


# Mating environments mediate the evolution of behavioral isolation during ecological speciation

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## Abstract

The evolution of behavioral isolation is often the first step toward speciation. While past studies show that behavioral isolation will sometimes evolve as a by-product of divergent ecological selection, we lack a more nuanced understanding of factors that may promote or hamper its evolution. The environment in which mating occurs may be important in mediating whether behavioral isolation evolves for two reasons. Ecological speciation could occur as a direct outcome of different sexual interactions being favored in different mating environments. Alternatively, mating environments may vary in the constraint they impose on traits underlying mating interactions, such that populations evolving in a “constraining” mating environment would be less likely to evolve behavioral isolation than populations evolving in a less constraining mating environment. In the latter, mating environment is not the direct cause of behavioral isolation but rather permits its evolution only if other drivers are present. We test these ideas with a set of 28 experimental fly populations, each of which evolved under one of two mating environments and one of two larval environments. Counter to the prediction of ecological speciation by mating environment, behavioral isolation was not maximal between populations evolved in different mating environments. Nonetheless, mating environment was an important factor as behavioral isolation evolved among populations from one mating environment but not among populations from the other. Though one mating environment was conducive to the evolution of behavioral isolation, it was not sufficient: assortative mating only evolved between populations adapting to different-larval environments within that mating environment, indicating a role for ecological speciation. Intriguingly, the mating environment that promoted behavioral isolation is characterized by less sexual conflict compared to the other mating environment. Our results suggest that mating environments play a key role in mediating ecological speciation via other axes of divergent selection.

**Keywords:** *Drosophila*, premating isolation, reproductive isolation, sexual conflict, sexual isolation, sexual selection

## Lay Summary

Behavioral isolation occurs when individuals preferentially mate with others from their own population, a key step in the formation of new species. We studied a set of experimental fruit fly populations to gain insights into factors affecting the evolution of behavioral isolation. Surprisingly, we found that evolving in different mating environments did not drive the evolution of behavioral isolation directly. Rather, there was strong evidence that adaptation to different-larval environments caused behavioral isolation, but this divergent larval selection was not sufficient on its own. If both populations had evolved in a highly simplified mating environment (one of two used here), then behavioral isolation did not evolve, whereas a more complex mating environment was conducive to its evolution. Our results suggest that mating environments may play a key role in constraining or facilitating speciation via other sources of divergent selection.

## Introduction

Behavioral isolation reduces gene flow between populations and is thought to be a common first step in initiating speciation (Coyne & Orr, 2004). Despite the importance of behavioral isolation to speciation, much remains unknown about the factors that promote or hinder its evolution. Behavioral isolation is the result of evolved differences in traits underlying both intra- and inter-sexual mating interactions. These mating interactions can be affected by the environment in which they occur. For instance, the environment may impact the frequency of encountering potential mates, the sending and receiving of signals, and the

range and effectiveness of possible responses to said signals (Ryan & Cummings, 2013; Servedio & Boughman, 2017). Through such impacts, the mating environment may affect the expression of existing behavioral isolation (e.g., premating isolation is stronger when mating occurs in the presence vs. absence of alternative host plants in gall wasps; Egan et al., 2012). Mating environment may also affect the evolution of behavioral isolation, and this is our focus here.

There are three non-exclusive scenarios by which mating environment could impact the evolution of behavioral isolation between two populations. In the first, two populations experience

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different mating environments that cause divergent selection on mating phenotypes, and behavioral isolation therefore evolves via the process of ecological speciation. We refer to this as “ecological speciation by mating environment.” For example, stickleback females typically prefer males displaying red nuptial coloration. However, in populations that have evolved to mate in light environments with limited transmission of red wavelengths, female preference and male nuptial collaboration have evolved differently, resulting in increased behavioral isolation between populations evolved in different light environments (Boughman, 2001). Compared to other axes of environmental variation, differences in mating environment may be particularly conducive to ecological speciation because divergent selection can act directly on traits that give rise to behavioral isolation.

Scenarios two and three involve cases in which two populations are evolving in similar mating environments so divergence, if it occurs, arises indirectly via some other process. Mating environments may nevertheless be important because some may be more conducive than others to the evolution of behavioral isolation. In scenario two, divergence results from the unique identity of mutations that happen to increase first in frequency in each population—a process known as “mutation-order speciation” (Schluter, 2009). This could cause two populations to follow unique evolutionary trajectories in response to sexual selection and intersexual conflict, generating behavioral isolation between them (Coyne & Orr, 2004; Gavrillets, 2014; Gavrillets & Hayashi, 2005; Lande, 1981; Schluter, 2009). Mating environments may be key to determining whether such a coevolutionary process occurs, and the identity of the traits involved when it does. For example, in high-predation environments, the diversification of sexual displays may be constrained, and hence behavioral isolation is unlikely. In contrast, isolated populations evolving in low-predation environments are free to diverge in sexual signals and preferences, resulting in behavioral isolation from one another.

In the third scenario, behavioral isolation evolves due to divergent selection arising from ecological differences unrelated to the mating environment (i.e., “ecological speciation but not by mating environment”). As in mutation order, the mating environment may nevertheless impact the extent of divergence and whether behavioral isolation arises from this. For example, adaptation to different-larval host plants may result in evolved changes to biochemical pathways affecting the adult pheromones (Chung & Carroll, 2015). In mating environments in which female preference for pheromones is important, behavioral isolation could evolve, but only between populations adapted to different-larval host plants. In contrast, in mating environments in which females focus on visual or auditory displays, or where males have evolved to circumvent female preference, then divergence in pheromones will have little influence on behavioral isolation. The mating environment is not the direct cause of behavioral isolation but rather permits its evolution only if other drivers are present.

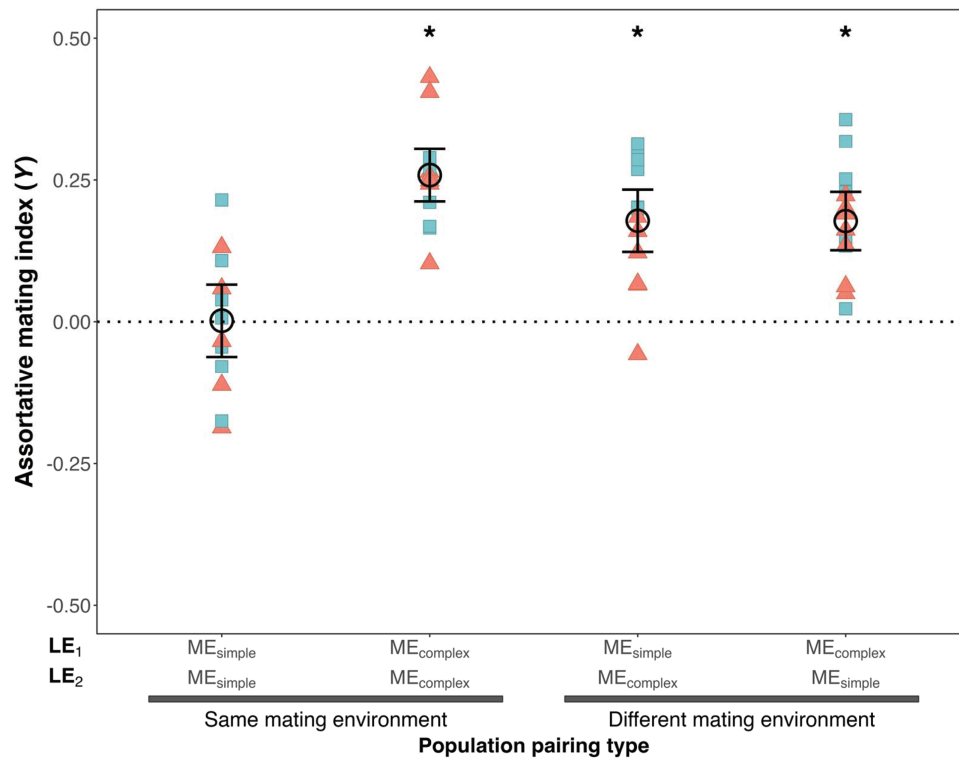
Here we explore these ideas by taking advantage of a long-term evolution experiment in *Drosophila melanogaster* that manipulates mating environment. Specifically, we use a set of 28 experimental populations, each of which evolved under one of two mating environments and one of two larval environments in a factorial design. These populations were originally created to test the effect of the mating environments on the rate of adaptation to novel larval environments (Yun et al., 2018). Here we are interested in the effect of these different mating and larval environments on the evolution of behavioral isolation. The two larval environments differed with respect to food and temperature, which were arbitrarily chosen ecological dimensions to which the

populations could adapt (and behavioral isolation could arise as a by-product). The two mating environments were chosen because they differ with respect to how mating interactions occur and thus selection from these interactions is likely to differ. We refer to these as the “simple” and “complex” mating environments (hereafter  $ME_{\text{simple}}$  vs.  $ME_{\text{complex}}$ ) because the former was higher density and had less spatial complexity than the other. The simple environment was characterized by more frequent male–female interactions and mating as well as substantially greater male-induced harm to females (Yun et al., 2017, 2019, 2021). Thus, the simple and complex environments can be viewed as differing with respect to the relative importance of sexual conflict.

With 28 populations, there are 378 possible pairs that could be tested for behavioral isolation which would be logistically infeasible. We therefore take a targeted approach, using three experiments to gain insights into sources of variation in behavioral isolation. We first address whether mating environments themselves are a source of divergent selection that drives the evolution of behavioral isolation as a by-product (i.e., “ecological speciation by mating environment”). That is, we ask whether behavioral isolation is more likely between two populations from different mating environments than between two populations from the same mating environment. We find that behavioral isolation exists and that adaptation to different mating environments was not required for its evolution. Given that behavioral isolation is present between populations that have evolved in the same mating environment, we then turn our attention to the other processes by which it can evolve. Has it occurred via a mutation-order process, or has some more deterministic process such as divergent ecological selection played a role? Specifically, we test whether behavioral isolation is more likely between populations that evolved in different rather than the same-larval environments, holding mating environment constant.

## Methods and results

We use populations from a previously described evolution experiment (Yun et al., 2018, 2019, 2021). Seven replicate populations were derived from a common lab stock and were maintained under each factorial combination of larval and mating environment, yielding  $7 \times 2 \times 2 = 28$  populations total (Supplementary Figure S1). Each generation during the adult phase of their life cycle was comprised of four groups of 35 males and 35 females. For the simple mating environment ( $ME_{\text{simple}}$ ), adults interacted in standard *Drosophila* culture vials containing 10 mL of food media. For the complex mating environment ( $ME_{\text{complex}}$ ), adults interacted in 1.65-L cylindrical plastic Ziploc food storage containers, each of which included five separate food patches and two pipe cleaners protruding from the lid into the interior space. After 6 days, males were discarded and 105 randomly chosen females from among the four groups for a given population were distributed among seven vials for egg laying. Each population was assigned to one of two larval environment treatments: (a) cornstarch-yeast medium and a 5 h heat shock at 37 °C to 3-day-old larva; (b) commel larval medium supplemented with 5% NaCl and a constant 28 °C. Following eclosion, adults were collected and placed in the appropriate mating treatment for the next generation. Behavioral isolation was quantified in three separate experiments conducted at generations ~74, 108, and 122. Prior to each experiment, all populations were reared on ancestral food, under common mating conditions, for two generations. An overview of the design of the behavioral experiments is provided below; further details are in Supplementary material.



**Figure 1.** Assortative mating index ( $Y$ ) among different types of population pairs. All pairs involved populations from different-larval environments ( $LE_1$  and  $LE_2$ ). Circles represent the average  $Y \pm 1$  SE within a population pairing type. There is significant heterogeneity in  $Y$  among pairing types (one-way ANOVA:  $F_{1,11} = 8.75$ ,  $p = .013$ ). In addition, each pairing type was separately assessed for behavioral isolation; asterisks denote  $Y$  values significantly different from zero ( $p < .05$ , one-sample  $t$ -tests). Squares/red triangles represent trials where males and females from the same population were marked with different/same food coloring, respectively.

## Experiment 1

To test for ecological speciation by mating environment, we performed mating trials using 196 unique pairs from the set of 28 populations (Supplementary Figure S2). Half of these pairs involved populations that evolved in different mating environments (i.e., one  $ME_{simple}$  and one  $ME_{complex}$ ); the other half involved populations that both evolved in the same mating environment (i.e., either  $ME_{simple}$  or  $ME_{complex}$ ). All pairs consisted of populations that had evolved in different-larval environments. In each replicate trial, 30 virgin flies of each sex from each population (120 flies total) were placed in a cage and the first 20 mating pairs were removed. Prior to beginning each trial, flies had been marked by feeding them colored yeast so we could identify the type of fly in each mating pair (Supplementary Figure S2). Behavioral isolation was quantified using the margin-free index  $Y$  based on the cross-product ratio of the  $2 \times 2$  contingency table of the number of matings of each male and female combination.  $Y$  ranges from  $-1$ , indicating perfect disassortative mating, to  $+1$  indicating perfect assortative mating, with zero indicating non-assortative mating (Bishop et al., 1975).

We found significant heterogeneity in behavioral isolation among different types of population pairs (one-way analysis of variance [ANOVA] of  $Y$ :  $F_{3,24} = 26.6$ ,  $p < .0001$ ; a general linear mixed model yielded the same result; Supplementary Material), with significant positive (and reasonably symmetrical; Supplementary Figure S5) assortative mating in all population pairing types in which at least one population had evolved in the complex mating environment,  $ME_{complex}$  (Figure 1). Notably, there was no support for ecological speciation by mating environment, under which behavioral isolation would be stronger between populations that

evolved in different mating environments compared to those from the same mating environment. Rather, the highest point estimate for average assortative mating was for pairs where both populations had evolved in the  $ME_{complex}$  treatment. The stark difference in behavioral isolation between  $ME_{simple} - ME_{simple}$  pairs compared to  $ME_{complex} - ME_{complex}$  pairs instead suggests that the complex mating environment is more conducive to its evolution.

## Experiment 2

Experiment 1 showed that divergent mating environments did not drive the evolution of behavioral isolation, implying some other process was responsible. This could be a mutation-order process such as intersexual coevolution along unique evolutionary trajectories in independent populations, or divergent ecological selection between larval (rather than mating) environments. We cannot distinguish between these from experiment 1 because all pairs in that assay involved populations from different-larval environments. To address this, we conducted 224 replicate mating trials (like those in experiment 1) involving 13 pairs of populations, all from the complex mating environment. Seven of these involved pairs of populations adapted to different-larval environments and six involved pairs adapted to the same-larval environment (three pairs where both populations were from each larval environment, respectively; Supplementary Figure S3). Behavioral isolation was again quantified using the index  $Y$ .

Behavioral isolation was significantly stronger in different-larval environment pairings than same-larval environment pairings (one-way ANOVA of  $Y$ :  $F_{1,11} = 8.75$ ,  $p = .013$ ). In the different-larval environment pairings, assortative mating was significantly greater than zero, but in the same-larval environment

pairings, it was not (Figure 2). These results offer no support for a mutation-order process as there is no assortative mating between populations evolved in the same-larval environment. In contrast, the results are consistent with behavioral isolation evolving via divergent selection between larval environments (i.e., ecological speciation by larval environment).

In analyzing these results, we noticed an unexpected effect of the red and blue food-colored yeast, we fed the flies ahead of time so they could be distinguished during the assay. Among the different-larval environment pairs, assortative mating was only present when males and females from the same population were marked with different colors (i.e., red females with blue males and vice versa). This color effect was not evident in same-larval environment trials despite the same color combinations used, indicating that color combinations alone did not produce assortative mating. Analysis of a statistical model including a factor indicating whether males and females from the same population were colored the same or differently revealed a significant interaction between this factor and “population pairing type” (i.e., same- vs. different-larval environment; Supplementary Table S2). This suggests that assortative mating exists between populations from different-larval environments, but it can be obscured by some color manipulations. Notably, we observed assortative mating in experiment 1 for the same types of population pairs (i.e., pairs of ME<sub>complex</sub> populations from different-larval environments) and no color effect was evident (Supplementary Table S1; Figure 1), though different colors (red and green) were used for marking in that experiment.

### Experiment 3

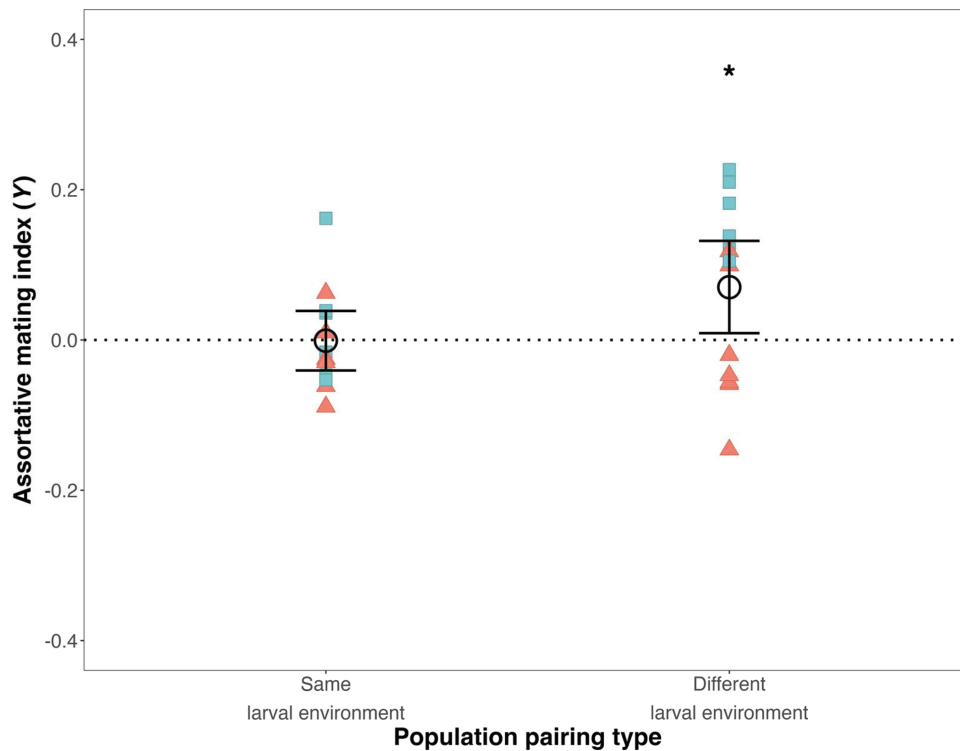
As another test of mutation-order vs. ecological speciation by larval environment, we performed a third experiment in which we quantified assortative mating using a different approach. In

experiment 3, we performed trials in which an individual female was placed with two males, one from the female’s own population (“homospecific male”) and one from another population (“heterospecific male”); the heterospecific male was from a population that had evolved in either the same- or different-larval environment as the female. Thus, as in experiment 2, there are both “same-larval environment” and “different-larval environment” trials. In this experiment, females did not need to be color-marked. As in experiment 2, only flies from the 14 populations that evolved in the complex mating environment were used. A total of 1,190 assays were performed in which mating was recorded (Supplementary Figure S4). Behavioral isolation was quantified as the proportion of homospecific matings.

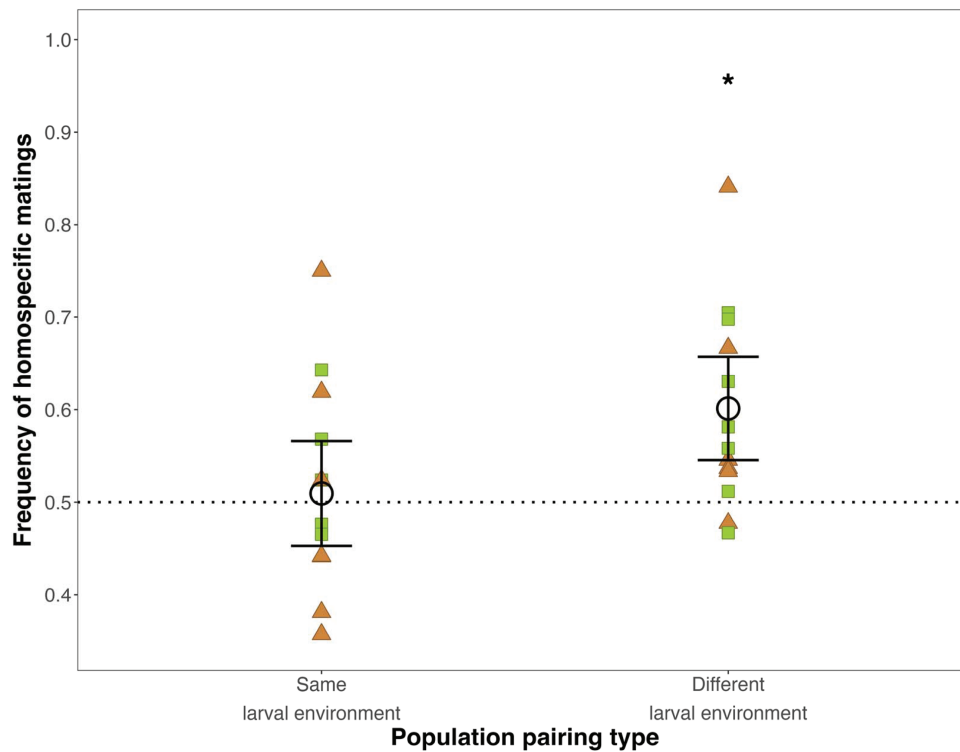
Positive assortative mating was significantly greater in different-larval environment trials than in same-larval environment trials (binomial generalized linear mixed model, Wald  $\chi^2 = 9.72$ ,  $df = 1$ ,  $p = .0018$ ; Supplementary Table S3). In the latter case, there was no evidence of assortative mating: females were not more likely to mate with a homospecific male than a heterospecific male that evolved in the same-larval environment (quasibinomial glm: intercept = 0.04,  $t_{1,df} = 0.37$ ,  $p = .718$ ). In contrast, females were more likely to mate with their homospecific male than a heterospecific male that evolved in a different-larval environment (intercept = 0.41,  $t_{1,df} = 3.35$ ,  $p = .006$ ). These results echo experiment 2 in providing support for ecological speciation by larval environment and not mutation order. There is no evidence that these results were sensitive to color marking of males (Figure 3; Supplementary Table S4).

### Discussion

There is extensive evidence demonstrating that reproductive isolation can evolve as a by-product of ecologically divergent



**Figure 2.** Assortative mating index (Y) among population pairs evolved in the same- or different-larval environments (LE). Black circles denote average  $Y \pm 1$  SE within a population pairing type and asterisks indicate values significantly different from zero ( $p < .05$ , one-sample t-tests). Squares/triangles represent trials where males and females from the same population were marked with different/same colors, respectively.



**Figure 3.** Frequency of homospecific mating among population pairs evolved in the same- or different-larval environments (LE). Black circles represent the average frequency of homospecific mating  $\pm$  1 SE and asterisks denote values that are significantly different from the null expectation of 0.5 ( $p < .05$ , quasibinomial generalized linear models). Orange triangles/green squares denote which larval environment females were from.

selection (i.e., ecological speciation; Coyne & Orr, 2004; Fry, 2009; Schluter, 2009), but factors that may promote or hamper it have received much less attention. Here we evaluated the evolution of behavioral isolation in response to two axes of divergent selection: different-larval environments and different adult mating environments. Our experiments showed that divergent larval selection was necessary, but not sufficient, for the evolution of behavioral isolation. In contrast, there was no evidence that evolving in different mating environments caused behavioral isolation. Though differences between mating environments did not directly drive the evolution of behavioral isolation (“ecological speciation by mating environment”), there was clear evidence that mating environments had a strong influence on the evolution of assortative mating; for pairs evolving in contrasting larval environments, behavioral isolation was present when both had evolved in the complex mating environment, but it was absent in pairs where both evolved in the simple mating environment.

Why was our complex mating environment more conducive to the evolution of behavioral isolation than our simple mating environment? There are at least two possibilities. The first is that mating environments themselves impact the rate and extent of adaptation. There is a longstanding hypothesis that sexual selection promotes adaptation (Darwin, 1859; Rowe & Rundle, 2021), and sexual selection may vary by mating environments. Because behavioral isolation evolves as a by-product of adaptation, it may also be affected by mating environment. Our populations were originally created to test the effect of the mating environments on adaptation to their larval environment. Larval fitness assays in generations 14 and 50 showed that the  $ME_{\text{complex}}$  populations were better adapted than the  $ME_{\text{simple}}$  populations (Yun et al., 2018), but subsequent assays in generations 110+ revealed that this difference no longer existed. Experiment 1 of the current study

was conducted at generation 74, so the extent of any differential adaptation is unclear.

The second possibility is that mating environments differ with respect to the traits that mediate sexual interactions, and this has consequences for the evolution of behavioral isolation. In our case, we know that sexual interactions and mating are more frequent in the simple than the complex environments (e.g., estimated mating rate was approximately double; Yun et al., 2017, 2019). We also know that  $ME_{\text{complex}}$  males have evolved to be less harmful to females than  $ME_{\text{simple}}$  males: when assayed in vials, there was an ~17% relative reduction in female fitness under high vs. low exposure to  $ME_{\text{simple}}$  males, compared to only a ~2% reduction using  $ME_{\text{complex}}$  males (Yun et al., 2021). This implies that sexual conflict is less important in the complex environment, presumably because females have more control over sexual interactions and can more easily avoid or escape male harassment (*sensu* Zuk et al., 2014). As such, there may be greater scope for females to choose among males and behavioral isolation may result if preferences pleiotropically diverge between populations in response to local adaptation. In contrast, in the simple mating environment, females may be less able to express preferences because of constant male harassment, making behavioral isolation less likely.

One interpretation of our results is that divergence of traits involved in mating interactions can have different consequences in different mating environments, with behavioral isolation arising in some but not in others. Such an effect of mating environment has been seen in natural populations. For example, behavioral isolation between benthic and limnetic sticklebacks in Enos Lake was eliminated following the introduction of signal crayfish, and this may have been due to changes in their mating environment (Taylor et al., 2006). The absence of crayfish

was presumably a permissive environment to the evolution of behavioral isolation; if crayfish had been present in the lake from the beginning, limnetics and benthics would presumably never have diverged. Likewise, in cichlid fish in Lake Victoria, previously existing behavioral isolation generated by differences in color has been compromised by decreases in water clarity (Seehausen et al., 1997), suggesting that clear, but not turbid, water is a mating environment permissive to the evolution of behavioral isolation in this group.

Above we have speculated that the reason that the simple environment is non-conducive to the evolution of behavioral isolation is because it is characterized by more male harm and less female control, i.e., a more prominent role for sexual conflict. This contrasts with theoretical models that have proposed sexual conflict as an engine of speciation (Gavrilets, 2000; Gavrilets et al., 2001; Rice, 1998; reviewed in Gavrilets, 2014). Experimental evolution in the dung fly *Sepsis cynipsea*—a system with evidence of male harm—showed that the frequency of between-population mating was lower among populations that evolved under “normal” conditions rather than under enforced monogamy (Martin & Hosken, 2003), which removes sexual conflict (but also other forms of sexual selection) and selects against male harm. Moreover, the effect was greater in populations maintained at high densities where conflict is presumed to be more intense. However, similar studies on *Drosophila* (Bacigalupe et al., 2007; Wigby & Chapman, 2006) and bulb mites (Plesnar-Bielak et al., 2013) failed to find similar results. Arnqvist et al. (2000) showed that diversification rates are higher in polyandrous versus monandrous paired clades of insects. Though they interpreted this as evidence of the importance of sexual conflict in promoting speciation, other aspects of sexual selection likely also differ between the two types of clades. Moreover, similar analyses in other taxa failed to find a similar result (e.g., Gage et al., 2002; Morrow et al., 2003).

Though much attention has been given to the idea that sexual conflict can promote speciation, it has also been suggested that sexual conflict could instead have the opposite effect. Sexual conflict has been predicted to inhibit the evolution of reproductive isolation in systems where males are able to coerce females to mate beyond the optimal rate for females (Parker & Partridge, 1998). Though that model was developed in the context of parapatry or secondary contact, the underlying logic is similar to what we have proposed for our “allopatric” experiment: female preferences, which may otherwise drive the evolution of behavioral isolation, may be rendered ineffective in systems with high levels of male coercion. The physical environment in which mating occurs may often play a key role in mediating the importance of different traits in mating interactions and how much male coercion occurs.

Here we employed a framework that makes a clear distinction between mating and non-mating environments. This is reflective of some species, but it would be difficult to employ in others that lack distinct mating vs. non-mating environments. In such cases, it would be hard to determine whether behavioral isolation arose because different mating environments caused direct selection on mating phenotypes, or because mating phenotypes diverged as a by-product of other sources of divergent ecological selection. Identifying the mating phenotypes that have diverged, and testing the evolutionary processes responsible, may be the best way forward in such situations.

Finally, while a number of past lab evolution experiments have shown that behavioral isolation can evolve as a by-product of ecologically divergent selection, others have failed to find this (Coyne & Orr, 2004; Fry, 2009; Rice & Hostert, 1993). Among those

that have, many are based on a single assay. In those studies in which repeat assays were performed, results were not always consistent (reviewed in Florin & Ödeen, 2002). This is perhaps unsurprising given that behavior can be highly sensitive to various (often unknown) factors. Though not all aspects of the work reported here were assessed in separate experiments, our study provides uncommonly robust support for the existence of behavioral isolation. In three separate experiments, we detected positive assortative mating between populations from the complex mating environment adapting to different-larval environments. These experiments differed in a variety of ways: (a) different generations of flies used in each experiment; (b) experimenters and institution where the work was performed differed in experiment 1 vs. 2 and 3; (c) assay type differed in experiments 1 and 2 (multiple choice) vs. experiment 3 (female choice). This provides a particularly robust demonstration of the earliest stages of behavioral isolation during ecological speciation.

## Supplementary material

Supplementary material is available online at *Evolution Letters*.

## Data and code availability

Data and code for our statistical analyses are available on Dryad <https://doi.org/10.5061/dryad.2547d7wzp>.

## Author contributions

H.D.R., A.F.A., and T.S.B. designed the research; M.-L.S. and K.E.K. performed experiment 1 and T.S.B. performed experiments 2 and 3. T.S.B., H.D.R., and A.F.A. analyzed the data and wrote the article.

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*Conflict of interest:* The authors declare no conflict of interest.

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