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Female, but not male, nematodes evolve under experimental sexual coevolution

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Coevolution between the sexes is often considered to be male-driven: the male genome is constantly scanned by selection for traits that increase relative male fertilization success. Whenever these traits are harmful to females, the female genome is scanned for resistance traits. The resulting antagonistic coevolution between the sexes is analogous to Red Queen dynamics, where adaptation and counteradaptation keep each other in check. However, the underlying assumption that male trait evolution precedes female trait counteradaptation has received few empirical tests. Using the gonochoristic nematode *Caenorhabditis remanei*, we now show that 20 generations of relaxed versus increased sexual selection pressure lead to female, but not to male, trait evolution, questioning the generality of a male-driven process.

1. Introduction

Antagonistic coevolution arises when a conflict between two opponents results in evolutionary adaptation to one another. One opponent has an advantage over the other by evolving a trait, while the other needs to coadapt to this trait. The Red Queen theory predicts that this process of counter adaptations is necessary to preserve the coexistence of the two opponents.

In sexually antagonistic coevolution, males and females are the two opponents that have different mating optima [1]. As males are more prolific in their gamete production and compete for access to female gametes [2], a suite of traits has evolved to outcompete other males and persuade females to mate [3]. Male competition and manipulation can manifest through physical and chemical traits. In most promiscuous systems, males use seminal fluids to manipulate competitors and females [4]. One prominent example is *Drosophila* males that manipulate females to produce and allow fertilization of more eggs after a given mating [5–7] or to postpone remating [8]. These manipulations are considered costly to females by lowering their fitness when being promiscuous [9–11]. As a consequence, selection favours females that express resistance traits against such manipulations. Females can evolve reduced susceptibility in response [12,13]. It has also been suggested that female counteradaptations select for further trait elaboration (or novel traits) in males to keep pace and adapt in return [14,15]. This argument is rooted in the strong selection pressure on males by male–male and sperm competition. Yet few empirical tests exist that demonstrate that male offensive traits evolve before female resistance traits.

A powerful way to test this fundamental assumption of male–female coevolution is experimental evolution [14,16–19]. Such experiments involve two stages. First, experimentally elevated or relaxed sexual conflict is used to generate measurable changes in trait expression. Second, males and females of different selection regimes are crossed and the fitness of the different matings is compared. If coevolution or adaptation has taken place, relaxed sexual conflict lines should have less resistant females and less competitive (and therefore less harmful) males relative to the enhanced conflict treatments. A series of experimental evolution studies have been performed on different model organisms to test the prediction of male and female coevolution. In *Drosophila*, manipulation of sex ratios over generations has led to changes in male behaviour [20], seminal fluids [16] and the resulting differences in fitness resulting from male treatment [13]. For females, remating rates [16], longevity [21] and lifetime reproductive success [21,22] were affected

within a few tens of generations [22]. Also, in beetles such as *Tribolium* or *Callosobruchus*, male- and female-biased sex ratios affected both male and female traits at the same time [23,24]. In these studies, males and females both reacted to the treatment and reduced or enhanced their ability to manipulate, compete or resist according to the strength of selection.

A promising model system in sexual selection and evolution experiments is offered by terrestrial nematodes of the genus *Caenorhabditis* [25]. They are easy to maintain and have a short generation time (3 days). The most investigated species is the largely selfing hermaphrodite *C. elegans* [26–28], which has not only been shown to coevolve antagonistically with microparasites in laboratory experiments within 50 generations, showing the first changes already after a fraction of that time [29,30], but also has been studied in the context of sperm competition [31]. Larger sperm was the result of increased sperm competition.

We used the gonochoristic species *C. remanei* to test whether a series of reproductively relevant male traits evolve before female traits under sexually antagonistic coevolution. *Caenorhabditis remanei* female fitness decreases with the number of mating partners and therefore represents a perfect fit for our experiment [32]. We intensified sexual conflict to females above the laboratory baseline (1 : 1 sex ratio) for 20 generations by imposing a 1 : 5 (polyandry, PA) sex ratio and relaxed it using a 5 : 1 (polygyny, PG) sex ratio. Additionally, our design allows for mate choice for the males in the PG treatment and females in the PA treatment. Sexually antagonistic coevolution theory predicts that increasing male–male competition in the PA treatment should result in an increase in male harm and female resistance, while males and females in the PG treatment should be less harmful and less resistant in the final assays.

2. Material and methods

(a) Model organism

Caenorhabditis remanei was kept at 19°, with 24 h light and 60 ± 10% humidity. Under these conditions, the generation time is approximately 4 days. Populations were maintained on agar with OP50 *Escherichia coli* as described in [33], with a higher agar concentration as in [34].

Prior to the onset of the experiment a genetically diverse starting population (SP8) was created. The laboratory strains SB146 (Freiburg, Germany), PB206 (Wright State Woods, USA) and MY31 (Tübingen, Germany) were crossed in a fully factorial design and tested for fertility. Offspring from all crossings were pooled and maintained for eight generations to adapt to laboratory conditions. SB146 and PB206 were both obtained from the Caenorhabditis Genetics Center, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440).

(b) Experimental evolution

In order to start the experimental evolution, we synchronized SP8 by bleaching; only eggs survive using this procedure [33]. After two developmental days, we assigned 960 nematodes to two different treatments (PG/female-biased 1 : 5 and PA/male-biased 5 : 1), each replicated in four lines. Replicates within treatments therefore consisted of 120 individuals each and were subdivided into 20 mating groups, separate Petri dishes containing six individual nematodes during the phase of copulation/reproduction. In the female-biased treatment these mating groups were formed by one male and five females (1 : 5 PG) and in the male-biased treatment mating groups were formed by five males and one female (5 : 1 PA; figure 1). After 2 days of copulation and egg laying, all 20 individual mating groups per replicate were pooled and synchronized by bleaching. Virgin offspring were then randomly assigned to a new set of 20 mating groups as outlined above. We repeated this protocol for 20 generations and froze a sub-sample of starved nematodes of each generation. All Petri dishes were randomly coded and randomly spatially arranged to allow for a design completely blind to the observer, to prevent any bias.

Finally, frozen nematodes of the 20th generation were thawed and left for three generations under laboratory conditions prior to conducting the final assays.

(c) Final assays

(i) Sperm size

Ten virgin, adult males from each replicate line and treatment (PA: line A, B, C and D; PG: line A, B, C and D) were transferred to physiological sperm medium [35] and cut in the middle of the body with a needle from an insulin-syringe to isolate the testis. The sample was gently squeezed between slide and cover slip to release individual sperm cells (methods adjusted to K. F. LaMunyon 2008, personal communication). Pictures were taken under a Leica microscope with 630× magnification with a mounted Leica camera. The volume of 20 (spherical) sperm cells per individual male was calculated by means of ImageJ v. 1.41o.

(ii) Plug size

After mating, the male secretes the copulatory plug onto the entrance of the female vulva. To determine plug size, we chose particular, evenly distributed combinations of replicates of the PG treatment, the PA treatment and the SP8, resulting in 32 different mating combinations, each replicated eight times (figure 2). A virgin male was placed 0.8 cm away from a virgin female on a 3 cm Petri dish and was removed after the first mating. Images of freshly mated females were taken within 20 min after mating and the two-dimensional area of the plug was measured using ImageJ v. 1.41o.

(iii) Soporific effect

The soporific effect is induced by males and visible as a paralysis of the female during mating. The same individuals and experimental set-up as in the plug size assay were used (figure 2). The time estimation of the soporific effect started with the insertion of the male spiculae into the female vulva, which is a clear starting point for our measurement and approximately represents the moment of transferring the soporific substance. We ended our measurement at the moment the female started to grind food again and started crawling, which was independent from spiculae or male position. The Petri dishes containing the experimental animals were coded blindly so that no observer biases were possible.

(iv) Lifetime reproductive success I

We used the same mating combinations of treatments, replicate lines and the SP8 as in the plug size assay (figure 2), and replicated these combinations eight times. Five females were kept with five males on individual Petri dishes and were transferred to fresh Petri dishes every day until females laid fewer than 10 eggs per day, maximally 7 days. The plates with eggs were kept for another 2 days under laboratory conditions until larvae hatched and were then frozen at −80°C. Subsequently, to count daily offspring production, Petri dishes were thawed again.

(v) Lifetime reproductive success II

We repeated the 'lifetime reproductive success I' assay under PA conditions (five males : one female), resembling the PA treatment design of the initial experimental evolution. We replicated each combination 15 times (figure 2). This time only the first 3 days of

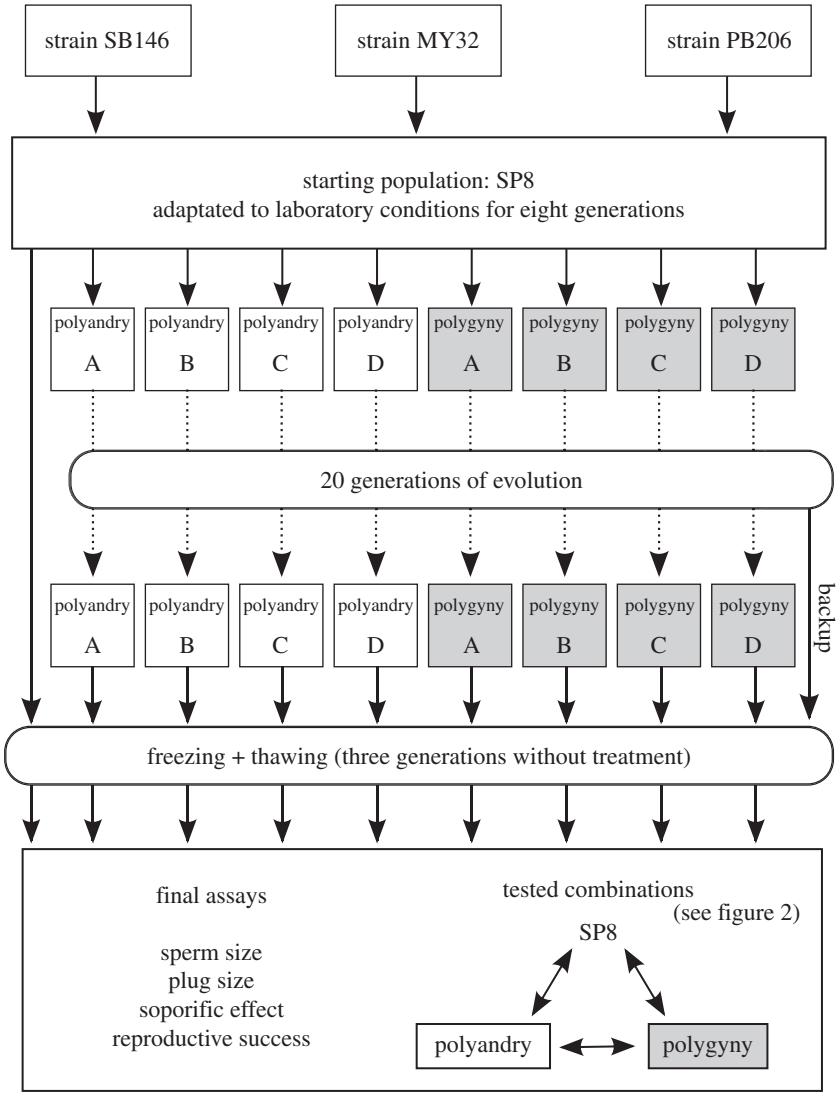

Figure 1. Scheme of the experimental evolution and the final assays. Three strains were crossed to form the starting population, which adapted to the laboratory for eight generations. The subsequent starting population was randomly split into two treatments with four replicates each. After 20 generations (see Material and methods), final assays were performed by comparing the two treatments with each other and with the starting population.

reproduction were analysed and the offspring were not counted on plates, but washed off into 1.5 ml Eppendorf tubes and frozen for counting at a later date.

(d) Statistics

All datasets were analysed with nested two-way ANCOVA models (plug size, soporific effect and reproductive success I + II) or nested ANOVA models (sperm size and body size) with selection replicate (replicate line of treatment) as a random effect to be able to account for all the variation within and between crosses of treatments (figure 2). We examined the effects of sex ratio/treatment (PA, PG and SP8) for each sex separately as well as their interaction on plug size, soporific effect and lifetime reproductive success. For the sperm size measurements, the mean of several sperm cells per male was calculated prior to the analysis. All analyses were done using JMP v. 9.0 and R v. 2.13.1.

3. Results

(a) Sperm size

After experimental evolution, sperm size did not differ between the PA treatment, the PG treatment and the starting population ($p = 0.460$; table 1).

(b) Plug size

Plug size varied strongly within treatments, but did not show significant differences between the two treatments (PA and PG) and the SP8 irrespective of whether the males or females came from the PA or PG treatment or the SP8 (table 1).

(c) Soporific effect

The soporific effect differed significantly for females of different treatments (PA, PG and SP8). However, neither the treatment of the male nor the interaction of female treatment and male treatment affected the soporific effect. Females that evolved in the polyandrous treatment showed reduced duration of the soporific effect independent of the male they were mated to. These females started crawling away from their mating partner sooner and terminated the mating event earlier than females from the polygynous treatment and the SP8 ($p < 0.001$; table 1 and figure 3).

(d) Lifetime reproductive success I

In the assays with an equal sex ratio of five males and five females, the treatment of the female, irrespective of the male treatment, had a significant effect on the number of produced

Table 1. Results of the final assays. Plug and sperm size did not change during the experimental evolution or between the treatments. Changes were detectable in the soporific effect assay and in the lifetime reproductive success. Only female treatment had a significant influence on the outcome. A more detailed table is available as the electronic supplementary material.

trait	source	*n* total	d.f.	*F* ratio	*p*-value
sperm size	male treatment	90	2	0.89	0.460
plug size	male treatment	258	2	0.11	0.838
	female treatment		2	0.27	0.749
	male × female		4	1.12	0.352
soporific effect	male treatment	258	2	0.39	0.710
	female treatment		2	4.58	<0.001
	male × female		4	0.51	0.448
lifetime reproductive success I	male treatment	185	2	0.42	0.347
	female treatment		2	7.67	0.011
	male × female		4	0.07	0.959
lifetime reproductive success II	male treatment	393	2	0.34	0.659
	female treatment		2	4.72	<0.001
	male × female		4	0.21	0.788

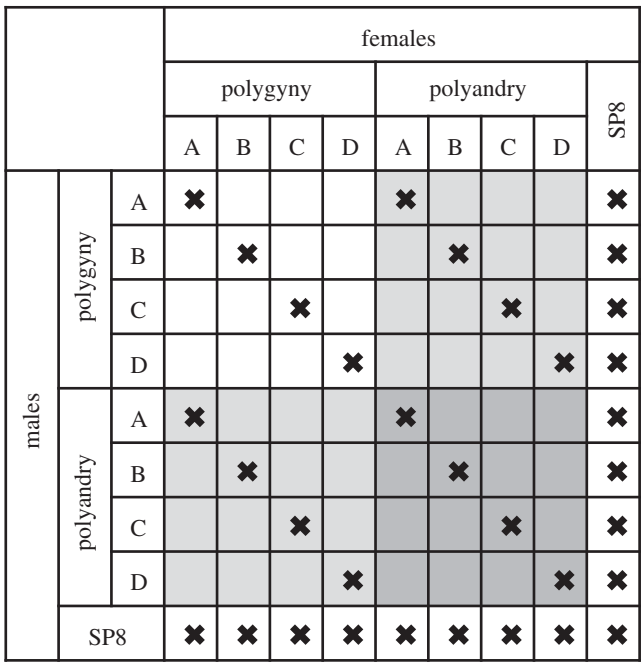

Figure 2. Combinations for the final assays. To reduce the number of combinations, only males and females of certain replicates were combined. In total, all replicates were tested within replicate lines and between one replicate line of the other treatment (i.e. crosses between line PA A and PG A would include matings of PG A males with PA A females, and PA A males with PG A females) and the SP8 control. Additionally, every replicate was combined with itself as a control. Crossed boxes indicate the chosen combinations for final assays.

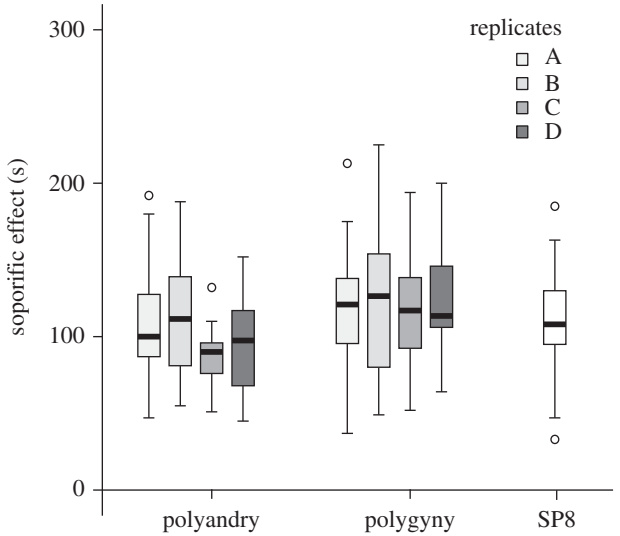

Figure 3. Results of the soporific effect assay. Females of the PA treatment (irrespective of the male they were mated with) awoke earlier after being immobilized by the male during mating. Box plots show mean and quartiles of the data. The different shades of grey represent the different replicate lines (A, B, C and D) of the two sex ratio treatments (PA and PG). Populations with the same shade were crossed for the final experiments.

offspring. Polyandrous females produced more offspring than females from the polygynous treatment or SP8 ($p = 0.011$; table 1). The treatment of males and the interaction of female treatment with male treatment had no effect on lifetime reproductive success.

(e) Lifetime reproductive success II

Similarly to the 'lifetime reproductive success I' results, female treatment had a significant influence on the amount of produced offspring in the 'lifetime reproductive success II' assay. Polyandrous females produced more offspring than females from the polygynous treatment. However, females of the SP8 showed highest LRS, contradicting our initial expectations ($p < 0.001$; table 1). Neither male treatment nor an interaction of male and female treatment affected offspring production.

4. Discussion

None of the measured male traits, but all of the female traits, evolved under our sexually antagonistic experimental evolution design. Importantly, maternal effects cannot explain our results because all replicates were kept under neutral conditions for three generations due to thawing of frozen populations. This

result—that female (but not male) reproductive traits evolve under sexual conflict—demonstrates that the assumed male-first, female-follow scenario may not be universal. Specifically, we found that females from the PG treatment were more sensitive to the sedative substances that males transfer to immobilize females [36] compared with females from the PA lines. Similarly, lifetime reproductive success under both PA and 1 : 1 sex ratio showed a female, but not male, response to selection. Increased female resistance under PA in terms of longevity was earlier documented for *Drosophila melanogaster*, while males did not differ in their ability to harm females [12]. This study on *Drosophila* is the only example where females (but not males) reacted to experimental evolution in reproductive success, but the authors explained that the reaction in males to the treatment might be masked by the experimental procedure. For our experiment, we cannot find such an explanation.

From a female's point of view, successful reproduction in populations with a manipulated sex ratio can be seen as adaptation to a new environment. Those females that were able to adapt faster to the new conditions had a higher reproductive success and populations were able to evolve through time. PA females generally had a higher reproductive success than PG females in the final lifetime reproductive success assays irrespective of the male treatment they were mated to. This effect can potentially be due to enhanced adaptation speed of PA females. Allowing for sperm competition, and cryptic and non-cryptic female choice, a polyandrous sex ratio can lead to increased variation in male reproductive success, and therefore to a more rapid adaptation [37,38]. The question remains whether we strictly tested sexual selection with our experimental design or, by allowing the latter explanation, also included natural selection. Nevertheless, all traits we tested were fitness measurements and/or are known to be related to fitness.

One of these fitness-related traits is the size of sperm. In *C. elegans*, higher risk of sperm competition selected for an increase in sperm size [31,39]. For its relative, we expected, but did not find, that males under PA evolved larger sperm. One explanation could be that *C. remanei* already reached their maximum sperm size and an increase would result in less competitive or even non-functional sperm cells. Otherwise, selection requests genetic diversity to work and sperm size could be fixed in *C. remanei* due to strong competition not allowing for any genetic variation.

Copulatory plugs can be produced by the male, and are mostly thought to prevent females from remating [40,41], but can also be produced by females themselves to reduce the number of matings [42]. In *C. remanei*, the function of the copulatory plug is not clear. While Barker [43] found an effect in *C. elegans* and Palopoli *et al.* [44] for *C. remanei*, Timmermeyer *et al.* [34] did not find a remating delay or a reduction in female mating rate but a reduction in female fitness in unplugged females. Irrespectively, if the effect of plugging is positive or negative for males or females, in this study, plug size did not evolve in *C. remanei* males under PA or PG conditions. Instead, we found a high variability in plug size, which contraindicates a strong selection for plug size. Nevertheless, we did not test plug composition or other attributes of this secretion, which could have changed during experimental evolution.

Still, it is theoretically possible that males evolved a trait that is not detectable in our experiment. We chose the most important traits that have shown to be relevant in previous experiments. By measuring lifetime reproductive success, we tried to include traits that are invisible to us but could harm the female and would therefore be important in coevolution. But as we only detected changes in female lifetime reproductive success, it is unlikely that such traits evolved and made a difference for male–male competition or female–male interactions.

As our populations were rather small, one might suggest that drift could have affected the results. We agree that there is a risk due to small population size, but still drift should have affected both males and females, and is rather negligible as the population size was the same for both the PA and the PG treatments. Additionally, all replicates of the treatments showed the effect in the same direction (the soporific effect assay, for example, which is unlikely to be explained by drift in general) [19].

However, our experimental design does not correct for the number of X chromosomes present in both treatments. Female *C. remanei* have two X chromosomes, males have one. Therefore, evolutionary processes that include selection on this sex chromosome can act differently in the two sexes [45], known as the faster-X effect [46]. This imbalance can explain our results, as females could potentially evolve twice as fast as males. If this effect has such a high potential, what effect might it have in natural populations with balanced sex ratios? *Caenorhabditis remanei* populations consist of males and females in the same numbers, which also means the X chromosome/autosome ratio is always in favour of females, giving them the potential to always be faster than males. Further investigation is necessary to assess the consequences for *C. remanei*.

We conclude that males were less sensitive to sexual conflict during 20 generations of experimental evolution, irrespective of the underlying mechanisms. Reasons for that might be low genetic variation and/or that male trait evolution is constrained because traits may be more costly to males, as well as the effects the X chromosomes might have. Thus, females might respond quicker to changes in selection regimes in our gonochoristic model system, which might differ from observations made in other systems. However, the process of male–female coevolution might not be as straightforward and predictable as expected by theory.

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