Labile Sex Expression and the Evolution of Dioecy in Ophryotrocha Polychaete Worms

(Article begins on next page)

05 May 2024

UNIVERSITÀ DEGLI STUDI DI TORINO

1

2

This is an author version of the contribution published
on: Questa è la versione dell'autore
dell'opera: [**Evolutionary Biology DOI: 10.1007/s11692-014-**
9297-0]

The definitive version is available at:

La versione definitiva è disponibile alla URL:

[**http://link.springer.com/article/10.1007/s11692-014-9297-0**]

11

Labile sex expression and the evolution of dioecy in *Ophryotrocha*

polychaete worms

14

15

16

17

18   S. Meconcelli[§], M. C. Lorenzi[§] , G. Sella[§]

19

20

21   [§] Department of Life Sciences and Systems Biology, Università di Torino, Turin, Italy.

22

23

24

25

26

27   *correspondence:*   Stefania Meconcelli, Department of Life Sciences and Systems Biology,

28   Università di Torino, Via Accademia Albertina 13, 10123 Turin, Italy. Tel.: +39 011 670

29   4511; e-mail: stefania.meconcelli@unito.it

30

31   Short title: Labile sex expression in *Ophryotrocha* worms

32

33

34

Abstract

Labile sex expression is considered to play a key role in the evolution of breeding systems and in the transition from hermaphroditism to dioecy, according to the evolutionary models proposed for plants. While in hermaphrodites sex allocation within the individual can be plastically adjusted in response to social environment, in dioecious species it is predicted to be fixed. However, labile sex expression in the form of gender plasticity can still be present in dioecious species of animals with environmental sex determination. It is still unclear how gender plasticity is involved in the evolution of breeding systems and what its role is in the transition from hermaphroditism to dioecy. We assessed the degree of plasticity in gender expression in three dioecious species of polychaete worms of the genus *Ophryotrocha*. We found sexual polymorphism and plasticity in sex expression during the juvenile phase to be a response to social environment. The majority of juveniles reared with an adult female or male expressed the gender opposite of that of the partner, so as to form heterosexual pairs. On the basis of these findings we outline a possible evolutionary pathway of the transition from hermaphroditism to dioecy in the genus *Ophryotrocha*.

Keywords:

Gender plasticity; pseudohermaphrodite; monoecy; evolutionary transition; environmental sex determination

Introduction

Labile sex expression is widespread among plants and animals (Charnov and Bull 1977; Korpelainen 1990,1998; Delph and Wolf 2005). Natural selection is expected to favor organisms with labile sex expression when individual fitness as a male or female is strongly influenced by environmental factors and when parents cannot predict in which environment the offspring will live (Charnov and Bull 1977).

63   Given their lack of mobility, plants are highly exposed to environmental variations and are consequently

64   more prone to adapt to different environments plastically (Bazzaz 1991). Indeed plants are often characterized by

65   labile sex expression in response to different environmental conditions (Freeman et al. 1980). As a consequence

66   of this high lability in sex expression, there is a large variety of breeding systems in plants in addition to dioecy

67   and hermaphroditism – namely, gynodioecy, androdioecy and subdioecy (or trioecy) (Renner and Ricklefs

68   1995; Ehlers and Bataillon 2007). These latter breeding systems are considered to represent intermediate stages

69   in the evolutionary transition between hermaphroditism and dioecy (Charlesworth and Charlesworth 1978;

70   Freeman 1997; Delph 2005; Barrett 2013). For this reason, labile sex expression is considered to have an

71   important role in the evolution of breeding systems and in the transition from hermaphroditism to dioecy

72   (Freeman 1997; Delph and Wolf 2005; Crossman and Charlesworth 2013).

73   In animals, labile sex expression in the form of plasticity in gender expression is generally observed

74   when the mechanism of sex determination is environmental (Charnov and Bull 1977; Mankiewicz et al. 2013).

75   Environmental sex determination involving phenotypic plasticity in gender is common in invertebrates (Leonard

76   2013), while in vertebrates it has been found only in fishes and reptiles (Bull 1983; Godwin et al. 2003; Sarre et

77   al. 2004). The environmental factors which influence sex expression in invertebrates, fish and reptiles are both

78   abiotic (e.g., temperature, photoperiod, nutrition, density, pH, UV light, metabolic products, salinity and light)

79   and biotic (e.g., parasites, exposure to the opposite sex, social cues and host characteristics in parasitoids) (Bull

80   1983; Korpelainen 1990; Godwin et al. 2003; Sarre et al. 2004).

81   Adaptation of sex expression to the environment is also a common feature of hermaphroditic plants and

82   animals (Charnov 1977). Both are able to allocate reproductive resources to female and male function in

83   response to environmental conditions, such as population size or mating opportunities (Pannell 1997; Charnov

84   1977; Korpeleinen 1998; Schärer 2009; Schleicherová et al. 2014).

85   Sex allocation theory mainly focuses on species with fixed sex expression, while several species display

86   labile sex expression. According to sex allocation theory, dioecious species are only able to change their

87   offspring sex ratio (Charnov 1982; Schärer 2009). Therefore, within the individual, the expression of gender and

88   sex allocation are predicted to be fixed, independent of group size variations and uninfluenced by mating

89   opportunities.

90   In contrast with this prediction, plasticity in gender expression can still be present in dioecious species

91   that have a hermaphroditic ancestor and environmental sex determination, at least in the developmental stage

92   (Korpelainen 1998). Little is known about the degree of plasticity in gender expression in dioecious species of

93   animals with environmental sex determination. In some of these species, plasticity in gender expression during

94   the juvenile phase can be elicited by the gender of a conspecific adult. If that adult represents the only social

95   environment that the juvenile will experience, as in a low density population, we can expect that the juvenile will

96   be able to express the gender opposite to that of the adult. There are several examples among invertebrates of

97   this kind of influence on gender expression: the marine worm *Bonellia viridis* (Echiura) (Bacci 1965; Leutert

98   1975; Agius 1979; Berec 2005), the siboglinid worms of the genus *Osedax* (Vrijenhoek et al. 2008), the

99   crustacean parasites *Pachypygus gibber* (Copepoda) (Hipeau-Jacquotte 1978; Becheikh et al. 1998; Michaud et

100   al. 2004), *Ione thoracica* (Isopoda) and *Stegophryxus hyptius* (Isopoda), some parasitic species of mermithids

101   (Nematoda) (Parenti 1965) and the dioecious species of the marine polychaete worms of the genus *Ophryotrocha*

102   (Rolando 1984).

103   In the genus *Ophryotrocha* there are dioecious, simultaneously hermaphroditic and sequentially

104   hermaphroditic species and all of them show a large extent of labile sex expression in response to social

105   conditions. Therefore this genus presents us with a target model system for studying the plasticity of gender

106   expression from an evolutionary perspective. For example, in the sequential hermaphroditic species *O. puerilis,*

107   when pairs of two females are formed, one of the two worms, usually the youngest one, changes to the male sex,

108   so as to form a heterosexual pair (Åkesson 1974; Pfannenstiel 1975, 1977; Kegel and Pfannenstiel 1983;

109   Berglund 1986). In the dioecious species *Ophryotrocha labronica* and other *Ophryotrocha* dioecious species,

110   sex expression in a juvenile is influenced by the presence of a sexually mature worm so that the juvenile will

111   develop the sex opposite to that of its partner significantly more often than expected (Bacci et al. 1979; Rolando

112   1983, 1984). Conversely, abiotic environmental factors have no influence on gender expression (Åkesson 1975;

113   Prevedelli et al. 1998; Prevedelli and Simonini 2001). Moreover, some *Ophryotrocha* dioecious species cannot

114   be defined as purely dioecious. The presence of four sexual phenotypes (i.e. pure male, male with a few oocytes,

115   pure female, and female with a few sperm) has been reported repeatedly (Pfannenstiel 1976; Rolando and Giorda

116   1982; Rolando 1983; Lorenzi and Sella 2013). Lorenzi and Sella (2013) interpret this sexual polymorphism as a

117   vestigial trait of an ancestral hermaphroditic state, which was inferred from phylogenetic analyses based on

118   morphological and molecular markers (Dahlgren et al. 2011; Thornhill et al. 2009).

119   As opposed to plants, in animals it is still unclear how gender plasticity is involved in

120   the evolution of breeding systems and what its role is in the transition from hermaphroditism

121   to dioecy. Therefore the study of the variation of plasticity in the expression of the sexual

122   phenotypes may help to identify a possible evolutionary pathway of the evolution of dioecy

123 from a hermaphroditic ancestor. If plastic sex allocation in response to social group size is one

124 of the main advantages of hermaphroditism over dioecy (Schärer 2009), we can expect a

125 reduction or a loss of plasticity in sex allocation in the transitions from hermaphroditism to

126 dioecy. This reduction of plasticity could be manifested as a decrease in the ability of sensing

127 and/or responding to environmental stimuli, or as a reduction of the time-window when

128 plasticity can be expressed. In the present study, we tested for variations in the degree of

129 gender plasticity of juveniles and adults in three sexually dioecious species of *Ophryotrocha*

130 worms – *Ophryotrocha labronica, Ophryotrocha robusta* and *Ophryotrocha macrovifera,*

131 according to the social environment they were exposed to – i.e the presence of an adult male

132 or female. The three species have similar morphology and reproductive biology but they

133 differ in some genomic aspects (*O. macrovifera* and *O. labronica* have a different number of

134 chromosomes compared to *O. robusta* (Robotti et al. 1991); and the genome size of *O.*

135 *macrovifera* is twice that of the other two species (Sella et al. 1993)). The three species

136 diverge also in their geographical distribution (Simonini 2009; Paxton and Åkesson 2010).

137   In the current study, ee found that plasticity in gender expression in the three species

138 was confined to the juvenile stage, that four sexual phenotypes (pure males, pure females,

139 males with a few oocytes and females with a few sperm) were expressed in the populations of

140 the three species and that, in the adult phase, individuals expressed only one of the four sexual

141 phenotypes. The presence of sexual polymorphism among adults together with plasticity in

142 the sex expression of juveniles allowed us to outline the transition from ancestral

143 simultaneous hermaphroditism to dioecy via monoecy (i.e. a situation where the

144 hermaphroditic organism has distinct female and male gonads) as the most likely evolutionary

145 pathway (Freeman et al. 1997; Golenberg and West 2013).

146

147 **Materials and methods**

148

Study species and animal rearing

The external morphology and life cycle parameters of *O. labronica, O. robusta* and *O. macrovifera* are only slightly different (Table 1). In the three species mating is achieved by pseudo-copulation, a process of external fertilization in which partners reach close physical contact before releasing their gametes (Westheide 1984). Eggs are released in water and are enveloped by a transparent mucous cocoon, through which egg development can be easily observed. Females grow faster than males and reach sexual maturity at a body size larger than that of males. Both sperm and oocytes originate from the same clusters of primordial germ cells and then mature freely floating in the coelom (Pfannenstiel and Grünig 1982; Brubacher and Huebner 2009). Ripe oocytes can be easily seen from the transparent body walls, while unripe oocytes and sperm can only be observed after intense manipulations of worms. Sexual dimorphism consists of a wider prostomium and a larger and thicker upper jaw in males than in females. These traits, together with presence of visible oocytes, make it easy to distinguish males from females by visual inspection. In addition, males have more rosette glands than females. Rosette glands are located dorsally one per segment on the posterior segments of the body. The rosette glands have been described for all the three species (Paxton and Åkesson 2010), but their function has never been investigated. They can be easily observed under a phase-contrast microscope (250X). Sexual dimorphism in secondary sexual traits such as prostomium and jaw size and shape allowed us to distinguish only two sexual phenotypes, male and female, although four sexual phenotypes (pure female, pure male, male with oocytes and female with sperm) can be identified in these worms by also looking at the types of gametes present in every individual.

In *Ophryotrocha* species, the sex determining mechanism and sex ratio control are supposed to be polygenic (Bacci 1978; Premoli et al. 1996). Polygenic systems are known to be very sensitive to various environmental effects (Falconer 198; Bull 1983). However in *Ophryotrocha* species, abiotic environmental factors such as temperature, photoperiod, salinity, artificial or natural marine water and diet do not influence gender expression (Åkesson 1975; Prevedelli et al. 1998; Prevedelli and Simonini 2001).

Ophryotrocha species occur interstitially, at relatively low density in shallow, nutrient-rich waters (Thornhill et al. 2009). *Ophryotrocha labronica* has a cosmopolitan worldwide distribution (Paxton and Åkesson 2010) and inhabits both harbors and brackish water environments (Simonini 2009). *O. macrovifera* is much rarer than *O. labronica*. It was found in only a few localities along the Mediterranean sea and the North Atlantic coasts (Paxton and Åkesson 2010; Simonini 2009). *O. robusta* is endemic to the Mediterranean sea, where it occurs only in a few localities (Paxton & Åkesson, 2010, Simonini, 2009). Because of the low mobility of these

178 worms, different populations are supposed to be quite reproductively isolated (Lanfranco and Rolando 1981;

179 Sella and Robotti 1986).

180   All experiments were carried out using laboratory populations established several years ago starting from

181 large samples of worms collected from the wild (*O. macrovifera* from Chioggia, Italy (2006), *O. labronica* from

182 Alamitos Beach, Long Beach, California, USA (2005) and *O. robusta* from Porto Empedocle, Italy (2010)).

183 Animals were reared in 30 ml bowls with filtered artificial marine water (33 psu) at a constant temperature of 21

184 °C and fed with spinach *ad libitum*.

185

186 **Experimental design**

187   To test how the presence of an adult male or female influences the expression of the

188 sexual phenotype in juveniles in the three species, we set up 55 pairs of parents (20 pairs of *O.*

189 *labronica*, 20 pairs of *O. macrovifera* and 15 pairs of *O. robusta*). From the offspring of these

190 pairs we selected 330 juveniles (6 per pair) (hereafter "experimental worms") as soon as they

191 had a body length of 3 segments with setae. The selected juveniles were assigned to three

192 treatments (2 experimental worms of each family per treatment) (Figure 1): 1) juvenile paired

193 with an adult female, 2) juvenile paired with an adult male, and 3) juvenile isolated as a

194 control. We expected experimental worms to develop the gender opposite to that of their

195 partner. Therefore, we expected sex ratio in treatment 1) and 2) to differ from the sex ratio in

196 our control treatment. Adult males and females (hereafter "partners") used in treatments 1)

197 and 2) were obtained from the progeny of 108 pairs (36 per species) and were all of the same

198 age (21 days). When the experimental worms reached a clear sexual differentiation, we sexed

199 them. They were sexed according to the presence of visible oocytes in females and of a

200 prostomium and an upper jaw larger in males than in females.

201   To test the effect of the presence of an adult male or female on the expression of the

202 sexual phenotypes of sexually mature individuals of the three species, we used a subsample of

203 the sexually mature experimental worms and formed 87 homosexual pairs by pairing each of

204 them with a partner. If gender plasticity is still present in the adult stage, we can expect

205 worms in homosexual pairs to be stimulated to produce gametes of the sex opposite to that of

206 their partner's. Ninety heterosexual pairs were set up as controls. To check for the presence of

207 oocytes in males and sperm in females, we needed to kill worms. Therefore we formed these

208 pairs relying on external sexual dimorphism only, thus without distinguishing pure females

209 from females with sperm and pure males from males with oocytes. Pairs were reared for a

210 time interval that allowed all the heterosexual pairs to lay at least two egg masses. We

211 guessed that those homosexual pairs in which at least one of the partners had both oocytes and

212 sperm would have had the opportunity to lay at least one egg mass in that same time interval.

213   All experimental worms were eventually checked for sperm in females or oocytes in

214 males. To check for the presence of sperm, worms were gently squeezed between two slides,

215 so that sperm oozed out of the parapodia, and were observed by phase-contrast microscopy

216 (250X). Oocytes can be easily identified from the transparent body walls of the worms at

217 250X magnification. Females that had sperm and males that had oocytes were classified as

218 pseudohermaphrodites, because generally in these worms only one type of gamete is

219 functional (Baldi et al. 2009; Lorenzi and Sella 2013). In a subsample of worms (n = 184; 64

220 from treatment 1, 57 from treatment 2 and 63 from treatment 3), we measured the

221 developmental time to sexual differentiation as the number of days from the stage of 3

222 segments with setae to sexual maturity.

223   In order to check for a correlation between sexual phenotype and number of rosette

224 glands (Lorenzi and Sella 2013; Paxton and Åkesson 2010), we also measured the number of

225 rosette glands and the number of segments with setae (as an estimate of body size) in the

226 same subsample. Measures were taken under phase-contrast microscopy (250X).

227 **Statistical analysis**

228   We first focused on sex ratio, i.e., the effect of social environment during the juvenile phase on worm sex

229 expression. We tested whether the sex ratio (i.e., the frequencies of sexual phenotypes in experimental worms)

230   differed according to treatment in the juvenile phase using a Generalized Linear Mixed Model (GLMM) with

231   binomial distribution. Sex was assigned based on external morphology, therefore juveniles became either males

232   (pure males and males with oocytes) or females (pure females and females with sperm). Predictor variables

233   included species and social environment (i.e. juvenile + male, juvenile + female, isolated juvenile). The sibship

234   of every experimental worm was added as a random blocking to control for similarities in the proportion of the

235   different sexual phenotypes within families. Since the sex of worms was not significantly affected by treatment

236   during the adult phase, in the GLMM we used all the data obtained from the 330 juveniles that entered the

237   experiment.

238   Then, we focused on how many juveniles matured the gender opposite to their partner's. Using a Generalized

239   Linear Model (GLM) with Poisson error distribution and a log link function, we analyzed the difference between

240   the number of experimental worms that matured the gender opposite to their partner's and the number of

241   experimental worms that matured the same gender as their partner's (heterosexual pairs vs. homosexual pairs). In

242   this statistical analysis pseudohermaphrodites (males with oocytes and females with sperm) were therefore

243   excluded. The same statistical analysis was used to compare the number of pseudohermaphrodites among the

244   three social environments and species.

245   Using a Generalized Linear Mixed Model (GLMM) with Poisson error distribution and a log link

246   function, we also analyzed the developmental time (i.e., the number of days that passed from the stage of 3

247   segments with setae to the sexual differentiation stage). Predictor variables included sexual phenotype, species

248   and social environment. The sibship of every experimental worm was handled as the random factor. Three

249   different GLMMs, one for every sexual phenotype (males, females and pseudohermaphrodites), were made to

250   compare the developmental times among the three social environments. As in the previous analysis, predictors

251   were species and social environment, while sibship was a random factor. We used the results of these statistical

252   tests only to assess differences in developmental times between social environments within the same sexual

253   phenotype.

254   For all the analyses, we followed a model selection process based on Aikaike's information criterion

255   (AIC), which is a measure of model fit. AIC was recorded from models including all possible combinations and

256   interactions of effects, and we selected the model having the lowest AIC (Quinn and Keough 2002). In the

257   GLMM and GLM with Poisson error distribution we also checked for overdispersion.

258   We assessed whether the proportion of sexual phenotypes in the adult phase differed between homo-

259   and hetero-sexual pairs using a 2×4 contingency table (Chi-squared test).

260 Finally, we analyzed the number of rosette glands using a Generalized Linear Model with Poisson error

261 distribution and a log link function. To analyze the number of rosette glands, we used the following factors as

262 explanatory variables: species, sexual phenotype, social environment and body size. Model selection and

263 statistical assumptions were checked, as described for the previous analysis.

264 All statistical analyses were performed using the software SPSS 20.

265

266 **Results**

267

268 **Type and frequency of sexual phenotypes of the experimental worms**

269 In the three species, we found four sexual phenotypes, i.e. 39.3% pure males, 35.6% pure females, 19.1%

270 females with sperm and 6.0% males with oocytes. The frequencies of males (pure males and male with oocytes)

271 and females (pure females and females with sperm) were not significantly different among species and were

272 significantly affected by the gender of the adult to which juveniles were exposed (Table 2 and Figure 2). The

273 interaction between these two predictors was removed after checking it was non-significant in a preliminary

274 analysis, which suggested that the social environment had the same impact on the juveniles of the three species.

275 Statistical comparisons show that the difference in sex ratio among "social environments" is due mainly to the

276 difference between the environment "juvenile+ female" and the other two social environments (Table 2),

277 indicating female as the sex able to affect juvenile sexual development.

278 When juveniles reached sexual maturity, they formed true heterosexual pairs with their adult partner

279 (pure male + pure female) (47.5%) significantly more often than true homosexual pairs (pure male + pure male

280 or pure female + pure female) (31.1%) (GLM with Poisson error distribution: d.f. = 2, $\chi^2_{(Wald)}$ = 19.56, P < 0.001;

281 heterosexual pairs (pure male + pure female) vs homosexual pairs (pure male + pure male or pure female + pure

282 female), B = 0.42, $\chi^2_{(Wald)}$ = 6.55, P = 0.01). The remaining pairs (21,4%) were composed of at least one male

283 with oocytes or one female with sperm. In the subsequent analysis, we merged these two intermediate

284 phenotypes together to form the experimental group of pseudohermaphrodites, since females with sperm and

285 males with oocytes were relatively rare phenotypes. The number of pseudohermaphrodites depended

286 significantly on species and social environment (Figure 3) (GLM: species, $\chi^2_{(Wald)}$ = 25.74, d. f. = 2, P < 0.001;

287 social environment, $\chi^2_{(Wald)}$ = 25.74, d. f. = 2, P <0.001). The number of pseudohermaphrodites was significantly

288 higher when juveniles developed in isolation than when they developed together with males (B = 0.75, $\chi^2_{(Wald)}$ =

289 25.69, P < 0.0001) or with females (B = 0.27, $\chi^2_{(Wald)}$ = 4.41, P = 0.036).

290

Developmental time to sexual maturity

The developmental time of juveniles was significantly different among species and sexual phenotypes, but juveniles of the three species adjusted their developmental time to social conditions in a similar way, although sexual phenotypes responded differently to social environment (Table 3). The developmental time of juveniles that expressed the same gender of their adult partner was significantly longer than that of juveniles which expressed the gender opposite to that of their partner (Table 3 and Figure 4). Overall, juveniles that developed in isolation had developmental times which were generally intermediate compared to the developmental times of their conspecifics exposed to adults. The large variations between species and phenotypes do not allow to identify clear, common effects of isolation on developmental times (Figure 4).

Expression of the sexual phenotypes of sexually mature worms

No differences were observed in the number of sexual phenotypes between worms in homosexual pairs and worms in heterosexual pairs during the adult phase ($\chi^2 = 0.43$, d.f. = 3, $P = 0.93$). Pairing off with a worm of the same sex did not stimulate the production of gametes of the opposite sex. In those homosexual pairs that were composed of two females, worms occasionally laid eggs. Egg laying occurred in 4 out of 16 homosexual pairs of females in *O. robusta*, in 2 out of 39 pairs in *O. macrovifera* and in 5 out of 32 pairs in *O. labronica*. Therefore in those homosexual pairs at least one of the partners was a female with sperm. We do not know whether fertilized eggs were the result of a self-fertilization process or whether the homosexual pairs were functionally heterosexual pairs.

Rosette glands

The number of rosette glands was positively associated to body size and varied significantly between species and sexual phenotypes, but no interaction between the two factors was found (Figure 5). In all the three species the number of rosette glands was larger in males than in females and pseudohermaphrodites (GLM: species, log-

316   likelihood chi-square $(G^2) = 19.87$, d. f. $= 2$, $P < 0.001$; sexual phenotype, $G^2 = 80.20$, d. f. $= 2$, $P < 0.0001$;

317   social environment, $G^2 = 5.64$, d. f. $= 2$, $P > 0.05$; body size, $G^2 = 170.7$, d. f. $= 1$, $P < 0.0001$). The number of

318   rosette glands was significantly different between males and females ($B = -0.39$, $\chi^2_{(Wald)} = 1.88$, $P < 0.0001$),

319   males and pseudo-hermaphrodites ($B = -0.52$, $\chi^2_{(Wald)} = 0.69$, $P < 0.0001$), while it was not different between

320   females and pseudo-hermaphrodites ($B = -0.13$, $\chi^2_{(Wald)} = 2.64$, $P = 0.10$). This means that only two sexual

321   phenotypes, male and female, can be distinguished according to the number of rosette glands.

322

323   Discussion

324

325   Our results showed that social environment – i.e. the presence of a sexually mature

326   partner – influenced the expression of the sexual phenotype in juveniles of the *Ophryotrocha*

327   dioecious species. The effect was documented 1) by variations of the frequencies of sexual

328   phenotypes according to the social environment. Indeed juveniles tend to develop so as to

329   form heterosexual pairs.. Furthermore the absence of a partner stimulated the production of

330   pseudohermaphroditic sexual phenotypes. Indeed pseudohermaphrodites were significantly

331   more common among isolated juveniles than among juveniles reared with adults of either sex.

332   The effect of social environment was also documented 2) by the significantly different

333   developmental times to the onset of sexual maturity of juveniles. Juveniles which have

334   matured the same gender of their adult partner needed longer time to reach sexual maturity

335   than juveniles which had matured the gender opposite to that of their partner's in all three

336   species.

337   Sex expression was influenced by social conditions only during the juvenile phase for all

338   the three species. This can be expected in species whose populations have largely fluctuating

339   densities and live in patchy environments, such as intertidal communities do (Sella and

340   Ramella 1999; Prevedelli et al. 2005). During the adult phase, frequencies of sexual

341   phenotypes were no longer influenced by the social environment, as expected in species that

342 underwent selective pressures for sexual specialization towards dioecy. *Ophryotrocha*

343 dioecious species are therefore another example of labile gender maturation of juveniles in

344 response to the presence of a sexual mature partner, in addition to those reported by Leutert

345 (1975), Berec (2005); Bacci (1965), Agius (1979), Hipeau-Jacquotte (1978), Beckeickh et al.

346 (1998), Michaud et al. (2004), Parenti (1965) and Vrijenhoek et al. (2008).

347   Although the three species differ from each other in their geographical distribution,

348 genome structure and life cycle, they did not differ in their degree of plasticity in sexual

349 expression at the end of the juvenile phase. Looking both at the propensity of juveniles to

350 develop the gender opposite to that of their partner's and to vary in their developmental time

351 according to their response to social conditions, the three species behaved in a similar way (as

352 shown from the absence of statistical interactions involving species as a predictor variable).

353 This interspecific homogeneity can be due either to the phylogenetic proximity (Dahlgren

354 2001) or to maintenance of plasticity in sex expression during development as an adaptive

355 response to common selective forces.

356   Not all experimental worms reacted in the same way to the social environment:

357 31.12% of juveniles matured the same gender of their partner. Nevertheless, they showed a

358 longer developmental time than that of juveniles which developed the gender opposite to that

359 of their adult partner's. This result suggests that in *Ophryotrocha* worms the degree of sensing

360 and/or responding to stimuli from adult partners is also influenced by genetic variations

361 between individuals. In a similar way social environments influence juveniles sexual

362 development differently: looking at the external morphology of experimental worms only

363 adult females are able to influence the sex of juveniles (Figure 2). However when looking at

364 gametes production we can asses also a influence of adult males on juveniles sexual

365 development since the number of juveniles developed to pseudohermaphrodites is lower when

366 juveniles are paired with males compare to isolated juveniles (Figure 3). According to these

367 results,, the most recent theories about phenotypic plasticity (West-Eberhard 2003; Ah-King

368 and Nylin 2010; Golenberg and West 2013), identify two factors involved in determining the

369 final sexual phenotype: 1) variations in the sequences of regulatory genes responsible for the

370 control of alternative developmental pathways and 2) environmental stimuli.

371   The results of our experiment made it possible for us to outline a possible evolutionary

372 pathway of the evolution of dioecy from a hermaphroditic ancestral state in *Ophryotrocha*. In

373 plants, the transition from hermaphroditism to dioecy is thought to have evolved through two

374 main distinct pathways (Ehlers and Bataillon 2007): from hermaphroditism via gynodioecy to

375 dioecy and from hermaphroditism via monoecy to dioecy. Gynodioecy refers to the

376 coexistence in a population of two sexual phenotypes, i.e. pure females and individuals

377 having both sexual functions (within the same flower or in separate flowers), while monoecy

378 refers to plants having both sexual functions in separate male and female flowers within the

379 same individual (Ehlers and Bataillon 2007). In animals the distinction between individuals

380 having both sexual functions either within the same flower or in separate flowers translates

381 respectively to syngonic (the same gonads producing both male and female gametes) or

382 digonic (distinct male and female gonads in the same individual) simultaneous

383 hermaphrodites (Vega-Frutis et al. 2014).

384   The pathway through gynodioecy (Charlesworth and Charlesworth 1978; Delph and

385 Wolf 2005) is based on two mutational events. Starting from a population of hermaphrodites,

386 a first mutation is responsible for the production of pure females, so that the remaining

387 hermaphrodites will be selected to plastically adjust their sex allocation and becoming

388 strongly male biased. A second mutation will then generate pure males that will spread and

389 outnumber the strongly male-biased hermaphrodites. This model relies on a genetic

390 assumption (the first genetic mutation) and does not include gene x environment interactions

391 (Freeman 1997). In species evolving through this pathway, gender expression should vary

392 only in hermaphrodites as a consequence of the presence of pure females rather than other

393 environmental conditions. Moreover, the model predicts that when pseudohermaphroditic

394 phenotypes are present, they belong to the male gender, i. e. the gender which did not undergo

395 the first genetic mutation determining male-sterility (Ehlers and Bataillon 2007).

396   In contrast, the pathway through monoecy (Renner and Ricklefs 1995) is based on

397 mechanisms of regulation of gender expression triggered by variations in environmental cues.

398 A mutation of the regulatory sequence of sex expression would determine the tendency to

399 express one gender only, setting the evolutionary stage of dioecy or subdioecy. At this stage

400 the sexual development of the organism is still directly dependent on the perception of

401 external environmental cues and therefore it will maintain its ability to adapt to environmental

402 variations   plastically.   Following   this   evolutionary   model,   during   the   transition,

403 pseudohermaphroditic phenotypes should be common and extreme phenotypes (pure male

404 and pure female) rare, since all individuals retain the ability to express both sexual

405 phenotypes (Freeman 1997).

406   Our results fit well a possible monoecy pathway in which both the influence of social

407 conditions on sex expression and the presence of pseudohermaphrodites can be explained. It

408 is difficult to classify the pseudohermaphroditic phenotypes of dioecious species as syngonic

409 or digonic, since only clusters of germ cells, and no true gonads, are present. They are

410 hermaphroditic phenotypes with strong male- or female-biased sex allocation, and with rare

411 gametes of the opposite sex. However, simultaneous hermaphroditic species of this genus also

412 have spatially separate male and female sections (in the first 2-3 body segments these

413 hermaphrodites produce only sperm, while in the remaining segments they produce only

414 oocytes) (Åkesson 1974; Schleicherová et al. 2014). Therefore, they resemble digonic rather

415 than syngonic simultaneous hermaphrodites.

416   In plants, the main selective force favoring the transition to dioecy via monoecy is

417   sexual specialization (Freeman 1997 and references therein). In animals, selective pressures

418   leading to sexual specialization are poorly known (but see Weeks 2012). In the populations of

419   the hermaphroditic ancestor of the dioecious *Ophryotrocha* species, selection for sexual

420   specialization would have been responsible for the appearance of pseudohermaphrodites (in

421   which both types of gametes are present but only one type is functional) and then of pure

422   males and pure females. One may wonder why pseudohermaphrodites still coexist with pure

423   males and pure females in the existing populations of *Ophryotrocha*. According to Ehlers and

424   Bataillon (2007) and Lorenzi and Sella (2013) selection for sexual specialization may become

425   less strong or ineffective when pseudohermaphrodites are strongly biased towards one of the

426   two genders. In the *Ophryotrocha* dioecious species, the dichotomy between sexual

427   dimorphism at the morphological level and sexual polymorphism at the gamete level is

428   illustrated well by the number of rosette glands. This sex-related trait allowed us to

429   distinguish only two reproductive morphs (males and females), while at the gamete level four

430   sexual phenotypes exist (pure male, pure female, male with oocytes and female with sperm).

431   If we can find out more precisely what the function of rosette glands is, we can more easily

432   understand what the selective pressures are that act for sexual specialization and hence drive

433   the evolution of dioecy in this genus.

434

438

References

440 Agius, L. (1979). Larval settlement in the echiuran worm Bonellia viridis: settlement on both
441   the adult proboscis and body trunk. *Marine Biology, 53*(2), 125-129.

442 Ah-King, M., & Nylin, S. (2010). Sex in an evolutionary perspective: just another reaction
443   norm. *Evolutionary biology, 37*(4), 234-246.

444 Åkesson, B. (1974). Reproduction and Larval Morphology of Five Ophryotrocha Species
445   (Polychaeta, Dorvilleidae). *Zoologica Scripta, 2*(4), 145-155.

446 Åkesson, B. (1975). Reproduction in the genus Ophyotrocha (Polychaeta,
447   Dorvilleidae).[Conference paper]. *Pubblicazioni della Stazione Zoologica, Napoli*.

448 Bacci, G. (1965). Sex determination. Pergamon Press, Oxford.

449 Bacci, G. (1978). Genetics of sex determination in Ophryotrocha (Annelida, Polychaeta).
450   *Marine organisms: genetics, ecology, and evolution. Plenum Press, New York*.

451 Bacci, G., Lanfranco, M., Mantello, I., & Tomba, M. (1979). A new pattern of
452   hermaphroditism (inducible hermaphroditism) in populations ofOphryotrocha
453   labronica (Annelida Polychaeta). *Experientia, 35*(5), 605-606.

454 Baldi, C., Cho, S., & Ellis, R. E. (2009). Mutations in two independent pathways are
455   sufficient to create hermaphroditic nematodes. *Science, 326*(5955), 1002-1005.

456 Barrett, S. C. (2013). The evolution of plant reproductive systems: how often are transitions
457   irreversible? *Proceedings of the Royal Society B: Biological Sciences, 280*(1765),
458   20130913.

459 Bazzaz, F. (1991). Habitat selection in plants. *American Naturalist, 137*(supplement: hab).

460   Becheikh, S., Michaud, M., dé ric Thomas, F., Raibaut, A., & Renaud, F. (1998). Roles of

461   resource and partner availability in sex determination in a parasitic copepod.

462   *Proceedings of the Royal Society of London. Series B: Biological Sciences, 265*(1402),

463   1153-1156.

464   Berglund, A. (1986). Sex change by a polychaete: effects of social and reproductive costs.

465   *Ecology, 67*(4), 836-845.

466   Brubacher, J. L., & Huebner, E. (2009). Development of polarized female germline cysts in

467   the polychaete, Ophryotrocha labronica. *Journal of morphology, 270*(4), 413-429.

468   Bull, J. J. (1983). *Evolution of sex determining mechanisms*: The Benjamin/Cummings

469   Publishing Company, Inc.

470   Charlesworth, B., & Charlesworth, D. (1978). A model for the evolution of dioecy and

471   gynodioecy. *American naturalist*, 975-997.

472   Charnov, E. L., & Bull, J. (1977). When is sex environmentally determined?

473   Crossman, A., & Charlesworth, D. (2013). Breakdown of dioecy: models where males acquire

474   cosexual functions. *Evolution*.

475   Dahlgren, T. G., Åkesson, B., Schander, C., Halanych, K. M., & Sundberg, P. (2001).

476   Molecular phylogeny of the model annelid Ophryotrocha. *The Biological Bulletin,*

477   *201*(2), 193-203.

478   Delph, L. F., & Wolf, D. E. (2005). Evolutionary consequences of gender plasticity in

479   genetically dimorphic breeding systems. *New Phytologist, 166*(1), 119-128.

480   Ehlers, B. K., & Bataillon, T. (2007). 'Inconstant males' and the maintenance of labile sex

481   expression in subdioecious plants. *New Phytologist, 174*(1), 194-211.

482   Falconer, D. (1981). Introduction to Quantitative GeneticsLongman Inc. *New York, NY*.

483   Freeman, D., Harper, K., & Charnov, E. (1980). Sex change in plants: old and new
484   observations and new hypotheses. *Oecologia, 47*(2), 222-232.

485   Freeman, D. C., Doust, J. L., El-Keblawy, A., Miglia, K. J., & McArthur, E. D. (1997).
486   Sexual specialization and inbreeding avoidance in the evolution of dioecy. *The*
487   *Botanical Review, 63*(1), 65-92.

488   Godwin, J., Luckenbach, J. A., & Borski, R. J. (2003). Ecology meets endocrinology:
489   environmental sex determination in fishes. *Evolution & development, 5*(1), 40-49.

490   Golenberg, E. M., & West, N. W. (2013). Hormonal interactions and gene regulation can link
491   monoecy and environmental plasticity to the evolution of dioecy in plants. *American*
492   *journal of botany, 100*(6), 1022-1037.

493   Hipeau-Jacquotte, R. (1978). Existence de deux formes sexuelles mâles chez le copépode
494   ascidicole Notodelphyidae Pachypygus gibber (Thorell, 1859). *Comptes Rendus de*
495   *I'Academie des Sciences, Paris*, 287 D**,** 253-256.

496   Kegel, B., & Pfannenstiel, H.-D. (1983). Evaluation of the pair-culture effect inOphryotrocha
497   puerilis (Polychaeta: Dorvilleidae). I. Pair-culture effect and sex ratio. *Helgoländer*
498   *Meeresuntersuchungen, 36*(2), 205-213.

499   Korpelainen, H. (1990). Sex ratios and conditions required for environmental sex
500   determination in animals. *Biological Reviews, 65*(2), 147-184.

501   Korpelainen, H. (1998). Labile sex expression in plants. *Biological Reviews, 73*(2), 157-180.

502   La Greca, M., & Bacci, G. (1962). Una nuova specie di Ophryotrocha delle coste tirreniche
503   (Annelida, Polychaeta). *Italian Journal of Zoology, 29*(1), 7-18.

504 Lanfranco, M., & Rolando, A. (1981). Sexual races and reproductive isolation in
505   Ophryotrocha labronica La Greca and Bacci (Annelida, Polychaeta). *Italian Journal of*
506   *Zoology, 48*(3-4), 291-294.

507 Leonard, J. L. (2013). Williams' Paradox and the role of phenotypic plasticity in sexual
508   systems. *Integrative and comparative biology, 53*(4), 671-688.

509 Leutert, R. (1975). Sex-determination in Bonellia. In *Intersexuality in the animal kingdom*
510   (pp. 84-90): Springer.

511 Lorenzi, M. C., & Sella, G. (2013). In between breeding systems: neither dioecy nor
512   androdioecy explains sexual polymorphism in functionally dioecious worms.
513   *Integrative and comparative biology, 53*(4), 689-700.

514 Mankiewicz, J. L., Godwin, J., Holler, B. L., Turner, P. M., Murashige, R., Shamey, R., et al.
515   (2013). Masculinizing effect of background color and cortisol in a flatfish with
516   environmental sex-determination. *Integrative and comparative biology, 53*(4), 755-
517   765.

518 Michaud, M., de Meeûs, T., & Renaud, F. (2004). Environmental sex determination in a
519   parasitic copepod: checking heterogeneity and unpredictability of the environment.
520   *Marine Ecology Progress Series, 269*, 163-171.

521 Pannell, J. (1997). Variation in sex ratios and sex allocation in androdioecious Mercurialis
522   annua. *Journal of Ecology, 85*(1), 57-69.

523 Parenti, U. (1965). Male and female influence of adult individuals on undifferentiated larvae
524   of the parasitic nematode *Paramermis contorta*. *Nature,* 207, 1105-1106.

525   Paxton, H., & Åkesson, B. (2010). The Ophryotrocha labronica group (Annelida,
526   Dorvilleidae)—with the description of seven new species. *Zootaxa, 2713*, 1-24.

527   Pfannenstiel, H.-D. (1975). Mutual influence on the sexual differentiation in the protandric
528   polychaete Ophryotrocha puerilis. In *Intersexuality in the animal kingdom* (pp. 48-56):
529   Springer.

530   Pfannestiel, H.-D. (1976). Ist der Polychaet Ophryotrocha labronica ein proterandrischer
531   hermaphrodit? *Marine Biology, 38*(2), 169-178.

532   Pfannenstiel, H.-D. (1977). Experimental analysis of the "Paarkultureffekt" in the protandric
533   polychaete, *Ophryotrocha puerilis* Clap. Mecz. *Journal of Experimental Marine*
534   *Biology and Ecology, 28*(1), 31-40.

535   Pfannenstiel, H.-D., & Grünig, C. (1982). Yolk formation in an annelid (*Ophryotrocha*
536   *puerilis*, polychaeta). *Tissue and Cell, 14*(4), 669-680.

537   Prevedelli, D., & Simonini, R. (2001). Effects of diet and laboratory rearing on demography
538   of Dinophilus gyrociliatus (Polychaeta: Dinophilidae). *Marine Biology, 139*(5), 929-935.

539   Prevedelli, D., & Vandini, R. Z. (1998). Effect of diet on reproductive characteristics of
540   Ophryotrocha labronica (Polychaeta: Dorvilleidae). *Marine Biology, 132*(1), 163-170.

541   Quinn, G. P., & Keough, M. J. (2002). *Experimental design and data analysis for biologists*:
542   Cambridge University Press.

543   Renner, S. S., & Ricklefs, R. E. (1995). Dioecy and its correlates in the flowering plants.
544   *American Journal of Botany*(5), 596-606.

545   Robotti, C., Ramella, L., Cervella, P., & Sella, G. (1991). Chromosome analysis of 9 species
546   of Ophryotrocha (Polychaeta, Dorvilleidae). *Ophelia*, 625-632.

547   Rolando, A. (1984). The sex induction hypothesis and reproductive behaviour in four
548   gonochoristic species of the genus Ophryotrocha (Annelida Polychaeta). *Monitore*
549   *Zoologico Italiano-Italian Journal of Zoology, 18*(4), 287-299.

550   Rolando, A., & Giorda, R. (1982). Male intersexes in Ophryotrocha labronica La Greca &
551   Bacci (Annelida Polychaeta). *Monitore Zoologico Italiano-Italian Journal of Zoology,*
552   *16*(1), 67-73.

553   Sarre, S. D., Georges, A., & Quinn, A. (2004). The ends of a continuum: genetic and
554   temperature- dependent sex determination in reptiles. *Bioessays, 26*(6), 639-645.

555   Schleicherová, D., Sella, G., Meconcelli, S., Simonini, R., Martino, M., Cervella, P., et al.
556   (2014). Does the cost of a function affect its degree of plasticity? A test on plastic sex
557   allocation in three closely related species of hermaphrodites. *Journal of Experimental*
558   *Marine Biology and Ecology, 453*, 148-153.

559   Schärer, L. (2009). Tests of sex allocation theory in simultaneously hermaphroditic animals.
560   *Evolution, 63*(6), 1377-1405.

561   Sella, G., & Ramella, L. (1999). Sexual conflict and mating systems in the dorvilleid genus
562   Ophryotrocha and the dinophilid genus Dinophilus. In *Reproductive Strategies and*
563   *Developmental Patterns in Annelids* (pp. 203-213): Springer.

564   Sella, G., Redi, C., Ramella, L., Soldi, R., & Premoli, M. (1993). Genome size and karyotype
565   length in some interstitial polychaete species of the genus Ophryotrocha
566   (Dorvilleidae). *Genome, 36*(4), 652-657.

567   Sella, G., & Robotti, C. (1991). Heterozygote deficiency at the phosphoglucose isomerase
568   locus in a tyrrhenian population of *Ophryotrocha-labronica* (Polychaeta, Dorvillidae).
569   *Ophelia*, 641-645.

570 Simonini, R., Massamba-N'Siala, G., Grandi, V., Prevedelli, D. 2009. Distribution of the
571   genus *Ophryotrocha* (Polychaeta) in Italy: new reports and comments on the biogeography
572   of Mediterranean species. *Vie et Milieu*, 59, 79–88.

573 Thornhill, D. J., Dahlgren, T. G., & Halanych, K. M. (2009). Evolution and ecology of
574   Ophryotrocha (Dorvilleidae, Eunicida). *Annelids in modern biology*, 242-256.

575 Vega- Frutis, R., Macías- Ordóñez, R., Guevara, R., & Fromhage, L. (2014). Sex change in
576   plants and animals: a unified perspective. *Journal of evolutionary biology, 27*(4), 667-
577   675.

578 Vrijenhoek, R., Johnson, S., & Rouse, G. (2008). Bone- eating Osedax females and their
579   'harems' of dwarf males are recruited from a common larval pool. *Molecular ecology,
580   17*(20), 4535-4544.

581 Weeks, S. C. (2012). The role of androdioecy and gynodioecy in mediating evolutionary
582   transitions between dioecy and hermaphroditism in the Animalia. *Evolution, 66*(12),
583   3670-3686.

584 West-Eberhard, M. J. (2003). *Developmental plasticity and evolution*: Oxford University
585   Press.

586 Westheide, W. (1984). The concept of reproduction in polychaetes with small body size:
587   adaptations in interstitial species. *Fortschr. Zool, 29*, 265-287.

588

589

590 **Figure legends**

591

592   **Figure 1** Experimental set up. Juveniles (n = 330) were randomly assigned to one of three

593   treatments: 1. juvenile paired with an adult female 2. juvenile paired with an adult male 3.

594   juvenile isolated. When juveniles reached a clear sexual differentiation, a subsample of these

595   sexually mature worms were screened to verify the presence of sperm (in females) or oocytes

596   (in males). The remaining worms were used to form homosexual pairs (n = 87) or

597   heterosexual pairs (n = 90). At the end of the experiment all the worms were checked for

598   sperm in females or oocytes in males.

599

600   **Figure 2.** Relative frequencies of males (including males andmales with oocytes) and females (including females

601   and females with sperm) in every of the three social environments (juvenile paired with a male, with a female or

602   isolated). 55.9% of juveniles became males when paired together with females, while only 38.3% developed as

603   males in pair with an adult male. In a similar way, 61.7% of juveniles developed as females when they

604   developed together with males, while 44.1% became females in pair with females. Juveniles in isolation

605   developed 58.8% as females and 41.2% as males.

606

607   **Figure 3.** Frequencies (%) of lpseudohermaphrodites (female with sperm and male with oocytes) in *O.*

608   *labronica, O. macrovifera* and *O. robusta* depending on the social environment (juveniles paired with a

609   male, a female or isolated).

610

611   **Figure 4.** Variations in the developmental time (days) to sexual maturity in *O. labronica, O.*

612   *macrovifera* and *O. robusta* under the effect of the social environment (juveniles paired with a

613   male, a female or isolated) paneled seperately for every sexual phenotype (females, males and

614   pseudohermaphrodites). The graph shows the means ± SE.

615

616   **Figure 5.** Variations in the number of rosette glands relative to body size depending onsexual phenotypes

617   (female, male, pseudohermaphrodite), paneled separetely for *O. labronica, O. macrovifera* and *O. robusta*.

618

619

620

621

622

623

624

625

626

627

628

Table 1. Main differences in the life cycle of the three tested species (mean ± SD)

	Ophryotrocha labronica	*Ophryotrocha robusta*	*Ophryotrocha macrovifera*
N. Eggs/cocoon	116 ± 46	134 ± 51	76 ± 33
N. segments with setae at hatching	2 ± 1	0	2 ± 1
N. segments with setae at ♂ definitive upper jaw appearance	15 ± 2	15 ± 2	14 ± 2
N. segments with setae at ♀ Oocytes appearance	16 ± 2	14 ± 2	14 ± 2
time from hatching to ♂ definitive u.jaw appearance (days)	22 ± 5	28 ± 8	21 ± 7
time from hatching to ♀ oocytes appearance (days)	20 ± 4	26 ± 6	18 ± 6

629

630

631

632

642 **Table 2.** Results of the GLMM testing for the effect of species and social environment on the
643 sex ratio.

644

645

Predictor	Comparisons		*P*
species		$F_{2,293} = 0.76$	0.468
social environment		$F_{2,293} = 4.54$	0.011
	"J+♂" *vs* "J+♀"	$t = -2.74$	0.006
	"J+♂" *vs* "isolated J"	$t = -0.39$	0.698
	"J+♀" *vs* "isolated J"	$t = 2.43$	0.016
Random effect			*P*
sibship		$z = 1.75$	0.080

646

647

648

649

650

651

Table 3. Results of the GLMMs testing a) the effect of species, social environment and sexual phenotype on the developmental time to sexual maturity; b) the effect of the social environment for each type of sexual phenotype (J = Juvenile).

655

a)	Predictor		P
	species	$F_{2,173} = 11.79$	<0.001
	social environment	$F_{2,173} = 0.46$	0.630
	sexual phenotype	$F_{2,173} = 4.61$	0.011
	social environment X sexual phenotype	$F_{2,173} = 6.35$	<0.001
	Random effect		**P**
	sibship	$z = 2.63$	0.008

b)	Predictor	Comparisons		P
Females	social environment		$F_{2,43} = 3.75$	0.032
		"J+♂" vs "J+♀"	$t = 2.74$	0.009
		"J+♂" vs "isolated J"	$t = 1.27$	0.210
		"J+♀" vs "isolated J"	$t = -1.69$	0.098
Males	social environment		$F_{2,79} = 9.26$	<0.001
		"J+♂" vs "J+♀"	$t = 3.79$	<0.001
		"J+♂" vs "isolated J"	$t = -3.54$	0.001
		"J+♀" vs "isolated J"	$t = -0.32$	0.754
Pseudoherm.	social environment		$F_{2,47} = 1.64$	0.206

656