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Experimentally evolved and phenotypically plastic responses to enforced monogamy in a hermaphroditic flatworm

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Abstract

Sexual selection is considered a potent evolutionary force in all sexually reproducing organisms, but direct tests in terms of experimental evolution of sexual traits are still lacking for simultaneously hermaphroditic animals. Here, we tested how evolution under enforced monogamy affected a suite of reproductive traits (including testis area, sex allocation, genital morphology, sperm morphology and mating behaviour) in the outcrossing hermaphroditic flatworm *Macrostomum lignano*, using an assay that also allowed the assessment of phenotypically plastic responses to group size. The experiment comprised 32 independent selection lines that evolved under either monogamy or polygamy for 20 generations. While we did not observe an evolutionary shift in sex allocation, we detected effects of the selection regime for two male morphological traits. Specifically, worms evolving under enforced monogamy had a distinct shape of the male copulatory organ and produced sperm with shorter appendages. Many traits that did not evolve under enforced monogamy showed phenotypic plasticity in response to group size. Notably, individuals that grew up in larger groups had a more male-biased sex allocation and produced slightly longer sperm than individuals raised in pairs. We conclude that, in this flatworm, enforced monogamy induced moderate evolutionary but substantial phenotypically plastic responses.

Introduction

Sexual selection has long been argued to shape the evolution of male and female reproductive traits in all sexually reproducing organisms (e.g. Darwin, 1871; Charnov, 1979; Andersson, 1994). In particular, sexual selection is expected to promote the evolution of traits that affect the outcome of intrasexual competition and of those involved in mate choice (reviewed in Birkhead & Pizzari, 2002; Parker, 2014). Following the Darwin–Bateman paradigm (Bateman, 1948; Dewsbury, 2005; Parker & Birkhead, 2013; Janicke *et al.*, 2016), male reproductive success is typically expected to be subject to intense pre- and post-copulatory episodes of intrasexual competition (i.e. precopulatory male–male competition and sperm competition, respectively). In contrast, female reproductive success is often considered to be mainly determined by the resources available for egg production (Bateman, 1948; Williams, 1966; but see Tang-Martinez, 2010), so that females are assumed to be under stronger selection to choose the most rewarding mate during pre- and post-copulatory episodes of mate choice compared to males (i.e. precopulatory female choice, Kirkpatrick, 1987; and cryptic female choice *sensu,* Thornhill, 1983; but for male choice see, for example, Wedell *et al.*, 2002; Clutton-Brock, 2007). As a consequence, the fitness return of a particular mating may differ between males and females, which can generate sexual conflict (Chapman *et al.*, 2003) that is thought to favour the coevolution of female resistance and male persistence traits (Arnqvist & Rowe, 2005; Lessells, 2006; Parker, 2006).

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There is plentiful empirical support for the idea that sexual selection is a potent evolutionary force shaping reproductive traits, primarily involving three lines of evidence. First, descriptive studies and controlled experiments have revealed that intrasexual competition and mate choice can have profound fitness consequences in both sexes and that the outcome of these processes depends on a range of morphological, physiological and behavioural traits (e.g. Andersson, 1982; Hotzy *et al.*, 2012; Sekii *et al.*, 2013). Second, comparative studies have explored the impact of sexual selection on reproductive traits at the macro-evolutionary scale, providing evidence that male and female traits often coevolve (e.g. Koene & Schulenburg, 2005; Rönn *et al.*, 2007; Schärer *et al.*, 2011) and that the strength of sexual selection is evolutionarily linked to the expression of certain reproductive traits across species (e.g. Stockley *et al.*, 1997; Bjork & Pitnick, 2006). And third, experimental evolution studies have been used to document the evolution of reproductive traits under varying intensities of sexual selection at the micro-evolutionary scale (e.g. Hosken & Ward, 2001; Pitnick *et al.*, 2001; Edward *et al.*, 2010; Firman & Simmons, 2010). The vast majority of these studies have been carried out in separate-sexed animals, despite the fact that sexual selection theory is thought to provide valid predictions for all sexually reproducing organisms, including simultaneous hermaphrodites (i.e. organisms which produce male and female gametes at the same time).

Darwin initially doubted that sexual selection could occur in simultaneous hermaphrodites (Darwin, 1871) and some authors have suggested, based on theoretical grounds, that sexual selection is weaker in simultaneous hermaphrodites than in separate-sexed organisms (e.g. Morgan, 1994; Greeff & Michiels, 1999; reviewed in Arnqvist & Rowe, 2005). For instance, Morgan (1994) argued that Fisherian runaway selection is less likely to operate in hermaphrodites because viability selection on male traits reduces female preferences for these traits. Nevertheless, based on more recent empirical work, it is now widely acknowledged that sexual selection and sexual conflict also operate in simultaneous hermaphrodites (Charnov, 1979; Michiels, 1998; Anthes, 2010; Schärer *et al.*, 2015). In particular, several authors have speculated that sexual selection in simultaneous hermaphrodites is more shifted towards the post-mating arena compared to gonochorists. This is because in a hermaphroditic population, every conspecific is a potential mating partner, which reduces precopulatory competition for mates and mate choice (Greeff & Michiels, 1999). Ultimately, the shared interest of both partners to donate sperm is likely to translate into a higher mating propensity especially in reciprocally mating hermaphrodites, which may eventually lead to an increased sperm competition intensity and higher potential for cryptic female choice compared to gonochorists (Michiels, 1998; Schärer & Pen, 2013).

Studying sexual selection in hermaphrodites has particular relevance for our understanding of how these organisms allocate their reproductive resources, owing to their unique capability of maximizing their fitness by directly adjusting their own sex allocation (rather than the sex ratio of offspring, as occurs in gonochorists). Specifically, sex allocation theory for simultaneous hermaphrodites predicts that in the absence of sexual selection (e.g. under selfing or strict monogamy), any investment into traits that favour an individual's male competitive mating and/or fertilization success could be more profitably channelled into the female sex function (reviewed in Charnov, 1982; Schärer, 2009; West, 2009). For instance, under conditions with no scope for sperm competition (e.g. monogamy), theory predicts a minimal investment into sperm production. This is because the transfer of large ejaculates will impose competition between the own sperm of a given donor (a phenomenon termed 'local sperm competition' or hereafter 'LSC'; Schärer, 2009), which is not beneficial from the sperm donor's perspective and therefore expected to select for female-biased resource allocation whenever the number of mating partners is small (Charnov, 1980; Fischer, 1981; Greeff *et al.*, 2001; Schärer & Pen, 2013).

Empirical work on simultaneously hermaphroditic animals has revealed a high potential for post-copulatory sexual selection by documenting intense sperm competition (Pongratz & Michiels, 2003; Janicke & Schärer, 2009a; Pélissié *et al.*, 2012) and evidence for cryptic female choice (e.g. Bishop *et al.*, 1996). In addition, comparative studies have demonstrated that male and female reproductive traits do not evolve independently from each other (Koene & Schulenburg, 2005; Anthes *et al.*, 2008; Schärer *et al.*, 2011), suggesting underlying sexual conflict (reviewed in Schärer *et al.*, 2015). Moreover, empirical studies found that varying levels of intrasexual competition and/or mate choice evoke a phenotypically plastic response in sex allocation (e.g. Trouvé *et al.*, 1999; Janicke *et al.*, 2013) and comparative approaches showed that sex allocation is correlated with proxies of LSC across species (Petersen, 1991; reviewed in Schärer, 2009). However, to our knowledge, no studies have been reported that test for an effect of sexual selection on reproductive traits in simultaneously hermaphroditic animals at a micro-evolutionary scale using an experimental evolution approach (but see Dorken & Pannell, 2009 for a study on plants).

Here, we aim to fill this gap by providing the first experimental evolution study testing for an effect of sexual selection on reproductive traits in a simultaneously hermaphroditic animal, the free-living flatworm *Macrostomum lignano*. Previous work provides evidence that pre- and post-copulatory sexual selection operate in *M. lignano*. For instance, a behavioural study suggests precopulatory mate choice based on a preference for

mating with bigger individuals (Janicke *et al.*, 2012). Moreover, the number of mating partners can be high under certain social conditions (Janicke & Schärer, 2009a; Janicke *et al.*, 2013) suggesting intense sperm competition characterized by moderate second male sperm precedence (Marie-Orleach *et al.*, 2014). In this study, we manipulated the intensity of sexual selection by rearing worms over 20 generations of experimental evolution in either monogamous (i.e. pairs) or polygamous (i.e. groups of eight individuals) lines. In a final assay, each selection line was subjected to both social environments (i.e. pairs and groups of eight individuals), which allowed us to test for both experimentally evolved and phenotypically plastic responses to enforced monogamy on various morphological and behavioural reproductive traits. Under the assumption that monogamy relaxes sexual selection and sexual conflict, we predicted that enforced monogamy would promote the evolution and the phenotypically plastic expression of a more female-biased sex allocation, resulting in reduced expression of costly male traits in favour of female fecundity traits. Many of the studied traits have previously been demonstrated to show a phenotypically plastic response to group size, including body size (Janicke *et al.*, 2013), testis size (Schärer & Ladurner, 2003), ovary size (Schärer *et al.*, 2005), the number of copulations (Janicke & Schärer, 2009b) and the number of post-copulatory sucks (Janicke & Schärer, 2009b). Therefore, our study also aims to shed light on whether phenotypic plasticity promotes or hampers the genetic adaptation to novel environments – a question that is subject of highly controversial debate in evolutionary biology (e.g. Ghalambor *et al.*, 2007, 2015; Draghi & Whitlock, 2012; Kopp & Matuszewski, 2014).

Methods

Study organism

The free-living flatworm *Macrostomum lignano* (Macrostomorpha, Platyhelminthes) is an obligately outcrossing simultaneous hermaphrodite of the intertidal meiofauna of the Northern Adriatic Sea (Schärer & Ladurner, 2003; Ladurner *et al.*, 2005). Very little is known about the natural history of these worms. However, systematic sampling in the field revealed that density can be high and may vary considerably at a small spatial scale (e.g. K. Sekii, personal observation). Moreover, the majority of individuals caught in the field have allosperm in storage (e.g. 269 of 353 individuals [76.2%] sampled near Lignano, Italy; T. Janicke, P. Sandner, D.B. Vizoso, L. Schärer, personal observation) suggesting that worms copulate regularly in their natural environment. Stock cultures are maintained at 20 °C in glass Petri dishes filled with f/2 medium (Andersen *et al.*, 2005) on a 14-h : 10-h day/night cycle and fed with the algae *Nitzschia curvilineata*. Under these conditions, juveniles hatch approximately 5 days after egg laying and worms become sexually mature at an age of 13 days. Thus, the generation time is about 18 days (Schärer & Ladurner, 2003).

The worms are transparent, allowing noninvasive measurement of a number of morphological traits, such as testis size, ovary size and the shape of the male copulatory organ (hereafter called 'stylet'; Schärer & Ladurner, 2003; Janicke & Schärer, 2009a). Worms copulate frequently (usually more than five times per hour), and copulations are reciprocal meaning that both individuals mate in the male and female sex function simultaneously (Schärer *et al.*, 2004; Marie-Orleach *et al.*, 2013). However, the extent to which reciprocal mating also translates into reciprocal sperm transfer has not yet been tested. After mating, worms often exhibit a so-called suck behaviour, which has been proposed to enable worms to remove received ejaculate components out of their sperm storage organ (Schärer *et al.*, 2004; Vizoso *et al.*, 2010; Marie-Orleach *et al.*, 2013), but direct empirical evidence in support of this hypothesis is lacking. The sperm of *M. lignano* has a complex morphology with distinct parts that are easily recognizable under light microscopy: a rapidly undulating section (termed feeler) in the front, followed by the body, where most vesicles are packed, and the nucleus-containing shaft. Two types of appendages are also visible, a pair of stiff lateral bristles that extend backwards between the body and the shaft, and a cluster of short microtubules, termed brush, that protrude at the posterior end of the shaft (Willems *et al.*, 2009; for hypotheses regarding their function, see Vizoso *et al.*, 2010). A previous study documented substantial interindividual variation in the length of some of these traits and that their measurement is highly repeatable (Janicke & Schärer, 2010).

Worms used for this experiment were obtained from an outbred laboratory culture called LS1 (see also Marie-Orleach *et al.*, 2013). This culture was established in 2003 from two independent cultures that were initiated from several hundred worms collected at site UV in Bibione and site PS on the Isola di Martignano near Lignano Sabbiadoro, Italy (see Ladurner *et al.*, 2005 for a map of the sample locations). Worms from these two independent cultures were crossed together in a controlled way, and they have since been maintained in a metapopulation structure of twelve Petrie dishes to maintain genetic diversity. Every generation each dish is started from 100 juvenile worms (for a total population size of 1200), with five migrants exchanged between six specific pairs of dishes. Owing to the high density of approximately 100 worms per dish, the ancestral population was maintained under rearing conditions that were likely to impose intense pre- and post-copulatory sexual selection.

Predictions for evolutionary responses to experimental evolution

For most of the above-mentioned morphological and behavioural traits, we can formulate predictions for how enforced monogamy leads to selection on these traits based on previous studies. First and foremost, sex allocation, measured in terms of testis and ovary size, has been found to shift towards the male sex function in a phenotypically plastic way when individuals experience increased sperm competition by growing up in larger social groups (e.g. Schärer & Ladurner, 2003; Janicke *et al.*, 2013). Moreover, testis size has been shown to predict sperm-transfer success (Janicke & Schärer, 2009a) and male reproductive success (Marie-Orleach *et al.*, 2016). Hence, we predict that enforced monogamy selects for smaller testes and larger ovaries, resulting in more female-biased sex allocation and higher female fecundity. Second, stylet curvature has been documented to covary with sperm-transfer success (with stylets that were more curved away from the seminal vesicle conferring higher success; Janicke & Schärer, 2009a), and stylet size has been shown to be positively correlated with male reproductive success (Marie-Orleach *et al.*, 2016). Thus, on the assumption that the optimal stylet morphology is more costly for the bearer, we expect selection on stylet morphology to be relaxed under enforced monogamy, leading to shorter stylets that are more convex with respect to the seminal vesicle. Third, higher mating rates have been found to translate into more sired offspring in a competitive context (Sekii *et al.*, 2013; Marie-Orleach *et al.*, 2016) suggesting directional selection for high mating rates under polygamy. Moreover, the suck behaviour has been argued to potentially serve as a cryptic female choice and/or female resistance trait, as it may permit the removal of unwanted sperm or harmful substances received from the sperm donor (Vizoso *et al.*, 2010). Thus, on the assumption that the suck behaviour is a costly trait, we predict the evolution of lowered suck frequency under enforced monogamy. Finally, even though we are currently lacking empirical evidence that sperm morphology is under sexual selection in *M. lignano*, several hypotheses have been formulated in order to explain its complex structure as a response to sperm competition and/or cryptic female choice (Vizoso *et al.*, 2010; Schärer *et al.*, 2011). Specifically, Vizoso *et al.* (2010) hypothesized that sperm bristles prevent sperm from being removed from the female sperm storage organ during the post-copulatory suck behaviour and/or to hinder rival sperm from getting anchored in the epithelium of the female sperm storage organ. In both scenarios, longer bristles are likely to be beneficial under sperm competition, so that we expect relaxed selection on bristle length under enforced monogamy. Furthermore, in several separate-sexed organisms, sexual selection has been demonstrated to favour the evolution of longer sperm (reviewed in Snook, 2005; but see Crudgington *et al.*, 2009) so that we could expect a reduction of total sperm length under enforced monogamy. Note that all of the above-mentioned predictions also hold for the expected phenotypically plastic response to group size (see, e.g. Schärer & Ladurner, 2003; Janicke & Schärer, 2010).

Experimental design

The experiment was initiated in September 2007 and lasted for 20 generations, involving the consistent imposition of one of two different selection regimes. In the monogamy treatment, all individuals of a selection line were kept in pairs, whereas in the polygamy treatment, all individuals were kept in groups of eight individuals (hereafter called 'octets'). Previous studies have demonstrated that this range of group size manipulation induces considerable variation in the number of mating partners (i.e. Janicke & Schärer, 2009a; Janicke *et al.*, 2013) so that the two selection regimes are expected to differ greatly in the magnitude of sexual selection and sexual conflict. Specifically, pre- and post-copulatory sexual selection and sexual conflict can be considered to be relaxed in the monogamy treatment and intense in the polygamy treatment. Both selection regimes were applied to 16 independent replicate selection lines (i.e. 32 evolving selection lines in total), each of which comprised 48 founder individuals (Fig. 1a). This experimental design was used to maximize the number of biological replicates while still minimizing genetic drift (e.g. assuming an effective population size of $N_e = 96$, the inbreeding coefficient after 20 generations $F_t = 0.10$; Hartl & Clark, 2007).

For each selection line, we started a new generation by pooling all juveniles produced by the previous generation (total number of offspring produced per line averaged over 20 generations, mean ± SE [range]: monogamy, 175.4 ± 3.9 [64–380]; polygamy, 158.4 ± 3.2 [56–362]). Next, we randomly selected 48 juveniles and transferred them to 12-well tissue culture plates (TPP AG, Switzerland) where they were kept in either 24 pairs (for each monogamy treatment line) or six octets (for each polygamy treatment line). After 14 days of growth, we transferred all groups to fresh wells where the by-then adult worms were allowed to lay eggs for three subsequent days. We then removed all adult worms so that juveniles could hatch and develop without any interference of the parental generation. After 11 days of development, we pooled all the juveniles (i.e. of an age of 6–9 days after hatching) of all 48 parents and started the next generation. Thus, each generation cycle lasted for 28 days (Fig. 1b). On the assumption that worms become adult at an age of approximately 18 days after egg laying (Schärer & Ladurner, 2003), this set-up ensured that sexually mature individuals could interact for a period of

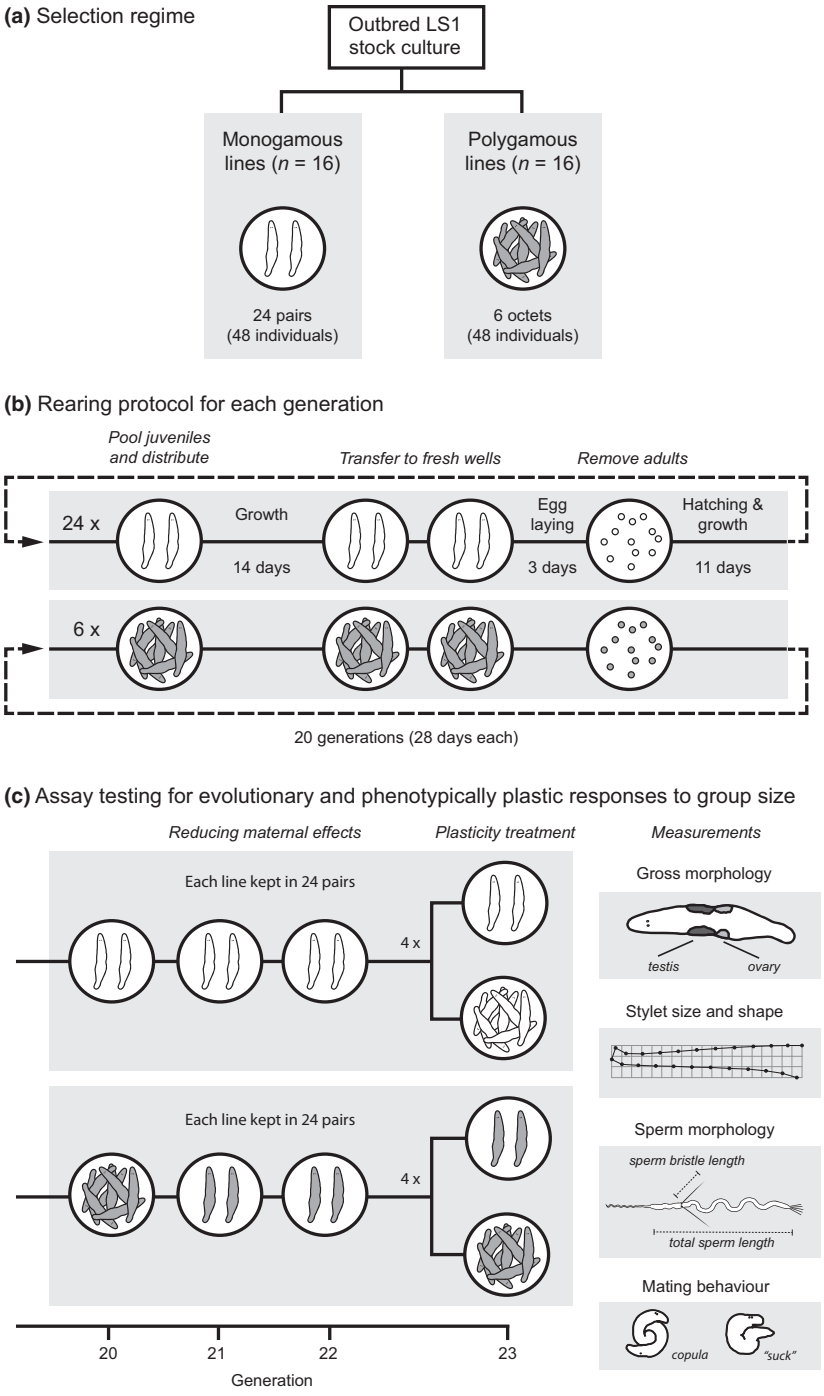

Fig. 1 Schematic illustration of the experimental design, including (a) the selection regime, (b) the specific rearing protocol and (c) the final assay.

7–10 days (depending on when exactly the egg has been laid by their mother within the 3 days of egg laying).

The experiment comprised 20 generations of experimental evolution, after which all 32 selection lines were held under the monogamy treatment for two generations to reduce potential maternal effects associated with the group size manipulation. In generation 23, we tested for a selection response. For this, we obtained 40 randomly selected offspring of each selection line from the 22nd generation and distributed them randomly among four pairs and four octets per line (i.e. 128 pairs and 128 octets in total), following the same protocol of the monogamy and polygamy selection regimes, respectively (Fig. 1c). This allowed us to test for effects of both the previous selection regime and the current

developmental environment, which would constitute evolutionary and phenotypically plastic responses to group size, respectively. Finally, from day 44 until day 47 after initiating the 23rd generation, we obtained morphological and behavioural measurements.

Morphological measurements

Gross morphology (i.e. body, testis and ovary area) and stylet morphology were measured for two individuals of each group (i.e. both worms of all pairs and two randomly selected worms of all octets, resulting in a total of 16 measured worms per line) following standard protocols (Schärer & Ladurner, 2003; Janicke & Schärer, 2009a). Briefly, worms were anesthetized in a 5 : 3 mixture of 7.14% $MgCl_2$ and f/2 medium for 10 min and then squeezed dorsoventrally to a fixed thickness of 35 μm between a microscope slide and the cover slip of a haemocytometer. We took digital micrographs of the entire body, the testes, the ovaries and the stylet with a Leica DM 2500 compound microscope (Leica Microsystems, Germany) and a digital video camera (DFK 41AF02, The Imaging Source Europe GmbH, Germany) at 40× magnification for body area and 400× magnification for testis area, ovary area and stylet shape. For image acquisition, we used BTV Pro 6.0b1 (http://www.bensoftware.com/). Measurements of body, testis and ovary area were obtained from the images using ImageJ 1.42k (http://rsb.info.nih.gov/ij/). Stylet size and shape were quantified using a geometric morphometrics approach (Zelditch *et al.*, 2004). Briefly, we defined 24 landmarks that were superimposed on each stylet image using tpsDig 2.10 (http://life.bio.sunysb.edu/morph/). Six of these landmarks were defined as fixed landmarks (two at the basis and four at the tip) and the remaining 22 as sliding semi-landmarks (Janicke & Schärer, 2009a). We then performed relative warp score (RWS) analyses using tpsRelw 1.45 (http://life.bio.sunysb.edu/morph/), for which the RWSs can be considered as principal components describing the variation in shape of the stylet. By definition, all RWSs are uncorrelated with each other and each one describes the variation of a particular shape characteristic around the consensus shape (Fig. S1a). The RWS analysis yielded 44 RWSs, of which the first three explained 75.7% of the total variation in the shape. The first relative warp score (RWS 1; explaining 46.9%) described the direction and the extent to which the stylet is curved (Fig. S1b). The second relative warp score (RWS 2; explaining 16.6%) described the width of the stylet (Fig. S1c) and the third relative score (RWS 3; explaining 12.3%) mainly described the orientation of the stylet tip (Fig. S1d). In our final analysis of stylet shape, we focus only on these three RWSs and on centroid size, which is the square root of the sum of squared distances between all landmarks to their common centroid (Zelditch *et al.*, 2004), and which can serve as an estimate of stylet size. All these morphological measurements have been shown to have reasonably high repeatabilities (Schärer & Ladurner, 2003; Janicke & Schärer, 2009a).

Sperm morphology was measured from one randomly selected individual out of each group (i.e. one individual out of each pair and each octet, resulting in a total of eight sampled individuals per line) following a protocol described in detail elsewhere (Janicke & Schärer, 2010). Briefly, for each individual, we took measurements of five sperm and used the averaged trait values in the analysis. Here, we focus on two sperm traits, namely total sperm length and length of the bristles (stiff lateral appendages), for which we had clear predictions.

Behavioural measurements

We tested for evolutionary and phenotypically plastic effects of group size on mating behaviour by recording the number of copulations and the number of post-copulatory sucks in paired worms. Specifically, for each selection line, we formed two pairs of individuals that had been raised in four independent pairs and two pairs of individuals that had been raised in four independent octets. From day 44 until 47 after starting the 23rd generation, we observed each day 32 pairs (in four observation chambers each containing eight pairs). In these observation chambers, worms were placed in drops of 3 μL artificial seawater (32‰ salinity) between two microscope slides (for more details, see Schärer *et al.*, 2004). Each chamber was filmed at 1 frame s^{-1} for 2 h using a digital video camera (Sony DFW-X700; Sony Broadcast & Professional, Köln, Germany), and we recorded movies in QuickTime format using BTV PRO 6.0b1. Movie capture started within 5 min after chamber assembly. Mating behaviour was scored by manual frame-by-frame analysis of the resulting QuickTime movies using KMPlayer version 1.5.1 (http://kmplayer.com). For each pair, we assessed the total number of copulations and the number of sucks per copulation within the 2-h mating trials.

Female fecundity

We assessed female fecundity by counting juveniles of the four pairs and the four octets of each selection line that were produced between day 31 and 39 after starting the 23rd generation. All juveniles were counted on day 48, and all estimates were divided by group size, so that values are expressed as the per capita number of juveniles produced during 8 days.

Statistical analyses

In total, we measured gross morphology for 510 individuals sampled from 256 social groups (i.e. four pairs

and four octets for each of the 32 selection lines). Data on sperm morphology were obtained from 185 individuals (for total sperm length) and 181 individuals (for sperm bristle length). Finally, mating behaviour was assessed for 127 pairs and fecundity for 249 social groups. Deviations from the original sample sizes of 512 (for gross morphology and stylet shape), 256 (for sperm morphology and fecundity) and 128 (for mating behaviour) resulted from pipetting errors, losses during preparation or poor digital photographs that did not allow reliable measurements. All losses were approximately balanced across the selection regimes and plasticity treatments.

We ran linear mixed effects models (LMM) with selection regime, developmental environment and their interaction as fixed factors and with line (i.e. lines 1 through 32) defined as a random factor nested in selection regime (Schielzeth & Nakagawa, 2013). Hence, we tested for (i) an evolutionary response to the selection regime, (ii) a phenotypically plastic adjustment in response to group size (hereafter called plasticity treatment) and (iii) the evolution of phenotypic plasticity (indicated by a significant selection regime × plasticity treatment interaction term). We quantified the fraction of variance explained by random and fixed effects following the guidelines by Nakagawa & Schielzeth (2013). For almost all morphological traits, we measured two randomly chosen individuals of each social group and used the mean estimate of both worms for the final analysis to avoid pseudo-replication. Note that modelling individual trait values (instead of group means) with identity of the social group defined as an additional random factor produced qualitatively identical results. In total, we ran LMMs for 15 reproductive traits: body area, testis area, ovary area, residual testis area (residuals from a linear regression of testis area on body area), residual ovary area (residuals from a linear regression of ovary area on body area), sex allocation (testis area divided by total gonad area), total sperm length, sperm bristle length, centroid size of the stylet, RWS 1, RWS 2 and RWS 3, the number of copulations, the number of sucks per copulation and female fecundity. We tested for effects on residual testis and residual ovary area because both testis and ovary area typically scale linearly with body area (e.g. Schärer & Ladurner, 2003) and their residuals provide an estimate of the investment into the male and female function relative to body area (Janicke & Schärer, 2009a). We used log-transformed values for the number of copulations and the number of sucks per copulations to fulfil assumptions of normality.

As outlined in section 'Predictions for evolutionary responses to experimental evolution', we expected directional selection under enforced monogamy for most of the measured traits. However, for completeness, we also tested for stabilizing selection by comparing the variances in trait expression between monogamous and polygamous lines. For instance, the absence of pre- and post-copulatory sexual selection may lead to an increased variance in sexually selected traits under monogamy as supported by comparative studies on sperm morphology in gonochorists (Calhim *et al.*, 2007; Fitzpatrick & Baer, 2011). First, we computed for each of the 15 measured reproductive traits the mean value of each independently evolving line. Based on these values, we assessed the coefficient of variation for monogamous ($N = 16$) and polygamous ($N = 16$) lines. We then used bootstrapping (10 000 iterations) to obtain 95% confidence limits and tested for differences in the coefficient of variation between selection treatments using permutation tests (10 000 permutations). We report two-sided *P*-values signifying the proportion of sampled permutations for which the difference in the coefficient of variation was larger than or equal to the observed difference (Sokal & Rohlf, 2012, pp. 121).

All statistical analyses were carried out using R, version 3.2.0 (R Core Team 2015). Given that we were not interested in testing the universal null hypothesis of no differences between monogamous and polygamous lines but had instead distinct *a priori* predictions for most of the traits tested, we interpret our findings based on *P*-values uncorrected for multiple testing (Perneger, 1998; see also Moran, 2003 for other objections against corrections for multiple testing). Values are given as means ± 1SE unless otherwise stated.

Results

Selection history significantly affected two of the 15 measured reproductive traits (Table 1; Figs 2–4). There was no indication for an effect of the selection regime on the two parameters for which we arguably had the strongest predictions, testis area and ovary area (Fig. 2b,e). Similarly, we also found no selection response for residual testis area and residual ovary area (Fig. 2c,f). As a consequence, sex allocation was unaffected by the selection regime (Fig. 2d). However, worms evolving under polygamy were found to have sperm with longer bristles (Fig. 3b) and stylets with a straighter tip (Fig. 3f). Apart from these effects on mean trait values, we found no differences in the coefficient of variation of reproductive traits among selection treatments except for sperm length, which showed more phenotypic variance in the monogamy treatment (Table S1). However, this effect of the selection history on variance in total sperm length was only statistically significant when tested in pairs (Table S1).

The developmental environment induced, sometimes considerable, phenotypically plastic responses in six of the measured traits, namely body area, residual testis area, sex allocation, sperm length, RWS 1 and the number of sucks per copulation (Table 1). Specifically, worms raised in pairs were smaller, had smaller testes

Table 1 Effects of selection history, plasticity treatment and their interaction on morphological and behavioural reproductive traits in *Macrostomum lignano*. Results of generalized linear mixed models are shown. For summary statistics of variance components and statistical testing of the random factor, see Table S2. Statistically significant effects at $\alpha < 0.05$ highlighted in bold.

	Selection regime			Plasticity treatment			Selection × Plasticity		
Trait	$d.f._{num}$, $d.f._{den}$	*F*	*P*	$d.f._{num}$, $d.f._{den}$	*F*	*P*	$d.f._{num}$, $d.f._{den}$	*F*	*P*
Body area	1, 30	2.94	0.097	1, 222	38.27	**< 0.001**	1, 222	0.09	0.764
Sex allocation	1, 30	0.46	0.503	1, 222	33.94	**< 0.001**	1, 222	0.26	0.613
Testis area	1, 30	0.74	0.398	1, 222	34.12	**< 0.001**	1, 222	0.01	0.962
Ovary area	1, 30	0.52	0.476	1, 222	0.29	0.590	1, 222	0.19	0.667
Residual testis area	1, 30	0.07	0.792	1, 222	9.77	**0.002**	1, 222	0.05	0.819
Residual ovary area	1, 30	0.11	0.745	1, 222	3.50	0.063	1, 222	0.26	0.611
Total sperm length	1, 29.6	0.09	0.770	1, 157.1	157.10	**0.001**	1, 157.1	0.06	0.810
Sperm bristle length	1, 28.3	6.07	**0.020**	1, 157.5	1.91	0.169	1, 157.5	0.13	0.714
Centroid size	1, 29.9	1.70	0.202	1, 192.7	3.21	0.075	1, 192.7	0.19	0.666
RWS 1	1, 29.3	0.04	0.837	1, 195.9	10.34	**0.002**	1, 195.9	0.03	0.870
RWS 2	1, 29.8	1.41	0.245	1, 193.3	1.91	0.168	1, 193.3	0.10	0.751
RWS 3	1, 29.5	8.32	**0.007**	1, 194.8	0.06	0.809	1, 194.8	0.13	0.718
Number of copulations	1, 30.0	0.27	0.607	1, 93.4	2.58	0.112	1, 93.4	0.00	0.978
Sucks per copulation	1, 23.8	0.09	0.772	1, 54.7	13.11	**0.001**	1, 54.8	0.06	0.814
Female fecundity	1, 30.0	2.55	0.121	1, 215.5	2.20	0.139	1, 215.5	0.02	0.879

RWS, relative warp score.

and therefore more female-biased sex allocation, produced shorter sperm, had a stylet that was more curved away from the seminal vesicle and sucked more frequently after copulation (Figs 2–4). None of the tested interactions between selection regime and plasticity treatment were statistically significant (Table 1), suggesting that phenotypic plasticity did not evolve differently in both selection regimes.

Discussion

This study provides moderate support for the hypothesis that sexual selection is a potent selective agent for the evolution of reproductive traits in simultaneous hermaphrodites. We found that two of 15 measured reproductive traits showed an evolutionary response to enforced monogamy after 20 generations of selection, whereas six traits responded in a phenotypically plastic way to group size. In the following, we first discuss the effects of the selection regime with particular emphasis on potential reasons why we only detected a selection response for some traits, and no selection response for those traits for which we arguably had the strongest predictions. Second, we briefly discuss the observed phenotypic plastic responses in the light of previous findings. Finally, we outline a research agenda that aims to advance our understanding of sexual selection in *M. lignano* by integrating phenotypic plasticity of sexually selected traits.

Evolutionary responses to group size

The main finding of this study is that sexual selection and/or sexual conflict affect the evolution of morphological reproductive characters in *M. lignano*. However, only two traits, namely the shape of the male copulatory organ (i.e. RWS 3), and sperm morphology (i.e. bristle length), were affected by our selection regime. Both of these traits have been argued to be involved in post-copulatory interactions in *M. lignano*. First, the shape of the male copulatory organ, in terms of a particularly curved stylet, has previously been found to correlate with sperm-transfer success (Janicke & Schärer, 2009a). The present study suggests that sexual selection favours stylets with a straighter tip, for which we have currently no mechanistic explanation. Second, sperm morphology has been speculated to be selected in the context of sexual conflict and/or sperm competition (Schärer *et al.*, 2004, 2011; Vizoso *et al.*, 2010). In accordance with our prediction, we found that monogamous lines evolved shorter sperm bristles compared to polygamous lines. However, in order to pinpoint the underlying mechanisms leading to the evolution of stylet shape and sperm morphology, further manipulative experimental approaches are required to study the fitness consequences of these trait values. Recent studies in *M. lignano* have used RNA interference of candidate genes to phenotypically engineer the function of reproductive traits (Sekii *et al.*, 2013; Arbore *et al.*, 2015), suggesting that such an approach might actually be feasible in this study system.

Apart from the detected differences in mean trait values, we found that the variance in total sperm length was reduced among polygamous lines suggesting stabilizing selection on sperm length in the presence of sexual selection. Such reduction of the variance on sperm length has also been documented in comparative

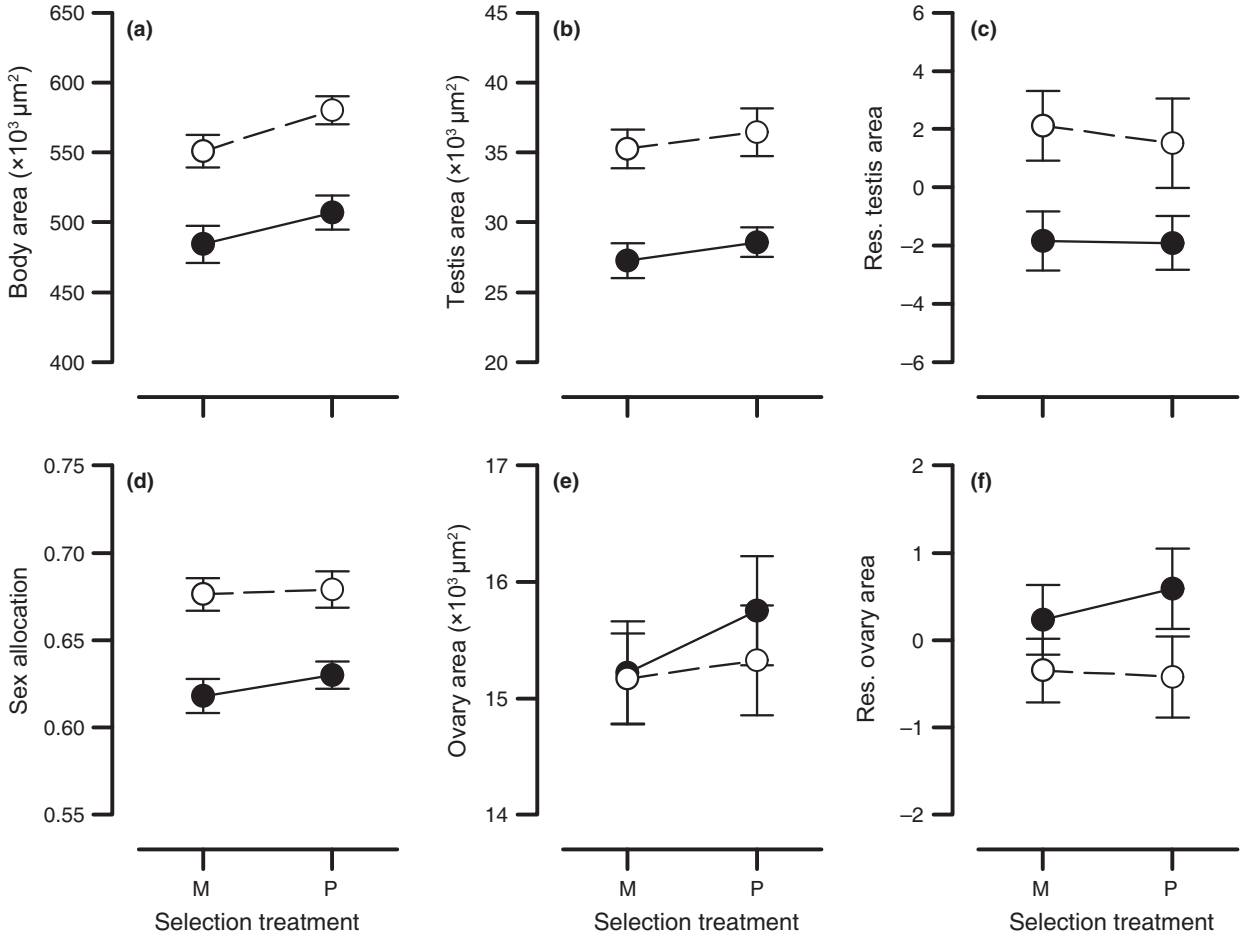

Fig. 2 Effects of enforced monogamy on gross morphology and sex allocation in *Macrostomum lignano*. The selection response is shown on the *x*-axis and the plasticity treatment is shown using different symbols (filled circles: monogamy; open circles: polygamy). Plots show raw data means and standard errors.

studies on birds (Calhim *et al.*, 2007) and insects (Fitzpatrick & Baer, 2011). However, in our case, the effect on variance in total sperm length was only found when worms were raised in pairs but not when tested in octets, which questions whether the observed difference in pairs actually reflects a response to selection or just a type I error.

Body area was the only tested trait that has been suggested to be under precopulatory sexual selection in *M. lignano* (Janicke *et al.*, 2012), but did not show a significant selection response. Together with our findings on stylet shape and sperm morphology, our results are therefore in line with the hypothesis that sexual selection in reciprocally mating simultaneous hermaphrodites operates primarily at the post-copulatory rather than the precopulatory level (Charnov, 1979; Michiels, 1998; Schärer & Pen, 2013; Marie-Orleach *et al.*, 2016). Surprisingly, sex allocation did not evolve under our selection regime. This was unexpected given that our experimental set-up should, according to sex allocation theory (Charnov, 1980, 1982), have favoured individuals with more female-biased sex allocation in the monogamy treatment (in terms of smaller testes, larger ovaries and higher female fecundity).

The lack of such a selection response in sex allocation, and in other traits for which we expected an evolutionary response (such as the number of copulations), might have methodological and/or biological reasons. One potential methodological reason explaining the absence of a selection response in sex allocation might be the lack of sufficient genetic variation in the laboratory culture that was used as the founder population for this experiment. Although we cannot fully reject this hypothesis, we have reasons to believe that the experiment began with a genetically diverse population. First, to form the first generation, we collected hatchlings from the outbred LS1 laboratory culture that has been kept at a population size of approx. 1200 individuals since it was initiated by crossing two spatially separated field populations, which should minimize the loss

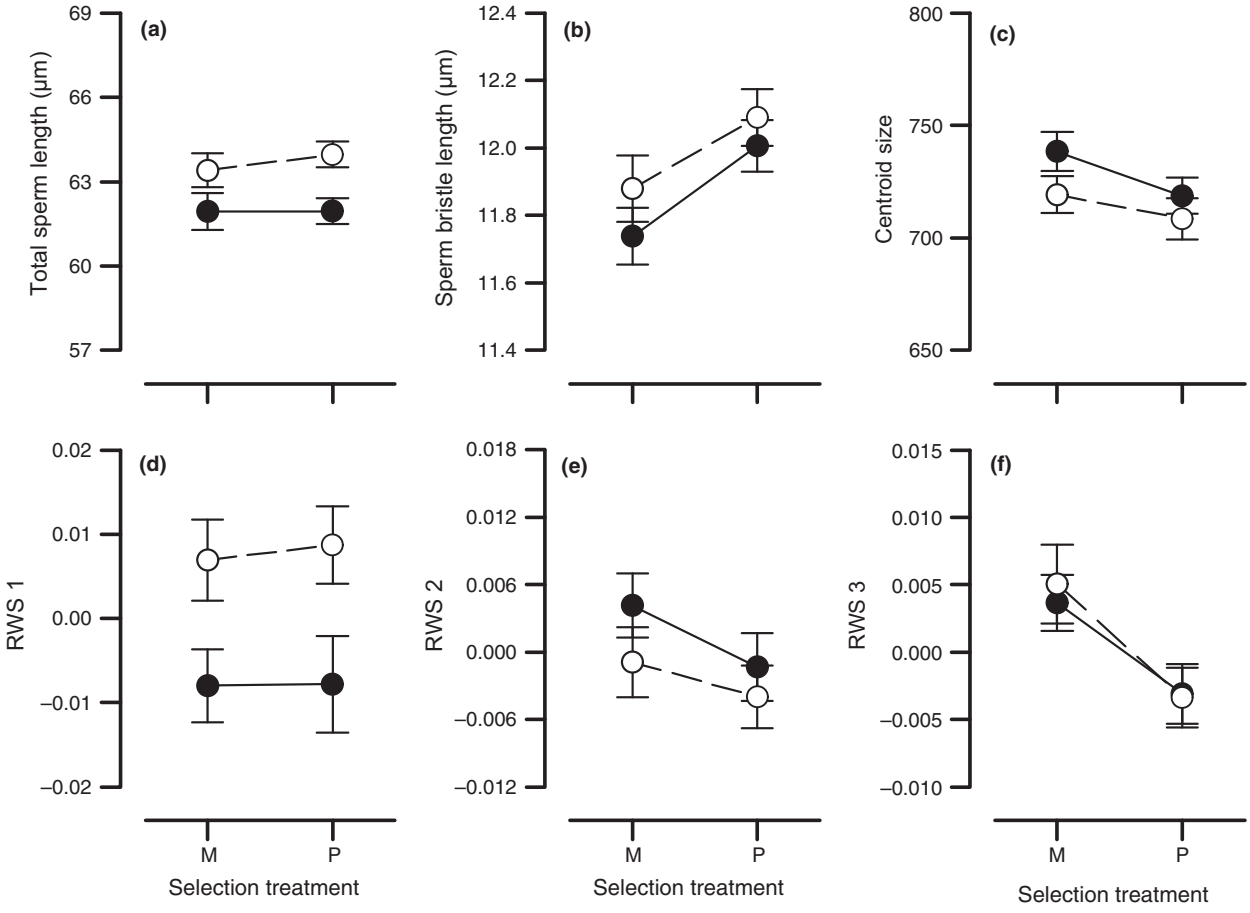

Fig. 3 Effects of selection regime and developmental environment (filled circles: monogamy; open circles: polygamy) on male reproductive morphology. Plots show raw data means and standard errors.

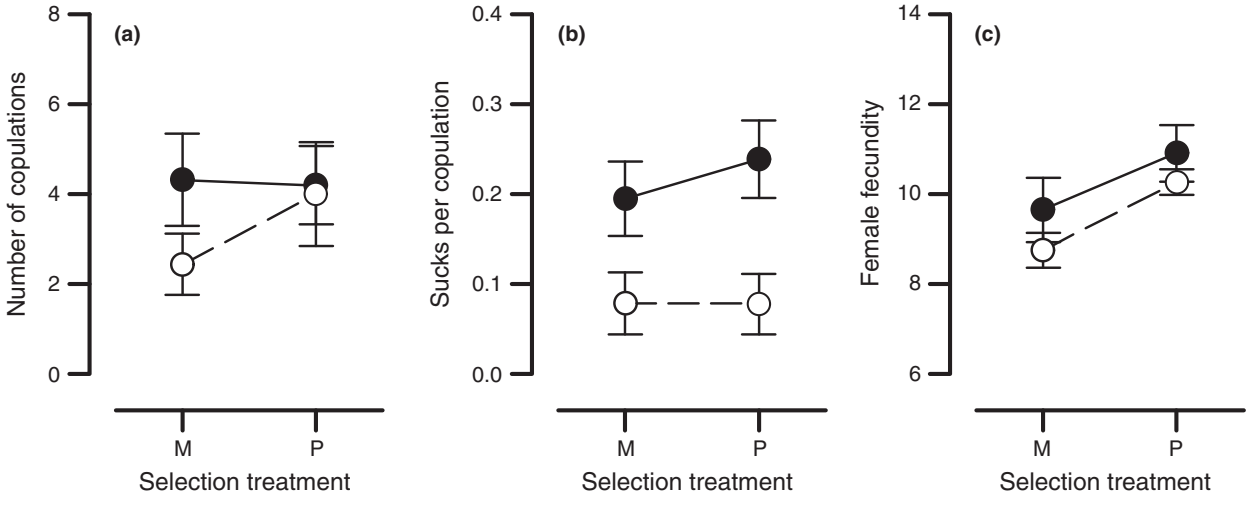

Fig. 4 Effects of selection regime and developmental environment (filled circles: monogamy; open circles: polygamy) on mating behaviour and female fecundity. Plots show means and standard errors of the raw data, but note that log-transformed data were used for the statistical analysis of the number of copulations and the number of sucks per copulation.

of genetic variation due to drift. Second, inbred lines derived from the same field populations show considerable variation in morphological and behavioural traits, suggesting substantial genetic variation in this culture (L. Marie-Orleach, N. Burri, P. Mouginot, A. Schlatter, D. B. Vizoso, N. W. Bailey and L. Schärer in preparation; D. B. Vizoso, unpublished data). And third, a subsequently performed quantitative genetic study using worms from the same culture indicated at least moderate broad-sense heritabilities in univariate analyses for some of the traits investigated here, namely body, testis and ovary area (see Appendix S2), suggesting that the LS1 culture contains standing genetic variation for these traits.

The absence of predicted evolutionary responses might also be due to our experimental set-up. First, 20 generations might have been too short to observe micro-evolutionary changes. We cannot completely reject this hypothesis, but several other experimental evolution studies focussing on the effect of sexual selection on reproductive characters have detected evolutionary responses after a similar number of generations of selection (e.g. Hosken *et al.*, 2001; Simmons & Garcia-Gonzalez, 2008; Cayetano *et al.*, 2011; Michalczyk *et al.*, 2011). Second, we might possibly have had insufficient statistical power to detect evolutionary responses of a similar magnitude as reported for other study systems. This seems unlikely, however, as we believe that our experiment represents one of the best-replicated experimental evolution studies focussing on the effect of sexual selection on reproductive characters that has been performed to date. In fact, many micro-evolutionary studies in separate-sexed organisms comprised only 2–4 independently evolving selection lines for each tested selection regime, but still found selection responses to enforced monogamy for a variety of traits including testis area (e.g. Pitnick *et al.*, 2001; Simmons & Garcia-Gonzalez, 2008; reviewed in Garcia-Gonzalez, 2011). Consequently, we presume that potential limitations of our experimental design alone cannot explain the lack of an evolutionary response of sex allocation.

Notably, most of the traits that did not evolve nevertheless showed a marked phenotypically plastic response to group size, which represents an interesting finding in the light of the ongoing debate on the role of phenotypic plasticity for the rate of adaptive evolution (reviewed in Price *et al.*, 2003; Ghalambor *et al.*, 2007; Kopp & Matuszewski, 2014). On the one hand, phenotypic plasticity has been argued to shield the genotype from effects of selection. Specifically, if adaptive plasticity allows the expression of a phenotype that matches the fitness optimum, phenotypic plasticity is expected to impede the evolution of a given trait (Price *et al.*, 2003). On the other hand, however, if phenotypic plasticity is adaptive but does not allow an optimal response (i.e. the fitness optimum cannot be reached), plasticity is predicted to accelerate the adaptive evolution of a trait. This is because phenotypic plasticity may allow a population to persist in a novel environment, whereas directional selection will favour genotypes exhibiting the most extreme phenotypes – a process called genetic assimilation (Waddington, 1953; West-Eberhard, 1989; Pigliucci *et al.*, 2006). Therefore, several authors have argued that moderate levels of phenotypic plasticity provide a mechanism for the most rapid genetic adaptation to novel environments (Price *et al.*, 2003; Ghalambor *et al.*, 2007; Draghi & Whitlock, 2012; Gomez-Mestre & Jovani, 2013). Our results suggest that phenotypically plastic traits (i.e. sex allocation, body area, total sperm length, the curvature of the stylet and the number of sucks) did not evolve under the tested conditions, whereas those that did evolve (i.e. sperm bristle length and orientation of the stylet tip) showed no phenotypically plastic response to group size. This is an unexpected outcome at least for sex allocation because it is questionable whether phenotypic plasticity allows individuals to attain an optimal sex allocation under enforced monogamy. Theory predicts that simultaneous hermaphrodites should show minimal investment into male reproduction in the absence of pre- and post-copulatory sexual selection (Charnov, 1980, 1982). However, this and previous studies found that *M. lignano* copulates readily and produces more sperm than needed to fertilize the partner's eggs when kept in pairs (e.g. Schärer & Ladurner, 2003; Schärer & Vizoso, 2007; Janicke & Schärer, 2009b). This may reflect an adaptation to anticipate future sperm competition in highly fluctuating and unpredictable social environments. Alternatively, it suggests that the phenotypically plastic adjustment to group size is not sufficient to attain the evolutionary optimal sex allocation. Further empirical work exploring the fitness landscape, the genetic architecture and the cost of phenotypic plasticity for all measured traits is certainly needed to better understand why the tested phenotypically plastic traits did not evolve (see Outlook section below).

Phenotypically plastic response to group size

For a number of traits that did not show an evolutionary response, we detected, sometimes considerable, phenotypic plasticity. First of all, individuals raised in the polygamy treatment had larger testes (both absolute and relative to body area), leading to more male-biased sex allocation compared to individuals raised in the monogamy treatment as already documented previously for *M. lignano* (e.g. Schärer & Ladurner, 2003; Janicke *et al.*, 2013). By contrast, we only found a tendency for higher female fecundity in pairs compared to octets. Hence, our study does not confirm earlier findings showing that individuals in pairs have significantly higher female fecundity than individuals in larger groups (Schärer *et al.*, 2005; but see Schärer & Ladurner, 2003). However, individuals in octets grew bigger

compared to individuals in pairs (as reported in previous studies; e.g., Schärer & Janicke, 2009; Janicke *et al.*, 2013), suggesting that the former could, for some unknown reasons, acquire more resources, which might have masked the predicted effect of group size on female fecundity.

Interestingly, worms in octets produced slightly longer sperm compared to worms in pairs. This contrasts with an earlier study that provided no support for phenotypic plasticity in sperm morphology in response to group size (Janicke & Schärer, 2010). However, the earlier experimental test had less statistical power (i.e. sample size was $N = 24$ individuals each in pairs vs. octets, compared to $N = 91$ pairs and $N = 94$ octets here). In the present study, sperm of individuals raised in pairs were only ~ 3% shorter compared to sperm of individuals raised in octets. This effect size is below the threshold of 5% that we were able to detect in the previous study (Janicke & Schärer, 2010). Until now, phenotypic plasticity in sperm length in response to sperm competition intensity has been reported in only a few cases, including studies on birds (Immler *et al.*, 2010), insects (Morrow *et al.*, 2008) and broadcast-spawning ascidians (Crean & Marshall, 2008). Nevertheless, our observed difference of ~ 3% is relatively small and to date there is no evidence that longer sperm confer benefits to its bearer in terms of higher sperm competitiveness and/or higher persistence to overcome post-copulatory female choice in *M. lignano*. Hence, further studies are needed to infer whether longer sperm observed under sperm competition represent an adaptive phenotypically plastic response.

For the first time in *M. lignano*, we observed phenotypic plasticity in stylet shape, with individuals in octets having a stylet that is more concave with respect to the position of the seminal vesicle. A previous study suggested that stylets that are more convex with respect to the seminal vesicle are associated with higher sperm-transfer success (Janicke & Schärer, 2009a), which may question the adaptive significance of our observed plastic response to group size.

Finally, the social environment also affected mating behaviour, with worms in pairs showing more post-copulatory sucks than worms in octets. This was already observed earlier (Janicke & Schärer, 2009b) and might be due to the fact that individuals in smaller groups usually have larger seminal vesicles (e.g. Schärer & Ladurner, 2003; Janicke *et al.*, 2010) allowing them to donate a greater amount of sperm per copulation, which might then be countered by more frequent sucks (Vizoso *et al.*, 2010; Marie-Orleach *et al.*, 2013).

Outlook

In this study, we found selection responses on traits presumed to be subject to post-copulatory sexual selection, after 20 generations of experimental evolution. Sex allocation did not evolve, so that to date, we are still lacking experimental evidence for an effect of male–male competition on sex allocation in simultaneously hermaphroditic animals at the micro-evolutionary scale (but see Dorken & Pannell, 2009 and Macke *et al.*, 2011 for plants and gonochoristic animals, respectively). Many of the tested traits showed a phenotypically plastic but no evolutionary response to group size. Given the controversy over the role of phenotypic plasticity for adaptive evolution, it seems important to also publish studies that fail to find predicted evolutionary shifts in plastic traits. To better understand the reported results in the context of sexual selection in simultaneous hermaphrodites and the role of phenotypic plasticity for adaptive evolution, we propose the following research agenda. First, we need more powerful experimental tests of selection differentials for the tested reproductive traits to predict the fitness optimum for a given trait along a sexual selection gradient (e.g. Marie-Orleach *et al.*, 2016). This will not only reveal whether plasticity is adaptive and effective enough to match the phenotype to the fitness optimum, but will also allow to specify the shape of the fitness landscape, which has also been argued to affect the interplay between phenotypic plasticity and adaptive evolution (Borenstein *et al.*, 2006). Second, detailed information on the genetic architecture is certainly required to predict the rate of adaptation and the evolutionary trajectories of the tested reproductive traits. In particular, we need to assess the genetic variance–covariance (G) matrix and quantify the major axis of genetic variation (g_{max}) of all tested traits to illuminate the alignment of phenotypically plastic and evolutionary responses (e.g. Lind *et al.*, 2015). Third, we need to quantify the costs associated with the observed phenotypically plastic adjustments to group size, which is essential to understand the evolution of phenotypic plasticity itself. Finally, to provide the most rigorous test of whether plasticity expedites or hampers adaptive evolution, we need to compare the evolutionary responses by selection lines that differ in their reaction norms (e.g. Teotónio *et al.*, 2009; Schaum & Collins, 2014).

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Appendix S1. Supplementary tables and figures.

Table S1. Comparison of the coefficient of variation (CV) between selection regimes.

Table S2. Summary report of the variance explained by General Linear Mixed Models including selection regime, developmental environment and their interaction defined as fixed factors and line (i.e. lines 1 through 32) defined as a random factor.

Figure S1. Visualization of geometric morphometrics of male copulatory organ shape in *M. lignano*.

Appendix S2. Documentation of standing genetic variation in the LS1 culture.

Table S3. Family effects and broad-sense heritability estimates for body size, testis size and ovary size of *M. lignano*.

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