Evolution of sex allocation plasticity and its predictors in a flatworm genus

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Abstract

Local sperm competition (LSC), which occurs when related sperm compete for access to a given set of eggs, can influence sex allocation (SA) in simultaneous hermaphrodites. Different factors that have been predicted to affect LSC—and hence, optimal SA—include the mating strategy (such as reciprocal mating vs. hypodermic insemination) and the ability to self-fertilize. Moreover, the level of LSC experienced could vary temporally and/or spatially, favouring the evolution of SA plasticity. However, it is unknown how the mating strategy and ability to self-fertilize can influence SA and SA plasticity. Here, we explored this by collecting estimates of SA and SA plasticity for seven species of the flatworm genus, *Macrostomum*, and performing analyses while controlling for phylogeny. While we found substantial interspecific variation in SA, neither the mating strategy nor the ability to self-fertilize had a significant effect on SA. Next, we estimated standardized effect sizes for SA plasticity in response to i) the presence of mating partners and ii) the strength of LSC for each species, and tested if the mating strategy or ability to self-fertilize predicted these effect sizes. We found considerable interspecific variation in SA plasticity, with some species being highly plastic and others showing no significant plasticity. Moreover, self-fertilization predicted SA plasticity with respect to the presence of mating partners, with SA plasticity being lower for self-fertilizing species. Finally, we showed that interspecific variation in SA is nearly three times higher than intraspecific variation, suggesting that SA estimates in field-collected specimens can, at least to some degree, be interpreted as representing interspecific rather than intraspecific variation. Our study suggests that both SA and SA plasticity can evolve rapidly, with self-fertilization being an important predictor for the latter in *Macrostomum*.

Keywords

phenotypic plasticity, sperm competition, self-fertilisation, reciprocal mating, hypodermic insemination, local mate competition

63 ## *Introduction*

64 Sex allocation (SA) theory in simultaneous hermaphrodites predicts the optimal allocation of
65 resources towards the male versus female function (Charnov 1982). Local sperm competition
66 (LSC), which can be thought of as the inverse of sperm competition (Parker 1970, 1998),
67 occurs when related sperm (usually from the same individual) compete for access to a given
68 set of eggs, and it can influence the optimal SA in simultaneous hermaphrodites (Schärer
69 2009; Schärer and Pen 2013). An example of a factor that can affect LSC, and hence the
70 optimal SA, is the mating group size (Charnov 1980, 1982), denoted as K+1, where K is the
71 number of sperm donors from which a sperm recipient receives sperm at the time its eggs are
72 fertilized. Specifically, Charnov's mating group size model predicts an influence of the
73 mating group size on SA, such that the optimal proportion of resources allocated to the male
74 function in an individual is predicted to increase with the number of sperm donors that are
75 present in a local mating group.

76 The link between optimal SA and LSC can be visualised in terms of male fitness gain curves,
77 which describe how much fitness is gained through the male function per unit resource
78 investment (Charnov 1982; Schärer 2009). In small groups, e.g. under monogamy, LSC is
79 necessarily very high, since the competing sperm are maximally related to each other,
80 resulting in the male fitness gain curve saturating quickly. Thus, any investment into the
81 production of more sperm than are required to fertilize the partner's eggs is a waste of
82 resources, as the related sperm simply compete amongst themselves for fertilizations. Instead,
83 these resources could be more profitably invested into the individual's own female function
84 (Charnov 1982), since the female fitness gain curve is often assumed to be non-saturating and
85 linear (Schärer 2009). Consequently, under monogamy, a simultaneous hermaphrodite is
86 expected to have a female-biased SA (Charnov 1980, 1982). But as the mating group size
87 increases—with more competitors contributing sperm to a given recipient—an individual's

88 sperm are more and more competing with unrelated sperm. So it now pays off to invest in the
89 male function to gain a greater share of the fertilizations, and the male fitness gain curve is
90 predicted to linearize, with a subsequent shift towards a more equal SA (Charnov 1982;
91 Schärer 2009; Schärer and Pen 2013).

92 An interesting question that arises then is whether and how interspecific variation in
93 reproductive biology, including different mating strategies or the ability to self-fertilize, could
94 influence the strength of LSC and hence the optimal SA. For example, hermaphrodites often
95 evolve different mating strategies in response to sexual conflict over mating roles, i.e. over
96 whether to mate as a sperm donor and/or as a sperm recipient (Charnov 1979; Michiels 1998;
97 Schärer et al. 2015). One such mating strategy is reciprocal mating (also called reciprocal
98 copulation), in which both partners mate in both mating roles, and thus donate and receive
99 sperm simultaneously (Michiels 1998; Schärer et al. 2015). This strategy can lead to
100 individuals being quite willing to engage in mating in order to get an opportunity to donate
101 sperm, and this general willingness likely results in reduced precopulatory sexual selection.
102 The ensuing higher mating rates could in turn result in increased sperm competition (and thus
103 a decrease in LSC) and more intense postcopulatory sexual selection. However, the presence
104 of certain postcopulatory processes could also increase LSC, e.g. by leading to the removal of
105 sperm of one or multiple donors from competition (Schärer 2009). Indeed, theoretical studies
106 have predicted that different postcopulatory sexual selection processes, such as cryptic female
107 choice, sperm digestion, and sperm displacement, can result in changes in LSC and hence the
108 predicted optimal SA (Charnov 1996; Pen and Weissing 1999; van Velzen et al. 2009;
109 Schärer and Pen 2013).

110 Moreover, it has been suggested that this intense postcopulatory sexual selection and sexual
111 conflict can lead to selection for traits and mating strategies that allow a donor to bypass these
112 postcopulatory processes and fertilize the eggs more directly (Charnov 1979; Michiels 1998).

113 One such mating strategy is forced unilateral insemination, in which one partner mates only in
114 the male role and donates sperm, e.g. via traumatic or hypodermic insemination (Lange et al.
115 2013; Reinhardt et al. 2015), while the other only mates in the female role, likely often
116 against its interests (Charnov 1979; Michiels 1998; Schärer et al. 2015). It is currently
117 difficult to predict the direction and magnitude of changes in LSC resulting from such a shift
118 in mating strategy—and the effects that such shifts may have on optimal SA—since we still
119 know relatively little about the particular postcopulatory processes involved in these mating
120 strategies. In species with reciprocal mating there may be some control over who or how
121 many partners an individual receives sperm from, and there could be strategies that lead to the
122 (possibly selective) removal of received sperm (van Velzen et al. 2009), possibly leading to
123 increased LSC. In species with hypodermic insemination such control might be more limited,
124 since there sperm function in a less controlled environment in which sperm from multiple
125 donors are more likely to mix randomly in something more closely resembling a fair-raffle-
126 type sperm competition, which would be expected to lower the strength of LSC (Schärer and
127 Janicke 2009).

128 Species that employ self-fertilization might differ from species that primarily outcross, since
129 self-fertilization will generally increase the strength of LSC and hence reduce the fitness
130 returns from investing in the male function, thus favouring the evolution of a more female-
131 biased optimal SA compared to outcrossing species (Charlesworth and Charlesworth 1981;
132 Charnov 1982, 1987), which is supported by empirical work in both plants and animals
133 (Lemen 1980; Schoen 1982; Charnov 1987; McKone 1987; Johnston et al. 1998). In general,
134 we would expect any process that has an effect on LSC to affect the shape of the male fitness
135 gain curve, and hence the optimal SA (Schärer 2009).

136 While most SA models investigate the effect of LSC on SA over evolutionary timescales,
137 LSC can also vary temporally or/and spatially within an individual's lifetime. In

138 hermaphrodites, altering the SA in response to the current conditions can influence the
139 immediate reproductive success of an individual, favouring the evolution of SA plasticity.
140 Indeed, plastic SA has been suggested to be one of the advantages of hermaphroditism over
141 separate-sex species (Charnov 1982; Michiels 1998, 1999). However, for species that do not
142 often experience variation in LSC during their lifetime, we may not expect high levels of
143 plasticity, particularly if there are costs to plasticity (DeWitt et al. 1998; Auld et al. 2010;
144 Siljestam and Östman 2017). Interestingly, while SA plasticity has now been documented in
145 many hermaphroditic species (Schärer 2009), its evolution is still comparatively poorly
146 understood, with few studies having investigated variation in SA plasticity across animals
147 species in a controlled experimental context (Schleicherová et al. 2014). Moreover, as SA can
148 vary both within-species (e.g. due to phenotypic plasticity) and between-species (e.g. due to
149 differences in mating strategy over evolutionary timescales), it is interesting to examine the
150 relative magnitude of the interspecific versus intraspecific variation in SA.

151 An excellent model system for testing the effect of the mating strategy and the ability to self-
152 fertilize on SA and SA plasticity is the free-living flatworm genus *Macrostomum*
153 (Macrostomorpha, Platyhelminthes). This genus contains many species exhibiting one if at
154 least two different mating strategies; one involving reciprocal mating and the other
155 hypodermic insemination (Brand et al., in prep.; Schärer et al. 2011). In reciprocally mating
156 species, a facultative postcopulatory 'suck' behaviour has been observed that has been
157 hypothesised to remove received ejaculate (Singh et al., in prep.; Schärer et al. 2004a, 2011;
158 Vizoso et al. 2010; Marie-Orleach et al. 2013). Remarkably, no such postcopulatory suck
159 behaviour has been documented in five hypodermically inseminating species, which
160 presumably exhibit forced unilateral mating, with sperm being hypodermically injected into
161 the partner via a needle-like male copulatory organ (Schärer et al. 2011). Interestingly,
162 hypodermic insemination is also associated with a suite of morphological traits that

163 potentially allow self-fertilisation (Ramm et al. 2012, 2015; Giannakara and Ramm 2017),
164 although self-fertilization has recently also been documented in at least one reciprocally
165 mating species, *M. mirumnovem* (Singh et al. 2020b).

166 Here, we collected data on SA estimates from a range of experiments performed in seven
167 *Macrostomum* species (including in a new and currently still undescribed species,
168 *Macrostomum* sp. 22). The experimental layout generally consisted of raising worms in three
169 different group sizes (isolated, pairs or octets) and then obtaining estimates of SA once the
170 worms had matured. To facilitate SA plasticity comparisons between species, we calculated
171 standardised effect sizes for SA plasticity in response to i) the presence of mating partners
172 (i.e. comparing isolated worms vs. worms with partners) and ii) the strength of LSC (i.e.
173 comparing paired worms vs. octet worms). Using SA estimates and SA plasticity effect sizes,
174 we then examined if the mating strategy and ability to self-fertilize predicted SA or SA
175 plasticity, while accounting for the phylogenetic interrelationships. Lastly, considering the
176 interspecific SA differences and the extensive SA plasticity shown by some of the species, we
177 examined how much SA varies within-species versus between-species by partitioning the SA
178 variation into its intraspecific and interspecific components. This would also allow us to more
179 confidently interpret SA variation observed in field-collected worms as at least partially
180 representing interspecific differences.

181

182 *Materials and Methods*

183 *Study species*

184 To examine how SA and SA plasticity evolves across the *Macrostomum* genus, we gathered
185 data on SA and SA plasticity in seven *Macrostomum* species: three that are hypodermically
186 inseminating, *M. pusillum* Ax 1951 (Giannakara and Ramm 2017), *M. hystrix* Ørsted 1843

187 sensu Luther 1905 (Winkler and Ramm 2018), and *Macrostomum* sp. 22 (reported in the
188 Supplementary Information S1); and four that are reciprocally mating, namely *M. janickei*
189 Schärer 2019, *M. cliftonensis* Schärer & Brand 2019, and *M. mirumnovem* Schärer & Brand
190 2019 (Schärer et al. 2020), plus multiple studies in *M. lignano* Ladurner, Schärer,
191 Salvenmoser & Rieger 2005 (see below). *M. mirumnovem* and all three hypodermically
192 inseminating species can self-fertilize (Ramm et al. 2012, 2015; Giannakara and Ramm 2017;
193 Singh et al. 2020; this study). We classified species as either reciprocally mating or
194 hypodermically inseminating using both behavioural and morphological data. Behavioural
195 data showed the presence of both reciprocal mating and the postcopulatory suck behaviour in
196 *M. lignano* (also see Schärer et al. 2004a), *M. janickei*, *M. cliftonensis*, and *M. mirumnovem*
197 (Schärer et al. 2020), while neither of these behaviours were observed in *M. pusillum*,
198 *M. hystrix*, and *Macrostomum* sp. 22 (Singh et al., in prep.). In addition, the classification of
199 species using morphological traits, which is known to be correlated with the mating strategy
200 in *Macrostomum* (Schärer et al. 2011), corroborated our above classification based on
201 behaviour. Briefly, morphologically, species were classified as reciprocal mating or
202 hypodermically inseminating depending on their male and female genital morphology, the
203 sperm morphology, and the location of (received) allosperm (Brand et al., in prep.).

204 ***SA estimates and SA plasticity effect sizes across species***

205 For most species the experimental procedure was similar to what we describe for
206 *Macrostomum* sp. 22 (Supplementary Information S1), i.e., worms were raised from juveniles
207 in three different group sizes (isolated, pairs or octets) until adulthood, when testis and ovary
208 size was measured in one randomly chosen worm per replicate. The SA was estimated as
209 testis size/(testis size+ovary size). In some experiments, the density of worms was varied
210 independently of group size by using two enclosure sizes (small and large) (for details and
211 sample sizes see Supplementary Table S2). We expect that the experiments are nevertheless

212 comparable, since all experiments where density was included as a factor revealed that it did
213 not have a significant effect on our estimate of SA.

214 For each experiment, we calculated the average SA across the group sizes, weighted by the
215 sample size for each group size. For SA plasticity estimates, we calculated standardised effect
216 sizes, using Cohen's d (Cohen 1988) with Hedges correction for small sample size (Hedges
217 1981), including confidence intervals (Howell 2011) of the effect sizes using the R package
218 'effsize' (Torchiano 2017), to facilitate interspecies comparisons. For each species, we
219 calculated an effect size for SA plasticity to assess the effect of the presence of mating
220 partners (i.e. comparing isolated worms vs. worms with partners) and the strength of LSC (i.e.
221 comparing worms in pairs vs. worms in octets). Pairs represent conditions with high LSC
222 whereas octets represent conditions with low LSC.

223 For *M. lignano*, we collected datasets from multiple published (up to 25th November 2019)
224 experiments, and calculated SA and SA plasticity effect sizes for each of them separately. In
225 total, we found two and six studies, respectively, where we could extract effect of the
226 presence of mating partners (Schärer and Janicke 2009; Ramm et al. 2019) and the strength of
227 LSC (Schärer and Ladurner 2003; Schärer et al. 2004b; Janicke and Schärer 2009; Janicke et
228 al. 2013; Marie-Orleach et al. 2014; Ramm et al. 2019). For Schärer and Ladurner (2003), we
229 calculated SA as testis size/(testis size+ovary size), since that study only gives values for
230 testis and ovary size, but not SA. We excluded one previous study (Janicke and Schärer 2010)
231 from our analysis, since the worms used in that study came from the same experiment as
232 Janicke and Schärer (2009), and so these were not independent datasets (we used the latter
233 study since it had more replicates). In addition, in Marie-Orleach et al. (2014) we combined
234 the data from the two different inbred lines (HUB1 and DV1), since they did not differ in any
235 of the traits measured. Also, note that in Schärer and Janicke (2009), there are only isolated
236 and paired worms and hence the estimated effect size for the presence of mating partners does

237 not include the effect of octets. This should not have a large effect on the calculated effect
238 size, since in most cases the difference between isolated and pairs is much larger than the
239 difference between paired and octet treatments (Figure 1).

240 For *M. lignano*, a weighted average across experiments was used to estimate the weighted
241 SA, and estimated SA plasticity effect sizes (Turner and Bernard 2006) for both the presence
242 of mating partners and the strength of LSC. For SA, we took the average of the weighted SA
243 across experiments, weighted by the total sample size of that experiment. To weight each
244 effect size, it was multiplied by the inverse of its variance (inverse variance weight, IVW),
245 which allowed us to take into consideration the differences in sample size across experiments,
246 namely:

247 $$Hedges's\ g^* = \frac{\sum_{i=1}^{n} Hedges's\ g_i \times IVW_{Hedges's\ g_i}}{\sum_{i=1}^{n} IVW_{Hedges's\ g_i}}$$

248 And its standard error was calculated as:

249 $$SE_{g^*} = \sqrt{\frac{1}{\sum_{i=1}^{n} IVW_{Hedges's\ g_i}}}$$

250 ***Evolution of SA and SA plasticity***

251 For the phylogenetically corrected analyses of the SA and SA plasticity effect sizes, we used a
252 trimmed (*ape* package, Paradis et al. 2004) ultrametric phylogenetic tree of the *Macrostomum*
253 genus, which is based on large-scale phylogenomic analyses of *de novo* transcriptomes (C-IQ-
254 TREE, Brand et al., in prep.). We estimated the phylogenetic signal using Blomberg's K
255 (*picante* package, Blomberg et al. 2003; Kembel et al. 2010) and Pagel's λ (*phytools* package,
256 Pagel 1999; Revell 2012), for SA and both SA plasticity effect sizes. For both estimates of
257 phylogenetic signal, a value close to zero is suggestive of phylogenetic independence, while a
258 value close to 1 suggests that the traits evolve under Brownian motion. In our case, the

259 estimates of phylogenetic signal for SA showed a phylogenetic signal, while the two SA
260 plasticity effect sizes did not differ significantly from 0 (Supplementary Table S3). Since our
261 sample sizes are small, the likelihood ratio tests used to assess Pagel's λ can be unreliable
262 (Boettiger et al. 2012), as the asymptotic properties of maximum-likelihood estimation may
263 not hold for small sample sizes. Thus, we present both phylogenetically corrected analyses
264 and analyses without correcting for phylogeny.

265 For the phylogenetically corrected analyses, we compared the fit of different character
266 evolution models (i.e. Brownian motion, Ornstein–Uhlenbeck, and Early-burst) (*geiger*
267 package, Harmon et al. 2008) for SA and each effect size, and found that for each case
268 Brownian motion was the best character evolution model (see Supplementary Table S4).
269 Thus, we used Brownian motion as the preferred model for the subsequent analysis. Using
270 phylogenetic generalized least squares (PGLS) regressions and a Brownian motion model of
271 character evolution (*nlme* package, Pinheiro et al. 2014), we tested if the mating strategy
272 (reciprocal mating vs. hypodermic insemination) or self-fertilization (presence or absence)
273 predict SA, or the two SA plasticity effect sizes (presence of mating partners and strength of
274 LSC). PGLS allows us to account for the phylogenetic non-independence of observations
275 resulting from common evolutionary history of species. And for the analyses without
276 correcting for phylogeny, we calculated Wilcoxon rank-sum tests to assess if the mating
277 strategy or self-fertilization predicted the differences in SA or SA plasticity effect sizes.

278 Finally, to divide the observed variance in SA into a within-species and a between-species
279 component, we used the SA (weighted SA for *M. lignano*) for each group for each species
280 (thus, 3 observations per species), and fit a linear mixed model (*lme4* package, Bates et al.
281 2015) using species as a random effect and calculated the percentage of variance explained by
282 species.

283 All statistical analyses were carried out using R, version 3.4.1 (R Core Team 2017) unless
284 stated otherwise.

285 ## *Results*

286 ### *SA and effect sizes of SA plasticity for all species*

287 There was substantial variation in overall SA across the seven species (Figure 1,
288 Supplementary Table S2), both with respect to the mating strategy (solid vs. stippled lines)
289 and self-fertilization (circles vs. triangles). Two of the three hypodermically inseminating
290 species (i.e. *Macrostomum* sp. 22 and *M. pusillum)*, exhibited a low SA. Similarly, and in line
291 with our predictions, three of the four self-fertilizing species (*Macrostomum* sp. 22,
292 *M. pusillum* and *M. mirumnovem*), exhibited a relatively low SA, likely indicating that SA in
293 these species was female-biased. Interestingly, the exception to this pattern was *M. hystrix*,
294 which had the highest SA of all the studies species. There was also interspecific variation in
295 the effect size estimates of SA plasticity, even among relatively closely related species
296 (Figure 2, Supplementary Table S2). *M. cliftonensis* and *M. lignano* exhibited the highest SA
297 plasticity in response to the presence of mating partners and the strength of LSC, respectively.
298 In addition, *M. lignano* exhibited SA plasticity across all experiments and while the
299 magnitude varied somewhat, the confidence intervals overlapped, indicating that the estimates
300 were fairly consistent across experiments.

301 ### *Evolution of SA and SA plasticity*

302 The PGLS models (see Supplementary Table S3 and S4 for more details) showed that neither
303 the mating strategy ($t_{7,5}$ = -0.39, P = 0.70) nor the ability to self-fertilize ($t_{7,5}$ = -1.03, P =
304 0.34) significantly predicted SA. And similar to SA, the mating strategy also did not
305 significantly predict the SA plasticity effect size, neither due to the presence of mating
306 partners ($t_{7,5}$ = 0.94, P = 0.39) nor the strength of LSC ($t_{7,5}$ = 0.23, P = 0.82). In contrast, self-

307 fertilization significantly predicted SA plasticity with respect to the presence of mating
308 partners ($t_{7,5}$ = -3.07, P = 0.03), but not due to the strength of LSC ($t_{7,5}$ = -0.57, P = 0.59). SA
309 plasticity with respect to the presence of mating partners was lower for selfing species
310 (compare selfing vs. non-selfing species of green symbols in Figure 2). And essentially the
311 same results were observed in the analyses without correcting for phylogeny using Wilcoxon
312 rank-sum tests (Supplementary Table S5).

313 Lastly, the linear-mixed model (Intercept = 0.55±0.05, $t_{\text{-value}}$ = 10.38, 21 observations for 7
314 species) showed that between-species variance explained 73.6% of the total SA variance
315 (interspecific variance = 0.0177, and residual variance = 0.0063), suggesting that SA variance
316 between-species is nearly three times larger than variance within-species.

317 *Discussion*

318 Our study showed that there was substantial interspecific variation in both SA and SA
319 plasticity, also among relatively closely related species, suggesting that both SA and SA
320 plasticity can evolve rapidly in the *Macrostomum* genus. Moreover, although the mating
321 strategy did not predict SA or either of the SA plasticity effect sizes, self-fertilizing species
322 had a lower SA plasticity in response to presence of mating partner. In the following, we
323 discuss these findings in some detail.

324 *SA and effect sizes of SA plasticity for all species*

325 Very few studies have explored predictions of SA theory across multiple species in
326 hermaphroditic animals in the context of phenotypic plasticity (Hoch and Levinton 2012;
327 Schleicherová et al. 2014), with most studies focussing solely on intraspecific comparisons
328 (Schärer 2009). Hoch and Levinton (2012) tested Charnov's (1980, 1982) mating group size
329 model in two species of acorn barnacles, *Semibalanus balanoides* and *Balanus glandula,* by
330 varying both the number and density of competitors in a natural setting. They showed that

331 both species exhibit increased allocation in absolute, but not relative, male allocation
332 (estimated using the sum of the mass of testes, sperm, seminal vesicle, and penis) at higher
333 densities. Interestingly, the species differed in how they responded to the treatments in terms
334 of their female allocation (estimated using egg mass), with both the number and density of
335 competitors having an effect in *S. balanoides*—although in the opposite direction than
336 predicted by the model, while there was no effect in *B. glandula.* The authors proposed that
337 this might result from interspecific differences in life-history traits. In contrast to the acorn
338 barnacles, a study in three related polychaete worms, *Ophryotrocha diadema, O. adherens*
339 and *O. gracilis*, showed that while there were interspecific differences in the plasticity in
340 female allocation (using multiple estimates, such as resource investment in eggs, total number
341 of egg cocoons, and time interval between egg laying) in response to different numbers of
342 mating partners, there was no significant plasticity in the male function (estimated as the
343 number of sperm produced by an individual) across the species (Schleicherová et al. 2014).
344 The authors proposed that the magnitude of plasticity depended on the species-specific costs
345 of the sex function, and also on the mating system of the species. So, while both of these
346 studies document interspecific variation in SA plasticity, they lacked the statistical power to
347 explore whether interspecific differences in reproductive traits may affect the level of SA
348 plasticity.

349 Our results showed that both SA and SA plasticity varied considerably across the studied
350 *Macrostomum* species. Both hypodermically inseminating and self-fertilizing species tended
351 to show a low (i.e. more female-biased) SA, with the notable exception of *M. hystrix*.
352 *M. hystrix* represents an independent origin of hypodermic insemination in the reciprocally
353 mating clade (Schärer et al. 2011), and it might therefore still retain characteristics of its
354 reciprocally mating ancestors, which in the current sample of species appear to have relatively
355 high (i.e. more male-biased) SA (see Figure 1), and our analyses suggested some level of

356 phylogenetic signal in SA. Moreover, it has been argued that hypodermic insemination may
357 lead to sperm competing more in a fair-raffle-type scenario than with reciprocal mating
358 (Schärer and Janicke 2009), which might lead to a SA that more closely matches the group
359 size. In addition, *M. hystrix* is known to reach very high densities in the field (L. Schärer,
360 pers. obs.), which could also favour the evolution of a high SA.

361 *M. lignano* and *M. janickei,* which are sibling species capable of hybridization (Singh et al.
362 2020a), show clear differences in SA plasticity in response to the strength of LSC, with
363 *M. lignano* being more plastic (although the 95% confidence intervals overlap compared to
364 two of the *M. lignano* studies). Variation in SA plasticity in species with similar reproductive
365 biology could stem from different environmental conditions experienced by the species. For
366 example, evolution of SA plasticity might not be favoured in species that inhabit relatively
367 stable environments, especially if the maintenance of plasticity is costly (DeWitt et al. 1998;
368 Auld et al. 2010; Siljestam and Östman 2017). Even in the absence of maintenance costs,
369 there could still be production costs of plasticity, though if the benefits of adjusting SA
370 outweigh these costs, then phenotypic plasticity could still be maintained. This cost-benefit
371 ratio of SA plasticity can vary across species (Van Buskirk 2002; Steiner 2007) and
372 environments (Ratikainen and Kokko 2019), leading to the retention or loss of plasticity in a
373 given species. Moreover, such costs of SA plasticity might play an important role in both the
374 evolution and the maintenance of simultaneous hermaphroditism (St. Mary 1997), and could
375 also constrain changes in SA leading to a suboptimal SA. A study in *O. diadema* showed that
376 there were no large short-term fitness costs of sex adjustment for the species (Lorenzi et al.
377 2008), while in *M. lignano* production costs of SA plasticity have been documented (Sandner
378 2013), with an alternating group size environment leading to a lower hatchling production
379 compared to a stable group size environment. We currently do not have data on production
380 costs of plasticity in *M. janickei* when exposed to a stable vs. fluctuating environment.

381 Across all species, the estimates of plasticity effect sizes are larger for the presence of mating
382 partners than for the strength of LSC (number of mating partners), and for *M. cliftonensis* and
383 *M. janickei*, the confidence intervals of the two effect size estimates do not overlap. This
384 suggests that the increase in SA going from paired to octet groups is not as drastic as going
385 from isolated worms to worms in larger groups. A similar phenomenon has been seen in a
386 freshwater snail, *Lymnaea stagnalis*, where paired snails showed higher expression of six
387 seminal fluid protein (SFP) genes compared to isolated snails, while the SFP expression of
388 snails in larger (6 individuals) groups was similar to that of paired snails (Nakadera et al.
389 2019). In our study, this situation could potentially arise if in the presence of multiple partners
390 in octets, worms do not only increase their testis size, but also their rate of sperm production
391 per unit testis size (Schärer and Vizoso 2007), which in *M. lignano* has been shown to occur
392 due to an increased speed of spermatogenesis (Giannakara et al. 2016). If such effects occur
393 generally (but see Giannakara & Ramm 2017 for *M. pusillum*, a species that lacks SA
394 plasticity, see also below), we might underestimate male allocation when solely measuring
395 testis size. Alternatively, there could also be increased investment in other components of the
396 male function (Schärer and Pen 2013), e.g. seminal fluid production (Patlar and Ramm 2019;
397 Patlar et al. 2019; Ramm et al. 2019), or mating behaviour (Janicke and Schärer 2009). A
398 study in *O. diadema* showed that increased mating opportunities are accompanied by higher
399 investment into male function, such as aggressive behaviour (Santi et al. 2018).

400 ***Evolution of SA and SA plasticity***

401 The SA plasticity effect sizes did not differ significantly between species exhibiting the
402 reciprocal and hypodermic mating strategies, while self-fertilizing species had a lower SA
403 plasticity (in response to presence of mating partner, but not the strength of LSC). Interesting
404 questions that arise then are about the causes of the variation in SA and SA plasticity between
405 the self-fertilizing species. While there has been both theoretical and empirical work

406 exploring the effect of self-fertilization on SA in plants (Lemen 1980; Charlesworth and
407 Charlesworth 1981; Schoen 1982; Charnov 1987; McKone 1987; Brunet 1992), there have
408 been few such studies in animals (Johnston et al. 1998). One possible explanation for
409 variation between self-fertilizing species could be if the species differ in their rate and pattern
410 of self-fertilization, which could have an effect on both optimal SA and SA plasticity. For
411 example, in obligatorily self-fertilizing species, we would expect that they do not increase
412 their SA with group size, since LSC would be expected to remain high irrespective of the
413 group size. On the other hand, for species that exhibit facultative self-fertilization, we might
414 expect SA plasticity despite a low overall SA.

415 Interestingly, our results do conform somewhat to this pattern, with *M. pusillum*—which has
416 been hypothesised to be obligatorily self-fertilizing (Giannakara and Ramm 2017)—showing
417 both the lowest overall SA and no SA plasticity. Its congener, *Macrostomum* sp. 22, showed a
418 similarly female-biased SA in isolated and paired worms, but significant SA plasticity
419 between worms in pairs and octets. If worms shifted from self-fertilization in isolation to only
420 outcrossing in pairs, then the strength of LSC would not be expected to change, while the
421 strength of LSC would clearly be expected to drop from worms in pairs to octets, a scenario
422 that would match the observed SA plasticity patterns. This could suggest that
423 *Macrostomum* sp. 22 may only self in isolation. In contrast, *M. hystrix*—thought to be a
424 preferentially outcrossing species, with studies showing delayed selfing, at least in some
425 populations, as well as costs of self-fertilization (Ramm et al. 2012; Giannakara and Ramm
426 2020)—was found to be more plastic than *M. pusillum.*

427 Finally, our results showed that there was nearly three times more variation in SA between-
428 species than within-species in our experiments. This is remarkable, since we saw extensive
429 plasticity in SA in certain species, such as across the multiple experiments in *M. lignano*.
430 Thus, this result suggests that we can interpret variation in SA among field collected worms

of different *Macrostomum* species as being at least partially due to interspecific variation, even if they come from different social environments. This therefore facilitates the study of the evolution of SA patterns across species that cannot currently be cultured in the laboratory (Brand et al., in prep.). Collectively, our results suggest that SA and SA plasticity can evolve rapidly in *Macrostomum*, with self-fertilization being an important predictor for the latter.

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607 ***Figures***

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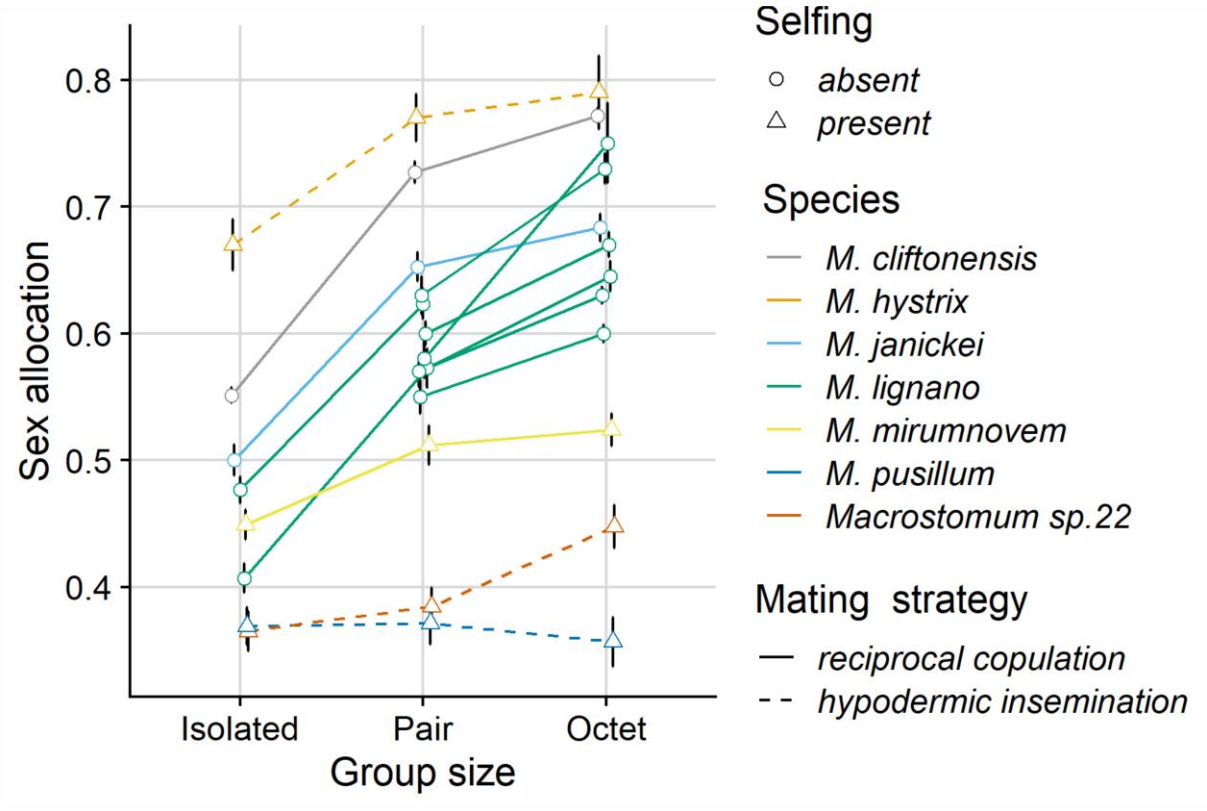


609

610 Figure 1. Effect of group size on estimates of sex allocation in seven different *Macrostomum*

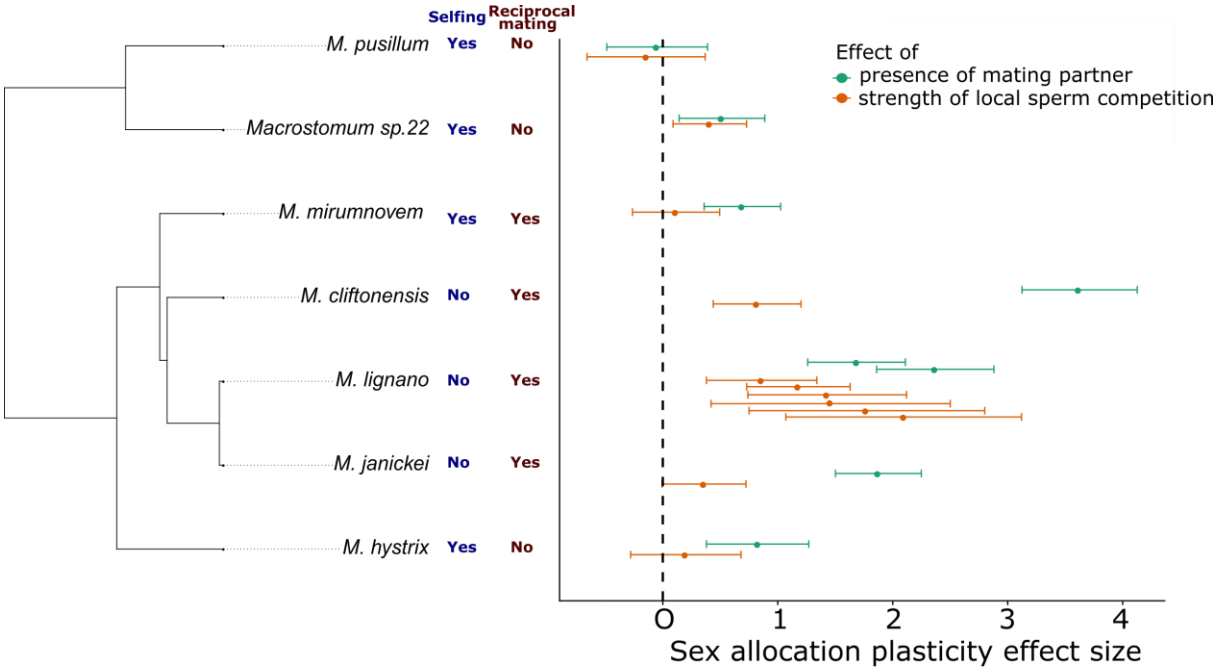
611 species (given by different colours). The line types represent the two mating strategies and the

612 symbols represent the ability to self-fertilize in *Macrostomum*. The plots show means and

613 standard errors of the raw (untransformed) data. Note that for *M. lignano*, data from seven

614 independent experiments are shown.

615



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617 Figure 2. Standardized SA plasticity effect sizes for the effect of the presence of mating
618 partners (i.e. comparing isolated worms vs. worms with partners) and the strength of LSC (i.e.
619 comparing worms in pairs vs. octets) in seven *Macrostomum* species, along with data on
620 whether the species exhibit self-fertilization or/and reciprocal mating. Note that for
621 *M. lignano*, data from multiple experiments are shown. The error bars represent 95%
622 confidence intervals.