Evolution of mate harm resistance in females from *Drosophila melanogaster* populations selected for faster development and early reproduction

Tanya Verma[1], email id: tanya17@iiserbpr.ac.in

Susnato Das[2], email id:daskpa201@gmail.com

Saunri Dhodi-Lobo[3], email id: dhodi.lobo@students.iiserpune.ac.in

Ashish Mishra[4], email id: ashish.akm1999@gmail.com

Soumi Bhattacharyya[5] email id: soumisayantan2000@gmail.com

Bodhisatta Nandy[1]*

[1]Indian Institute of Science Education and Research Berhampur, Odisha, India

[2]Institute of Animal Welfare and Animal Husbandry, Friedrich-Loeffler-Institut, Celle, Germany

[3]Indian Institute of Science Education and Research Pune, Maharashtra, India

[4]National Institute of Science Education and Research, Odisha, India

[5]Indian Institute of Science Education and Research Kolkata, West Bengal, India

*Corresponding author, email address: nandy@iiserbpr.ac.in, ORCID iD: 0000-0002-9588-0316

Authors Contribution:

BN and TV conceptualized the study, designed the experiments, analysed and interpreted the results, and prepared the manuscript. SD helped with some parts of manuscript writing at earlier stage. TV, SD, SDL, AM, SB executed the experiments, including data collection.

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Abstract

Interlocus sexual conflict is predicted to result in sexually antagonistic coevolution between male competitive traits, which are also female-detrimental, and mate harm resistance (MHR) in females. Though such antagonistic coevolution has been experimentally shown, little is known about its connection with life-history evolution. Here, we investigated the evolution of MHR in a set of experimentally evolved populations selected for faster development and early reproduction. Previously we showed the reduction of harming ability of males in these populations. Here, we measured mortality and fecundity of females in these populations and those of their matched controls under different male exposure conditions. As predicted by the coevolution theory, we observed that the evolved females were more susceptible to mate harm - suffering from significantly higher mortality under continuous exposure to control males. We used fecundity data to show that this higher mortality in evolved females is unlikely due to cost of reproduction *per se*.

Keywords: Interlocus sexual conflict, sexually antagonistic coevolution, life history evolution, cost of reproduction, post-mating response in females

Introduction

Evolutionary interests of sexes are often not aligned leading to evolutionary conflict over traits with sexually antagonistic fitness effects (1). In one form of such conflict, commonly referred to as interlocus sexual conflict, expression of male-benefitting traits (for example, courtship and mating behavioural traits) reduces female fitness as an incidental side effect (2–4). Effect of such antagonistic male effect, often referred to as mate harm, constitutes a significant portion of female cost of reproduction (5). Theories predict evolution of female counter measures that allow females mitigate such cost of mate harm – potentially resulting in sexually antagonistic coevolution (6). While interlocus conflict has been reported in a wide diversity of animals in the form of mate harm (7,8) male-female coevolution has also been shown in a number of studies (9,2,10–17). Though sexual conflict has emerged as one of the most exciting areas of investigation, with an ever increasing body of empirical evidence and a solid theoretical framework, it is now very important to integrate it in the broader framework of life history evolution as conflict related traits including MHR can only evolve within the life-history constrains such as, cost of reproduction and reproductive lifespan (18).

In *Drosophila melanogaster*, a classical model for interlocus conflict, exposure to males increases female mortality due to persistent courtship (19), and effects of the seminal fluid proteins transferred during a copulation (5,20). Resistance traits, collectively hereafter referred to as mate-harm resistance (MHR), can include behavioural and physiological traits such as, mating rejection (21), avoidance of male encounter by finding refuges (22), alteration in production of proteins and peptides that respond to male seminal fluid proteins (23). There is now ample evidence for evolution of female resistance (11,24). If there is a substantial cost of expressing MHR, life-history theories would predict a trade-off between these and other traits such as somatic maintenance. However, if expression of MHR leads to reduction in reproductive cost in general, detecting cost of MHR *per se* might be difficult. Alternatively, cost of MHR can be indirectly inferred from the evolutionary reduction in MHR when selection on such traits is removed (11,24).

Mital et al. (2021) reported a reduction in MHR in females in populations of *D. melanogaster* experimentally evolved for faster development and early reproduction, possibly due to a change in breeding system to near-monogamy (25,26). In a similar study, we showed reduction in mate mating behaviour, including mate harming ability (27). Our results seem to suggest roles of body size evolution as well as that of the changes in the breeding ecology, most notably extreme shrinkage of effective adult reproductive life (27). Here we extend our investigation to incorporate the evolution of MHR in females of the faster developing and early reproducing populations, and their controls.

If maintaining MHR is costly, populations should divest in MHR if it does not have any fitness benefit. Therefore, since selection for early reproduction resulted in reduced interlocus conflict (27), MHR in females from these populations should evolve reduced MHR. Such females should thus be more susceptible to mate harm. To test this theory, we measured mortality rates under virgin (i.e., non-reproducing), single mating (i.e., limited male exposure, but reproducing), and continuous male exposure (i.e., reproducing under constant male presence) of evolved and control females. To differentiate between the cost of male exposure *per se* and cost of reproduction, we also measured female reproductive output. The above-mentioned traits were measured using a standard control population male to equalise the male effects.

Materials and methods

We used a set of experimentally evolved *D. melanogaster* populations, ACOs and their paired controls, COs to conduct the experiments. Detailed information on these populations can be found in the supplementary information. Briefly, ACO populations are subjected to selection for faster pre-adult development and early reproduction, and have an extremely short effective adult life of 24-36 hours. ACOs were derived from COs in 1989 by Chippindale et al. (1997). While COs have a discrete generation cycle of 28 days, ACOs are maintained on a 9-day discrete generation cycles. There are five replicate ACOs, derived from five replicates of COs. By the time the following experiments were

conducted, ACO populations had evolved for >1200 generations. Driven by strong experimental selection, ACO populations have evolved markedly faster pre-adult development, smaller body size, and shorter adult lifespan (28,29). For the purpose of this investigation, three replicates of the population sets were randomly selected. All assays described below were conducted with ACO_1, ACO_2, ACO_3, ACO_4 and their paired control CO populations. All experimental flies were generated from a subset of the stock populations, after one generation of common garden rearing to equalise non-genetic parental effects.

Mate harm resistance assay setup:

In *D. melanogaster*, MHR can be measured by comparing female mortality under limited and extended exposure to males (11,24,30). Females with lower MHR are expected to show sharper increase in mortality under extended male exposure compared to those with higher MHR.

Assay vials were set up with 1-2 day old virgins (see supplementary information). The 45 vials, each having ten virgin females from a population, were randomly assigned to three assay conditions - virgin, single exposure, and continuous exposure such that each assay condition consisted of an initial count of 15 vials. The experimental vials were set up by introducing flies in fresh food vials. For the virgin assay condition, females were held without any male exposure for the entire assay duration. Single exposure and continuous exposure vials were set up by introducing 10 virgin control (i.e., CO) males along with the ten experimental females in a fresh food vial. For the single exposure vials, matings were manually observed and after a single round of mating, sexes were separated under CO_2-anaesthesia to discard the males. The females were then returned back in the same vials. For the continuous exposure treatment, males and the females were kept together in the same vials till the end of the assay. To ensure similar handling of flies across all treatments, flies under virgin and continuous exposure treatments were also exposed to anaesthesia. Throughout the experiment, except sorting of sexes, all other fly handling were done without anaesthesia. All vials were maintained for twenty days and the flies in each vial were flipped to fresh food vials every alternate day. For all vials regardless of assay condition, mortality in

females was recorded daily until day 20. Our previous observation suggests that the difference in effects of mate harm on female mortality can be detected in the first twenty days of adult life (27). In addition, this period represents early-to-mid life in this system, most relevant to both control (CO) and experimental (ACO) population ecology. Further, the difference in age-dependent mortality rate between the two selection regimes has minimal impact on mortality difference within this duration (data not shown). Dead flies were aspirated out during vial-to-vial flips. In the continuous exposure assay condition, in case a female fly was found dead in a vial, along with the dead female, a male was also removed from the same vial to maintain a 1:1 sex ratio.

Female fecundity was recorded twice a week starting from the onset of the assay until day 20 (i.e., day 1, 3, 6, 9, 12, 15, 18, and 20). On each of these days, flies were flipped to a fresh food vial (hereafter referred to as a fecundity vial) and were left undisturbed for 24 hours. Following this, the flies were transferred to a fresh food vial, while the fecundity vial was frozen immediately to prevent further development of the already deposited eggs. The number of eggs laid in a fecundity vial was counted under microscope. Fecundity count was carried out for single exposure and continuous exposure treatments. Per capita fecundity, calculated as total number of eggs in a vial divided by the number of females alive in that vial on that given day, from individual vials was taken as the unit of analysis. A few vials were removed from the assay for a variety of reasons, including accidental escape, a few females failing to mate, etc. The final sample size throughout the entire experiment was 13-15 vials per population.

<u>Data analysis:</u>

Female survivorship was analysed using Cox's Proportional hazards model. Selection regime (levels: ACO and CO) and assay condition (levels: virgin, single exposure and continuous exposure) were modelled as fixed factor and block as random factor using R package `Coxme` (31). Cox partial likelihood (log-likelihood) estimates across selection regimes were compared.

Per capita fecundity was analysed in two ways. Cumulative fecundity i.e., per capita fecundity pooled across all eight age classes was analysed to compare to total early-to-mid life reproductive output of the females. In addition, age-specific per capita fecundity was analysed to compare the age related pattern of reproduction. The latter was done only for the continuous exposure set to minimise model complication. Both cumulative female fecundity and age-specific fecundity data were square root transformed before analysis. A linear mixed effect model was fitted to the transformed data. `lme4` package (32) and `lmerTest` (33) in R version 4.2.1 (R Core Team, 2022). In the cumulative fecundity model, selection regime (levels: ACO and CO), assay condition (levels: single exposure and continuous exposure) and their two-way interactions as fixed factors, block as a random factor. In the analysis of age-specific per capita fecundity, selection regime and age (levels: 1, 3, 6, 9, 12, 15, 18, 20) were the fixed factors, and block and all interaction terms involving block were modelled as random factors. All models are mentioned in the supplementary information. Post-hoc pairwise comparisons using Tukey's HSD method were performed with the package `Emmenas` (34). The ANOVA table was obtained following Satterthwaite's method using type III sum of squares.

Results

Cox partial likelihood estimates suggested that the effects of selection regime, assay condition, and selection regime × assay condition interaction on female mortality were significant (Figure 1, Table 1). Pairwise comparisons indicated a significant difference in survivorship of ACO and CO females only under continuous exposure, with ACO females more than 9.5 times likely to succumb compared to CO females (estimated hazard ratio: 9.83).

The effects of selection regime and assay condition on cumulative fecundity were significant (Table 2). While females under continuous exposure had significantly higher fecundity regardless of the selection

regime, cumulative fecundity of ACO females was 27% less than that of the control CO females (Figure 2a). Age-specific fecundity analysis indicated significant effects of selection regime, and age (Figure 2b). However, we found a two-way and a three-way interaction term involving random block to be significant (Table S2). Hence, we analysed each block separately (see supplementary information, Table S1). Though across blocks the age-specific pattern seemed to vary, CO females generally showed higher per-capita fecundity in most age points (Figure S1). Fecundity on day 1 was of particular interest as ACO maintenance regime selects for fecundity at this age. Hence, we analysed day 1 fecundity separately, using a linear mixed model similar to that used to analyse cumulative fecundity. The results indicated significant effects of selection regime and assay condition, with CO females consistently showing higher fecundity (Table 2, Figure S2).

Discussion

Our results suggest that selection to faster development and early reproduction has led to the evolution of sexually antagonistic traits in both sexes. The evolved ACO population males were previously shown to be significantly less harming to their mates (27). Results of our MHR assay reported here suggest that ACO females are significantly more susceptible to continued male interaction. When held with males, ACO females showed close to ten times higher mortality rate compared to that of the control (CO) females in the same condition. Such higher mortality is unlikely due to the difference in baseline rate of ageing as we did not find any difference in mortality rate of non-reproducing, and singly mated reproducing females of the two selection regimes. Further, ACO females were found to be consistently less fecund, regardless of the length of male exposure, and age. Hence, higher mortality rate of ACO females mentioned above is unlikely due to an increased cost of reproduction *per se* but a result of reduction of MHR.

If MHR is costly to express, it is expected to be constrained by the resource availability (35). Females in resource deprived condition should therefore be limited in terms of their ability to resist mate harm. Such

condition dependence of MHR has been recently demonstrated (17,36). In addition, for reproducing females, the cost of producing progeny can further constrain resources available for other physiological processes - potentially making them vulnerable to stresses including mate harm. The evolved ACO females in our study are small in size (see supplementary information), and can thus be expected to be resource limited (37). However, they have a lower reproductive rate - hence, lower absolute investment in reproduction. Though it is difficult to assess relative reproductive investment, as evident from our data from the single mating treatment, there appears to be a baseline reduction in reproductive rate of ACO females. However, evidently this baseline difference in reproduction did not result in mortality rate difference, which is only evident under extended male exposure. In addition, there was no evidence that this difference in reproductive investment between the evolved ACO and control CO females was higher under continuous male presence. Hence, it is very unlikely that observed differences in susceptibility is a mere reflection of the size difference of the experimental females. Our conclusions are also in line with those of Mital et al. (2021) who used phenocopied females to demonstrate the size independent reduction in MHR.

Several experimental evolution studies have shown the evolution of MHR (2,10–14,16,17,24,38). Of these, only two have directly connected evolution of conflict related traits to life history traits such as, condition, adult lifespan, development time, and size (17,39). Though evolution of MHR is important for a population's survival (40), continuation of sexual selection (41), and maintenance of genetic variation (42), it cannot evolve in the vacuum of sexually antagonistic traits only. Our results is an important addition to the growing list of evidences suggesting that sexual conflict is subjected to a typical eco-evolutionary feedback process. Herein, breeding ecology sets the stage of sexual conflict and drives antagonistic coevolution between sexes, but life history affects such evolution by (a) setting physiological and genetic constraints, and (b) constraining breeding ecology. Hence, selection for life history traits such as, lifespan, reproductive schedule etc. should be important drivers of sexually antagonistic coevolution as such selection can impact breeding ecology and offset the fitness premium on sexually antagonistic traits.

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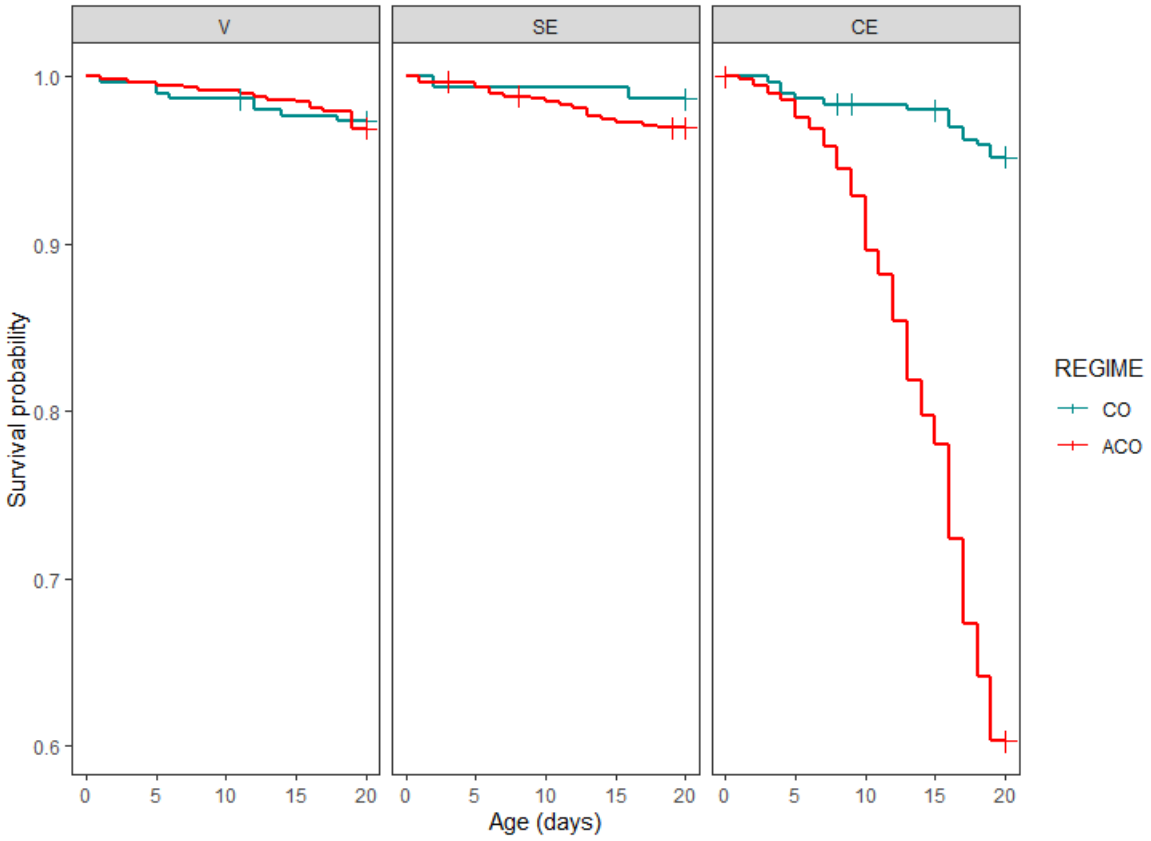

Figure1: Survivorship curves obtained from Cox proportional hazard analysis on the mortality of ACO (red line) and CO (dark cyan line) regime females held under virgin (V), single exposure (SE) and continuous exposure (CE) condition for 20 days during the assay. The differences between survivorship ACO and CO females were found to be nonsignificant under virgin and SE conditions. Under CE condition, ACO females showed significantly higher mortality rate.

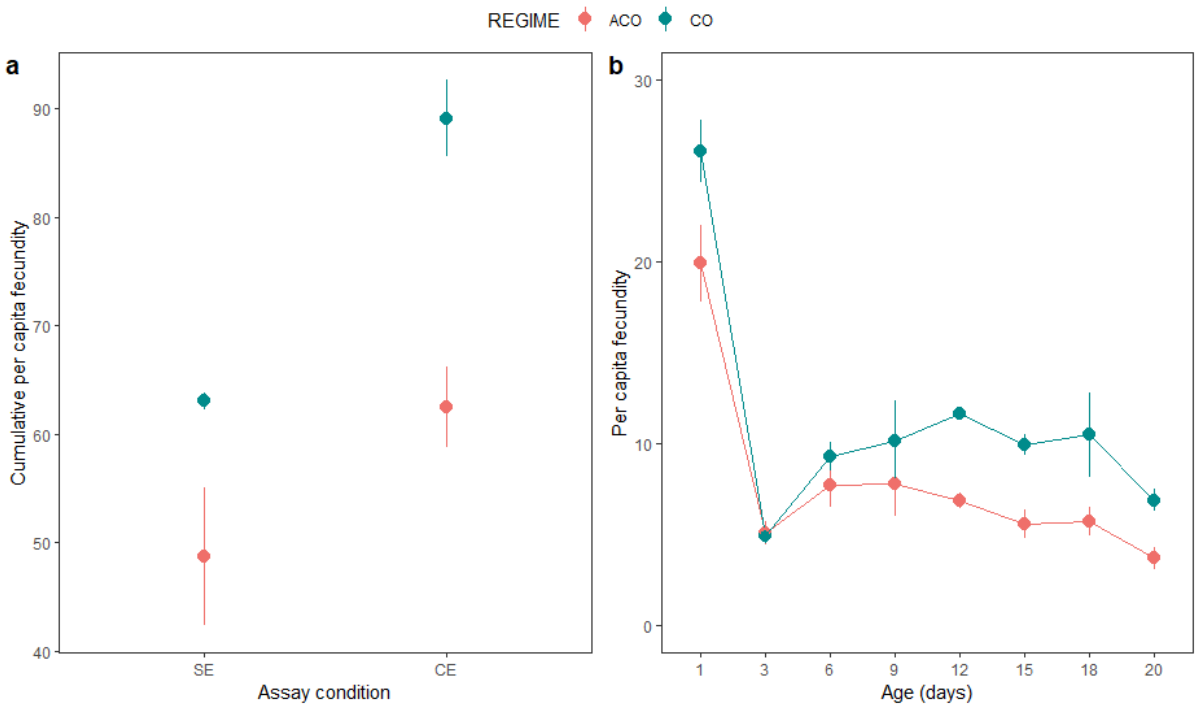

Figure 2: Results from the fecundity measurements. The panel shows (a) cumulative fecundity per capita, and (b) age-specific per capita fecundity across ACO and CO selection regime females held with control (CO) males. Age specific fecundity was analysed only for continuous exposure assay condition to minimise model complication. Filled circles and error bars represent means, and standard error respectively. Standard errors are calculated using block means (i.e., population means). Effects of selection regime, and assay condition on cumulative per capita fecundity were found to be significant. Effects of selection regime and age were found to be significant on age specific per capita fecundity.

Fixed Coefficients	**Hazard Ratios**	**Lower CI**	**Upper CI**	**z**	**p**
Selection Regime ACO	1.111	-0.737	0.947	0.24	< **0.001**
Assay condition SE	0.501	-1.891	0.509	-1.13	< **0.001**
Assay condition CE	1.831	-0.264	1.473	1.36	< **0.001**
Selection Regime ACO: Assay condition SE	1.975	-0.686	2.047	0.98	< **0.001**
Selection Regime ACO: Assay condition CE	8.702	1.171	3.156	4.27	< **0.001**
Random effects	**Variance**				
Block	0.0843				

Table 1: Output of mixed effect Cox proportional hazard model for analysis of female survivorship ACO and CO regime females held under virgin (V), single exposure (SE) and continuous exposure (CE) condition with ancestral CO males. Hazard ratios are relative to the default level for each factor which is set to 1. The default level for selection regime was 'CO', and the default level for assay condition was 'Virgin'. Lower CI and Upper CI indicate lower and upper bounds of 95% confidence intervals. Level of significance was considered to be $\alpha = 0.05$, and significant p-values are mentioned in bold font style

Trait	**Effect**	**SS**	**DF**	**MS**	**Den**	**F**	**p**

					DF		
Cumulative fecundity	Selection Regime (SR)	4054.8	1	4054.8	3.008	25.826	**0.015**
	Assay condition	5476.4	1	5476.4	2.987	34.881	**0.010**
	Selection Regime × Assay condition	583.0	1	583.0	3.016	3.713	0.149
Day 1 per capita fecundity	Selection Regime (SR)	301.22	1	301.22	5.649	11.673	**0.016**
	Assay condition	296.34	1	296.34	4.470	11.484	**0.023**
	Selection Regime × Assay condition	129.31	1	129.31	2.077	5.011	0.150
Age-specific per capita fecundity	Selection regime	14.201	1	14.2008	40.027	42.210	**<0.001**
	Age	48.574	7	6.9391	21.019	20.626	**<0.001**
	Selection regime × Age	5.194	7	0.7420	23.470	2.205	0.071
Body size	Selection Regime	1.1065		1.1065	235	1446.6	**<0.001**

Table 2: Summary of the results of linear mixed model (LMM) analysis of cumulative fecundity, day 1 per capita fecundity, age-specific per capita fecundity and body size using `lmerTest` function in R. Selection regime and assay condition in cumulative and day 1 per capita fecundity and regime and Selection regime in body size were modelled as fixed factors and block as a random factor. All tests were done considering $\alpha = 0.05$ and significant p-values are mentioned in bold font style.