On Male Harm: How It Is Measured and How It Evolves in Different Environments

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ABSTRACT: Males can harm the females that they interact with, but populations and species widely vary in the occurrence and extent of harm. We consider the merits and limitations of two common approaches to investigating male harm and apply these to an experimental study of divergence in harm. Different physical environments can affect how the sexes interact, causing plastic and/or evolved changes in harm. If harmful male phenotypes are less likely to evolve in situations where females have more control over sexual interactions, populations evolving in environments in which females have greater control should have less harmful males. We test this idea using experimental populations of *Drosophila melanogaster* that have evolved in either of two environments that vary in the extent to which females can avoid males or in a third environment without mate competition (i.e., enforced monogamy). We demonstrate an evolved reduction in harm in the absence of mate competition and also in a mate competition environment in which females have greater control. We also show a plastic effect in that otherwise harmful males are no longer so when tested in the environment in which females have greater control. Our results reveal the different perspectives provided by the two methods of studying harm.

Keywords: environmental complexity, mate competition, monogamy, polygamy, sexual conflict.

Introduction

Mate competition can lead to the evolution of traits that cause substantial harm to females (Parker 1979, 2006; Rice and Holland 1997; Morrow et al. 2003; Arnqvist and Rowe 2005; Lessells 2006). Numerous examples have been documented across the animal kingdom (Arnqvist and Rowe 2005), including injurious courtship and mating behaviors (Le Boeuf and Mesnick 1991; Rowe 1994; Stone 1995), damaging morphologies (Crudgington and Siva-Jothy 2000; Stutt and Siva-Jothy 2001), and toxic biochemical cocktails in male ejaculates (Liddle et al. 1995; Gems and Riddle 1996; reviewed in Simmons 2001). Male harm necessarily creates conflict between the sexes over the nature of their interactions, generating selection in females to reduce any harm they experience. This can lead to a process of sexually antagonistic coevolution that can have important consequences for the evolution of both male and female traits (Arnqvist and Rowe 2002), as well as how the sexes interact with one another (MaGurran and Seghers 1994*a*; Ortigosa and Rowe 2002; Muller et al. 2007), and that can potentially drive population divergence/speciation (Arnqvist et al. 2000; Gavrilets 2000; Martin and Hosken 2003*a*).

As male harm is at the crux of sexual conflict and its evolutionary consequences, it is key to develop a thorough understanding of its evolution and expression. Fundamental to the study of this subject is the ability to quantify harm in a way that allows comparisons across environments or evolutionary divergent populations. In obligately sexual gonochoristic species, males are necessary for females to reproduce. Male harm in such species refers to the idea that a female's fitness with a normal level of male exposure is reduced relative to what it would be with the minimal level of male exposure required to fertilize her eggs. Most empirical studies use one of two approaches (see below), yet there has been little discussion or comparison of these alternatives. One of our two main goals is to consider the merits and limitations of these two approaches. Our other major goal is to use these approaches to study how male harm evolved in different mating environments experienced by populations in a long-term evolution experiment.

One of the main approaches to studying harm (hereafter, "method 1") is to compare the fitness of females when exposed to one type of male versus another (e.g., Holland and Rice 1999; Gay et al. 2011; Arbuthnott et al. 2014; Łukasiewicz 2020). If females are less fit with type A males than with type B, then A males are interpreted as being

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more harmful than B males. However, this pattern could instead occur if B males are more beneficial to females than are A males. Method 1 can also be thought of as measuring the indirect genetic effect (IGE) of males on female fitness (Moore et al. 1997; Marie-Orleach et al. 2017). The major concern with this type of study is that it does not actually test which, if any, of the males are harmful; such inferences require other evidence. Thus, method 1 is best used in contexts—not just species—where harm is already known to occur. Even in species where harm has been documented, how males affect females and the net effect on female fitness can depend heavily on the context (e.g., biotic and/or abiotic conditions under which it is measured and the populations used; MaGurran and Seghers 1994*b*; Rowe et al. 1994; Bretman et al. 2009; Gosden and Svensson 2009; Fricke et al. 2010; Yun et al. 2017; García-Roa et al. 2019).

The second main approach ("method 2") compares the fitness of females when they receive normal (i.e., continuous) exposure to males versus when they receive limited exposure to males (e.g., Fowler and Partridge 1989; Partridge and Fowler 1990; Rönn et al. 2006; Harano 2015). Method 2 measures a "dosage" effect of males on female fitness. If females are more fit when exposure to males is limited, then this provides strong evidence that males are harmful. Though method 2 has often been used in studies designed to demonstrate the existence of harm caused by a single type of male in a single context, it has been underutilized for comparisons across contexts or populations. A nice feature of method 2 is that the resulting data can be used to quantify male harm (H) as the proportional reduction in a female's fitness under normal (e.g., high) exposure to males compared with what it is under low exposure:

$$H = (\bar{W}_{\text{low exposure}} - \bar{W}_{\text{high exposure}})/\bar{W}_{\text{low exposure}}. \qquad (1)$$

This dimensionless metric allows the magnitude of harm to be compared across contexts (e.g., among different female genotypes and/or environments), even if the intrinsic fitness of females varies among those contexts.

An important practical caveat of method 2, or any study of male harm that manipulates exposure, is that the results depend—typically in unknown ways—on the choice of exposure levels (i.e., what is a reasonable way to implement the "low"-exposure treatment?). An advantage of method 1 is that it can be performed using only the "normal" or natural level of exposure. If method 2 is used to compare the effects of different male types, we designate this as method 2–DMT to denote that different male types are incorporated into the design. Method 2–DMT is logistically demanding but automatically yields the data of a method 1 experiment. In our study, the two methods largely lead to similar inferences, but where they differ highlights the challenges of making conclusions from either method alone. Method 2–DMT can be viewed as offering an opportunity to investigate how IGEs of males differ across exposure levels (i.e., IGE × exposure interaction). This is not the primary lens through which we view our data, but we return to this perspective in "Discussion."

We are interested in understanding how male harm evolves in response to changes in the environment in which mate competition occurs. Male harm is intimately linked to mate competition because it arises from traits that are favored because they help males outcompete other males, maximizing their relative mating/fertilization success (Parker 1979). Because of this, if mate competition is eliminated, selection should act against males that harm their mate(s) and male harm is expected to decrease. In a landmark study, Holland and Rice (1999) tested this key prediction by evolving lab populations of *Drosophila melanogaster* for 34 generations under polygamy or enforced monogamy. In subsequent assays, they found that the reproductive rate of females was much higher when housed with males evolved under monogamy than those evolved under polygamy (i.e., method 1), consistent with expectation.

Drosophila melanogaster was a good choice for the experiment by Holland and Rice (1999) because previous work had shown that males are harmful in multiple ways. Using variants of method 2, early studies had indicated that males cause harm via both pre- and postmating mechanisms (Fowler and Partridge 1989; Partridge and Fowler 1990). Elegant manipulations showed that postmating harm was mediated by accessory gland proteins (Chapman et al. 1995). Much additional work since has addressed various aspects of male harm in *D. melanogaster* (e.g., Rice et al. 2006; Sirot et al. 2014; Hollis et al. 2019 and references therein), making it an influential model system that has raised the perceived importance of sexual conflict in animals more generally.

The compelling result of Holland and Rice (1999), along with a growing body of other evidence of male harm in other taxa (Arnqvist and Rowe 2005), is sometimes implicitly interpreted as indicating that male harm will—rather than may—evolve under polygamy (where mate competition is present) but not monogamy (where mate competition is absent). However, Zuk et al. (2014) pointed out that in many nonmonogamous species, sexual interactions are largely under the control of females. For instance, females in some taxa feed and mate in separate habitats, minimizing their exposure to males (e.g., Wiklund et al. 1993; García-González and Simmons 2005; Edvardsson 2007). Under such circumstances, females can have a large degree of control over mating interactions and hence can greatly limit the opportunity for male harm. One reason that populations or species may differ in the control that females have relates to the environment in which mating occurs. For instance, in environments where females can avoid or escape males,

the opportunity for precopulatory male harm may be greatly reduced.

Though *D. melanogaster* is one of the canonical examples of sexual conflict and male harm, most studies have been conducted in lab culture conditions that involve high densities of flies and environments that are spatially restricted and structurally simple and thus not representative of many natural environments (but see Byrne et al. 2008). Would male harm be an important aspect of sexual interactions in a lower-density, more complex environment that possibly offered females more control over sexual interactions? In a previous study (Yun et al. 2017), we used a population of flies adapted to typical lab culture conditions to assay male harm in two different environments (via a method 2 design). Corroborating classic fly studies, we found that exposure to males was harmful to females when the assays were conducted in a standard lab (i.e., "simple") environment. In contrast, there was little to no evidence of harm when assays were conducted in a lower-density, more structurally complex environment, presumably because females could easily evade males in that environment. Consistent with this, mating rates are lower in the complex compared with the simple environment (Yun et al. 2019).

Yun et al. (2017) illustrated plasticity in male harm but did not address how male harm evolves in populations maintained in such an environment. Would selection for success in mate competition in the complex environment inadvertently cause males to evolve phenotypes that harmed females even in that environment? Alternatively, might the greater control of females in that environment render harmful male phenotypes useless and costly, driving an evolutionary reduction in harm?

As part of a study originally designed to test how different mating environments affect adaptation and purging (Yun et al. 2018), we evolved 63 fly populations divided among three mate competition treatments: (i) enforced monogamy (i.e., "mate competition absent," MC_{absent}); (ii) mate competition in a simple environment (MC_{simple}); and (iii) mate competition in a complex environment ($MC_{complex}$). These populations provide the opportunity to investigate how male harm evolves in response to changes in the mate competition environment. We previously assayed adult male (and female) fitness of flies from each treatment factorially in each of the three mating environments (Yun et al. 2019). A few of those results are pertinent here. First, as expected, $MC_{complex}$ males adapted to the challenges of mate competition in the complex environment—that is, $MC_{complex}$ males were considerably more fit than MC_{absent} or MC_{simple} males when assayed in the complex mating environment. Second, the assays of male fitness conducted under the conditions of the enforced monogamy treatment are (conveniently) equivalent to a study of male harm using method 1. (Fitness assayed under the conditions of the other two mating treatments cannot be used to make inferences of male harm.) We observed that single females paired with individual MC_{simple} males had reduced fitness relative to single females individually paired with either MC_{absent} or $MC_{complex}$ males. This implies that MC_{simple} males are harmful relative to MC_{absent} males, matching the results of Holland and Rice (1999) and others (e.g., Martin and Hosken 2003*b*; Crudgington et al. 2005, 2010). Most interestingly, these results also imply that $MC_{complex}$ males are not harmful, despite evolving to be successful in a treatment where mate competition occurs.

While suggestive, that study was not intended as an investigation of the evolution of male harm and has several shortcomings in that regard. By virtue of its equivalence to a method 1 harm assay, the data do not truly test whether males are harmful. For example, we do not know whether $MC_{complex}$ males are (i) less harmful than MC_{simple} males but still harmful, (ii) not harmful at all, or (iii) beneficial to females. A second major caveat is that the data relevant to male harm come from only a single assay environment (i.e., that used in the enforced monogamy treatment). This means, for example, that those data offer no direct information about the potential harm caused by $MC_{complex}$ males when mating in the complex environment to which they have adapted. Males can affect females in a number of ways (e.g., disturbing foraging, causing damage during physical interactions, and manipulating their physiology and reproductive output via seminal fluid proteins). Given that the opportunity for, and fitness consequences of, these forms of harm are likely to vary across environments, it is plausible that males from different evolutionary treatments will express harm differently in each assay condition.

Here we perform a direct and more thorough assessment of evolutionary divergence in male harm among these three mating treatments. Using a method 2–DMT design involving low and high exposure to males, we measure harm of males from each mating treatment reciprocally in each of the three mating environments. Our assays are designed to closely match the conditions under which the experimental populations have evolved—that is, the impact of males on female fitness is assessed in a manner relevant to this evolutionary context. This experiment allows us to ask how harm has evolved in males from populations with different selective histories in these environments, how the expression of harm depends on the environment in which it is assayed for all of these types of males, and whether there is an interaction between evolutionary history and assay environment. As in earlier studies (e.g., Holland and Rice 1999; Martin and Hosken 2003*b*; Crudgington et al. 2005, 2010), we expect males in the monogamy treatment to evolve reduced harm. We are particularly interested in

the contrast between males evolved in the two treatments where mate competition occurs. Does male harmfulness evolve to lower levels in the complex mate competition environment where our previous study suggested that females have more control over sexual interactions? Alternatively, have males that adapted to the complex environment, including via increased reproductive success, managed to gain greater control and become harmful in that environment?

The experiment also allows us to make interpretations from both a method 1 and a method 2–DMT perspective. Taken together, our data indicate that males evolved in the absence of mate competition (i.e., under monogamy) or in the presence of mate competition in a complex environment are not very harmful, regardless of assay environment in which they are tested. In contrast, males evolved in the presence of mate competition in a simple environment are harmful but are not capable of expressing harm when tested in the complex environment. Differences between the interpretations arising from the different approaches hint that males may exert a mixture of positive and negative effects on females and that divergence has occurred in how females are affected by males; future studies will be required to understand these effects properly.

Material and Methods

Experimental Populations

A stock population (the "ancestor") of *Drosophila melanogaster* was originally collected from the Similkameen Valley, British Columbia, Canada, in 2005 by S. Yeaman. Since 2010, this stock has been maintained in standard *Drosophila* culture bottles containing 40 mL of cornmeal medium at 25°C, 12L : 12D photoperiod, and 50% relative humidity at a large population size (~3,000 adults) with discrete, nonoverlapping 2-week generations. In September 2014, 63 separate experimental *D. melanogaster* populations were created from this stock. The origin and maintenance of these experimental populations are described in detail in Yun et al. (2018). In brief, the 63 populations were divided equally into three "larval adaptation sets" (21 populations per set), with each set involving unique and novel larval rearing conditions (figs. S1, S2; figs. S1–S9 are available online). Within each larval adaptation set, the 21 populations experienced one of three different adult mating treatments every generation (seven populations per mating treatment), as described below. The 63 populations were initially created to examine how alternative mating regimes affect adaptation to novel abiotic environments (as reported in Yun et al. 2018). For the current project, however, we had no reason to expect differences in harm among larval adaptation sets. Rather, the three different adaptation sets simply serve as a level of replication for the primary factor of interest: adult mating treatment. Larval adaptation set was included in our statistical models for completeness, and results were generally consistent among adaptation sets.

The three adult mating treatments were as follows: mate competition absent (MC_{absent}), mate competition in a small and structurally simple mating environment (MC_{simple}), or mate competition in a larger and more structurally complex environment ($MC_{complex}$). For a given population, mate competition was removed by randomly assigning 140 single male-female pairs to separate wide straws, while mate competition was permitted by creating four replicate groups of 35 males and 35 females each and putting these separately into either standard *Drosophila* culture vials (MC_{simple}) or 1.65-L cylindrical plastic Ziploc food storage containers ($MC_{complex}$). Each container housed five separate food patches (i.e., small cups with plastic barriers further subdividing the surface of the food) and had two pipe cleaners protruding from the lid into the interior space (see fig. S1 of Yun et al. 2019). Adults interacted for 6 days in their respective mating treatments, after which 105 surviving females were randomly chosen and distributed among seven vials to lay eggs for 24 ± 3 h. Females were then discarded, and the surface of the food was scraped to yield ~200 eggs per vial. Eleven days later, emerging adult offspring were collected and stored in holding vials separately by sex (35 flies per vial) for 3 days (to produce a 21-day generation time) before repeating the above mating protocol for the next generation. Each larval adaptation set was maintained on a 3-week nonoverlapping generation, with each set offset from the others by 1 week.

Male Harm Assays

Male harm was assayed separately for each larval adaptation set after 66–72 generations of experimental evolution. Within each adaptation set, male harm was quantified in a factorial design by assaying the survival and fecundity of females under both low (periodic) and high (continuous) exposure to males while manipulating the identity of the males (i.e., the experimental population from all three evolutionary mating treatments) and the environment in which the exposure occurred (i.e., single-pair straw, multifly vial, or multifly container). We refer to the latter as the "assay mating environment" to distinguish it from the "evolutionary mating treatment" under which a given population evolved (i.e., MC_{absent}, MC_{simple}, or $MC_{complex}$). Other than the intended manipulations of male exposure and assay mating environment, these assays were designed to closely mimic normal maintenance conditions and hence fitness during experimental evolution (figs. S3, S4).

This experimental design yields data on female fitness under both high and low exposure to males of different

types, allowing both method 1 and method 2–DMT analyses. A method 1 analysis compares females exposed to different male types under a single exposure level. Typically, method 1 approaches have been applied using "standard" or high exposure to males, but it can be performed using any exposure level. Therefore, for completeness, we separately analyze data from both our low and our high male exposure levels using a method 1 approach. For the method 2–DMT analysis, male harm was quantified as the proportional reduction in a female's fitness under high compared with low exposure (i.e., H; eq. [1]) to a given type of male in a specific assay environment, and values for H were then compared among male types and assay environments. The statistical analyses are described below.

For each larval adaptation set, we used males from six of the seven populations from each of the three evolutionary mating treatments, yielding 18 experimental populations per set and 54 populations in total. The seventh population within each evolutionary mating treatment was used to collect females such that males were never tested with females from their own population. For each larval adaptation set × assay mating environment combination, males from the 18 experimental populations were tested with females that had evolved in that mating environment. For example, within larval adaptation set 1, males from the 18 experimental populations (six populations each from the MC_{absent}, MC_{simple}, and $MC_{complex}$ evolutionary mating treatments) were used in low and high male exposure treatments with females from the seventh MC_{absent} population when tested in the single-pair straw assay mating environment, with females from the seventh MC_{simple} population when tested in the multifly vial assay mating environment, and with females from the seventh $MC_{complex}$ population when tested in the multifly container assay mating environment. The rationale for this choice of females is that male harm in a given environment was assayed using females that were adapted to that environment but that had not coevolved with any of the males (i.e., females and males were never from the same population). Males from the ancestral population were also assayed within each mating environment simply as an external reference point.

In all cases, females spent 6 days in their respective assay mating environment, mirroring their normal maintenance during experimental evolution. At the end of the sixth day, female survival was recorded and fecundity was subsequently determined for a subset of the survivors (see below). In the multifly vial and multifly container assay mating environments, three replicates were set up using males from each experimental population (25 replicates for the ancestor) in each assay mating environment, with each replicate consisting of 35 males and 35 females. In the low-exposure treatment, these 35 females were held with 35 males for 5 h in a vial on days 1, 4, and 6. This 5-h window occurred between 10 a.m. and 3 p.m. approximately, with the exact timing being consistent across mating treatments within a given larval adaptation set each generation; this was during the lights-on period of the 12L : 12D cycle. Outside of these exposure periods, males were removed and the 35 females were held together in their appropriate assay mating environment (i.e., in a vial or a container). Males were stored on fresh food outside of the exposure periods and were used with the same replicate each time. In the high-exposure treatment, the 35 females experienced the same 5 h exposure to 35 males in a vial on days 1, 4, and 6 of the mating phase (with the same timing as above), but the males remained present outside of these periods when the females were held in their respective assay mating environment, meaning that females in these replicates were continually exposed to males. At the end of the 6 days, males were discarded, the number of surviving females was recorded, and random pairs of females were placed in each of seven fresh cornmeal media vials for 20 h for oviposition. Fourteen days later, we counted the number of adults that had emerged in each vial. In a small number of replicates, one out of the two females died during the egg-laying period; these were treated in the analysis in the same way as other replicates (mortality during egg laying contributed to variation in female fecundity rather than survival).

In the single-pair straw assay mating environment, we tested 30 males from each experimental population (90 ancestral males). In the low-exposure treatment, these 30 males were held together with 30 females for 5 h in a vial on days 1, 4, and 6 of the mating phase, as with the other assay mating environments above. Outside of these exposure periods, the males were removed and stored in a fresh vial while the females were held individually in straws. In the high-exposure treatment, the 30 males and 30 females were again held together to experience the same 5 h exposure in a vial on days 1, 4, and 6, but a single male accompanied each female outside of this period when the females were held separately in straws. At the end of the 6 days, males were discarded, the number of surviving females was recorded, and random pairs of females were placed in each of 10 fresh vials for 20 h for oviposition. Fourteen days later, we counted the number of adults that had emerged in each vial.

Within a given larval adaptation set, all the flies were maintained for two generations in a common larval and mating environment (mass culture in vials containing ancestral cornmeal media at 25°C) before conducting the assay (to minimize plastic effects of mating treatment environment). In all larval adaptation sets and assay mating environments, females were handled in the same way in the high- and low-exposure treatments and received similar exposure to CO_2 anesthesia. To reduce variation in sex ratio during the 6-day mating phase, dead males were replaced

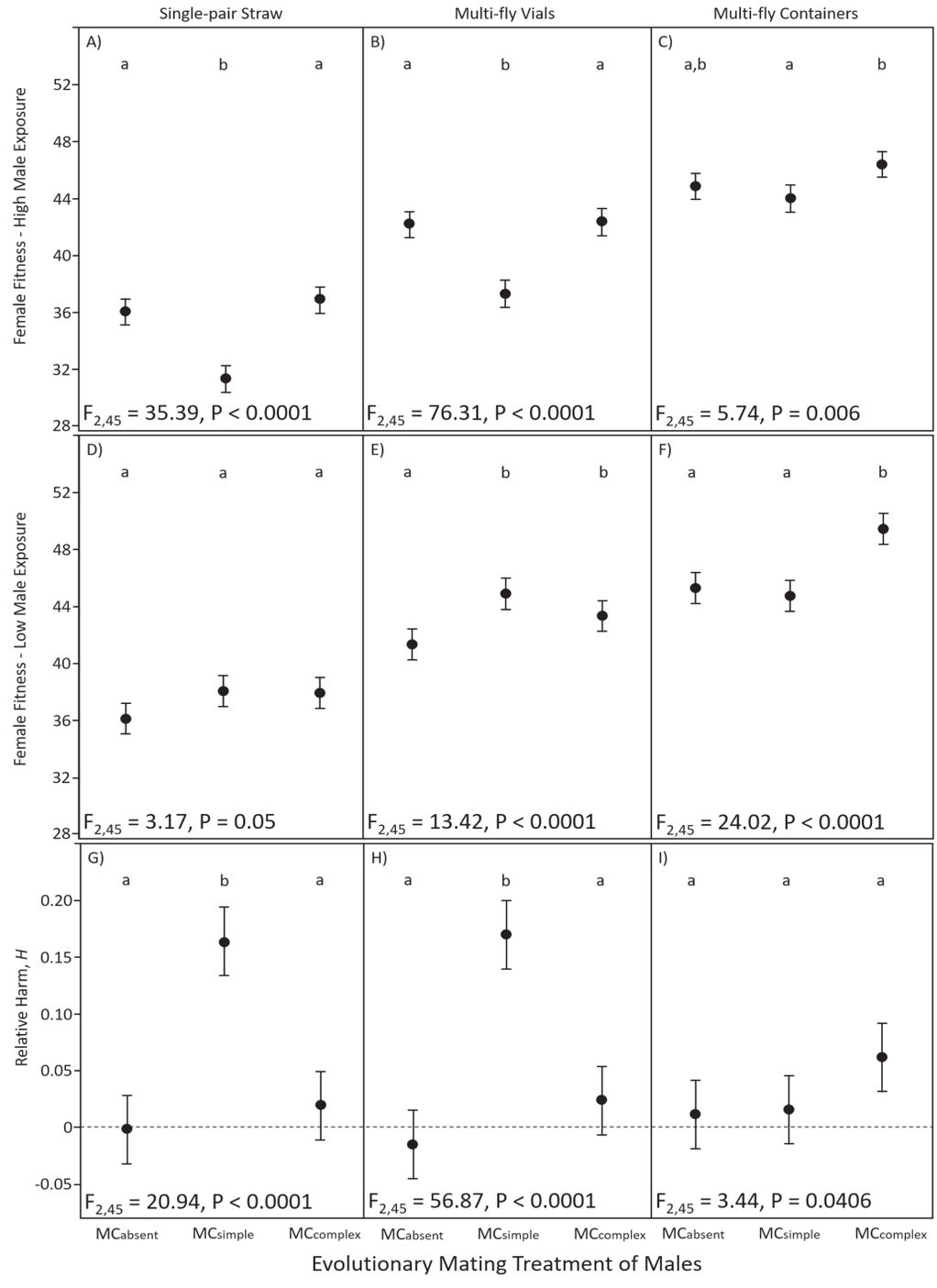

Figure 1: Effect of males from the different evolutionary mating treatments on females when tested in each of three assay mating environments (columns). Male effects are quantified in terms of (i) female fitness under high (i.e., continual) male exposure, corresponding to a method 1 analysis (top row, *A–C*); (ii) female fitness under low male exposure (middle row, *D–F*); and (iii) relative male harm, *H* (bottom row, *G–I*), measured as the relative reduction in female fitness under low versus high exposure (see eq. [1]) and corresponding to a method 2–DMT analysis. Points are least square means (±95% confidence interval) depicting average effects across the three adaptation sets, treating populations as replicates. Lowercase letters denote results of post hoc comparisons (Tukey's honestly significant difference) following a separate two-way ANOVA within each panel testing the effect of evolutionary mating treatment, adaptation set, and their interaction. The *F* and *P* values are for the main effect of evolutionary mating treatment.

with live males from the same population and dead females with brown-eye mutant females (these mutant females were ignored when calculating survival and did not have their fecundity measured). All the food present in the mating arenas (e.g., straw, vial, or container) was made with 25% more agar to reduce liquefaction caused by developing larvae.

Statistical Analyses

Female fitness was calculated as the product of the average survival (proportion alive after the 6-day mating treatment) and the average fecundity across all vials using males from a given population, at a given exposure level, when tested in a given assay mating environment. This treats populations (from which the assay males originated) as the unit of replication, with each population being tested in each of the three assay mating environments and at both exposure levels. For the method 1 approach, we analyzed the data from low and high male exposure separately. In each case, variation in female fitness was analyzed using a general linear mixed model, fitted via restricted maximum likelihood, that included fixed effects of the mating treatment under which the males had evolved, assay mating environment in which they were tested, larval adaptation set, and the two- and three-way interactions among these and employing partial (type III) tests of these effects. Population from which the males were derived was included as a random effect to account for repeated measures of the same population. The effect of evolutionary mating treatment varied by assay mating environment when analyzing female fitness under both low and high male exposure (see "Results"), so differences among mating treatments were subsequently tested separately by assay mating environment using a two-way ANOVA that included mating treatment, larval adaptation set, and their interaction.

For method 2–DMT, the proportional reduction in female fitness owing to continuous (vs. low) exposure to males—hereafter, "relative harm" (*H*)—was calculated following equation (1), again treating populations (from which the assay males originated) as the unit of replication. As above, female fitness was calculated as the product of the average survival and the average fecundity across all vials for each of these combinations. Variation in *H* was analyzed using the same general linear mixed model described above. The effect of evolutionary mating treatment again varied by assay mating environment (see "Results"), so the same simplified two-way ANOVA was also fit separately by assay mating environment.

Results

The results below focus on the main effects of mating treatment, averaged across adaptation sets, because there is no a priori reason to expect male harm to vary among larval adaptation sets. The adaptation sets exist because our experimental populations were originally created to test the effects of the mating treatments on adaptation across three larval adaptation sets (Yun et al. 2018). Larval adaptation set and interactions with it were nevertheless included in our analyses, and we note cases in which the effect of mating treatment varies among adaptation sets.

Method 1: Variation in Female Fitness under High Exposure to Males

Female fitness varied significantly under continual (i.e., high) exposure to males from the different evolutionary mating treatments, and this effect depended on the mating environment in which the assay was performed (i.e., assay mating environment × evolutionary mating treatment interaction; $F_{4,90} = 7.32$, $P < .0001$; fig. 1*A*–1*C*; table S1; tables S1–S3 are available online). Given this, we analyzed each assay mating environment separately. In single-pair straws, average female fitness varied significantly among evolutionary mating treatments ($F_{2,45} = 35.39, P < .0001$), with females performing significantly worse when exposed to males that evolved in the simple mate competition environment (i.e., MC_{simple} males) compared with when they were exposed to males that evolved either in the absence of mate competition or in the complex mate competition environment (i.e., MC_{absent} and $MC_{complex}$ males; fig. 1*A*). This effect was consistent across all three larval adaptation sets (i.e., no significant adaptation set × evolutionary mating treatment interaction; fig. S5*A*–S5*C*). Results were qualitatively the same when female fitness was assayed in the multifly vials (fig. 1*B*). Females again had significantly lower average fitness when exposed to males from the MC_{simple} evolutionary mating treatment compared with when they were exposed to males evolved in either the MC_{absent} or the $MC_{complex}$ mating treatments ($F_{2,45} = 76.31$, $P < .0001$). Although the magnitude of this effect varied significantly across larval adaptation sets, the main pattern was always the same—that is, females were least fit when housed with MC_{simple} males (fig. S5*D*–S5*F*). Results differed somewhat when female fitness was assayed in the multifly containers. Evolutionary mating treatment was again significant ($F_{2,45} = 5.74$, $P = .006$), although differences in average female fitness among treatments were smaller and females did best when housed with males that evolved in this mating environment ($MC_{complex}$; fig. 1*C*). This effect of evolutionary mating treatment was consistent across larval adaptation sets (fig. S5*G*–S5*I*).

Method 1: Variation in Female Fitness under Low Male Exposure

Female fitness varied significantly under low exposure to males from the different evolutionary mating treatments.

This effect again depended on the mating environment in which the assay was performed (i.e., assay mating environment × evolutionary mating treatment interaction; $F_{4,90} = 9.92$, $P < .0001$; fig. 1*D*–1*F*; table S2), so we proceed to analyze each assay environment separately. In single-pair straws, females had slightly lower fitness on average when exposed to males that evolved in this environment (i.e., MC_{absent} males) compared with males that evolved in the other mating treatments (i.e., MC_{simple} and $MC_{complex}$ males; fig. 1*D*), a difference that approached significance ($F_{2,45} = 3.17$, $P = .0515$). This effect was consistent among larval adaptation sets (fig. S6*A*–S6*C*). When assayed in multifly vials, female fitness varied significantly with male type ($F_{2,45} = 13.42$, $P < .0001$), with females doing best on average with males that evolved in this environment (i.e., MC_{simple}) compared with males from the other two mating treatments (MC_{absent} and $MC_{complex}$; fig. 1*E*). This effect varied significantly among larval adaptation sets, although in all three adaptation sets females had higher fitness with MC_{simple} than with MC_{absent} males (fig. S6*D*–S6*F*). In multifly containers, female fitness again varied significantly with male type ($F_{2,45} = 24.02$, $P < .0001$) and females again did best when exposed to males that evolved in this environment (i.e., $MC_{complex}$ males) compared with when they were exposed to MC_{absent} or MC_{simple} males (fig. 1*F*). This effect was consistent across larval adaptation sets (fig. S6*G*–S6*I*).

Method 2–DMT: Variation in Relative Male Harm (H)

Relative male harm, *H* (the proportional reduction in female fitness under high vs. low exposure to a given male type in a particular assay environment; eq. [1]), varied significantly among assay mating environments and was lower overall in the complex environment relative to the other environments (fig. 2; table S3). Male harm also differed among evolutionary mating treatments, although this effect depended on the assay mating environment (i.e., assay mating environment × evolutionary mating treatment interaction; $F_{4,90} = 14.20$, $P < .0001$; fig. 1*G*–1*I*; table S3).

Analyzing separately by assay mating environment, the patterns of variation in *H* were very similar to those for the method 1 analysis of female fitness under high male exposure above (fig. 1). In single-pair straws, male harmfulness to females varied significantly among evolutionary mating treatments ($F_{2,45} = 20.94$, $P < .0001$); males that evolved in the simple mate competition environment (i.e., MC_{simple} males) were significantly more harmful to females than males from either of the other mating treatments (i.e., MC_{absent} and $MC_{complex}$ males; fig. 1*G*). This effect was consistent across all three larval adaptation sets (fig. S7*A*–S7*C*). Results were qualitatively

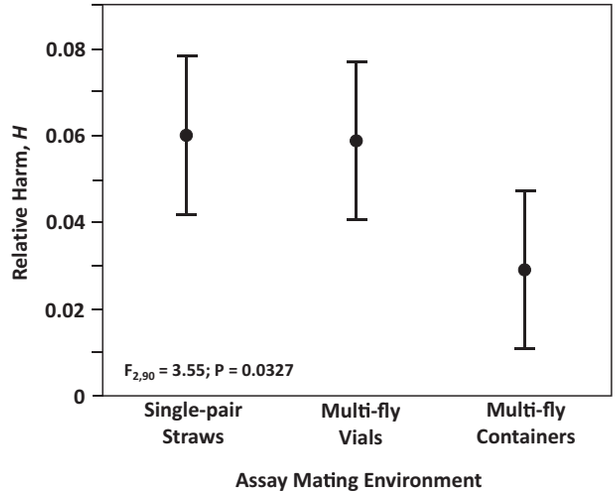

Figure 2: Variation in relative harm (*H*; see eq. [1]) among the three assay mating environments, corresponding to a method 2–DMT approach. Values are least square means (±95% confidence interval) from the linear model in table S3 (available online) and depict the main effect of assay mating environment while adjusting for the effects of adaptation set and evolutionary mating treatment, treating male populations as replicates.

the same when male harm was assayed in the multifly vials (fig. 1*H*); MC_{simple} males were again significantly more harmful than either MC_{absent} or $MC_{complex}$ males ($F_{2,45} = 56.87$, $P < .0001$), and this effect was also consistent among larval adaptation sets (fig. S7*D*–S7*F*). In both straws and vials, harm by MC_{simple} males was significant (95% confidence intervals [CIs] do not overlap zero; fig. 1*G*, 1*H*), whereas that by the other two male types was not.

In the multifly containers, values of *H* tended to be low on average (fig. 2). Moreover, variation among male types was low though still significant ($F_{2,45} = 3.44$, $P = .0406$; fig. 1*I*). The highest and only significant value of *H* (95% CI does not overlap zero; fig. 1*I*) was associated with $MC_{complex}$ males, though this result requires careful interpretation in light of our method 1 results (see "Discussion"). The effect of evolutionary mating treatment was consistent across larval adaptation sets (fig. S7*G*–S7*I*). Finally, visual inspection of the two underlying components of female fitness across all three mating environments revealed that variation in male harm arose in large part from its effects on female fecundity as opposed to female survival (figs. S8, S9).

Discussion

We are interested in the evolution and expression of male harm in alternative environments that vary in mate competition and the physical conditions under which it occurs. The contrast between simple and complex mating environments is of interest for three related reasons. First, it

is likely representative—at least in spirit—of temporal or spatial variation in real mating environments experienced by some species. Second, Zuk et al. (2014) argued that sexual conflict will be less important in systems where females have more control over intersexual interactions, and male harmfulness should decline under such circumstances, especially if traits causing harmfulness are costly (Parker 1979). Our complex environment was designed in part to increase female control (females can readily hide or fly away and have a choice of where to feed and lay eggs), and past studies suggest that this was achieved, as intersexual interactions, mating rates, and male harm are all lower in the complex compared with the simple environment (Yun et al. 2017, 2019). Third, *Drosophila melanogaster* has been influential in the study of male harm and sexual conflict, yet standard lab conditions in which populations are usually maintained and studied are likely unrepresentative of many natural settings in which this species occurs. While our environments cannot be considered natural, our results shed light on the importance of environment for the evolution and expression of male harm in this influential model system for the study of sexual conflict.

For the above reasons, we had the primary goal of comparing the divergence of male harm among mating treatments, but doing so necessitates a method to quantify harm. Two common approaches have been taken in the literature: the first compares the fitness of females under normal exposure to different male types (method 1, high exposure), while the second contrasts how females perform under low versus high exposure. Our second goal was to consider the advantages and limitations of these two approaches, which we have done in the introduction, and to compare the interpretations arising from them when applied to our primary goal.

There are two major patterns that dominate the variation in male harm in our data. The first concerns the expression of male harm in different mating environments: harm is greatly reduced in the complex mating environment (fig. 2; see also Yun et al. 2017). This conclusion requires a method 2–DMT approach because absolute female fitness can vary among environments for reasons unrelated to male effects, severely limiting the inferences that can be drawn from method 1 with respect to how the environment mediates harm. The second major pattern pertains to the evolutionary divergence of harm among mating treatments: when assayed in an environment in which harm can be expressed (i.e., single-pair straws and multifly vials; fig. 2): males evolved with mate competition in a simple environment (i.e., MC_{simple}), characteristic of the majority of *Drosophila* lab populations, are quite harmful, whereas MC_{absent} and $MC_{complex}$ males are not. From a typical method 1 (high-exposure) perspective, we see that females perform significantly worse when held with MC_{simple} than with other males. This pattern can be interpreted as MC_{simple} males being more harmful, but logically it could instead indicate that they are simply less beneficial than other males. Using method 2–DMT, we can see that the MC_{simple} males are indeed harmful, whereas other males are not.

Other studies have previously reported that fly populations maintained without mate competition (i.e., enforced monogamy) evolve male phenotypes that are less harmful than those maintained with mate competition under standard lab conditions (Holland and Rice 1999; Martin and Hosken 2003*b*; Crudgington et al. 2005, 2010). Though mate competition may be necessary for the evolution of male harm, our results show that it is not sufficient. Males from the $MC_{complex}$ treatment evolved very low levels of harm (fig. 1), even though such males evolved in the presence of mate competition and are highly successful mate competitors in their own and other mating environments, as we have previously demonstrated (Yun et al. 2019). (MC_{absent} males are also not harmful but, in contrast, are poor mate competitors.) The complex environment presumably allows females more control over sexual interactions. Consequently, males that have evolved harmful phenotypes in other environments (e.g., MC_{simple} males) are unable to exert harm when assayed in the complex environment (fig. 2; see also Yun et al. 2017). A priori, it was unclear whether populations experiencing ongoing mate competition in the complex environment would evolve males with elevated or reduced levels of harm in response. These results are consistent with the claim that male harm is not likely to be a prominent feature in systems where females have greater control over sexual interactions (Zuk et al. 2014).

In sum, $MC_{complex}$ males are highly successful mate competitors (Yun et al. 2019) and yet they express little to no harm in any assay environment (fig. 1). We can only speculate why harmfulness of $MC_{complex}$ males is reduced compared with MC_{simple} males. First, there may be selection in the multifly containers against males wasting time and effort in precopulatory harassment of uninterested females, given that such females can readily escape and hide (i.e., an energetic and opportunity cost to male harassment with little potential gain). Second, mating rates are lower in containers than in vials (Yun et al. 2019), presumably because females can avoid or escape from males. If a female is unlikely to remate, a male's fitness becomes more closely aligned with his current mate, selecting against harmful postcopulatory effects.

In the discussion thus far, we have highlighted the advantages of method 2–DMT over method 1 in terms of inferential power. With respect to evolutionary divergence, the inferences from method 1 (high exposure) and method 2–DMT are largely consistent. (This is not surprising

because both use the data from the high-exposure treatment.) However, there is a discrepancy worth discussing, as it illuminates a concern with relying entirely on the relative harm measure *H* from method 2–DMT. Consider the harmfulness of MC_{simple} and $MC_{complex}$ males when assayed in multifly containers. Under high male exposure, females have slightly higher fitness with $MC_{complex}$ than with MC_{simple} males (fig. 1*C*). Ignoring the possibility that males could be beneficial, a standard method 1 interpretation would be that MC_{simple} males are somewhat more harmful than $MC_{complex}$ males. However, the inference from method 2–DMT is very different. There is significant variation in *H* among male types in this environment (fig. 1*I*), and females experienced a small but significant decrease in relative fitness with increased exposure to $MC_{complex}$ males ($H = \sim 5\%$). In contrast, for MC_{simple} males *H* was not significantly different from zero. To summarize, method 1 (high exposure) indicates that MC_{simple} males are more harmful than $MC_{complex}$ males in this assay environment, whereas method 2–DMT suggests the opposite.

Inspection of female fitness under low male exposure in the multifly containers (fig. 1*F*) sheds light on this discrepancy. Under low male exposure, females perform significantly better when paired with $MC_{complex}$ than with MC_{simple} males. However, the relative decrease in female fitness with increased male exposure is greater (i.e., *H* is larger) for $MC_{complex}$ than for MC_{simple} males, even though females have higher absolute fitness with $MC_{complex}$ males when comparing at both exposure levels. While $MC_{complex}$ males may be weakly harmful in this mating environment, it does not seem appropriate to consider them more harmful than MC_{simple} males.

Unlike method 1, method 2–DMT requires two exposure levels. In most cases, including our own, this involves a low-exposure treatment created by imposing a potentially unnatural restriction of sexual interactions. While results dependent on such manipulations should be regarded with caution, they can also reveal underlying biological complexity. One hypothesis for the above discrepancy between the inferences from method 1 and method 2–DMT recognizes that males can impact females in a variety of ways (e.g., physically, via chemosensory signals in mating interactions, and biochemically, via a cocktail of seminal fluid proteins after mating) and females likely evolve in response to these male "inputs." Females may evolve to resist male effects that are particularly damaging and even to take advantage of particular male inputs to benefit their own reproduction. For example, in several taxa seminal fluid proteins appear to be used by females as a reliable cue to coordinate their reproductive physiology with the availability of sperm (Sirot et al. 2014). Males likely exert a mix of positive and negative effects on females, even in systems where the net effect of high male exposure is harmful (Wiklund et al. 1993; Rönn et al. 2006). In our case, it is possible that in the container assay, the beneficial effects of $MC_{complex}$ males is reaped with limited exposure whereas the deleterious effects grow with increased exposure, while other male types may provide a weaker set of both beneficial and deleterious effects. Alternatively, the other male types might provide stronger deleterious effects than $MC_{complex}$, and these are fully realized even under low exposure. Regardless of the explanation, the more general issue is that, while testing for harm in a given context is reasonably straightforward (i.e., does increased male exposure reduce female fitness?), greater care must be given when comparing harm among contexts (e.g., among males types or environments) because differences in relative fitness can be misleading about patterns in absolute fitness.

Much of the variation that we observe in *H* among male types is due to variation in female fitness under high exposure, which is why method 2–DMT and method 1 (high exposure) lead to largely consistent interpretations. Nonetheless, male type also affects female fitness under low exposure and additional insight may be gained by considering this. Interestingly, the pattern of variation in female fitness under low male exposure does not follow the narrative discussed above. For example, in straws and in vials MC_{simple} males are the most harmful when considering either *H* or fitness under high exposure. Under low exposure, one might thus expect females to do worse when housed with MC_{simple} males than with either of the other male types, though the difference might be small because exposure is more limited. However, in vial assays, the data do not match this expectation, as females do significantly better with MC_{simple} males than with either other male type (fig. 1*E*). What should we make of this? As noted above, females experience a variety of physical, behavioral, and biochemical inputs from males during mating and are likely to evolve and potentially coevolve in response to these. The net effect of a male may depend on the extent to which females have adapted to that male's set of inputs in the mating environment in which the interaction occurs. Our experiment was not formally designed to test for female adaptation/coadaptation to males, although the assays in each mating environment did use females adapted to that environment. The increased performance of vial-adapted females under lower exposure to vial-evolved males (compared with other males; fig. 1*E*) may indicate that these females are somewhat reliant on stimuli provided by MC_{simple} males and the stimuli provided by other male types with only low exposure is insufficient. However, we emphasize that these possible "beneficial male effects" and "female adaptation effects" are small compared with the strong signature of net harm observed in straw and vial assays.

We employed the method 2–DMT design to measure *H*, but as we have just discussed, this relative measure needs to be interpreted with caution and with inspection

of the underlying components. Another way to view the data is as a study of the IGE of different male types on female fitness at each of two exposure levels. The results in figure 1*A*–1*C* versus 1*D*–1*F* show clear examples of IGEs (i.e., different males affect females differently), the effect of exposure level (i.e., female fitness is often reduced by high vs. low exposure), and their interaction. As discussed above, in some cases this interaction is very strong (e.g., in vial assays, females have higher fitness with MC_{absent} than with MC_{simple} males under high exposure, but the reverse is true under low exposure; fig. 1*B* vs. 1*E*). This ANOVA-type perspective offers an assumption-free description of the data. Of course, that description requires interpretation—using the same principles guiding our earlier discussion—to relate the observed patterns to the motivating questions about male harm.

Our goal was to gain a broader understanding of variation in male harm, and our results suggest that there is a strong plastic effect of the environment on this. However, our experiment used different females in each assay mating environment, so in principle, the variation in harm among mating environments could be due to differences in the physical environment and/or the female type. We strongly suspect that it is the former because our current results closely mirror those of an earlier study that assayed male harm in vials and in containers using males and females from a single population (Yun et al. 2017). Such plastic effects are not surprising: harmful male behaviors have been shown to vary in response to various ecological factors in several taxa, including water striders (Rowe et al. 1994), damselflies (Gosden and Svensson 2009), and guppies (MaGurran and Seghers 1994*b*).

Our experiment also allowed us to investigate how harm evolves in response to being maintained in different environments that directly affect the expression of harm. Data from a variety of other taxa show that aspects of sexual conflict can vary with population density, sex ratio, the availability of refuges, resource levels, predation risk, and other factors that in turn are likely to vary with the physical environment (Arnqvist 1992; Rowe 1992, 1994; Krupa and Sih 1993; MaGurran and Seghers 1994*b*; Gosden and Svensson 2009; Karlsson et al. 2010). In water striders, some of these factors (e.g., population density, predation risk) have also been associated with the among-population evolution of phenotypes underlying conflict (Perry and Rowe 2018). The extent to which environmental variables that directly affect the expression of male harm also influence the evolution of harm is an important topic for future research.

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Statement of Authorship

A.F.A., H.D.R., and L.Y. conceived the experiments, and L.Y. collected the data. L.Y., H.D.R., and A.F.A. contributed to data analysis and interpretation, and A.F.A. and H.D.R. wrote the manuscript with input from L.Y.

Data and Code Availability

Data have been deposited in the Dryad Digital Repository (https://doi.org/10.5061/dryad.9cnp5hqht; Rundle et al. 2021).

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