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**Demographic costs of sex allocation: hermaphrodites perform better in sparse populations**

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**Running title:** reproductive load in simultaneous hermaphrodites

39 Abstract

40 *Ophryotrocha diadema* is an outcrossing, simultaneous hermaphroditic polychaete with  
41 external fertilization. In isolated pairs, mature worms take turn contributing eggs upon the  
42 condition that their partners reciprocate egg donation. In dense populations, these worms do  
43 not reciprocate. Instead, they strongly compete for mating in their preferred male role and  
44 produce few eggs. This plastic sex allocation may result in an overall different reproductive  
45 performance: mean individual reproductive output will be larger in sparse than in dense  
46 populations. We tested this hypothesis by measuring the individual reproductive output  
47 (paternal *and* maternal offspring) of worms in sparse and dense replicated populations. In  
48 dense populations, mean individual reproductive output was fourfold lower than that in sparse  
49 populations. We hypothesise that such dramatic demographic costs are potentially widespread  
50 in outcrossing simultaneous hermaphrodites with external fertilization and plastic sex  
51 allocation. The reproductive output of hermaphroditic organisms is a function of population  
52 density (i.e., the number of conspecifics) and studies on population growth and reproductive  
53 performance should take this effect into account.

54

55 **Keywords:** reproductive load, tragedy of the commons, population size, mating, *Ophryotrocha*  
56 *diadema*

57

58

**Comment [CR1]:** I would suggest not to include this in the keyword: it is mentioned at the very end of the study, only

**Comment [MCL2]:** We would prefer to leave it here: there are only few examples of this phenomenon in the literature. This paper could be more easily picked up by searching engines and cited, if this term appears in the keywords

59

## 60 Introduction

61 Simultaneous hermaphrodites have two sexual functions and partition their reproductive  
62 resources between them. Sex allocation theory predicts that they plastically adjust the proportion of  
63 resources allocated to each sex as a function of mating group size (Charnov 1982). Theory predicts  
64 that in sparse populations, where monogamous pairs may form, hermaphrodites increase their  
65 investment in eggs and limit the male function to the production of the few sperm needed to fertilize  
66 their partners' eggs. In dense populations, hermaphrodites divert resources from egg production and  
67 invest more into the male function. Experiments or observations on different hermaphrodites have  
68 tested this theory and have often found overall support for it, although the patterns of resource  
69 allocation adjustments are highly species-specific (Raimondi & Martin 1991; Trouvè et al. 1999;  
70 Schärer & Janicke 2009; Locher & Baur 2002; Hughes et al. 2002; Tan et al. 2004; Brauer et al.  
71 2007). In some model systems, results show that sex allocation in hermaphrodites is plastic (i.e. it  
72 changes as a function of mating opportunities), as predicted by theory. For example, the  
73 hermaphroditic polychaete worms *Ophryotrocha diadema* Åkesson 1976 (Annelida: Polychaeta:  
74 Dorvilleidae) have plastic female allocation that they adjust to mating opportunities, trading-off  
75 with their investment in the male function. When mating opportunities are common (as in dense  
76 populations), worms reduce their egg production drastically and compete for mating in the male  
77 role; when mating opportunities are rare (as in sparse populations), they invest proportionally more  
78 resources in egg production and, in the absence of competitors, reduce their investment into the  
79 male function (Lorenzi et al. 2005, 2006). Sex allocation adjustments are the effect of sexual  
80 selection acting on both sexual functions in hermaphrodites (Lorenzi and Sella, 2008; Anthes et al.  
81 2010). These adjustments are typically hermaphroditic traits, and could explain why population  
82 growth rates (as measured in dense, lab populations) are higher in gonochoric than hermaphroditic  
83 species (Prevedelli et al. 2006).

**Comment [MCL3]:** We prefer to start with the definition of hermaphroditism and introduce the fact that some hermaphrodites alternate between sex roles later on in the manuscript, because in the specific literature, scientists distinguish between functions and roles. E.g. each simultaneous hermaphrodite has a male function but chooses whether it will play the male role depending on its partner(s) condition. We stay stuck to this definition throughout the manuscript.

**Comment [MCL4]:** Sperm has two plural forms either sperm or sperms but the first is more common in the specific literature

**Deleted:**

**Comment [MCL5]:** We prefer to use the word adjustment because we already quoted this ability at line 52

**Comment [MCL6]:** We would prefer to stay with "common". In the first review round, Referee #2 suggested the use of the word "common" (see referee #2, who suggested the following: Minor point #5): "L63 consider 'common' instead of high (and rephrase the stuff in parentheses)"

**Comment [CR7]:** Explain: it is rather unclear!!

**Comment [MCL8]:** We clarified the text

85 If we assume a fixed budget for reproductive resources, we expect that hermaphrodites in large  
86 populations would use the same amount of resources for egg production than hermaphrodites in  
87 small populations, devalued of the resources diverted to increase the male function. Then we should  
88 find that hermaphrodites in large populations have a lower mean reproductive success than those in  
89 small populations. This reduced reproductive output should, in turn, affect population growth. We  
90 tested this hypothesis in the outcrossing simultaneously hermaphroditic polychaete worm *O.*  
91 *diadema* by measuring individual reproductive output of focal worms in sparse and dense  
92 populations.

93

#### 94 **Material and methods**

95 The animal model

96 *O. diadema* (Annelida, Polychaeta, Dorvilleidae) is a polychaete worm originally found in the  
97 sediments of Californian harbors. Sampling from natural populations suggests that populations have  
98 low densities (Premoli & Sella 1995). For example, only few *O. diadema* individuals were isolated  
99 among hundreds of worms of a gonochoric *Ophryotrocha* species in the Pacific Coast (pers. comm.  
100 by B. Åkesson to G.S.) and 0.1–6.6 individuals per kg<sup>-1</sup> of mussel clusters were collected in the  
101 Mediterranean Sea (Simonini, pers. comm. to M.C.L.) (Schleicherová et al. 2013).  
102 These worms are outcrossing simultaneous hermaphrodites with external fertilization. Before  
103 maturing as hermaphrodites, they have a protandrous phase during which they can fertilize the eggs  
104 laid by hermaphrodites (Sella & Lorenzi 2003). Then, they mature as hermaphrodites, and can both  
105 fertilize their partners' eggs or lay eggs, but play one single role at each mating event. Eggs are laid  
106 in jelly cocoons and develop into larvae that leave their cocoons 8 days later and mature into  
107 simultaneous hermaphrodites in approx. 45 days. Mature hermaphrodites reproduce iteroparously  
108 for 7-10 weeks (Åkesson 1976, 1982).  
109 In isolated pairs, worms take turns in laying cocoons of 20-25 eggs every third day (Sella 1985,  
110 1988). When more than two worms are present, they adjust their sex allocation by investing

**Comment [CR9]:** If mean egg production of hermaphrodites were similar in sparse and dense populations, then the amount of resources allocated to increase the male function could reduce the reproduction contribution, and hermaphrodites in dense populations would reproduce less.

**Comment [MCL10]:** We changed the previous sentence to clarify its meaning, following comment CR9

**Deleted:** Polycheta

**Comment [MCL11]:** We used "Californian" following the suggestion by referee #2 (see Minor comments, L78 remove 'which was' and make it 'Californian')

**Comment [MCL12]:** A few *O. diadema* worms were found among hundreds of worms of a (single) gonochoric species.

112 proportionally more resources into the male function (Lorenzi et al. 2005, 2006), mate  
113 promiscuously (Sella & Lorenzi 2000) and can share the paternity of a single egg-cocoon with other  
114 hermaphrodites (Lorenzi et al. 2013). Sex allocation adjustments are not costly in the short term  
115 (Lorenzi et al. 2008) and polychaetes sense the number of conspecifics and/or potential mates  
116 through waterborne chemical cues (Schleicherová et al. 2006, 2010; Minetti et al., 2013).

### 117 ***Experimental procedure***

118 Data were gathered from focal worms. The 'focal' worms were identified through the colour of  
119 their eggs. In mature worms, eggs can be easily detected through the transparent body wall as  
120 either yellow or whitish eggs. In these worms, a dominant Y allele determines a yellow-egg  
121 phenotype, while the recessive y allele determines a white-egg phenotype (Sella and Marzona  
122 1983). By means of this genetic marker, we can identify focal worms in a group and ascribe their  
123 progeny. The focal worms had yellow eggs and their mates white eggs.

124 We carried out the experiment in glass bowls filled with 10-ml artificial sea-water and kept in a  
125 thermostatic chamber at 20°C. Once a week water was replaced in the bowls and worms were fed  
126 with spinach *ad libitum*.

127 To obtain a sufficient number of worms for the experiment, 24 pairs of yellow-phenotype worms  
128 and 40 pairs of white ones were cultivated separately and allowed to reproduce. Their offspring  
129 supplied the virgin, newly mature, yellow- and white-phenotype worms of same age to be used for  
130 the experiment. At sexual maturity, two worms from each yellow-phenotype offspring (n = 48  
131 worms, hereafter, 'focal worms') were randomly assigned either to sparse populations (population  
132 size = 2; the population consisting of one focal, yellow-phenotype worm and one white-phenotype  
133 partner, n = 24 replicates) or to dense populations (population size = 12, consisting of one focal,  
134 yellow-phenotype worm and 11 white-phenotype potential partners, n = 24 replicates). With such a  
135 matched-sample design, each worm in the sparse population served as a control for its sibling in the  
136 dense population.

**Comment [MCL13]:** We clarified the text and added details.

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**Comment [CR14]:** Are worms under crowdy or sparse conditions kept in same volume?

**Comment [MCL15]:** All bowls had a volume of 10 ml (this info is reported at line 109)

138 Experimental populations were checked daily for 12 days. At the first check, focal worms' body  
139 size was measured as the number of chaetigerous segments. At each check, we recorded the number  
140 of yellow (laid by focal worms) and white cocoons (laid by focal partner/s), and the number of eggs  
141 per cocoon. Adult worms were removed from the bowls on day 9.

142 Reproductive output of focal worms was quantified by rearing offspring until they were sexually  
143 mature. Indeed, in large populations multiple potential "fathers" were present and paternity of the  
144 progeny could be assigned to either the focal worms or one of their rivals only after the progenies  
145 were sexually mature. When these worms matured and had eggs in their coeloms, they expressed  
146 their yellow or white phenotypes and we assessed their paternity (i.e., about 45 days after egg  
147 laying). Following Åkesson (1976), this marker is neutral, since there is no difference in worm  
148 mortality rates before sexual maturity.

149 The ratio between the total number of cocoons produced in sparse populations and that produced in  
150 dense populations was approx 1:2, leading to more larvae per unit of volume in the dense  
151 population bowls. Therefore, to standardize rearing conditions, on day 9 the volume of sea water  
152 was doubled in the dense population bowls.

153 The total reproductive output of focal worms was estimated as the number of offspring (both  
154 maternal *and* paternal offspring) that on maturity had the yellow phenotype. Focal worms without  
155 offspring were included in the calculations.

156 In order to control for the potentially confounding effect of differential egg-mortality in sparse and  
157 dense populations, we estimated egg mortality as the average proportion of eggs that disappeared  
158 from the cocoons in each bowl (with respect to the laid eggs).

159

### 160 *Statistical analyses*

161 Some replicates were excluded from [calculations](#) for various reasons (e.g., some worms died  
162 altering population size). By using related worms in sparse and dense populations, we reduced the  
163 overall variability due to genetic differences (Howell 2010). We used a linear mixed model (LMM)

**Comment [MCL16]:** We clarified the text and added details.

**Comment [MCL17]:** We clarified the text as requested

**Deleted:** caluclations



165 to assess the significance of the differences in reproductive output between pairs of siblings in  
166 sparse and dense populations (dependent variable: reproductive output; within-subject factor:  
167 population size; random factor: family ID; covariate: body size).  
168 Probabilities were two-tailed. Statistical analyses were performed using SPSS 20.0 statistical  
169 package (SPSS Inc, Chicago, IL).

170

## 171 **Results**

172

### 173 ***Reproductive output of focal worms***

174 Focal worms had a dramatically lower reproductive rate in dense populations, compared to that in  
175 sparse populations, with a fourfold reduction in their reproductive output (mature maternal +  
176 paternal offspring) (Fig 1). The difference was highly significant, indicating that reproductive  
177 output in simultaneous hermaphrodites was strongly affected by population size (LMM, population  
178 size:  $F_{1,35.79} = 55.557$ ,  $P < 0.0001$ ; body size:  $F_{1,34.99} = 4.721$ ,  $P = 0.037$ ). The significant relationship  
179 between body size and reproductive output which emerged in the LMM occurred only in dense  
180 populations (Spearman's rho, in dense populations:  $\rho = 0.496$ ,  $P = 0.022$ ; in sparse populations:  
181  $\rho = 0.133$ ,  $P = 0.545$ ). Body size advantage in dense populations was not associated with the  
182 female function (correlation between body size and egg production in dense populations:  $\rho =$   
183  $0.180$ ,  $P = 0.460$ ; in sparse populations:  $\rho = 0.288$ ,  $P = 0.231$ ). This suggests that larger  
184 hermaphrodites in dense populations might have a higher reproductive output because they were  
185 more successful in the competition for the male role.

186 Egg mortality was not significantly different between sparse and dense populations (Wilcoxon test,  
187  $Z = 73.00$ , total  $n = 20$ ,  $P = 0.376$ ), suggesting that it did not affect the results (median proportion of  
188 eggs which disappeared in sparse populations: 5.56 % vs 4.86% in dense populations).

189

## 190 **Discussion**

Comment [MCL18]: We clarified the sentence

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Comment [MCL19]: We deleted the sentence that should have been moved to discussion. We left a short comment at the end of the sentence, to help the reader to understand the meaning of these results..

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194 In this study we document that population size (i.e. the number of conspecifics) affects the  
195 reproductive output of simultaneous hermaphrodites as they produce four times more offspring in  
196 sparse than in dense populations. We interpret these results as a consequence of the fact that  
197 hermaphrodites have a plastic sex allocation which they adjust to mating opportunities. As  
198 population size increases, mating opportunities increase as well and hermaphrodites adjust their sex  
199 allocation in favour of the male function at the expense of the female function.

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200 The reduced reproductive output of the worms in dense vs. sparse populations could be the result of  
201 uncontrolled density-dependent effects (e.g., mortality, oophagy, etc.) rather than a response to  
202 population size (e.g., the number of conspecifics). However, this hypothesis is not supported by  
203 evidence. First, egg mortality did not differ between sparse and dense populations. Second, a  
204 previous study documented that *O. diadema* worms had a higher egg production in sparse than in  
205 dense populations, irrespective of any density-dependent effects such as metabolite accumulation or  
206 encounter probability (Lorenzi et al. 2005). Furthermore, in other experiments, we simulated large  
207 population size, so that pairs of worms perceived cues as if population size were larger than two,  
208 and they reduced their egg output according to the perceived, and not the real, population size  
209 (Schleicherová et al. 2006, 2010). All these observations support the hypothesis that worms reduce  
210 their egg output as population size increases.

Comment [MCL20]: We clarified the difference between density-dependent effects and population size

Comment [MCL21]: We used the same term as above, to highlight the difference between density-dependent effects and population size

211 It could be argued that, if worms decrease egg production in dense populations, the competition for  
212 mating as males should increase and worms with more female-biased allocation will gain higher  
213 reproductive success. Whilst this might be true in the short term, it might be disadvantageous in the  
214 long term, because fecundity often trades off with lifespan (Stearns & Hoekstra 2000). Indeed,  
215 hermaphrodites which skip the female role for long time periods live longer (Di Bona et al. 2010).

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216 Mating in sparse populations is associated with small mating groups, i.e., low numbers of partners  
217 and few or no rivals over the male role. In small mating groups, hermaphrodites invest large  
218 proportions of their reproductive resources into eggs, trade eggs with their partners and take turns in  
219 the two sexual roles (Sella, 1985; Sella & Ramella 1999). This is an evolutionary solution to the

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224 conflict on sex roles, since both partners prefer to play the cheaper male role than the expensive  
225 female role (Leonard 1993, 2005, 2006; Di Bona et al. 2010). In natural contexts, outcrossing  
226 hermaphrodites may be constrained to monogamous mating regimes when they live in very sparse  
227 populations, as *O. diadema* (Sella & Ramella 1999; Simonini, personal communication). In other  
228 hermaphroditic species, the sizes of the populations are large but hermaphrodites are trapped in  
229 monogamous mating regimes by other life-history traits. For example, the serranid fish,  
230 *Hypoplectrus nigricans* is an outcrossing hermaphrodite which mates monogamously (Fischer  
231 1980). Here, monogamy is constrained by the short spawning period (few hours per day), which  
232 reduces the chances that paired partners desert: reproductive gains from deserting the partner may  
233 be low, if most partners are paired.

234 In present study, worms in dense populations reduced their reproductive output to less than 30%  
235 when compared to worms in sparse populations. Similarly, *Plasmodium chabaudi* adjust their sex  
236 allocation in response to the presence of unrelated conspecifics. Reece et al. (2008) directly  
237 manipulated mating-group sex ratio of these malaria parasites and measured the resulting  
238 reproductive output as the number of zygotes produced. As predicted by sex allocation theory,  
239 mating output was maximized at intermediate sex ratios, indicating that sex allocation in this  
240 malaria parasite is likely to be under stabilizing selection and reproductive output was maximized at  
241 female-biased sex ratios.

242 Overall, our study shows that the potential individual advantages in fitness due to opportunistic sex  
243 allocation are countered at the population level when populations are dense; opportunistic sex  
244 allocation is advantageous to the individual, but disadvantageous to the population, whose  
245 reproductive rate declines. Accordingly, Prevedelli et al. (2006) found that dense populations of  
246 hermaphrodites had a demographic disadvantage compared to gonochorists. Here, we highlight that  
247 the demographic disadvantage of hermaphrodites is mainly due to their adaptive ability to adjust  
248 their sex allocation to mating group size and, ultimately, to population size. In this perspective, our  
249 study is an example of the tragedy of the commons (Hardin 1968), where traits which are

**Comment [MCL22]:** We changed the term using the words used by Reece et al use in their Nature paper.

**Comment [CR23]:** A bit confused. To be rephrased.

**Comment [MCL24]:** We added the explanation

250 advantageous at the individual level reduce population fitness. For example, strong cannibalism of  
251 larvae and pupae by adult flour beetles is adaptive at individual level but impairs population growth  
252 (Wade 1977). Similarly, hyperaggressive water-strider males gain a slightly higher mating success  
253 than less aggressive males but reduce overall group mating in their pond (Chang & Sih 2013). More  
254 specifically, sexual selection can diminish population reproductive rates of *Drosophila* populations  
255 by imposing a “reproductive load” (Holland & Rice 1999). The reproductive load highlighted in  
256 *Drosophila* was caused by antagonist sexual selection and intersexual conflicts inherent to  
257 promiscuity. Similarly, sex allocation adjustments are promoted by sexual selection acting on the  
258 two sexes of simultaneous hermaphrodites (Lorenzi & Sella 2008; Anthes et al. 2010; Leonard  
259 2013).

260 We highlight that the demographic advantage of hermaphroditism in sparse populations (relative to  
261 dense populations) is the bare outcome of sex allocation adjustments in hermaphrodites where the  
262 two sexual functions interfere with each other and resources are traded off between the male and  
263 female function (Lorenzi et al. 2006). Therefore, we expect that the results we obtained here could  
264 be obtained in other hermaphroditic systems as well, where the two sexual functions act in  
265 opposition and resources are partitioned between the male and the female function on the basis of  
266 population size. We hypothesise that such dramatic demographic costs of sex allocation are  
267 potentially widespread in outcrossing simultaneous hermaphrodites with external fertilization. If the  
268 reproductive output of hermaphroditic organisms is a function of population size, population growth  
269 studies (and their practical applications) should take the effect of sex allocation into account.

270

**Comment [MCL25]:** This is the term used by Chang and Sih 2013

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273 **Acknowledgements**

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278 University of Turin (ex 60% to M.C.L. and G.S.).

279

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371

372 Figure legend

373 Figure 1. The reproductive output of focal worms (maternal and paternal offspring) in sparse and  
374 dense populations (mean  $\pm$  s.e.).

