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**In between breeding systems: neither dioecy nor androdioecy explain sexual polymorphism in functionally dioecious worms**

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Abstract

Related species share genetic and developmental backgrounds. Therefore separate-sex species that share recent common ancestors with hermaphroditic species may have hidden genetic variation for sex determination that causes some level of lability of expression of gender. Worms of the polychaete species *Ophryotrocha labronica* have separate, dimorphic sexes and their ancestor was hermaphroditic. *O. labronica* has a worldwide distribution and populations may differ in the degree of gender specialization. We analyzed the extent to which *O. labronica* had fixed or labile expression of gender. We found that there were up to four different sexual phenotypes, namely, pure males, males with oocytes, pure females, and females with sperm; the relative frequency of these sexual phenotypes varied in three geographically-distant populations. These sexual morphs had either male or female morphology. However, populations differed in the extent to which worms were sexually dimorphic. In the less dioecious-like population (in which pure males and females were virtually absent, all worms had both oocytes and sperm and sexual dimorphism was relatively weak), males with oocytes had slightly plastic female allocation that depended on mating opportunities – a clearly hermaphroditic trait. Males with oocytes and females with sperm were not functional hermaphrodites. They only used one type of gametes to reproduce and in this respect they probably differed from many cases of inconstancy of gender described in the literature. We consider these populations as novel examples of intermediate states between androdioecy and dioecy. This study contributes to our understanding of breeding systems as continuous gradients rather than as distinct clear-cut alternatives.

Introduction

   Dioecy and hermaphroditism are the two most common breeding systems in plants and animals. Organisms with separate sexes invest resources in a single sexual function and may show sexual dimorphism, while hermaphroditic organisms invest resources in two sexual functions and are monomorphic. From an evolutionary perspective, dioecy and hermaphroditism may be viewed as endpoints along a continuum of sexual systems (Lloyd 1976; Ah-King and Nylin 2010; Avise 2012), that includes gynodioecy (hermaphrodites + females), androdioecy (hermaphrodites + males) and trioecy or subdioecy (males + females + rare hermaphrodites). Sexual phenotypes can vary along these pathways in a quantitative way (Lloyd 1972, 1980; Delph 2003). Indeed, plasticity of gender may play important roles in the evolution of breeding systems.

   The plasticity of expression of gender is widely present in populations of flowering plants because plants develop continuously during their whole life. Therefore, botanists tend to interpret gender from a functional viewpoint as a quantitative character rather than a qualitative one (Lloyd 1972, 1980; Delph 2003). In plants, a sexual polymorphism depending on the social environment has been recognized, in which, apart from truly unisexual individuals, other individuals with labile sex expression were present (Lloyd 1976; Delph and Lloyd 1991; Delph and Wolf 2005; Pannell 1996). Labile expression of gender has been viewed in plants as the result of variation in environmental resources (Delph and Wolf 2005), in mating environment (Ehlers and Bataillon 2007), and in population density (Pannell 1997).

   Animals also display a variety of breeding systems (Leonard 2010). However, instances of intermediate breeding systems between purely hermaphroditic or purely dioecious systems are rare (Weeks 2012; Avise 2012) and therefore in animals it is more difficult than in plants to trace the evolutionary steps that lead from hermaphroditism to dioecy or vice versa. In hermaphrodites, expression of gender depends on how reproductive resources are allocated to the male and female functions. Sex-allocation theory predicts that simultaneously hermaphroditic organisms have a female-biased allocation when the mating group is small but which shifts to a more male-biased allocation when the mating group is large (Charnov 1982; Fischer 1984; Schärer 2009). The ability to adjust reproductive resources to mating groups sets the stage for the evolution of separate sexes. Separate sexes evolve if the male and female functions compete for a common budget of resources (and, of course, if the appropriate disruptive selective pressures persist in time) (see Lorenzi and Sella 2008; Anthes et al. 2010). The negative covariation between male and female allocations is a

pre-requisite for gender specialization (Charlesworth and Charlesworth 1978; Ehlers and Bataillon 2007).

 Among the various hermaphroditic model species in which sex-allocation theory has been tested, the simultaneously hermaphroditic polychaete worms in the genus *Ophryotrocha* conform rather well to theoretical predictions. These hermaphrodites show a very strong investment in the female function and a relatively much smaller investment in the male function (Sella and Ramella 1999). Indeed, these worms produce relatively high numbers of large eggs and just a few aflagellate sperm that they deposit on their partner's eggs (Oug 1990). They are obligate outcrossers and they reciprocally exchange eggs during successive pseudocopulations. As predicted by sex-allocation theory, these hermaphroditic worms opportunistically adjust their sexual investment according to their partner's investment (Sella and Lorenzi 2000) and to opportunities for mating. In isolated pairs, sex allocation is strongly female biased. As opportunities for mating increase, these hermaphrodites drastically reduce the amount of resources allocated to the female function, i.e. to egg production (Lorenzi et al. 2005). This does not translate into a corresponding variation in sperm production, possibly because the need for aflagellate sperm is always very low in pseudocopulation. The resources spared from egg production are likely invested in expensive agonistic behaviors during competition for mates (Lorenzi et al. 2006). Eventually, 2% of hermaphrodites function as males-only and never lay their eggs (Di Bona et al. 2010).

 The transition between hermaphroditism and dioecy occurred only once in the genus *Ophryotrocha* and produced dioecious species (Dahlgren et al. 2001). However, in the dioecious and sexually dimorphic species *Ophryotrocha labronica*, different populations might not be purely dioecious, as they exhibit different levels of lability in expression of gender. Indeed, *O. labronica* was collected in the Mediterranean Sea and classified initially as hermaphroditic (La Greca and Bacci 1962) but later as dioecious (Åkesson 1970, 1972a, b; Premoli et al. 1996) or dioecious with some hermaphrodites (Bacci et al. 1979). This species has a worldwide distribution and, across populations, both females that reproduced by selfing (Zunarelli Vandini 1967; Sella and Zambaldi 1985; Åkesson and Paxton 2005; Martino 2012), and males that produced never-to-lay oocytes (Åkesson and Paxton 2005) were found.

 We tested whether these contrasting reports on the reproductive modes of *O. labronica* were hiding a mixed breeding system in which males and/or females coexisted with hermaphrodites. In three geographically distant populations we found males with oocytes and females with sperm. Therefore, we tested whether these worms were able to shift their resources between sexual functions according to opportunities for mating, as hermaphrodites do. Finally, we tested whether the worms with gametes of two sexes were functional hermaphrodites.

**Material and methods**

**Study species and sex related traits**

*Ophryotrocha labronica* La Greca and Bacci (1962) is included in a large, coherent, informal monophyletic group, the *O. labronica* group (Åkesson and Paxton 2005; Paxton and Åkesson 2010). This species is a 4-mm-long, benthic, marine worm, with ubiquitous distribution (Paxton and Åkesson 2010); it lives among fouling organisms in eutrophic waters such as those of harbors. Worms are sexually dimorphic with phenotypic males and females (Paxton and Åkesson 2007). In *Ophryotrocha* species, sperm and oocytes derive from the same type of germ cells, which can differentiate rather flexibly. Females grow faster than males and reach their sexual maturity at a body size of 11-12 chaetigerous segments, while males are sexually mature at 7-8 chaetigerous segments. Males have thicker jaws (and wider prostomium) than do females. These traits allow easy identification of gender at low magnification. At higher magnification, when worms are kept immobile, another sexually dimorphic trait is appreciable - the dorsal median rosette glands – which are more abundant in the posterior body segments of males than in those of females (Paxton and Åkesson 2010). The function of the rosette glands is unknown. Worms were reared in 10-mL bowls filled with marine water (33 psu) and kept in thermostatic cabinets at 21°C. We kept worms in resource-rich bowls, feeding them with spinach *ad libitum*. We changed water in the bowls every third day to avoid accumulation of catabolites.

We used three populations. One was from the Mediterranean harbor of Genova (Italy), and two were from California - Alamitos Beach (Long Beach) and San Diego. Since species identification is difficult in the *O. labronica* group (Paxton and Åkesson 2010), we checked whether these populations were interfertile by setting up 72 reciprocal interpopulation crosses with all possible combinations of sex and population. Seventy-four percent of them produced fertile $F_1$ and fertile $F_2$. The probability of reproducing successfully did not depend on which populations were crossed or on which population supplied the male or the female worm (factor population: Wald $\chi^2 = 1.338$, d. f. = 2, P = 0.512; factor male/female worm: Wald $\chi^2 = 0.796$, d. f. = 1, P = 0.372; the interaction term was removed because it had no significant impact on the comparison).

**Experimental set up**

Sex-related traits in isolated worms

We obtained worms from 30 pairs of parents per population and reared their same-age brood up to sexual maturity. Within every brood, we sexed worms by visually inspecting their external morphology without any manipulation of the worms (stereomicroscope, 10 X magnification). We classified worms as males when they had 1) relatively small body size, 2) large jaws, and 3) no visible oocytes in the coelom. We classified worms as females when they had 1) relatively large body size, 2) small jaws, and 3) oocytes visible in the coelom through the transparent body wall.

Among the sexed worms, we randomly chose a total of 48 males and 59 females of the three populations (Alamitos Beach: 20 males and 22 females; San Diego: 15 males and 22 females; Mediterranean Sea: 15 males and 13 females) and reared worms in isolation (1 worm/bowl) for three weeks. By isolating worms we aimed to control for differences in maturation rates between sexes and populations and for potential social effects on expression of gender (e.g., Rolando 1984). After the isolation period, we checked for presence of sperm and oocytes in the coelom of the worms. Screening for sperm or oocytes required heavy manipulations of worms. Worms were gently squeezed between two slides, so that sperm oozed from the body walls and both sperm and oocytes in the coelom were counted under a phase-contrast light microscope (400 X magnification). Sperm are aflagellate (Morrow 2004) and have a diameter of $3 - 4$ μm (Berruti et al. 1978; Troyer and Schwager 1979); oocytes have a diameter of $120 - 130$ μm (Paxton and Åkesson 2010).

Based on whether worms had male or female gametes, we re-classified putative males and females in four categories:

1) pure males (i.e. worms with male external morphology that lacked any female allocation and had only sperm in the coelom)

2) males with oocytes (i.e. worms with male external morphology and both sperm and at least one oocyte of any maturation phase in the coelom);

3) females with sperm (i.e. worms with female external morphology and both oocytes and at least one sperm in the coelom).

4) pure females (i.e., worms with female external morphology that lacked any male allocation and had only oocytes in the coelom).

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We were aware that the presence of oocytes is easier to detect than the presence of sperm, particularly when sperm were very rare. Therefore, the frequency of pure females could be overestimated. We also used the number of chaetigerous segments as a measure of body size and we counted the number of rosette glands in each worm.

We checked whether worms initially classified as males were re-classified as males (either pure males or males with oocytes) and whether worms initially classified as females were re-classified as females (either pure females or females with sperm). The correlation between the first and the second classification by sex was equal to 1 in the San Diego and Mediterranean populations and was highly significant for the Alamitos Beach population, in which two worms initially classified as males turned out to be pure females (Spearman's rho, rho = 0.908, n = 42, P < 0.0001).


Pure males and males with oocytes under high and low opportunities for mating


Among males, our analyses highlighted that there were pure males and males with oocytes in almost a 1:1 ratio in one population. This opened an interesting question. Since egg production consumes more resources than does sperm production in *Ophryotrocha* (Di Bona et al. 2010), males investing in eggs might be less fit than pure males, unless they were plastic. Therefore, we tested whether these sexual phenotypes were plastic and whether the frequencies of the two types of males depended on opportunities for mating. We also tested whether males with oocytes adjusted their sex allocation in accordance with opportunities for mating. In hermaphroditic *Ophryotrocha* worms, oocyte production is extremely plastic and worms adjust it opportunistically in relation to opportunities for mating (Lorenzi et al. 2005, 2006). We tested whether the males with oocytes in *O. labronica* were actually hermaphrodites (or had kept some of the plasticity in sex allocation of their hermaphroditic ancestors) and plastically changed their female allocation depending on the opportunities for mating, as hermaphrodites do. Therefore we tested whether the males with oocytes had relatively low female allocation when they had multiple opportunities for mating and multiple mate competitors. Vice versa, we expected that males with oocytes had relatively high female allocation when each of them was paired to a single female (limited opportunities for mating and no mate competitors).

For these reasons, we exposed non-sibling, same-age, newly-mature, virgin males to one of two levels of opportunities for mating:

Low opportunities for mating (1 male + 1 female) for three weeks (Alamitos Beach: n = 23 pairs; San Diego: n = 31 pairs; Mediterranean Sea: n = 22 pairs).

High opportunities for mating (3 males + 3 females) for three weeks (Alamitos Beach: n = 27 groups; San Diego: n = 20 groups; Mediterranean Sea: n = 27 groups).

During the three weeks, bowls were checked once a week. Whenever we found egg cocoons, we took notes and removed the cocoon(s) from the bowl. At the end of the three-week period, we tested whether males had only sperm or both oocytes and sperm in their coelom (using the procedure explained above) and we counted their rosette glands. We also measured sex allocation in males with oocytes as follows:

We measured female allocation by checking for oocytes in the coelom and we assigned a score to every male with oocytes as follows:

score 1: small oocytes (stage II);

score 2: medium oocytes (stage III);

score 3: large oocytes (stage IV);

score 4: between 30 and 50 oocytes ready for oviposition (stage V),

score 5: > 50 oocytes ready for oviposition (stage V).

The scale is based on oogenesis stages II-V according to Pfannenstiel and Grünig (1982).

We measured male allocation by checking for sperm in the coelom (following the procedure described above). Two observers counted sperm and their counts were averaged for data analysis. Observations by both observers were blind with respect to the two groups and measures were highly repeatable (Spearman rank correlation test, rho = 0.87, P < 0.001).


Reproduction in isolated worms and in same-sex pairs of worms


We tested whether worms were able to self or outcross as hermaphrodites. We did this by isolating worms and by setting up pairs of worms of the same sex (Table 1). We checked whether non-sibling, same-age, newly-mature, virgin males and females (sexed on the basis of external morphology) selfed or outcrossed during 60 days, which is approximately 2/3 of their average lifespan (Martino 2012). When worms laid cocoons, we reared them separately to check whether embryos developed to adulthood.  As a control, reproductive  success of heterosexual pairs was measured in the pairs kept under low opportunities for mating for 3 weeks.


**Statistical analyses**

Data on the frequencies of sexual phenotypes were analysed with Pearson's $\chi^2$ or Wald $\chi^2$. We obtained the latter when we had > 1 factor and used Generalized Linear Models for binomially

distributed data (link function = logit). The number of rosette glands was analysed with a Generalized Linear Model for Poisson distributed data (link function = log) with body size as a covariate, to control whether body size affected the number of rosette glands. Male allocation (sperm number) was analysed with a Mann-Whitney test. Female allocation in males with oocytes was measured as the frequency of worms with a given female allocation, out of five classes of female allocation (see above). These counts were analyzed with a Generalized Linear Model for Poisson distributed data (link function = log). Males with oocytes were checked to see whether female and male allocations, as well as the number of rosette glands, were correlated. Correlations were measured in males with oocytes across both low and high mating opportunities in the three populations. We did not apply Bonferroni corrections to correlation probability values to avoid over-inflation of Type II error (Nakagawa 2004).

**Results**

Sex-related traits in isolated worms

<u>Frequency of sexual phenotypes</u>

There were multiple sexual phenotypes in these populations. Many worms that we had classified either as males or females according to their external morphology ultimately had gametes of the opposite sex in their coeloms, so that pure males and pure females were rare (Fig. 1). The worms with both male and female gametes had normal external morphology (shape of the jaw and of the rosette gland), which suggested that they were not gynandromorphs. The frequencies of the four sexual phenotypes (pure males, pure females, males with oocytes, and females with sperm) were significantly different among populations (Pearson $\chi^2 = 22.937$, n = 107, d. f. = 6, P = 0.001).

In the Mediterranean population, all four phenotypes were present and the two most common were pure males and females with sperm. Worms with gametes of both sexes, i.e., males with oocytes and females with sperm, overall amounted to 64%, and this figure raised to 94% in the San Diego population and to 95% in the Alamitos Beach population, where these phenotypes were the most common. Pure males were either very rare or absent in the other two populations (< 5% for San Diego, 0% for Alamitos Beach). Occasional pure females (< 5%) were present in the Alamitos Beach population, but this measure could be overestimated (see methods).

<u>Rosette glands</u>

The number of rosette glands varied among sexual phenotypes in different ways across populations (interaction population by sexual phenotype: total n = 106, Wald $\chi^2$ = 19.405, d. f. = 3, P = 0.0002, covariate body size: $\chi^2$ = 5.382, d. f. = 1, P = 0.020) (Fig. 2). In the Alamitos Beach population, all worms had roughly similar numbers of rosette glands (total n = 42, Wald $\chi^2$ = 1.545, d. f. = 2, P = 0.462; covariate body size: $\chi^2$ = 0.897, d. f. = 1, P = 0.343), so that this trait was not dimorphic (range of number of rosette glands: males with oocytes = 2 - 6; females with sperm = 0 - 7; the only two pure females had 2 and 4 rosette glands). In contrast, in the San Diego population, worms had different numbers of rosette glands depending on whether they were pure males (range of rosette numbers = 6 - 7), males with oocytes (range = 0 - 6) or females with sperm (range = 0 - 5) (total n = 36, Wald $\chi^2$ = 17.898, d. f. = 2, P = 0.0002; covariate body size: $\chi^2$ = 3.483, d. f. = 1, P = 0.062). Therefore, the number of rosette glands was sexually dimorphic in this population and its value increased from females with sperm to pure males, i.e. with increasing maleness. In the Mediterranean population, sexual dimorphism in rosette glands was even more marked than in the San Diego population. Pure males had more rosette glands (range: 6 - 8)  than did males with oocytes (range: 2 - 6) and males with oocytes had more rosette glands than did females with sperm (range 0 - 1; the only one pure female had no rosette glands) (total n = 28, Wald $\chi^2$ = 16.371, d. f. = 2, P = 0.0003; body size: $\chi^2$ = 2.642, d. f. = 1, P = 0.104).

It is worth noting that males with oocytes and females with sperm were phenotypically distinct worms in the San Diego and Mediterranean populations. We classified them as males and females respectively on the basis of external morphology, and they also differed in the number of rosette glands (San Diego: Wald $\chi^2$ = 7.340, d.f. = 1, P = 0.007; Mediterranean: $\chi^2$ = 19.041, d. f. = 1, P = 0.0001). In contrast, we classified males with oocytes and females with sperm as distinct phenotypes in the Alamitos Beach population, but they ultimately had similar numbers of rosette glands ($\chi^2$ = 1.175, d. f. = 1, P = 0.278).

Pure males and males with oocytes under high and low opportunities for mating

Frequency of sexual phenotypes

There were significant differences among populations - but not between mating opportunities - in the proportion of pure males versus males with oocytes (total n = 259, factor population: Wald $\chi^2$ = 29.012, d. f. = 2, P < 0.0001; factor mating opportunities: $\chi^2$ = 0.958, d. f. = 1, P = 0.329; the interaction term was removed because it had no significant impact on the comparison).

Plasticity in sex allocation

Males with oocytes significantly differed between populations in their female allocation in relation to mating opportunities (total n = 226, factor population: Wald $\chi^2$ = 17.384, d.f. = 2, P < 0.0001; factor mating opportunities: $\chi^2$ = 0.037, d. f. = 1, P = 0.847; and interaction population by mating opportunities: $\chi^2$ = 4.567, d. f. = 2, P = 0.102) (fig. 3). In the Alamitos Beach population, males with oocytes slightly adjusted their female allocation to the mating opportunities they encountered, by increasing it when mating opportunities were low and decreasing it when mating opportunities were high. These differences were marginally significant (Wald $\chi^2$ = 3.686, d. f. = 1, P = 0.055). In contrast, worms from the San Diego and Mediterranean populations did not adjust their female allocation to mating opportunities, possibly because their female allocation was negligible (San Diego: $\chi^2$ = 1.008, d. f. = 1, P = 0.315; Mediterranean population: $\chi^2$ = 0.300, d. f. = 1, P = 0.584).

Male allocation did not vary with mating opportunities in any population (Mann-Whitney test, Alamitos Beach: U = 791.5, total n = 98, P = 0.703; San Diego: U = 611.0, total n = 78, P = 0.687; Mediterranean Sea: U = 115.5, total n = 50, P = 0.168).

Correlation between sex-related traits

 In males under high and low mating opportunities, the larger the male allocation, the smaller the female allocation, and this negative correlation was highly significant (Table 2).

In the males from the Mediterranean population, the higher the male allocation, the higher the number of rosette glands. In contrast, in males from the Alamitos Beach the number of rosette glands was not related to the degree of male allocation (Table 2). Worms from the San Diego population were intermediate in this trait; the lower the number of rosette glands they had, the higher the female allocation, but there was no clear relation between the number of rosette glands and the male allocation (Table 2).

Reproduction in heterosexual pairs of worms

Of the pairs of worms kept under low mating opportunities, all reproduced successfully within three weeks (100%) (Fig. 4). These pairs were composed of a female with sperm and either a pure male or a male with oocytes. This suggests that all sexual phenotypes were fertile.

Reproduction in same-sex pairs of worms

Only six of 76 (8%) same-sex pairs of females (total number of females: n = 152) produced fertile broods in two months (Fig. 4). In contrast, none of 90 (0%) same-sex pairs of males produced a brood in the same time period (Fig. 4).

Reproduction in isolated worms

Although 16 out of 56 females that were kept in isolation for two months laid eggs, only one produced a fertile brood (2%) (Fig. 4). This female was from the San Diego population. In contrast, none of the 55 males in isolation produced a brood (0%) (Fig. 4). There was no significant difference between the frequency of females that produced fertile broods in isolation (1 out of 56) and the frequency of females that produced fertile broods in same-sex pairs (6 out of 152; 4%) ($\chi^2$ = 0.588, d. f. = 1, P = 0.443), suggesting that all these brood might have been produced by rare selfing females. Overall, the rarity of broods obtained from isolated worms or from same-sex pairs suggests that males with oocytes and females with sperm were not functionally hermaphroditic.

**Discussion**

Our results document that worms of three *O. labronica* populations had up to four different sexual phenotypes, namely, pure males, males with oocytes, pure females, and females with sperm. We consider these populations to be examples of intermediate states between androdioecy and dioecy. This variety of sexual phenotypes is novel in animals and the makes this species uniquely suited to study the dynamics of the evolution of breeding systems.

In the pure males and in the very rare pure females, morphological gender and functional gender coincided (i.e., pure males had male external morphology and produced only sperm and pure females had female external morphology and produced only oocytes). In contrast, males with oocytes and females with sperm had gametes of both sexes but used only the gametes of one sex for reproduction, i.e. the gametes of their morphological sex (except for the rare females with sperm that selfed). This suggests that most of these worms were not functionally hermaphroditic, as they gained fitness through one functioning sex only. For this reason, on the basis of a functional criterion of gender (Lloyd 1980; Pannell 2002; Delph 2003), they cannot be classified as hermaphrodites. Nor can they be classified as pure males or pure females, as they had gametes of two sexes. Moreover, their external morphology was generally dimorphic in secondary sexual traits (i.e., females with sperm resembled females in body size and jaw shape, and males with oocytes resembled males in the same traits). Secondary sexual traits helped us in identifying multiple sexual

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phenotypes beyond males, females and hermaphrodites. Because an extensive literature defines these worm populations as composed of males and females (see for example Paxton and Åkesson 2010), we called them females with sperm and males with oocytes.

The relative frequency of males with oocytes, females with sperm, pure males, and pure females varied among populations. Nearly all the females had sperm in all three populations. In contrast, in the Mediterranean population, about half of the males were pure and the other half had oocytes, whereas pure males were nearly absent in both Californian populations.

The combination of sexual phenotypes that we described (summary in Table 3) does not fit any of the types of breeding systems described in the literature on animals. In the reviews of breeding systems, animal populations that are neither dioecious nor hermaphroditic are generally composed of functional hermaphrodites and pure males (androdioecy) or pure females (gynodioecy) or of functional hermaphrodites plus pure males and pure females (trioecy or subdioecy) (Avise and Mank 2009; Weeks 2012). If we had checked the worms' gender by screening for gametes only, we would have classified these populations as androdioecious. However, our data show that functional hermaphrodites were virtually lacking in these populations as worms were generally unable to selfThe difficulty in classifying the breeding systems of these populations is also increased by the fact that they differed in the frequency and characteristics of their sexual phenotypes. In the Alamitos Beach population, pure males were absent, so that nearly all worms had gametes of both sexes (although they appeared as either males or females) and had similar numbers of the sex-related rosette glands. In contrast, in the Mediterranean population, pure males were common and worms were extremely dimorphic in the number of rosette glands. The San Diego population was intermediate in these respects. Pure males were present, but were very rare; sexual phenotypes were dimorphic in the number of rosette glands, but not so strongly as in the Mediterranean population. In our view, these populations are a rare example of what were thought to be ephemeral, intermediate states between androdioecy and dioecy. These states involve the retention of some allocation to the opposite sex. The breeding system of these worms is quite unique and is different from the breeding systems described in plants as transitional  (in which invasion of pure females in populations of hermaphrodites is  the key event in the evolutionary trajectory from hermaphroditism to dioecy, Charlesworth 1984).

The current evolutionary models of the transition from hermaphroditism to dioecy consider androdioecy and gynodioecy as intermediate states along a two-step process that includes two mutational events (Charlesworth and Charlesworth 1978; Delph and Wolf 2005). A first mutation determines, for example, male-sterility in some hermaphrodites and creates gynodioecious populations. A second mutation determines female-sterility in the extant hermaphrodites and creates

dioecious populations. The states in between the two mutational events - androdioecy and gynodioecy – are considered ephemeral and rare at best. However, this evolutionary pathway may oversimplify the range of natural variation in sexual phenotypes.

Indeed, sexual morphs are reported in the literature other than fertile males, females, and hermaphrodites. In some plant species, males with fixed male expression coexist with males with labile sex expression (called "inconstant males" i.e., male genotypes than can also reproduce via the female function) (Lloyd 1976; Ehlers and Bataillon 2007). In the androdioecious herb *Mercurialis annua*, the hermaphrodites that coexist with males might be actually females with "inconstant" sex expression (Dorken and Pannell 2009). Males of several gynodioecious plants listed by Lloyd (1976) contribute a considerable number of ovules and show high inconstancy. In subdioecious populations of *Wurmbea dioica*, plants with hermaphrodite flowers are male plants displaying sex inconstancy (Barrett 1992). These examples come from plants that maintain flexibility in their sex expression throughout their lives via the undifferentiated meristematic cells. Although there may be more of this kind of "inconstancy" in animals than is generally recognized, in most animals sex expression is canalized during embryonic development (Jesson and Garnock-Jones 2012). This may limit the plasticity of sex expression in animals, but rare examples that vaguely resemble plants' inconstant-sexes are known. For example, in a scleractinian coral, populations are composed of female colonies that release eggs and male colonies that release both sperm and eggs – yet, those eggs are non-viable (Baird et al. 2009). In the clam shrimp *Eulimnadia texana* there are males that also produce some eggs (Weeks et al. 2006). In the iteroparous *Ophryotrocha* worms, gonads are very simple and gametes complete their development at each reproductive bout as free germ cell cysts in the worm's coelom, thus possibly maintaining some plasticity (Brubacher and Huebner 2011). The frequencies of inconstant males are highly variable both between and within populations of plant species (e.g., 0 - 80%) (Ehlers and Bataillon 2007). Similarly, our data show that in the three worm populations there were male and female worms with labile sex expression (males with oocytes and females with sperm) and their frequency varied among populations. However, inconstant males in plants can produce fruits (Ehlers and Bataillon 2007), whereas *O. labronica* males with oocytes never laid eggs and females with sperm only occasionally selfed. In our view, these three populations may represent three different stages along the evolutionary trajectory between androdioecy and dioecy.

Recent available phylogenetic analyses support the hypothesis that hermaphroditism was the ancestral state in the genus *Ophryotrocha* (Heggøy et al. 2007; Thornhill et al. 2009) and indicate that the transition from hermaphroditism to dioecy occurred only once in the genus (Dahlgren et al. 2001). In this scenario, we can make the hypothesis that females with sperm and males with oocytes

exhibited vestigial traits of an ancestral hermaphroditic state. This is not surprising, since hermaphroditic traits were already reported for dioecious *Ophryotrocha* species (Pfannenstiel 1976; Bacci et al. 1979; Rolando 1982; Åkesson 1984; Premoli et al. 1996). Actually the three populations may represent three steps in the pathway from hermaphroditism to dioecy. The Alamitos Beach population retains hermaphroditic traits. The worms rarely had fixed sexual phenotypes. In fact, most worms had labile sex expression. The worms were weakly dimorphic (see Fig. 2) and most importantly, males with oocytes exhibited a tendency to plastically adjust their female allocation to mating opportunities. Plasticity in sex allocation is a trait expected in hermaphrodites (Schärer 2009) and plasticity in female allocation is strongly expressed by the hermaphroditic *Ophryotrocha* worms (Lorenzi et al. 2005). Among the three populations, the Mediterranean population retains the least of the hermaphroditic traits. Half of the morphological males had fixed male phenotype (although the other half of morphological males and all females had labile sex expression). The worms were strongly dimorphic and males with oocytes did not exhibit any plasticity in their female allocation. The worms from the San Diego population were intermediate in these traits. Males with fixed sex expression were more common than in the Alamitos Beach population, but rarer than in the Mediterranean population. Sexual dimorphism was more pronounced than in the Alamitos Beach population but less than in the San Diego one. Finally, males with oocytes had no plastic female allocation under our resource-rich experimental conditions.

The selective forces that favor the transition from hermaphroditism to dioecy are poorly known in animals, in which the transition occurred more often through androdioecy than through gynodioecy (whereas the opposite occurred in plants, Weeks 2012). In plants, the predominant view that inbreeding avoidance is one of the selective forces in the evolution of breeding systems (e.g., Charlesworth and Charlesworth 1978; Ehlers and Bataillon 2007) has been enriched by the speculation that sexual specialization may also play a role (e.g., Eppley and Pannell 2007). In animals, sexual specialization might be one of the main factors in the dynamics of breeding-system evolution (Puurtinen and Kaitala 2002; Eppley and Jesson 2008). In this view, the transitions from hermaphroditism to dioecy appear to be associated with size specialization between the sexes (Weeks 2012). Actually, in *O. labronica* we distinguish males from females on the basis of shape and size  of jaws and body. Future studies should also focus on behavioral traits potentially associated to specialization of gender. For example, mate competition might involve a specialization of the male gender in fighting abilities. Fighting abilities are already exhibited  in the hermaphroditic species  *O. diadema*. In this species hermaphrodites fight as they compete with

rivals to mate in the male role (Lorenzi et al., 2006), which is the preferred role (Di Bona et al. 2010).

In hermaphroditic species both of plants and animals, trade-offs between male and female investment are evident as negative genetic correlations between male and female reproductive outputs. These trade-offs allow/enhance the process of sexual specialization and therefore may facilitate the evolution of dioecy from hermaphroditism (Ashman 1999; Olson and Antonovics 2000). Remarkably, in our study we did find that in the males with oocytes of all three populations, the male and the female allocations were negatively correlated. If these phenotypic correlations are indicative of underlying genetic correlations, this means that, in situations in which strong allocation to the male function gave higher fitness reward, disruptive selection would select against males with oocytes and in favor of pure males.

The retention of some female function in males with oocytes and the retention of some male function in females with sperm need some explanation. The adaptive value of sexual phenotypes with labile sex expression is being debated (Ehlers and Bataillon 2007). If producing two types of gametes instead of one comes at a cost, the sexual morphs with gametes of two sexes (males with oocytes and females with sperm) should be selected against, rather than maintained in natural populations. We do not know what costs males with oocytes and females with sperm paid for their double allocation and further studies are needed. However, oocytes are much more expensive than sperm in these worms (Di Bona et al., 2010). Therefore, it is reasonable to hypothesize that selection for saving resources from egg production may have been stronger than that for saving resources from sperm production. This would explain why all the females retained some allocation to sperm production. In contrast, among males, many retained some allocation to the female function but several did not exhibit this trait. In plants, disruptive selective forces that act during the transition from hermaphroditism to dioecy may become less effective when hermaphrodites are already strongly biased towards one sex (Dorken and Pannell 2009). Similarly, in worms, males with oocytes and females with sperm might be under weak selection pressures to become pure sexes.

## Acknowledgements

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