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Correlated evolution of male and female reproductive traits drive a cascading effect of reinforcement in *Drosophila yakuba*

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Selection against maladaptive hybridization can drive the evolution of reproductive isolation in a process called reinforcement. While the importance of reinforcement in evolution has been historically debated, many examples now exist. Despite these examples, we typically lack a detailed understanding of the mechanisms limiting the spread of reinforced phenotypes throughout a species' range. Here we address this issue in the fruit fly *Drosophila yakuba*, a species that hybridizes with its sister species *D. santomea* and is undergoing reinforcement in a well-defined hybrid zone on the island of São Tomé. Within this region, female *D. yakuba* show increased postmating-prezygotic (gametic) isolation towards *D. santomea* when compared with females from allopatric populations. We use a combination of natural collections, fertility assays, and experimental evolution to understand why reinforced gametic isolation in *D. yakuba* is confined to this hybrid zone. We show that, among other traits, *D. yakuba* males from sympatric populations sire fewer progeny than allopatric males when mated to allopatric *D. yakuba* females. Our results provide a novel example of reinforcement acting on a postmating-prezygotic trait in males, resulting in a cascade of reproductive isolation among conspecific populations.

1. Introduction

Reinforcement can drive the evolution of strong prezygotic reproductive isolation through natural selection acting against the production of maladapted, infertile, or inviable hybrids [1]. Because reinforcement acts through selection against hybrids, it occurs where species hybridize and can result in 'reproductive character displacement' (RCD); a pattern of stronger reproductive isolation in sympatric versus allopatric regions of a species' range [2]. While historically controversial [3,4], reinforcement has now been observed in a wide range of taxonomic groups, including fungi [5,6], animals [7–9], and plants [10,11]. Reinforcement could, therefore, be common during the 'completion' of speciation [12,13].

Despite an increase in our understanding of reinforcement, important aspects of the process remain underexplored. For example, we generally lack an understanding of how variation in levels of reinforced reproductive isolation (RRI) across a species' range, and a pattern of RCD, is maintained. One explanation for the maintenance of RCD is that reinforcement favours phenotypes that are selectively disfavoured in allopatry, confining 'reinforced' alleles to areas where hybridization occurs [14–16]. Under this scenario, reinforcement can drive incidental increases in levels of reproductive isolation between conspecific populations (i.e. the 'cascade reinforcement' hypothesis [14,15,17]). Recent work supports this hypothesis and suggests that phenotypes favoured by reinforcing selection in sympatry are often disadvantageous in allopatry [18–23]. The types of disadvantages can be diverse; however, because reinforcement frequently acts on traits involved in premating isolation, costs tend to manifest as a reduction in the ability to solicit potential mates when 'sympatric' phenotypes are found in allopatry [18–22,24]. To date, all reported cases of fitness costs associated with phenotypes involved in RRI come from studies focusing on individual traits affecting premating reproductive isolation (e.g. calls in frogs [20] or cuticular hydrocarbons in insects [19]).

Reinforcement is not restricted to premating traits, and postmating-prezygotic barriers can also be involved (e.g. interactions between reproductive tracts and gametes [6,25]). Just as occurs with other traits under the influence of sexual selection, postmating-prezygotic traits can influence female and male fitness in different ways and lead to sexual conflict and coevolution between the sexes [26,27]. For example, ejaculate traits that directly increase male fitness can have deleterious effects on female fitness [28,29] and previous work in *Drosophila* fruit flies has demonstrated ongoing coevolution between the female reproductive tract and male ejaculate [30,31]. Alternative explanations for correlated evolutionary responses in male and female traits include correlated selective pressures or genetic linkage. Independent of the specific mechanism(s), a correlated evolutionary response to reinforcing selection in both sexes could have cascading effects on levels of reproductive isolation between conspecific populations because 'sympatric' traits may become mismatched with conspecific 'allopatric' traits (e.g. female preferences and/or male signals).

Drosophila species that hybridize and are undergoing reinforcement [7,9,24,25] provide an opportunity to test the mechanisms maintaining a pattern of RCD despite ongoing gene flow between conspecific populations. *Drosophila yakuba* represents one such species: it is widespread throughout sub-Saharan Africa and, on the island of São Tomé, is mostly found in low-altitude (below 1 450 m), semi-dry habitats commonly associated with humans [32], but hybridizes with its sister species, *Drosophila santomea*, in a narrow hybrid zone on the mountain of Pico de São Tomé. Previous work has shown that female *D. yakuba* from this hybrid zone show higher postmating-prezygotic isolation towards males of *D. santomea* than do *D. yakuba* females from outside the hybrid zone [25]. Stronger reproductive isolation between sympatric *D. yakuba* and *D. santomea* is selectively advantageous because hybrid male offspring are sterile and, therefore, costly to produce [33].

The specific trait underlying RRI between *D. yakuba* and *D. santomea* is unknown; however, Matute [25] showed that sympatric female *D. yakuba* lay fewer eggs when mated with *D. santomea* than do allopatric females. This finding suggests that reinforcement in *D. yakuba* drives the evolution of postmating-prezygotic traits that affect how the female's reproductive tract, or eggs, interact with sperm from *D. santomea*. Matute [25] also used experimental evolution to show that reinforcement in *D. yakuba* can occur rapidly under laboratory conditions. Given the relatively short time frame of these experiments (10 generations), they show that the genetic variation required for gametic isolation to evolve is segregating within natural populations of *D. yakuba*. There is currently no evidence for reinforcement in *D. santomea* [25].

Here, we explore whether *D. yakuba* lines that show RRI from *D. santomea* also exhibit reduced fertility when crossed with conspecific individuals collected from allopatric populations. We first measure levels of fertility within populations of *D. yakuba* collected along an altitudinal transect on Pico de São Tomé when crossed with conspecific genetic backgrounds derived from sympatric and allopatric populations of *D. yakuba*. These data allow us to test the 'cascade reinforcement' hypothesis and ask whether *D. yakuba* found in sympatry with *D. santomea* show lower fertility when mated to conspecifics with 'allopatric' genotypes and vice versa. Second, we use experimental evolution to test whether the evolutionary response to reinforcing selection in populations of *D. yakuba* can lead to a correlated decrease in male fertility when mated to conspecific females from allopatric regions of the species' range. We predict that if reinforcement drives the correlated evolution of female and male traits, we will observe reduced fertility in conspecific crosses between two individuals that differ with respect to whether they are from populations found in sympatry or allopatry with *D. santomea*.

2. Material and methods

(a) Isofemale lines collected along an altitudinal transect on São Tomé

We carried out fertility assays (described below) using 100 isofemale lines collected from 10 sites (10 lines per site; see electronic supplementary material, Appendix S1, S2, and table S1 for details), equally distributed with respect to altitude, along an altitudinal transect on the island of São Tomé. These sites started at low elevations where only *D. yakuba* were collected (i.e. the seven low-altitude 'allopatric' sites) and finished at higher elevations where we observed hybrids between *D. yakuba* and *D. santomea* (i.e. the three high-altitude 'sympatric' sites).

Because *D. yakuba* only co-occur and hybridize with *D. santomea* at high altitudes it is difficult to disentangle the effect of reinforcement from other factors that might influence the traits we assay in this study. To address this issue, we sampled *D. yakuba* from 10 sites on the island of Bioko, spanning the same altitudinal range as the transect on São Tomé. Bioko is located to the northeast of São Tomé and while *D. yakuba* can be collected here, *D. santomea* is absent. Our goal was to use these data as a type of natural control and test whether altitude and geography affect levels of fertility between populations of *D. yakuba*, independent of the presence of *D. santomea*. Below we focus on data collected from the São Tomé transect and briefly summarize parallel analyses and results for the Bioko transect. All methods applied to the São Tomé transect were applied in parallel to the Bioko transect (see the electronic supplementary material for details).

(b) Female fertility with *Drosophila santomea*

To measure heterospecific fertility, we collected virgin *D. yakuba* females less than 8 h after eclosion from each of the 100 São Tomé isofemale lines, maintained them in isolation from males for 4 days, and then gave them the opportunity to mate with heterospecific *D. santomea* males (line SYN2005). We carried out these matings by combining an individual male and an individual female in a 100 ml vial containing cornmeal food. We observed all matings for 1 h and obtained a total of 15 mated pairs per line ($N = 1\,500$ females). After mating, we removed males from the vials and allowed each female to oviposit for 10 days, transferring each female to a fresh vial every 24 h. The number of eggs produced over 10 days was taken as an estimate of fertility.

(c) Female fertility with conspecific males with different genetic backgrounds

If reinforcing selection acting in sympatry indirectly affects levels of fertility between conspecific populations, as predicted by the 'cascade reinforcement' hypothesis, we expect that levels of fertility between conspecific matings will be lower when females and males are 'mismatched' with respect to their genetic background. To measure levels of conspecific fertility, we counted the number of eggs that a female produced when mated to different, but controlled, conspecific male genetic backgrounds. We first measured baseline levels of fertility within each isofemale line by crossing females from the same 100 focal lines described above with males from the same line. To measure conspecific fertility when mated to sympatric *D. yakuba* genotypes, we crossed females from each focal line to males from two 'tester' lines derived from females collected in 2005 from the centre of the São Tomé hybrid zone (BOSU1250.5 [sympatric$_{\mathrm{BOSU1250.5}}$] and SA1 [sympatric$_{\mathrm{SA1}}$]; see the electronic supplementary material for validation with other sympatric genotypes). To measure female fertility when mated to allopatric *D. yakuba* genotypes, we crossed females from the 100 focal lines with males from two allopatric tester lines: Täi18 (allopatric$_{\mathrm{Täi18}}$), collected in the Täi forest (Liberia) and SJ2 (allopatric$_{\mathrm{SJ2}}$), collected in the lowlands of São Tomé. We followed the same mating procedure described for heterospecific matings and obtained 15 mated females from each of the 100 focal line × male genotype combinations ($N = 9\,000$ females). We then counted the number of eggs produced by each female as described in the Methods Section for 'Female fertility with *D. santomea*'. We focused on the number of eggs laid rather than other measures of fertility, such as the proportion of fertilized eggs, because the proportion of laid eggs that are fertilized and viable does not vary between *D. yakuba* with sympatric or allopatric genetic backgrounds (electronic supplementary material, table S3).

(d) Male fertility with conspecific females with different genetic backgrounds

To test whether the fertility of males derived from sympatric and allopatric populations varies when crossed with 'sympatric' or 'allopatric' female genotypes, we crossed male *D. yakuba* from each focal line to females of the same tester stocks used to assess female fertility: sympatric$_{\mathrm{BOSU1250.5}}$, sympatric$_{\mathrm{SA1}}$, allopatric$_{\mathrm{Täi18}}$, and allopatric$_{\mathrm{SJ2}}$. As with females, we collected virgin males and allowed them to age for 4 days before crossing them with virgin females of the four tester lines. We then estimated the fertility of a given cross by counting the number of eggs produced by mated females as described above.

(e) Statistical analyses

We used a combination of generalized linear mixed models (GLMMs) and Spearman's rank correlations to test whether heterospecific female fertility, conspecific female fertility, and conspecific male fertility varied with respect to the genetic backgrounds of the two individuals involved in a cross. First, we modelled heterospecific female fertility (number of eggs produced per female) as a function of the altitude a female was collected from (a proxy for distance from the hybrid zone and the likelihood of reinforcement). We included isofemale line as a random effect in this model to control for genetic variation found within each sample site. Second, we modelled conspecific female fertility as a function of the altitude a female was collected from, the genetic background of the male line used in the cross (i.e. either sympatric or allopatric), and the interaction between altitude and male genetic background. We included focal isofemale line and male tester line as random effects to account for genetic variation observed within each sampling site and between male tester lines, respectively. Third, we modelled male fertility as a function of altitude, the genetic background of the female tester line used in the cross, and the interaction between altitude and female genetic background. We included the isofemale line of the focal male and the genotype of the female line he was crossed with as random effects. We fitted each of these GLMMs assuming Poisson distributed error with the 'glmer' function in the lme4 R library [34]. We predicted that if reinforcing selection has cascading effects on levels of conspecific fertility we should see a significant interaction between the location a focal line was collected from (i.e. altitude) and the genetic background to which it was crossed on levels of fertility. To assess the significance of this interaction we used likelihood ratio tests (LRT, 1 d.f.) which compared models that included versus excluded the altitude × genetic background interaction. For each full model, we report coefficients of determination (R^2) as the proportion of variation in observed fertility that was explained by the model-predicted levels of fertility.

We used Tukey's *post hoc* contrasts to determine differences in fertility between different types of matings. We predicted that sympatric *D. yakuba* would show decreased fertility when mated with allopatric conspecifics and vice versa. For female fertility this analysis resulted in 15 contrasts: females were classified as sympatric or allopatric and males as within-line, sympatric, or allopatric. For male fertility, the analysis resulted in six contrasts: males and females were classified as sympatric or allopatric.

Finally, we used Spearman's rank correlations to test the directionality of relationships between the altitude a line was collected from and fertility in the different types of crosses. Under the cascade reinforcement hypothesis, we predicted that populations of *D. yakuba* found in sympatry with *D. santomea* would show lower fertility when mated to *D. santomea*, higher fertility when mated to conspecific tester lines with sympatric genetic backgrounds, and lower fertility when mated to conspecific tester lines with allopatric genetic backgrounds.

(f) Correlations between heterospecific, conspecific female and conspecific male fertility

We next tested whether the traits responding to reinforcing selection in sympatry also affected levels of conspecific fertility. We predicted that if there is a correlated response to reinforcing selection in male and female *D. yakuba*, a decrease in heterospecific fertility should result in a concomitant decrease in fertility with allopatric *D. yakuba* (negative correlation). By contrast, *D. yakuba* showing higher heterospecific fertility should also show higher fertility with conspecific allopatric genotypes (positive correlation). We also tested whether mean female and male fertility of an isofemale line was correlated when mated to either conspecific allopatric genotypes or conspecific sympatric genotypes. All correlations were assessed using Spearman's rank correlation tests in R [34].

(g) Experimental sympatry

Among the lines we sampled on São Tomé, we found that as RRI from *D. santomea* increased (i.e. fertility decreased) fertility between male *D. yakuba* and allopatric conspecifics decreased (Spearman's $\rho = 0.90$; figure 2*a*). To test whether this decreased male fertility could evolve as a correlated effect of reinforcing selection, we carried out 10 generations of experimental evolution, selecting against hybridization between *D. yakuba* and *D. santomea*. We kept 23 populations of *D. yakuba*, derived from an outbred allopatric laboratory population, in experimental sympatry with *D. santomea* (see electronic supplementary material for details). Experimental populations consisted of 500 *D. yakuba* (equal sex ratio) and 500 *D. santomea* (equal sex ratio). Each generation, we collected virgin flies from the experimental populations within 10 h of eclosion (once they had obtained adult pigmentation), discarded hybrids (recognized by their abdominal pigmentation), and reconstituted experimental sympatry by combining 500 *D. yakuba* from each experimental population with 500 *D. santomea* from stock populations. Twenty-three control populations of *D. yakuba* were maintained in parallel and contained the same number of conspecifics but lacked *D. santomea*.

We compared levels of female fertility when mated to *D. santomea*, male fertility with sympatric$_{\mathrm{BOSU1250.5}}$ females, and male fertility with allopatric$_{\mathrm{Täi18}}$ females at the onset of the

Table 1. Summary of models testing for an interaction between altitude and the genetic background of a 'tester' individual used to carry out a cross (G_m for males and G_f for females; sympatric/allopatric) on levels of fertility in 100 *D. yakuba* lines sampled along an altitudinal transect on the island of São Tomé. See electronic supplementary material, table S6 for a parallel analysis with *D. yakuba* from Bioko. $p < 0.000001$ is highlighted in italics; Exp. dev., proportion of residual deviance explained by altitude (heterospecific matings) or the interaction between altitude and the genetic background of the tester line used in a given cross.

details of models					
type of fertility	fixed effects	random effects	LRT (χ^2)	exp. dev. (%)	R^2
heterospecific	altitude	isof. line	*539.11*	5.51	0.95
conspecific (female)	altitude + G_m + (altitude × G_m)	isof. line + male line	*638.13*	0.90	0.18
conspecific (male)	altitude + G_f + (altitude × G_f)	isof. line + female line	*3707.7*	6.51	0.34

experiment and following 10 generations of evolution using generalized linear models (GLMs) with Poisson distributed error. To test whether female fertility with *D. santomea* and male fertility with allopatric or sympatric females changed over the course of the experiment, we used LRTs that compared models that included generation as a fixed effect to 'null' models lacking this effect. Models were fitted using the 'glm' function in R [34]. Finally, we used Spearman's rank correlation to compare whether there was a correlation between female fertility with *D. santomea* and conspecific male fertility when mated to allopatric *D. yakuba* females.

3. Results

(a) Female fertility with *Drosophila santomea*

We found that levels of gametic isolation between *D. yakuba* females and *D. santomea* males vary across the altitudinal transect as predicted by reinforcement: the altitude that an isofemale line was collected from explains her fertility when mated to male *D. santomea* ($p < 0.000001$; table 1 and figure 1*a*) and heterospecific fertility is negatively correlated with altitude (Spearman's $\rho = -0.98$; $p < 0.000001$; table 2). While these results are based on crosses carried out with males from a single *D. santomea* line, they are consistent with previous work providing evidence for reinforcement acting in populations of *D. yakuba* on the island of São Tomé [25]. We observed no evidence for reinforcement along the 'control' transect on the island of Bioko (electronic supplementary material, table S5).

(b) Female fertility with conspecific males with different genetic backgrounds

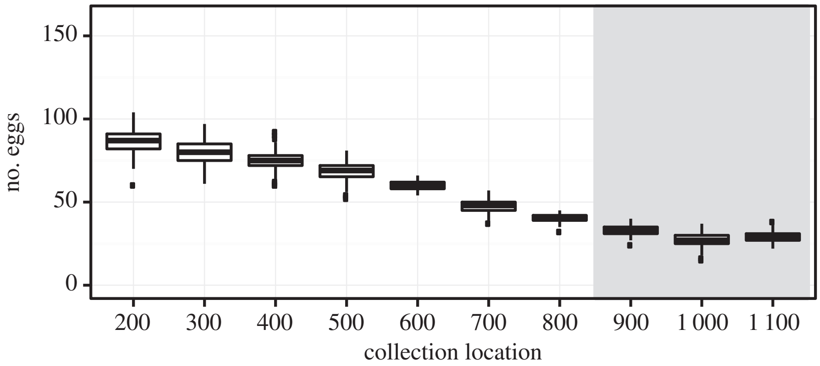
In contrast with female fertility when mated to heterospecific *D. santomea* males, baseline levels of fertility within an isofemale line did not differ across the altitudinal transect ($p = 0.237$; table 2). Conspecific female fertility when crossed with controlled male genotypes did, however, vary depending on the interaction between the collection location of the female line and whether a female was crossed with a sympatric or allopatric male (LRT, $\chi^2 = 638.19$; $p < 0.000001$; table 1; figure 1*b*). We also found a positive correlation between fertility and the location that a female was collected from when crossed with sympatric male *D. yakuba* (Spearman's $\rho = 0.37$; table 2) and a marginally significant negative correlation between fertility and altitude when females were crossed with allopatric *D. yakuba* (Spearman's $\rho = -0.23$; table 2). These correlations do not exist on the island of Bioko (electronic supplementary material, table S7).

Linear contrasts revealed that the effect of the collection location × male genotype interaction was driven by allopatric female *D. yakuba* having low fertility when crossed with sympatric conspecifics (electronic supplementary material, table S4). Specifically, allopatric females had lower fertility when crossed with sympatric males than when crossed with allopatric males, or males from the same line (mean fertility = 74.29 versus 88.74 and 90.50 eggs per female, respectively). We also observed lower levels of fertility in allopatric female × sympatric male crosses when compared with sympatric females that were crossed with allopatric males (88.44 eggs per female), sympatric males (83.74 eggs per female), and males from the same line (88.24 eggs per female). Together, these results indicate that allopatric female genotypes will be at a selective disadvantage in sympatric regions of *D. yakuba*'s range because they have lower fitness when they mate with sympatric males.

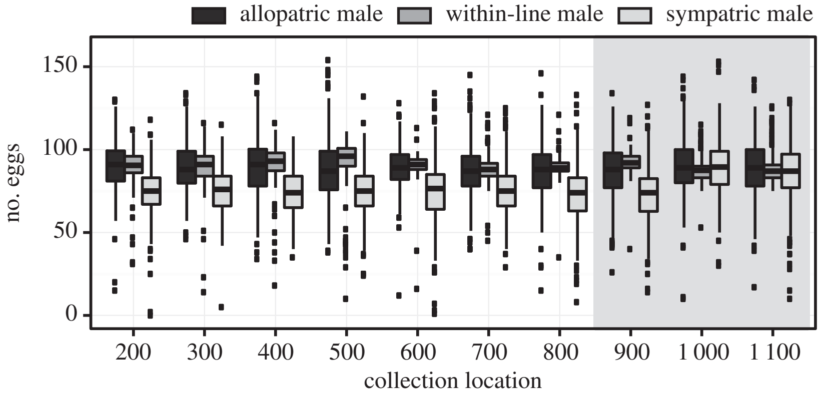
(c) Male fertility with conspecific females with different genetic backgrounds

We next tested whether reinforcing selection acting in sympatry affected levels of conspecific male fertility by mating males from each focal line (100 lines total) with females from the same four tester lines used to assess conspecific female fertility. As with female fertility, we found a significant effect of the interaction between the location a male was collected from (i.e. altitude) and female genotype on levels of fertility (LRT, $\chi^2 = 3\,707.7$; $p < 0.000001$; table 1; figure 1*b*). We also found a positive correlation between male fertility with sympatric female genotypes and altitude (Spearman's $\rho = 0.74$; table 2; figure 1*c*, light grey boxes) and a strong negative correlation between male fertility with allopatric female genotypes and altitude (Spearman's $\rho = -0.91$; table 2; figure 1*c*, dark grey boxes). Both of these correlations are absent in samples collected from the island of Bioko (Spearman's $\rho =$ 0.06 and 0.10, respectively; $p > 0.1$; electronic supplementary material, table S7).

Linear contrasts comparing male fertility in the different types of crosses show that the collection location × female background interaction can be explained by lower fertility in crosses that are mismatched with respect to their genetic background: allopatric males had higher fertility when crossed to allopatric female genetic backgrounds (mean fertility = 90.02 eggs per female) than when crossed to sympatric female genetic backgrounds (87.50 eggs per female) and sympatric males had higher fertility with sympatric female genetic backgrounds (91.67 eggs per female) compared with allopatric (*a*) fertility with *D. santomea*



(*b*) conspecific female fertility



(*c*) conspecific male fertility

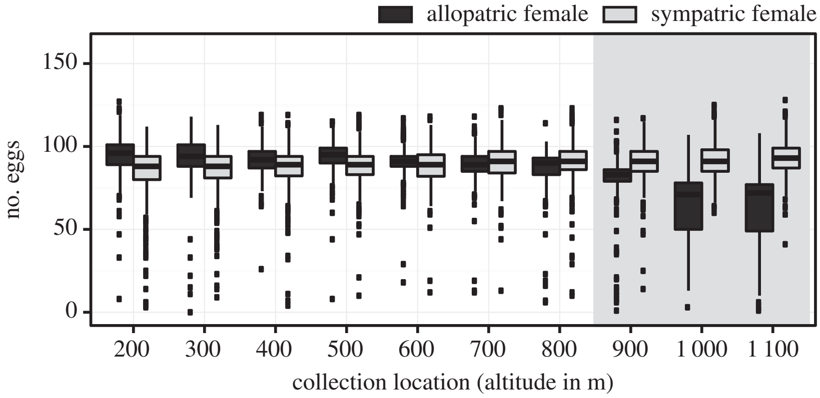


Figure 1. Levels of heterospecific, conspecific female and conspecific male fertility along a transect on São Tomé. (*a*) Isofemale lines collected from the hybrid zone show lower fertility with *D. santomea* males than females from outside the hybrid zone. (*b*) Fertility of female *D. yakuba* when crossed with conspecific males is lowest when allopatric females (collection locations below 900 m) were mated to sympatric male genotypes (light grey boxes; see text). (*c*) Fertility of *D. yakuba* males when crossed with conspecific females with controlled genetic backgrounds is lowest for males from the hybrid zone (sympatric genotypes) when they are mated with female *D. yakuba* from allopatric tester lines (dark grey boxes). On the other hand, males from allopatric populations show higher fertility when mated to allopatric females (leftmost dark grey boxes compared with right boxes; see text). Grey polygons in each panel demarcate the location of the hybrid zone and collection locations are given as the altitude of a site (in metres above sea level).

females (71.61 eggs per female; see electronic supplementary material, table S5). Consistent with results for females, the lowest level of fertility we observed was between sympatric male *D. yakuba* and allopatric conspecifics (figure 1*b*,*c*).

(d) Correlations between heterospecific, conspecific female and conspecific male fertility

If reinforcing selection has correlated effects on levels of fertility between conspecific populations, we predicted that interspecific fertility would be correlated with conspecific fertility when crossed with allopatric (positively correlated) or sympatric (negatively correlated) genetic backgrounds. Consistent with this prediction, the mean interspecific fertility of a *D. yakuba* line is positively correlated with mean fertility when crossed with allopatric conspecifics (Spearman's $p = 0.22$ and 0.90 for female and male fertility, respectively; figure 2*a*) and negatively correlated with mean fertility when crossed with sympatric conspecifics (Spearman's $p = -0.38$ and -0.72 for female and male fertility, respectively; figure 2*b*). We also observed significant correlations between mean female and male fertility when mated to both allopatric and sympatric genotypes (Spearman's $\rho = 0.21$ and 0.37, respectively; figure 2*c*). The only correlation that was significant among isofemale lines collected on the

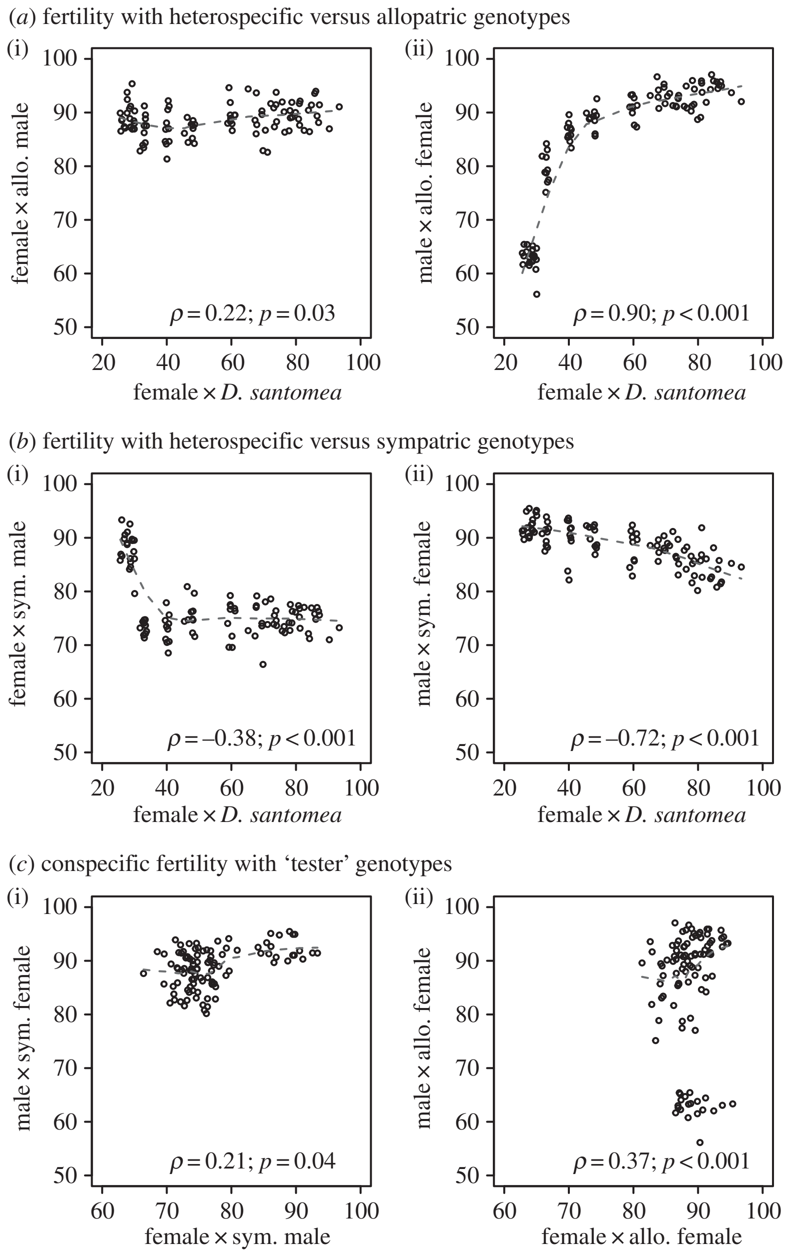


Figure 2. Correlation of fertility between different genetic combinations in natural populations of *D. yakuba*. (*a*) Heterospecific fertility is weakly correlated with fertility between female *D. yakuba* and allopatric male genotypes (i) but strongly correlated with fertility between male *D. yakuba* and females with allopatric genotypes (ii). (*b*) Heterospecific fertility is negatively correlated with fertility between female *D. yakuba* and sympatric male genotypes (i) and with fertility between male *D. yakuba* and sympatric female genotypes (ii). (*c*) Male and female fertility within lines is positively correlated when mated with conspecific tester lines having either 'sympatric' or 'allopatric' genetic backgrounds (i and ii, respectively). Dashed lines in all panels are LOWESS smoothers and are included for illustrative purposes only. Spearman's rank correlations (ρ) are given in each panel (see text). Fertility is reported as the numbers of eggs produced by an inseminated female and the axis labels denote the cross-type being considered (focal individual × controlled genetic background). See electronic supplementary material, table S8 for parallel analysis of *D. yakuba* from the island of Bioko.

island of Bioko was between fertility when mated to *D. santomea* and male fertility with sympatric female *D. yakuba* (electronic supplementary material, table S8). However, this relationship was positive (Spearman's $\rho = 0.26$), the opposite of what we observed along the São Tomé transect.

(e) Experimental sympatry

Collectively, the results presented above suggest that reinforcing selection acting in sympatry can incidentally drive differentiation—and generate reproductive isolation—between conspecific populations of *D. yakuba*. We used experimental evolution to test whether male *D. yakuba* could evolve reduced conspecific fertility with either allopatric$_{\text{Täi18}}$ or sympatric$_{\text{BOSU1250.5}}$ *D. yakuba* females in populations subject to 10 generations of reinforcing selection. Confirming that there was an evolutionary response to reinforcing selection, female *D. yakuba* from the experimental populations laid fewer eggs when mated to *D. santomea* males after 10 generations of selection against hybrids (Poisson GLM, LRT: $\chi^2 = 2\,767.7$; d.f. = 1; $p < 0.000001$, figure 3*a*). Reinforcing selection also resulted in conspecific differentiation: when mated with allopatric$_{\text{Täi18}}$ females, male fertility significantly decreased over the course of the experiment (LRT: $\chi^2 = 743.9$; d.f. = 1; $p < 0.000001$, figure 3*b*) and there was a significant correlation between levels of female fertility with heterospecific

Table 2. Correlations between a *D. yakuba* line's collection altitude and fertility when crossed with *D. santomea* (heterospecific), *D. yakuba* from populations found in sympatry with *D. santomea* (sympatric), and *D. yakuba* from populations found in allopatry from *D. santomea* (allopatric). See electronic supplementary material, table S7 for parallel analysis with *D. yakuba* from Bioko.

type of fertility	Spearman's ρ	*p*-value
female crossed with *D. santomea*	−0.98	<0.001
within-line	−0.12	0.237
female crossed with sympatric male	0.37	0.001
female crossed with allopatric male	−0.23	0.022
male crossed with sympatric female	0.74	<0.001
male crossed with allopatric female	−0.91	<0.001

D. santomea and male fertility with conspecific allopatric$_{\text{Täi18}}$ females at the end of the experiment (Spearman's $\rho = 0.69$; $p < 0.000001$; figure 3*c*). By contrast, we found no change in male fertility when mated to sympatric$_{\text{BOSU1250.5}}$ females (LRT: $\chi^2 = 1.06$; d.f. = 1; $p = 0.304$; figure 3*b*) and no correlation between heterospecific fertility and fertility in matings with conspecific sympatric$_{\text{BOSU1250.5}}$ females (Spearman's $\rho = -0.046$, $p = 0.115$). Finally, we did not observe any change in fertility in control populations (female fertility: LRT: $\chi^2 = 0.54$; d.f. = 1; $p = 0.462$; male fertility: LRT: $\chi^2 = 0.89$; d.f. = 1; $p = 0.347$) and overall levels of fertility following conspecific matings remained the same (LRT: $\chi^2 = 2.43$; d.f. = 1; $p = 0.12$; figure 3*a*). These results provide experimental evidence that when *D. yakuba* females evolve gametic isolation from *D. santomea*, males also show a correlated reduction in fertility with conspecific females from allopatric populations.

4. Discussion

In this study, we used the drosophilid flies *D. yakuba* and *D. santomea* to test the hypothesis that reinforcement can have indirect effects on levels of fertility between conspecific populations. We report three pieces of evidence that together support this hypothesis. First, sympatric lines of *D. yakuba* have reduced fertility when mated with male *D. santomea* when compared with allopatric *D. yakuba*. Second, sympatric male *D. yakuba* show reduced fertility when mated with allopatric female *D. yakuba* (tables 1 and 2; figure 2). Third, when we experimentally evolved populations of *D. yakuba* in the presence of *D. santomea* and imposed selection against maladaptive hybridization with *D. santomea*, populations that evolved stronger RRI from *D. santomea* also evolved reduced conspecific fertility when (experimentally sympatric) males were mated with allopatric females (figure 3).

(a) Correlated evolution of male and female reproductive traits

Our results provide evidence that reinforcement can drive the correlated evolution of postmating-prezygotic traits in both sexes. This finding is nuanced: while we observed reduced fertility in crosses between allopatric female and sympatric male genotypes, we did not observe a concordant reduction in fertility between sympatric females and allopatric males, a pattern that would be predicted by simple one-to-one coevolution. Instead of strict one-to-one coevolution, the patterns of fertility we observed could be explained by different (and independent) traits responding to reinforcing selection in female and male *D. yakuba*. It is well known that postcopulatory sexual selection can occur as a result of both (or either) intra and inter-sexual interactions [35], and selection can act on multiple traits including sperm morphology, the number of sperms, female sperm storage, and/or seminal proteins [36,37]. A hypothetical mechanism explaining our result is, therefore, that a sympatric male sperm (or seminal fluid) trait is under selection in sympatric *D. yakuba*, resulting in a mismatch between this male trait and female traits found in allopatry. For example, sperm traits in sympatric *D. yakuba* could be responding to selection generated by interactions occurring with the sperm of *D. santomea* in multiply inseminated *D. yakuba* females. While this male sperm trait evolves, the trait used by sympatric female *D. yakuba* to detect conspecifics remains unaltered, and a second trait (used to detect heterospecific sperm and limit its ability to fertilize eggs or stimulate ovipositioning) is evolving during reinforcement.

Our results provide indirect evidence supporting the hypothesis that different traits are responding to reinforcing selection in the sexes. First, the geographical cline in female fertility with *D. santomea* appears much broader than sympatric male fertility with allopatric conspecifics (cf. figure 1*a* with the light grey boxes in figure 1*b* and the dark grey boxes in figure 1*c*). Second, the relative change in the strength of gametic isolation from *D. santomea* is larger than the reduction in fertility with allopatric conspecifics during experimental evolution (cf. the black boxes in figure 3*a* to those of figure 3*b*). Work in other systems has shown that the same traits likely underlie both RRI and within-species mate discrimination [24]; however, to our knowledge, *D. yakuba* represents the first example of selection associated with reinforcement driving the evolution of different postmating prezygotic traits in females and males. Future work is needed to develop a better understanding of the specific traits, and genes, involved in reinforcement in *D. yakuba*.

(b) Cascading effects of reinforcement

Despite the specific mechanism(s) underlying reduced fertility in *D. yakuba* being unknown, our results predict that the movement of adaptive 'sympatric' alleles into allopatric populations will be constrained due to mismatches between sympatric male and allopatric female traits. Reinforcement in *D. yakuba*, therefore, adds to the growing number of examples demonstrating that locally adaptive phenotypes subject to reinforcing selection can have costs outside of regions of sympatry [18–24]. These examples provide evidence that the pattern of RCD frequently described in cases of reinforcement can be actively maintained by selection acting on 'reinforced' alleles between allopatric and sympatric conspecific populations.

Phenotypic differentiation between sympatric and allopatric populations can be the result of a variety of processes that do not involve selection acting directly against 'reinforced' alleles in allopatry. We assessed four alternative explanations in the populations we studied here and found it unlikely that conspecific differentiation results from (i) local adaptation to temperature (an environmental factor varying with altitude), (ii) forms of reproductive isolation other than gametic interactions, (iii) genetic differentiation due to geographical

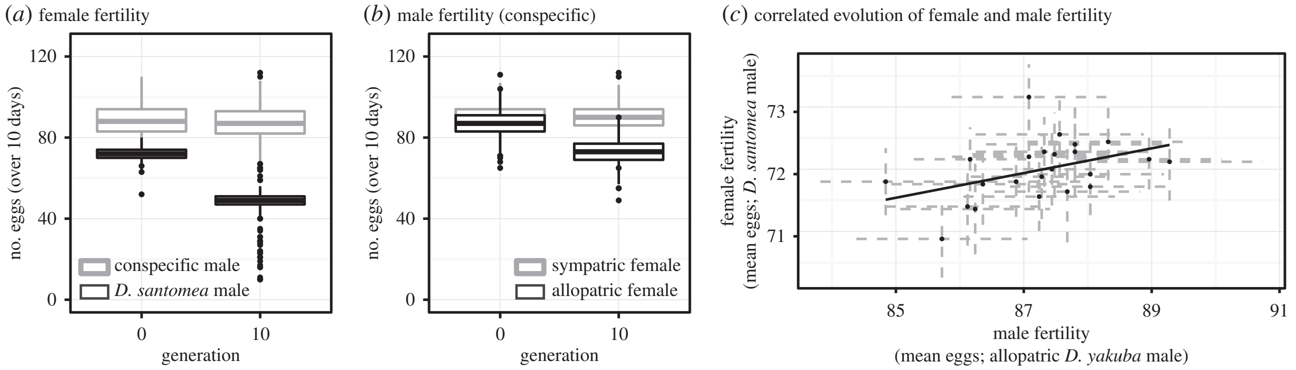


Figure 3. Experimental evidence that gametic isolation and decreased male fertility coevolve after experimental sympatry with *D. santomea*. (*a*) After 10 generations of experimental sympatry, allopatric *D. yakuba* lines evolved enhanced RRI (black boxes) with no change in conspecific fertility (grey boxes). (*b*) The same evolved lines also showed a decrease in male fertility when males were mated to an allopatric tester line of *D. yakuba* (black boxes) but no decrease in fertility when mated to females from a sympatric tester line of *D. yakuba* (grey boxes). (*c*) Within experimental populations, the degree of RRI that evolved over 10 generations ('female fertility'; smaller values represent higher levels of isolation) was correlated with levels of male fertility with allopatric *D. yakuba* females. Points in (*c*) represent means for each of 23 experimental populations and dashed grey lines represent ± 1 standard error (s.e.). Control populations showed no change in gametic isolation from *D. santomea*, female fertility with conspecific males, male fertility when males were mated with allopatric *D. yakuba*, or male fertility when mated with sympatric *D. yakuba* (see main text).

isolation, or (iv) large chromosomal inversions limiting admixture between demes (see the electronic supplementary material). Interestingly, we did observe non-zero genetic differentiation (median $F_{ST} = 0.0503$; electronic supplementary material) among isofemale lines derived from females sampled from opposite ends of the altitudinal transect, suggesting that reinforcing selection acting in sympatry—and selection against sympatric alleles in allopatry—could help drive genetic differentiation among conspecific populations; a hypothesis that warrants further investigation.

5. Conclusion

Reinforcement acts on traits involved in prezygotic isolation and may be similar to processes such as speciation by 'magic' traits [38] and sensory drive [39] in its ability to promote speciation. For example, speciation by magic traits, sensory drive, and reinforcement all affect prezygotic traits either through pleiotropy or tight genetic linkage (magic traits), directly (sensory drive), or indirectly (reinforcement). The common thread shared by these three processes is that selection does not directly favour reproductive isolation between conspecific populations; instead, reproductive isolation evolves as an incidental effect of selection acting on other traits (or through pleiotropy). Reinforcement could, however, be unique in its ability to simultaneously drive speciation between the species directly involved and conspecific populations differentiating as an incidental effect of the process. Future work is needed to determine whether these cascading effects of reinforcement have long-lasting consequences for speciation or are transient patterns that are eventually erased by intraspecific gene flow.

Ethics. Sampling in São Tomé and Equatorial Bioko was done under permits issued by government authorities.

Data accessibility. Dryad (http://dx.doi.org/10.5061/dryad.g5rq3); SRA: SRP077753.

Authors' contributions. A.A.C. and D.R.M. designed the study, D.R.M. and A.V. performed experiments, all authors contributed to analysing the data, and A.A.C. and D.R.M. wrote the manuscript.

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