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RESEARCH ARTICLE

# Self-fertilization, sex allocation and spermatogenesis kinetics in the hypodermically inseminating flatworm *Macrostomum pusillum*

**Athina Giannakara and Steven A. Ramm***

## ABSTRACT

The free-living flatworm genus *Macrostomum* is an emerging model system for studying the links between sex allocation, sexual selection and mating system evolution, as well as the underlying developmental and physiological mechanisms responsible for wide intra- and inter-specific variability in reproductive phenotypes. Despite compelling comparative morphological evidence of sexual diversity, detailed experimental work on reproductive behaviour and physiology in *Macrostomum* has so far been largely limited to just two species, *M. lignano* and *M. hystrix*, an obligate and a preferential outcrosser, respectively. In this study, we establish that a third species, *M. pusillum*, exhibits a combination of reproductive traits strikingly different from both of its congeners. Unlike *M. lignano*, we demonstrate that *M. pusillum* does not adjust sex allocation or the speed of spermatogenesis to the prevailing social group size. *Macrostomum pusillum*'s relatively simple sperm morphology likely explains the short spermatogenesis duration we report, and is linked to a hypodermically inseminating mode of fertilization, which we show also means that these worms are capable of self-fertilization. Surprisingly, and unlike *M. hystrix*, selfing in isolated worms commences after only a short (if any) delay compared with the onset of reproduction in grouped individuals, with little evidence of differential inbreeding depression in 'isolated' progeny. These combined results suggest that, in nature, *M. pusillum* may be regularly selfing, in contrast to the congeners studied to date. Our findings highlight the rapid and correlated evolution of reproductive traits, and reinforce the utility of the genus *Macrostomum* for understanding the evolutionary and developmental mechanisms responsible for this diversity.

**KEY WORDS: Hermaphroditism, Hypodermic insemination, Self-fertilization, Sex allocation, Spermatogenesis, Sperm competition**

## INTRODUCTION

Self-fertilization (selfing) occurs as a form of reproduction in a variety of hermaphroditic organisms and has evolved independently multiple times in animals and plants (Jarne and Charlesworth, 1993; Goodwillie et al., 2005; Jarne and Auld, 2006). Various factors have been suggested to favour the evolution of selfing (Goodwillie et al., 2005), but most research to date has focused on two main aspects (Busch and Delph, 2012). First, as initially proposed by Fisher (1941) and recently confirmed experimentally in a plant species (Stone et al., 2014), a gene responsible for selfing should automatically be selected and thus increase in frequency when emerging in a primarily outcrossing population because of its higher transmission rate (transmission advantage hypothesis). Second, from an ecological perspective, selfing can act as a reproductive assurance mechanism when mate availability is low (Darwin, 1876; Jain, 1976), as is the case for many organisms (e.g. Jarne et al., 1991; Kalisz et al., 1999; Tsitrone et al., 2003b; Schjørring, 2004; Noel et al., 2016), including the hermaphroditic flatworm *Macrostomum hystrix* (Ramm et al., 2012).

Contrasting these transmission and reproductive assurance advantages, the major downside of selfing is that it can result in the production of offspring with lower fitness than the parental generation, i.e. inbreeding depression (Charlesworth and Charlesworth, 1987). Two main genetic mechanisms have been proposed to explain the occurrence of inbreeding depression: the partial dominance and the overdominance hypotheses (Roff, 2002; Charlesworth and Willis, 2009; but see Li et al., 2008), which explain the reduced fitness of inbred individuals either through increased homozygosity of deleterious recessive alleles or a loss of (beneficial) heterozygosity, respectively.

Inbreeding depression in the progeny, then, appears to be the major selective force acting against the evolution of self-fertilization (Charlesworth and Charlesworth, 1979). Nevertheless, under regular selfing, harmful alleles can be gradually purged from the population over successive generations (Porcher and Lande, 2016), as indeed has recently been demonstrated in the simultaneously hermaphroditic snail *Physa acuta* (Noel et al., 2016). Theoretical models for the evolution of self-fertilization (Fisher, 1941; Lande and Schemske, 1985) therefore predict two evolutionarily stable mating system strategies for hermaphroditic organisms: (1) 'preferential selfing', i.e. predominant selfing with low inbreeding depression, a strategy which becomes adaptive once selfing has purged deleterious recessive alleles from the population (Gutiérrez et al., 2001; Meunier et al., 2004; Caplins and Turbeville, 2015); and (2) 'preferential outcrossing', i.e. predominantly outcrossing with high inbreeding depression (e.g. Tsitrone et al., 2003b; Ramm et al., 2012), a strategy that is adaptive because it maintains population heterozygosity at high levels. However, mixed mating systems with intermediate selfing rates, although rare, occur as well; their existence has been argued to depend strongly on ecological circumstances (Goodwillie et al., 2005; Jarne and Auld, 2006).

One way in which organisms sometimes attempt to balance the costs and benefits of outcrossing versus selfing is through so-called delayed selfing, i.e. performing the latter only if the former proves difficult. Theory predicts an optimal waiting time before commencing self-fertilization, related to the intensity of inbreeding depression in the progeny, the ability to re-allocate reproductive resources to later fecundity and the probability of finding a partner versus dying as the individual ages (Tsitrone et al., 2003a). Empirical evidence for delayed selfing in line with these predictions comes from a variety of hermaphroditic organisms,

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including plants (Kalisz et al., 1999; Qu et al., 2007), freshwater snails (Tsitrone et al., 2003b; Auld & Henkel, 2014; but see Escobar et al., 2007), cestodes (Schjørring, 2004, but see Schärer & Wedekind, 1999) and the flatworm *M. hystrix*, which delays reproduction for up to 50% of the average age at first outcrossing, when forced to self (Ramm et al., 2012).

Apart from the decision between outcrossing and self-fertilization, another crucial aspect in a simultaneous hermaphrodite's life is its sex allocation, i.e. the allocation of reproductive resources into male and female reproduction (Ramm, 2017; Schärer and Ramm, 2016). Sex allocation theory investigates how an organism should partition its reproductive resources in order to gain maximal fitness in a given ecological context (Charnov, 1982). Specifically in simultaneous hermaphrodites, it predicts the optimal investment of reproductive resources into the two sex functions (usually measured empirically as the size of the gonads or other sex-specific traits), assuming a linear trade-off between male and female allocation and different fitness gain curves for each sex function (Schärer, 2009). The evolution of sex allocation in simultaneous hermaphrodites is considered to have been largely shaped by sperm competition (Charnov, 1979, 1982, 1996). As has been demonstrated in a variety of species, hermaphrodites respond to higher levels of sperm competition by shifting their reproductive investment more towards the male function in order to increase their chances in reproduction over their competitors (Schärer and Ladurner, 2003; Tan et al., 2004; Hart et al., 2011; but see Baeza, 2007).

Free-living flatworms of the genus *Macrostomum* make an excellent model system for studying the evolutionary biology of sex in hermaphrodites. The genus itself contains ca. 200 described species (see http://turbellaria.umaine.edu/) differing widely in various aspects of their reproduction, such as mating behaviour, sperm and genital morphology, and sex allocation (Schärer et al., 2011; Janssen et al., 2015; Ramm, 2017). Many of these traits exhibit evidence of rapid and correlated evolution, with one important distinction being the transition between those species that mate by reciprocal copulation versus those that mate by hypodermic insemination (Schärer et al., 2011), as we explain in more detail below. Apart from the wide inter-specific diversity within the genus, another highly advantageous aspect is that individual flatworms are transparent, meaning many traits relevant to reproduction, such as the size of the testes and ovaries, can be measured *in vivo* (Vizoso et al., 2010; Marie-Orleach et al., 2014, 2016). Although morphological diversity across the *Macrostomum* genus is by now quite well documented (Schärer et al., 2011; Janssen et al., 2015), detailed investigations of the biology of sex have to date been conducted primarily in just two highly contrasting species, as we now briefly summarise.

By far the best-studied *Macrostomum* species to date is *M. lignano* (Ladurner et al., 2005; Wasik et al., 2015), an emerging model organism in stem cell, ageing, regeneration and sexual selection research (Ladurner et al., 2008; Mouton et al., 2009; Vizoso et al., 2010; Wasik et al., 2015). *Macrostomum lignano* is an obligately outcrossing simultaneous hermaphrodite that mates by reciprocal copulation, meaning both mating partners donate and receive sperm during each mating interaction (Schärer et al., 2004). It has a complex sperm morphology (Vizoso et al., 2010; Schärer et al., 2011) and is highly responsive to social environmental conditions, plastically adjusting both its sex allocation (Schärer and Ladurner, 2003; Janicke et al., 2013) and various sperm production traits such as testicular proliferative activity (Schärer et al., 2004), sperm production rate (Schärer and Vizoso, 2007) and spermatogenesis speed (Giannakara et al., 2016), to match the prevailing social group size and thus sperm competition level.

By contrast, the related species *M. hystrix* mates by hypodermic insemination, injecting much simpler sperm through the body wall of the mating partner (Schärer et al., 2011). However, in the extended absence of mating opportunities, isolated *M. hystrix* individuals can switch to self-fertilization (Ramm et al., 2012) by hypodermic self-insemination (Ramm et al., 2015), despite significant inbreeding depression in the progeny (Ramm et al., 2012).

Our study species, *M. pusillum*, is an understudied, more distantly related congener which appears quite similar to *M. hystrix* in terms of sperm and stylet morphology and hypodermic insemination mating mode, although these traits have apparently evolved independently in the two species (Schärer et al., 2011). We therefore initially hypothesized that *M. pusillum* is more likely to be capable of self-fertilization like *M. hystrix* than an obligate outcrosser such as *M. lignano*. If so, a further question concerns the frequency of selfing compared with outcrossing. We therefore first tested whether *M. pusillum* can indeed self-fertilize, and then performed a series of experiments to shed light on its plastic response to different social group sizes, the predictions for which differ according to the frequency of selfing.

If *M. pusillum* prefers to outcross, as appears to be the case for *M. hystrix*, enforced selfing is expected to negatively affect offspring fitness because of inbreeding depression (offspring produced by isolated parents are inbred but those produced by grouped parents are most likely outbred); to prevent this, isolated individuals should delay reproduction in the expectation of outcrossing matings (Tsitrone et al., 2003a). Additionally, social group size is likely to represent mating group size, and thus worms in larger groups should exhibit higher mating rates owing to more mating encounters. In turn, mating group size is expected to represent sperm competition level, as is the case for other *Macrostomum* species (Janicke and Schärer, 2009; Janicke et al., 2013). *Macrostomum pusillum* worms should, therefore, be able to plastically adjust both their sex allocation and sperm production speed accordingly to match the current sperm competition conditions. Alternatively, if *M. pusillum* is a preferential (or exclusive) selfer, this would mean that these worms might be routinely selfing, without obvious fitness costs, even in large social groups, thus rendering the three social group size treatments equivalent to each other in terms of optimal sex allocation. In that case, we can expect neither a sex allocation nor a spermatogenesis speed response to social group size, or a difference in mating frequency between isolated and grouped worms. Moreover, reproduction would be expected to commence on average at the same time regardless of social group size, and offspring produced in either social context should be equally fit.

Our experiments reveal that despite being quite similar to *M. hystrix* in terms of reproductive morphology, *M. pusillum* exhibits striking differences in its reproductive behaviour and plasticity compared with previously investigated *Macrostomum* species.

## MATERIALS AND METHODS

### Experimental design

We aimed at manipulating sperm competition level by keeping worms in different social group sizes: isolated, paired or in groups of eight. By monitoring the worms daily, we investigated their self-fertilization ability, estimated their age at first reproduction and tested whether enforced selfing induced a delay in reproductive

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onset. Additionally, we used morphological measurements of sex allocation traits and immunocytochemistry to track the course of spermatogenesis to test for an effect of sperm competition on those parameters. We furthermore assessed potential inbreeding depression in the progeny by recording the survival rates and productivity of the offspring of these worms. Finally, using the parental and offspring estimates for the age at first reproduction, we estimated reproductive onset heritability. A detailed description of the experimental procedures follows in the next sections.

### Study animal

*Macrostomum pusillum* (Ax, 1951) (Macrostomorpha, Platyhelminthes) is a hypodermically inseminating, free-living hermaphroditic flatworm distributed across a wide variety of habitats, including the North Sea, Mediterranean, Black Sea, Canada and Alaska (Ax and Armonies, 1990). Note, however, that recent molecular evidence for substantial divergence between Mediterranean and North Sea '*M. pusillum*' suggests that although individuals from these locations were morphologically indistinguishable, this species as currently constituted may actually contain multiple species (see Supporting Information to Schärer et al., 2011). For this study, we used worms initially collected from Lignano Sabbiadoro in Italy in 2006, maintained in the laboratory of L. Schärer (University of Basel, Switzerland) since that time, and cultured in our laboratory since 2014. Worms are kept in the laboratory under standard temperature and humidity conditions (~20°C and ~60%, respectively) on a 14 h:10 h light:dark cycle, in 32‰ artificial seawater (ASW) (Andersen et al., 2005) in Petri dishes and fed with the algae *Nitzschia curvilineata*, as per standard culturing techniques of the congener *M. lignano* (Schärer and Ladurner, 2003). The species has a short generation time, with adult worms being reproductively mature at 6–7 days old, typically laying two to three eggs per day which then hatch ca. 4 days later. Another major advantage of *M. pusillum* is its transparency, which allows the non-invasive observation and measurement of many internal structures *in vivo*, including those most relevant to reproduction, such as the paired testes and ovaries. At the same time, immunocytochemical protocols, such as BrdU labelling, routinely employed for its congener *M. lignano* (Schärer et al., 2007; Janicke and Schärer, 2009), can also be applied to *M. pusillum*, allowing tracking germ cell proliferation and differentiation and therefore the quantification of more dynamic reproductive traits, such as spermatogenesis kinetics.

### Treatment group formation

Approximately 300 adult worms from a mass *M. pusillum* culture were placed in a Petri dish containing 32‰ ASW and *ad libitum* algae to lay eggs, and were transferred to a new Petri dish under the same conditions every day. Three days after each transfer, each Petri dish was inspected for the presence of hatchlings for two consecutive days (by day 5 after egg laying, all hatchlings are expected to have hatched). Hatchlings were collected every day and randomly assigned to different social group sizes. Mating group size is a function of social group size in *M. lignano* (Janicke and Schärer, 2009; Janicke et al., 2013) and a typical way of inducing variation in mating group size and thus sperm competition level involves manipulating social group size. We followed the same practice for *M. pusillum*, reasoning that social group size could predict mating group size in this species as well, to form three treatment groups potentially differing in outcrossing opportunities and sperm competition level: 'isolated' (one worm – enforced selfing, no sperm competition), 'paired' (two worms – potential outcrossing, low potential sperm competition) and 'octet' (eight worms – potential outcrossing, high potential sperm competition) in individual wells of 24-well plates (TPP, Trasadingen, Switzerland), each containing ca. 800 µl 32‰ ASW. All worms within the same treatment group and plate were allocated on the same day and therefore were of the same age. Each plate contained eight isolated, eight paired and eight octet replicates in a balanced arrangement (six plates in total). Worms were fed *ad libitum* with *N. curvilineata* with octet wells receiving eight times more algae than isolated worms and four times more than paired worms. All replicates were transferred for the first time into new 24-well plates under the same conditions at 5 days of age (before they reach sexual maturity), and thereafter every 5 days for a total of three transfers before BrdU treatment (see Spermatogenesis assay, below). As usually occurs with similar experiments in *Macrostomum* flatworms, several worms were lost during transferring or died during treatment group formation, resulting in a reduction in group size. Thus, one pair replicate found with one missing worm and one octet replicate found with three missing worms on the third transfer had to be excluded from further processing. Seven octet replicates with only one missing worm, distributed evenly across the six plates, were processed as planned assuming that the absence of only one individual would have had no or only a minor effect on the perceived degree of sperm competition. By the third transfer, a total of $n_i$=48 worms remained assigned in 48 isolated replicates, $n_p$=94 worms in 47 pairs and $n_o$=369 worms in 47 octets, and these constitute the experimental worms for the spermatogenesis and sex allocation assays.

### Selfing ability and the onset of reproduction

In order to estimate reproductive onset in *M. pusillum*, all individual wells in each plate were inspected daily for the presence of hatchlings from day 5 after assignment until the experimental worms were treated with BrdU 10–13 days later (see Spermatogenesis assay, below); the first day a hatchling was observed in each individual well was recorded as the onset of reproduction for that particular replicate. Note that the onset of reproduction values were also available for the aforementioned excluded pair and octet replicates with missing worms because they started reproducing before the reduction in group size occurred, and also that three replicates (all from the isolated treatment) did not produce any hatchlings over the course of the experiment; thus the final reproductive onset estimates were calculated based on $n_i$=45, $n_p$=48 and $n_o$=48 replicates.

This assay additionally allowed us to investigate whether isolated worms are capable of self-fertilization and at what cost (see next section). Because the experimental worms were assigned into the isolated treatment directly on the day of birth and had no contact to another worm during the course of the experiment, any hatchlings found in these wells were inferred to be the product of self-fertilization.

### F1 survival and productivity

Assuming a higher probability of outcrossing in larger social groups (i.e. assuming the first scenario that *M. pusillum* is a preferential outcrosser), offspring produced by worms in octets and pairs can be expected to be more outbred than those produced by isolated worms. Thus assuming that selfing is costly for *M. pusillum*, offspring produced by isolated worms – and therefore the product of self-fertilization – are expected to suffer more intensely from inbreeding depression than outbred counterparts produced in the larger groups. To test for an effect of selfing on offspring quality, we measured two potential fitness indicators, namely, survival and

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early productivity, in the F1 generation. All experimental worm replicates in three out of six plates (for logistical reasons only the first three plates to have been formed were monitored) were observed daily for the presence of hatchlings until treated with BrdU (see Spermatogenesis assay, below). The first four hatchlings (F1 generation) observed in each individual well were immediately removed and isolated in separate wells (irrespective of the parental treatment) in 24-well plates containing 1 ml 32‰ ASW and 200 µl of a dense algae suspension. Three isolated experimental worm replicates failed to produce any hatchlings, thus reducing the final sample size for the survival assay to $N$=276 ($n_i$=84, $n_p$=96 and $n_o$=96 offspring). The sample size was slightly smaller for the productivity assay ($N$=269; $n_i$=81, $n_p$=94 and $n_o$=94 offspring) because seven offspring died during the experiment. To perform both assays, the isolated worms produced from isolated, paired or octet parents were monitored daily for 25 days; their survival status was assessed by daily inspection of the individual wells and their productivity by counting the number of hatchlings present (F2 generation) 5 days after the first hatchling was found in each particular well.

### Heritability of reproductive onset

To estimate the heritability of reproductive onset we monitored this parameter in the F0 parents and their F1 offspring but focused solely on the isolated treatment where we could be certain of the parental identity. As in the case of the experimental worms, the time point at which the first hatchling was observed in each F1 offspring well (F2 generation) was recorded as the reproductive onset for this individual. The heritability of reproductive onset was estimated by linear regression of the offspring reproductive onset (mean onset of up to four offspring per replicate, based on a total of 82 offspring from isolated parents) on the parental onset (based on 21 isolated experimental worm replicates, as three replicates did not produce any offspring).

### Spermatogenesis assay

An immunocytochemical assay based on BrdU (5-bromo-2′-deoxyuridine) labelling of germ cells in the testes and tracking of their differentiation status over time was employed to estimate the duration of spermatogenesis. By doing this for pairs and octets (for logistical reasons, only these two treatments were subjected to this assay), we could subsequently test for differences in the kinetics of spermatogenesis among those treatment groups possibly attributed to the potentially different sperm competition conditions in each group. BrdU is a synthetic nucleoside that incorporates in cells currently undergoing DNA replication, i.e. stem cells within the worms' bodies, including germ cells in the testes and ovaries, as is known for example in *M. lignano* (Ladurner et al., 2008). The BrdU labelling is a 'pulse-chase' experiment: 'pulse' refers to the initial application of the immunocytochemical agent and its incorporation into the dividing cells and 'chase' is the time window in which these cells continue to differentiate before their final staining and morphological evaluation. The appearance of labelled elongating spermatids in the worms' testes serves as a morphological marker of advanced spermatogenesis, while the presence of mature sperm in the seminal vesicle marks the process's end. In order to capture both stages, and based on preliminary data suggesting that in this species germ cells need ca. 3 days to reach the elongation stage and ca. 5 days to mature and move to the seminal vesicle where they are stored prior to mating (A.G. and S.A.R., unpublished observations), we chose a 'chase time' window of 2–5 days after BrdU treatment for the morphological evaluation.

#### BrdU pulse

At 15–18 days of age, all experimental worms were treated with BrdU in two batches, such that three consecutive plates were processed on the same day. All replicates were incubated in a 1:10 mixture of 50 mmol $l^{-1}$ BrdU (B5002, Sigma-Aldrich Biochemie, Hamburg, Germany) and 32‰ ASW (final concentration: 5 mmol $l^{-1}$) in separate wells of a 24-well plate for 30 min in the dark in their original group constitution. After three washing steps in 32‰ ASW, worms were returned to 24-well plates where they remained for 2, 3, 4 or 5 days depending on the randomly allocated 'chase time' to which they had been assigned. In this way, all eight group size×chase time combinations were represented by a maximum of two replicates per plate. On each 'chase day', two worms per replicate were randomly chosen for further processing: one was used for the sex allocation assay and the second for the fixation and visualization assay.

#### Fixation–visualization assay

For logistic reasons, the two worms originating from the two randomly selected independent replicates belonging to the same group size×chase time combination on each plate were processed together for the fixation–visualization step. The protocol was based on previous ones used for *M. lignano* (Schärer et al., 2007; Janicke and Schärer, 2009) with minor adjustments. First, worms were anaesthetized in a 1:1 mixture of $MgCl_2$ and 32‰ ASW for 15–20 min and then fixed for 60 min in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS). Afterwards, the fixed worms were washed three times in PBS-T (i.e. PBS plus 0.1% Triton X-100) for 15 min, and then transferred to PBS-T for a further 60 min. Next, they were permeated with 0.15 mg $ml^{-1}$ Protease XIV in PBS-T at room temperature with the activity of the protease being visually checked and stopped with cold 0.1 mol $l^{-1}$ HCl after approximately 45 min. The worms were then transferred in 2 mol $l^{-1}$ HCl for 60 min, washed with PBS-T, blocked for 30 min with BSA-T (i.e. PBS-T plus 1% bovine serum albumin) and incubated overnight in a 1:400 mixture of the primary rat anti-BrdU antibody (ab6326, Abcam, Cambridge, UK) and BSA-T at 4°C. On the following day, they were washed three times with PBS-T for 15 min and transferred into a 1:200 mixture of the FITC-conjugated secondary antibody [goat F(ab')2 anti-rat IgG, ab6115, Abcam] in BSA-T for 60 min in the dark. Worms were then washed three times in PBS-T for 15 min, briefly transferred to PBS for 1 min and then given a unique ID and mounted individually on a microscope slide in 22 µl of Vectashield Hardset (Vector Laboratories, Burlingame, CA, USA). In total, $N$=94 worms ($n_p$=47 and $n_o$=47) were successfully processed, comprising 11–12 worms per group size×chase time combination. Mounted worms were then observed under the microscope for the assessment of the elongation status of spermatids in the testes by the same researcher performing the fixation–visualization protocol, who was blind to the group size and/or chase time of each worm. The observations were done under epifluorescence at 400× magnification using a Nikon Ni-U microscope (Nikon, Düsseldorf, Germany). The elongation status of cells within the testis was scored as 0 when no BrdU-labelled elongated spermatids were observed, or 1 when BrdU-labelled elongated spermatids were observed in at least one testis. Some samples could not be scored, owing to poor staining quality, resulting in a final sample size of $N$=89 worms ($n_p$=44 and $n_o$=45), evenly distributed across the group size×chase time combinations.

### Sex allocation assay

To measure sex allocation in *M. pusillum*, we adapted standard techniques used for *M. lignano* (Schärer and Ladurner, 2003).

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Briefly, one randomly selected worm per replicate was placed on a microscope slide in a drop of 32‰ ASW and anesthetized by gradually adding 7.14% $MgCl_2$ to a final 1:1 concentration (total volume 40 µl). It was then squeezed dorsoventrally with a cover slip using two small plastic squares of standard thickness as spacers. Squeezed worms were observed under 100–400× magnification using a Nikon Ni-U microscope coupled to a ProgRes MFcool camera (Jenoptik, Jena, Germany) connected to a computer running ProgRes CapturePro software v2.7.6 (Jenoptik). Several digital photos of the body area, testes, ovaries and seminal vesicle area were captured and processed using ImageJ (http://imagej.nih.gov/ij/) in order to calculate the whole body area of each individual, the area of both testes and ovaries from which sex allocation (SA) was derived [SA=total testes area/(total testes area+total ovaries area)] as well as that of the seminal vesicle. Preliminary experiments confirmed that this results in repeatable estimates of the relevant parameters (based on a sample of 46 worms; intra-class correlation coefficient, $r_{bodysize}$=0.61, $r_{residualtestis}$=0.54, $r_{residualovary}$=0.68, $r_{SA}$=0.55, $r_{residualsv}$=0.63, all $P$<0.001). During both image acquisition and measuring, the observer was blind with respect to the worms' treatment group. The starting sample size was $N$=142 worms (47–48 worms per treatment group). However, several worms had to be excluded because they were crushed during processing, they appeared to be malformed or it was not possible to obtain a full set of testes and ovaries pictures for them. This resulted in a somewhat reduced final dataset for the sex allocation assay of $n_i$=31, $n_p$=32 and $n_o$=29 worms (a few more worms were excluded from the seminal vesicle measurements because of missing seminal vesicle pictures, leading to a final sample size of $n_i$=25, $n_p$=31 and $n_o$=27 for this parameter).

### Statistical analysis

The normality of the data was assessed by visual inspection of the residuals and linear models were employed for all analyses. For the onset of reproduction assay, age at first reproduction was treated as the continuous dependent variable, treatment group as the three-level predictor and plate as a random factor with six levels. The heritability of reproductive onset was estimated by linear regression of the mean offspring reproductive onset against parental onset focusing on the isolated treatment, where the parental identity was known. We followed a similar approach when testing for an effect of parental treatment group on offspring productivity, incorporating family as a random factor with 68 levels in the analysis. For the sex allocation assay, total testes area, total ovaries area, sex allocation and seminal vesicle area were separately treated as the dependent variables, treatment group as the predictor variable and plate as a random factor. Ovaries area varied significantly across plates, as did body area; the latter also correlated significantly with all measured parameters. This plate-related variation in ovary area was no longer significant when analysing the residuals from a regression on body area, while the outcome of the same approach for all other parameters did not differ from the initial results and is therefore not shown. Lastly, for the spermatogenesis kinetics analysis, we employed a binomial generalised linear model with a logit link function treating spermatid elongation status as the binomial response variable (1=elongation, 0=no elongation) and incorporating three explanatory factors: treatment group, chase day and their interaction. All analyses were performed in R (version 2.3.4) (R Foundation for Statistical Computing, Vienna, Austria), with the GLM implemented using the lme4 package (Bates et al., 2015). All raw data are provided in Table S1.

## RESULTS

### Self-fertilization but no selfing delay, and heritability of reproductive onset

*Macrostomum pusillum* worms are able to self-fertilize. Hatchlings were found in all but three of the 48 isolated replicate wells (ca. 94%) during the observation period. We employed an ANOVA to investigate whether isolated worms delay reproduction compared with those with outcrossing opportunities. As can be seen in Fig. 1A, there was significant variation in reproductive onset among treatment groups, with isolated worms starting to reproduce on average ca. 2 days later than octets and ca.1 day later than pairs (treatment group: $F_{2,138}$=22.75, $P$<0.001; plate: $F_{2,135}$=3.38, $P$<0.001). However, one must not neglect in this comparison an important bias in our experimental design, stemming from the constraint that because the worms were grouped, we always sampled the offspring of the fastest individual(s) to have reproduced in the

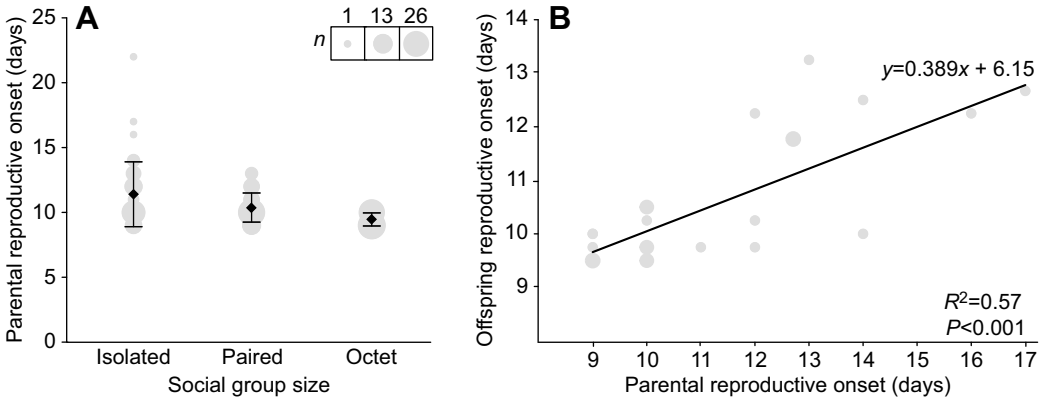


**Fig. 1. Delay in reproductive onset and heritability of reproductive onset under enforced selfing in *Macrostomum pusillum*.** (A) Worms forced to self-fertilize (isolated) exhibited a delay in the onset of reproduction compared with those given mating opportunities (paired and octet), with the difference between the two extreme groups at ca. 20%; but see also Results and Discussion ($n_i$=45, mean$_i$±s.d.=11.40±1.41, $n_p$=48, mean$_p$±s.d.=10.38±1.19, $n_o$=48, mean$_o$±s.d.=9.46±0.53; $F_{2,138}$=22.75, $P$<0.001). Data are means±s.d.; raw values are plotted in the background (grey circles) according to their frequency as specified in the legend showing the lowest, median and highest frequency observed. (B) A linear regression between parental ($N$=21 isolated parents) and offspring reproductive onset (mean value of max. four offspring per parent; $N$=82 offspring in total) estimated the heritability of the trait at ca. 0.57 ($F_{1,19}$=24.46, $P$<0.001). Note that overlapping data points (depicted as larger circles) occur in some cases because identical mean offspring onset values were shared by more than one parent with the same reproductive onset. Also note that the range of the parental values is larger in A compared with B, the reason being that the most extreme parental values were observed in the last three plates and these were only sampled for the parental onset of reproduction assay and not further.



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octets/pairs, thus ignoring any between-individual variation within each well and thus potentially inflating the differences between the treatment groups. To test whether this sampling bias alone could account for the differences we found between isolated and octet treatments, we therefore simulated 1000 octets by randomly sampling the onset values of eight worms from the isolated treatment 1000 times. We then kept the lowest onset value out of the eight randomly chosen in each of the 1000 iterations, from which we estimated the overall mean response of our simulated octet. The mean age at first reproduction in that case went down to 9.24 days, and to 10.17 days when following an equivalent procedure to simulate pairs, very close to the observed values of 9.46 days and 10.38 days actually observed for octets and pairs, respectively. This strongly suggests that differences between treatments in reproductive onset can be largely or even entirely attributed to the inherent observational bias in our experimental design, and thus that there is unlikely to be any true, substantial difference in reproductive onset depending on the availability of mating partners.

The heritability of reproductive onset was estimated using data from the isolated experimental worm replicates and their F1 offspring. A strong correlation was found between mean offspring and parental reproductive onset, with ca. 57% of the variation in offspring reproductive onset being explained by variation in parental onset ($F_{1,19}$=24.46, $P$<0.001; Fig. 1B).

### No evidence for differential inbreeding depression in the isolated progeny

We assessed offspring fitness in terms of both their survival and early productivity. Parental treatment group (and thus potentially inbreeding status of the offspring, under preferred outcrossing) had no effect on either parameter. For survival, mortality rates were very low overall, with just seven out of 276 F1 offspring (ca. 2.5%) dying during the experimental observation period (three produced by isolated, two by paired and two by octet worms). For productivity, we employed a general linear model; despite the fact that there was significant between-family heterogeneity in the number of offspring they produced within the same time window of 5 days ($F_{2,66}$=1.84, $P$<0.001), we found no significant systematic effect of parental treatment group on offspring productivity ($F_{2,273}$=1.34, $P$=0.26; Fig. 2).

### No between-treatment variation in sex allocation or seminal vesicle area

We found no impact of the treatment group, and hence the assumed level of sperm competition under preferred outcrossing, on either total testes area ($F_{2,89}$=0.98, $P$=0.38; Fig. 3A) or total ovaries area ($F_{2,89}$=0.65, $P$=0.52; Fig. 3B), nor therefore on sex allocation ($F_{2,89}$=0.21, $P$=0.81; Fig. 3C). Worms were overall highly female-biased (overall sex allocation, i.e. proportion male allocation: mean±s.e.=0.37±0.01). A significant plate effect was observed only in regard to ovaries area (total ovaries area: $F_{5,87}$=3.10, $P$=0.01; total testes area: $F_{5,87}$=1.38, $P$=0.24; sex allocation: $F_{5,87}$=1.10, $P$=0.37), which disappeared when analysing the residuals from a regression with body area, indicating that housing conditions might have differentially affected the worms' growth and, consequently, their female allocation. We measured seminal vesicle area as a potential indicator of mating rate (worms mating more often should tend to have fewer sperm reserves) in order to test for potential differences between treatment groups. A similar statistical approach found no variation among treatment groups in seminal vesicle size, which was highly variable (means±s.d., isolated=395.8±212 μm$^2$, paired=422.5±519.3 μm$^2$, octet=263.4±106.2 μm$^2$; treatment group: $F_{2,80}$=3.38, $P$=0.07; plate: $F_{5,77}$=0.90, $P$=0.48), and thus no difference in presumed mating rate.

### No plasticity in spermatogenesis kinetics among treatment groups

As expected, in both treatment groups, the probability of observing elongating spermatids in the worms' testes significantly increased with time (i.e. between chase days), confirming our initial time-window choice as appropriate for this assay ($\chi^2_{1,1}$=11.843, $P$<0.001). However, no difference among treatment groups

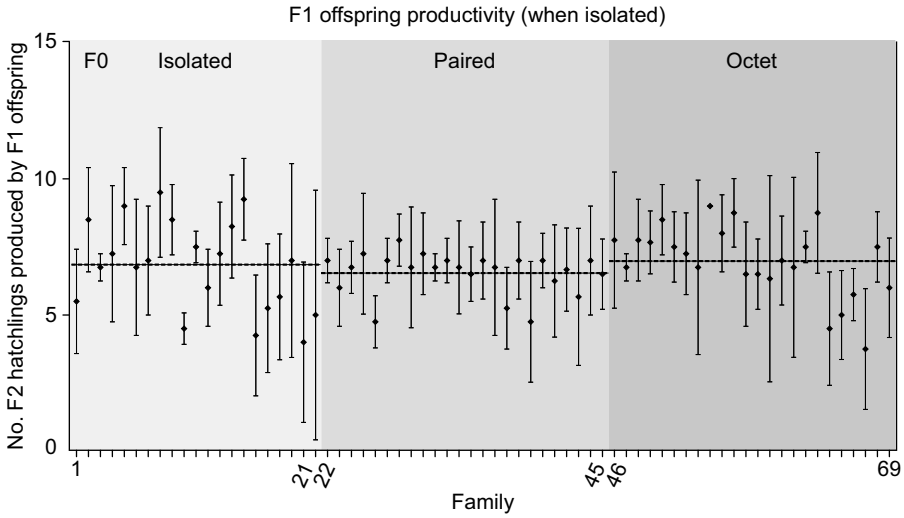


**Fig. 2. F1 offspring productivity according to parental social group size and individual family.** Up to four F1 offspring per family (i.e. individual experimental worm replicate, isolated, paired or octet) were removed at the day of birth, isolated in separate wells and monitored for up to 25 days. Individual productivity was estimated as the number of F2 hatchlings present in each F1 well 5 days after the first hatchling was found. Diamonds show the mean productivity of all four F1 offspring sampled per family and whiskers the standard deviation ($n_i$=81, mean$_i$±s.d.=6.85±2.5, $n_p$=94, mean$_p$±s.d.=6.54±1.56, $n_o$=94, mean$_o$±s.d.=6.98±2.08). Note that for family 54, s.d.=0, because all F1 offspring produced the same number of hatchlings. Families 1–21 were formed by isolated parental worms, 22–45 by paired and 46–69 by octet as indicated by the different background colour. Dashed lines within each coloured panel show the overall mean for each social group size. There was significant variation in productivity among F1 offspring originating from different families ($F_{1,66}$=1.84, $P$<0.001) but no difference depending on the parental social group size ($F_{2,273}$=1.34, $P$=0.26). Data are based on a total of 269 F1 offspring from 69 different families.

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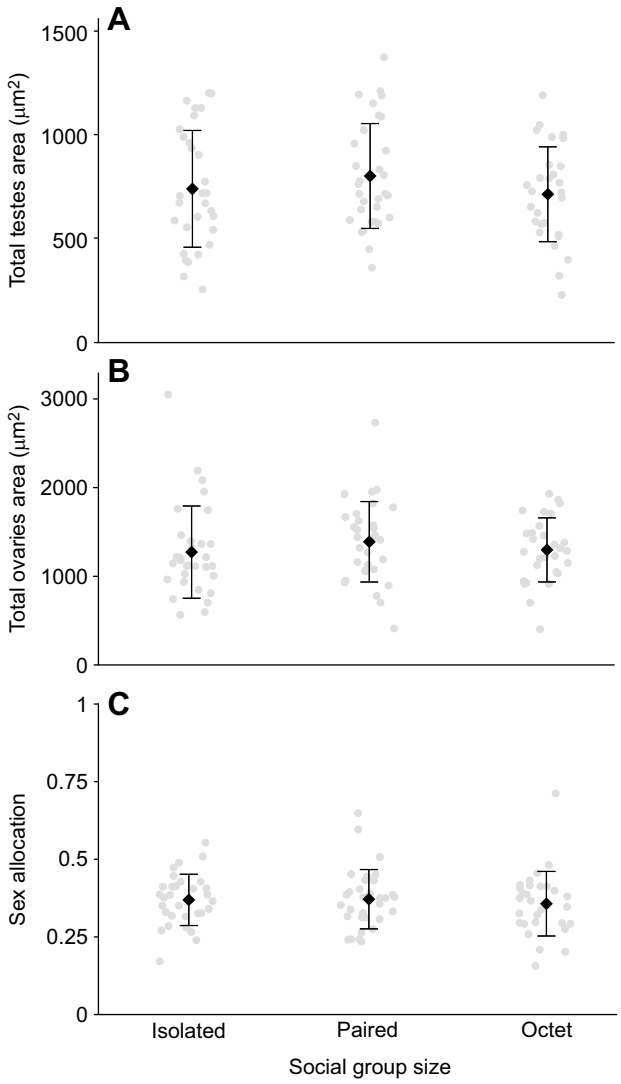


**Fig. 3. Reproductive resource allocation is not sensitive to potential sperm competition level in *M. pusillum*.** Worms kept either in isolation or in groups, and thus under potentially different sperm competition conditions, did not differ in (A) testes size ($mean_i$±s.d.=737.9±282.1 μm², $mean_p$±s.d.=800.0±253.4 μm², $mean_o$±s.d.=711.9±229.1 μm²; $F_{2,89}$=0.98, $P$=0.38), (B) ovaries size ($mean_i$±s.d.=1272.8±519.9 μm², $mean_p$±s.d.=1389.5±453.5 μm², $mean_o$±s.d.=1297.9±361.3 μm²; $F_{2,89}$=0.65, $P$=0.52) or (C) sex allocation ($mean_i$±s.d.=0.369±0.082, $mean_p$±s.d.=0.371±0.095, $mean_o$±s.d.=0.356±0.010; $F_{2,89}$=0.21, $P$=0.81). Diamonds show the mean and whiskers the standard deviation; raw data are plotted as grey circles in the background. Data are based on $n_i$=31, $n_p$=32 and $n_o$=29 worms.

was detected ($\chi^2_{1,1}$=0.007, $P$=0.93), nor was there a significant interaction between treatment group and chase day ($\chi^2_{1,1}$=0.129, $P$=0.72), suggesting that spermatogenesis progresses in a similar manner regardless of treatment group in *M. pusillum* (see Fig. 4 and Table 1 for statistical details).

Four days after BrdU administration, mature labelled sperm could be observed in the seminal vesicles of half of the worms in both treatment groups, and we could thus estimate the duration of spermatogenesis at ca. 4 days in this species.

## DISCUSSION

Our study reveals that *M. pusillum* exhibits a strikingly different combination of reproductive traits compared with either of its two

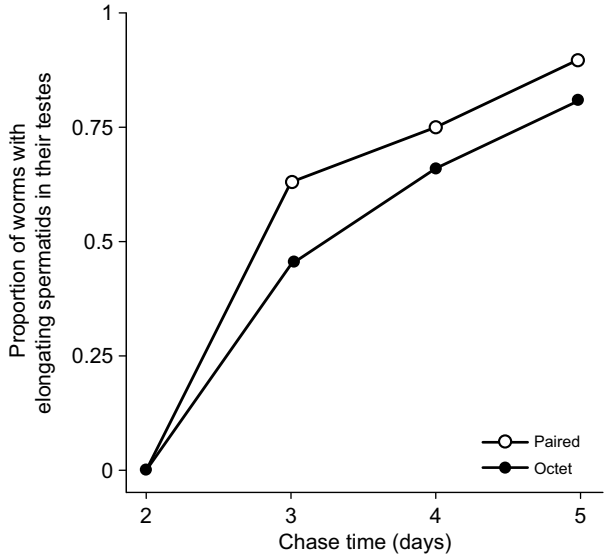


**Fig. 4. No variation in spermatogenesis speed between different potential sperm competition levels in *M. pusillum*.** Worms kept either in pairs or in octets (i.e. under low or high sperm competition level, respectively) were treated with BrdU, a synthetic nucleoside that incorporates in S-phase testicular germ cells (among all other cells) undergoing DNA replication. The testes of these worms were then observed 2–5 days after BrdU administration (chase time) for the presence of elongated spermatids as a sign of advanced spermatogenesis. The graph shows the proportion of paired and octet worms containing elongating spermatids within their testes on each chase day. Progressing in a similar manner in both treatment groups, spermatogenesis speed seems to be unaffected by potential variation in sperm competition level among treatment groups (GLM: chase day: $\chi^2_{1,1}$=11.843, $P$<0.001; treatment group: $\chi^2_{1,1}$=0.007, $P$=0.93; treatment group×chase day: $\chi^2_{1,1}$=0.129, $P$=0.72).

congeners investigated previously, namely: the ability to self, but little evidence of either delayed selfing or inbreeding depression, and no evidence for plasticity in either sex allocation or spermatogenesis kinetics in response to social group size. We first discuss each of these main results in turn, and then speculate as to why *M. pusillum* might exhibit such a combination of reproductive traits.

### Self-fertilization in *M. pusillum*

The ability of several *Macrostomum* flatworms, including *M. pusillum*, to hypodermically inseminate seems to relate to the possession of (1) a needle-like stylet able to pierce through another worm's body and inject sperm into the parenchyma and (2) morphologically simple sperm (i.e. sperm that is short and lacks bristles) that can presumably more easily travel through the parenchyma to the site of fertilization (Schärer et al., 2011). These adaptations to hypodermic insemination are likely also of assistance to the ability to self-fertilize (Ramm et al., 2015). Our study

**Table 1. Generalized linear model for the spermatogenesis assay**

| | Estimate | s.e. | $Z$ | d.f. | $\chi^2$ | $P$ |
|---|---|---|---|---|---|---|
| Intercept | −4.957 | 1.484 | −3.340 | | 11.155 | <0.001 |
| Chase day | 1.390 | 0.404 | 3.441 | 1 | 11.843 | <0.001 |
| Treatment group | −0.180 | 2.152 | −0.084 | 1 | 0.007 | 0.93 |
| Interaction | 0.220 | 0.612 | 0.360 | 1 | 0.129 | 0.72 |

The model treats the presence of elongating spermatids in the worms' testes as the binomial response variable and incorporates chase day, treatment group and their interaction as the explanatory terms. Data are based on $n_p$=44 paired and $n_o$=45 octet worms, evenly distributed across each group size×chase day combination.

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represents the first demonstration that *M. pusillum* can self-fertilize, rendering it the second species in the *Macrostomum* clade known to perform this type of mating, the other being *M. hystrix* (Ramm et al., 2012). How exactly *M. pusillum* engages in self-fertilization is currently unknown, but a recent study in the morphologically similar congener *M. hystrix* revealed that this occurs by hypodermic self-insemination into its body and head regions, presumably because the own anterior part of the body is more easily reached by the copulatory organ located in the tail (Ramm et al., 2015). Given their morphological similarities, we can speculate that an analogous mechanism operates in *M. pusillum*, though further experiments are needed to confirm whether this is the case, especially given their distant phylogenetic relationship within the clade (Schärer et al., 2011). This apparent link between hypodermic insemination and selfing ability – as well as the continued production of sperm even in isolated individuals – also speaks against other potential mechanisms of uniparental reproduction that might have explained our findings such as asexual parthenogenesis.

This brings us to a second remaining ambiguity about the reproductive biology of *M. pusillum*, namely, its propensity to self-fertilize. Because self-fertilization is often associated with inbreeding depression (Charlesworth and Charlesworth, 1987), but at the same time inbreeding depression can be purged by frequent selfing, theoretical models on the evolution and maintenance of self-fertilization (Lande and Schemske, 1985) predict that organisms should either exclusively self or outcross and only under certain ecological circumstances follow mixed mating strategies (Goodwillie et al., 2005). Through a series of complementary findings, our results would appear to point towards self-fertilization being a frequent reproductive strategy in these worms. We therefore cautiously conclude that *M. pusillum* may even be a preferentially self-fertilizing species, but note that this is our interpretation of a series of indirect results, and is not yet supported by the necessary genetic data.

### No selfing delay or differential inbreeding depression

The first indicator of frequent selfing in *M. pusillum* is the absence of a delay to commence selfing. Theory predicts that organisms for which self-fertilization exerts significant costs should delay reproduction under enforced selfing in case potential outcrossing opportunities occur, and such organisms are regarded as preferential outcrossers (Tsitrone et al., 2003a). Such a response has been shown to exist in *M. hystrix* (Ramm et al., 2012, 2015) and a wide range of other taxa (see Introduction). Superficially, the delay exhibited by *M. pusillum* when isolated resembles the response of a preferential outcrosser and seems to contradict our suggestion of a preferentially self-fertilizing species. However, such a delay (ca. 20%) would be moderate compared with that exhibited by *M. hystrix* (Ramm et al., 2012) and many snails (Tsitrone et al., 2003b; but see Escobar et al., 2007) and, most importantly, appears to be an experimental artefact, stemming from and fully accounted for by the sampling bias in our design as discussed in the Results. We can thus suggest with confidence that *M. pusillum* does not significantly delay reproduction under enforced selfing, supporting our argument in favour of a frequent self-fertilizing mode of reproduction in this species. Reproductive onset under selfing seems to have a strong genetic component in *M. pusillum*, with a heritability estimate of 0.57, while previous studies on the trait's heritability in self-fertilizing animals (Escobar et al., 2007; Ramm et al., 2012) and plants (Damgaard and Loeschcke, 1994) have yielded similar estimates, suggesting that selfing propensity is strongly genetically influenced in many animal taxa.

An additional clue of frequent selfing in *M. pusillum* is the absence of differential inbreeding depression in the offspring of isolated versus grouped worms. In the preferentially outcrossing congener of *M. pusillum*, *M. hystrix*, enforced selfing leads to high mortality and low reproductive capacity in the selfed progeny (Ramm et al., 2012). Similar responses (based on different indices) are exhibited by freshwater snails (Jarne et al., 1991) and plants (Schemske and Lande, 1985). The absence of any survival or early productivity disadvantage between offspring from isolated and grouped parents in *M. pusillum* is not really surprising, however, because we would only have expected to see such inbreeding depression if isolated and grouped worms really are reproducing exclusively by selfing and outcrossing, respectively, which we now think is not the case.

### No sex allocation or spermatogenesis speed adjustment to sperm competition

Further support for our frequent selfing interpretation comes from the absence of variation in sex allocation between treatment groups as well as the overall highly female-biased sex allocation of this species. Various studies in animals (see Schärer, 2009, for a review) and plants (Brunet, 1992) have demonstrated that hermaphroditic organisms respond to sperm (pollen) competition by allocating more of their reproductive resources towards the male function, as predicted by sex allocation (Charnov, 1982, 1996) and sperm competition theory (Parker, 1998). For example, *M. lignano* individuals increase testes size (Schärer and Ladurner, 2003; Janicke et al., 2013) and speed up spermatogenesis (Giannakara et al., 2016) when kept in large groups [social group size reflects mating group size in this species (Janicke et al., 2013)], presumably in order to cope with higher levels of sperm competition. We now know that this does not occur in *M. pusillum*. No variation in sex allocation or in sperm production speed was detected between worms kept in different social group sizes, which we would now interpret as evidence that these different social group sizes do not translate into (sufficient) differences in mating group size to cause sex allocation plasticity. If selfing is the regular or even exclusive mating system irrespective of social group size, the sex allocation response (or lack thereof) is entirely in keeping with sex allocation theory, as individuals simply have to produce sufficient, minimal numbers of sperm to be able to fertilize their own eggs. Correspondingly, sex allocation overall was substantially female-biased, in absolute terms of testis versus ovary size (which may admittedly be a poor measure of absolute bias; see Schärer, 2009), as well as being more female-biased than is usually estimated for the obligately outcrossing congener *M. lignano* (cf. Schärer and Ladurner, 2003; Schärer et al., 2005; Janicke et al., 2013). Additionally, and again in contrast to *M. lignano*, no variation in inferred mating frequency as approximated by the size of the seminal vesicle was detected among treatment groups, again questioning the link between social and mating group size in this species.

### Short spermatogenesis duration

Recent comparative studies in a variety of animal taxa (Schärer et al., 2008; Lüpold et al., 2009; Ramm and Stockley, 2010) have demonstrated a positive correlation between sperm length and spermatogenesis duration. This pattern seems to also apply to the *Macrostomum* clade, if we compare the two congeneric species for which data on spermatogenesis duration are currently available, *M. pusillum* and *M. lignano*. As we have shown here, it takes only 4 days for *M. pusillum* to complete spermatogenesis, i.e. 2 days less

than *M. lignano* (Schärer et al., 2007; Giannakara et al., 2016) and this variation likely stems from the differences the two species exhibit with respect to the length and complexity of the spermatozoa they produce (Schärer et al., 2011). However, investigating spermatogenesis kinetics in additional *Macrostomum* species is required to confirm this pattern across the genus.

## Outlook and conclusions

To conclude, we demonstrate for the first time the ability of the hermaphroditic flatworm *M. pusillum* to self-fertilize and provide evidence that this might be the preferred mating strategy in this species. This is based on a series of observations: (1) absence of differential inbreeding-related costs in the selfed versus potentially outcrossed progeny, (2) absence of a delay in the onset of reproduction under enforced selfing, (3) no plasticity in spermatogenesis speed or sex allocation and overall highly female-biased sex allocation, and (4) no variation in presumed mating rate between isolated and grouped worms. We acknowledge, however, that genetic evidence of selfing (e.g. from genotyping progeny arrays or estimating selfing rates in natural populations) is currently lacking. Moreover, collecting data from natural populations is especially important, as these worms have been kept in the laboratory for more than a decade, and more frequent selfing might potentially have evolved as an adaptation to the laboratory environment.

More generally, our study provides insights into the great reproductive diversity of the *Macrostomum* clade. Including *M. lignano*, an obligate outcrosser with extremely plastic sex allocation, *M. hystrix*, a preferential outcrosser that exhibits delayed selfing and substantial inbreeding depression, and *M. pusillum*, which we have now shown likely prefers self- over cross-fertilization and exhibits no sex allocation plasticity, this genus clearly contains species exhibiting a wide range of reproductive trait combinations. Many other species are yet to be studied, but are certainly variable in their reproductive biology (Schärer et al., 2011; Janssen et al., 2015), making *Macrostomum* an excellent group for both identifying the underlying genetic causes of divergence in sex allocation and spermatogenesis plasticity, and for exploring and identifying the ecological factors shaping mating system evolution.

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### Competing interests

The authors declare no competing or financial interests.

### Author contributions

S.A.R. conceived the study, A.G. performed the experiment and both authors contributed to statistical analysis and drafting the manuscript.

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### Supplementary information

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**Table S1**

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