Socio-sexual cues shape female diet choice in *Drosophila melanogaster*

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Male harassment can disturb female feeding behaviour and limit females' access to preferred foraging locations. However, it is not yet known how females trade off costs of sexual harassment or increased intrasexual competition against preference for dietary macronutrients when making foraging decisions. We used the fruit fly *Drosophila melanogaster* to investigate how female foraging decisions were affected by cues of conspecific presence and interactions with males. We assessed the strength of female choice for high-protein versus low-protein diet patches for foraging and oviposition in either single-sex groups, during direct interactions with males, or when exposed to cues of males or females ('restricted' conspecifics held within a transparent barrier, allowing auditory, visual and chemical cues but preventing physical contact and direct disturbance). Contrary to predictions, females did not avoid foraging or laying eggs on patches that had cues of male presence. Instead, females prioritised associating with restricted males, overriding harassment risk and diet preferences. In a subsequent experiment, we found that females were attracted to cues of conspecifics of either sex, even when those cues were derived from low-protein food patches. Female attraction to food patches with cues of conspecifics, for both feeding and reproduction, suggests that cues from socio-sexual partners are used in foraging decisions and can modulate, or sometimes override, diet quality preferences.

Keywords: *Drosophila melanogaster*, fruit flies, diet choice, foraging, harassment, sexual conflict, nutritional ecology, social nutrition.

Male harassment of females is often costly for females. Costs of sexual harassment include reduced fecundity (McLain & Pratt, 1999), increased predation risk (Rowe, 1994), and increased injury and mortality (Réale *et al.*, 1996; Mühlhäuser & Blanckenhorn, 2002). Sexual harassment also disturbs female feeding. For example, male harassment reduces female foraging time and efficiency in live-bearing fishes and in insects (Magurran & Seghers, 1994; Rowe, 1994; Stone, 1995; Griffiths, 1996; Pilastro *et al.*, 2003; Plath *et al.*, 2003; Tobler *et al.*, 2011; Teseo *et al.*, 2016).

Despite the documented impact of male harassment on female foraging behaviour, little is known about whether females dynamically change their nutrient intake in anticipation of, or in response to, sexual harassment. For example, females may feed on sub-optimal food patches either to avoid males, or because they have been directly displaced onto those diets by male pursuit. In many species, females express strong preferences related to diet macronutrient content (Simpson & Raubenheimer, 2012). Females often prefer a higher protein diet in comparison to conspecific males (Perry, 2011; Lee *et al.*, 2013; Camus *et al.*, 2018; Archer *et al.*, 2026). This preference aligns with optimal dietary intakes for female reproduction, which is generally maximised on relatively high protein diets (Lee *et al.*, 2008; Maklakov *et al.*, 2008; Fanson & Taylor, 2012; Solon-Biet *et al.*, 2015). Macronutrient content strongly affects female reproductive output; therefore, effects of male harassment on female diet choices and diet composition are expected to have important fitness consequences for females.

In this study, we tested whether the presence of males, and interactions with males, shifts females away from their dietary optima. We measured the preferences of *D. melanogaster* females for high-protein and low-protein food under different regimes of male exposure. To manipulate male harassment and social information about male presence, we exposed females directly (to enable interactions including harassment to occur) or indirectly (such that direct interactions and harassment were not possible) to individuals of the same or opposite sex. *D. melanogaster* is suitable for this study for several reasons: females show clear preferences for high protein diets and achieve higher reproduction on them (e.g., Jang & Lee, 2018), and well-established protocols exist for measuring diet preference. In

addition, male harassment of females is frequent and easily observed (Bastock & Manning, 1955; Lasbleiz *et al.*, 2006).

In the first experiment, there were four treatments: females were held in female-only groups, freely interacted with males in mixed sex groups or exposed to males 'restricted' within transparent circular arenas on either the high- or low-protein diet. We predicted that: (1) females in the same sex treatment would prefer the high protein diet, consistent with previous studies (e.g., Jang & Lee, 2018); (2) females would show altered foraging behaviour when in direct contact with courting, harassing males, due to physical disturbance; (3) females would avoid patches containing restricted males and thus show weaker or no preference for the high-protein diet containing restricted males. Part of this experiment revealed that females preferred diets on which male flies were restricted – even when male flies were restricted on the low-protein diet. Therefore, we reasoned that females might be attracted to the presence of conspecifics of either sex, perhaps because the presence of conspecifics provides social information indicating food availability or quality (reviewed by Lihoreau et al. 2015, Sulikowski 2017) or through benefits of being in a group (reviewed by Buxton *et al.*, 2020). To test this, we conducted a second follow up experiment as part of this same study, to disentangle the effects of social versus sexual cues on female diet preference. For this, females were held in female-only groups, exposed to restricted females or males on either the high- or low-protein diet.

METHODS

Fly stocks and rearing

Experimental flies were maintained on a standard sugar-yeast-agar (SYA) diet (50 g sucrose, 100 g brewer's yeast, 15 g agar, 30 ml Nipagin (10 % solution), 3 ml propionic acid, 970 ml water) and were derived from a large stock population of outbred wildtype *D. melanogaster* from the Dahomey line (Chapman *et al.*, 1994). To generate standardized parents of experimental flies (and limit parental carry-over effects), eggs were collected from the stock population in glass bottles containing 70 ml SYA medium and allowed to develop over a 10-day period. To generate experimental flies, eggs were collected from these parental generation flies using purple-grape-juice medium supplemented with live yeast paste. After 24 h, 50 first-instar larvae were transferred to glass vials containing 7 ml SYA medium. Experimental adults emerging from these cultures were collected as virgins using ice anaesthesia and housed in SYA vials in groups of 10 virgin males or of 20 females plus five males, to allow mating of focal females. Following a 3-day period, mated females were moved into new vials for 24 h before the start of the experiment. A subset of females was set aside in individual vials and allowed to lay eggs to confirm that females had mated (N = 54, 98 % of vials contained offspring). All flies were maintained, and experiments conducted, in a controlled environment at 50 % humidity and 25 °C, under a 12 h light-dark schedule.

High-protein and low-protein diets

Solid meridic diets were made following a recipe adapted from Piper and colleagues (2014; Supplementary Tables S1 and S2). Solid diets allowed us to set up diet patches within transparent arenas, and permitted flies to forage in more ecologically realistic conditions in comparison to liquid capillary feeding (e.g. Ja *et al.*, 2007). Diets were made to 1 L with equal amounts of cholesterol, lecithin, agar, water, preservatives, essential vitamins and salts, while amounts of bovine casein (P) and sucrose (C) were adjusted to alter the protein to carbohydrate (P:C) ratio: 4:1 P:C (96 g bovine casein and 24 g sucrose) and 1:4 P:C (24

g bovine casein and 96 g sucrose). Diets were autoclaved at 120 °C for 15 minutes and poured into trays at approximately 5 mm thickness. Diets were stored at 2 °C until use.

On the morning of observations, patches were cut from the 1:4 and 4:1 diets using a 26 mm diameter circular cutter. One patch from each diet was placed into each of the plastic test arenas (100 mm x 15 mm petri dish with a small hole in the side to allow entry of CO_2 gas for anaesthetisation). In both experiments, strips of transparent acetate film were formed into 150 mm diameter rings, which were pressed into each patch of food, so that roughly a third of the patch was also enclosed.

To create the restricted treatments, single-sex groups of 10 males or females were placed within one of the acetate barriers to prevent direct harassment and physical interaction with focal females. Rings were cut to 13 mm high to ensure close contact with the lid to prevent files escaping and evenly perforated using a mounted needle. This allowed visual, pheromonal and auditory cues of males to be detected by females in the wider arenas in the absence of direct contact (Bretman *et al.*, 2011; Saltz, 2011; Fowler *et al.*, 2022). To control for the presence of the acetate rings, all observation arenas contained two acetate enclosures, regardless of whether any flies were held within them. Arenas were then randomised using coded identifiers, to anonymise the treatments from the perspective of the observer, as far as possible.

Diet preferences

We first tested the diet preferences of focal mated females for high- versus low-protein diets following direct exposure to males (females and males moving freely), indirect exposure (males restricted to a portion of either food patch within an acetate barrier, preventing direct contact with females) or no exposure to males (Supplementary Figure S1a). The four experimental treatments were:

a) Single-sex female groups.
b) Freely interacting mixed sex groups.
c) Unrestricted females with males restricted on the preferred (4:1) diet.

d) Unrestricted females with males restricted on the non-preferred (1:4) diet.

Diet and oviposition preference data were collected across two experimental blocks. Observations of each group were taken over three days in block one (five observations on day one, three on day two and three on day three) and block two (five observations on day one, five on day two and six on day three). Oviposition preference data were collected from the first day of block two.

In the second experiment we tested the diet preferences of focal females for high- versus low-protein diets following indirect exposure to social groups of either sex (females or males restricted on either food patch within acetate arenas) or no exposure to social groups (Supplementary Figure S1b). The five experimental treatments were:

a) Single-sex females.
b) Unrestricted females with males restricted on the preferred (4:1) diet.
c) Unrestricted females with males restricted on the non-preferred (1:4) diet.
d) Unrestricted females with females restricted on the preferred (4:1) diet.
e) Unrestricted females with females restricted on the non-preferred (1:4) diet.

Data were collected from a single experimental block, with behavioural observations recorded over two days (four observations on day one and five on day two). Oviposition preference data were collected from the second day.

To verify that female location was a reliable proxy for foraging (i.e., feeding) choice, we conducted a supplementary experiment using media marked with red or blue dye (Supplementary Methods and Supplementary Figures S2-S4). These dyes can be visualised in female abdomens following ingestion (Ribeiro & Dickson, 2010). A control experiment to test the potential effect of the acetate rings themselves on female preferences was measured in an additional supplementary experiment (Supplementary Figure S5).

To start the data collection phase in the two main experiments, four-day old focal mated females were added to each of the arenas in groups of ten, using CO_2 anaesthesia

(experiment 1, block 1 $N = 52$ arenas; experiment 1, block 2 $N = 64$ arenas; experiment 2 $N = 45$ arenas). In all treatments, 10 females were added to the centre of each arena and had access to both diet patches. In the male restricted treatments (experiment one and two), 10 males were added to either the 4:1 or 1:4 patch enclosure (Supplementary Figure S1). In the mixed-sex treatment (experiment one), 10 males were added to the centre of the arenas alongside females. In the female restricted treatments (experiment two), 10 females were added to either the 4:1 or 1:4 patch enclosure. Flies were allowed to acclimatise inside arenas for one hour before observations began.

Behavioural observations of patch preference and extent of interactions including sexual harassment

Arenas were scan sampled for behaviour every 0.5 - 1 h, and the number of female flies on the 4:1 patch, the 1:4 patch, or not on the food (i.e., on the dish or acetate barrier) were recorded. A two-second video was recorded for each arena containing the mixed-sex treatment in order to assess the extent of female harassment. Harassment was recorded when male files engaged in courtship towards a female (licking, wing-vibrating, orientation, or chasing) (Bastock & Manning, 1955) that did not result in mating.

Flies remained in the arenas overnight after the first day of observations. Flies were anaesthetised using CO_2 in the morning of the second day to allow replacement of the food patches by fresh diet from the same food batches. It was important to renew the diets to prevent the effect of larvae on female patch preferences, as larval digestion could alter diet composition and contribute additional social cues. Following anaesthetisation, flies were allowed to acclimatise for one hour, before behavioural scans began following the same protocol as above. Food patch replacement and behavioural observations were repeated for a third day in experiment one.

Offspring development

Eggs laid by focal females onto diet patches were retained 24 h after behavioural observations began. Diet patches were placed in individual vials containing 7 ml SYA to standardise developmental conditions. The number of adult offspring that emerged from

each diet patch were recorded. In experiment two, photos of patches were also taken to record oviposition on the top surface of each patch and counted using ImageJ Fiji software (Schindelin *et al.*, 2012) (Supplementary Figure S6), before patches were similarly transplanted into individual SYA vials. All offspring were left to develop for 12 days and vials were subsequently frozen. Numbers of adult offspring per vial were counted to profile reproduction on each patch in the 24 h window (experiment one $N = 108$ patches, experiment two $N = 90$ patches).

Data analysis

All 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 models can be found in Supplementary Table S3. Linear mixed models were run using the R package glmmTMB (Brooks *et al.*, 2017). Model fit was checked and selection was done using the R packages DHARMa (Hartig, 2022) and Performance (Lüdecke *et al.*, 2021). Post-hoc Tukey tests were carried out using the estimated marginal means (EMMs) package emmeans (Lenth *et al.*, 2023).

a) Diet preference

The number of flies present on the 1:4 food, 4:1 food, or not on the food (on the sides of the acetate barriers or on the dish) was recorded over 11 and 13 observations in experiment one (block 1 and 2, respectively) and 9 observations in experiment two, in a repeated measures design. To assess how attracted focal females were to any food patch, we calculated a food preference score as the number of female flies on either food patch divided by the total number of focal females in the arena ($N = 10$ in all cases). This was calculated for each arena at each observation. Of those females choosing a diet, the proportion choosing 4:1 over 1:4 was also calculated, again for each arena at each observation.

To assess the effect of treatment on preference for food (versus non-food) and for 4:1 diet (versus 1:4), we ran linear mixed models using the R package glmmTMB (Brooks *et al.*,

2017) using preference scores as response variables, weighted by the number of flies observed on food per observation.

To account for repeated measures and nested levels in the experimental design, data were analysed within partially nested models (Supplementary Table S3). Day was nested within block (experiment one only), and observation was nested within day (checked using cross tabulation, with xtabs in the R package stats). Arena IDs per block were included as random effects in all diet models. Only one experimental block was carried out in the second experiment, therefore block was included as a fixed effect and day was included as a random effect in experiment one, and day was included as a fixed effect in experiment two. Estimated marginal means were extracted (Lenth *et al.*, 2023) from each linear model (with 95 % confidence intervals) and used in plots of preference score.

b) Offspring counts

The effect of treatment group on the number of offspring per patch was analysed separately for each diet type in two models: 1) the number of offspring from 4:1 patches was compared across treatment groups and 2) the number of offspring from 1:4 patches was compared across treatment groups. Data were analysed in linear models using the R package glmmTMB (Brooks *et al.*, 2017).

RESULTS

Results from the supplementary experiment supported our use of female presence on a solid diet as a proxy for diet choice: the colour of the diet patch that females were observed on was strongly associated with the colour of female abdomens (Supplementary Figures S1C, S3 and S4). Females showed a strong preference for the 4:1 over the 1:4 diet (Supplementary Figure S3), regardless of whether the 4:1 diet was dyed red or blue (diet colour: $\chi^2_1 = 0.2$, $P = 0.69$). In addition, the presence of acetate barriers alone resulted in a marginal decrease in preference for 4:1, but preference for 4:1 was still detectable when barriers were present (Supplementary Figure S1D and S5).

Experiment one - effect of direct versus indirect exposure to males on female diet choice

a) Female feeding was disrupted by direct disturbance from males

Female preference for food was significantly affected by treatment ($\chi^2_3 = 12.6$, $P < 0.01$). Females were significantly less likely to be found on food patches when freely mixing with males, as compared to when males were absent, or when males were restricted to the 4:1 patch (Figure 1; $P < 0.05$ in all cases). When the sexes could freely interact, males frequently harassed females: at least one incidence of harassment was observed in 83 % of observations in the mixed treatment ($N = 348$ observations). There was also a significant effect of block ($\chi^2_1 = 27.2$, $P < 0.001$), which was accounted for within the statistical model.

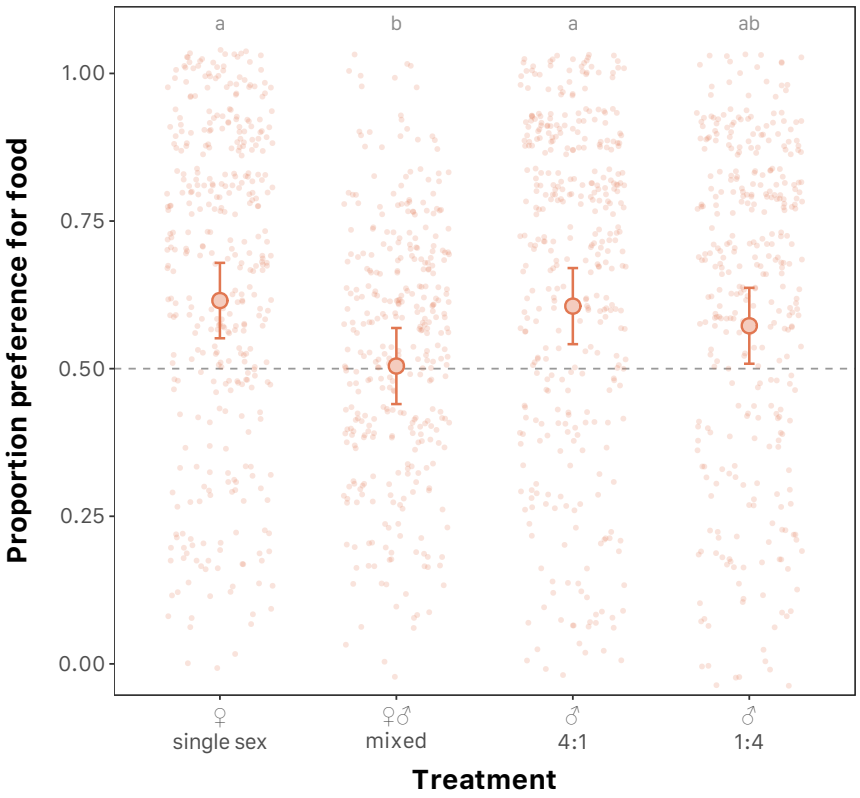

Figure 1

Foraging behaviour is significantly reduced during direct interactions with males but not by cues of restricted males. Shown is the proportion of females present on food between the four socio-sexual treatments: ♀ single sex (10 females), ♀♂ mixed sex (freely interacting 10 females and 10 unrestricted males), ♂ 4:1 (10 females with 10 males restricted to the 4:1 protein: carbohydrate diet patch), and ♂ 1:4 (10 females with 10 males restricted to the 1:4 protein: carbohydrate diet patch). Large circles represent estimated marginal means (± 95 % confidence intervals) extracted from the linear mixed model to account for repeated observations. Small circles represent raw data and 50% preference is marked by the grey, dashed line. Letters indicate significant differences from a post-hoc Tukey test.

b) Female diet choice varied with male location

Female diet choice was significantly altered according to the presence and location of males ($\chi^2_3 = 65.1$, $P < 0.001$) (Figure 2). Counter to expectations, female preference for the 4:1 diet increased when males were restricted on the 4:1 patches, compared with all other treatments (post-hoc Tukey tests of males on 4:1 versus females alone: $t_{1428} = 4.5$, $P < 0.001$, versus mixed-sex: $t_{1427} = 3.4$, $P > 0.01$, versus males on 1:4: $t_{1428} = 8$, $P < 0.001$). Similarly,

females also shifted their diet preference towards the 1:4 patch when males were restricted on those 1:4 patches, hence decreasing their preference for 4:1 compared with all other treatments (post-hoc Tukey tests for males on 1:4 versus single-sex: t_{1428} = -3.5, P > 0.01, versus mixed-sex: t_{1427} = -4.6, P < 0.001).

Counter to expectations (and the supplementary experiment results), there was no overall female preference in this experiment for the 4:1 diet in the single sex treatment. There was also no difference in preference for 4:1 between the single-sex and mixed-sex treatments (post-hoc Tukey test: t_{1428} = -1.1, P = 0.7) and no significant effect of block (χ^2_1 = 0.5, P = 0.5). Overall, the results suggest that female presence on any diet was reduced by direct contact with males, but that females were attracted to patches containing restricted males.

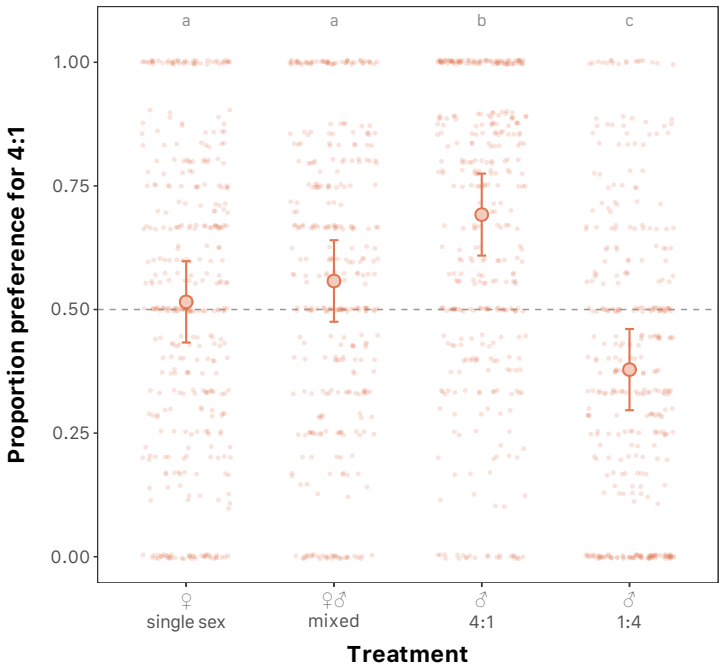

Figure 2

Female preference for the high-protein diet is significantly affected by cues of restricted males, but not by direct interactions with males. Shown is the proportion of females on the 4:1 (protein: carbohydrate) diet patch versus the 1:4 (protein: carbohydrate) diet patch between the four socio-sexual treatments in the first experiment. Female dietary preference for 4:1 diet for the four experimental treatments: ♀ single sex (10 females), ♀♂ mixed sex (freely interacting 10 females and 10 unrestricted males), ♂ 4:1 (10 females with 10 males restricted to the 4:1 patch), and ♂ 1:4 (10

females with 10 males restricted to the 1:4 patch). The number of females on the 4:1 patch was divided by the total number of flies on both patches to provide a proportion score of 4:1 preference. Large circles represent estimated marginal means (± 95 % confidence intervals) extracted from the linear mixed model to account for repeated observations and model weights. Small circles represent raw data and 50 % preference is marked by the grey, dashed line. Letters indicate significant differences from a post-hoc Tukey test.

c) Offspring production was highest on diet patches with restricted males

The number of adult offspring produced from each diet varied significantly with socio-sexual treatment, for both 4:1 and 1:4 diet patches ($\chi^2_3 = 15$, $P < 0.01$ and $\chi^2_3 = 24.3$, $P < 0.001$, respectively; Figure 3). The number of offspring produced on 4:1 diet was significantly higher when males were restricted on the 4:1 diet, compared to when males were restricted on the 1:4 diet (113 ± SE 13.9 versus 53 ± 9.3, respectively). Similarly, the number of offspring collected from 1:4 patches when males were restricted on the 1:4 patch was significantly higher than all other treatment groups. These results suggest that the presence of restricted males on a diet patch made it more attractive to females as an oviposition substrate, resulting in more offspring.

Overall, the mean total offspring on each diet patch was 85 ± 0.7 and 73 ± 0.6, for 4:1 and 1:4 respectively ($N = 64$ patches). Interestingly, the total number of offspring produced per arena (summed across both diets) remained consistent between the four treatments ($\chi^2_3 = 2.1$, $P = 0.6$; Supplementary Figure S7), suggesting that interactions with males did not limit females' ability to oviposit.

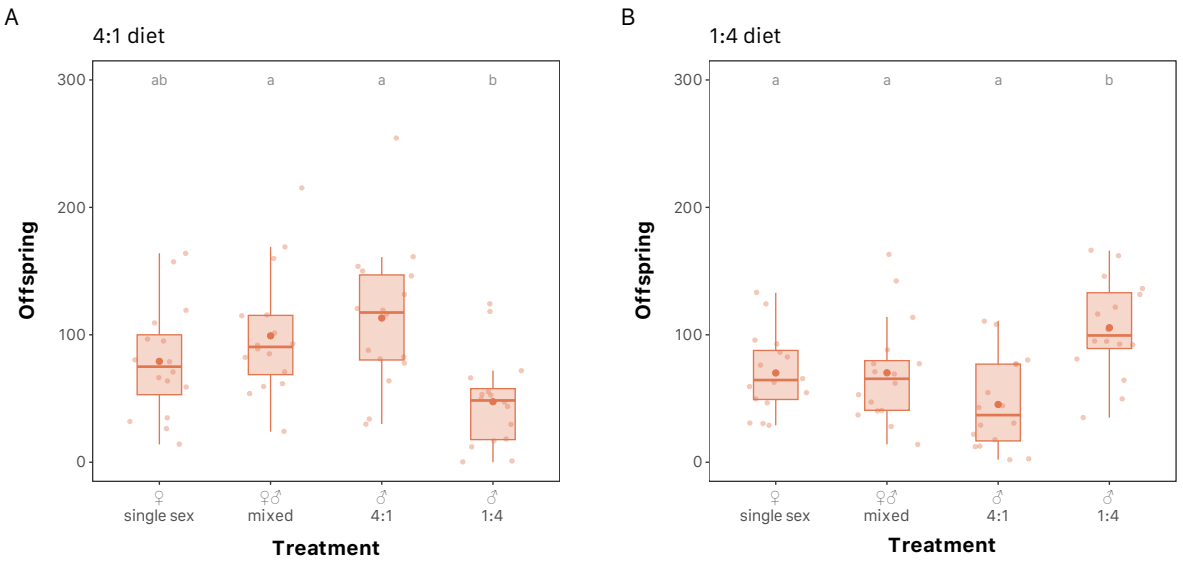

Figure 3

Female oviposition choice for the low-protein diet was significantly affected by cues of males. Shown is the number of offspring produced from 24h samples of eggs laid by females on the 4:1 (protein: carbohydrate) diet **(A)** or 1:4 (protein: carbohydrate) diet **(B)** between the four socio-sexual treatments: ♀ single sex (10 females), ♀♂ mixed sex (freely interacting 10 females and 10 unrestricted males), ♂ 4:1 (10 females with 10 males restricted to the 4:1 patch), and ♂ 1:4 (10 females with 10 males restricted to the 1:4 patch). Boxes represent interquartile range (IQR) with whiskers as 1.5 x IQR. Small circles represent raw data. Medians are presented as thick horizontal lines and means as large, filled circles. Letters indicate significant differences from post-hoc Tukey tests.

Experiment two - effect of indirect exposure to males or females on female diet choice

a) Female feeding was not altered by male or female cues

In the second follow-up experiment, we tested the effect of the sex of the restricted flies on female diet choice by including two additional treatments: ♀4:1 (unrestricted focal females with non-focal females restricted on the 4:1 patch) and ♀1:4 (unrestricted focal females with non-focal females restricted on the 1:4 patch). There was no effect of any of the restricted treatments on female preference for being on any food ($\chi^2_4 = 0.1$, $P = 1$; Figure 4).

The proportion of females on any diet patch differed between days ($\chi^2_1 = 710.4$, $P < 0.001$), which was accounted for within the statistical model.

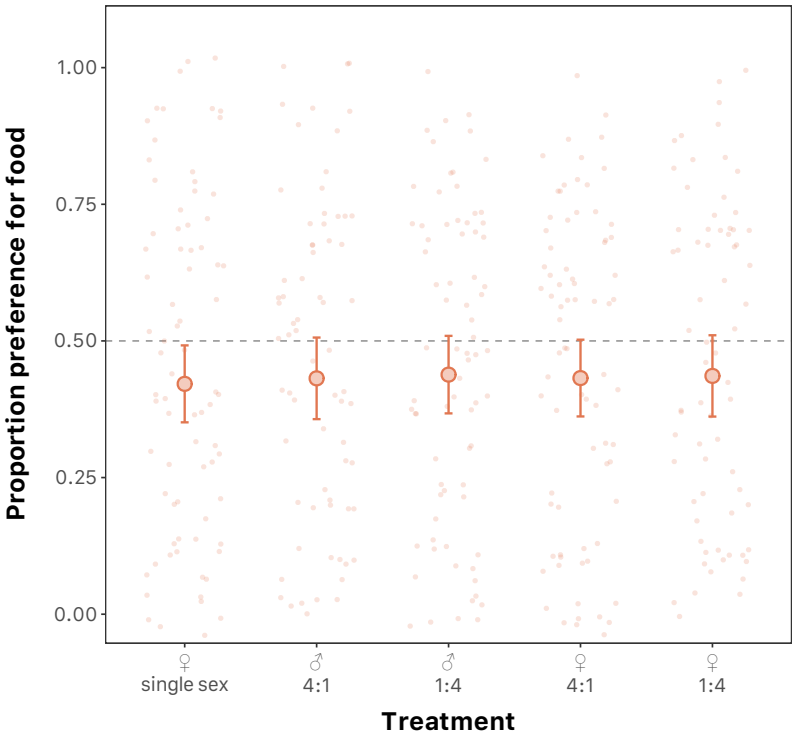

Figure 4

Foraging for either diet was not significantly affected by cues of males or females. Shown is the proportion of females present on food between the five socio-sexual treatments: ♀ single sex (10 females), ♂ 4:1 (10 females with 10 males restricted to the 4:1 protein: carbohydrate patch), ♂ 1:4 (10 females with 10 males restricted to the 1:4 protein: carbohydrate patch), ♀ 4:1 (10 females with 10 females restricted to the 4:1 protein: carbohydrate patch), and ♀1:4 (10 females with 10 females restricted to the 4:1 protein: carbohydrate patch). The total number of females on either food patch was divided by the total number of females (always 10) to provide an overall proportion score of food preference. Large circles represent estimated marginal means (± 95 % confidence intervals) extracted from the linear mixed model to account for repeated observations. Small circles represent raw data and 50 % preference is marked by the grey, dashed line. Letters indicate significant differences from a post-hoc Tukey test.

b) Female diet preference was affected by social cues

In the second experiment to test the effect of male and female cues on female foraging choices, females showed a significant preference for the 4:1 diet (Figure 5), as predicted. There was a significant impact of socio-sexual treatment on female preference for the 4:1 diet ($\chi^2_4 = 52.8$, $P < 0.001$), with preference shifted toward patches that contained restricted conspecifics of either sex. For example, when conspecifics were restricted on the 4:1 diet, preference for the 4:1 diet remained consistently high (single sex versus males on 4:1 and single sex versus females on 4:1; $t_{360} = -1.2$, $P = 0.7$ in both cases). Preference for the 4:1 diet decreased significantly when conspecifics were restricted on the 1:4 diet, suggesting a shift in preference toward the 1:4 diet. There was a significant effect of day on female diet preference accounted for within the statistical model ($\chi^2_1 = 50.7$, $P < 0.001$).

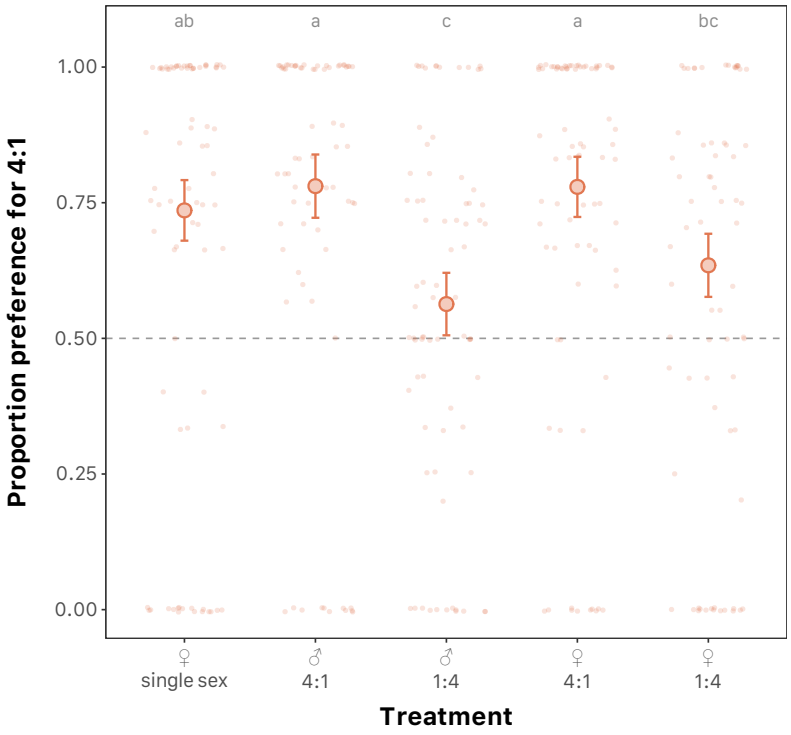

Figure 5

Female foraging preference for high-protein was significantly reduced when cues of males or females were present on low-protein. Shown is the proportion of females on the 4:1 (protein: carbohydrate) patch versus the 1:4 (protein: carbohydrate) patch between the five socio-sexual treatments: ♀ single sex (10 females), ♂ 4:1 (10 females with 10 males restricted to the 4:1 patch), ♂

1:4 (10 females with 10 males restricted to the 1:4 patch), ♀ 4:1 (10 females with 10 females restricted to the 4:1 patch), and ♀1:4 (10 females with 10 females restricted to the 4:1 patch). The number of females on the 4:1 patch was divided by the total number of flies on both patches to provide a proportion score of 4:1 preference. Large circles represent estimated marginal means (± 95 % confidence intervals) extracted from the linear mixed model to account for repeated observations and model weights. Small circles represent raw data and 50 % preference is marked by the grey, dashed line. Letters indicate significant differences from a post-hoc Tukey test.

c) Oviposition decisions were influenced by both diet and social cues

Consistent with the first experiment, numbers of offspring generated from the 4:1 or 1:4 patches were significantly dependent on socio-sexual treatment ($\chi^2_4 = 13.4$, $P < 0.01$ for offspring from 4:1 patches, $\chi^2_4 = 16.1$, $P < 0.01$ for offspring from 1:4 patches). More offspring were produced from 4:1 patches when there were cues of other females being present on those patches, compared with the absence of conspecific cues on any patches (single sex versus ♀4:1, Figure 6A). Similarly, more flies were produced from 1:4 patches when there were cues of conspecifics of either sex being present on those patches, compared with cues of females being present on 4:1 patches instead (Figure 6B).

The number of eggs laid on the surface of each patch closely aligned with the offspring results (Supplementary Figure S8). Overall, the mean total offspring on each diet patch was 154 (± 11) and 103 (± 10), for 4:1 and 1:4 respectively ($N = 45$ patches per diet). As in experiment one, the total number of offspring produced per arena (summed across both diets) was consistent among the five treatments ($\chi^2_4 = 4.5$, $P = 0.3$; Supplementary Figure S9).

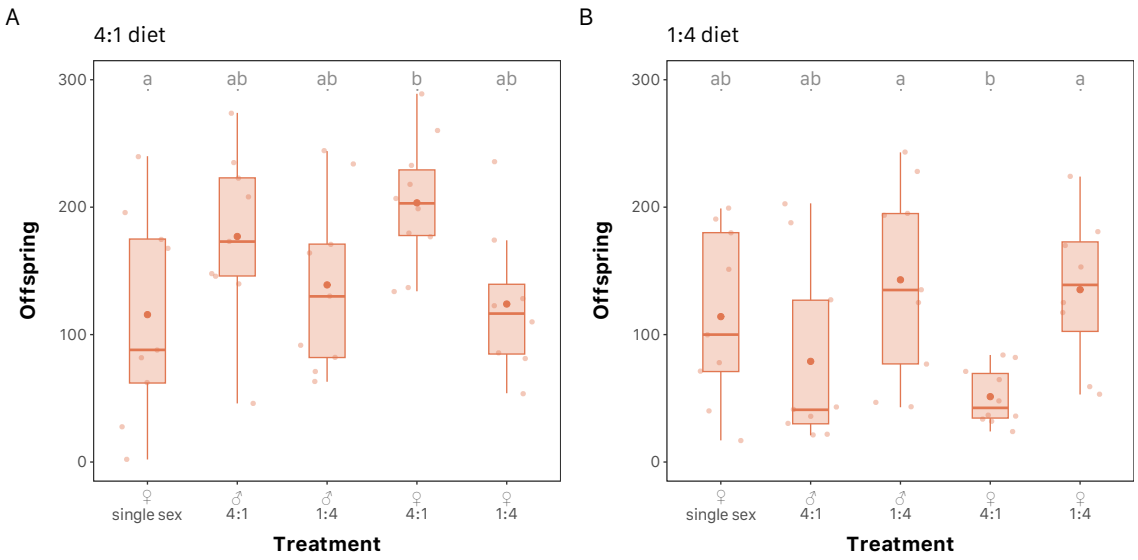

Figure 6

Female oviposition choice was significantly affected by cues of males or females. Shown is the number of offspring produced from 24h samples of eggs laid by females on the 4:1 (protein: carbohydrate) diet (A) or 1:4 (protein: carbohydrate) diet (B) between the five socio-sexual treatments: ♀ single sex (10 females), ♂ 4:1 (10 females with 10 males restricted to the 4:1 patch), ♂ 1:4 (10 females with 10 males restricted to the 1:4 patch), ♀ 4:1 (10 females with 10 females restricted to the 4:1 patch), and ♀1:4 (10 females with 10 females restricted to the 4:1 patch). Boxes represent interquartile range (IQR) with whiskers as 1.5 x IQR. Small circles represent raw data. Medians are presented as thick horizontal lines and means as large, filled circles. Letters indicate significant differences from post-hoc Tukey tests.

DISCUSSION

We tested whether female foraging and reproductive behaviour was altered in response to the risk of sexual harassment and the presence of socio-sexual cues. In the first experiment, we manipulated direct and indirect exposure of mated, female flies to male flies. Contrary to our prediction that females would avoid cues of male presence, females were attracted to diet patches that held restricted males. Surprisingly, this attraction was strong enough to reverse female preference for high-protein food, suggesting that attraction to socio-sexual cues overrode preferences based on macronutrient content. Results from the second experiment showed that females were attracted to diet patches on which conspecifics were present, regardless of the sex of those conspecifics. We also found that direct interactions with males drove females away from food patches altogether.

Females were attracted to cues of conspecifics

We predicted that females would avoid cues of male presence (prediction 3). However, there was a significant increase in female presence on food patches that held restricted males. Our second experiment showed that females were not attracted to restricted males specifically, but rather to food patches with cues of conspecifics of either sex. In fact, cues from conspecifics reversed female preference for high-protein food in the first experiment and weakened its strength in the second experiment. Previous studies have reported a similar preference for joining social groups in female *D. melanogaster* (Saltz, 2011; Lihoreau *et al.*, 2016a; Geiger & Saltz, 2020).

Female attraction to food patches with conspecifics might yield several benefits that are not mutually exclusive. One possibility is that food patches with conspecifics could provide social information on food availability or quality (Lihoreau *et al.*, 2018). There is evidence that foragers use social information in several species; for example, in cockroaches, *Blattella germanica* (Lihoreau *et al.*, 2010), desert locusts, *Schistocerca gregaria* (Günzel *et al.*, 2023) and our study species (Lihoreau *et al.*, 2016a; Geiger & Saltz, 2020). Another possibility is that group membership reduces predation risk, for example through the dilution effect (Lehtonen & Jaatinen, 2016). A related hypothesis is that females approach groups of conspecifics to dilute the risk of sexual harassment. For example, female eastern mosquito fish, *G. holbrooki*, move closer to conspecific females when a male, but not a

female, is presented, and females approach groups of males more often than they approach individual males, as male-male competition may reduce an individual's risk of harassment from males (Dadda *et al.*, 2005, 2008). If female attraction to food patches with conspecifics is adaptive, then benefits from associating with conspecifics must outweigh the costs of sexual harassment from males and of competition with other females for food and oviposition sites, as well as increased competition for offspring during development (Wertheim *et al.*, 2006).

Female attraction to cues of conspecifics might have been mediated by social odorants (reviewed by Wertheim *et al.*, 2005). Cuticular hydrocarbons in *D. melanogaster* act as both close- and long-range pheromones (Bartelt *et al.*, 1985; Dweck *et al.*, 2015), and males and females can emit species-specific attractant pheromones following transfer from the male to the female during mating (Bartelt *et al.*, 1985; Wertheim, 2005). This can lead to the formation of large aggregations (Wertheim *et al.*, 2006). In addition, social odorants interact with cues of nutritional content; females show a much lower preference for food odorants than do starved females, but addition of the *D. melanogaster* sex pheromone, cis-vaccenyl acetate, increased attractiveness of food odorants to fed females (Lebreton et al., 2015). Aggregation pheromones, and possible interaction with nutritional cues, may be a mechanism for our results in both the first and second experiments to attract focal females to diet patches, regardless of the sex of the emitter (Wertheim *et al.*, 2002, 2005; Lebreton *et al.*, 2015).

There was no strong preference for the 4:1 P:C diet when females were held in single-sex groups in the first experiment (prediction 1), which was contrary to our predictions and previous observations in female insects, including *D. melanogaster* specifically (e.g., Maklakov *et al.*, 2008; Lee *et al.*, 2013; Camus *et al.*, 2018, but see Lihoreau *et al.*, 2016b). It is unclear why females did not display a clear preference in this treatment. However, we did observe the expected strong preference for 4:1 P:C diet in both the preliminary experiment and the second experiment. The reason for the discrepancy is not clear.

In both experiments, the number of offspring from a food patch was highest when conspecifics were restricted on that food patch. This finding is in alignment with the results for female patch choice, suggesting that females simply laid eggs on the patches they fed

from. However, other studies have reported that female *D. melanogaster* preferences for oviposition and feeding can be uncoupled (Wertheim *et al.*, 2002; Lihoreau *et al.*, 2016b) and the macronutrient content of oviposition substrate chosen by females is important for larval development and survival (Rodrigues *et al.*, 2015). In this study, it is possible that social cues influenced oviposition decisions in addition to macronutrient content.

Direct interactions with males reduced female foraging but not oviposition

We expected to find disrupted foraging in the mixed-sex treatment, where males could physically disturb females (prediction 2). There was no evidence for a change in female diet choice when females interacted directly with males, compared with single-sex female groups. In contrast, changes to female diet choices in response to male harassment have been observed in the solitary bee *Anthophora plumipes*, in which females avoid nectar-rich flowers that are associated with high male harassment (Stone, 1995). In *D. melanogaster*, harassment alters female feeding choice via learning, because harassed females have less time to associate aversive stimuli with particular diets (Teseo *et al.*, 2016). However, we did observe females on either food significantly less often when females interacted directly with males. This result is consistent with observations that harassment physically disrupts feeding in other species (Magurran & Seghers, 1994; Rowe, 1994; Stone, 1995; Griffiths, 1996; Herberstein *et al.*, 2002; Pilastro *et al.*, 2003; Plath *et al.*, 2003; Tobler *et al.*, 2011; Teseo *et al.*, 2016). For example, in the mosquitofish, *G. holbrooki*, females take longer to initiate feeding and ate less per minute when a male was present (Pilastro *et al.*, 2003). In guppies (*Poecilia reticulata*) and in water strider (*Aquarius remigis*), females forage less when they are subject to frequent male harassment (Sih & Watters, 2005; Yang *et al.*, 2023). The observation of disruption to female food intake from male harassment in these diverse taxa suggests that this pattern might be widespread.

Although females spent less time on food patches in the mixed-sex treatment, we found that offspring production was similar to the single sex treatment, which suggests that sexual harassment did not affect oviposition rates. This result contrasts with a previous finding of reduced oviposition by female *D. melanogaster* under frequent male harassment (Teseo *et al.*, 2016). It is possible that a reduced opportunity for oviposition in the mixed sex treatment could have been masked by females re-mating, which might have increased

fecundity. However, any effect of re-mating is likely to have been minimal because female re-mating rate is low within 24 hours (Singh & Singh, 2004), as was noted observationally in this study. Therefore, further study of female and male movement is needed to identify finer-scale disruptions to feeding and oviposition from male harassment.

CONCLUSION

Our findings suggest that female feeding decisions are altered by the presence of social partners or their cues. It is surprising that socio-sexual cues weakened or reversed female preference for high-protein food, given the strong association between protein intake and reproduction, and the risk of male harassment, which may harm females and disrupt foraging. Further study manipulating the size and sex ratio of social groups and pheromonal cues (e.g., by artificially seeding cuticular extracts onto foods) in a diet choice assay would help to uncover the mechanisms underlying these observations. It would be fruitful to test whether female *D. melanogaster* experience a dilution of sexual harassment in larger groups (Pilastro *et al.*, 2003), and investigate the influence of collective decision making on female foraging behaviour under sexual harassment, by testing the feeding choices of smaller groups of focal females, or even individuals.

Data availability

URL to dataset in an online repository will be added to manuscript upon acceptance for publication.

Author contributions

M.C.S. conceptualisation, data curation, formal analysis, investigation, methodology, visualisation, writing – original draft, writing – review & editing. J.C.P. conceptualisation, funding acquisition, resources, supervision, writing – original draft, writing – review & editing. T.C. conceptualisation, funding acquisition, methodology, project administration, resources, supervision, writing – original draft, writing – review & editing.

Competing interests

The authors declare no conflicts of interest.

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Supplementary Information for Socio-sexual cues shape female diet choice in *Drosophila melanogaster*

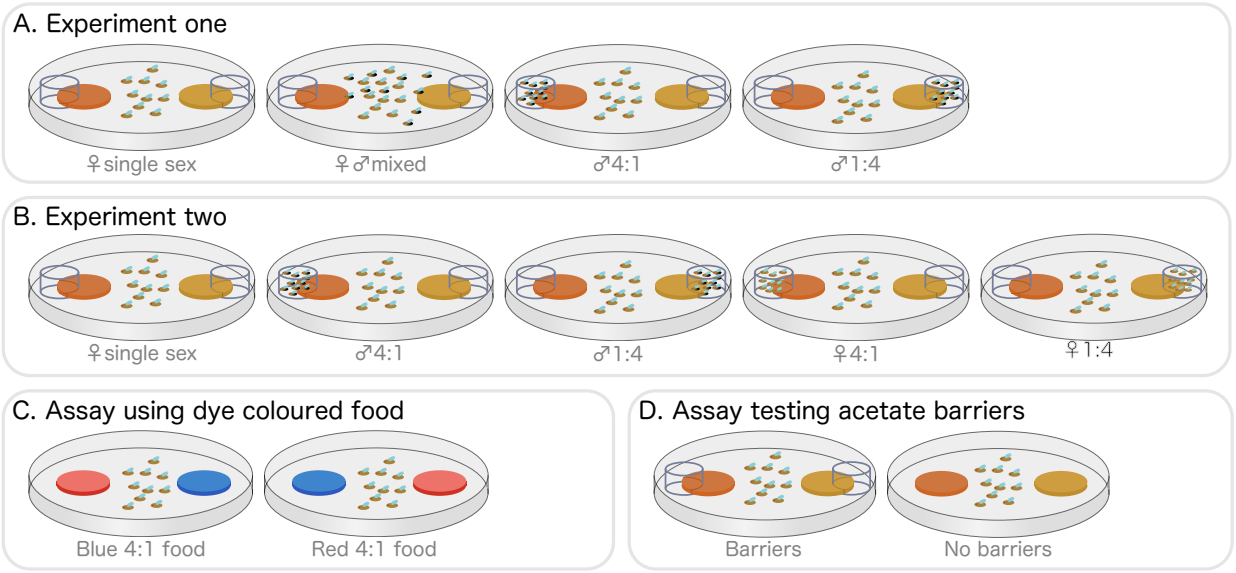

Figure S1.

Experimental design of the diet choice and behavioural assays. In all treatments and experiments, 10 mated females were added to Petri dishes containing a disc of 4:1 protein: carbohydrate and 1:4 protein: carbohydrate diet. Lighter fly icons represent female flies, and darker fly icons represent male flies. **(A)** Experiment one: ♀ single sex (10 females), ♀♂ mixed sex (freely interacting 10 females and 10 unrestricted males), ♂ 4:1 (10 females with 10 males restricted to the 4:1 diet patch), and ♂ 1:4 (10 females with 10 males restricted to the 1:4 diet patch). **(B)** Experiment two: ♀ single sex (10 females), ♂ 4:1 (10 females with 10 males restricted to the 4:1 diet patch), and ♂ 1:4 (10 females with 10 males restricted to the 1:4 diet patch), ♀ 4:1 (10 females with 10 females restricted to the 4:1 diet patch), and ♀ 1:4 (10 females with 10 females restricted to the 1:4 diet patch). **(C)** Assay using dye coloured food: blue 4:1 food (4:1 diet containing ingestible blue dye and 1:4 diet containing ingestible red dye) and red 4:1 food (4:1 diet containing ingestible red dye and 1:4 diet containing ingestible blue dye). **(D)** Assay testing acetate barriers: barriers (discs of 4:1 and 1:4 diet both hold perforated, empty acetate ring) and no barriers (neither discs of 4:1 or 1:4 diet hold an acetate ring).

Supplementary methods for assay using dye coloured food

For red and blue diets, 4:1 and 1:4 protein: carbohydrate diets were additionally dyed with red and blue pigment, which can be observed externally within fly guts following ingestion (Ribeiro & Dickson, 2010). 125 mg of powdered red dye (amaranth, Sigma A1016) and 50 mg of powdered blue dye (indigo carmine, Sigma 131164) were added to separate 250 ml aliquots of each diet, to create four diet types (blue 4:1, blue 1:4, red 4:1 and red 1:4). All diets were autoclaved at 120 °C for 15 minutes for sterilisation and poured into trays at approximately 5 mm thickness to set, as in the main experiment. Diets were stored at 2 °C until use.

Diets were cut using a 26 mm circular cutter, and one of each P:C content were added to each observation arena ($N = 28$), e.g., half of the arenas contained one patch of blue 4:1 and one of red 1:4, while the other half of arenas contained one patch of blue 1:4 and one of red 4:1, to control for colour of diet influencing female choice. Arenas were then randomised using coded identifiers, to anonymise the treatments from the perspective of the observer.

Behavioural observations of female patch preference

Three-day old, mated females were collected from a second generation of controlled rearing (by the method described in the methods section) and added to the blue and red food arenas in single sex groups of 10 using CO_2 anaesthesia. Arenas were then moved to 25 °C and left to acclimatise for 2 hours. Food patch preference scores were obtained for each arena by scan sampling the number of individuals on each of three locations (blue patch, red patch or dish) every 0.5 h, for 2.5 h.

After the last observation, focal female flies were anaesthetised using CO_2 and photos were taken of each individual's abdomen using a digital microscope camera (GXCAM, GT Vision Ltd; $N = 280$ photos). Camera settings were fixed for all photos. Photos were edited post-production to adjust contrast, with edits applied identically to all photos using the Mac

Photos app (Version 9.0, Apple Inc.). To determine abdomen hue, five observers were each provided with the photos in a randomised order in Microsoft PowerPoint and were asked to score the colour of each fly's abdomen using a reference colour score bar: 0 – no colour, 1 – blue, 2 – purple (mixed diet) or 3 – red (Supplementary Figure S2).

Analysis

The effect of diet colour on diet preference was analysed by using a binomial GLMM, in which the number of flies on each patch was combined using the cbind function. Arena ID and observation were included as nested random factors.

Location of females aligned with the colour of dye in their abdomen

Females in all treatments showed the strong, expected preference for the high protein 4:1 food (Supplementary Figure S3). This preference was observed regardless of the colour of the diet patches. Treatment (colour of the 4:1 food) did not affect the number of females on each diet patch ($\chi^2_1 = 0.2$, $P = 0.69$). The proportion of abdominal images scored as red/blue within each treatment also closely aligned to the observed preferences for 4:1 observed in the behavioural data (Supplementary Figure S4).

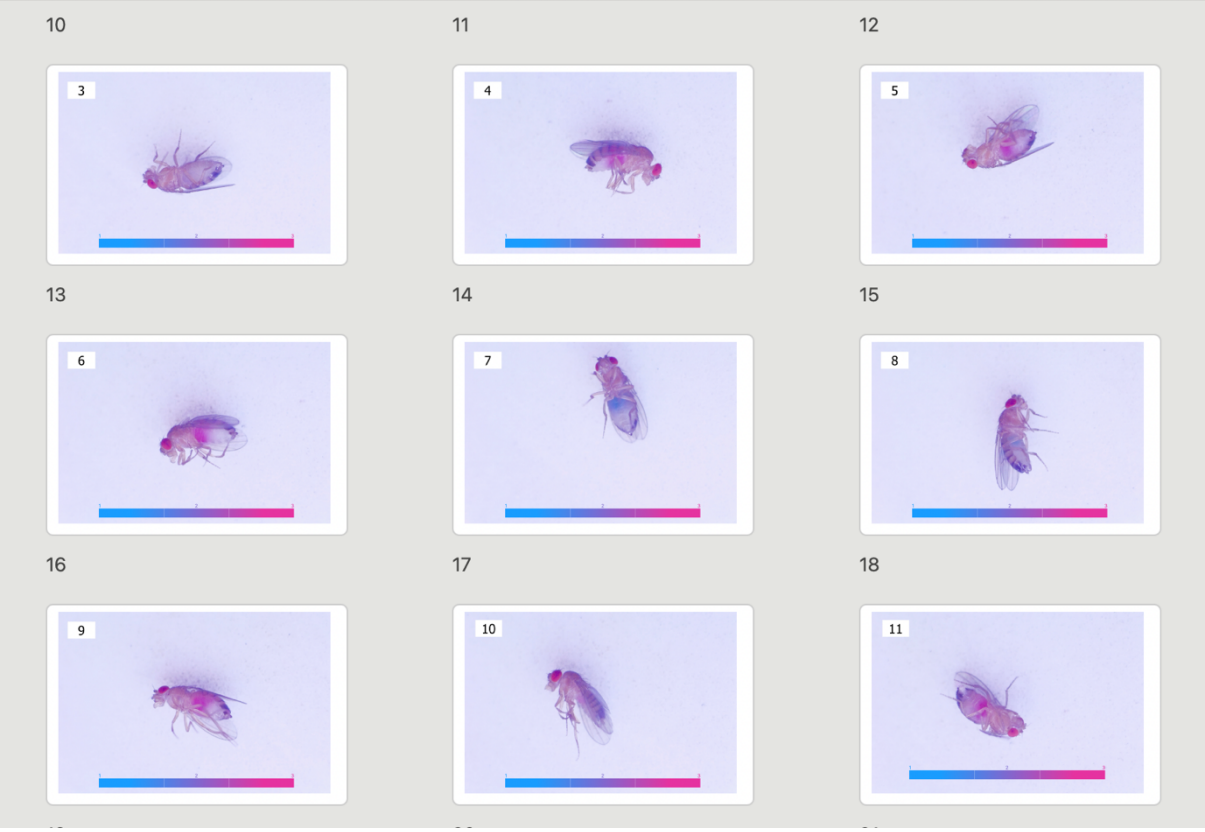

Figure S2

Subset of 9 example images scored during the diet preference for coloured patches assay. Images of female flies were presented to scorers on individual slides using Microsoft PowerPoint (version 16.86) and each image contained the reference colour scoring bar, and a randomised ID. Scorers were instructed to give each fly a score of 0 (no food), 1 (blue abdomen), 2 (purple abdomen) or 3 (red abdomen).

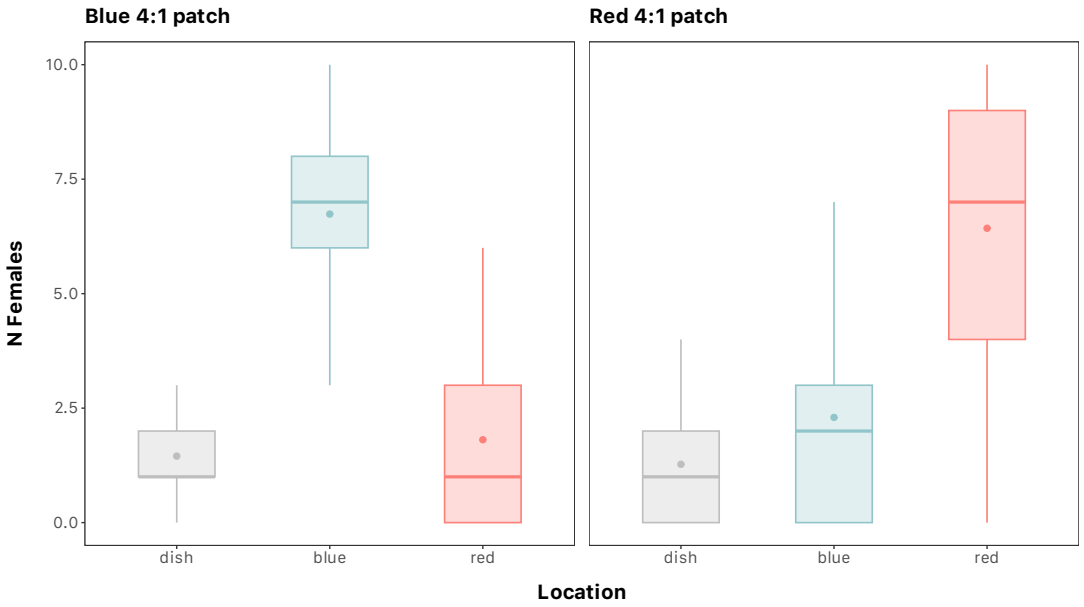

Figure S3

The number of female flies resting on the 1:4 and 4:1 blue and red diets or on the dish across six observations of 28 arenas. Boxes represent IQR with whiskers as 1.5 x IQR. Medians are presented as thick horizontal lines and means as large, filled circles.

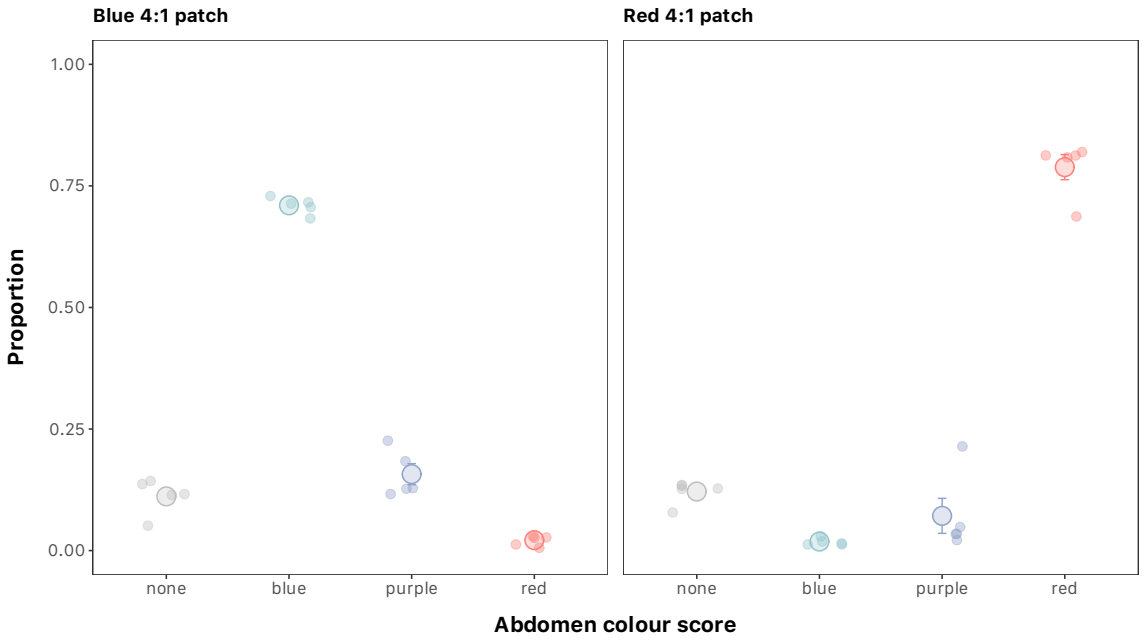

Figure S4

The proportion of scores assigned to photos of female flies after feeding in arenas with two patches of dyed diets (either blue 4:1 and red 1:4 patch, or red 4:1 and blue 1:4 patch) (N = 280 females). Scores were obtained from observers marking the colour of each fly's abdomen in photos as none, blue, purple or red. Large, coloured circles represent the average proportion of photos scored as either none, blue, purple, or red, with black, vertical lines as standard error. Small circles represent the proportion per observer.

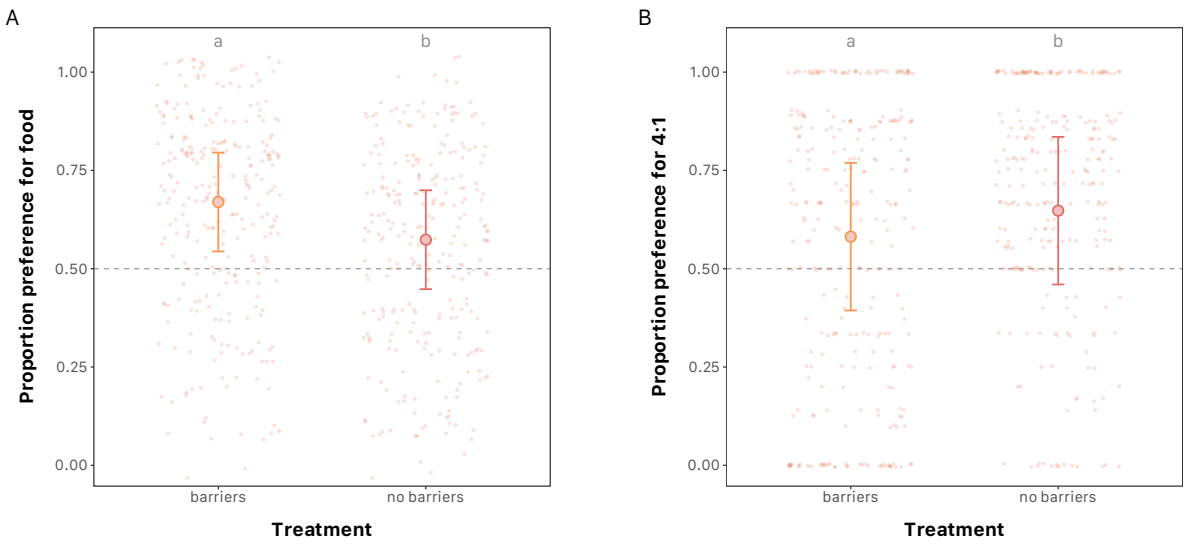

Figure S5

Pilot study testing the effect of transparent, perforated, acetate barriers on diet preference. 10 mated female flies were added to small arenas containing two solid diet patches (4:1 and 1:4, methods as reported main methods in experiment one and two). In half of the samples, acetate rings were added to both diets. Female location was recording using scan sampling. Large circles represent estimated marginal mean values (± 95 % confidence intervals) extracted from the linear mixed model to account for repeated observations and model weights. Raw, unweighted data from all observations is underlaid as small circles. Significance levels are represented as letters above each treatment to visualise results of pairwise comparisons between groups from a post-hoc Tukey test.

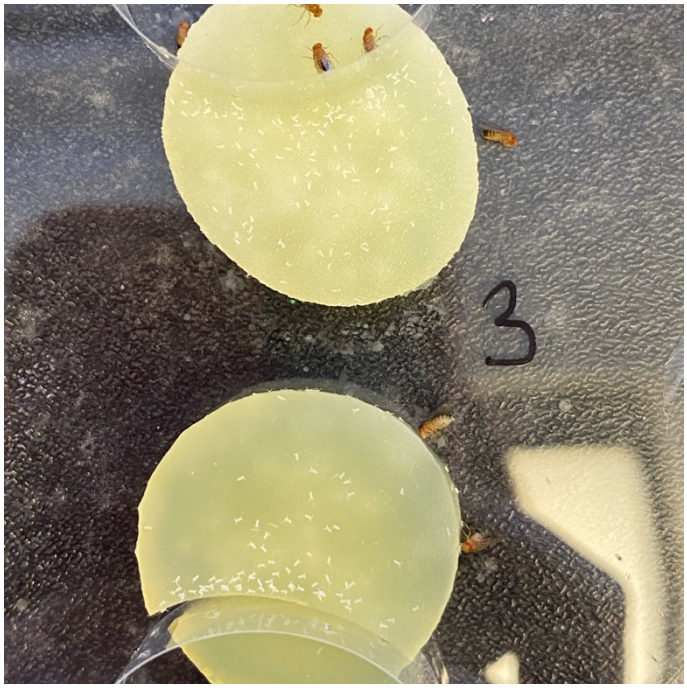

Figure S6

Example image of eggs laid on the surface of 4:1 and 1:4 diet patches, in an observation arena, by 10 females, 24 hours after behavioural observations started. Flies were anaesthetised using gaseous CO_2 (to allow removal of the patches to rear offspring in standard conditions, in vials containing 7 ml sugar-yeast-agar diet) at which point a photo was taken. Eggs were counted later using ImageJ Fiji software (Schindelin et al., 2012). Only visible eggs on the top surface of each patch were counted, to provide a proxy number of egg laying rates.

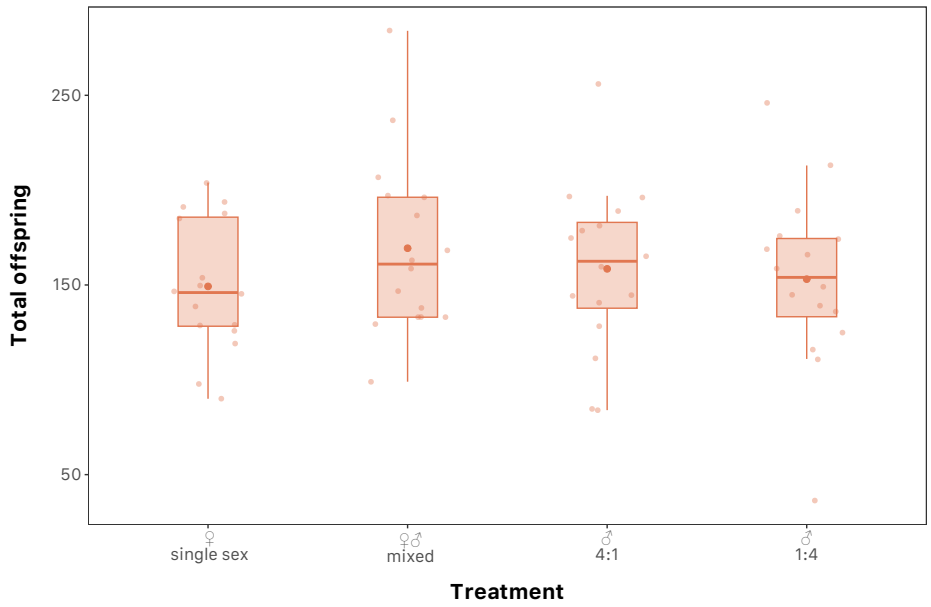

Figure S7

The total number of offspring produced across four socio-sexual treatments: alone; females remained alone, mixed; free non-focal males added to arena, ♂4:1; non-focal males added to restricted area on the 4:1 patch, and ♂1:4; non-focal males added to restricted area on the 1:4 patch (experiment one). Offspring numbers are summed across the two diet patches in each arena (individual values are represented by small circles overlaid on corresponding treatment). Boxes represent IQR with whiskers as 1.5 x IQR, medians are presented as thick horizontal lines and means as large, filled circles.

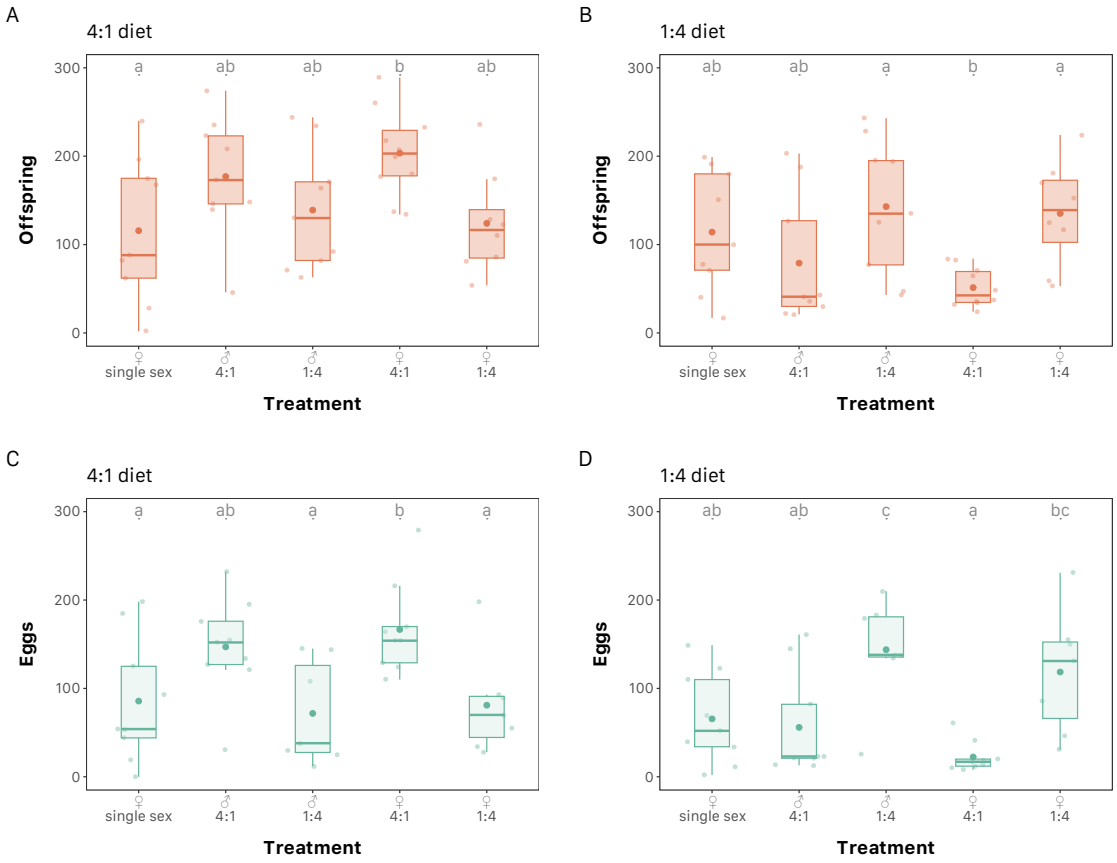

Figure S8

The total number of offspring produced on the 4:1 protein: carbohydrate **(A)** or 1:4 protein: carbohydrate **(B)** diets from 24h samples of eggs laid by females on the 4:1 **(C)** or 1:4 **(D)** diets (e.g., Supplementary Figure S6). Egg and offspring counts produced across five socio-sexual treatments in experiment two: ♀ single sex (10 females), ♂ 4:1 (10 females with 10 males restricted to the 4:1 patch), ♂ 1:4 (10 females with 10 males restricted to the 1:4 patch), ♀ 4:1 (10 females with 10 females restricted to the 4:1 patch), and ♀1:4 (10 females with 10 females restricted to the 4:1 patch). Individual values are represented by small circles overlaid on corresponding treatment. Boxes represent IQR with whiskers as 1.5 x IQR, medians are presented as thick horizontal lines and means as large, filled circles.

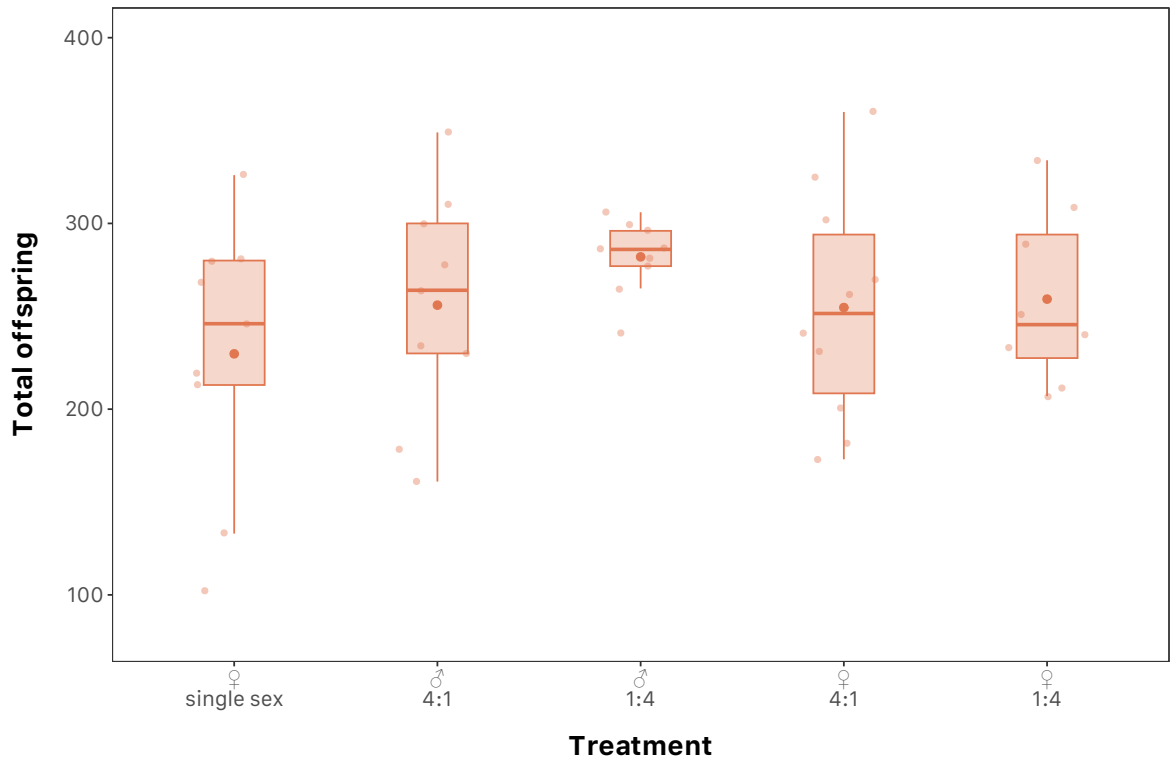

Figure S9

The total number of offspring produced across five socio-sexual treatments in experiment two: ♀ single sex (10 females), ♂ 4:1 (10 females with 10 males restricted to the 4:1 patch), ♂ 1:4 (10 females with 10 males restricted to the 1:4 patch), ♀ 4:1 (10 females with 10 females restricted to the 4:1 patch), and ♀1:4 (10 females with 10 females restricted to the 4:1 patch). Offspring numbers are summed across the two diet patches in each arena (individual values are represented by small circles overlaid on corresponding treatment). Boxes represent IQR with whiskers as 1.5 x IQR, medians are presented as thick horizontal lines and means as large, filled circles.

Table S1. Diet quantities required for 1.5 L of solid meridic diet of varying P:C ratios, based on a combined caloric concentration of 120 g/L.

P:C ratio	1:2	1:4	1:5	1:8	4:1
Casein (g)	60	36	30	20	120
Sucrose (g)	120	144	150	160	60
Cholesterol (g)	0.45	0.45	0.45	0.45	0.45
Lecithin (g)	6	6	6	6	6
Agar (g)	30	30	30	30	30
KH_2PO_4 (ml)	150	150	150	150	150
K_2HPO_4 (ml)	150	150	150	150	150
$MgSO_4$ (ml)	150	150	150	150	150
$NaHCO_3$ (ml)	150	150	150	150	150
Nucleic acid sol (ml)	150	150	150	150	150
De-ionised H_2O (ml)	300	300	300	300	300
Autoclave the mixture at this stage					
10% Nipagin-ethanol solution (ml)	15	15	15	15	15
Propionic acid (ml)	4.5	4.5	4.5	4.5	4.5
Vitamin mix (ml)	225	225	225	225	225
De-ionised H_2O (ml)	Top up to 1.5 L	Top up to 1.5 L	Top up to 1.5 L	Top up to 1.5 L	Top up to 1.5 L

Salt solutions contain 2 L de-ionised H_2O and each salt in solid form (14.22 g KH_2PO_4, 74.6 g K_2HPO_4, 12.4 g $MgSO_4$, 20 g $NaHCO_3$). Nucleic acid solution contains 11.4 g Uridine, 12.8 g Inosine, 2 litres de-ionised H_2O. Diet adapted from Piper and colleagues (Piper et al., 2014).

865 Table S2. Recipe to make 2 L vitamin mix

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Ingredient	Quantity
Thiamine (g)	0.0267
Riboflavin (g)	0.133
Nicotinic acid (g)	0.16
Ca Pantothenate (g)	0.222
Pyridoxine (g)	0.033
Biotin solution (ml)	133
Folic acid solution (ml)	133
De-ionised H2O (ml)	1733

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868 Biotin solution made from 0.01g Biotin and 500ml de-ionised H_2O. Folic Acid solution made from
869 0.119g Folic Acid, 80ml ethanol and 320ml de-ionised H_2O. Both made in 500ml conical flasks and
870 stored in fridge until use.

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875 Table S3

876 Statistical linear models used to analyse data reported in the main manuscript, with test statistic,
877 degrees of freedom and P value reported. All models were carried out in R version 4.0.4 (The R
878 Foundation for Statistical Computing, Vienna, Austria, http://www.r-project.org).

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Test	Model	Outcome
Experiment 1		
Food preference	glmmTMB(foodPref ~ treatment + block + (1|uniqueDay) + (1|uniqueObs) + (1|id_new), data)	a) Treatment χ^2_3=12.6, P=0.00565 b) Block χ^2_1=27.4, P<0.001
4:1 preference	glmmTMB(propPref ~ treatment + block + (1|uniqueDay) + (1|uniqueObs) + (1|id_new), weights = totalfood, data)	a) Treatment χ^2_3=65.1, P<0.001 b) Block χ^2_1=0.5, P=0.4924
4:1 offspring	glmmTMB(adult.offspring ~ treatment, data)	a) Treatment χ^2_3=15, P=0.00185
1:4 offspring	glmmTMB(adult.offspring ~ treatment, data)	a) Treatment χ^2_3=24.4, P<0.001
Total offspring	glmmTMB(total ~ treatment, data)	a) Treatment χ^2_3=2.1, P=0.5583
Experiment 2		
Food preference	glmmTMB(foodpreference ~ treatment + day + (1|observation) + (1|id), data)	a) Treatment χ^2_4=0.1, P=0.998 b) Day χ^2_1=710.4, P<0.001
4:1 preference	glmmTMB(prefprop ~ treatment + day + (1|observation) + (1|id), weights = totalfood, data)	a) Treatment χ^2_4=52.8, P<0.001 b) Day χ^2_1=50.7, P<0.001
4:1 offspring	glmmTMB(adult_offspring ~ treatment, data)	a) Treatment χ^2_4=13.4, P=0.009
1:4 offspring	glmmTMB(adult_offspring ~ treatment, data)	a) Treatment χ^2_4=16.1, P=0.002878
Total offspring	glmmTMB(total ~ treatment, data)	a) Treatment χ^2_4=4.5, P=0.3478
4:1 eggs	glmmTMB(n.eggs ~ treatment, data)	a) Treatment χ^2_4=20.1, P<0.001

1:4 eggs	glmmTMB(n.eggs ~ treatment, data)	a) Treatment χ^2_4=30.4, P<0.001
Total eggs	glmmTMB(total ~ treatment, data)	a) Treatment χ^2_4=9.3, P=0.05345
Barrier pilot		
Food preference, barrier pilot	glmmTMB(foodpreference ~ treatment + block + (1|day) + (1|observation) + (1|id), data)	a) Treatment χ^2_1=8.6, P<0.01 b) Block χ^2_1=0.6, P=0.436381
4:1 preference, barrier pilot	glmmTMB(highpreference ~ treatment + block + (1|day) + (1|observation) + (1|id), weights = totalfood, data)	a) Treatment χ^2_1=4.7, P=0.003275 b) Block χ^2_1=0.001, P=0.97

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