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

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Short title: Labile sex expression in *Ophryotrocha* worms

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).

Given their lack of mobility, plants are highly exposed to environmental variations and are consequently more prone to adapt to different environments plastically (Bazzaz 1991). Indeed plants are often characterized by labile sex expression in response to different environmental conditions (Freeman et al. 1980). As a consequence of this high lability in sex expression, there is a large variety of breeding systems in plants in addition to dioecy and hermaphroditism – namely, gynodioecy, androdioecy and subdioecy (or trioecy) (Renner and Ricklefs 1995; Ehlers and Bataillon 2007). These latter breeding systems are considered to represent intermediate stages in the evolutionary transition between hermaphroditism and dioecy (Charlesworth and Charlesworth 1978; Freeman 1997; Delph 2005; Barrett 2013). For this reason, labile sex expression is considered to have an important role in the evolution of breeding systems and in the transition from hermaphroditism to dioecy (Freeman 1997; Delph and Wolf 2005; Crossman and Charlesworth 2013).

In animals, labile sex expression in the form of plasticity in gender expression is generally observed when the mechanism of sex determination is environmental (Charnov and Bull 1977; Mankiewicz et al. 2013). Environmental sex determination involving phenotypic plasticity in gender is common in invertebrates (Leonard 2013), while in vertebrates it has been found only in fishes and reptiles (Bull 1983; Godwin et al. 2003; Sarre et al. 2004). The environmental factors which influence sex expression in invertebrates, fish and reptiles are both abiotic (e.g., temperature, photoperiod, nutrition, density, pH, UV light, metabolic products, salinity and light) and biotic (e.g., parasites, exposure to the opposite sex, social cues and host characteristics in parasitoids) (Bull 1983; Korpelainen 1990; Godwin et al. 2003; Sarre et al. 2004).

Adaptation of sex expression to the environment is also a common feature of hermaphroditic plants and animals (Charnov 1977). Both are able to allocate reproductive resources to female and male function in response to environmental conditions, such as population size or mating opportunities (Pannell 1997; Charnov 1977; Korpeleinen 1998; Schärer 2009; Schleicherová et al. 2014).

Sex allocation theory mainly focuses on species with fixed sex expression, while several species display labile sex expression. According to sex allocation theory, dioecious species are only able to change their offspring sex ratio (Charnov 1982; Schärer 2009). Therefore, within the individual, the expression of gender and sex allocation are predicted to be fixed, independent of group size variations and uninfluenced by mating opportunities.

In contrast with this prediction, plasticity in gender expression can still be present in dioecious species that have a hermaphroditic ancestor and environmental sex determination, at least in the developmental stage (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

from a hermaphroditic ancestor. If plastic sex allocation in response to social group size is one of the main advantages of hermaphroditism over dioecy (Schärer 2009), we can expect a reduction or a loss of plasticity in sex allocation in the transitions from hermaphroditism to dioecy. This reduction of plasticity could be manifested as a decrease in the ability of sensing and/or responding to environmental stimuli, or as a reduction of the time-window when plasticity can be expressed. In the present study, we tested for variations in the degree of gender plasticity of juveniles and adults in three sexually dioecious species of *Ophryotrocha* worms – *Ophryotrocha labronica, Ophryotrocha robusta* and *Ophryotrocha macrovifera,* according to the social environment they were exposed to – i.e the presence of an adult male or female. The three species have similar morphology and reproductive biology but they differ in some genomic aspects (*O. macrovifera* and *O. labronica* have a different number of chromosomes compared to *O. robusta* (Robotti et al. 1991); and the genome size of *O. macrovifera* is twice that of the other two species (Sella et al. 1993)). The three species diverge also in their geographical distribution (Simonini 2009; Paxton and Åkesson 2010).

In the current study, ee found that plasticity in gender expression in the three species was confined to the juvenile stage, that four sexual phenotypes (pure males, pure females, males with a few oocytes and females with a few sperm) were expressed in the populations of the three species and that, in the adult phase, individuals expressed only one of the four sexual phenotypes. The presence of sexual polymorphism among adults together with plasticity in the sex expression of juveniles allowed us to outline the transition from ancestral simultaneous hermaphroditism to dioecy via monoecy (i.e. a situation where the hermaphroditic organism has distinct female and male gonads) as the most likely evolutionary pathway (Freeman et al. 1997; Golenberg and West 2013).

Materials and methods

148

149 **Study species and animal rearing**

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

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

172 *Ophryotrocha* species occur interstitially, at relatively low density in shallow, nutrient-rich waters
173 (Thornhill et al. 2009). *Ophryotrocha labronica* has a cosmopolitan worldwide distribution (Paxton and Åkesson
174 2010) and inhabits both harbors and brackish water environments (Simonini 2009). *O. macrovifera* is much rarer
175 than *O. labronica*. It was found in only a few localities along the Mediterranean sea and the North Atlantic
176 coasts (Paxton and Åkesson 2010; Simonini 2009). *O. robusta* is endemic to the Mediterranean sea, where it
177 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)

differed according to treatment in the juvenile phase using a Generalized Linear Mixed Model (GLMM) with binomial distribution. Sex was assigned based on external morphology, therefore juveniles became either males (pure males and males with oocytes) or females (pure females and females with sperm). Predictor variables included species and social environment (i.e. juvenile + male, juvenile + female, isolated juvenile). The sibship of every experimental worm was added as a random blocking to control for similarities in the proportion of the different sexual phenotypes within families. Since the sex of worms was not significantly affected by treatment during the adult phase, in the GLMM we used all the data obtained from the 330 juveniles that entered the experiment.

Then, we focused on how many juveniles matured the gender opposite to their partner's. Using a Generalized Linear Model (GLM) with Poisson error distribution and a log link function, we analyzed the difference between the number of experimental worms that matured the gender opposite to their partner's and the number of experimental worms that matured the same gender as their partner's (heterosexual pairs vs. homosexual pairs). In this statistical analysis pseudohermaphrodites (males with oocytes and females with sperm) were therefore excluded. The same statistical analysis was used to compare the number of pseudohermaphrodites among the three social environments and species.

Using a Generalized Linear Mixed Model (GLMM) with Poisson error distribution and a log link function, we also analyzed the developmental time (i.e., the number of days that passed from the stage of 3 segments with setae to the sexual differentiation stage). Predictor variables included sexual phenotype, species and social environment. The sibship of every experimental worm was handled as the random factor. Three different GLMMs, one for every sexual phenotype (males, females and pseudohermaphrodites), were made to compare the developmental times among the three social environments. As in the previous analysis, predictors were species and social environment, while sibship was a random factor. We used the results of these statistical tests only to assess differences in developmental times between social environments within the same sexual phenotype.

For all the analyses, we followed a model selection process based on Aikaike's information criterion (AIC), which is a measure of model fit. AIC was recorded from models including all possible combinations and interactions of effects, and we selected the model having the lowest AIC (Quinn and Keough 2002). In the GLMM and GLM with Poisson error distribution we also checked for overdispersion.

We assessed whether the proportion of sexual phenotypes in the adult phase differed between homo- and hetero-sexual pairs using a 2×4 contingency table (Chi-squared test).

Finally, we analyzed the number of rosette glands using a Generalized Linear Model with Poisson error distribution and a log link function. To analyze the number of rosette glands, we used the following factors as explanatory variables: species, sexual phenotype, social environment and body size. Model selection and statistical assumptions were checked, as described for the previous analysis.

All statistical analyses were performed using the software SPSS 20.

Results

Type and frequency of sexual phenotypes of the experimental worms

In the three species, we found four sexual phenotypes, i.e. 39.3% pure males, 35.6% pure females, 19.1% females with sperm and 6.0% males with oocytes. The frequencies of males (pure males and male with oocytes) and females (pure females and females with sperm) were not significantly different among species and were significantly affected by the gender of the adult to which juveniles were exposed (Table 2 and Figure 2). The interaction between these two predictors was removed after checking it was non-significant in a preliminary analysis, which suggested that the social environment had the same impact on the juveniles of the three species. Statistical comparisons show that the difference in sex ratio among "social environments" is due mainly to the difference between the environment "juvenile+ female" and the other two social environments (Table 2), indicating female as the sex able to affect juvenile sexual development.

When juveniles reached sexual maturity, they formed true heterosexual pairs with their adult partner (pure male + pure female) (47.5%) significantly more often than true homosexual pairs (pure male + pure male or pure female + pure female) (31.1%) (GLM with Poisson error distribution: d.f. = 2, $\chi^2_{(Wald)} = 19.56$, $P < 0.001$; heterosexual pairs (pure male + pure female) *vs* homosexual pairs (pure male + pure male or pure female + pure female), $B = 0.42$, $\chi^2_{(Wald)} = 6.55$, $P = 0.01$). The remaining pairs (21,4%) were composed of at least one male with oocytes or one female with sperm. In the subsequent analysis, we merged these two intermediate phenotypes together to form the experimental group of pseudohermaphrodites, since females with sperm and males with oocytes were relatively rare phenotypes. The number of pseudohermaphrodites depended significantly on species and social environment (Figure 3) (GLM: species, $\chi^2_{(Wald)} = 25.74$, d. f. = 2, $P < 0.001$; social environment, $\chi^2_{(Wald)} = 25.74$, d. f. = 2, $P < 0.001$). The number of pseudohermaphrodites was significantly higher when juveniles developed in isolation than when they developed together with males ($B = 0.75$, $\chi^2_{(Wald)} = 25.69$, $P < 0.0001$) or with females ($B = 0.27$, $\chi^2_{(Wald)} = 4.41$, $P = 0.036$).

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-

likelihood chi-square (G^2) = 19.87, d. f. = 2, $P < 0.001$; sexual phenotype, $G^2 = 80.20$, d. f. = 2, $P < 0.0001$; 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 rosette glands was significantly different between males and females ($B = -0.39$, $\chi^2_{(Wald)} = 1.88$, $P < 0.0001$), males and pseudo-hermaphrodites ($B = -0.52$, $\chi^2_{(Wald)} = 0.69$, $P < 0.0001$), while it was not different between females and pseudo-hermaphrodites ($B = -0.13$, $\chi^2_{(Wald)} = 2.64$, $P = 0.10$). This means that only two sexual phenotypes, male and female, can be distinguished according to the number of rosette glands.

Discussion

Our results showed that social environment – i.e. the presence of a sexually mature partner – influenced the expression of the sexual phenotype in juveniles of the *Ophryotrocha* dioecious species. The effect was documented 1) by variations of the frequencies of sexual phenotypes according to the social environment. Indeed juveniles tend to develop so as to form heterosexual pairs.. Furthermore the absence of a partner stimulated the production of pseudohermaphroditic sexual phenotypes. Indeed pseudohermaphrodites were significantly more common among isolated juveniles than among juveniles reared with adults of either sex. The effect of social environment was also documented 2) by the significantly different developmental times to the onset of sexual maturity of juveniles. Juveniles which have matured the same gender of their adult partner needed longer time to reach sexual maturity than juveniles which had matured the gender opposite to that of their partner's in all three species.

Sex expression was influenced by social conditions only during the juvenile phase for all the three species. This can be expected in species whose populations have largely fluctuating densities and live in patchy environments, such as intertidal communities do (Sella and Ramella 1999; Prevedelli et al. 2005). During the adult phase, frequencies of sexual 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

results,, the most recent theories about phenotypic plasticity (West-Eberhard 2003; Ah-King and Nylin 2010; Golenberg and West 2013), identify two factors involved in determining the final sexual phenotype: 1) variations in the sequences of regulatory genes responsible for the control of alternative developmental pathways and 2) environmental stimuli.

The results of our experiment made it possible for us to outline a possible evolutionary pathway of the evolution of dioecy from a hermaphroditic ancestral state in *Ophryotrocha*. In plants, the transition from hermaphroditism to dioecy is thought to have evolved through two main distinct pathways (Ehlers and Bataillon 2007): from hermaphroditism via gynodioecy to dioecy and from hermaphroditism via monoecy to dioecy. Gynodioecy refers to the coexistence in a population of two sexual phenotypes, i.e. pure females and individuals having both sexual functions (within the same flower or in separate flowers), while monoecy refers to plants having both sexual functions in separate male and female flowers within the same individual (Ehlers and Bataillon 2007). In animals the distinction between individuals having both sexual functions either within the same flower or in separate flowers translates respectively to syngonic (the same gonads producing both male and female gametes) or digonic (distinct male and female gonads in the same individual) simultaneous hermaphrodites (Vega-Frutis et al. 2014).

The pathway through gynodioecy (Charlesworth and Charlesworth 1978; Delph and Wolf 2005) is based on two mutational events. Starting from a population of hermaphrodites, a first mutation is responsible for the production of pure females, so that the remaining hermaphrodites will be selected to plastically adjust their sex allocation and becoming strongly male biased. A second mutation will then generate pure males that will spread and outnumber the strongly male-biased hermaphrodites. This model relies on a genetic assumption (the first genetic mutation) and does not include gene x environment interactions (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.

In plants, the main selective force favoring the transition to dioecy via monoecy is sexual specialization (Freeman 1997 and references therein). In animals, selective pressures leading to sexual specialization are poorly known (but see Weeks 2012). In the populations of the hermaphroditic ancestor of the dioecious *Ophryotrocha* species, selection for sexual specialization would have been responsible for the appearance of pseudohermaphrodites (in which both types of gametes are present but only one type is functional) and then of pure males and pure females. One may wonder why pseudohermaphrodites still coexist with pure males and pure females in the existing populations of *Ophryotrocha*. According to Ehlers and Bataillon (2007) and Lorenzi and Sella (2013) selection for sexual specialization may become less strong or ineffective when pseudohermaphrodites are strongly biased towards one of the two genders. In the *Ophryotrocha* dioecious species, the dichotomy between sexual dimorphism at the morphological level and sexual polymorphism at the gamete level is illustrated well by the number of rosette glands. This sex-related trait allowed us to distinguish only two reproductive morphs (males and females), while at the gamete level four sexual phenotypes exist (pure male, pure female, male with oocytes and female with sperm). If we can find out more precisely what the function of rosette glands is, we can more easily understand what the selective pressures are that act for sexual specialization and hence drive the evolution of dioecy in this genus.

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590 **Figure legends**

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Figure 1 Experimental set up. Juveniles (n = 330) were randomly assigned to one of three treatments: 1. juvenile paired with an adult female 2. juvenile paired with an adult male 3. juvenile isolated. When juveniles reached a clear sexual differentiation, a subsample of these sexually mature worms were screened to verify the presence of sperm (in females) or oocytes (in males). The remaining worms were used to form homosexual pairs (n = 87) or heterosexual pairs (n = 90). At the end of the experiment all the worms were checked for sperm in females or oocytes in males.

Figure 2. Relative frequencies of males (including males andmales with oocytes) and females (including females and females with sperm) in every of the three social environments (juvenile paired with a male, with a female or isolated). 55.9% of juveniles became males when paired together with females, while only 38.3% developed as males in pair with an adult male. In a similar way, 61.7% of juveniles developed as females when they developed together with males, while 44.1% became females in pair with females. Juveniles in isolation developed 58.8% as females and 41.2% as males.

Figure 3. Frequencies (%) of lpseudohermaphrodites (female with sperm and male with oocytes) in *O. labronica, O. macrovifera* and *O. robusta* depending on the social environment (juveniles paired with a male, a female or isolated).

Figure 4. Variations in the developmental time (days) to sexual maturity in *O. labronica, O. macrovifera* and *O. robusta* under the effect of the social environment (juveniles paired with a male, a female or isolated) paneled seperately for every sexual phenotype (females, males and pseudohermaphrodites). The graph shows the means ± SE.

Figure 5. Variations in the number of rosette glands relative to body size depending onsexual phenotypes (female, male, pseudohermaphrodite), paneled separetely for *O. labronica, O. macrovifera* and *O. robusta*.

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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

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

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

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

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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

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