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Author for correspondence:
Steven A. Ramm
e-mail: steven.ramm@uni-bielefeld.de

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Hypodermic self-insemination as a reproductive assurance strategy

Steven A. Ramm[1,2], Aline Schlatter[1], Maude Poirier[1] and Lukas Schärer[1]

[1]Evolutionary Biology, Zoological Institute, University of Basel, Basel 4051, Switzerland
[2]Evolutionary Biology, Bielefeld University, Bielefeld 33615, Germany

SAR, 0000-0001-7786-7364

Self-fertilization occurs in a broad range of hermaphroditic plants and animals, and is often thought to evolve as a reproductive assurance strategy under ecological conditions that disfavour or prevent outcrossing. Nevertheless, selfing ability is far from ubiquitous among hermaphrodites, and may be constrained in taxa where the male and female gametes of the same individual cannot easily meet. Here, we report an extraordinary selfing mechanism in one such species, the free-living flatworm *Macrostomum hystrix*. To test the hypothesis that adaptations to hypodermic insemination of the mating partner under outcrossing also facilitate selfing, we experimentally manipulated the social environment of these transparent flatworms and then observed the spatial distribution of received sperm *in vivo*. We find that this distribution differs radically between conditions allowing or preventing outcrossing, implying that isolated individuals use their needle-like stylet (male copulatory organ) to inject own sperm into their anterior body region, including into their own head, from where they then apparently migrate to the site of (self-)fertilization. Conferring the ability to self could thus be an additional consequence of hypodermic insemination, a widespread fertilization mode that is especially prevalent among simultaneously hermaphroditic animals and probably evolves due to sexual conflict over the transfer and subsequent fate of sperm.

1. Introduction

Hypodermic insemination occurs when, rather than being directly transferred to the female reproductive tract during mating, sperm are injected following traumatic wounding of the mating partner, usually through the body wall into some extra-genital location [1,2]. Such an insemination route has been described in several separate-sexed animals (e.g. [3–8]), and seems especially prevalent in simultaneous hermaphrodites (e.g. [9–12]; reviews in [1,13]). It is usually thought to evolve as a response to sexual conflict, as a means through which the sperm-donating individual may be able to enforce mating, and thus (unilateral) sperm donation, and/or regain some control over the fate of its donated sperm (i.e. by by-passing adaptations in the recipient's reproductive tract that would otherwise process that sperm in a manner that could be sub-optimal for the sperm-donating individual) [5,9,14]. Here, we establish that an additional feature of hypodermic insemination in one simultaneously hermaphroditic animal is to enable self-insemination. This in turn enables facultative self-fertilization under conditions of low mate availability, a common feature of many simultaneously hermaphroditic plants and animals [15–17] that is often thought to evolve as a reproductive assurance strategy under ecological conditions that disfavour or prevent outcrossing [18–23].

The simultaneously hermaphroditic flatworm genus *Macrostomum* includes both species that reproduce by reciprocal copulation, in which each mating worm transfers sperm into the female antrum of its mating partner (e.g. *Macrostomum lignano* [24,25]), and species that reproduce by hypodermic insemination, in which mating can be unilateral and sperm are instead injected through the body wall into the parenchyma of the mating partner [26,27]. *Macrostomum hystrix*

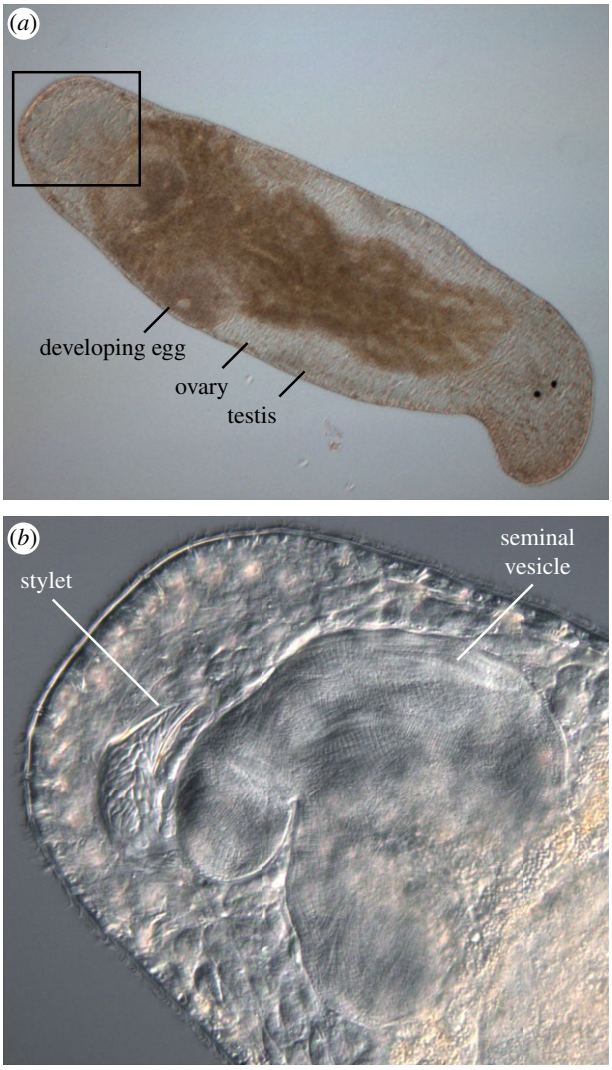

Figure 1. The male copulatory apparatus in *M. hystrix* includes a needle-like stylet for sperm injection. (*a*) An adult *M. hystrix* flatworm viewed under light microscopy, with both male (testis) and female (ovary) reproductive organs clearly visible through the transparent body wall. Approximate body length 1 mm. (*b*) Close-up of the tail region of the same worm (region highlighted in (*a*)) under differential interference contrast light microscopy, to illustrate the needle-like male copulatory stylet, and the seminal vesicle (the male sperm storage organ, here densely packed with sperm). (Online version in colour.)

(figure 1) falls into the second category, possessing a copulatory stylet (male copulatory organ) with a needle-like ending and sub-terminal opening, together with a simple sperm morphology that presumably facilitates their movement through the parenchyma to the site of fertilization following insemination [26]. Isolated *M. hystrix* individuals have recently been shown to be capable of self-fertilization [28], but only commence reproduction when induced to self in the extended absence of outcrossing opportunities after a substantial delay and at a significant cost (relative to outcrossing) [28], as expected from theory [20].

Owing to their transparency, received sperm can be readily observed in living worms with light microscopy, and both field-caught and mass-cultured worms often contain large numbers of received sperm in different body regions (electronic supplementary material, movie S1; S.A.R. & L.S. 2002–2015, personal observation). To test the hypothesis that adaptations to hypodermic insemination of the mating partner expressed under outcrossing [26] could also facilitate selfing via hypodermic self-insemination [28], we quantified the number and distribution of received sperm in worms raised under different social conditions designed to manipulate the likelihood of outcrossing versus selfing, first by keeping worms in stable social groups that either allowed or prevented outcrossing for *roughly* four weeks, and then by testing whether sperm distributions can change over shorter time scales by manipulating the social conditions of a subset of these worms for a further six days. Collectively, our results imply that isolated worms switching to self-fertilization do so by self-injecting sperm into more anterior regions of their own body, including or even predominantly into their own head.

2. Material and methods

(a) Study organism

Experimental subjects came from a mass culture of *M. hystrix* Ørsted 1843 *sensu* Luther 1905 (see electronic supplementary material of [26] for further taxonomic details), maintained at the Zoological Institute, University of Basel, from samples originally collected from the San Rossore Regional Park (43.684° N, 10.283° E) near Pisa, Italy, in May 2010. Worms are maintained at 20°C in glass Petri dishes containing 6‰ artificial seawater (ASW) and fed with the diatom *Nitzschia curvilineata*, under conditions that are similar to those used to maintain its congener *M. lignano* [29].

(b) Experimental design

Approximately 1000 adult worms from mass culture were distributed to 10 Petri dishes (approx. 100 worms per dish) containing algae and allowed to lay eggs. Hatchlings were collected in two batches, 10 and 14 days later, and randomly allocated to two treatment groups: an *isolated* treatment in which worms were kept alone in individual wells of a 24-well plate in ASW with ad libitum food (diatoms), and a *triplet* treatment in which worms were kept under identical conditions, but in groups of three worms per well. These treatments were designed to manipulate the breeding system, by preventing or enabling outcrossing, respectively. Worms were transferred to fresh wells prepared in the same way approximately two weeks later, and received sperm were scored (see §2c) for the first time 11–13 days later, meaning that at the time of the first scoring (designated 'scoring day 1'), the worms had been in their respective groups for 28–32 days. This time point was chosen because all worms were expected to be sexually mature (cf. [28]) and would therefore have had an opportunity to receive sperm. We began the scoring phase with $n = 31$ isolated and $n = 18$ triplet wells. However, because we observed that in fact some wells contained one or more apparently immature individuals, these wells were excluded immediately prior to or during scoring to avoid potential biases. In addition, some worm replicates were excluded due to worm mortality prior to or during scoring. Thus, realized sample sizes for the sperm scores on scoring day 1 were $n = 28$ isolated worms and $n = 12$ triplets (comprising 36 scored worms). Comparing these two treatment groups allows us to test the hypothesis that outcrossing and selfing worms differ in their received sperm distributions. In addition, for the isolated treatment, we counted the number of (selfed) hatchlings produced in all wells up to the end of the first scoring phase, to assess which of the isolated worms had already begun to self at the time when received sperm were scored.

In order to further test how rapidly sperm distributions might change in worms with or without outcrossing opportunities, we extended the experiment for a further 6 days. After measuring on scoring day 1, each replicate in the triplet treatment was subdivided into two new treatment groups: an isolated worm (designated *triplet-to-isolated*) now kept alone in a fresh well, and the

remaining pair of worms (designated *triplet-to-paired*) now kept together in a separate, fresh well. After a further 6 days (designated 'scoring day 2'), we then again scored the received sperm for triplet-to-isolated and triplet-to-paired worms. Final sample sizes on scoring day 2 were $n = 12$ matched pairs of observations comparing each of 12 worms in the triplet-to-isolated treatment to the corresponding two worms in each triplet-to-paired treatment. Given our finding that sperm distributions differ between outcrossing and selfing worms (see §3), this additional comparison further enables us to test whether a 6-day window without outcrossing opportunities is sufficient to alter the observed sperm distributions, as would be expected if received sperm are processed rapidly within the parenchyma of the worms.

(c) Received sperm scores

To obtain estimates of the amount of received sperm on both scoring day 1 and scoring day 2, worms were first anaesthetized in an approximately 1 : 4 $MgCl_2$: ASW solution (7.14% $MgCl_2$) for approximately 8 min [29,30]. Worms were then placed individually on a microscope slide in approximately 40 µl of liquid, covered using a 26 × 22 mm coverslip held in place by four small pieces of modelling clay at each corner, and the preparation pressed so as to slightly squeeze the worm. We then scored the number of sperm in three easily definable body regions: the head region (anterior to the testis), the gonad region (between the anterior tip of the testis and the posterior tip of the ovary) and the tail region (posterior to the ovary; figure 2*a*). Sperm scores were estimated using a five-point scale ranging from no sperm visible (0), to a few sperm present (1), to several sperm present in multiple places within the region (2), to over 50% of the region being full of sperm (3), to large numbers of sperm visible throughout the region (4). All scoring was performed by a single researcher, using the same microscope set-up (differential interference contrast observation using a Leica DM 2500 light microscope at 1000× magnification under oil immersion), and the scorer was blind to the treatment group of the worm being scored (i.e. one researcher prepared each slide knowing the origin of the worm, and a second researcher then independently scored the received sperm without that knowledge, effectively avoiding observer bias).

(d) Statistical analyses

To test for differences in the received sperm scores between the treatment groups, the statistical analysis needed to take into account that the three sperm scores per worm and the three worms per triplet are statistically dependent, and that the sperm scores we obtained have awkward distributional properties across the treatment groups, making parametric analyses problematic. We therefore devised a simple non-parametric test to address this and used a pilot experiment as a reference point to compare the observed sperm scores. Specifically, pilot data from a very similar experiment (see legend to electronic supplementary material, figure S1) had suggested that grouped worms usually exhibit a very clear sperm distribution, namely with received sperm scores being lowest in the head region (rank 3), intermediate in the gonad region (rank 2) and highest in the tail region (rank 1; electronic supplementary material, figure S1). We therefore used this expectation to derive a test statistic by comparing the observed distribution of sperm scores over the three regions in our experimental subjects to the expected distribution of sperm scores previously observed under outcrossing. To do so, we calculated a Spearman's rank correlation coefficient (r_s) between each independent replicate in our experiment to the expected rank distribution under outcrossing derived from the pilot data (i.e. values of r_s close to +1 suggest a distribution that is similar to the one observed among outcrossing worms in the pilot). Specifically, the match between the observed and expected distribution was measured as a single r_s-value for each isolated

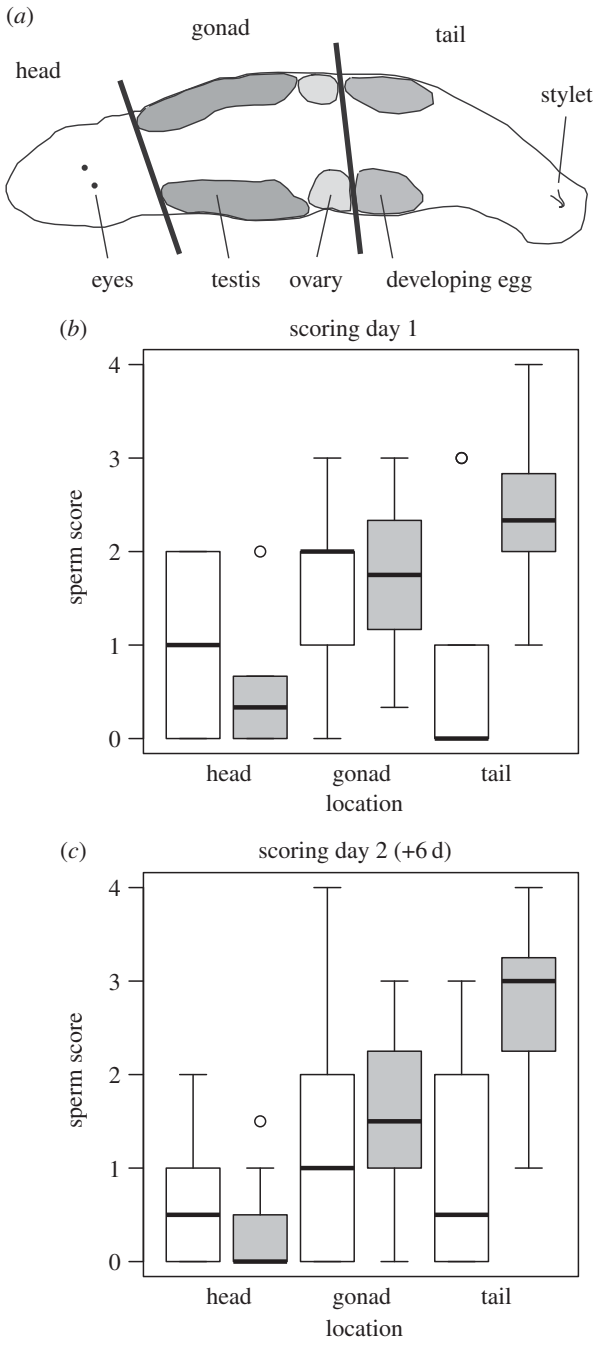

Figure 2. Experimental evidence for hypodermic self-insemination as the mechanism of selfing in *M. hystrix*. (*a*) We estimated sperm distributions in worms maintained under different social conditions designed to prevent or allow outcrossing, scoring numbers of received sperm in three body regions (head, gonad and tail) according to morphological landmarks, as shown in this schematic drawing of an adult worm. (*b*) Boxplots of received sperm scores in worms maintained in the isolated treatment group (open bars) compared with average received sperm scores from groups of worms maintained in triplets (filled bars). Note the radically differing sperm distributions under these two experimental conditions (see main text for statistical analysis of this difference). (*c*) Received sperm scores in worms that had previously been kept in the triplet treatment until scoring day 1, and that were then randomly split into an isolated (open bars) and paired (filled bars) treatment until scoring day 2 (6 days later; again, see main text for statistical analysis of the differing sperm distributions). In (*b*,*c*), each treatment–region combination is plotted as the median sperm score (bold horizontal line), the interquartile range (box), and the minimum and maximum values (whiskers); open circles represent outliers (more than 1.5× the interquartile range above the third quartile or below the first quartile).

worm and as the averages of the r_s-values of the individual worms within each triplet and paired replicate (to avoid pseudoreplication). These r_s-values were then compared across treatment groups. On scoring day 1, we used a Wilcoxon rank-sum test to compare the independent isolated and triplet replicates, and on scoring day 2 (6 days later) we used a Wilcoxon signed-rank test to additionally take into account the paired nature of these data (i.e. each set of triplet-to-isolated and triplet-to-paired replicates had been kept together until scoring day 1) and test the prediction that the worms in the triplet-to-paired treatment would correlate more strongly with the outcrossing expectation than triplet-to-isolated worms. Note that on scoring day 1, one replicate r_s could not be directly calculated due to no variation in sperm scores between body regions, and the same was true for six replicates on scoring day 2. In these cases, we assumed that $r_s = 0$, meaning the observed distribution did not correspond to the expected distribution under outcrossing ($r_s = 1$), but nor was the expected distribution reversed ($r_s = -1$).

3. Results

When assessed on the first scoring day after being kept in stable social groups for roughly four weeks, we found strikingly different distributions of sperm in isolated compared with triplet worms (Wilcoxon rank-sum test comparing r_s in isolated versus triplets: $n = 40$, $Z = 3.98$, $p < 0.0001$; figure 2*b*). Triplet worms contained large numbers of sperm, predominantly in the tail region, with a very high correspondence between the observed rank of sperm scores across the three body regions and that expected based on previous evidence from outcrossing worms (i.e. head < gonad < tail; median $r_{s\ triplets} = 0.89$, range 0.07 to 1). In stark contrast, isolated worms contained very few sperm in the tail but more sperm than triplets in the head region, resulting in a reversal in the correlation between the observed and expected distributions (median $r_{s\ isolated} = -0.5$, range -1 to 1). Such a pattern strongly suggests that outcrossing in triplet worms normally occurs through hypodermic insemination into the tail region of the worm, whereas self-fertilization in isolated worms is likely to be achieved through the extraordinary mechanism of hypodermic self-insemination, including or even exclusively into their own head region, from where the relatively simple and highly motile sperm [26] presumably then migrate to the site of fertilization. Indeed, within the isolated treatment group, there was a positive correlation between the received sperm score in the head and the number of selfed hatchlings each worm had produced prior to scoring day 1 ($r = 0.43$, $n = 28$, $p = 0.024$; figure 3), corroborating the link between the presence of received sperm outside the tail region and the onset of selfing.

To test whether individuals that had previously been able to outcross are induced to commence self-insemination when isolated, we experimentally manipulated the triplet worms for a further 6 days (see §2). Again, consistent with hypodermic self-insemination coinciding with the onset of selfing, newly isolated worms assessed on scoring day 2 now had more sperm in the head and fewer in the tail compared with paired worms with which they had previously been raised as triplets, resulting in significantly different sperm distributions (median $r_{s\ triplet\text{-}to\text{-}isolated} = 0$, range -1–1; median $r_{s\ triplet\text{-}to\text{-}paired} = 0.93$, range -0.25–1; Wilcoxon signed-rank test comparing r_s in triplet-to-isolated versus triplet-to-paired: $n = 12$ matched pairs, $S = -26$, $p = 0.04$; figure 2*c*). A slight difference with scoring day 1 was that isolated worms on day 6 appeared to have somewhat fewer sperm in the gonad region, perhaps indicating that 6 days of isolation is sufficient for allosperm to be removed from this region but not yet for these to be fully replaced by self-inseminated autosperm. Taken together with the results from scoring day 1, these data strongly imply that selfing in *M. hystrix* is achieved by hypodermic self-insemination into more anterior parts of the body than normally occurs under outcrossing, presumably due to anatomical constraints that limit the possible sites of autosperm injection to this region.

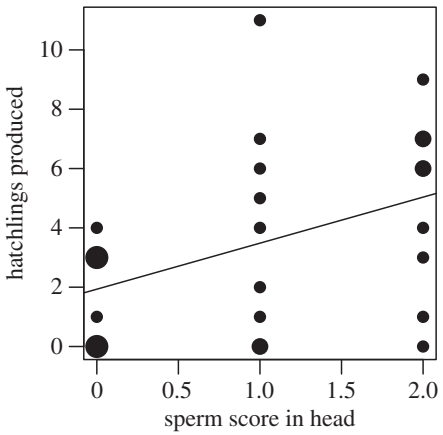

Figure 3. Self-insemination and the onset of self-fertilization. The plot shows the relationship between the received sperm score in the head and the number of hatchlings each isolated worm had produced by selfing. Sperm scores and number of hatchlings were recorded on scoring day 1 of the experiment, and there was a significant, positive correlation between the two traits (see main text for statistics).

4. Discussion

Several simultaneously hermaphroditic animals and plants have specific adaptations to avoid automatically selfing under conditions where outcrossing is favoured. For example, the close proximity of the anthers and pistils in many flowering plants and the joint male–female organization of the reproductive ducts in gastropods have necessitated the evolution of mechanisms that prevent selfing and promote cross-fertilization. These include self-incompatibility in plants [15] and some animal groups [30], together with structures for the prolonged storage of received allosperm [31] or its preferential utilization [30]. Here, we have discovered a mechanism of selfing in a taxon where it had previously been argued that the strict separation of male and female reproductive structures would render selfing impossible [32] (but see also [28]). This may indeed be the case for the congeneric species *M. lignano*, which is instead adapted to a mating behaviour involving 'reciprocal copulation' [24,26,33]. Nevertheless, the apparent costs of self-fertilization relative to outcrossing (as seen by reduced hatchling production and offspring survival) and substantial delay to commence reproduction exhibited by isolated *M. hystrix* individuals [28] suggest that outcrossing is the preferred mating system also in this species. If so, this means that the ability to facultatively self may have evolved as a secondary by-product of adaptations to hypodermic insemination (e.g. a needle-like copulatory stylet, and relatively simple sperm morphology) that are normally expressed in outcrossing worms, and probably arise due to sexual conflict over

sperm transfer and utilization [14,26]. It may also be relevant here that, compared with other species in the genus, *M. hystrix* appears to have very large testes and so presumably a high rate of sperm production (L.S. 2002–2015, personal observation), which would normally be adaptive under outcrossing due to numerical sperm competition over fertilization [34], but is not expected under high levels of selfing [35–38].

A further striking aspect of our study is the apparent location of self-insemination. While cephalo-traumatic secretion transfer via a hypodermic penile appendage was recently described in a sea slug [39], this is to our knowledge the first described case of individuals apparently injecting sperm into their own head or upper body region. While such a self-imposed trauma may not seem like the most obvious route to achieve self-fertilization, in reality the options in *M. hystrix* may be constrained by the fact that it is likely to be these regions that are most easily reached by the posteriorly located copulatory stylet. The sort of shortcut to self-fertilization seen in gastropods—with their shared male and female internal genitalia [30]—is precluded in this taxon by the strict separation of the male and female sex functions.

The fact that the sperm distributions in previously outcrossing worms that were isolated for 6 days quickly began to resemble those of isolated worms suggests that the dynamics of sperm utilization in *M. hystrix* may be quite rapid. Moreover, the fact that triplet worms contained very few sperm in the head implies that self-insemination in this region is a conditional response to isolation, and is usually avoided under conditions that enable outcrossing. The costs of sperm receipt under outcrossing and selfing have not been quantified, but it seems likely that, whether involving allo- or autosperm, the combined costs of puncture wounds and of receiving and processing large amounts of (normally) foreign material in the parenchyma could be substantial [3,40,41] (but see [42]), perhaps especially so under selfing because of the atypical site of insemination (cf. [43,44]). However, throughout this account, we employ the term 'hypodermic insemination' rather than 'traumatic insemination' because at this stage we do not know whether the wounding involved is costly, but note that these terms are used interchangeably in the recent literature [1]. Irrespective of the exact costs and benefits of such an insemination route under outcrossing, we can now identify an additional feature of hypodermic insemination in hermaphroditic animals, namely that it can facilitate self-fertilization under conditions predictive of low mate availability. Other simultaneous hermaphroditic animals possess adaptations to hypodermic insemination (e.g. [5,10,12,45]), and some of these are known to self (e.g. [40]). However, selfing can also be achieved by other means, even among free-living flatworms (e.g. [46]), and so it would now be very interesting to assess the extent to which hypodermic insemination and selfing ability co-evolve across a wider taxonomic range.

In summary, we have shown that hypodermic insemination in *M. hystrix* may also facilitate selfing. In the extended absence of outcrossing opportunities, this apparently occurs via the mechanism of hypodermic self-insemination of autosperm, including into the individual's own head region. We thereby establish a novel evolutionary link between sexual conflict and the evolution of animal breeding systems.

Data accessibility. The datasets supporting this article have been uploaded to Dryad (http://dx.doi.org/10.5061/dryad.dd34q).

Authors' contributions. S.A.R. and L.S. conceived the study, designed the experiments, carried out the statistical analyses and drafted the manuscript; A.S. and M.P. participated in experimental design, acquired the data and helped to draft the manuscript. All authors gave final approval for publication.

Competing interests. We declare we have no competing interests.

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