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# Can developmental plasticity shape sexual competition and promote reproductive isolation?

Kristin Hubakk<sup>1,2</sup>, Zachariah Wylde<sup>1,2</sup> and Russell Bonduriansky<sup>1,2,\*,0</sup>

<sup>1</sup>Evolution & Ecology Research Centre, University of New South Wales, Sydney, NSW 2052, Australia <sup>2</sup>School of Biological, Earth and Environmental Sciences, University of New South Wales, Sydney, NSW 2052, Australia

Corresponding author: School of Biological, Earth and Environmental Sciences, University of New South Wales, Sydney, NSW 2052, Australia. Email: r.bonduriansky@unsw.edu.au

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Environmental factors, such as dietary nutrients, can shape the expression of developmentally plastic sexual traits in many species. However, while there has been extensive research into the developmental plasticity of sexual traits at the individual level, the broader consequences of this variation at the population scale remain poorly understood. Here, we asked whether plastic responses to the developmental environment can shape sexual competition and initiate reproductive isolation between populations. We reared neriid flies, *Telostylinus angusticollis*, on nutrient-rich and nutrient-poor larval diets, generating adult flies that differed in body size and secondary sexual trait expression. We then investigated sexual competition in experimental populations from each developmental environment and tested for reproductive isolation between flies from mismatched environments. We found that, compared with poor-diet populations, rich-diet populations exhibited more frequent and escalated male-male combat and more frequent mating and mate-guarding. However, we found no evidence that sexual selection was affected by the developmental environment. Mismatched pairs were not less likely to mate within 1 h or produce viable offspring. Our findings suggest that developmental plasticity could generate dramatic differences in sexual competition between populations and could contribute to reproductive isolation.

Key words: developmental environment; larval diet; mating behavior; Neriidae; plasticity; sexual competition; sexual dimorphism; sexual selection; Telostylinus angusticollis.

#### Introduction

Experimental evidence from many species shows that the availability of nutrients in the developmental environment can affect the development of adult secondary sexual traits, and thereby impact adult behavior and, potentially, fitness (e.g. Andersson 1982; Emlen 1994, 1997; Zikovitz and Agrawal 2013; Perdigón Ferreira and Lüpold 2022). In insects, males that manage to acquire more nutrients during development tend to grow to a larger adult body size and express relatively larger secondary sexual traits, relative to nutrient-limited individuals (Cotton et al. 2004). Similarly, females raised on a nutrient-rich diet are often more attractive to males (Han et al. 2020), more fecund (Blanckenhorn 2000), and more choosy (Hunt et al. 2005). However, we have limited understanding of the consequences of plasticity at the population level (Forsman 2015) and, in particular, its potential to shape sexual interactions and the evolution of mating systems (Malek and Long 2019; Cattelan et al. 2020).

Many studies have investigated how the developmental environment affects sexual trait expression by manipulating nutrients in the developmental diet (Cotton et al. 2004). Such manipulations are typically used to simulate natural variation in resource patches exploited by individual larvae, and investigate the resulting variation in individual adult sexual traits and performance (e.g. Zikovitz and Agrawal 2013; Plesnar-Bielak et al. 2017; Edmunds et al. 2021). However, developmental environments also vary on macroecological scales, and different populations can, therefore, experience unequal access to nutrients (Chown and Gaston 2010). For example, different *Onthophagus* beetle populations can subsist on dung from different species of mammals (Emlen 1997). If different habitats provide different quantities or qualities of developmental resources then genetically similar populations could differ in average expression of developmentally plastic sexual traits. Could this result in differences among populations in patterns of reproductive behavior and sexual selection?

At the population level, the strong developmental plasticity of sexual traits has the potential to shape sexual competition by influencing the form, frequency, and outcome of male-male and male-female interactions. For example, nutrient-rich resource patches may produce males that have larger weapons and engage in more intense combat interactions (Sentinella et al. 2013), and females that are more choosy or resistant to mating (Hunt et al. 2005). Heightened expression of sexual traits and increased choosiness in resource-rich developmental environments, therefore, has the potential to lead to changes in the form or intensity of sexual selection by comparison with resource-limited environments. Few studies have investigated such populationlevel effects. Cattelan et al. (2020) subjected guppies to high- or

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low-nutrient adult diets over 15 d and then investigated sexual behaviors and quantified sexual selection in experimental replicate populations derived from each diet treatment. They found that diet treatment affected the frequency of mating attempts, sperm production, and body colouration. Moreover, low-nutrient diets increased the opportunity for sexual selection and altered the pattern of sexual selection on male sexual traits. Morimoto et al. (2017) manipulated larval density to generate variation in resource availability for Drosophila melanogaster larvae and then investigated courtship rates, reproductive rates, and survival rates. They found that experimental populations of adult flies derived from the low larval density treatment group (i.e. abundant developmental resources) exhibited higher rates of courtship as well as altered reproductive timing. Winkler and Janicke (2022) manipulated larval diet in Tribolium beetles and found that low-quality diet increased the opportunity for selection on both sexes.

Differences between populations in phenotypic means for traits such as sexual signals or body size could also interfere with mate recognition or create mechanical barriers to mating or fertilization, and thereby promote reproductive isolation. While most research on reproductive isolation has focused on genetic differences between populations (Kulmuni et al. 2020), environmental factors could contribute to reproductive isolation in the absence of genetic differentiation. For example, in *Drosophila*, there is evidence that mate recognition can be disrupted by differences in the diet and consequent changes in the gut microbiome (Sharon et al. 2010) (although see Leftwich et al. 2017), and mate compatibility can be affected by *Wolbachia* endosymbionts (Richardson et al. 2019). Alternatively, plastic effects on trait expression could weaken reproductive isolation between populations. For example, if small females have a reduced ability to resist large males, differences in mean body size between populations could impede gene flow in one direction (males from the small-bodied population mating with females from the large-bodied population) but facilitate gene flow in the other direction (males from the largebodied population mating with females from the small-bodied population). We still know little about the potential for plastic responses to the developmental nutritional environment to induce reproductive barriers between populations.

In this study, we used the neriid fly T. angusticollis to investigate whether differences in developmental nutrition have the potential to generate differences between populations in sexual competition and induce reproductive isolation. Telostylinus angusticollis aggregates and breeds on rotting bark of several tree species, such as native Acacia longofolia and introduced Erythrina × sykesii, in New South Wales and southern Queensland, Australia (Bonduriansky 2006). Males have relatively elongated heads, antennae, and legs, and use these traits as signals and weapons in combat over females and territories (Fig. 1) (Bonduriansky, 2006). Abundant nutrients during larval development enhance adult body size in both sexes and dietary protein enhances the relative size of male secondary sexual traits (Sentinella et al. 2013). Males are especially developmentally plastic, such that variation in larval nutrition can result in a more than 10-fold difference in adult male body size and a difference of at least 2.25 standard deviations in head elongation relative to body size (Bonduriansky 2007; Sentinella et al. 2013). Larval nutrition also affects male combat behavior (Bath et al. 2012) and copulatory behavior (Fricke et al. 2015; Wylde et al. 2019b). The larval nutritional environment, therefore, has the potential to shape sexual competition in T. angusticollis populations and to induce some degree of reproductive isolation between populations that experience differential access to nutrients.



**Fig. 1.** Telostylinus angusticollis demonstrating a) a mating, b) a male guarding a female as she oviposits, and c) male–male combat (Photos: R. Bonduriansky).

We manipulated the larval diet to generate nutrient-rich and nutrient-poor nutritional environments. These experimental treatments could represent 2 different types of host-trees that differ in the availability of macronutrients (carbohydrates and protein) for T. angusticollis larvae. Telostylinus angusticollis adults can disperse between different host-trees and habitat patches (Kawasaki et al. 2008), and anthropogenic introduction of novel host-tree species could also provide novel nutritional environments for larvae. We asked whether the developmental environment (larval diet) would affect the pattern and intensity of sexual competition. We also asked whether the developmental environment would alter the intensity or pattern of sexual selection by quantifying opportunity for sexual selection, and by examining the relation between individual male body size and mating success. We expected that increased expression of male secondary sexual traits would result in increased opportunity for sexual selection, and perhaps stronger sexual selection on male body size, in experimental replicate populations reared on a nutrientrich larval diet. Finally, we asked whether female-male pairs from mismatched larval diets would exhibit reduced propensity to mate (indicating partial or complete pre-mating reproductive isolation) or reduced fertilization rates (indicating partial or complete post-mating reproductive isolation).

### Methods

#### Rearing and culturing of flies

The individuals used in the experiments described below were second-generation individuals reared from *T. angusticollis* collected from Fred Hollows Reserve in Randwick, New South Wales, Australia (33°54′44.04″S, 151°14′52.14″E). The flies were housed in population cages with moist cocopeat and were given 3 separate petri dishes containing brown sugar, yeast, and oviposition medium. The oviposition medium consisted of a nutrient-rich diet (as described below) that had been left to mold for approximately 4 ds and then mixed to encourage oviposition. The cages were sprayed with reverse osmosis (RO) water every second day. To generate focal flies for experiments, eggs were collected from oviposition medium in stock cages and transferred to 500 mL jars containing 200 mL of either a nutrient-rich diet ("rich

diet") or a nutrient-poor diet ("poor diet") to represent developmental environments differing in availability of macronutrients. Approximately 20 eggs were transferred to each container, with 19 containers for each diet treatment (N = 760 eggs in total). The rich diet consisted of 32.9 g of soy protein (Nature's Way Instant Natural Protein) and 41.3 g of brown sugar (Coles Brown Sugar), whereas the poor diet consisted of 5.5 g of soy protein and 6.9 g of brown sugar, mixed with 1 L dry cocopeat (coconut husk shavings), and approximately 800 mL RO water (Sentinella et al. 2013). Virgin adults were separated within 24 h after emergence into 12 L plastic tanks by sex and diet, with approximately 20 males or 25 females per tank (N = 18 tanks in total). Tanks contained adult flies of similar age, with flies from multiple larval containers combined randomly in adult tanks. Each tank contained a layer of moist cocopeat for humidity, and petri dishes containing an excess of brown sugar, yeast, and oviposition medium that were replaced approximately every 7 d. The tanks were sprayed with RO water every second day. Flies, larval medium containers, and experiments were all kept and conducted in a controlled lab environment with a temperature of 25 °C (±2 °C). The focal flies were used in 2 separate experiments, as described below.

### Sexual competition experiment

We observed a total of 13 experimental replicate populations (i.e. unique groups of 5 males and 5 females combined and observed inside an experimental arena) from each larval diet (N = 26 replicate populations in total). Each replicate population was created by combining 5 virgin females and 5 virgin males (all of similar age and reared on the same larval diet, and each male marked with a different color as described below) together in a transparent plexiglass arena  $(26 \times 36 \times 20 \text{ cm})$  with a mesh sleeve (Fig. 2). Replicate populations were comprised of focal individuals drawn randomly from the same adult tank (when possible), and focal individuals were not re-used in this experiment. Focal flies were between 14 and 45 d old when used in experiments. Flies reared on the poor larval diet emerged ~3 d later on average than flies reared on the rich larval diet, as typically observed in this species (Bonduriansky 2007; Hooper et al. 2017). However, since T. angusticollis can live for >130 d in the laboratory (Hooper et al. 2017), all focal flies were relatively young when used in experiments



Fig. 2. Experimental design to investigate sexual competition in populations of flies reared on rich (red, top) and (blue, bottom) poor larval diets.



Fig. 3. Experiment design to test for reproductive isolation between flies reared on rich (red, large symbols) and (blue, small symbols) poor larval diets.

and the small average difference in adult age between rich- and poor-diet focal flies is unlikely to have substantially influenced our results. The arena was designed to simulate natural mating aggregations on rotting tree bark, where females oviposit and feed and males compete for matings (Bonduriansky 2006, 2007). The bottom of the arena contained a layer of moist cocopeat, and a petri dish (5cm diameter) filled with oviposition medium was placed in the center. The arena was illuminated with a broad-spectrum light source placed above the oviposition medium to encourage interaction.

In order to identify individual males in replicate populations, the focal males were anesthetized with  $CO_2$  using a Flystuff Benchtop Flow Buddy System (59-122BCU, Genesee Scientific, USA) with Ultimate Fly Pad and Gun, and a spot of enamel paint (Tamiya Color Enamel Paint, Japan) was applied to the thorax, with each of the 5 males in each replicate population marked with a different color. The males were placed in individual vials to allow the paint to dry and for recovery from the anesthesia, and then kept in tanks with other males (as described above) for at least 24 h before being allocated to replicate populations for the experiment.

During each day of the experiment, we collected data on one rich-diet replicate population and one poor-diet replicate population using the same dish of oviposition medium. Each replicate population was left in the arena for 30 min to acclimatize and was then observed for 1 h. For each individual male, we recorded the duration and number of matings and mating attempts, number and duration of guarding bouts after mating, number of male wing flicking bouts, number and duration of combat interactions, and number of times the male was rejected by a female. Mating was recorded when a male positioned himself above or behind a female and mounted the female for at least 20 s (Bath et al. 2012; Wylde et al. 2019b). Mating duration was quantified as the time in seconds between a male mounting a female and removing his genitalia from the female's oviscape (Wylde et al. 2019b) (Fig. 1a). A mounting interaction that lasted less than 20 s was counted as a mating attempt. Female rejection was recorded when a female kicked a male or flew and/or ran away when a male attempted to mate with her (Bath et al. 2012). Guarding was identified as a male standing above a female after mating and using the span of his legs to enclose the female (Bonduriansky 2006) (Fig. 1b). Malemale combat interactions were recorded when 2 males used their forelegs and/or body to strike each other (Bath et al. 2012) (Fig. 1c). Wing flicking involved males flicking their wings at another individual (Wylde et al. 2019b).

#### Reproductive isolation experiment

To determine whether individuals reared on different larval diets can mate and produce offspring, males, and females were paired in all possible combinations (poor male with rich female, poor male with poor female, rich male with poor female, and rich male with rich female), with 23 replicates of each combination (Fig. 3). To ensure that the males were sexually experienced, males from the sexual competition experiment were re-used in the reproductive isolation experiment. However, all females were virgins. Each pair was placed in a 500 mL container with moist cocopeat on the bottom and a small petri dish of oviposition medium. After the female and male flies were combined, they were left to adjust for 10 s, and then observed for 1 h. We recorded latency to mate, duration and number of matings, number of mating attempts, and female rejection behaviors. Here, female rejection was evidenced by a female wing-flicking or kicking a male as he tried to mate, or when a female ran/flew away from a male during a mating attempt. On each day of the experiment, we set up one replicate of each of the 4 larval diet combinations (Groups 1 - 4 in Fig. 3). A broad-spectrum light source was positioned above the containers throughout the experiment.

After 1 h, the male was removed from the container and the female was left to lay eggs. Each oviposition dish was checked for eggs every day for a maximum of 6 d after the pairing was conducted. After 6 d, the female was removed and frozen for body size measurement. For each pair, 20 eggs (where possible) were transferred to a container with 200 mL of standard larval diet consisting of 11 g soy protein and 13.8 g brown sugar per 1 L of dry cocopeat, and 800 mL of water (Sentinella et al. 2013). After the first adult emergence in each container, the container was left for 10 d and then the emerged flies were counted. The larval containers with eggs but with no emerging flies were left for 40 d before they were discarded.

#### Body size measurement

The focal males used in the sexual competition experiment and reproductive isolation experiment, and females used in the reproductive isolation experiment, were frozen at -20 °C and then used to quantify body size. One wing from each individual was removed, mounted on a microscope slide using double-sided tape, and then covered with cling-wrap. The wings were then photographed at a magnification of 6.3× using a Leica MC170 HD camera mounted on a Leica MS5 stereo-microscope (Wetzlar, Germany). The linear distance from the intersection of the R<sub>2+3</sub>

wing vein with the wing margin to its intersection with the  $\rm R_s$  wing vein was measured using ImageJ software (Schneider et al. 2012).

#### Statistical analysis

All statistical tests were carried out using R (4.0.3) (R\_Core\_Team 2020). To verify that our larval diet manipulation resulted in the expected effects on adult phenotype, we modeled wing length using a linear model with larval diet, sex, and their interaction as fixed effects. Although we did not keep track of family identity or larval container, the flies were derived from eggs laid in stock tanks containing numerous flies and eggs were distributed among 38 larval containers. Effects of genotype and shared (container-specific) environments are, therefore, unlikely to have biased the results of this analysis.

In the sexual competition experiment, some males did not fight or interact with females, such that the individual data set was zero-inflated. Thus, analysis was carried out on means (for duration of mating, combat interactions, guarding) or total counts (number of matings, guarding bouts, female rejections, combat interactions, and wing flicks) for replicate populations. General and generalized linear mixed models were fitted to the replicate population means or sums using the R package lme4 (Bates et al. 2015), with each response variable modeled separately. Models included larval diet as a fixed effect, and day as a random block effect (but the random effect of day yielded extremely small variance components and was removed from 2 models to facilitate model fit: see Table 1). Combat duration was log-transformed to improve model diagnostics. For over-dispersed count data, an observation-level random effect (unique code for each replicate population) was included. For Gaussian models, effects were tested using t-tests with Satterthwaite's degrees of freedom using the package LmerTest (Kuznetsova et al. 2017). For Poisson models, effects were tested using z-tests. For rejection behaviors by females, the model failed to converge and a Wilcoxon matched-pairs test was used instead to compare data from rich- verses poor-diet replicate populations within days.

Mating success skew represents the opportunity for sexual selection (Jones 2009; Cattelan et al. 2020). To estimate mating success skew, we calculated coefficients of variation of the number of matings by individual males within replicate populations. The coefficients of variation were then compared using a Gaussian mixed-effects model with larval diet as the fixed effect and day as a random block effect. To test for and compare sexual selection on male body size in rich-diet vs. poor-diet replicate populations, we used a Gaussian model of individual male mating success (number of matings) as a function of male body size, with larval diet and body size as fixed effects and day as a random block effect. For this analysis, individual male mating success and body size (wing length) were both standardized (z-transformed) within replicate populations. In this model, a main effect of male body size would indicate overall sexual selection on male body size, whereas a larval diet × body size interaction would indicate a difference between larval diet treatment groups in sexual selection on male body size. Note that the main effect of larval diet is not meaningful in this model because standardization within replicate populations brings the effect estimate to ~0.

To test for reproductive isolation, models were fitted with male larval diet, female larval diet, and their interaction as fixed effects and day as a random block effect. An environmentally induced reproductive barrier would be indicated by a reduced propensity to mate or produce viable offspring by flies reared on mismatched diets, detectable statistically as a male larval diet × female larval diet cross-over interaction. Mating outcome and female rejection behavior were modeled as binomial (0 or 1) response variables. A binomial model was also used to investigate treatment effects on egg-to-adult viability, represented for each replicate pair as a matrix of successes (number of eggs that resulted in adult flies) and failures (number of eggs that did not result in adult flies). For over-dispersed binomial data, an observation-level random effect (unique code for each pair) was included in the model. Latency to mate was modeled with Gaussian error.

## Results

## Effects of larval diet on body size

Sexual size dimorphism was female-biased on the poor larval diet but male-biased on the rich larval diet (Sex × Larval Diet interaction: Estimate = 1.116, F = 84.61, df = 214, P < 0.0001; Fig. 4). Flies reared on the rich larval diet were larger than flies reared on the poor larval diet overall (main effect of larval diet: estimate = 0.511, df = 214, F = 390.49, P < 0.0001), and within both females (Tukey test: wing length difference = 0.51 mm, P < 0.0001) and males (Tukey test: wing length difference = 1.63 mm, P < 0.0001). Although we did not quantify body shape in this study, visual inspection suggested that our larval diet manipulation resulted in a

**Table 1.** Results of analyses for response variables from the sexual competition experiment, based on means (for the duration of mating, guarding, and combat) or counts (number of matings, guarding bouts, rejection responses by females, wing flicks by males, combat bouts) from each replicate population. For the number of guarding bouts per mating, the number of matings was included as a fixed covariate. For the number of guarding bouts observed in each replicate population and total combat duration per male, the models failed to converge unless the random effect of day (which yielded extremely small variance components for these response variables) was dropped. The number of rejections by females was analyzed using a Wilcoxon matched-pairs test.

Response variable	Error distribution	Diet effect estimate	Diet effect S.E.	Test statistic	Р
Matings	Poisson	0.664	0.169	z = 3.928	<0.0001
Mating duration	Gaussian	7.066	11.431	t = 1.420	0.1820
Guarding bouts	Poisson	1.754	0.408	z = 4.297	< 0.0001
Guarding duration	Gaussian	70.606	12.917	t = 5.466	0.0001
Rejections by females				V = 29.5	0.2780
Wing flicks by males	Poisson	-1.108	0.470	z = -2.359	0.0183
Combat duration	Gaussian	20.901	5.737	t = 3.643	0.0013
Combat bouts	Poisson	2.795	0.514	z = 5.435	< 0.0001

substantial difference in male body shape between rich and poor larval diet treatment groups, as observed in previous studies on this species (e.g. Bonduriansky 2007; Sentinella et al. 2013).





#### Sexual competition experiment

Mating frequency was higher in rich-diet replicate populations than in poor-diet replicate populations (Table 1; Fig. 5a). Mating duration was not affected by larval diet (Table 1; Fig. 5b), but males in rich-diet populations engaged in more frequent and prolonged guarding after mating (Table 1; Fig. 5c, d). There was no difference between poor- and rich-diet replicate populations in the frequency of rejection behaviors by females (Table 1; Fig. 5e). Males in poor-diet replicate populations flicked their wings more frequently than did males in rich-diet replicate populations (Table 1; Fig. 5f), directing their wing-flicks at other males as well as females, sometimes while mounting other males. Males in rich-diet replicate populations spent more time engaging in combat interactions (Table 1; Fig. 5g), which were often brief but sometimes quite prolonged and escalated (range: 2-279 s), involving both males rising to a near-vertical posture and striking each other repeatedly with their bodies, legs, heads, and antennae. By contrast, males in poor-diet replicate populations seldom engaged in combat, and fully escalated combat bouts were never observed (range: 2-20 s). Males in rich-diet replicate populations also engaged in more frequent combat bouts (Table 1; Fig. 5h).

There was no clear difference in male mating skew (coefficient of variation in individual male mating success within replicate populations) between rich-diet and poor-diet replicate populations (larval diet effect estimate = -0.332, t = -1.946, P = 0.0762; Fig. 6a). Larger males achieved more matings in both rich-diet and poor-diet replicate populations (body size effect estimate = 0.238, t = 2.001, P = 0.0476), and the effect of body size on male mating success was equally strong in rich-diet and poor-diet replicate populations (larval diet × body size interaction effect estimate = -0.308, t = -0.778, P = 0.444; Fig. 6b).



**Fig. 5.** Sexual behaviors and mating in experimental replicate populations of *T. angusticollis* reared on rich (red, right boxes) versus poor (blue, left boxes) larval diets in the sexual competition experiment. Box plots show the median, inter-quartile range (boxes), and non-outliner data range (whiskers). Points represent replicate population means or total counts. An asterisk ("\*") denotes a significant difference between larval diet treatment groups for the response variable, whereas "NS" denotes the lack of a statistically significant effect of larval diet treatment.

#### **Reproductive isolation experiment**

There was a male larval diet × female larval diet interaction for female rejection behavior (Table 2; Fig 7a), driven especially by frequent rejection of poor-diet males by rich-diet females. The interaction of male larval diet × female larval diet was near-significant for latency to mate (Table 2; Fig. 7b), suggesting that individuals reared on mismatched larval diet took longer to start mating than individuals reared on the same larval diet. There was no support for an interaction of male × female larval diet on whether mating occurred within 1 hour (Table 2; Fig. 7c), but a tendency for reduced mating probability for poor-diet females (Table 2; Fig. 7c). The interaction of male larval diet × female larval diet was also not supported for egg-to-adult viability (Table 2; Fig. 7d).

## Discussion

Our study provides proof-of-principle evidence that the effects of the developmental nutritional environment on plastic sexual traits could lead to differences among populations in patterns of sexual competition and contribute to reproductive isolation. A few previous studies have investigated population-level effects of the developmental environment (Morimoto et al. 2017; Cattelan et al. 2020; Winkler and Janicke 2022), but our study is the first (to our knowledge) to do so in a species exhibiting strongly plastic morphological secondary sexual traits. Our sexual competition experiment revealed striking differences in male-male and male-female interactions between experimental replicate populations reared on nutrient-rich versus nutrient-poor larval diets. In rich-diet replicate populations, male-male combat was frequent and prolonged, the mating rate was high, and males frequently guarded females after mating. In poor-diet replicate populations, escalated combat and mate guarding were rare but males engaged in frequent wing-flicking, females often rejected males, and the mating rate was lower. Our reproductive isolation experiment provided evidence that differences in developmental environment can create pre-copulatory barriers to reproduction.

Female–male pairs from mismatched larval diets tended to take longer to mate than pairs from the same larval diet treatment, and poor-diet males were frequently rejected by rich-diet females. Our findings suggest that a change in developmental conditions could lead to an immediate shift in sexual interactions and competition, supporting the idea that developmental plasticity could promote evolution and diversification (West-Eberhard 2005; Pfennig et al. 2010; Forsman 2015; Levis and Pfennig 2021).

While some of our findings are predictable from individuallevel effects of larval diet on adult traits, other effects emerge from interactions between individuals. For example, the lack of mate-guarding in poor-diet replicate populations may be explained by the small body size of males relative to females. When T. angusticollis males mate-guard, they stand above the female, using their forelimbs to fight off rival males as the female oviposits (Bonduriansky 2006). As previously shown in this species (see Bonduriansky 2007), larval diet manipulation reversed the direction of sexual size dimorphism: when reared on a poor larval diet, the mean body size of males was smaller than that of females (3.8 mm vs. 4.1 mm wing length, respectively), suggesting that the poor-diet males were physically unable to guard females after mating; by contrast, when reared on a rich larval diet, males were larger than females (5.4 mm vs. 4.8 mm wing length, respectively), enabling guarding. Males respond more strongly than females to larval diet manipulation in this species and many other insects because male body shape is sexually selected and males reared on a nutrient-rich larval medium are able to invest more in morphological secondary sexual traits (Emlen 1994; Cotton et al. 2004; Bonduriansky 2007). Mate-guarding could also be more advantageous in rich-diet populations if such populations experience more intense sperm competition, as suggested by their higher mating rate (Parker 2020). Consistent with this idea, a previous study showed that rich-diet males ejaculate strategically, transferring more ejaculate when mating second, while poor-diet males do not adjust their ejaculate in response to sperm competition risk (Wylde et al. 2019b). Intense sperm competition



**Fig. 6.** a) Coefficient of variation in individual male mating success within replicate populations in the sexual competition experiment (rich-diet replicate populations shown in red on the right, poor-diet replicate populations shown in blue on the left). Box plots show the median, inter-quartile range (boxes), and non-outliner data range (whiskers). Points represent coefficients of variation for each replicate. b) The relationship between standardized male mating success and standardized male body size in rich-diet replicate populations (red, shallower slope) and poor-diet replicate populations (blue, steeper slope), with points representing individual males and solid lines representing separate least-squares regression lines for rich (red) and poor (blue) larval diet treatments.

Table 2. Results of analyses for response variables from the reproductive isolation experiment. A Gaussian model was used for latency
to mating. Binomial models were used for female resistance (0 = no resistance observed; 1 = at least one female resistance behaviour
observed), mating (0 = no mating occurred; 1 = mating occurred), and egg-to-adult viability (0 = egg failed to produce an adult; 1 = egg
produced an adult fly). In all models, the day was included as a random block effect.

Response	Effect	Estimate	Estimate S.E.	Test statistic	Р
Female resistan	ce				
	Male larval diet	0.8270	0.9417	z = 0.878	0.3800
	Female larval diet	2.2258	0.8942	z = 2.489	0.0128
	Male × Female larval diet	-3.0528	1.3067	z = -2.336	0.0195
Latency to mati	ng				
	Male larval diet	383.17	377.66	t = 1.015	0.3135
	Female larval diet	666.22	377.66	t = 1.764	0.0817
	Male × female larval diet	-1014.78	534.09	t = -1.900	0.0612
Mating					
	Male larval diet	-1.3938	0.8987	z = -1.551	0.1209
	Female larval diet	-1.6301	0.8908	z = -1.830	0.0672
	Male × female larval diet	1.6301	1.1316	z = 1.441	0.1497
Egg-to-adult via	bility				
	Male larval diet	-0.3696	1.2298	z = -0.301	0.7640
	Female larval diet	-0.8389	1.3339	z = -0.629	0.5290
	Male × female larval diet	-1.8123	1.8986	z = -0.955	0.3400

and last-male sperm precedence are associated with frequent mate-guarding in other insects, such as the fly *Antocha saxicola* (Adler and Adler 1991).

Rich-diet males also participated in more frequent and longer combats compared to poor-diet males. Rich-diet males are large and possess exaggerated secondary sexual traits, such as elongated, spiny forelegs and elongated head and antennae, that they use in combat over females and territories at aggregation sites, whereas poor-diet males are small and have a female-like body shape (Bonduriansky 2007). When poor-diet males engaged in a combat interaction, it was typically very brief (~1 s), and they were rarely seen defending the oviposition site (petri dish) in the experimental arena. By contrast, this study and previous studies have found that rich-diet T. angusticollis males fight to defend females and oviposition sites (Bath et al. 2012; Adler and Bonduriansky 2013). Male-male combats may be very damaging for rich-diet males, influencing their somatic state and survival. We did not observe any mortality in our experimental arenas but, in natural populations, males that develop on nutrient-rich larval diets may die sooner (Adler et al. 2016). In natural populations that develop on nutrient-rich substrates, it is possible that high male mortality resulting from damaging combat interactions would result in female-biased operational sex ratios and thereby affect the intensity of sexual competition (Adler and Bonduriansky 2013; Hooper et al. 2017). Thus, the strong effect of the developmental environment on the frequency and intensity of male-male combat not only has the potential to generate differential selection on male phenotypes but could also affect population demography.

While poor-diet males rarely engaged in escalated combat, they appeared to utilize other competitive tactics. For example, we found that males in poor-diet replicate populations engaged in frequent wing flicking, sometimes while mounting other males. Subordinate *T. angusticollis* males have female-like cuticular hydrocarbon (CHC) profiles (Wylde et al. 2019a). If poor-diet males are also female-like in their CHC profiles, male-male mounting in poor-diet replicate populations could result from difficulty identifying potential mates in such populations. Alternatively, males could mount other males as a dominance behavior (Scharf and Martin 2013). Wing flicking is also employed by large, dominant males (Wylde et al. 2019a), and is likely to produce both visual and auditory cues with signaling functions (Jonsson et al. 2011). Interestingly, in poor-diet replicate populations, females were often observed rejecting males and such rejections often triggered wing flicking by males. Wing flicking might, therefore, function in courtship, or as a strategy to overcome female rejection. Wing flicking has also been observed in *T. angusticollis* females, directed either at other females or at males, perhaps as part of rejection behavior (Wylde et al. 2019a).

We predicted that increased expression of secondary sexual traits would result in increased opportunity for sexual selection, and perhaps stronger sexual selection on male body size, in rich-diet replicate populations, but our findings did not support these predictions. As noted above, poor-diet males appeared to use other tactics, such as scramble competition, which could also enable some individuals to achieve high mating success (Hooper et al. 2017). Our results suggest that the very different patterns of sexual competition observed in rich-diet versus poor-diet replicate populations can result in similar opportunities for sexual selection. Likewise, although we found a positive effect of body size on the number of matings achieved by individual males within replicate populations, the effect of body size on mating success did not differ between rich-diet and poor-diet replicate populations. While a stronger effect of body size on male mating success might be expected in rich-diet replicate populations, where males engage in frequent and intense combat, failure to observe such an effect could reflect low variance in body size within larval diet treatments or, alternatively, trade-offs with large body size. Previous studies have shown that males reared on a nutrient-rich larval diet age faster and are more fragile than males reared on a nutrient-poor larval diet (Adler et al. 2016; Hooper et al. 2017). It is, therefore, possible that the largest males in rich-diet replicate populations were more senescent or more prone to injury in combat than other males. Nonetheless, it is likely that the striking differences in sexual competition between rich-diet and



**Fig. 7.** Reproductive outcomes from male and female larval diet combinations in the reproductive isolation experiment: a) proportion of pairings where the female was observed rejecting the male; b) latency to mate; c) proportion of pairings where mating occurred; d) egg-to-adult viability (proportion of eggs that produced adult flies). Points represent means and whiskers show standard errors of the mean.

poor-diet replicate populations would exert differential selection on other aspects of male phenotype, such as body shape, chemical signals, mate choice, or sperm (e.g. see Cattelan et al. 2020; Kustra and Alonzo 2020). We only explored pre-copulatory sexual selection in this study, but post-copulatory competition, involving sperm competition or cryptic female mate choice (Devigili et al. 2013), could also generate sexual selection and contribute to net opportunity for sexual selection. Indeed, Cattelan and colleagues (2020) found that the nutritional environment altered the relative magnitudes of pre- and post-copulatory sexual selection on male guppies.

Our reproductive isolation experiment investigated both pre- and post-mating isolation mechanisms. However, our experimental assays (where females were paired with males for 1 h inside a small container) did not provide females with an opportunity to fly away from males, eliminating a key mechanism of mating avoidance. Consequently, some pairs that mated in our experiment might not have done so in a natural setting. Nonetheless, we also quantified pre-mating behavioral outcomes (i.e. female resistance and latency to mating) because female resistance behaviors or delay in mating would probably result in reduced probability of mating in a natural environment. For pairs that mated, we quantified egg-to-adult viability as a measure of fertilization rate in order to test for post-mating isolation.

Low egg-to-adult viability can indicate complete or nearly complete post-mating isolation. This could occur if individuals reared on mismatched diets (rich male/poor female, poor male/ rich female) had difficulty mating, resulting in low rates of sperm transfer, or if different developmental environments resulted in epigenetic incompatibility, resulting in reduced viability of embryos, larvae, or pupae. The interaction of female diet × male diet was not supported for egg-to-adult viability, suggesting a lack of post-mating isolation. However, pre-mating mechanisms also have the potential to generate reproductive isolation. In insects, the fit between the male and female genitalia can affect the ability to mate and transfer sperm. In *T. angusticollis*, the sizes of male genitalic traits are affected very little by body size or larval diet (Wylde and Bonduriansky 2020). This low plasticity of genitalia could be an adaptation that enables males to mate with any female, regardless of female body size (Eberhard et al. 1998). This might explain why we found no evidence of an interaction of male larval diet × female larval diet on mating outcome. The low plasticity of genitalic traits, and lack of opportunity for females to escape from males in small containers during hour-long pairings, probably enabled mating to occur in female–male pairs from both matched and mismatched developmental environments.

Pre-mating isolation could also result from rejection behaviors by females and/or males (Nanda and Singh 2012; Barerra et al. 2024). Indeed, our results showed that rich-diet females often exhibited rejection behaviors when paired with poor-diet males, and suggested that individuals from mismatched developmental environments took longer to mate (although this interaction was marginally non-significant). Although these behavioral responses did not ultimately prevent mating in our small experimental containers, increased latency to mate and high incidence of rejection behaviors is likely to affect probability of mating in the wild (see Gröning and Hochkirch 2008; Yun et al. 2017). Telostylinus angusticollis experience a high mortality rate in the wild as a result of predation and other factors (Kawasaki et al. 2008), suggesting that any delay in mating is likely to be costly. Moreover, wild females have ample opportunity to escape from unattractive males. Our results thus suggest that pre-mating mechanisms such as mate rejection could result in partial reproductive isolation between T. angusticollis natural populations that experience different developmental environments. However, to determine the consequences of mate rejection for reproductive isolation, future studies could use larger observation arenas and/or more complex environments that provide more opportunities for females to escape and hide from males (see Yun et al. 2017; Malek and Long 2019).

Our results suggest that the effects of the developmental environment are not limited to males, but also extend to female behavior. Although our larval diet manipulation had a much weaker effect on female body size than on male body size (Fig. 4), the effect on females was nonetheless highly significant and consistent with previous studies on this species (Bonduriansky 2007; Sentinella et al. 2013). Previous studies on T. angusticollis found that females generally prefer rich-diet males with exaggerated traits (Fricke et al. 2015). Our finding that rich-diet females tended to reject poor-diet males suggests that high condition enhances female choosiness in this species. Female condition appears to affect mating strategies, including choosiness, in many insects and spiders (Hunt et al. 2005). For example, in fall field crickets, Gryllus pennsylvanicus, high-condition females were more choosy and took longer to make a mate choice compared with low-condition females (Judge et al. 2014). Similarly, poor-diet female Schizocosa wolf spiders did not discriminate between richdiet and poor-diet males, whereas rich-diet females mated more frequently with rich-diet males (Hebets et al. 2008).

Our findings could have relevance for the hypothesized "phenotype first" process in adaptive evolution, where novel environments induce plastic changes in phenotypes and selection on these phenotypes can then lead to genetic evolution and diversification (West-Eberhard 2005; Pfennig et al. 2010; Levis and Pfennig 2021; Pilakouta and Ålund 2021). In *T. angusticollis*, dispersal to a novel host-tree species and resulting change in larval nutrition could lead to an immediate plastic change in sexual competition as well as some degree of immediate reproductive isolation. If the environmental differences between natural populations, and resulting differences in the expression of developmentally plastic traits, persist for multiple generations, such differences could potentially initiate the evolution of novel sexual traits and tactics (see Barerra et al. 2024). Examples of this process in natural populations are rare (see Day et al. 1994), perhaps because of the difficulty of decoupling plastic effects of developmental environment from genetic differences. Our proof-of-principle evidence suggests that a plastic response to distinct developmental environments should be considered as a potential cause of phenotypic differences between natural populations, as well as a potential initiator of reproductive isolation and ecological speciation (Rundle and Nosil 2005; Nosil et al. 2009).

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## Author contributions

Kristin Hubakk (Conceptualization [Equal], Data curation [Lead], Formal analysis [Equal], Investigation [Lead], Methodology [Equal], Visualization [Equal], Writing—original draft [Lead]), Zachariah Wylde (Methodology [Supporting], Supervision [Supporting], Writing—review & editing [Supporting]), and Russell Bonduriansky (Conceptualization [Equal], Formal analysis [Equal], Funding acquisition [Lead], Methodology [Equal], Project administration [Lead], Supervision [Lead], Visualization [Equal], Writing—review & editing [Lead])

# Data availability

Analyses reported in this article can be reproduced using the data provided by Hubakk et al. (2024).

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