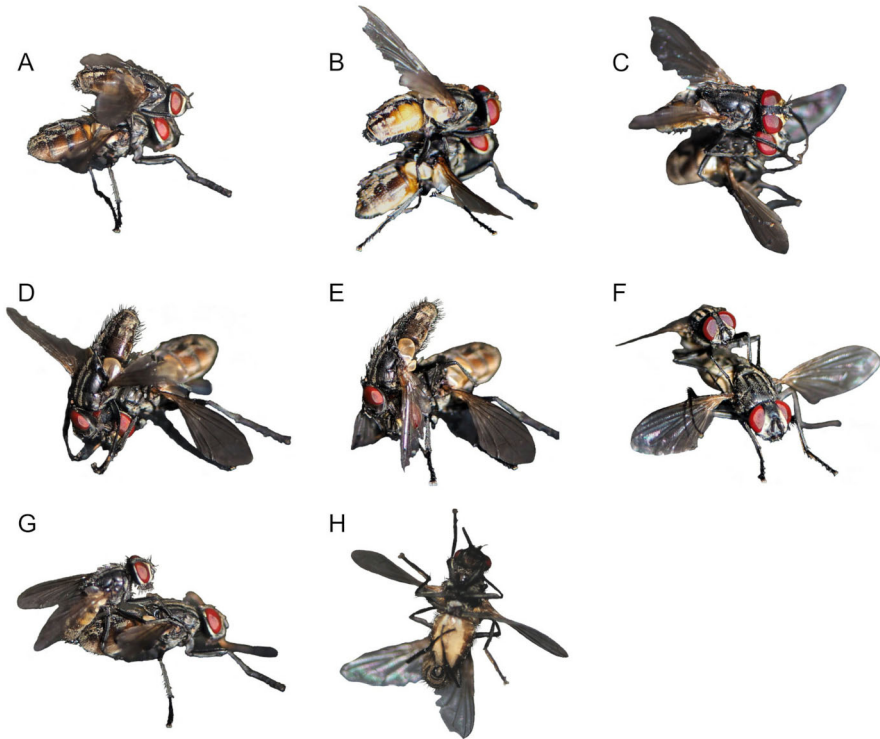
first 5 min of recording were not scored. Fly courtship and mating behaviour were first video recorded for 6 h. The observation period was then extended to 24 h to obtain more data on mating behaviour. As 24 h video recordings were not feasible for storage and analysis, flies were instead photographed once every minute for 24 h. The first 10 min of the 24 h observation periods were still video recorded to observe courtship behaviour in detail. Courtship data from 6 h and 24 h observation periods were pooled whereas mating behaviour data stems only from 24 h observations.

### *2.3. Housefly courtship behaviour*

Male houseflies exhibit several distinct courtship behaviours, usually in a certain order of steps (Colwell & Shorey, 1975; Meffert & Regan, 2002; Figure 1). First, courtship is initiated by a male jumping on a female, referred to as a strike (STRIKE). In response, a female moves her wings out of the way by spreading them perpendicular to the substrate she is standing on (WING OUT). During the strike the male can buzz his wings at virtually any moment (BUZZ). While mounted, the male moves forward positioning his head directly above the female's head (FORWARD). In this position, the male can lift the female's forelegs using his own forelegs (LIFT). The male can also hold his wings over the female's head in about 180° in this position (HOLD). After courtship a male moves backwards on the female to touch her genitalia with his own. Upon rejection a male either dismounts or performs courtship again.

To assess the effect of group sex ratio on housefly courtship and mating behaviour, we scored (1) the number and duration of male housefly courtship steps; (2) mating propensity, i.e., whether copulation occurred within the observation period; (3) mating latency (measured as time until the 1st copulation and time in between subsequent copulations); (4) remating of individual flies; and (5) the duration of each copulation. As males use about 40 min to transfer seminal fluid proteins during mating, copulation duration was taken as a proxy for the quantity of seminal fluid proteins allocated (Leopold et al., 1971b).

Despite using dyes to mark individuals it was often not feasible to identify individuals during courtship bouts, as they lasted only a few seconds. All courtship behaviour was therefore assessed on a group level rather than an individual level, i.e., courtship behaviours were not assigned to specific individuals but rather reflect how males in a sex ratio generally behaved. Please



**Figure 1.** Housefly courtship behaviour. Courtship is initiated after a males has spotted and/or touched a female with a male jumping on the female (STRIKE, A–H), which it is continued by steps such as the male moving forward to the female’s head (FORWARD, A), buzzing his wings (BUZZ, B), lifting the female’s forelegs with his own (LIFT, C and D) and the male holding his wings over the female’s head (HOLD, E). In response to a male initiating courtship a female spreads her wings (WING OUT, F). After successful courtship the male moves backwards on the female to initiate mating (G and H).

note that all other data is based on individual metrics. Determination of copulation duration was started if no other courtship behaviours were observed 60 s after a male attempted courtship. Copulation durations under 20 min were considered pseudo-copulations and excluded, as housefly copulations generally last about 70 min (Leopold et al., 1971a). Sporadically, genitalia of a mating pair failed to dislodge, presumably because they were stuck. This could be inferred from mating flies moving in other/opposite directions but remaining attached to each other. As it was impossible to observe when copulation was terminated in these instances, these pairs were excluded from data analysis. Courtship behaviour was observed for the first 10 min of recording

(after the acclimation period), mating latency and duration were observed for 24 h. To analyse courtship behaviours, videos were played back at 20% of their original speed. Videos were analysed with ‘Behavioural Observation Research Interactive Software’ (BORIS) software (Friard & Gamba, 2016).

#### 2.4. *Body size estimation*

To test for the effect of body size on copulation behaviour, flies were collected after mating assays. They were anaesthetised with CO<sub>2</sub> and placed individually in Eppendorf tubes (1.5 ml) and stored at 18°C. Right wings of individuals were gently dissected at the point of articulation with the thorax and mounted between microscope slides and coverslips using Euparal medium (Carl Roth, Germany, Karlsruhe). Wing images were captured using a digital camera Moticam 2000 (Motic Europe, Spain, Barcelona) connected to a stereomicroscope (Stemi SV617, Zeiss, Jena, Germany). Landmarks positioned at 17 vein intersections and terminations (Ludoški et al., 2014) were collected using TpsDig (version 2.31) (Rohlf, 2017a) and expressed as *x*- and *y*-coordinates in a Cartesian space (Figure A1 in the Appendix). Coordinates were superimposed using a generalized Procrustes analysis in tpsRelw (version 1.5) (Rohlf, 2017b) and wing centroid size was extracted. Centroid size, an isometric estimator of size, was calculated from the square root of the sum of the squared distances between the centre of the object (i.e., wing centroid) and its landmarks (Bookstein, 1991; Zelditch et al., 2004).

#### 2.5. *Statistical analysis*

All data analysis and visualisation was carried out in R (version 4.0.2) (R Development Core Team, 2022) and RStudio (version 1.2.5033) (RStudio Team, 2022) using the ‘betareg’ (Cribari-Neto & Zeileis, 2010), ‘cowplot’, ‘ggbeeswarm’, ‘ggfortify’ (Tang et al., 2016), ‘multcomp’ (Hothorn et al., 2008), ‘tidyverse’ (Wickham et al., 2019), ‘lmtree’ (Zeileis & Hothorn, 2002), ‘sjPlot’, ‘emmeans’, ‘factoextra’, ‘readxl’, ‘EnvStats’ (Millard, 2013) and ‘viridis’ packages.

Courtship indices (CI) were calculated by summing the total time any male spent courting (i.e., ‘STRIKE’), divided by the duration of the observation period (10 min). CI per male was additionally calculated by dividing CI by the number of males in the treatment. Mating behaviours (latencies and copulation durations) and body size were normalized by subtracting the mean and dividing by the standard deviation; body sizes were normalized

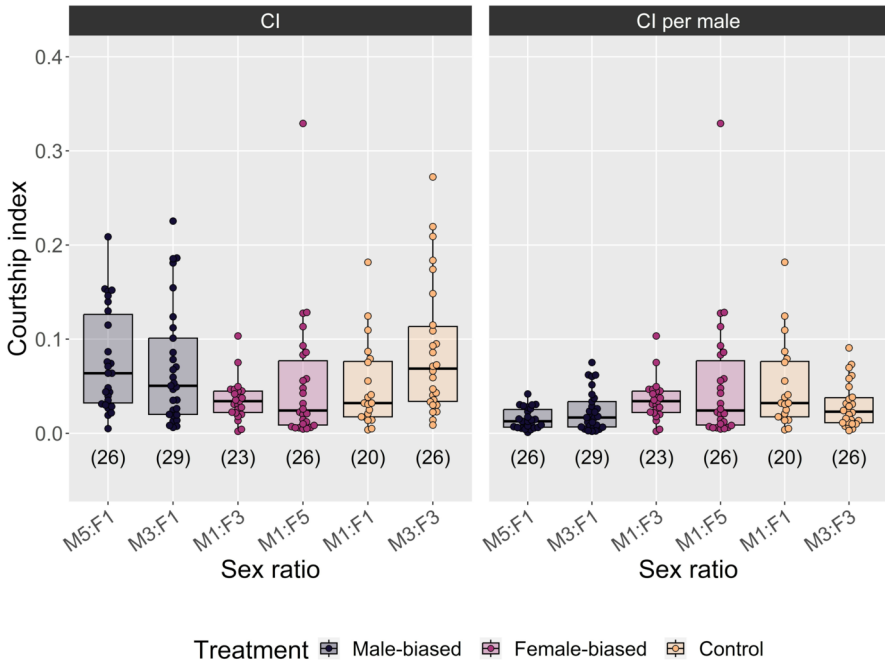
separately for males and females. Additionally, for each mating we obtained the body size differences (female size–male size), which was subsequently normalized as well.

The courtship indices (CI) were analysed using generalised linear models with a beta distribution and treatment as sole predictor. The average duration of courtship behaviours were analysed using a Principal Component Analysis and a linear model with treatment as sole predictor variable. Linear models were also used to analyse male- and female mating latency and copulation duration, with treatment and mating status as predictor variables. The effect of body size on copulation duration was also assessed with a linear model, with the body size difference and male- and female mating status and their interactions as predictor variables. Details on the specific analyses performed are presented in more detail per subject in the results section. Where applicable, post-hoc testing was done by pairwise comparisons between the control treatments versus the male-biased treatment to discover the effect of male–male competition, the control treatments versus the female-biased treatments for multiple mating partner effects, and the M1:F1 control versus the M3:F3 control treatments to assess the effect of group size.

### 3. Results

#### 3.1. Courtship performance

Over 1300 courtship bouts were analysed in detail in the 10-min observation period, with at least 100 bouts per sex ratio. The number of replicates per sex ratio were M5:F1 ( $N = 26$ ), M3:F1 ( $N = 29$ ), M1:F3 ( $N = 23$ ), M1:F5 ( $N = 26$ ), M1:F1 ( $N = 20$ ) and M3:F3 ( $N = 26$ ) (Figure 2). Males were expected to reduce courtship effort in male-biased groups but increase effort in female-biased groups. Generalized linear models with a beta distribution were fitted with the Courtship Index (CI) or CI per male as response variable and treatment as sole predictor variable. Full models were compared to reduced models to test for significant effects using likelihood ratio testing. The courtship index (CI) and CI per male were significantly affected by the social condition (group  $\chi^2 = 15.288$ ,  $p = 0.0092$ ; per male:  $\chi^2 = 14.634$ ,  $p = 0.012$ ). The CI per male was lower in both male-biased and the M3:F3 group compared to the female-biased and M1:F1 groups (Figure 2). The CI per male was significantly lower in the male-biased M5:F1 group compared to the M1:F1 control ( $z = 3.128$ ,  $p = 0.0139$ , Figure 2). The CI per group



**Figure 2.** Courtship index as a function of group size and sex ratio. Courtship index per treatment (A) and per male per treatment (B). Boxplots are depicted for male-biased (blue, left 2), female-biased (pink, middle 2) and control treatments (orange, right 2). Labels on the *x*-axis indicate the sex ratio of the treatment (males:females). Sample sizes are indicated beneath the boxplots in brackets.

was significantly lower in the female-biased M1:F3 ( $z = -2.793, p = 0.038$ ) and M1:F5 groups ( $z = -2.967, p = 0.0232$ ) compared to the M3:F3 control group. Overall, the CI per group increased with an increase in the number of males, but at the same time the CI per male decreased as the number of males increased.

To assess whether males altered the structure of their courtship behaviour depending on sex ratio, the average time males spent on particular courtship steps was recorded. Linear models were fitted to the average time spent on any courtship step with treatment as sole predictor variable. There was no significant effect of sex ratios on any courtship steps. Moreover, a principal component analysis (PCA) of the average duration of courtship behaviour revealed no difference in the distribution of values between treatments (Figure A2 in the Appendix). This indicates that males do not alter their effort

on these specific courtship steps, nor the general composition of courtship behaviour according to group sex ratios.

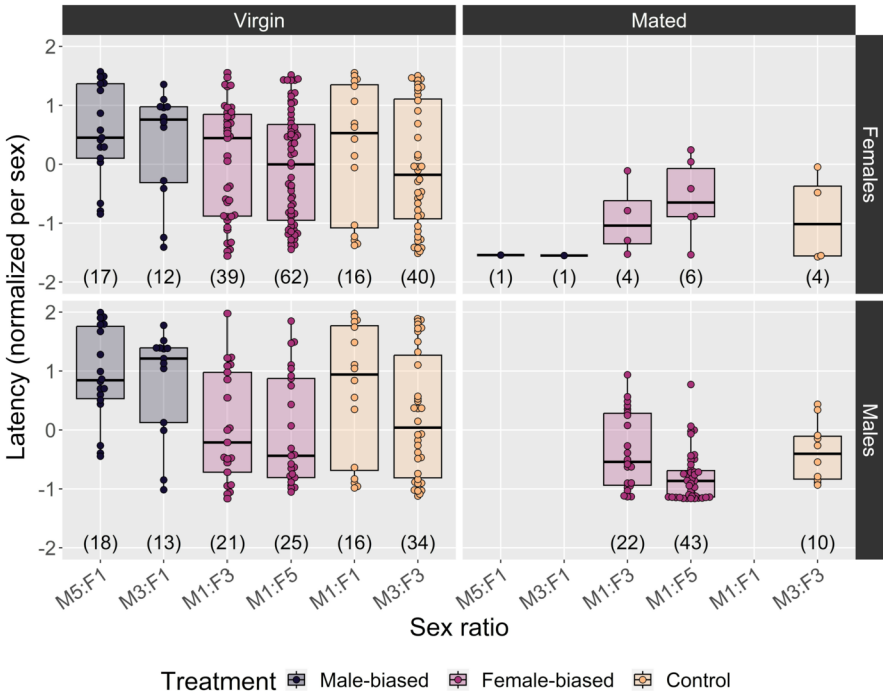
### 3.2. Mating behaviour — latency

A total of 257 copulations were observed. The number of replicates per sex ratio were M5:F1 ( $N = 20$ ), M3:F1 ( $N = 15$ ), M1:F3 ( $N = 28$ ), M1:F5 ( $N = 30$ ), M1:F1 ( $N = 21$ ) and M3:F3 ( $N = 26$ ). Housefly copulation behaviour and mating status (i.e., virgin, 1st remating, 2nd remating, etc.) was assessed during an observation period of 24 h. Mating latencies were standardized as described under “Statistical analysis”. Linear models were then fitted using treatment and mating status (virgin/mated) of the mating pair as the predictor variables, with separate models fitted on mating latencies in males and females. Full models were compared to reduced models to test for significant effects. Treatment and male mating status had significant effects on male mating latency (treatment:  $\chi^2 = -12.617$ ,  $p < 0.001$ ; male mating status:  $\chi^2 = -19.259$ ,  $p < 0.001$ ), whereas female mating latency was only affected by female mating status ( $\chi^2 = -12.027$ ,  $p < 0.001$ ). Opposite of the expectation, male mating latency in male-biased and M1:F1 control groups was higher compared to the female-biased and M3:F3 control groups (Figure 3). Male mating latency in the male-biased M5:F1 condition was significantly higher compared to the M3:F3 control ( $z = 2.978$ ,  $p = 0.0215$ ), whereas the latency of the female-biased M1:F5 group was lower compared to the M1:F1 control ( $z = -3.124$ ,  $p = 0.0136$ ). In other words any social condition containing just 1 female showed higher mating latencies than groups containing more than 1 female.

Virgin female mating latency was higher in female-biased groups compared to virgin male mating latency in female-biased groups (Figure 3). This is likely explained by the only male being present in female-biased groups already being engaged in copulation with one of the females, thus increasing mating latency for the other virgin females present who must wait for the completion of mating. Female remating rarely occurred in male-biased groups (2 out of 62 matings) during the 24 h observation period, and never in the M1:F1 control group (Figure 3), confirming observations from previous studies (Leopold, 1970; Arnqvist & Andrés, 2006).

### 3.3. Mating behaviour — copulation duration

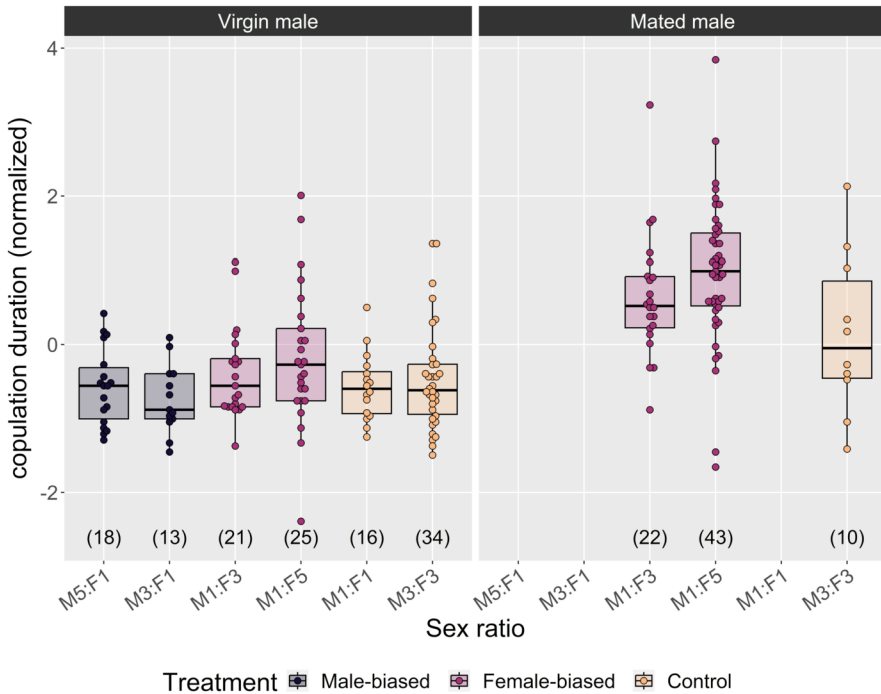
Males were expected to increase copulation duration in male-biased groups and decrease in female-biased groups. To assess the effect of sex ratio on



**Figure 3.** Mating latency per sex and mating status as function of group size and sex ratio. Female mating latencies are depicted on top, males below. Left graphs display latency until the 1st (virginal) mating and right graphs show latency until each subsequent mating. Boxplots are depicted for male-biased (blue, left 2), female-biased (pink, middle 2) and control treatments (orange, right 2). Mn:F<sub>n</sub> indicates the sex ratio of the treatment. Sample sizes are indicated beneath the boxplots in brackets.

copulation duration, a linear model was fitted on the standardized copulation durations with the effect of treatment, male mating status and female mating status as predictor variables. Full models were compared to reduced models to test for significant effects. Both treatment and male mating status had a significant effect ( $\chi^2 = -7.81, p = 0.031, \chi^2 = -32.63, p < 0.001$ , respectively). Contrary to our hypothesis, copulation durations in the M1:F5 group were significantly longer compared to the M3:F3 control group ( $z = 2.853, p = 0.0134$ , Figure 4); no other groups differed in copulation duration.

Copulations were also expected to be positively correlated with increased body size differences between mating pairs. The effect of body size on copulation duration was also assessed using linear models. Models were fitted on the copulation duration data with normalized body size difference, male mat-



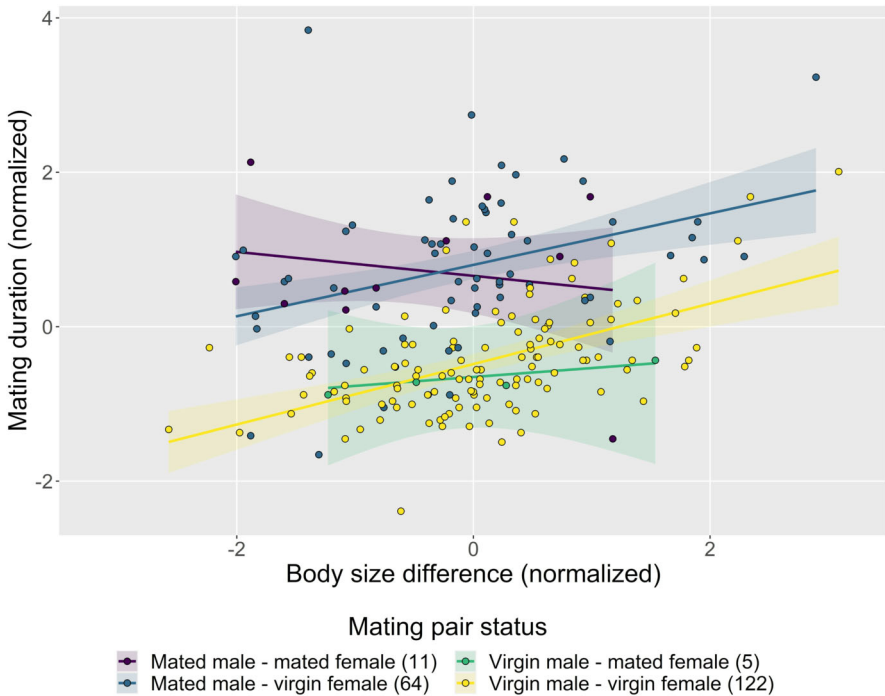
**Figure 4.** Standardized copulation duration as function of male mating status and sex ratio. Left depicts the copulation duration of virgin males, right of mated males. Boxplots are depicted for male-biased (blue, left 2), female-biased (pink, middle 2) and control treatments (orange, right 2). Mn:F<sub>n</sub> indicates the sex ratio of the treatment. Sample sizes are indicated beneath the boxplots in parentheses.

ing status, female mating status, and the interactions between these variables as the predictor variables. Both male status ( $t = -3.154$ ,  $p = 0.002$ ) and the interaction between the body size difference and female status ( $t = 2.198$ ,  $p = 0.029$ ) were significant. Male virginal copulation durations were shorter than non-virginal copulation durations (Figure 5). Copulations with virgin females lasted longer with larger body size differences, but body size difference itself was not a significant predictor for the copulation duration (Figure 5).

#### 4. Discussion

We investigated the effect of male and female-biased adult sex ratios on housefly mating behaviour to determine which social environment might be





**Figure 5.** Standardized male copulation duration related to centroid body size difference between females and males. Each regression line depicts a specific mating status combination of males and females. Sample sizes are indicated between brackets in the legend.

most suited for mass-rearing of houseflies. Male-biased sex ratios exhibited a higher courtship index per group, but a lower courtship index per male. Males did not differ in their courtship repertoire between sex ratios. Male mating latency was higher in male-biased sex ratios than in female-biased sex ratios, whereas copulation duration was longer in female-biased sex ratios. Copulation duration was positively correlated with the body size difference between both sexes for virgin females.

Similar to Carrillo et al. (2012), we found that males did not alter their courtship repertoire, i.e., the specific courtship steps and their duration, based on the adult sex ratio. Male houseflies are however able to modify their courtship performance. Males decreased the intensity (duration behaviour/duration observation period) of their BUZZ behaviour in their second courtship attempt with the same female (Aragaki & Meffert, 1998). Also, males from strains with a different courtship repertoire were able to

accommodate to female preference from a foreign strain by altering the intensity of their FORWARD and LIFT behaviours (Meffert & Regan, 2002). Interestingly, in both cases the female response, i.e., their WING OUT behaviour, to courting males was not altered. This indicates that female preference was unaltered, at least during the observation period. Male courtship repertoire may therefore be mostly dependent on female preference, which possibly varies between strains. Males may have performed similar courtship steps in different sex ratios because female preference was similar across sex ratios.

Although the courtship steps were similar between all sex ratios, the overall CI per male was lower in male-biased groups, but the inverse for the total CI per group. In other words, males in male-biased sex ratio individually spent less time courting, but cumulatively spend a higher total time on courtship compared to males in female-biased sex ratios. Male *Rhodeus ocellatus* and *R. sericeus* fish reduced courtship behaviour under higher intrasexual competition and instead increased effort on chasing away male competitors (Kano, 2000) or sperm competition (Mills & Reynolds, 2003) respectively. Male-biased sex ratios also lead to reduced courtship delivery but increased mating duration in *D. melanogaster* (Dore et al., 2020), whereas in the medfly *Ceratitis capitata* males increased courtship delivery but experienced lower paternity (Leftwich et al., 2012). This suggests a trade-off between a male's relative investment in specific mating behaviours. If the benefit of courtship behaviour is reduced under increased intrasexual competition in houseflies, males may have reduced their courtship rate accordingly. Alternatively, males in male-biased sex ratios may have encountered fewer females, which would result in lower courtship rates (Weir et al., 2011). Yet the unaltered courtship repertoire and reduced courtship index per male suggests that male houseflies do not compensate (fully) for a lower female encounter rate by increasing investment in activity and mate searching. Possibly, males lowered courtship rate and instead allocated more effort to post-copulatory traits like ejaculate investment.

A lower CI in female-biased sex ratios may translate to lower male harassment of females. As male harassment can negatively affect female fitness by reducing female longevity or foraging time (Gomez-Llano et al., 2018), reduced male harassment may increase female reproductive output. Male harassment indeed appears prevalent in houseflies as both sexes suffered

from increased wing damage and reduced longevity in male-biased compared to equal sex ratios, likely through increased mating and/or physical activity (Ragland & Sohal, 1973). Also, in the Tsetse flies *G. f. fuscipes* and *G. pallidipes* a 1:4 female-biased sex ratio had the highest offspring production as well as the lowest mortality rate due to lower male harassment (Desa et al., 2018). The reduced CI in female-biased sex ratios may therefore aid reproductive output in mass rearing contexts as female longevity is expected to be increased, resulting in a higher per capita productivity.

There may be a link between the higher courtship index per male and lower mating latency in female-biased sex ratios compared to male-biased sex ratios. Both males and females seem to assess their potential mating partner during courtship (Meffert & Regan, 2002; Shin et al., 2003) and considering that female houseflies exhibit particularly low remating rates they are likely choosy (Riemann et al., 1967; Riemann & Thorson, 1969; Leopold et al., 1971a; Arnqvist & Andrés, 2006). The initiation of copulation appears to be under female control, as female houseflies need to extend their ovipositor to initiate copulation (Degrugillier & Leopold, 1973) and male courtship often does not lead to copulation. Mating latency therefore ultimately depends on the rate at which a female accepts a copulation. If a male has less time or opportunity to court a female, e.g., due to intra-sexual competition in male-biased sex ratios, females may have fewer opportunities to assess the potential mating partners, which increases the mating latency. In a female-biased sex ratio a male likely suffers less from intra-sexual competition and females have fewer options in terms of mating partners, which may then reduce female choosiness (Souroukis & Murray, 1994; Passos et al., 2014; Villarreal et al., 2018) and the latency to copulation. Lower mating latencies may further increase productivity as less time is lost prior to fertilization and oviposition lowering reproduction time. A female-biased sex ratio may then not only increase reproductive output due to reduced male harassment, but also because of lower mating latencies.

A female-biased sex ratio may further improve reproduction performance through its effect on the duration of copulation, which is commonly taken as a proxy for the quantity of ejaculate transferred from the male to the female. Copulations lasted longer in the M1:F5 female-biased sex ratio compared to the M3:F3 control, which suggests that males allocated more seminal fluid proteins to females in female-biased sex ratios. Seminal fluid proteins benefit

females by increasing both fecundity and lifespan, though often at the opportunity cost of remaining monogamous (Arnqvist & Andrés, 2006; Carrillo et al., 2012). As the inhibitory effect of seminal fluid proteins depends on their dosage (Riemann & Thorson, 1969), we expected that males would allocate more to females in male-biased sex ratio to prevent potential sperm competition. Males can allocate more ejaculate in response to increased sperm competition risk (García-González & Gomendio, 2004; Bretman et al., 2009; Parker & Pizzari, 2010; Garbaczewska et al., 2013; Abraham et al., 2015; Esfandi et al., 2020). The increased copulation duration in a female-biased sex ratio is therefore somewhat confusing. Considering that increased allocation of seminal fluid proteins may increase female fecundity (Arnqvist & Andrés, 2006), males may have allocated more to females in the M1:F5 group as a form of parental investment because of the high paternity security due to the absence of sperm competition risk. Because of the depletion of seminal fluid proteins over multiple matings and correlated increase in female remating (Leopold et al., 1971a), a female-biased sex ratio will likely result in increased female remating frequency. Moreover, increased allocation of seminal fluid proteins per mating results in males becoming depleted of seminal fluid proteins sooner. This may further increase the frequency of female remating, which could add to female reproductive output depending on the balance between reduced transfer per mating and increased number of matings.

Apart from sex ratio, body size also influenced copulation duration. Body size difference between a mating pair was positively correlated with the duration of copulation for virgin females. Larger body size generally correlates with greater fitness in insects (Beukeboom, 2018); in houseflies, larger females experience increased fecundity (Black IV & Krafur, 1987), and males prefer to mate with larger females (Shin et al., 2003). Males that achieve a copulation with a relatively larger mate may therefore be inclined to allocate more seminal fluid proteins during the copulation, thus increasing copulation duration. Alternatively, smaller males may require more time to transfer equal amounts of ejaculate as larger males, so that they need to increase the copulation duration to achieve the same effect as larger males can achieve in a shorter time. If smaller males indeed invest more seminal fluid proteins per copulation it may be beneficial to induce larger body size differences between mating partners, preferably by increasing female size as it is correlated with fecundity (Black IV & Krafur, 1987).

We have assumed that copulation duration is positively correlated with seminal fluid proteins allocation. Many studies have assumed a positive correlation between the copulation duration and ejaculate investment (Esfandi et al., 2020). Positive correlations have indeed been reported between copulation duration and ejaculate investment, e.g. increased sperm transfer in the golden egg bug *Phyllomorpha laciniata* (García-González & Gomen-dio, 2004) and seminal fluid protein allocation in *D. melanogaster* (Wigby et al., 2009), but empirical support for the generalized assumption is lacking (Esfandi et al., 2020). In fact, male *D. melanogaster* (Garbaczewska et al., 2013) increase sperm allocation while decreasing the mating duration in response to the presence of rival males, showing that copulation duration is not always a good proxy for sperm/seminal fluid allocation. In *A. fraterculus* and *Ephestia kuehniella* males increase sperm allocation in the presence of rival males but show no effect on mating duration (Abraham et al., 2015; Esfandi et al., 2020). Furthermore, Carrillo et al. (2012) report that female houseflies experienced increased fitness in male-biased sex ratios possibly caused by increased allocation of seminal fluid proteins. An increase in copulation duration in houseflies may therefore not necessarily reflect ejaculate investment.

Another potential problem of assuming a correlation between mating duration and ejaculate investment is the male strategy of extending mating duration as a form of pre- or post-copulatory mate guarding in some species (Parker, 1974; Vitta & Lorenzo, 2009; Kuramitsu et al., 2019; But-terworth & Wallman, 2022). As there may be no (further) ejaculate transfer during mate guarding it could lead to overestimations of ejaculate invest-ment if such a strategy occurs. Male houseflies did not prolong courtship STRIKES in male-biased sex ratios and abandoned unsuccessful courtship attempts quickly. Males also did not interact more in male-biased sex ratios (Carrillo et al., 2012). This suggests that male houseflies may not exhibit pre-copulatory mate guarding. Housefly copulation duration is correlated with the expenditure of seminal fluid proteins in a quantitative manner (Rie-mann et al., 1967; Leopold et al., 1971a) and seminal fluid proteins generally inhibit females from remating (Riemann et al., 1967), which suggests that males may not need to guard a mated female from other males. Our finding that males in the M1:F5 group exhibited increased copulation duration also does not match with the mate-guarding behaviour hypothesis, as there were no males to guard against in this group. Male houseflies may therefore not

perform mate-guarding post-copulatory either. Yet, considering the potential issues of taking copulation duration as a proxy for ejaculate investment more research is required. Future research on male housefly ejaculate investment and its relation to copulation duration in response to sex ratio biases may be beneficial to ascertain the potentially beneficial effects of a female-biased sex ratio on housefly mating behaviour and fitness.

Our results suggest that female-biased sex ratios are most useful for mass-rearing of houseflies due to several mechanisms, as (1) a reduced courtship performance may translate to reduced male harassment, which might enhance female survival; (2) lower mating latencies which may reduce production time; and (3) increased copulation duration may reflect increased seminal fluid proteins allocation and hence enhance female fecundity. It is however still unclear under what sex ratio the productivity would be optimized. Our results show that a 1:5 ratio has the strongest effects compared to a 1:3 ratio, but we did not investigate the effects on offspring production. Male houseflies generally remate but the frequency drops with each consecutive mating (Leopold et al., 1971a), so a too strong female bias may result in unmated females. In Tsetse flies a 1:4 female-biased ratio is considered optimal for production (Desa et al., 2018); it seems plausible a similar ratio applies to houseflies as well, but future research is required to find the optimal sex ratio.

Another point of attention is that it is still unclear to what extent the effects of different adult sex ratios apply at different scales, in particular under mass-rearing conditions as opposed to the small-scale experimental numbers used here. Future research should establish the effects of adult sex ratio on reproductive behaviour on a larger scale like the population level. In our study the operational sex ratio deviated from the adult sex ratio once a mating occurred. As the adult and operational sex ratio could influence mating behaviour differently (Kokko & Jennions, 2008; Jennions & Fromhage, 2017), this deviation might have had some effect. Future research should therefore consider to more clearly separate or possibly retain similar adult and operational sex ratios. Other future directions for research could be to study the effect of different sex ratios on female oviposition behaviour, on population productivity (perhaps on different time scales) and population dynamics (e.g. sex-specific mortality). Altogether, our results highlight the potential for sex ratio biases to have indirect effects on the productivity of insect rearing.

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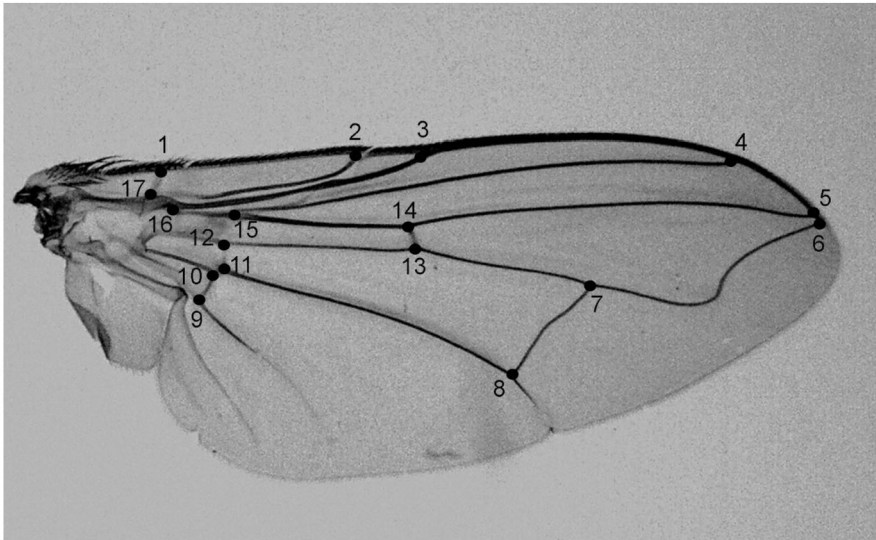
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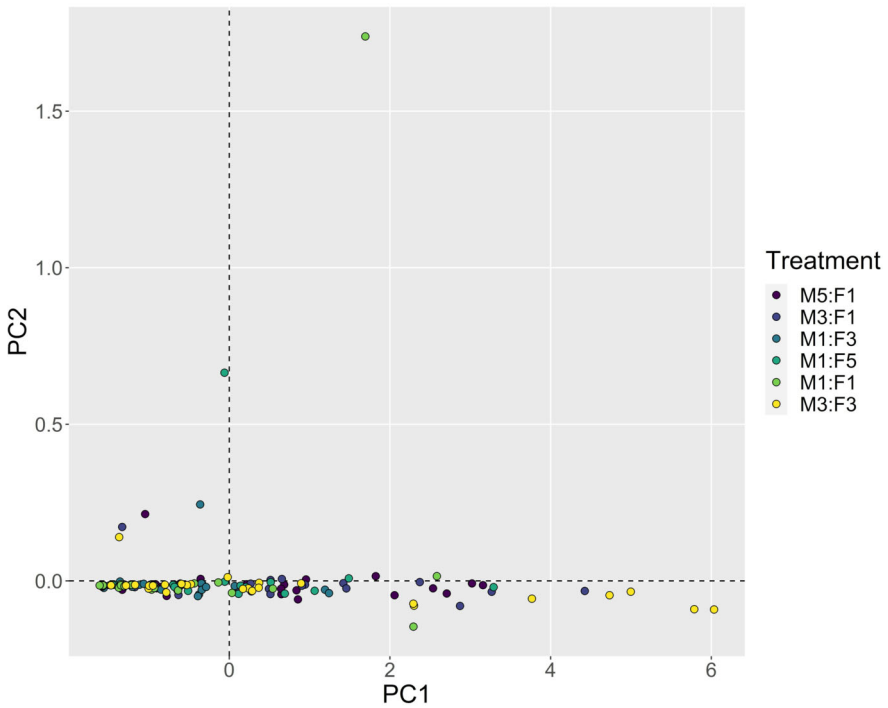
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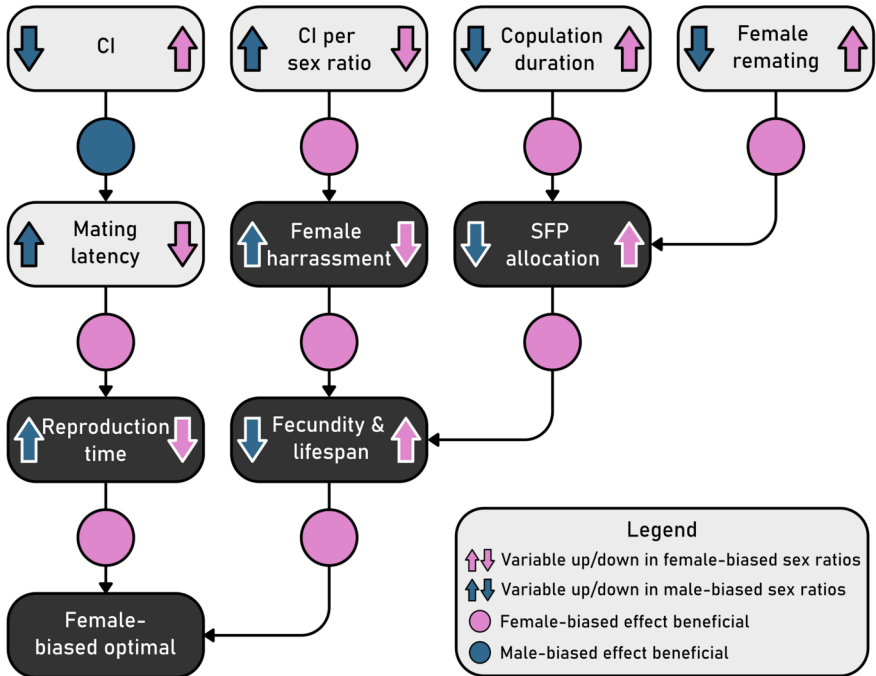
## Appendix



**Figure A1.** Example of a housefly wing with 17 landmarks added to wing intersections and terminations using TpsDig.



**Figure A2.** Principal component analysis of courtship behaviour under different social conditions. Principal components 1 and 2 are depicted on the *x*- and *y*-axis, respectively. Dots are coloured according to sex ratio (see legend).



**Figure A3.** Overview of the effect of sex ratios on different housefly behaviours and the potential for these to affect (mass-) rearing productivity. Boxes with light grey background depict results from this study; dark grey boxes indicate results from previous studies. Arrows indicate how the variable was affected by the male-biased (blue) and female-biased (pink) sex ratio; for dark grey boxes, the arrow direction is imputed based on previously-described effects. Circles indicate if the beneficial effect of a variable is under male- (blue) or female-biased (pink) sex ratios. Black arrows show the connection between variables.