Evolution of reduced mate-harming tendency of males in *Drosophila melanogaster* populations selected for faster life history

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Bodhisatta Nandy and Tanya Verma conceptualized the study, designed the experiments, and wrote the manuscript. Tanya Verma, Harish Kumar Senapati, Anuska Mohapatra, Rakesh Kumar Muni executed the experiments. Tanya Verma, Purbasha Dasgupta and Bodhisatta Nandy analysed and interpreted the results.

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Abstract

Detrimental effect of males on female, often termed mate-harm, is a hallmark of sexual conflict. Allowed to evolve unchecked, mate harming traits are predicted to bring down average fitness of a population, unless mitigated by the evolution of resistance in females. In addition, life history may also modulate sexual conflict, but the mechanism is not clearly understood. Here we investigated the evolution of mate-harm in a set of experimentally evolved laboratory populations of *Drosophila melanogaster* wherein a faster aging has evolved in response to >1000 generations of selection for faster development and early reproduction. We quantified mortality and fecundity of Oregon R females held with evolved (ACO) and ancestral males (CO) to show that the evolved males are significantly less detrimental to their mates. We compared our results from the ACO males with that from a phenocopied version of the ancestral regime (CCO) to show that only part of the observed difference in mate-harm can be attributed to the evolved difference in body size. We further show that the reduction in mate harming ability evolved despite an increase in courtship activity, especially early in life. We discuss the causative role of an evolved reproductive schedule and altered breeding ecology.

Keywords: Sexual antagonism, Faster aging, Life history traits, Courtship behaviour, Sexual selection.

Introduction

In many sexually reproducing species, the traits that maximize Darwinian fitness in males, may cause detrimental side-effects to their mates resulting in sexual conflict (Parker 1979; Johnstone and Keller 2000; Chapman et al. 2003; Arnqvist and Rowe 2005; Parker 2006; Queller and Strassmann 2018). Herein, both sexes are selected to evolve sex specific traits often resulting in antagonistic co-evolution between the sexes (Parker 1979; Rice 1992; Civetta and Singh 1995; Rice 1996; Arnqvist and Rowe 2002, 2005; Rönn et al. 2007), which is expected to lead to the evolution of costly male competitive traits, which are sexually antagonistic (SA) as well. Theories of sexual conflict (SC) explicitly predict the evolution of mate-harming ability in males (Jiang et al. 2011; Nandy et al. 2013b) and the resulting evolution of female resistance (Wigby and Chapman 2004; Nandy et al. 2013a; Dougherty et al. 2017; Chapman 2018; Rostant et al. 2020). However, in absence of female counter evolution in response to the evolution of increasingly competitive (and hence, harmful) males, SC can lead to the extinction of a population/species, an outcome dubbed as the "tragedy of commons" (Le Galliard et al. 2005; Rankin and Kokko 2006; Rankin et al. 2007). Populations subjected to SC can avert this debilitating outcome by evolving female resistance (Holland and Rice 1999; Wigby and Chapman 2004; Friberg 2005; Rankin et al. 2011; Dougherty et al. 2017; Snow et al. 2019). There is a second way in which a population can avert the tragedy of commons. The cost of the SA traits, and their genetic and phenotypic correlations with other life history traits (Wedell et al. 2006; Bonduriansky et al. 2008; Adler and Bonduriansky 2014; Lemaître et al. 2020) can, in theory, limit the evolution of such traits. Life history itself can constrain the evolution of SA traits by putting restrictions on the mating system and breeding ecology. Empirical tests of such predictions are too few to draw any generalized conclusion.

SC often involves males coercing or manipulating females to mate at a sub-optimally high rate (Koene 2012). Males, in such cases, use morphological and/or behavioural traits such as, persistent courtship, extravagant display, traumatic insemination, and even deception (Arnqvist and Rowe 2005). In addition to the physical toll, a physiological/chemical harassment may also be imposed on the females by the seminal fluid proteins transferred by the males in many species (Poiani 2006). For example, in bumblebee (*Bombus terristris*), grasshoppers (*Gomphocerus rufus*), and fruit flies (*Drosophila melanogaster*) accessory gland proteins/peptides transferred by males during copulation are known to reduce female mating receptivity, increase egg production immediately after mating and reduce lifespan (Chapman et al. 1995; Wolfner 1997; Hartmann and Loher 1999; Baer et al. 2000; Wolfner 2002). Though detrimental to the females, these traits are thought to be male

adaptations to competition for mating and/or fertilization. However, they are often costly to express and bear. For example, synthesis of a functional ejaculate imposes a non-trivial energetic cost on the males (Dewsbury 1982; Andersson 1994; Arnqvist and Rowe 2005). Cordts and Partridge (1996) found that increased courtship reduces longevity in male *Drosophila melanogaster*. Clutton-Brock and Langley (1997) showed that males pay longevity cost due to mating and courtship activity in tsetse flies (*Glossina morsitans morsitans*). Evidently, the cost is also expressed as increased mortality rate due to physical wear and tear (Clutton-Brock and Langley 1997; Stutt and Siva-Jothy 2001; Tatarnic et al. 2006), increased susceptibility to pathogenic infections (Jennions and Petrie 2000; Arnqvist and Rowe 2005; Rönn et al. 2007), and/or environmental stresses (García Roa et al. 2019; Vincent et al. 2020), increased risk of predation (Magnhagen 1991). Several other studies have also shown that investment in manipulative or defensive traits may come at the cost of survival (Promislow 1992; Pitnick et al. 2001; Adler and Bonduriansky 2011, 2014; Duxbury et al. 2017).

Much of the above-mentioned cost is expressed in the form of trade-offs between SA traits and other life history traits (Bonduriansky et al. 2008). Allocation of resources in somatic maintenance has been conjectured to constrain the potential for the reproductive traits, including those involved in SC (Partridge and Farquhar 1981; Stearns 1989; Cordts and Partridge 1996; Clutton-Brock and Langley 1997). This would imply that populations having longer lifespan and slower rate of aging, may not be able to increase investment in SA traits even if there is selection favouring SA traits (Rose and Charlesworth 1981; Ernsting and Isaaks 1991; Kirkwood and Rose 1991; Service 1993; Tatar et al. 1993; Kotiaho 2001; Hunt et al. 2004). Similarly, populations with faster aging and shortened lifespan due to reduced investment in somatic maintenance are expected to have the potential to invest more in SA traits, especially when there is ample selection for male competitive ability. Though, variation in lifespan and aging rate among males appears to reflect variation in reproductive investment (Alcock 1996; Cordts and Partridge 1996; Clutton-Brock and Langley 1997; Prowse and Partridge 1997; Hunt et al. 2004; Bonduriansky and Brassil 2005), whether such correlation can also be extended to SA male traits is not clear.

Apart from the trade-offs, life history itself can modulate the extent of SC in a population by (a) constraining the breeding ecology, and (b) modulating the degree to which the sexes interact (Parker 2006; Wedell et al. 2006; Long et al. 2010). In sexual species, reproductive schedule may constrain the breeding ecology. For example, a semelparous species with only one breeding season may effectively be monogamous and hence, experience reduced sexual conflict (Montrose et al. 2004; Clutton-Brock 2017; Griffith 2019). Long, Pischedda et al. (2010)

showed that even in *Drosophila* laboratory populations, which are effectively semelparous, timing of mating from the semelparous breeding window, could significantly alter the sexually antagonistic outcomes in females. As sexual antagonism depends on the physical interaction between the sexes, factors that affect male-female encounter rate are expected to be important determinants of the intensity of SC in a population. This has been explicitly shown in the experimental microcosm systems of *D. melanogaster*, where complexity of the physical environment and access to refuge were found to bring down SC (Byrne et al. 2008; Yun et al. 2017). More recently, García□Roa, Chirinos et al. (2019) showed that in *Drosophila melanogaster* the intensity of SC to decline at elevated temperature by reducing coercive male interaction on females, such as, persistent courtship and mating attempt. The importance of intersexual interaction in SC has been beautifully demonstrated in the natural populations and mesocosm system of damselflies of the genus *Calopteryx*. (Gomez□Llano et al. 2018) have shown that male induced harassment in *C. spendens* was reduced by the presence of heterospecific males of the species *C. virgo*, simply because survival of heterospecific males reduced the male-female interaction in *C. splendens*. Animals with prolonged breeding life should have greater scope of intersexual interactions and hence, higher scope of SC. Importantly, cost of mating, often an indication of sexual antagonism and the amount of resource allocation in reproductive and sexual traits, may be a function of the breeding system. In mammals, males under polygamous mating system on an average pay higher cost of competition than under monogamous mating system whereas for females it is similar under both types of mating systems. Bonduriansky (2014) showed that change in background mortality rate, resulting from increased predation pressure, could result in a short-term relaxation of SC. Population with low female life expectancy, increased mate harm by males is expected to bring down the average fitness of the population, resulting in a situation resembling the hitherto reported "tragedy of commons" (Rankin and Kokko 2006). Under such scenario selection, can in principle, favour males that induce less harm to females. Therefore, there are myriad ways in which life history, i.e., scheduling of reproduction and mortality throughout the life of an animal, can affect SC in a population.

Here, we investigated the evolution of SA male traits in a set of experimentally evolved populations of *D. melanogaster* having substantially faster pre-adult development and reduced lifespan compared to their ancestors due to selection for faster development and early reproduction for over 1000 generations. These populations – ACO_{1-5}, have been extensively investigated for a range of life history traits since early 1990's. The ACO flies, both males as well as females, are known to have >18% reduced mean lifespan (Chippindale et al. 1997) compared to the ancestral CO flies. In effect, ACO life history has been selected for a "live fast-die

young" strategy (see Methods section for details). We therefore argue that the ACO-CO system is an ideal system to investigate the problem developed in the previous paragraphs. In addition to being a well-documented system of populations where divergence of life history traits is well ascertained. Burke et al. (2010) also established the genomic basis of much of these reported divergences. In the present study, we investigated the divergence between ACO and CO with respect to the mate-harming ability and related components of reproductive behaviour in males. We specifically asked – do males of the ACO populations differ from the ancestral males in terms of their mate harming ability? A reasonable assumption is that the faster aging ACO males should have the ability to invest more in competitive traits, especially early in life because the ACO regime imposes selection for early life fitness components. There is substantial scope of sexual selection in this regime. Therefore, with significant reduction in female life expectancy and increased scope of early life scramble competition among males for mating success, how did ACO populations escaped tragedy of commons? In order to investigate these two fundamental questions, we measured the extent of mate-harm imposed on the females by the experimentally evolved males. We further investigated qualitative and quantitative differences in courtship behaviour of the ACO and CO males. We used standard laboratory line Oregon R females as a common background. Importantly, we compared all the measured traits across the evolved and ancestral populations at five different age-points to assess the importance of evolution of the age-specific expression of the SA traits under altered life history.

Methods

As mentioned above, the investigation reported here used ten laboratory populations of *Drosophila melanogaster* – five ACO populations and their matched ancestral CO populations. These populations were kindly provided to us by Prof. Michael R. Rose of University of California, Irvine, USA. They were maintained for 20 (CO) and 80 (ACO) generations in our laboratory before starting the experiment. The details of population history can be found in (Chippindale et al. 1997). A brief description is provided in Figure S1 in the supplementary information. On November 1991, selection for accelerated development were initiated on five replicate populations – $CO_{1\text{-}5}$ (subscript refers to replicate identity, (Rose et al. 1992). These populations are maintained on a four-week discrete generation cycle, under 24-hours light, 25 ^{o}C (± 1), ~80% relative humidity on standard banana-jaggery-yeast medium. Larval density is controlled at ~70 per 8ml medium in each culture vial. 40 such vials constitute a population. On day 12 following egg culture, adults from all 40 vials are transferred to a population cage. The CO flies take about 9-10 days to complete pre-adult development and

therefore, by day 12 virtually all surviving flies finish development and are in adult stage. A population cage is supplied with banana food on a petri dish. From day 14 of the generation cycle, an old food plate is replaced with a fresh one every alternate day until day 24. On day 26, food plate smeared with live-yeast paste (paste made with water) is produced in the cage, replacing the old food. Approximately 48 hours following this, i.e., on day 28, oviposition substrate (two pieces of banana food) is given inside the cage and a window of 18 hours is allowed for oviposition. Eggs are collected from these ovipositions substrates, and cultured in fresh food vials to start the next generation. The derived ACO populations employed a selection paradigm that involved strong selection for faster pre-adult development (Chippindale et al. 1997) in addition to a much reduced, viz., approximately 24 hours of adult life. Briefly, the ACO populations are maintained under a 9-day discrete generation cycle. The pre-adult development takes about 7-8 days, following which the adult flies are transferred to a population cage. This cage is provided with standard banana food pieces presented as oviposition substrate seeded with *ad-lib* amount of live-yeast paste. Eggs are collected following 24 hours of introducing the flies into the cage, to start the next generation. All other components of the ecology of the ACO regime are identical to those of the CO regime. The ACO populations had passed through >1200 generations by the time the following experiments were conducted.

Generation of experimental flies:

All experiments described here were done following the standard paradigm of experimental evolution assays in which both ACO and CO populations were passed through one generation of standard rearing, included a 14-day rearing schedule for CO's and 13-day rearing schedule for ACO's, to equalize the non-genetic parental effects. All experimental flies were reared at a density of 70 eggs per 8ml medium in a vial under the standard population maintenance regime described above. In all assays, to account for the difference in development time between ACO and CO flies (Chippindale et al. 1997), ACO eggs were collected two days following the collection of the CO eggs. This roughly synchronized the emergence of adult flies. Hereon, age of the adult flies refers to post-eclosion age, in days, unless mentioned otherwise.

We adopted an experimental design in which all male traits including the extent of sexually antagonistic effect of the experimental males was assessed against a common female background. Females from the standard *D. melanogaster* line Oregon R were used for this purpose. Eggs were collected from Oregon R line and females

were raised in the same manner as that followed for the CO and ACO males stated above, including the larval density of 70 per 8 ml food in a vial. All experimental females were age-matched with the experimental males.

Past selection has resulted in a considerable reduction in the body size of the ACO flies in both sexes (Passananti and Matos 2004; Burke et al. 2010), also see body weight data in the results section. Since smaller males are known to be less harming to their mates (Pitnick and García–González 2002; Friberg and Arnqvist 2003), the difference in body size between ACO and CO males was a confounding factor in our mate-harm assay. We adopted a conservative strategy by introducing an additional treatment in our mate-harm assay. In this treatment, we used smaller CO males having comparable body size as ACO males (hereafter referred to as phenocopied CO males, or CCO males). The phenocopied CO males were generated by growing them at the larval density of 240 per 3ml food in a standard vial. All the assays mentioned in later sections except courtship frequency and components of courtship behaviour were conducted using males belonging to three regimes – ACO, CO and CCO. The dry body weight at eclosion of the CCO males were not identical to that of the ACO males (see dry body weight results below). However, both ACO and CCO males were found to be smaller compared to the ancestral CO males by a comparable degree, approximately 50-54% lighter than the CO males. To put it simply, the evolved ACO males in our experiments were compared with the ancestral males as well as ancestral males which were phenocopied for smaller body size. We argue that if the difference in a trait between ACO and CO regimes is qualitatively identical to that observed between the CCO and CO regimes, the ACO-CO difference can be majorly attributed to the evolved body size difference. For example, if both ACO and CCO males induce reduced mate harm to the experimental females compared to that induced by the CO males, the evolved difference in the ACO males' harming ability can be attributed to the smaller body size.

All the adult flies used in the experiments mentioned below were collected as virgins. At the onset of eclosion (emergence from pupal shell), virgin males and females were collected within 4-6 hours of eclosion. Flies were held in groups of 10 individuals per vial with ample food until the assay setup with alternate day food-change. All adult collections were done under light CO_2 anaesthesia. The design and details of the individual assays are mentioned below. A generalized experimental plan is depicted in Fig.1.

<u>Assay Setup:</u>

When adult flies were 1-2 days old, the assay vials were set up by combining males and females in food vials. Each of these vials, therefore, had ten males and ten females. 2 sets of vials (each set has 10 vials) were set up for a given population. The vials were left undisturbed for one hour and the flies were allowed to mate. Mating was visually observed. No video recording was done. Vials where all ten females did not successfully copulate were removed, leaving the final number of vials at this stage at 18-20 for each population. The entire set of these vials were divided into two subsets – (a) Single Exposure (SE: 8-10 vials) and (b) Continuous Exposure (CE: 8-10 vials) treatments. In the SE set, after completion of the first round of mating, sexes were separated under light CO_2 anaesthesia, and females were retained in the same vial while the males were discarded. The CE set of vials was retained without separating the sexes. CE flies was also exposed to light CO_2 anaesthesia to equalize the handling across the two sets. The SE and CE vials were maintained, with alternate day food-change, for twenty days. In these twenty days, mortality, fecundity and behaviour components were recorded (see below). Throughout the experiment, except sorting of sexes, all other fly handling including combination of sexes and transfer of flies from spent vial to fresh food vials were done without anaesthesia.

Fecundity and mortality in test females:

Female mortality was recorded daily for the all twenty days of the assay. Dead flies were removed from the vials by aspiration. Fecundity was recorded in approximately five day intervals, on day 1, 5, 10, 15 and 20. On each of these days, flies (only females for SE and both sexes for CE set) from a vial were transferred to a fresh food-vial (hereafter, fecundity vial) and allowed to lay eggs for a duration of ~24 hours after which they were transferred again into a fresh food-vial to continue the assay (except day 20 count). The fecundity vials were then frozen immediately to stop further development. The eggs were later counted under a microscope. Per capita fecundity (PCF) on a given assay-day was calculated for a vial by dividing the total number of eggs in that vial by the number of females alive at the start of that day. PCF values were taken as the unit of analysis. For the analysis of the mortality data, proportion of females (i.e., out of a total ten in a vial) that were recorded dead at the end of the 20-day period in a vial, i.e., cumulative female mortality, was taken as the unit of analysis.

Courtship frequency:

Courtship frequency (CF) was measured as the average number of courtship bouts a male was found to perform per unit time. The CE vials were used to measure CF, and hence, Oregon R females were used as a common female background against which CF of ACO and CO males were measured. Since body size is not known to

systematically affect CF, CCO males were excluded from this assay. CF observations were done on day 2, 6, 11, 16 and 20 following mating set-up. During an observation, a given vial was measured four times in the following manner. A randomly picked male was observed for 15 seconds – during these fifteen seconds the number of bouts of independent courtship (see later) events were counted. This was repeated four times for a vial. These four counts constituted one observation for that vial. An average for one such observation was calculated by dividing the total count by four, yielding an observation value (C_i). The observation on a given vial was repeated every 90 minutes on a given assay day (i.e., day-2, 6, 11, 16 and 20) for four times ($n_c = 4$). This resulted in four C_i values. Mean courtship frequency (CF) was calculated for a replicate vial using the following function:

$$CF = \frac{\sum C_i}{n_c}$$

The observations were manual and did not involve any video recording. As the ACO and CO flies are visibly different, blind-folded observation did not have any utility and hence, was not adopted. Observers were well-trained to spot any of the following components of courtship behaviour: oriented toward female, following/chasing female, wing vibration, genitalia licking and attempted copulation (von Schilcher and Dow 1977). Performance of any of these behavioural components is counted as one bout of courtship. During the observation, 'two independent bouts of courtship' is defined as the courtship performed to two different females by the same male or behaviour belonging to two separate courtship sequence.

<u>Pattern of courtship behaviour in ACO and CO males:</u>

To investigate the qualitative difference in courtship behaviour ACO and CO regimes, we observed and quantified different components of courtship ritual in males in a separate trial. Courtship behaviour in male *D. melanogaster* is characterised by a complex series of discrete courtship components. In this assay, we quantified the frequencies of the five discrete components of courtship – (1) oriented toward female, (2) following/chasing female, (3) wing vibration, (4) genitalia licking, (5) attempted copulation (Ruedi and Hughes 2008). In addition to these five courtship components, males usually show three additional behavioural states – motionless (m), randomly moving (rm), copulation (c), which are not part of the courtship ritual. The observation vials were set up by introducing a 1-2 day old virgin male (either ACO or CO) and a 3-4 day old virgin Oregon R female in a fresh food vial without using anaesthesia. We started courtship observation after approximately 90 minutes from the initial introduction of the pair to the observation vial. This duration is usually sufficient for all females to undergo a single copulation. During observation, the behavioural state of the male in an observation vial was

recorded by instantaneous scans. A given male was observed every 30 seconds for 30 minutes, resulting in a total of 60 observations for a male. This assay was done for all ten populations, i.e., all five replicate population pairs ($ACO_{1\text{-}5}$ and corresponding CO replicate populations). In each population, 60 males were observed. Multiple trained observers (co-authors and volunteers) carried out the observations manually. However, for a given male all 60 observations were carried out by a single observer. Vials were randomly assigned among the observers to minimize observer bias in the data. Courtship component frequency (CCF: proportional contribution of a given component to the courtship behaviour in a treatment) was calculated by using the following definition:

$$CCF = \frac{(total\ count\ of\ a\ component)}{(total\ number\ of\ observations) - (total\ count\ of\ m, rm, c)}$$

Thus, the assay resulted in 60 CCF values for each of the five courtship components, for a population. These values were used as the unit of analysis.

<u>Statistical analysis:</u>

Cumulative female mortality data were analysed using three-factor mixed model Analysis of Variance (ANOVA) where Male regime (levels: CO, ACO and CCO) and male exposure type (levels: SE and CE) were modelled as fixed factors, and block (level: 1-5) was modelled as a random factor. Dry body weight, and CCF were analysed using two-factor mixed model ANOVA, with Male regime and block modelled as fixed and random factors respectively. As female mortality and CCF data were calculated as proportion values, analysis was performed following arcsine square root transformation (Sokal and Rohlf 1995; Zar 1999). All multiple comparisons were done using Tukey's Honestly Significant Difference (HSD). All these analyses were done using Statistica (Tibco Software Inc., version 13.3).

Fecundity (i.e., Per Capita Fecundity, PCF) data was analysed in two ways. First, PCF pooled across the five age classes, i.e., cumulative fecundity was analysed using three-factor mixed model ANOVA with male regime and exposure type (i.e., SE or CE) were modelled as fixed factors, and block was treated as a random factor. Secondly, to assess the effect of continued exposure to males on female fecundity, age-specific PCF was analysed only for the CE set. Initial analysis indicated significant block-to-block variation (see Results section and supplementary information, Table S3). Hence, each block was separately analysed using a linear mixed-effect model in R version 3.6.1 using `lme4` package (R Development Core Team, 2019) (Bates et al. 2015) and `lmerTest` (Kuznetsova et al. 2017). In the model, PCF was modelled as response variable; male regime, age,

and their two-way interactions as fixed factors; Vial id (replicate vial identity) as a random factor. Following model was used for analysis:

PCF~Male regime + age +Male regime:age + (1|Vial id)

To analyse the effect of male regime and age on courtship frequency, CF (Courtship Frequency) was also analysed using `lmerTest` and `lme4` package in R. CF was modelled as response variable with male regime, age and their two-way interaction as fixed factors and block including all its interactions as random factor. Vial id (replicate vial identity) nested within block was fitted as a random factor. Analysis indicated that block and its interactions were not significant contributors to the overall variance. Detailed analysis with random effects is provided in supplementary information (Table S6). The following Linear-mixed model was used to analyse CF:

CF ~ Male regime + age + Male regime:age + (1|Block/Vial id) + (1|Block:Male regime) + (1|Block:age) + (1|Block:Male regime:age)

Post-hoc analysis was done using Tukey's HSD using `emmeans` package (Lenth 2018).

Results:

Both the fixed factors, i.e., regime and male exposure type, were found to have significant effects on cumulative female mortality (Table 1). In addition, the effect of the male regime × male exposure type interaction was also found to be significant (Table 1). Tukey's HSD indicated that under SE condition, the differences in cumulative female mortality across the three male regimes were not statistically significant. The recorded mortality under SE was generally low (cumulative mortality <10%). Under CE condition, however, it was substantially higher, especially when the females were held with CO males (>24% higher compared to either ACO or CCO males, Fig. 2a). The difference between ACO and CCO treatments was not significant. As the analysis indicated a significant effect of male regime × male-exposure type × block three-way interaction (Table 1), the results from each block were analysed separately using two-factor ANOVA (male regime and male-exposure type as fixed factors). The detailed outcome of these analyses can be found in the supplementary material (Table S5). Briefly, block 3 failed to show any effect of the regime, results from all other blocks were qualitatively identical.

Dry body weight of males (ACO, CCO and CO) were measured at eclosion. We found significant effect of regime ($p < 0.001$, Table 1). Pairwise comparisons using Tukey's HSD showed that both ACOs and CCOs are significantly smaller than COs (weight in mg, mean ± standard error of mean, ACO: 0.162 ±0.003; CO: 0.271 ±0.004; CCO: 0.131 ±0.005). Qualitatively the pattern of dry body weight was similar in all the blocks, but we found significant interaction of regime × block. Hence, summary table of each block is provided in supplementary information (Table S1, Fig. S2).

Results of the three-factor mixed model ANOVA revealed significant effects of male regime, male exposure type, and male regime × male exposure type interaction on cumulative fecundity (Table 1). Multiple comparisons using Tukey's HSD indicated that under single mating exposure (i.e., SE), the three regime did not differ significantly from each other. Under CE condition, females held with ACO males were found to have ~13.65% higher cumulative PCF compared to the females held with CO males. Cumulative PCF of females held with CCO males were statistically not different from that of the females held with CO males (Fig.2b). Results of the linear mixed model analysis on age-specific PCF of the females in the CE set were more complex (see supplementary information). While in each block significant effects of male regime, age and male regime × age interaction were detected, there was little consistent trend across blocks (Table S4). However, in at least three of the five age points, females held with ACO males showed a significantly higher PCF compared to the other two treatments, particularly at later age points (Fig. S3).

Linear mixed model analysis on the courtship frequency (i.e., CF) results showed significant effects of male regime, age, and regime × age interaction (Table 2). Tukey's HSD results showed that courtship frequency of ACO males is significantly higher, particularly at early age classes than the CO males (Fig. 3a). Two factor mixed model ANOVA performed on the CCF results indicated an interesting effect of male regime. Male regime was not found to have a significant effect on four of the five courtship components (Table 3). However, we found a significant male regime effect on attempt-to-copulation ($p = 0.029$), where ACOs showed ~15% higher copulation attempts compared to COs (Fig. 3b, Table 3).

Discussion

Our results showed that ACO males are clearly far less harassing compared to the ancestral CO males. The females held with ACO males showed significantly less mortality and higher fecundity during the assay period.

Interestingly, ACO males were found to be more active in courtship, especially early in life. This age-specific pattern of change in courtship activity is consistent with the theory of selection for early life fitness components in the ACO males. Despite several previous investigations linking courtship activity to the level of mate-harm caused by *D. melanogaster* males, ACO males were found to be substantially less harming, in spite of having higher courtship activity.

Though faster aging ACO males are expected to have the potential to invest a greater proportion of their resources in competitive (and hence, sexually antagonistic) traits, their impact on female mortality and fecundity was quite contrary to this prediction. One potential explanation of this observation is that the ACO males are substantially smaller and are hence, less harming - measured in terms of the mates' survival rate and reproductive output. Such an effect of body size on harming ability of the males in this system has been previously shown (Pitnick and García–González 2002). Our results, however, suggest a more complex picture. Based on the ACO vs. CCO comparison, we could attribute only the mortality effect to the reduced size of the ACO males. However, the fecundity effect of the ACO males was quite unique. Average cumulative fecundity of females held with the ACO males was found to be significantly higher than that with CO males. However, no significant difference was found between cumulative fecundity of females held with CO and CCO males. Thus, it is reasonable to deduce that the difference in reproductive output of the experimental females held with ACO males and those held with CO males was unlikely to be an outcome of the size difference between the males from the two regimes. Thus, either due to reduction is size or due to changes in traits unrelated to size, or both, ACO males are significantly less harming to their mates.

D. melanogaster males are known to physically coerce females through persistent courtship (Fowler and Partridge 1989). Multiple lines of evidence have also shown the correlation between the degree of mate-harm and intensity of courtship behavior (Nandy et al. 2013b; MacPherson et al. 2018). For example, Nandy et al. (2013b) experimentally evolved a set of populations under male biased operational sex ratio that resulted in increased harming ability in males, along with increased courtship frequency. MacPherson et al. (2018) showed that females seemed to avoid mate harm when they were held in complex holding chambers with ample hiding opportunities, possibly by escaping direct exposure to persistent courtship. Different components of courtship behaviour have been found to have ample genetic variation both in natural and laboratory populations of *Drosophila* (Markow and Hanson 1981; Gromko 1987; Ritchie and Gleason 1995; Colegrave et al. 2000; Snook

et al. 2005; Dai et al. 2008). There has been ample evidence suggesting evolvability of courtship behaviour in *D. melanogaster* (Bedhomme et al. 2008; Nandy et al. 2013b). However, how evolution of courtship behaviour can contribute to the evolution of mate harming ability of the males is not clear. The fact that ACO males, in our study, were found to be less harming despite being more active in courting females, is a direct challenge to the conventional wisdom that draws a one-on-one connection between courtship and physical component of mate harm. Not only ACO males courted females more frequently, they also attempted copulation more often - a component of courtship that appears to be more coercive. Hence, our results indicate that the relationship between courtship behaviour and mate harm is more complex than previously anticipated.

In addition to persistent courtship, seminal fluid proteins (Sfp) transferred to females during copulation have been shown to bring down female survival rate (Chapman et al. 1995; Wigby and Chapman 2005; Wigby et al. 2020). Changes in Sfp content of the ejaculate can potentially explain reduction in harming ability of the ACO males. At this point we do not have data on Sfp to directly test this hypothesis. However, in a separate assay, we observed that copulation between an ACO male and a female from the corresponding population is significantly shorter compared to that between a pair from a CO population (see SI for more details, Fig. S4 and Table S7, mean CD in minutes ±SEM, ACO: 16.15 ±0.657; CO: 20.32 ±0.623). Variation in copulation duration has been correlated with variation in the amount of Sfp transferred (Singh and Singh 2004; Friberg 2006; Bretman et al. 2009; Bretman et al. 2010). In principle, reduction in post-copulatory sexual selection can bring about changes in Sfp and/or copulatory traits in ACOs. Monogamy and female biased operational sex ratio have already been shown to lead to reduction in both harming ability and investment in certain attributes of seminal fluid content including Sfp (Holland and Rice 1999; Wigby and Chapman 2004; Crudgington et al. 2005; Linklater et al. 2007). However, it is unrealistic to suggest that our ACO populations have a monogamous breeding system and hence, post-copulatory sexual selection is absent. Our observations suggest that females in ACO populations undergo remating quite regularly (Fig. S5). However, the breeding ecology of this regime is vastly different from the ancestral regime. In contrast to the ancestral CO regime, where breeding life is approximately 19 days of adult life, ACO flies get 24-36 hours to reproduce after becoming adults. In this incredibly short breeding life, males are selected to be reproductively active and invest heavily on competitive traits that maximize mating success. Our courtship frequency results support this hypothesis. However, given that there is strong last male sperm precedence in this species (Manier et al. 2010) and ample female remating, the exact nature and intensity of post-copulatory sexual selection is not clear.

In an independent investigation on a similar set of experimentally evolved populations, (Mital et al. 2021a; Mital et al. 2021b) found that the intensity of sexual selection and the degree of sexual antagonism to be lower in populations selected for faster development and very early reproduction. The parallel between our results and those of (Mital et al. 2021a; Mital et al. 2021b) is somewhat expected, and is a robust proof in support of our theory. However, even more interesting is the difference. Whereas (Mital et al. 2021a) could almost entirely assign the reduction in sexual antagonism in their FEJ populations to the reduction in body size, our results point to a different explanation. Comparison of the FEJ and the ACO regime is interesting. In ACO regime, due to a fixed egg-to-adult development time window of eight days, selection for faster pre-adult development has not been strong for the last several hundred generations. However, FEJ regime has a strong directional selection for faster development. As a result, flies under the FEJ regime have probably evolved a more extreme reduction in size, amounting to resource deprivation that did not allow the evolution of male competitive traits, including courtship (Mital et al. 2021a). In addition, whereas the FEJ regime practically ensures life-long monogamy, we observed substantial amount of promiscuity in ACO flies. Hence, it is possible that ACO males were specifically selected for traits that maximize mating and fertilization success in such a way that reduces incidental mate-harm. Such results clearly show the importance of an incredibly nuanced breeding ecology of animals, and its connection to life history.

Theories have long predicted a connection between life history and sexual conflict. Most often, the trade-offs between costly SA traits and one or more life history trait(s), most notably somatic maintenance, has taken the centre stage in such discussions (Bonduriansky et al. 2008; Maklakov and Lummaa 2013; Hooper et al. 2017). However, if the aim is to understand and predict the evolutionary outcome of changes in life history on SA traits, perhaps a more important question is, how a given alteration in life history affects the breeding system, and by extension, the ecology of sexual selection. The secondary outcomes in that case can affect a wider range of sexually antagonistic traits, potentially independent of trade-offs. Our results on the reduced mate harming ability in ACO males is evidence in support of this thesis.

Rankin and Kokko (2006, 2007) very explicitly showed a seemingly obvious outcome of SC, viz., population extinction due to unchecked evolution of sexually antagonistic male traits. This was previously ignored by most sexual conflict investigations. Perhaps, the ignorance is expected as SC is almost always viewed as an arms'

race and hence, counter adaptation in females is fundamental to the theory. In line with such a reasoning, many empirical investigations have reported strong female counter adaptation to mate harm imposed by male (Wigby and Chapman 2004; Nandy et al. 2014). As we have argued above, life history can also be a potent constraint on the evolution of mate harm. However, there is little literature on this subject. To the best of our knowledge, the present study on the ACO males is one of the very few empirical evidence of evolutionary reduction in mate-harming ability. The results presented here should be of general importance in explaining the variation in the intensity of sexual antagonism across species.

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Figure captions:

Fig.1 Schematic representation of the design main assay. Experimental subsets were generated from stock populations following one generation of standardization (common garden rearing). Experimental males were generated in the following manner: CO subset was used to generate CO experimental males and CCO males (phenocopied to ACO, these were generated by growing larvae at a density of 240/3ml of standard food) and ACO males were generated from ACO stock. Oregon R females were generated separately under standard conditions as common female for all three regime males. On assay day the whole set-up was divided into two male-exposure condition i.e. single exposure, SE (where males and females are allowed to mate once) and continuous exposure, CE (where males and females were housed together after first mating). Behaviour assays were then carried out for 20 days where female mortality was recorded every day, fecundity and courtship frequency was noted at every 5-day interval). All vials were flipped into fresh food vial at every alternate day

Fig. 2 Effect of exposure to treatment males (ACO/CCO/CO) on female mortality and fecundity. (a) Proportion of females died by the end of the 20-day assay period (cumulative female mortality), under the two male-exposure conditions – single exposure (SE) and continuous exposure (CE). The vertical bars indicate the mean across all replicate populations. Error bars represent the standard errors of means (SEM); (b) Average per capita fecundity of experimental Oregon R females exposed to treatment males across all five age classes, under different male exposure types SE and CE. Means were calculated over five replicate populations. Only relevant multiple comparisons, which were done using Tukey's HSD, are shown. Significant differences are marked with horizontal line and an asterix (*)

Fig. 3 Results of the courtship behaviour assays. (a) Courtship frequency (CF: bouts of courtship per observation) was measured for the 20-days assay period on days – 2, 6, 11, 16, and 20 in the CE vials of the main assay. (b) Courtship component frequency (CCF: proportional contribution of a courtship component) of five components of courtship ritual were measured in a separate assay, where ACO and CO males were held with Oregon R females. The vertical bars indicate the mean across all five replicate populations. Error bars represent the standard errors of means (SEM). Only relevant multiple comparisons are shown in the figure. Significant differences are marked with horizontal line and an asterix (*)

Table 1 Summary of results of three-factor ANOVA on cumulative female mortality, cumulative fecundity, and two-factor ANOVA on dry body weight of males at eclosion. Mortality analysis was done on arcsine square root transformed values. Regime and male-exposure type are taken as fixed factor and block as random factor in female mortality and cumulative fecundity analysis. Regime is taken as fixed factor and block as random factor in dry body weight analysis. All tests were done considering α=0.05 and significant p-values are mentioned in bold font style

Trait	Effect	SS	DF	MS	Den DF	Den MS	F	p
Female mortality	Male regime (MR)	2.580	2	1.290	8.005	0.080	16.075	**0.001**
	Male exposure type (MET)	6.933	1	6.933	4.002	0.070	98.434	**<0.001**
	Block	0.831	4	0.208	0.371	0.037	5.636	0.550
	MR × MET	4.560	2	2.280	8.004	0.114	20.032	**0.001**
	MR × Block	0.642	8	0.080	8.000	0.114	0.705	0.684
	MET × Block	0.282	4	0.070	8.002	0.114	0.619	0.661
	MR × MET × Block	0.911	8	0.114	265.000	0.047	2.401	**0.016**
	Block	2.580	2	1.290	8.005	0.080	16.075	**0.001**
Dry body weight	Male regime	0.543	2	0.271	8.000	0.004	65.570	**<0.001**
	Block	0.037	4	0.009	8.000	0.004	2.217	0.157
	Male regime × Block	0.033	8	0.004	135.000	0.001	6.781	**<0.001**
Cumulative fecundity	Male regime (MR)	206.30	2	103.150	8.001	12.371	8.338	**0.011**
	Male exposure type (MET)	170.890	1	170.890	4.000	14.423	11.849	**0.026**
	Block	1322.53	4	330.630	6.482	21.919	15.085	**0.002**
	MR × MET	104.850	2	52.430	8.002	4.877	10.750	**0.005**
	MR × Block	98.990	8	12.370	8.000	4.877	2.537	0.105
	MR × Block	57.690	4	14.420	8.002	4.877	2.958	0.090
	MR × MET × Block	39.020	8	4.880	267.000	3.044	1.602	0.124

Table 2 Summary of the results of linear mixed model (LMM) analysis of courtship frequency using `lmerTest` function in R. Regime and age were modelled as fixed factors and block as a random factor. All tests were done considering α=0.05 and significant p-values are mentioned in bold font style

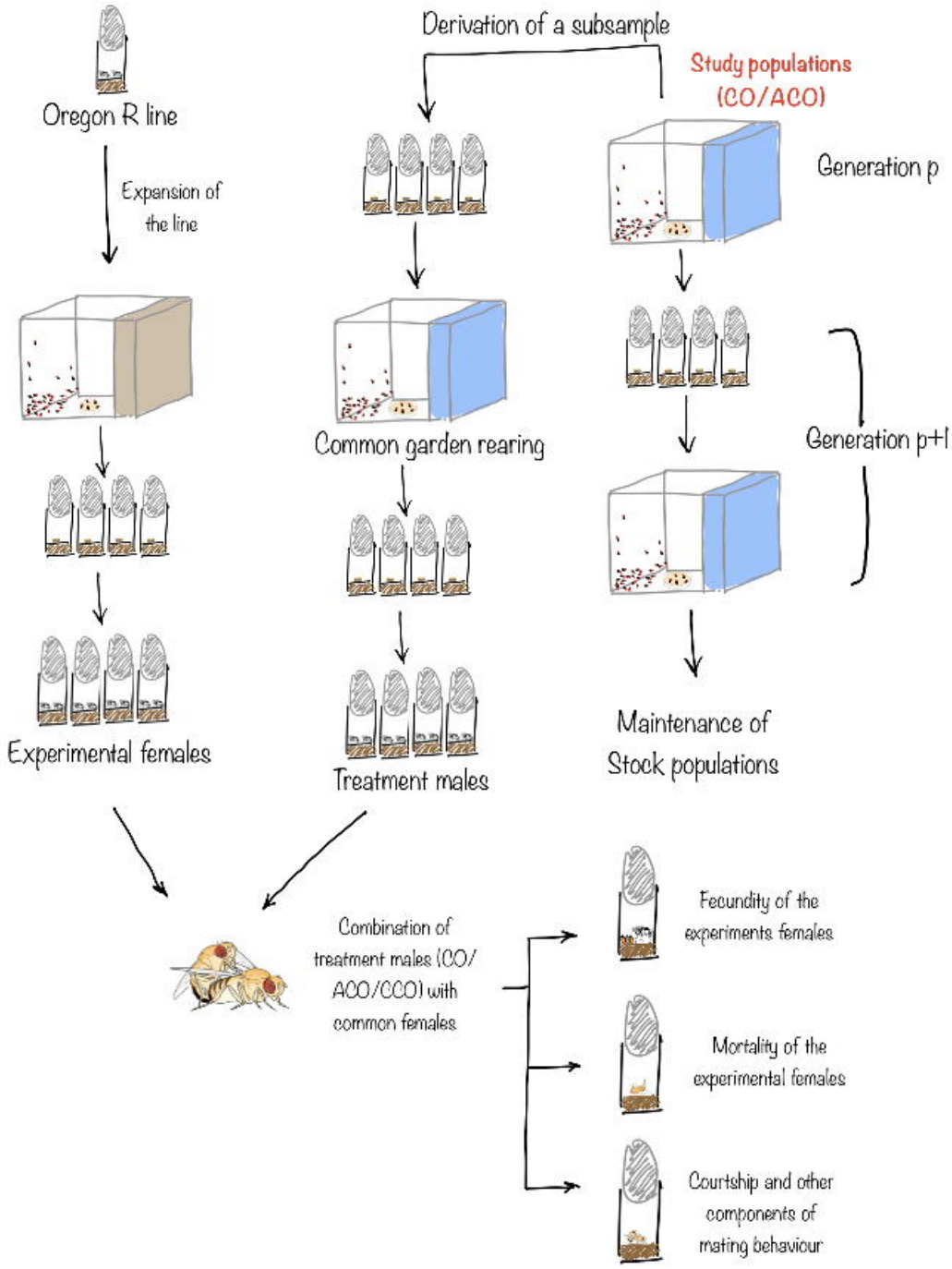
Effect	SS	DF	MS	Den DF	F	p
Male regime	12.472	1	11.422	480.06	143.125	**<0.001**
Age	5.927	4	1.437	479.990	17.005	**<0.001**
Male regime × Age	9.206	4	2.301	479.990	26.441	**<0.001**

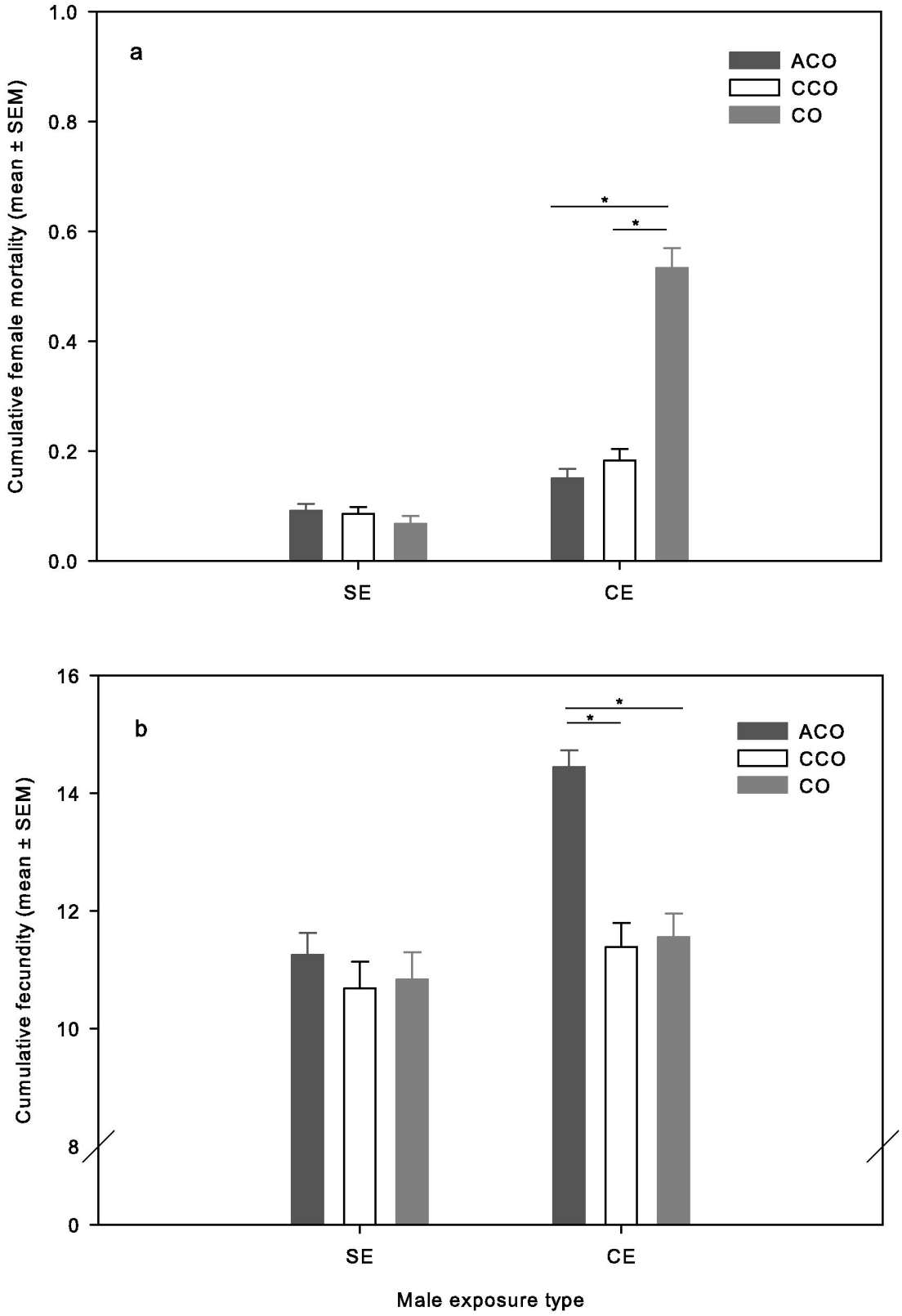
Table 3 Summary of results of two-factor ANOVA on courtship component frequency (CCF). Male regime was modelled as a fixed factor and block as a random factor. Analysis done on arcsine square root transformed values. All tests were done considering α=0.05 and significant p-values are mentioned in bold font style

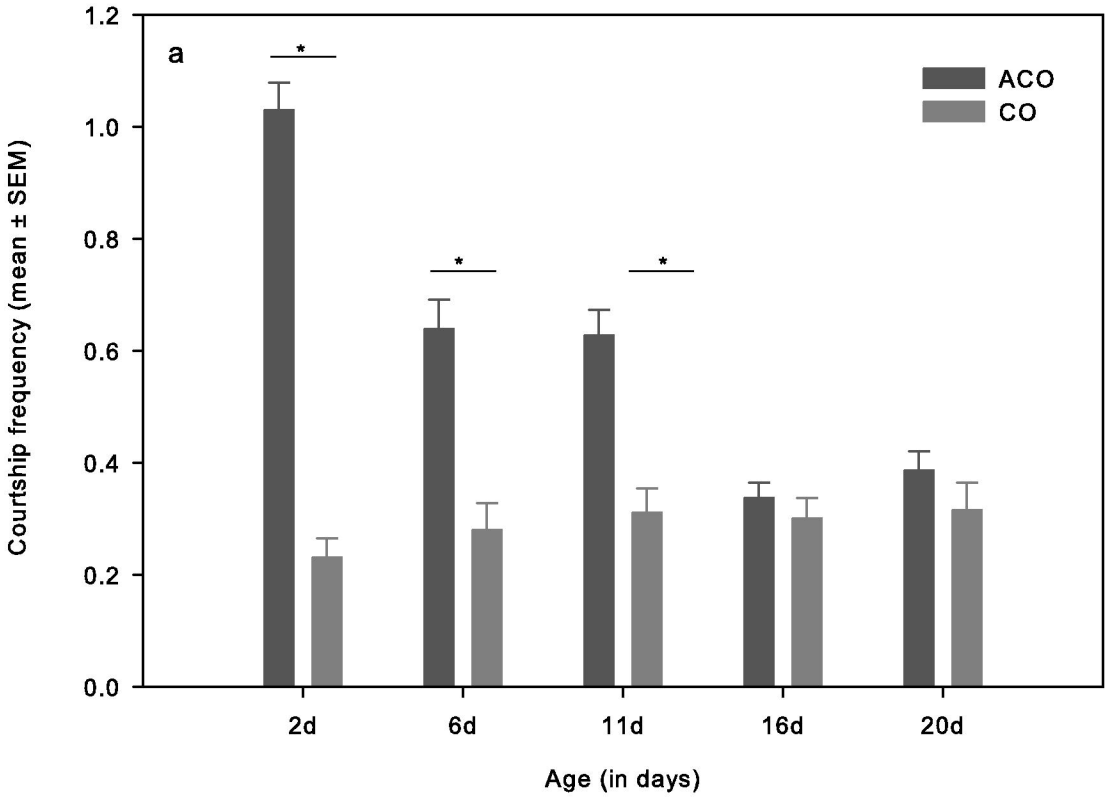
Trait	Effect	SS	DF	MS	Den DF	Den MS	F	p
Orientation	Male regime	2.255	1	2.255	4.002	0.456	4.947	0.090
	Block	5.485	4	1.371	4.000	0.456	3.007	0.156
	Male regime × Block	1.824	4	0.456	501.000	0.195	2.340	0.054
Chasing	Male regime	0.084	1	0.084	4.015	0.047	1.782	0.252
	Block	0.738	4	0.184	4.000	0.047	3.935	0.106
	Male regime × Block	0.188	4	0.047	501.000	0.160	0.292	0.883
Wing vibration	Male regime	0.358	1	0.358	4.004	0.177	2.017	0.228
	Block	3.605	4	0.901	4.000	0.177	5.079	0.072
	Male regime × Block	0.710	4	0.177	501.000	0.178	0.995	0.410
Licking	Male regime	0.371	1	0.371	4.007	0.065	5.744	0.074
	Block	0.688	4	0.172	4.000	0.065	2.665	0.183
	Male regime × Block	0.258	4	0.064	501.000	0.098	0.661	0.619
Attempt to copulation	Male regime	0.717	1	0.717	4.008	0.042	16.989	**0.014**
	Block	0.438	4	0.110	4.000	0.042	2.599	0.189
	Male regime × Block	0.169	4	0.042	501.000	0.079	0.534	0.711

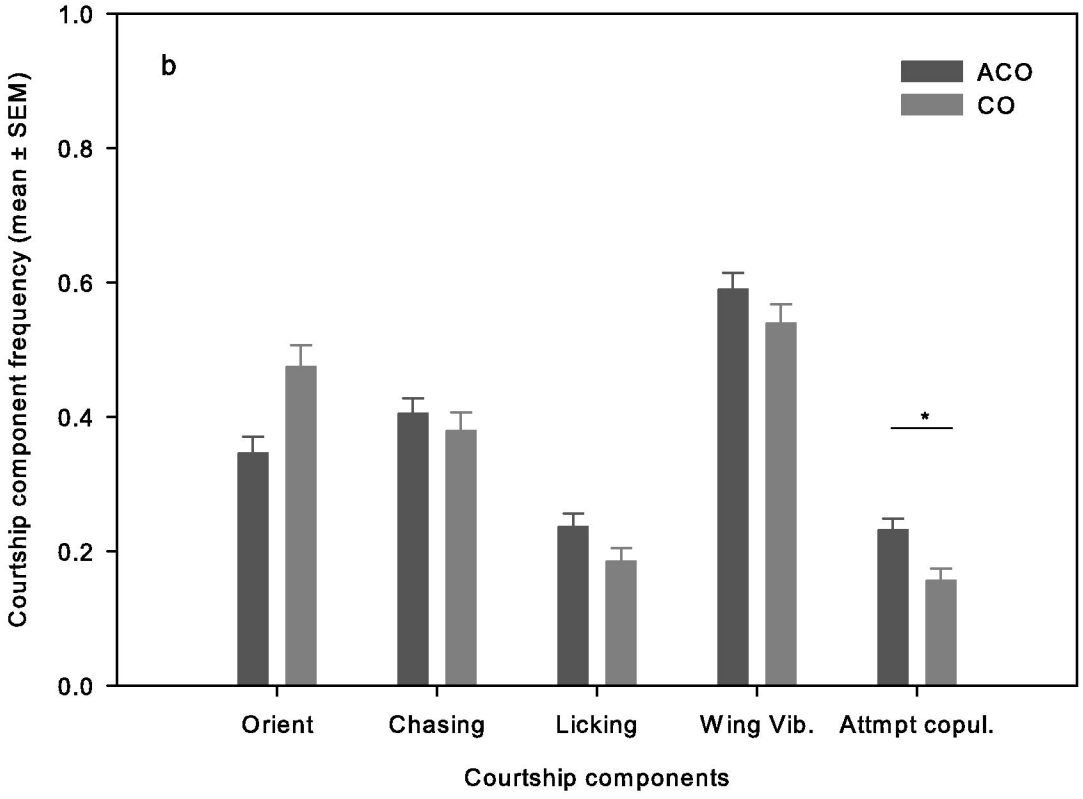
755

756


Derivation of a subsample
Study populations
(CO/ACO)
Oregon R line
Expansion of
the line
Generation p
Common garden rearing
Generation p+1
Experimental females
Treatment males
Maintenance of
Stock populations
Combination of
treatment males (CO/
ACO/CCO) with
common females
Fecundity of the
experiments females
Mortality of the
experimental females
Courtship and other
components of
mating behaviour


a
ACO
CCO
CO
Cumulative female mortality (mean ± SEM)
1.0
0.8
0.6
0.4
0.2
0.0
*
*
SE
CE
b
ACO
CCO
CO
Cumulative fecundity (mean ± SEM)
16
14
12
10
8
0
*
*
SE
CE
Male exposure type


a
ACO
CO
Courtship frequency (mean ± SEM)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
*
2d
6d
11d
16d
20d
Age (in days)


b
ACO
CO
Courtship component frequency (mean ± SEM)
1.0
0.8
0.6
0.4
0.2
0.0
*
Orient
Chasing
Licking
Wing Vib.
Attmpt copul.
Courtship components