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# Cooperation and competition as drivers of the evolutionary transition from simultaneous hermaphroditism to separate sexes in *Ophryotrocha* worms

Laura Picchi

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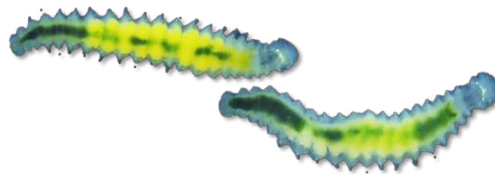
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**Cooperation and competition as drivers of the  
evolutionary transition from simultaneous  
hermaphroditism to separate sexes in  
*Ophryotrocha* worms.**



Directeur de thèse :

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*“Despite the Victorians’ reputation for prudishness, nineteenth-century natural philosophers spent lots of time watching animals mate”*

J. Knight, in Sexual stereotypes, *Nature* (2002)



# Coopération et compétition comme éléments moteurs de la transition évolutive de l'hermaphroditisme vers des sexes séparés chez les vers du genre *Ophryotrocha*

## Résumé

Chez les animaux, l'hermaphroditisme et le gonochorisme sont tous deux apparus plusieurs fois de manière indépendante. Cependant, les voies évolutives impliquées dans la transition entre les systèmes sexuels sont en grande partie inconnues. L'objectif de cette thèse était donc d'étudier la transition évolutive de l'hermaphroditisme vers des sexes séparés chez les vers du genre *Ophryotrocha*, en s'intéressant en particulier à la manière dont la coopération et la compétition entre individus peuvent déterminer l'allocation de ressources reproductives chez les hermaphrodites, soit en favorisant l'évolution de sexes séparés ou en stabilisant l'hermaphroditisme. Nos résultats ont révélé que les vers hermaphrodites échangent réciproquement des œufs, une forme de coopération qui favorise une allocation des ressources sexuelles biaisée en faveur de la fonction femelle et qui stabilise ainsi l'hermaphroditisme. Toutefois, lorsque les hermaphrodites sont exposés à une forte compétition pour l'accouplement, ils transfèrent les ressources de la fonction femelle vers la fonction mâle, ce qui diminue la production d'œufs et les soins parentaux, mais augmente la motilité et l'agressivité. À des niveaux élevés de compétition pour l'accouplement, motilité et agression peuvent améliorer la recherche et la monopolisation du partenaire, favorisant ainsi la propagation des hermaphrodites biaisés en faveur de la fonction mâle, promouvant ainsi l'évolution de mâles purs et, ensuite, de femelles pures. Enfin, nous avons montré que les hermaphrodites prêts à s'accoupler en tant que mâles uniquement (sans œufs matures) préfèrent des partenaires hermaphrodites prêts à s'accoupler en tant que femelles, qui, en revanche, n'expriment aucune préférence. Globalement, ce travail montre que la coopération et la compétition sont tous deux des facteurs importants dans la répartition des sexes chez les hermaphrodites et suggère que des changements dans l'environnement social ont pu jouer un rôle central dans l'évolution des systèmes sexuels chez les vers du genre *Ophryotrocha*.

**Mots clés :** systèmes sexuels, compétition pour la reproduction, allocation sexuelle, polychètes

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## Cooperation and competition as drivers of the evolutionary transition from simultaneous hermaphroditism to separate sexes in *Ophryotrocha* worms

### Abstract

In animals, both simultaneous hermaphroditism and gonochorism originated independently several times. Yet the evolutionary pathways involved in the transition between sexual systems are largely unknown. The aim of this thesis is therefore to investigate the evolutionary transition from hermaphroditism to separate sexes in *Ophryotrocha* worms, focusing on how cooperation and competition between individuals shape hermaphrodite sex allocation, either promoting the evolution of separate sexes or stabilizing hermaphroditism. Our results revealed that hermaphroditic worms reciprocally exchange egg-clutches, a form of cooperation that favor a female-biased sex allocation and stabilizes hermaphroditism. However, when hermaphrodites are exposed to high level of mate competition, they reallocate resources from the female to the male function, decreasing egg production and parental care and increasing motility and aggression. Under high mate competition level, motility and aggression may improve mate searching and partner monopolization and may favor the spread of male-biased hermaphrodites, promoting the evolution of pure males and, later, pure females. Finally, we showed that hermaphrodites ready to mate as males only (without ripe eggs) prefer hermaphrodites ready to mate as females as partners, which, in contrast, do not express any preference. Overall, this work shows that both cooperation and competition are important factors in hermaphrodite sex allocation, and suggests that changes in the social environment may have played a central role in the evolution of sexual systems in *Ophryotrocha* worms.

**Key words:** sexual systems, mate competition, sex allocation, polychaetes

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# Preface

The chapters of this thesis constitute a set of papers in preparation for submission, submitted or published in peer-reviewed journals.

## Publications included in this thesis

### Book chapters

**Picchi L.**, Lorenzi M.C. (in press). Polychaete worms on the brink between hermaphroditism and separate sexes. In: *Transitions between Sexual Systems* (Leonard J.L. ed). Springer Verlag.

### Peer reviewed journals

**Picchi L.**, Cabanes G., Ricci-Bonot C., Lorenzi M.C. (2018). Quantitative matching of clutch size in reciprocating hermaphroditic worms. *Current Biology* in press.

Santi M., **Picchi L.**, Lorenzi M.C. (2018). Dynamic modulation of reproductive strategies in a simultaneous hermaphrodites and the preference for the male role. *Animal Behaviour* in press.

Lorenzi M.C., Araguas A., Bocquet C., **Picchi L.**, Ricci-Bonot C. 2018. Courtship as a war of attrition in a simultaneous hermaphrodite. *Animal Biology* in press.

### Talks at meetings

**Picchi, L.**, Perrin L., Maury C., Lorenzi M.C. Gender-related behaviours in hermaphrodites and the transition to separate sexes. SHOW: Simultaneously Hermaphroditic Organisms Workshop, Paris (France) 14-16 February March 2018.

**Picchi, L.**, Ricci-Bonot, C., Cabanes, G., Lorenzi M.C. Reciprocal Egg-trading in a simultaneous hermaphrodite. 14th International Congress on Invertebrate Reproduction and Development, Italy, 2017 (Napoli, 29-30 August, 2017 – Firenze, 1-2 September, 2017).

**Picchi, L.**, Ricci-Bonot, C., Lorenzi M.C. Egg reciprocation in a simultaneous hermaphrodite. Annual meeting of the Société Française pour l'Étude du Comportement Animal SFECA. Gif-sur-Yvette, France, April 2017.

**Picchi, L.**, Ricci-Bonot C., Lorenzi M.C. Outcrossing hermaphrodites: solving the conflict through reciprocation. SHOW: Simultaneously Hermaphroditic Organisms Workshop, Lund (Sweden) 8-10 March 2017. Abstract Book p. 8.



### **Posters at meetings**

**Picchi L.**, Ricci-Bonot C., Cabanes, G., Lorenzi M.C. Dummies can do it: conditional egg reciprocation and numerical matching of egg clutches in pairs of polychaete worms. Evolution, II Joint Congress on Evolutionary Biology, Montpellier (France) 19 - 22 August 2018.

**Picchi L.**, Ricci-Bonot C., Lorenzi M.C. Parental care and reciprocal egg-exchange in a simultaneous hermaphrodite. International student meeting Ecology & Behaviour, Chizé (France) 19 - 23 June 2017.

### **Meeting organization**

**SHOW: Simultaneously Hermaphroditic Organisms Workshop**, Paris (France) 14-16 February March 2018 (in collaboration with Céline Bocquet and Maria Cristina Lorenzi).

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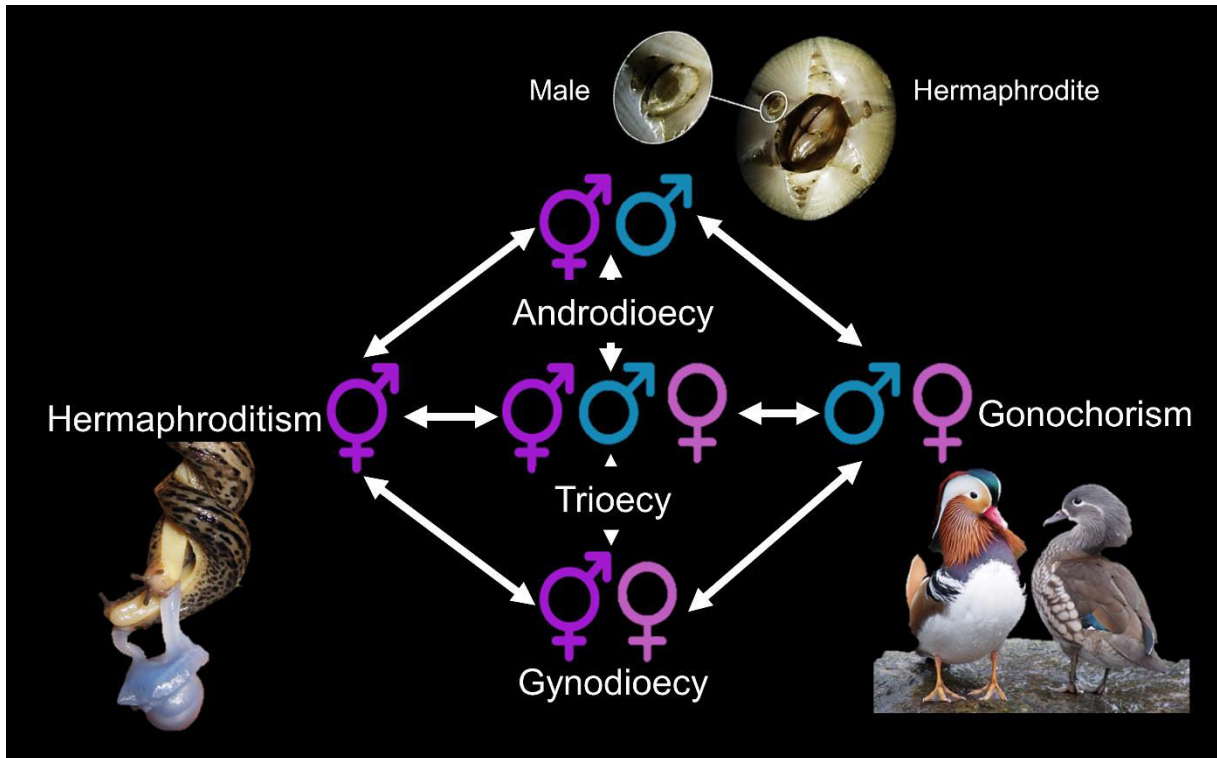
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# **Introduction**

## **Sexual systems and William's paradox**

Sexual systems are defined as the patterns of allocation towards male and female function across individuals in a species (Leonard 2013). According to this definition, we can recognize two main sexual systems, which are relatively more common and evolutionary stable: 1) gonochorism (also called dioecy) in which individuals have separate sexes and allocate their reproductive resources either to the male or to the female function; 2) hermaphroditism, in which each individual allocates resources to both sexual functions and is, therefore, able to reproduce through both sperm and eggs in a lifetime. Hermaphroditic organisms can further be divided in simultaneous hermaphrodites, when they are able to reproduce as males and females in a single breeding season, and in sequential hermaphrodites, when they reproduce as males during a part of their life and as females during the other. In animals, gonochorism is the most common sexual system, with about 95% of species where individuals are either male or female since their birth (e.g., virtually all insects and vertebrates other than fish); hermaphroditism, even if rare (about 5% of the species), is widespread among metazoans and is present in 70% of the animal phyla (Jarne and Auld 2006). In contrast, the vast majority of flowering plants are hermaphrodites (about 94% of angiosperms), though gonochorism is found in 43% of angiosperm families (Renner 2014) and is dominant in 8 out of 12 gymnosperm families (Walas et al. 2018).

Gonochorism and hermaphroditism can be viewed as the two endpoints along a continuum of sexual systems, which include intermediate steps, such as gynodioecy, in which populations consist of pure females and simultaneous hermaphrodites; androdioecy, with populations consisting of pure males and simultaneous hermaphrodites; and trioecy, in which simultaneous hermaphrodites, males and females coexist in the same population (Charlesworth and Charlesworth 1978; Delph and Wolf 2005; Avise 2012; Leonard 2013). These “intermediate” sexual systems are rare and (likely) evolutionary less stable compared to gonochorism and simultaneous hermaphroditism, but may represent key steps in the evolutionary transitions between sexual systems (Delph 2009, and Käfer et al. 2017 in plants; Weeks 2012 in animals; Figure 1).



**Figure 1:** Potential evolutionary pathways between sexual systems with examples of one hermaphroditic, one androdioecious, and one gonochoric species (from left to right: *Limax maximus*, photo by Spleines at en.wikipedia; *Chelonibia testudinaria*, adapted from Lin et al. 2015; *Aix galericulata*, photo by Francis C. Franklin at en.wikipedia).

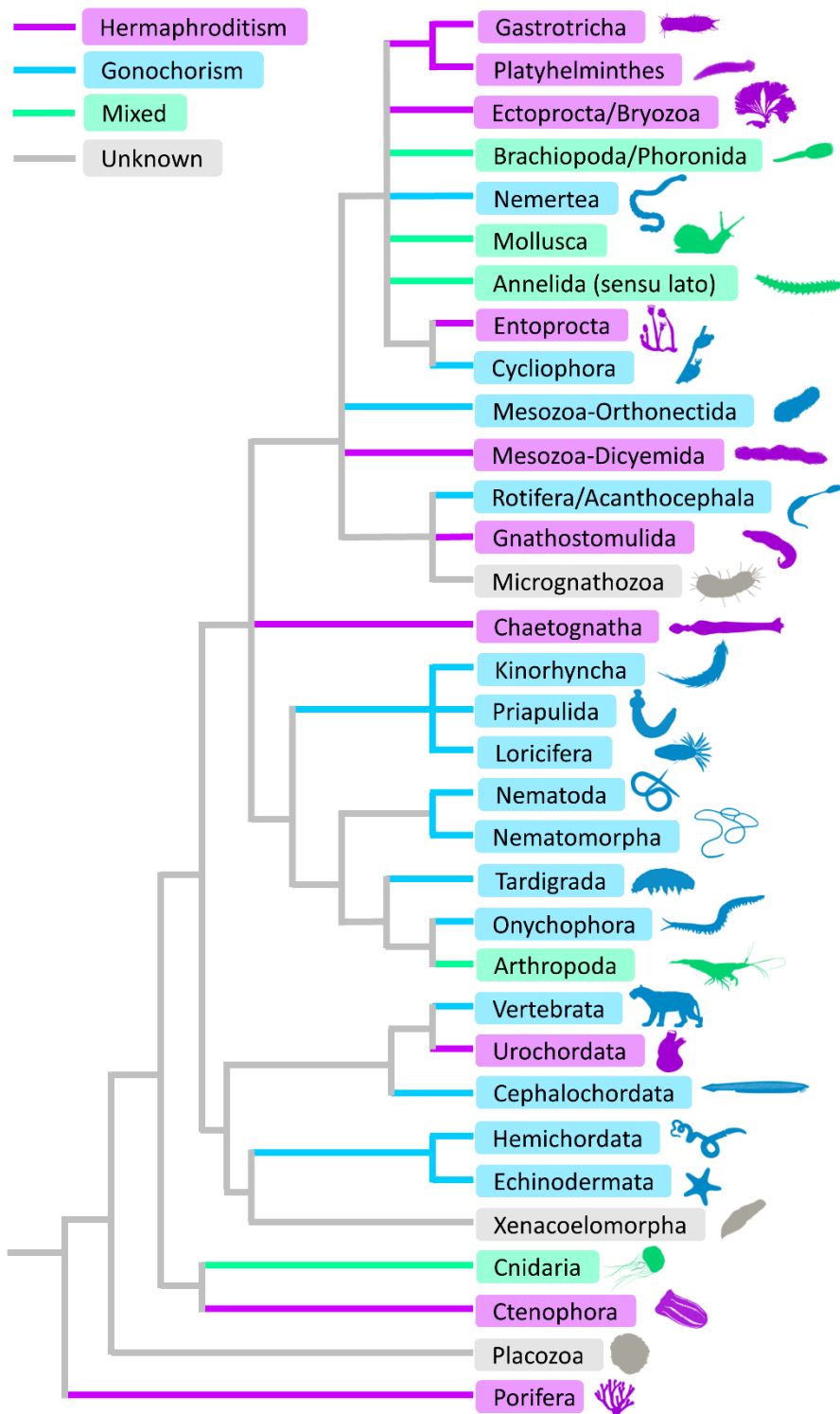
Indeed, both gonochorism and simultaneous hermaphroditism originated independently several times. In animals, for example, phylogenetic analyses suggest that simultaneous hermaphroditism represents the ancestral state, followed by at least 10 – 20 transitions from hermaphroditism to gonochorism (and *vice versa*) when considering the class to phylum taxonomic levels (Iyer and Roughgarden 2008; Eppley and Jesson 2008). Likewise, in plants, gonochorism originated independently 871 to 5000 times (Renner 2014) and often reverted to hermaphroditism (Käfer et al. 2014, 2017).

The transition between sexual systems has received extensive attention in the botanical literature, leading to the formulation of clear hypotheses on the evolutionary pathways and mechanisms involved in the origin of gonochorism and simultaneous hermaphroditism and to the collection of abundant evidence in support to these hypotheses (Charlesworth 2006; Barrett 2013; Pannell in press). Unfortunately, less attention has been given to this transition in the zoological community, where the mechanisms promoting the transition between sexual systems are still unclear (Weeks 2012). In order to understand the evolution of sexual systems in animals, we can identify two major questions that are still unanswered (Leonard in press);



- 1) which are the factors that determine the distribution of sexual systems among the animals?
- 2) which are the evolutionary pathways from one sexual system to the other?

The first question is often referred to as Williams' paradox (Leonard 1990; 2013; in press) since it was Williams (1975) who firstly pointed out that, while sexual systems are expected to be sensitive to ecological factors (see below), they seem to be more strongly linked to phylogeny than to ecology. Among animals (but also plants), there are whole clades (phyla or classes) that are almost entirely gonochoric and others that are mainly hermaphroditic (Figure 2). For example, insects (the largest taxon of animals in terms of number of species) are all gonochorists, even if they live in a large variety of ecological condition (suffice it to say that if Hexapoda were not considered, the proportion of hermaphroditic animals would rise from 5% to 30%, Jarne and Auld 2006). Similarly, in the large phylum Platyhelminthes, simultaneous hermaphroditism is the dominant sexual system (Ghiselin 1974; Leonard 2013). On the other hand, other taxa are more labile, such as the phylum Annelida, with heartworms and leeches that are mainly hermaphroditic and the polychaetes that are mainly gonochoric (see **chapter 2**). The same variation in the evolutionary stability of sexual systems can be found at lower taxonomic level. Gonochorism and simultaneous hermaphroditism (and sequential hermaphroditism) can indeed be present in the same genus [e.g. *Hydra* (Siebert and Juliano 2017), and *Ophryotrocha* (**chapter 2**)]; other genus, instead, have only one sexual systems, even if the different species face different ecological conditions. The genus *Lysmata*, for example, is entirely simultaneously hermaphroditic (as it is its sister group), though some species are free-living (a condition that should favor gonochorism) and others are symbiotic (which is expected to favor hermaphroditism) (Baeza 2013).

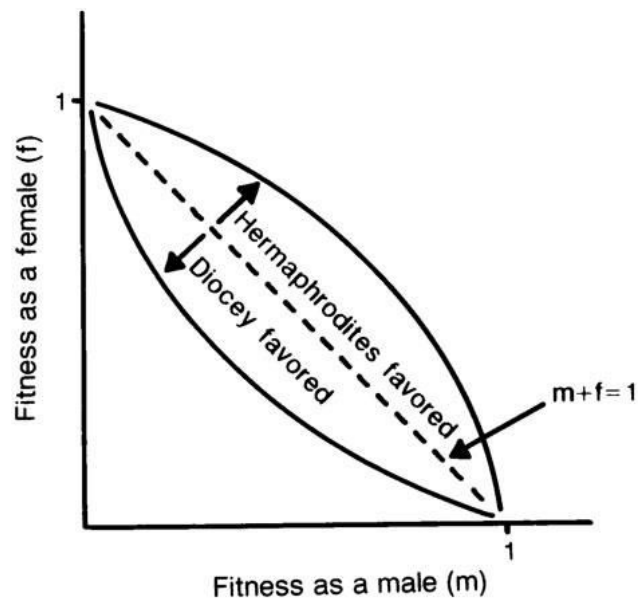


**Figure 2:** Distribution of gonochorism and hermaphroditism in the major animal phyla. Data on sexual systems adapted from Leonard 2013. Phylogenetic relationship adapted from Telford et al. 2015. Animal silhouettes from Phylopic [credits from the top: Noah Schlottman and David J Patterson; no credit; Noah Schlottman and Hans De Blauwe; no credit; Mali'o Kodis and Rebecca Ritger; no credit; no credit; Mali'o Kodis; Mali'o Kodis, P. Funch, and R.M. Kristensen; no credit; Michelle Site; no credit; Matthew Hooge and T. Michael Keese; Michelle Site; Michelle Site; Michelle Site; no credit; Michelle Site; Michelle Site; Eduard Solà Vázquez and Yan Wong; no credit; no credit; Maija Karala; Zimices; Mali'o Kodis and Melissa Frey; no credit; Michelle Site; no credit; no credit; Michelle Site; no credit; Mali'o Kodis; Mali'o Kodis and Derek Keats.]

Overall, the distribution of sexual systems strongly suggests that gonochorism and simultaneous hermaphroditism are evolutionary more stable in certain taxa than in others, but the causes of this stability remain unclear. To understand why sexual systems are conservative in only certain taxa, it is crucial to investigate the selective pressures driving the transition between sexual systems.

### **Selective advantages of simultaneous hermaphroditism and gonochorism**

The selective advantages of simultaneous hermaphroditism over gonochorism and *vice versa* have been the focus of important theoretical works. According to one of the earliest hypotheses, hermaphroditism should be favored under low population density, as it offers some kind of reproductive assurance, doubling the likelihood of finding a suitable partner and allowing (in many species) self-fertilization (Tomlinson 1966; Ghiselin 1969); the evolution of separate sexes, indeed, may be driven by the avoidance of inbreeding depression, which can negatively affect the fitness of the offspring of self-fertilizing hermaphrodites (Charlesworth and Charlesworth 1978). In their seminal paper “Why be an hermaphrodite?”, Charnov and colleagues (1976) proposed an elegant mathematical model aimed at predicting which sexual system should be favored on the basis of the trade-off relations between the fitness in the male function and that in female function in outcrossed hermaphrodites. According to this model (further developed in Charnov 1982), when the curve describing the relation between the fitness in the male and the female function is convex, hermaphroditic mutants can invade a gonochoric population; on the other hand, if the curve is concave, hermaphroditism cannot be stable and gonochorism should be favored (Figure 3).



**Figure 3:** Charnov's (1982) graph showing different trade-off relations between the fitness in the male function and the fitness in the female function. Reproduced from Leonard (1990).

The biological question is therefore to understand which factors shape the relationship between the two sexual functions. Charnov and colleagues (Charnov et al. 1976; Charnov 1979; 1982) proposed several factors that account for a concave curve: 1) Low reproductive resource overlap: when organisms use different resources for egg and sperm production, or male and female investments occur at different times, investing resources in one sexual function will have a weaker effect on the investment in the other; 2) Saturating fitness gain curves: when, over a certain threshold, a further increase in the resources allocated to one sexual function does not translate into an increase in the reproductive success through that sexual function, it is advantageous to invest the remaining resources in the other sexual function [for example, low mate encounter rates can cause the male fitness gain curve to saturate, as egg availability would limit reproductive success through the male function – the low population density hypothesis (Tomlinson 1966; Ghiselin 1969)]; 3) Cost sharing: whenever organisms have organs/structures that are linked to both the fitness in the male and in the female function, investing resources in those structures will benefit male and female reproductive success, resulting in a convex curve (for example, flowers in insect-pollinated plants contribute both pollen dispersal and ovule fertilization, so that individual with “better” flowers are visited more often by insects and, therefore, both give and receive more pollen). Conversely, gonochorism should be favored whenever it pays off to specialize in one sexual function, since increasing investment in one sexual function strongly affects the reproductive success in the other (i.e., the relation between male and female fitness is represented by a

concave curve). Factors favoring a concave curve are: 1) high mating encounter rate, which account for high population density, high motility and high mate search efficiency (Puurtinen and Kaitala 2002; Eppley and Jesson 2008; Iyer and Roughgarden 2008), and 2) high fixed costs, i.e., a considerable investment in reproductive structures, such as genitalia (Charnov 1982; Leonard 2013).

Finally, simultaneous hermaphroditism might be advantageous because the ability to produce both sperm and eggs at the same time allows individuals to adjust the relative investment in the male versus the female function in response to environmental changes, that is, hermaphrodites have a plastic sex allocation (Charnov 1982; Michiels 1998; Schärer 2009).

Sexual systems should, therefore, be highly sensitive to changes in the social environment, which, acting on sex allocation pattern, can trigger the evolutionary transition between simultaneous hermaphroditism and separate sexes.

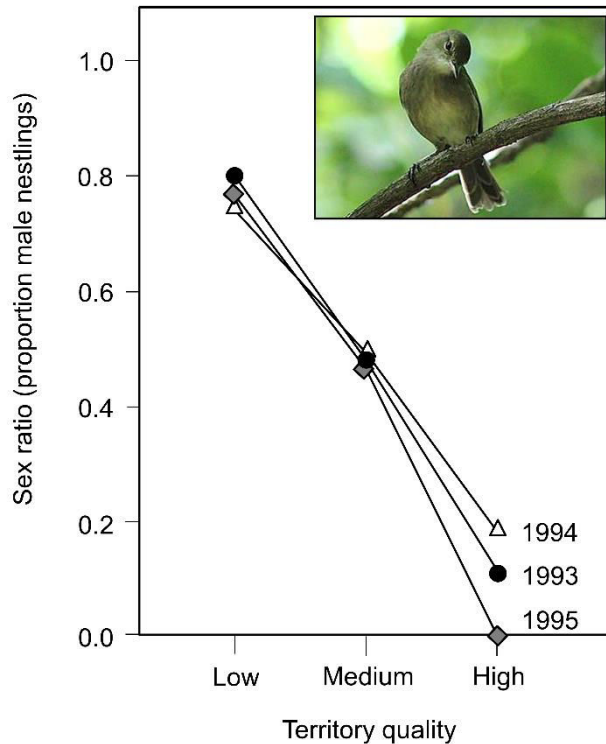
## **Fisher's condition and "extraordinary sex ratios": the role of cooperation and competition in shaping sex allocation**

Sex allocation theory (Charnov 1982; further reviewed in Hardy 2002; West 2009) aims at predicting the optimal resource allocation to female versus male reproduction in sexually-reproducing organisms, investigating different traits depending on the sexual systems. In gonochoric species, sex allocation studies mainly concern maternal decisions over the offspring sex ratio; in sequential hermaphrodites, the timing and direction of the sex change; and, in simultaneous hermaphrodites, the relative allocation to the female versus the male function. The additional aim of sex allocation theory is to understand under which environmental conditions gonochorism and hermaphroditism are evolutionary stable and which conditions favor intermediate sexual systems.

As West (2009, p.14) pointed out, "the basic null model for sex allocation theory" is provided by the theory of equal investment proposed by Fisher (1930), on the basis of the mathematical model developed by Düsing (1884, but see Edwards 1998). Fisher (1930) provided an elegant explanation for why, in gonochoric species, it pays off to produce an equal number of sons and daughters. The fundamental argument of Fisher's theory is that, despite mothers and fathers do not invest an equal amount of resources in the offspring, the genetic contribution to the next generation is evenly shared by the two parents (each

individual has exactly one mother and one father). At the population level, this implies that the average female fitness must be equal to the average male fitness, that is, males and females have equal reproductive value. If the sex ratio is biased towards one sex, however, the reproductive value of the more common sex will be reduced, while that of the rarer sex will be enhanced, increasing the fitness of those parents which produce an excess of the rarer sex. The number of individuals belonging to the rarer sex should therefore rise in the population, favoring a sex ratio of 50:50.

Fisher's theory, however, relies on some strong assumptions (reviewed in West 2009), among which, the lack of cooperative and competitive interactions between relatives is of particular interest for the purpose of this thesis. If relatives interact, both male- and female-biased sex ratios can be favored as they reduce competition or enhance cooperation between related individuals. Depending on whether individuals compete or cooperate with each other, we can identify two processes favoring a biased sex ratio: Local Resource Competition (LRC), when the production of individuals belonging to one sex increases the competition between relatives for limiting resources (Clark 1978) and Local Resource Enhancement (LRE), in which the production of one sex increases the fitness of its relatives. The Seychelles warbler represents a perfect example of how LRC and LRE can bias the offspring sex ratio. In this bird, males usually disperse while females may stay at their natal nest as helpers. Komdeur and colleagues (1997) showed that in low-quality territories, where LRC is likely to affect fitness more strongly than LRE, the offspring sex ratio is extremely male biased, that is, there is overproduction of the dispersing sex. Conversely, in high-quality territories, where resource competition is scarce, warblers produce significantly more females than males, which will stay at the nest as helpers, enhancing the mother's fitness (Figure 4).



**Figure 4:** Sex ratio of nestlings produced by Seychelles warbler pairs as a function of the quality of the breeding territory (results from Komdeur et al. 1997). Reproduced from Komdeur (2012). Photo by Dominik Tefert at de.wikipedia.

A particularly well-known example of LRC is Local Mate Competition (LMC), firstly proposed by Hamilton (1967) in his seminal paper “Extraordinary Sex Ratio” to explain the relative high frequency of female-biased sex ratio in insects and mites. In species where males compete mainly with their brothers for access to females, it pays off from the mother’s perspective to invest more resources in daughters than in sons. Indeed, reducing the investment in sons does not affect the mother’s fitness via the sons’ reproductive success, as related males would compete with each other, while it increases the mother’s fitness via the daughters’ reproductive success, as the resources spared from male production are reallocated to female production (Taylor 1981).

### Sex allocation in simultaneous hermaphrodites

Local Mate Competition plays a key role in shaping sex allocation also in simultaneous hermaphrodites (see Charnov 1979; 1982 for the theoretical framework), though in these organisms, competition concerns mainly related sperm rather than individuals, and it has been

proposed to term this process Local Sperm Competition (Schärer 2009). When simultaneous hermaphrodites live in isolation (self-fertilizers) or in monogamous pairs (outcrossers), they are expected to allocate a minimum amount of resources to the male function to re-invest them in the female function (Charnov 1982; Schärer and Pen 2013). In such conditions, indeed, the sperm produced by one individual will compete for egg fertilization only with other sperm produced by the same individual (related sperm); therefore, once a hermaphrodite has produced enough sperm to guarantee the fertilization of the available eggs, any further investment in sperm production will no longer translate in an increase in fitness via the male function, that is, the male fitness gain curve saturates. As for Local Mate Competition, a lower allocation to the male function allows reducing competition among related sperm, and makes resources available for the female function.

However, if the size of the mating group increases, so does sperm competition, as the sperm produced by one individual will compete for egg fertilization with the sperm produced by other individuals. Under high level of sperm competition, investing more in sperm production increases siring success, and it pays off from the hermaphrodite's perspective to reallocate resources from the female to the male function.

Sperm competition changes therefore the shape of the male fitness gain curve shifting the point at which a further investment in the male function does not translate in an increase in reproductive success. In theory, if sperm competition is sufficiently high, the curve may not saturate any longer favoring hermaphrodites which invest their reproductive resources entirely in the male function, setting the scene for the transition to separate sexes.

In the last years, abundant empirical evidence has been collected in support of sex allocation theory in simultaneous hermaphrodites. For example, hermaphroditic species which are expected to mate in monogamous pairs exhibit female-biased sex allocation (e.g., *Ophryotrocha diadema*, Sella 1990; *Hypoplectrus nigricans*, Fischer 1981). Furthermore, simultaneous hermaphrodites plastically adjust their sex allocation to the size of the social group, reducing the investment in the female function and/or increasing that in the male function when group size increases (reviewed in Schärer 2009).

It seems therefore likely that processes such as LRC, shaping male and female fitness gain curves, play a key role in the evolutionary transition between sexual systems. Pannell (in press) suggested an association between gonochorism in plants and certain traits which reduce LRC. For instance, wind-pollinated plants may often have evolved separate sexes (Friedman 2011), as wind-pollination is likely to reduce Local Mate Competition among related pollen.



Similarly, producing fleshy fruits, which favor seed dispersal, may reduce Local Resources Competition and favor the evolution of gonochorism (Renner and Ricklefs 1995).

Evidence for a strong association between mate competition and sexual systems comes also from fish. Erisman and colleagues (2013) show that sexual systems strongly correlate with the mating systems (and, thus, with the level of sperm competition) in teleost fish, so that gonochorism is more common in species which spawn in group than in species that spawn in pair.

### **Evolutionary pathways from hermaphroditism to gonochorism and *vice versa***

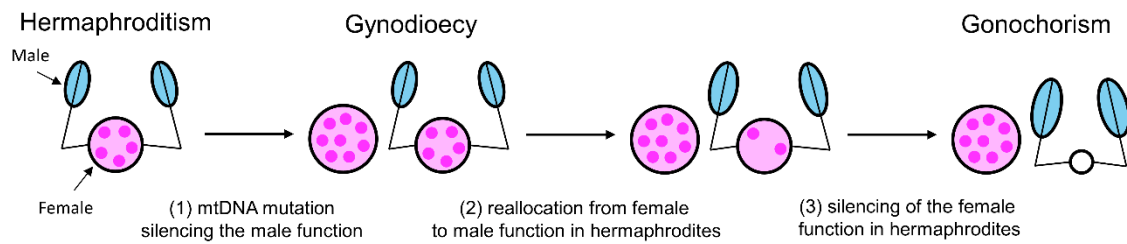
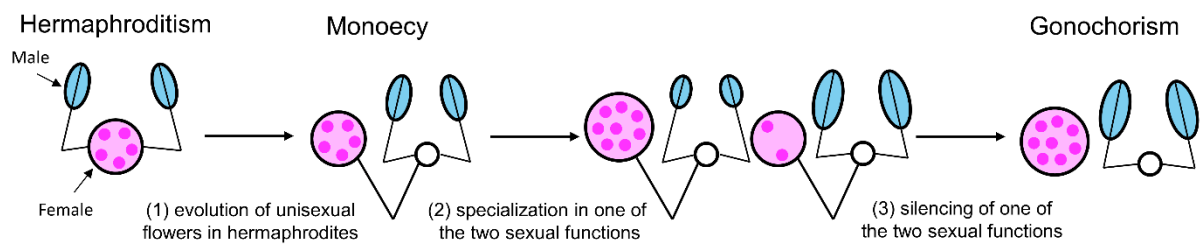
As mentioned before, the evolutionary transition between sexual systems has received more attention in plants than in animals, and, while in the latter the pathway from hermaphroditism to gonochorism and *vice versa* is still unclear, two main pathways have been proposed for angiosperms (reviewed in Pannell in press).

According to the first evolutionary pathway, separate sexes originated from hermaphroditic ancestor via gynodioecy, an intermediate step in which the population is composed by simultaneous hermaphrodites and pure females (reviewed in Delph and Wolf 2005; Charlesworth 2006; Delph 2009; Figure 5a). The first step of this pathway is the emergence of male-sterile mutants (i.e., pure females) in the hermaphroditic population. [Male-sterility is determined by a mutation in the mtDNA that causes inability to produce viable pollen grains (cytoplasmic male sterility) (Lewis, 1941; Touzet and Meyer, 2014)]. Under appropriate conditions (e.g., sufficiently high population density and/or inbreeding depression), male-sterile individuals can spread in the population since they reallocate resources from the male to the female function, and their offspring will not suffer inbreeding depression. Once pure females have spread in the population (gynodioecy), they will impose a selective pressure on the remaining hermaphrodites to invest progressively less resources in the female function and more to the male function. Eventually, hermaphrodites will allocate all of their reproductive resources to the male function, and the population will consist only of pure females and pure males (gonochorism). In support of this pathway, in plants, males of gonochoric species often retain the ability to produce a small amount of seeds and fruits (Ehlers and Bataillon 2007; Pannell in press).

Such gonochoric species can then revert to hermaphroditism via androdioecy (pure males + simultaneous hermaphrodites) when females gain male fertility through nuclear DNA mutation(s) or labile sex expression, that is, the ability of a genotype to express different sexual phenotypes depending on the environment (in this case, females are able to restore pollen production under pollen- or mate-limiting conditions) (Charlesworth 2006; Delph 2009).

Another possible evolutionary pathway is the one that leads from hermaphroditism to gonochorism via monoecy, i.e., simultaneous hermaphrodites with separate male and female flowers (reviewed in Barrett 2002; Figure 5b). This pathway is less well studied, although phylogenetic comparative analyses suggest that it may have occurred frequently, since monoecy is often associated with dioecy within genera (Renner and Ricklefs 1995). In contrast to the pathway via gynodioecy, here, the driving force for the evolution of separate sexes is expected to be sexual specialization rather than inbreeding avoidance. The monoecy pathway likely involves the evolution of unisexual flowers due to sterility mutations (Barrett 2002); monoecious plants may then spread in a population because of reduced pollen-stigma interference, which increases seed quantity and/or quality, but also because unisexual flowers allow a more plastic sex allocation in variable environments and a more exact sex allocation in constant environments (de Jong et al. 2008). Once monoecy has spread in the population, disruptive selection on male and female allocation can favor individuals that produce more male flowers at the expense of the female ones and *vice versa*, promoting individual specialization in one sexual function (either the male or the female one).

The evolutionary pathway between sexual systems is much less clear in animals, where a male (or female)-sterility mutation has never been described (Budar et al., 2003) and where intermediate sexual systems are extremely rare (Weeks 2012).

**(a) Gynodioecy pathway****(b) Monoecy pathway**

**Figure 5:** Schematic representation of the evolutionary pathways from hermaphroditism to gonochorism in plants. Evolution of gonochorism via invasion of the hermaphroditic population by (a) pure females (gynodioecy), and (b) hermaphrodites with unisexual flowers; and consequent sexual specialization. Female organs are represented in pink and male organs in light blue. Adapted from Delph and Wolf (2005).

## Thesis outline

The aim of this thesis is to provide new insights on the evolutionary transition between hermaphroditism and separate sexes, using the marine polychaete worms of the genus *Ophryotrocha* as study model. In particular, we will focus our attention on 1) how hermaphroditism can be maintained in these worms, and 2) how separate sexes might have evolved from hermaphroditic ancestors.

**Chapter 2** is meant to be the natural continuation of the introduction. In this chapter, we firstly give a general overview of sexual systems in polychaete worms and a more detailed description of the biology of the study organisms, the *Ophryotrocha* worms. We then present the results of experimental and observational studies which are particularly interesting for the purpose of this thesis and discuss them in relation to the evolution of sexual systems. Eventually, in the light of the results presented, we propose a hypothetical pathway from hermaphroditism to separate sexes in the genus, in which we suggest that mate competition, biasing sex allocation towards the male function, may have favored the emergence of pure

males (androdioecy) in ancestral hermaphroditic populations, which then selected for hermaphrodites with a female-biased sex allocation, favoring the evolution of pure females (gonochorism).

Throughout the manuscript we thus focus our attention on how the interactions between individuals can shape sex allocation in hermaphroditic worms, favoring the maintenance of hermaphroditism or promoting the evolution of separate sexes.

For example, egg-trading, the conditional exchange of egg clutches, may promote a female-biased sex allocation, which prevents pure-sex individuals from invading the hermaphroditic population. However, the spread of reciprocity among animals is hotly debated and it remains unclear whether hermaphrodites are actually capable of conditionally reciprocate eggs. In **chapter 3** we show that, in hermaphroditic worms, giving eggs is conditional upon receiving them from the partners and that worms are capable of evaluating the amount of eggs received and they lay accordingly. These results suggest that in the tested species egg-trading can play a crucial role in the maintenance of hermaphroditism.

**Chapter 4** and **5**, instead, are focused on how mate competition/mating opportunities shape sex allocation. Experimental evidence highlights that hermaphroditic *Ophryotrocha diadema* worms have a plastic sex allocation, although the data collected until now do not fulfill the assumptions of sex allocation theory. Indeed, worms kept in groups lay significantly less eggs compared to worms kept in pairs (i.e., they decrease their allocation to the female function), but they produce the same amount of sperm (i.e., male allocation does not vary). Therefore, to get a better understanding of plasticity in sex allocation, in **chapter 4**, we test to what extent hermaphroditic worms are flexible and reversible in their sex allocation, exposing the worms to weekly changes in mating opportunities and measuring not only their egg production, but also their behavior. Results show that worms are able to rapidly adjust their egg production in both directions and change their behavior according to mating opportunities: they are more aggressive at high mating opportunities.

Since the results of **chapter 4** highlight that *Ophryotrocha diadema* might invest their resources not only in gamete production, but also in reproductive behaviors, in **chapter 5** we investigate whether hermaphrodites exhibit male- and female-related behaviors and whether these behaviors trade-off with each other. Results show that parental care (a putative female behavior) and motility (a putative male behavior) are adjusted to mating opportunities in opposite directions and that worms performing more parental care move significantly less and *vice versa*, suggesting that there is a trade-off between these gender-related behaviors. To further confirm that these behaviors are sex-specific, we observe a closely-related species

with separate sexes, highlighting that males move more, while female cared more for their eggs.

Interestingly, motility has been associated with the evolution of separate sexes since it can improve mate search efficiency and thus increase the mate encounter rate. Therefore, in **chapter 7**, we test whether hermaphroditic and separate sex worms differ in their responsiveness when presented with a choice between a potential partner and food-enriched water or when offered the choice between two conspecifics. In this chapter, we further test whether hermaphrodites prefer worms with ready-to-lay eggs to worms without eggs, and whether their preference depends on their egg maturation level (whether the worm has or has not ready-to-lay eggs). Overall, this study shows that worms with separate sexes are more responsive than hermaphrodites to cues from potential partner, further suggesting that higher motility and mate searching efficiency may have been selected favorably during the transition from hermaphroditism to separate sexes. Furthermore, the results of this experiment highlight that egg maturation level (of both the tested worms and the potential partners) plays a central role in hermaphroditic mate preference.

In **chapter 8**, we summarize and discuss the results, focusing on the potential effects of phenotypic plasticity on the evolutionary processes. Eventually, we integrate the new results with those reviewed in **chapter 2** and provide an updated scenario on how separate sexes might have evolved from hermaphroditic ancestors in *Ophryotrocha* worms.

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# 2.

## **Polychaete worms on the brink between hermaphroditism and separate sexes**

Laura Picchi and Maria Cristina Lorenzi

In *Transitions between sexual systems*. Springer, in press.

## Abstract

Polychaetes (i.e., non-clitellate annelids) display one of the highest diversity of reproductive traits among marine invertebrates, due to the wide variety of habitats they have colonized and the relative simplicity of their reproductive system. Among polychaetes (i.e., non-clitellate annelids), the genus *Ophryotrocha* is one of the best studied models in sexual system investigations and is providing several clues as to how the evolutionary transition from hermaphroditism to gonochorism may have occurred. Within the genus, there are both hermaphroditic and gonochoric species but many species exhibit traits intermediate between the two sexual systems. Hermaphroditic species are able to plastically adjust their sex allocation to current mating opportunities, by diminishing the proportion of resources invested into the female function and increasing those invested into the male function, when mating opportunities increase (although the enhanced investment in the male function is expressed in behavioural traits – such as mate competition - rather than increased sperm production). Occasionally, hermaphrodites specialized in the male function are found in hermaphroditic populations (functional-male hermaphrodites), which might represent a first step towards gender specialization. On the other hand, in gonochoric (sexually dimorphic) species, multiple sexual phenotypes have been described, with males which produce oocytes, and females which produce sperm, which are likely to be vestigial hermaphroditic traits. The existence of functional-male hermaphrodites in hermaphroditic populations, and of males and females which produce both eggs and sperm in gonochoric species suggest that in *Ophryotrocha* the transition from hermaphroditism to gonochorism occurred via androdioecy, that is, through an evolutionary trajectory involving an intermediate stage where both hermaphrodites and males are present.

## Introduction

Annelida, commonly known as segmented or ringed worms, is a large (around 17,000 recognized species; Zhang 2011) and ecologically diverse phylum. In the last years, thanks to next-generation sequencing, annelids phylogeny has dramatically changed: polychaetes have turned out to be a paraphyletic taxon and have been mostly included in the two major clades Errantia and Sedentaria, the latter comprising also Clitellata (Weigert and Bleidorn 2016). The placement of Clitellata within the clade Sedentaria basically makes Polychaeta synonymous to Annelida. However, we usually use the term “*polychaetes*” to distinguish non-clitellate annelids from clitellates and, for the purposes of this review, we will use the term “*polychaetes*” to indicate non-clitellate annelids, referring to the traditional name.

Polychaetes (i.e., non-clitellate annelids) are a common annelid taxon of the marine benthos, often dominant both as number of species and as number of individuals. They are important components of hard-bottom and pelagic communities as well. This group is well diversified from a systematic point of view, with more than 85 accepted families (Rouse and Fauchald 1997; Brusca and Brusca 2003) and has a large variety of life strategies. In particular, these worms display one of the highest diversity of reproductive traits among marine invertebrates, probably at least partly due to the relative simplicity of their reproductive system (Wilson 1991; Giangrande 1997). Polychaetes indeed generally lack permanent gonads (or other complex reproductive organs) and gametes usually develop through proliferations of cells from the peritoneum. Gametes are then released as gametogonia or primary gametocytes in the coelom, where they mature before being released by means of gonoducts, coelomoducts, nephridia or by simple rupture of the body wall (Brusca and Brusca 2003). This relative simple reproductive system is subjected to few phylogenetic morphological constraints and thus allows the evolution of a wide variety of reproductive modes (Wilson 1991; Giangrande 1997).

Some species of polychaetes have asexual reproduction (most also with sexual reproduction), which is typically accomplished by subdivision of the body into parts and the subsequent regeneration of the missing body segments (Schroeder and Hermans 1975). The body may be divided in two or more parts, as is the case of some species of the genus *Dodecaceria* (Cirratulidae), which are able to regenerate entire individuals from single segments (Schroeder and Hermans 1975; Petersen 1999; Brusca and Brusca 2003).

The most common form of reproduction in polychaetes is sexual reproduction (George and Hartmann-Schröder 1985). Whereas oligochaetes and leeches are mostly hermaphroditic, polychaetes typically have separate sexes. Both simultaneously and sequentially hermaphroditic species exist in about 25 different families (Giangrande 1997; Schroeder and Hermans 1975). Self-fertilization has been reported for several species (*Neanthes (Nereis) limnicola*, Smith 1958; Fong and Pearse 1992; *Laonome albicingillum*, Hsieh 1997; *Terebrasabella heterouncinata*, Finley et al 2001; *Capitella* sp. Y, Méndez 2006) even if none are known to self-fertilize routinely (Knowlton and Jackson 1993).

The relative simplicity of their reproductive system probably allowed these worms to respond to ecological pressures by evolving a large variety of sexual systems (i.e., the pattern of distribution of male versus female functions across individuals in a species, following Leonard 2013) and reproductive strategies, which often differ even in sibling species (Wilson 1991).

One of the most fascinating examples of the extreme adaptability is the case of the gonochoric species *Dinophilus gyrociliatus*. This small interstitial species likely lives in low-density populations, at least temporarily, as do most of the species belonging to interstitial fauna (Westheide 1984). A peculiar mating system has been described in this species, that may be advantageous in low density populations: *D. gyrociliatus* is sexually dimorphic both in its adult morphology and life-history traits and in egg size; males are dwarf, have a very short lifespan relative to females and develop from eggs smaller than those that produce females (Martin and Traut 1987; Sella and Ramella 1999). Soon after hatching from eggs, males inseminate their immature sisters within the protective egg-capsule where male and female develop. Then males die before females leave the egg-capsule as larvae (Traut 1969; Sella and Ramella 1999). This peculiar mating system produces one of the most skewed sex ratio in the animal kingdom (Charnov 1987), and the free living populations are composed uniquely of females. The costs of finding mates are likely high in low-density populations; mating as larvae within the egg-capsule eliminates these costs and may represent an evolutionary solution for separate sex animals, enabling them to find mates in very sparse and structured populations. Such a female-biased sex-ratio has been described also for many species of fig wasps (Agaoninae) in which there is a low level of competition for mates between non-siblings since brothers usually fertilize their sisters, as for *D. gyrociliatus* (Hamilton 1979; West 2009).

On a broader level, polychaete species living in unfavourable environments (including interstitial species) are generally characterized by having a small body size and a relatively short life span and this is linked to a rapid attainment of sexual maturity, a low fecundity and the presence

of parental care; on the other hand, species that are highly competitive and dominate the community are usually characterized by long life span, very high fecundity and high dispersal capability, with planktonic larvae able to cover long distances (Sveshinkov 1985, in Giangrande 1997).

Hermaphroditism is relatively common in sessile tubicolous worms such as the Sabellidae and the Serpulidae and is often associated with small body size, as in the case of interstitial species such as those belonging to the genus *Ophryotrocha* (Dorvellidae) (Westheide 1984; Giangrande 1997). However, hermaphroditism is the predominant sexual system in a few polychaete families (for instance in the Spirorbidae, Schroeder and Hermans 1975; Kupriyanova et al. 2001). More often, polychaetes have separate sexes and hermaphroditic species are often rare in polychaete families (e.g. *Macellicephalo violacea* is the only hermaphroditic species in a family (Polynoidea) rich in gonochoric species, Wirén 1907; Rouse and Pleijel 2001). It can be argued that many of these species are described after morphological investigations of small sample sizes which may underestimate some sexual systems, e.g. sequential hermaphroditism, where individuals produce either sperm or eggs at a given stage of their life and may be wrongly assumed to have separate sexes.

Gonochorism is considered the ancestral sexual system in polychaetes, and hermaphroditism is a secondary acquisition in this perspective (Prevedelli et al. 2006). The presence of a few hermaphroditic species within mainly gonochoric genera suggests that the transition from gonochorism to hermaphroditism occurred several times within the polychaetes, and at least once in the opposite direction. In a population of *Parasabella microphthalmus* (*Demonax microphthalmus* of previous authors) both hermaphrodites and pure females were collected by Gregory (1905) and the closest ancestor of this species seems to be a hermaphrodite (Weeks 2012); this suggests that in *P. microphthalmus* a pure female mutant evolved from a hermaphroditic ancestor. In contrast, the genus *Ophryotrocha* is typically composed of separate sex species (Sella and Ramella 1999), but recent phylogenetic analyses have proposed that simultaneous hermaphroditism is the ancestral sexual system in the genus, where pure males and pure females may have evolved from hermaphroditic individuals (Heggøy et al. 2007; Thornhill et al. 2009). Therefore, the transition between hermaphroditism and separate sexes occurred repeatedly and in both directions within the polychaetes, as in several genera both sexual systems are documented. Table S1 (in supplementary materials) reports a list of genera where at least two different sexual systems are described, highlighting how often the transition between sexual systems occurred in these worms.

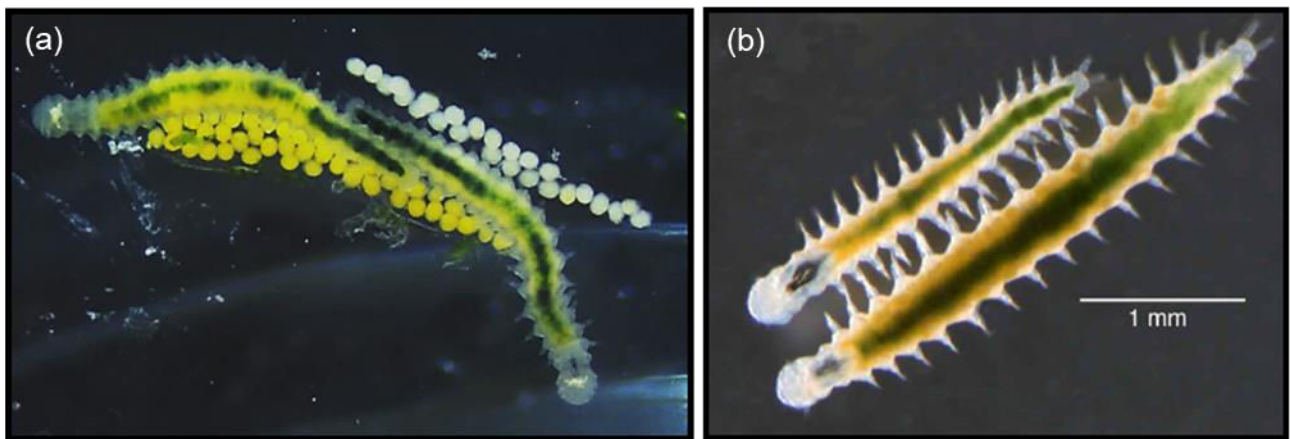
The presence of several transitions between hermaphroditism and gonochorism in both directions and the lack of morphological constraints in polychaete reproductive system makes these

worms an ideal group to study how different selective pressures shape sexual systems, in animals which have colonized a wide variety of habitats.

### ***Ophryotrocha* as a study model**

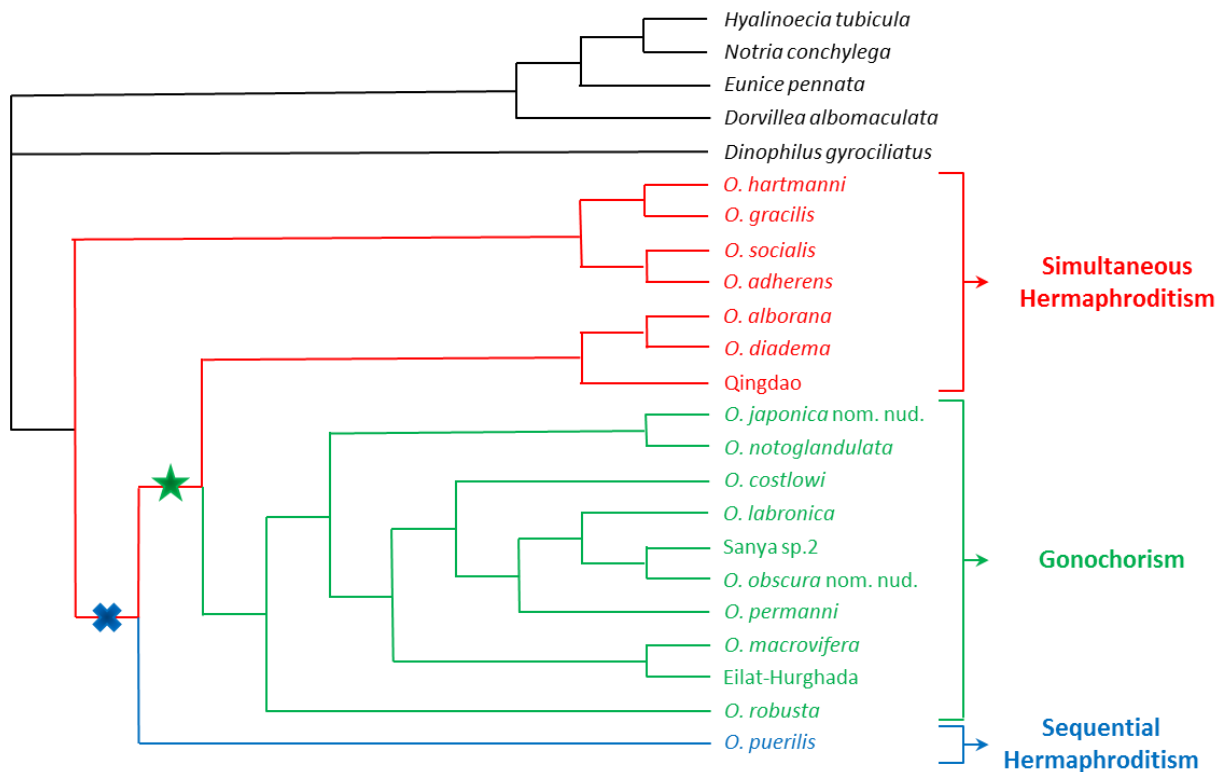
The genus *Ophryotrocha* (Dorvellidae) was described for the first time in 1869 by Claparède and Metschnikoff (1869), and since then more than 40 species belonging to this taxon have been described, with several new species discovered in recent years (Wiklund et al. 2012; Ravara et al. 2015).

Among polychaetes, the genus *Ophryotrocha* (Figure 1) is an ideal model for the study of the transition between sexual systems. Some species are simultaneous hermaphrodites, others are gonochoric, and at least one species is a sequential hermaphrodite (*O. puerilis*) (Dahlgren et al. 2001). Recently, viviparous species have been reported, whose sexual systems are still unclear (Paxton and Åkesson 2010; Wiklund et al. 2012). Therefore, a large variety of sexual systems occurs within the same genus. The analyses of the phylogeny of this taxon show that within the genus *Ophryotrocha* the transition between sexual systems happened only once and from hermaphroditism to gonochorism, since the former represents the ancestral situation (Figure 2; Dahlgren et al. 2001).



**Figure 1:** The hermaphroditic species *Ophryotrocha diadema* (a) and the gonochoric species *Ophryotrocha robusta* (Paxton and Åkesson, 2010) (b).

Comparing the sexual patterns of closely related species allows one to minimize the differences in reproductive traits (e.g., morphology, physiology, etc.) and highlights the effect of ecological variables on the evolution of sexual systems.



**Figure 2:** Phylogeny of the *Ophryotrocha* genus, highlighting the diversity of sexual systems. The star represents the hypothetical transition from simultaneous hermaphroditism to gonochorism; the cross represents the hypothetical transition from simultaneous hermaphroditism to sequential hermaphroditism (modified from Dahlgren et al, 2001).

Furthermore, all the *Ophryotrocha* species whose sexual system was studied are obligate outcrossers (i.e., they need a partner to successfully reproduce) and thus they are not able to self-fertilize. This could represent an advantage in the understanding the transition between sexual systems: avoidance of inbreeding (selfing) indeed has been proposed as one of the main advantage of gonochorism over hermaphroditism both in plants and animals (Ashman 2006; Leonard 2013); the absence of selfing in *Ophryotrocha* species means that the avoidance of inbreeding depression is not a selective force at work in this system and make it to throw light on other selective forces favouring a sexual system over another.

The worms in this taxon also have technical advantages as study models. Most of them are easy to rear in the laboratory and have relatively short generation times. They release eggs protected in a transparent cocoon, and egg development can be followed at low magnification, which allows for easy measures of individual reproductive success.

In addition, in one simultaneously hermaphroditic species (*O. diadema*), an albino phenotype for the colour of the eggs exists. The presence of the albino phenotype, jointly with the fact that



worms have transparent body walls, permits an easy estimate of the reproductive investment in the female role, since maternity can be easily assigned through the colour of the eggs (which are white in albino worms and yellow in wild phenotype worms). A dominant Y allele codes for the yellow egg color, while the recessive y allele codes for the white egg color (Sella and Marzona 1983). Because of their Mendelian inheritance, this genetic marker makes it possible to identify a focal worm in a group, and to identify its progeny (cfr. Lorenzi et al. 2014). (Following Åkesson 1976, the egg-color marker is neutral).

The worms of the genus *Ophryotrocha* are typically small (between 1.5 and 5 mm) and widespread, being distributed from the tropics to the poles. Generally, these worms are part of the infauna and inhabit a large variety of marine habitats (Thornhill et al. 2009): polluted harbours (Åkesson 1976; Simonini and Prevedelli 2003); deep-sea whale-fall and wood-fall habitats (Wiklund et al. 2012); and hydrothermal vents (Blake and Hilbig 1990; Wiklund et al. 2012).

As for their reproductive biology, *Ophryotrocha* worms mate through “pseudocopulation”, in which the fertilization is external but the two individuals stay in close contact to each other (Westheide 1984). Pseudocopulation generally follows a long and time-consuming (4.5h or more) courtship (Åkesson 1973; Westheide 1984; Rouse 1999). One or both parents generally take care of the eggs, which are released in a protective cocoon or a tube of mucus, parchment-like material, or loose jelly (Sella and Ramella 1999). Parental care usually consists of the adult(s) resting on the egg cocoon or moving back and forth possibly to clean it (Premoli and Sella 1995).

However, detailed behavioural studies on *Ophryotrocha* worms are still lacking to make it possible a thoroughly description of their mating behaviour.

### ***Ophryotrocha simultaneous hermaphrodites***

Within the genus *Ophryotrocha* eight species of simultaneous hermaphrodites (often referred to as just hermaphrodites along the text) have been described up to now (Sella and Ramella 1999; Pleijel and Eide 1996). All of them are obligate outcrossers (i.e., they do not self), and for a few of them the mating system has been studied thoroughly (*O. diadema*: Sella 1985, 1988, 1990, 1991; Sella and Lorenzi 2000; Lorenzi et al. 2005; *O. gracilis*: Westheide 1984; Sella et al. 1997; and *O. socialis*: Ockelmann and Åkesson 1990). All these species are part of the interstitial fauna, as they live among the detritus and fouling fauna of polluted harbours. There is no clear information about the density of the populations in the wild, but it is expected to be low, as it is usually in meiobenthic species (Svedmark 1964; Clark 1978; Westheide 1984) (except *O. socialis*, in which several worms

live together in a common system of mucous tubes where they perform communal breeding; Ockelmann and Åkesson 1990). Occasionally, population size is expected to increase locally, creating clustered dense populations, at least for short periods (Sella and Ramella 1999). The production of mucus trails, which worms build as they crawl on the substrate, likely favours a clustered spatial distribution, as individuals can probably follow the trails produced by conspecifics and form small groups, at least temporarily (Sella and Ramella 1999).

Living at low population density may represent an important selective pressure in shaping reproductive traits, as is the case of the aforementioned *Dinophilus gyrociliatus*. Low density is also one of the main ecological factors favouring the maintenance of simultaneous hermaphroditism as hermaphrodites have a double likelihood to encounter suitable partners relative to separate sex animals (Tomlinson 1966; Ghiselin 1969; Puurtinen and Kaitala 2002). In the meanwhile, occasional bursts of population density may have favoured the ability to adjust sex allocation (i.e., the ability to modify the proportion of resources allocated to the female and to the male function) in hermaphrodites, and indeed female sex allocation adjustments are easily seen in this taxon (e.g., Lorenzi et al. 2005; Schleicherová et al. 2014).

### *Protandry*

Another common trait in hermaphroditic species is a protandrous adolescent phase, during which the worms are still immature, but can already produce (and use) sperm. Sella and Lorenzi (2003) have shown that the young worms in their protandrous phase delay the age when they become hermaphrodites if they have the opportunity to fertilize eggs (produced by mature partners). This result was interpreted as a clue that sperm production is costly for these tiny, immature worms (i.e., there is a physiological trade-off between the resources invested in courting and fertilizing egg-cocoons on one side, and those invested in body growth on the other, Sella and Lorenzi 2003), but it could also be viewed as an adaptive reproductive choice which involve long-term sex allocation adjustments. When the opportunity to fertilize eggs is not zero, young worms may delay the age of full sexual maturity (i.e., the age at which they also invest in the female function), thus spending a longer part of their lives as males, especially if the male role is the preferred sexual role in these hermaphroditic worms (see below).

### *Sexually mature hermaphrodites*

Up to now, the majority of studies on *Ophryotrocha* hermaphrodites have focused on the sexual system of *O. diadema*. In this species, sexual life begins as soon as the worms reach a body length of six segments and start producing sperm in the third and fourth body segment (Sella 1985). The

full sexual maturation will be achieved about 40 days later, when worms will reach a body length of about 14-15 segments and will start producing eggs from the fifth segment onwards although continuing to produce sperm as well.

### *Mating*

When they mate, hermaphroditic *Ophryotrocha* worms have unilateral mating which means that at each mating one individual plays the female role - and spawns eggs - and the other plays the male role - and releases sperm. Eggs are protected in a mucus cocoon and generally both parents stay nearby in the next day taking care of the egg-cocoon, creating a kind of nesting site (Premoli and Sella 1995).

Mating was described by Westheide (1984) in the hermaphrodite *O. gracilis* as “pseudocopulation”, a relative complex behavioural sequence where two partners stay in physical contact, with the worm in the “male” role attached to the “tail” (i.e., the distal segments) of the worm in the “female” role. The “female” produces a mucous cocoon all around its body, which partially covers the head and neck of the male worm (whose anterior body segments are the sperm-producing segments). Westheide suggested that sperm release might precede the release of eggs within the mucous cocoon, because the worm playing the male role was seen leaving the egg cocoon before the worm playing the female role. However, this was not confirmed in two separate, video-recorded sequences of mating behaviour in *O. diadema*, where the worms playing the male role were the last worms to leave the cocoon (Santi M., personal observation; Lescher A., personal observation; and also Meconcelli S. in *O. adherens*).

One reason why mating requires physical contacts between partners may be that sperm are aflagellate in the genus (Morrow 2004) and thus may need to be “deposited” on the eggs, within the egg cocoon. However, we do not know whether worms mate by pseudocopulation because sperm are immotile or, vice versa, if sperm have lost their tail because worms mate by pseudocopulation. Competition for mating may occur and multiple paternity within a single egg-cocoon has been reported (Lorenzi et al. 2014) which makes it difficult to explain why aflagellate sperm have evolved.

In *O. diadema*, pairs are preferentially formed between sexually mature (e.g. ovigerous) hermaphroditic worms and the two partners regularly alternate their sexual roles for long time periods, behaving once as a female and once as a male (Sella 1985, 1988). This particular mating behaviour was first described by Fisher (1980) in the serranid fish *Hypoplectrus nigricans* and is called “egg trading”.

*Egg trading and the preferred sexual role*

When simultaneous hermaphrodites mate unilaterally, a conflict arises between partners due to the different pay-offs for the two sexual roles in term of costs and benefits (Charnov 1979; Leonard 2006; Schärer et al. 2014). According to “Bateman’s principle” (Bateman 1948; Charnov 1979; Anthes et al. 2010) an individual should prefer to mate in the male role, as sperm are cheaper than eggs to produce, and mating as a female offers the same fitness returns at higher costs. The preference for the male role should be shared by all the individuals of a population.

The preference for the male role may be not universal among hermaphroditic organisms in general (Leonard 2006), but some clues suggest that *O. diadema* hermaphrodites might have a preference for mating as males. Indeed, mature hermaphrodites 1) abandon their reciprocating partners for worms with riper eggs (Sella and Lorenzi 2000); 2) mate repeatedly in the male role and have a longer lifespan if they lay eggs less often (Di Bona et al. 2010); and 3) make a smaller investment into the female function and aggressively compete for mating in the male role, when mating opportunities are high (Lorenzi et al. 2005, 2006).

If all individuals in the population share the same preference for one of the two sexual roles, a conflict arises when two potential partners meet, as both may aim to play the preferred role. The long courtship which has been described in hermaphroditic species may indeed represent the time two partners need to “agree” on which role they are going to play. An evolutionary solution to the conflict about sexual roles is conditional reciprocity, which occurs when hermaphrodites regularly alternate their sexual roles over successive reproductive bouts (Leonard 2006). Indeed, *O. diadema* mature hermaphrodites are long-lived (about 3 months, according to Di Bona et al. 2010) and they alternate their sexual roles up to 40 times during this period (Di Bona et al. 2015). In this view, egg-trading individuals mate in the less preferred role to get access to mating in the preferred role (Axelrod and Hamilton 1981; Leonard 2005).

*Why trading eggs?*

Egg trading is a crucial behavioural mechanism in the study of the evolution of sexual systems in hermaphrodites. This form of cooperation not only solves the sexual conflict that arises between two simultaneously hermaphroditic mates, but, as a recent mathematical model by Henshaw et al. (2015) proposes, it also shows how the presence of egg traders in a population permits the maintenance of the hermaphroditism even when the selective pressures favouring this sexual system (i.e., low mating rate) are no longer acting. With this model the authors show that the egg-trading

behaviour increases the fitness value of eggs, as eggs can be traded for opportunities to mate in the male role. In this way, egg-trading favours a female-biased sex allocation that prevents pure females from invading a trading population.

Empirical support to this model come from the chalk bass *Serranus tortugarum* in which formation of monogamous pairs that perform long-term reciprocation is maintained in relatively high-density social groups (Hart et al. 2016).

### *Cheating*

Reciprocal egg trading is evolutionarily stable only under a low level of cheating (that is, when individuals attempt to mate repeatedly in the preferred role, and avoid mating in the less preferred one only rarely) and therefore some mechanism to guard against non-reciprocating individuals is expected to evolve. In *O. diadema* several mechanisms preventing cheating have been demonstrated. First, individuals are able to recognize the sexual phenotype of their partners (i.e., they behave differently when paired with mature hermaphrodites with ready-to-lay eggs, mature hermaphrodites without ready-to-lay eggs or adolescent males), and, depending on their partners' readiness to lay eggs, hermaphrodites changed their egg-production rate accordingly. Sella (1988) and Premoli and Sella (1995) have shown that the time interval between two successive spawnings by the same hermaphrodite is significantly longer when it is paired with a non-reciprocating individual than when it is paired with a mature reciprocating hermaphrodite, which is able to trade eggs. In other words, mature hermaphrodites release egg cocoons more often when they alternate egg-laying with their partners (average time interval between two spawnings by the same individual when hermaphrodites are paired with hermaphrodites:  $2.97 \pm 0.2$  days; when hermaphrodites are paired with adolescent males:  $5.2 \pm 0.2$  days; and when paired with non-mature hermaphrodites:  $5.4 \pm 0.36$  days). Furthermore, the study by Sella (1988) also documented that when hermaphrodites can choose between mature hermaphrodites and adolescent males, the former are generally preferred and adolescent males (i.e., non-reciprocating individuals) are rejected as mates.

### *Egg parcelling*

Since egg reciprocation between partners is non-simultaneous (i.e., at each mating, one individual releases eggs and the other releases sperm), but successive egg-layings follow with a delay, there is the risk that the partner fertilizes eggs and then deserts (cheating). A way to minimize fitness losses is to offer only a part of the mature eggs (i.e., egg parcelling). By doing this, not only could cheaters be identified at a lower cost (only a part of the eggs produced are fertilized by the

non-reciprocating partner), but also the fitness of reciprocating individuals will be higher. If they continue to reciprocate they have the opportunity to fertilize more eggs.

Egg parcelling has been described in several serranid fishes of the genus *Hypoplectrus* and *Serranus* (Fischer and Petersen 1987; Petersen 2006). A recent field study on the chalk bass *Serranus tortugarum* shows that parcel number is coordinated within pairs, whereas there is almost no correlation between parcel number and fecundity; this suggests that egg parcelling may represent a means to assess equality in resource exchange within a pair and thus a way of communication between partners (Hart et al. 2016).

Egg parcelling has never been described among *Ophryotrocha* hermaphrodites. However, comparing the number of eggs per cocoon and the time interval between two consecutive spawning by the same individual in hermaphroditic and gonochoric species, a strategy similar to the egg parcelling, as described in fish, may occur in hermaphroditic worms. Indeed, simultaneously hermaphroditic species lay smaller cocoons but at a higher rate, compared to gonochorist species (Premoli and Sella 1995) (for example, the simultaneously hermaphroditic *O. diadema* lays about 25 eggs per cocoon every 3 days, while the gonochoric *O. labronica* lays about 130 eggs each 11 days; Premoli and Sella 1995), although hermaphroditic species do not seem morphologically constrained to produce smaller clutches. Simultaneous hermaphroditic *Ophryotrocha* species might parcel eggs by maturing fewer eggs at a time and spawning them relatively more frequently than related gonochoric species.

#### *Population density, mate competition and sex allocation*

As mentioned above, the species of the genus *Ophryotrocha* are expected to live in sparse populations. In such conditions, mate searching is likely to be time and resource consuming, making it relatively disadvantageous for a paired hermaphrodite to desert its partner and break an established pair bond (cfr. Sella and Lorenzi 2000). When pairs are monogamous, any sperm will likely compete with related sperm from the same donor to fertilize the clutch of eggs. Under high rate of Local Sperm Competition (*sensu* Schärer 2009), it is advantageous for sperm donors to bias their sex allocation in favour of the female function, since producing large numbers of sperm does not offer any return in terms of fitness. In *O. diadema*, *O. gracilis* and *O. hartmanni* the biomass of the body area involved in the female function is 80% of the total gonadal biomass (Premoli and Sella 1995; Sella et al. 1997). This leads to one of the lowest sperm redundancy estimates among those known both for vertebrates and invertebrates (Cohen 1975), as *O. diadema* produces less than one hundred sperm per egg (Sella 1990).

In turn, the female-biased sex allocation reduces the advantages of deserting a partner, since hermaphrodites cannot mate frequently as males due to the relative scarcity of sperm. This stabilizes the egg-trading behaviour. If mating opportunities increase, the competition to fertilize eggs rises between unrelated sperm and hermaphrodites could gain an advantage in switching their sex allocation into investing relatively more resources in the male role at the expense of the female role.

Lorenzi et al. (2005) have shown that *O. diadema* worms adjusted their female sex allocation in response to reproductive competition: the absolute number of laid cocoons significantly decreased when individuals were reared with several conspecific mature hermaphrodites rather than when they were reared in isolated pairs. The diminished female allocation occurred irrespective of density-related stressors (Lorenzi et al. 2005) and was a response to species-specific signals or cues (Schleicherová et al. 2006), supporting the hypothesis that the sex-allocation adjustment is a response to mate competition and not a density effect (e.g. competition for food; oxygen depletion, etc.). Additionally, sex-allocation adjustments have been shown to be extremely rapid (i.e., worms adjusted their sex allocation to current mating opportunities in 5 days) and have no costs in the short term (Lorenzi et al. 2008).

In contrast, sperm production seems weakly or not at all adjusted to mating opportunities (Lorenzi et al. 2005; Schleicherová et al. 2014). The lack of clear evidence for sperm allocation adjustments can be partially due to the limited amounts of sperm in these species and to the technical difficulties in counting the aflagellate sperms, but it may also be a reasonable finding. Increasing the number of sperm, when sperm are aflagellate, and thus immotile, may not be a rewarding strategy: if sperm are immotile the relative position of the worms during sperm release may have a larger effect on fertilization success than the number of sperm released by each individual. This may explain why worms increase their allocation to the male function allocating more resources to expensive and risky behaviours (such as increasing aggressive interactions for access to mating) that likely enhance male reproductive success, rather than barely increasing the number of sperm, which are actually unable to “compete” with other sperm because they cannot move (Lorenzi et al. 2006). As the absolute number of eggs changes depending on mating opportunities, whereas sperm number does not, the relative proportion of resources invested in the two sexual functions, that is, sex allocation, is highly plastic in the species (Lorenzi et al. 2005).

Furthermore, in *O. diadema*, Di Bona et al. (2010) described a particular phenotype with a marked male-biased sex allocation: in a laboratory population the authors found individuals that were morphologically hermaphrodites (i.e., they had oocytes in their coelom) but they behaved as

males (i.e., they did not lay eggs) for a long time-period (21 days) or (rarely) for their entire life (functional-male phenotype). The frequency of the functional-male worms in the population changed as a function of mating opportunities: functional-males were more frequent under a promiscuous mating regime (i.e. when 4 mature hermaphrodites were reared together) than under a monogamous regime (i.e. when 2 mature hermaphrodites were reared in isolated pairs).

In many hermaphroditic fishes (e.g. chalk bass) individuals frequently engage in ‘streaking’, that is they intrude into other spawning pairs and release sperm attempting to gain more matings in the male role (Fisher 1984; Hart et al. 2010); this behaviour is more frequent in high density populations (Hart et al. 2010). The presence of functional males in *O. diadema* may suggest the existence of some analogous behaviour in this species, but, at the moment, there is no experimental evidence for it.

This ability to adjust sex allocation to environmental conditions is a key trait in the theoretical speculations as to the evolutionary transition between hermaphroditism and gonochorism (Charlesworth and Charlesworth 1978; Delph and Wolf 2005) and could set the stage for the evolution of separate sexes from hermaphroditic ancestors in *Ophryotrocha* worms (Lorenzi and Sella 2013).

### **Ophryotrocha sequential hermaphrodites**

Sequential hermaphroditism is expected to be favoured when the reproductive success of an individual as a male - or as a female - is strongly related to its body size (or its age) and the relationship between body size and reproductive success is different for the male and the female sex (Ghiselin 1969, 1974; Charnov 1982; Warner 1988). For example, if individuals with a large body size have a higher reproductive success as females than as males because female reproductive success increases with body size (but male reproductive success does not), it will be advantageous for small individuals to be males and change into females once they will reach a large body size, assuming relatively low costs for sex reversal. Anyway, according with the theory of “quantitative gender” proposed by Klinkhamer and Jong (Klinkhamer et al. 1997; Klinkhamer and Jong 2002; Cadet et al. 2004), sequential hermaphroditism is rarely more adaptive than gradual changes in sex allocation associated with size/age (e.g. the simultaneous hermaphrodite *O. diadema* has a protandrous adolescent phase and gradually allocates more resources to the female role while aging). Klinkhamer and Jong distinguish between two types of size effects: “direct” effects, which are benefits of size *per se* (e.g., the ability of a tall wind-pollinated tree to spread pollen over a larger



area) and “budget” effects, in which benefits are due simply to greater energetic resources (e.g., the ability of a larger fish to produce both more sperm and more eggs). When “direct” effects of size differ for male and female function and are more important than “budget” effects, sex change is expected to be adaptive (Leonard 2013). This seems to be the case for the only known (protandrous) sequential hermaphroditic species of the genus *Ophryotrocha*. Indeed, Berglund (1986, 1990) demonstrated that reproductive success was significantly related to body size for females, but not for males in *O. puerilis* and that large males fertilized significantly fewer eggs than small males because females preferred to mate with small males (Berglund, 1990). Berglund, 1990 and Premoli and Sella (1995) made the hypothesis that females prefer small males because of the lower prospective of a conflict with the partner over sexual roles, as large males are more likely to change sex to females. Mating preferences represent indeed a classical example of “direct” effects of size (Cadet et al. 2004) which make sex change adaptive.

The cost of sex reversal in *O. puerilis* is expected to be relatively low (as measured by the time necessary to change from one sex to the other: 2-5 days, Monahan 1988; Premoli and Sella 1995). *O. puerilis* worms start to produce sperm at a body size of about 9 segments and generally change to females when they are 15 to 20-segments-long (Bacci 1951). Sex reversal is genetically determined (Sella 1980) and controlled by hormones (Pfannenstiel and Grothe 1988) but there is also a social effect: females can inhibit egg production in males and smaller females, and this effect is mediated by hormones (Pfannenstiel 1973; Grothe and Pfannenstiel 1986).

*O. puerilis* is also one of few sequential hermaphroditic species that change sex repeatedly during their lifetime. This phenomenon has been also described in *Ostrea* oysters where individuals are males when they are brooding and females after egg hatching (Coe 1932; Chaparro and Thompson 1998), and in several species of fishes (Munday et al. 2010; Kuwamura et al. 2011). Among fish the case of the blue-banded goby (*Lythrypnus dalli*) is particularly interesting: in this species individuals are histologically and anatomically simultaneous hermaphrodites but they behave either as males or as females in relation to their relative body size (St. Mary 1993).

Berglund (1986, 1990) has interpreted alternating sex change in *O. puerilis* in terms of sex-allocation theory: males invest few resources in gamete production, as sperm are cheaper to produce than eggs, and thus males can grow faster than females. As a consequence, after some spawnings, a small male and a large female will reach the same body size and will change sex simultaneously, the male changing to female, and the female reverting to male (a phenomenon known as “Paar-Kultur”- pair-culture - effect, Hartmann and Huth 1936). Interestingly worms reared together for a long time period (more than one month) changed sex so often that they started to behave as

simultaneous hermaphrodites which regularly alternated egg laying and egg fertilization on short time intervals (every 3 days, Berglund 1986). We do not know whether pairs are stable for long time periods in the wild, but we do know that population density is often very low in this species, making long-term pair living a realistic option in this species (Premoli and Sella 1995).

### **Ophryotrocha gonochorists**

In the genus *Ophryotrocha* (and generally in polychaetes; Schroeder and Hermans 1975) most species are gonochoric, and only 9 out of more than 40 known species are hermaphroditic (either simultaneous or sequential hermaphrodites). This suggests that, in these animals, male reproductive success does not saturate with increasing investment and expressing only one sexual function often leads to high fitness returns (Charnov 1982); we may try to speculate why.

*O. labronica* is the best studied gonochoristic species in this genus. It is characterised by a clear sexual dimorphism where males have a wider prostomium and thicker jaws compared to females. Females grow faster than males and become sexually mature at a body length of about 12 segments, whereas males start to produce sperm at a smaller body size (Paxton and Åkesson 2007; Lorenzi and Sella 2013).

*O. labronica* worms share their habitat with *O. puerilis* and probably eat the same food. So why is one species gonochoric and the other a sequential hermaphrodite? One possible explanation is that in *O. labronica* both female and male reproductive success are related to body size, making sex reversal costly. However, Berglund (1991) showed that when worms were reared in pairs, female reproductive success increased with body size in *O. labronica*, whereas male reproductive success was not correlated with body size, as it occurs in *O. puerilis*. If *O. labronica* worms were reared in triplets composed of a female, a small male and a large male, pairs were preferentially formed by a female and a large male. Unfortunately, Berglund's experiment did not make it possible to disentangle the effect of male-male competition (i.e., large males are better competitors) from that of female choice (i.e., females prefer large males), but his results showed that large males had higher fitness returns than small ones and had nothing to gain from changing sex.

In laboratory mass cultures, *O. puerilis* populations typically have strongly male-biased sex ratios (Berglund 1991; Premoli and Sella 1995), whereas usually *O. labronica* populations have even, or slightly female-biased, sex ratios (Åkesson 1970; Sella and Bona 1993). These species-specific sex ratios may contribute to selection in favour or against sex reversal. Skewed sex ratio in *O. puerilis* can favour sex reversal if turning female is advantageous because it will end the

competition with the others males. In contrast, the relatively more balanced sex-ratios in *O. labronica* populations may impose no pressure in favour of changing sex.

## **Intermediate steps between truly hermaphroditic and gonochoristic species in Polychaetes**

In the preceding paragraph, we reasoned only about purely gonochoric or purely hermaphroditic species. Although these two sexual systems are the two most common sexual systems in the animal kingdom, they are the two endpoints along a continuum, which includes intermediate steps such as gynodioecy (i.e., hermaphrodites + females), androdioecy (i.e., hermaphrodites + males) and trioecy or subdioecy (i.e., hermaphrodites + females + males) (Delph and Wolf 2005; Charlesworth and Charlesworth 1978; Avise 2012). In their paper, Delph and Wolf (2005) well summarized the evolutionary pathway that lead from a hermaphroditic population to a gonochoric one via gynodioecy, but similar steps could be imagined for the pathway via androdioecy. According to Delph and Wolf (2005), the first step along this pathway occurs when in a hermaphrodite population a male-sterile mutant appears which is able to play the female role only; if female mutants have even a slightly higher fitness compared to hermaphrodites, they will spread in the population. When the population is dimorphic for gender (hermaphrodites + females), hermaphrodites will have higher fitness returns through male function; in this way, hermaphrodites will be favoured if they allocate relatively more resources to the production of male gametes, until they totally suppress the female function, which leads to a gonochoric population. A study from Dorken and Pannell (2009) provided experimental evidence for the androdioecy pathway: they allowed populations of the hermaphroditic plant *Mercurialis annua* to evolve either in the presence of pure male individuals or in populations composed entirely of hermaphrodites. After a few generations, the average male allocation of hermaphrodites had changed in different directions depending on whether pure males were present or not, confirming that pure-sex mutants produce a selective pressure on the hermaphrodite sex allocation.

Identifying and studying populations that display these intermediate sexual systems could not only highlight the selective pressures that favour one sexual system over another (i.e., the adaptive value), but also help to identify the mechanisms underlying the transition (i.e., sexual system evolution).

Unfortunately, relatively few species with sexual systems intermediate between hermaphroditism and gonochorism have been described in animals (Weeks 2012), although recently sexual phenotypes intermediate between hermaphroditism and gonochorism have been reported in *Ophryotrocha* worms.

### **First evidence for intermediate sexual phenotypes in *Ophryotrocha* worms**

In the oldest literature on *Ophryotrocha* worms, the sexual system of some species was controversial. Now we know that multiple sexual phenotypes exist in several species, and that populations differ in the relative frequency of these phenotypes, which clarifies why old descriptions of the sexual system of such species were confused, with authors often contradicting their own findings. For example, evidence for intermediate sexual systems was reported by Bacci (1951) for the species that we now describe as the sequential hermaphrodite *O. puerilis*. Bacci observed that in this species (where small worms are males and switch to female when they reach a large body size), a small percentage of short females and large males existed and he also reported one male who died at a very large body size without changing to female. In a successive publication, Bacci (1964) argued that small females and large males could actually belong to another species. We will never know which version was the correct one, but both could be correct if populations vary widely in their sexual traits.

### **Four sexual phenotypes in *O. labronica***

In the genus *Ophryotrocha*, androdioecious or gynodioecious populations have not been reported up to now. However, evidence for intermediate steps between hermaphroditism and gonochorism now exist.

Probably one of the most informative cases is *O. labronica*, an apparently gonochoric species that actually has four different sexual phenotypes: pure males, males with oocytes, females with sperm and pure females (Lorenzi and Sella 2013; Meconcelli et al. 2015a). This species was first described as hermaphroditic on the basis of morphological observations (Parenti 1960; La Greca and Bacci 1962) and later as gonochoric with some hermaphroditic individuals (Bacci et al. 1979), which documents that these authors found individuals with eggs and sperm.

Intriguingly, in Lorenzi and Sella (2013) males with oocytes and females with sperms could not be defined as either hermaphrodites or separate-sex individuals because they were sexually

dimorphic, functioned as one-sex individuals, but produced both male and female gametes (for a functional criterion of gender, cfr. Lloyd 1980; Pannell 2002; Delph 2003) (Lorenzi and Sella 2013). Indeed, females with sperm had the secondary sexual traits that typically identified females in the species (i.e., they had relatively large body size, small head and small jaw size), whereas males with oocytes were phenotypically males (they had relatively small body size, large head and large jaw sizes) (cfr. Paxton and Åkesson 2007). However, a screening for the gametes in the coelom showed that males with oocytes and females with sperm produced both sperm and eggs (Lorenzi and Sella 2013). The special combination of sexual dimorphism and production of two types of gametes suggested Meconcelli et al. (2015a) to classify them as pseudo-hermaphrodites.

Lorenzi and Sella (2013) discovered these four phenotypes in two American and one Mediterranean populations (i.e. Alamitos Beach, LA, CA; San Diego, CA; Genova, IT) and they documented that each population had distinct proportions of the four phenotypes. Pure females were very rare (virtually absent) in all populations, whereas the frequency of pure males, males with oocytes, and females with sperm varied significantly between the American and the Mediterranean populations. Males with oocytes and females with sperm made up nearly the entire Californian populations (95 % at Alamitos beach and 92 % in San Diego) and were roughly equally represented. In contrast, females with sperm and pure males were the two most common phenotypes in the Mediterranean population, the latter representing about one third of the entire population.

The four sexual phenotypes also differed between each other in another morphological trait, possibly a secondary sexual trait: the number of rosette glands, which are dorsal and epidermal structures located in the posterior segments of adult worms. These glands occur in many *Ophryotrocha* species (Pleijel and Eide 1996) and, in gonochoric species, are typically more abundant in males than in females (Paxton and Åkesson 2010). In *O. labronica*, the number of rosette glands varied among sexual phenotypes and it did so at a different extent across the three populations; rosette glands were significantly more abundant in male than in female phenotypes in the Mediterranean and San Diego populations, whereas they were in roughly similar numbers in the worms in the Alamitos Beach populations (Lorenzi and Sella 2013). The function of the rosette glands is unknown, but they have been associated to mating and spawning and might be involved in the release of sexual pheromones (cfr. Paxton and Åkesson 2007).

From a functional point of view, all sexual phenotypes of *O. labronica* are fertile and are able to reproduce when reared in heterosexual pairs (that is, when they are paired with a partner exhibiting the opposite sex, based on external morphology). Very few females were able to produce brood when paired with another female (only 8%) or when kept alone (only 2%) for two months. In

contrast no male in homosexual pairs or in isolation did so. This suggests that sperm in females are functional and may be used to self or fertilize the eggs of another female (at least occasionally), whereas oocytes may be not functional in males (Lorenzi and Sella 2013). Self-fertilization in this species was already reported by Parenti (1960)

Since egg production is reasonably costly (a single egg-cocoon accounts for approx. 1/3 of the worm body volume, and egg production trades-off with lifespan, Di Bona et al. 2010), Lorenzi and Sella (2013) have also checked whether virgin males with oocytes adjusted their sex allocation in response to mating opportunities, as hermaphrodites typically do. Again, the results of the experiment showed that the degree of plasticity in sex allocation strongly varied among populations. The worms from Alamitos Beach – the most “hermaphroditic-like” population – were able to plastically adjust their sex allocation to current mating opportunities: males reared under high mating opportunities had relative less oocytes in their coelom than males reared with only one partner, while no plastic adjustment was reported for males from the San Diego and the Mediterranean populations – the latter being the most “separate-sex” population.

In conclusion, *O. labronica* is not a strictly gonochoric species, since four different sexual phenotypes exists in at least three geographically separated populations. Yet, this species cannot be defined as either androdioecious or trioecious, as males with oocytes and females with sperm cannot function as hermaphrodites (with the exception of a few females which were able to fertilize their own eggs or those of a female partner). Instead, this species can be viewed as an intermediate step in the transition from hermaphroditism to gonochorism along a hypothetical androdioecious pathway (as defined by Delph and Wolf 2005, see above), where the gametes of the opposite sex in males with sperm and in females with oocytes are vestigial traits of an ancestral hermaphroditic state. However, one observation is not congruent with the expectations from the theoretical models of transitions, namely the fact that in Delph and Wolf model no more than three sexual phenotypes should coexist, whereas in *O. labronica* four sexual phenotypes co-exist. Dorken and Pannell (2009) reasoned that selection on gamete production might weaken towards the end of the pathway leading to separate sexes, when hermaphrodites are already strongly biased toward one sex. This could explain why *O. labronica* females retain sperm: selection for switching off sperm production may be relaxed, because these females already have a disproportionate investment in the female function, compared to their investment in rare sperm (Lorenzi and Sella 2013).

The same four sexual phenotypes have also been reported in two other, so-called gonochoric, species (*O. robusta* and *O. macrovifera*, Meconcelli et al. 2015a) suggesting that this might be a relatively common trait in the genus. In a broader view, examples of intermediate sexual systems

have been described also in the mainly hermaphroditic genus *Indodidymozoon* (Platyhelminthes), in which two species (*I. pearsoni* and *I. suttiei*) shows traits intermediate between hermaphroditism and gonochorism: female individuals have apparently non-functional testis and male individuals have apparently non-functional female reproductive organs (Anderson and Cribb 1994). As in the case of *Ophryotrocha* functional gonochoric species seem to have evolved from a hermaphroditic ancestor (Anderson and Barker 1998). Further examples came from crustaceans, where in gonochoric species individuals with traits belonging to the two sexes are often found: this is the case of *Cherax quadricarinatus*, where individuals, functionally male and with a male external morphology, have both female and male genital openings (Sagi et al. 1996); and the case of *Gammarus minus*, where functional females may present genital papillae (a male sexual characteristic) (Glazier et al. 2012). Eventually in crustacean a population entirely composed by individuals with intermediate traits has been described for the species *Parastacus pilimanus*, in which individuals have both female and male gonopores externally, but only one type of gonad internally (either female or male gonad) (Rudolph and Verdi 2010).

The three populations of *O. labronica* were largely different from each other in the relative frequency of the sexual phenotypes as well as in the expression of secondary sexual traits (rosette gland numbers) and, although to a small extent, in their degree of plasticity in sex allocation. For this reason, these three populations can be viewed as three different steps along the hermaphroditism-gonochorism continuum. From this perspective, the Alamitos Beach population retained the most hermaphroditic traits; the population was largely composed of individuals producing both type of gametes and having the lowest degree of sexual dimorphism as for the number of rosette glands. Furthermore, males with oocytes were able to plastically adjust their sex allocation in response to mating opportunities, a typical hermaphroditic trait (cfr. Charnov 1982; Schärer 2009). On the other hand, the Mediterranean population retained the least hermaphroditic traits, with a half of the morphological males expressing a pure (i.e., unisexual) male phenotype. Worms in the Mediterranean population were strongly sexually dimorphic and were unable to adjust their sex allocation to current mating opportunities (Lorenzi and Sella 2013). Finally, worms from the San Diego population were intermediate in these traits.

We can imagine the following evolutionary scenario for the “gonochoristic end” of the evolutionary pathway between hermaphroditism and gonochorism. Before a complete separation of sexes is achieved, *Ophryotrocha* worm populations might typically be composed of up to four sexual phenotypes, where the pseudo-hermaphroditic phenotypes (males with oocytes and females with sperm) have already evolved some degree of sexual specialization in their external

morphology (i.e., sexual dimorphism) but may still produce both eggs and sperm and retain some ability to adjust their sex allocation to environmental conditions (mainly social conditions, i.e., mating opportunities). The three *O. labronica* populations described in Lorenzi and Sella (2013) might represent three steps with different degrees of sex specialization. Yet, other questions can be raised. First, we may ask at which extent the sexual phenotype is influenced by environmental factors during development. Second, we can ask where the four sexual phenotypes come from, or which hermaphroditic trait enabled the appearance of a minimum level of sexual specialization in the would-be separate sex.

### **Labile sex expression**

Sexually labile species have the potential to produce male, female or both kind of gametes depending on the influence of different environmental factors (cfr., for plants, Korpelainen 1998). This form of lability is expected to play a central role in the evolution of sexual systems, with sexually labile species representing an intermediate stage between hermaphroditism and gonochorism (Leonard 2013). Diggle and Miller (2013) have shown that in *Solanum* plants a fixed sexual expression can evolve from a phenotypically plastic sex expression. Some *Solanum* species were able to produce both hermaphrodite and male flowers and the production of male flowers increased when resources were abundant; however, in other species of the same genus, male flower production was not plastic and did not vary with resource abundance. The authors found that plasticity in sex expression was the ancestral trait and a fixed production of male flowers evolved where resources were predictable and abundant. This suggests that there might be a continuum between plastic sex allocation in hermaphroditic species (see above) and a fixed sex expression in separate-sex species. Indeed, sex expression in gonochoric *Ophryotrocha* species still exhibits some level of plasticity.

### **Plasticity in sex expression during development**

Plasticity in sex expression during the juvenile phase has been found in three so-called gonochoric species of the genus *Ophryotrocha* (i.e. *O. labronica*, *O. robusta*, *O. macrovifera* Rolando, 1984; Meconcelli et al. 2015a). Meconcelli et al. (2015a) showed that in these species juvenile worms which were paired with females developed as males significantly more often than juveniles paired with males or reared in isolation. That is, there was a social effect on sex



expression, where the presence of an adult female triggered larvae to develop as males. Intermediate sexual phenotypes (i.e. females with sperms and male with oocytes) were present in all three species and their frequency changed in response to social environment: as expected, juveniles reared in isolation displayed intermediate sexual phenotypes more often than juveniles paired with adults. The presence and the gender of an adult also affected the developmental time of juveniles; in fact, in *O. labronica*, *O. robusta*, *O. macrovifera* the individuals that developed the same gender as that of their partner needed a significantly longer time period to mature sexually than individuals that matured the sex opposite to that of their partner. In contrast, the effect of the social environment was virtually null during the adult phase, when individuals reared in homosexual pairs did not change to another sex, suggesting that sex expression was labile only during juvenile development (Meconcelli et al. 2015a).

Overall the study by Meconcelli et al. (2015a), jointly with other studies (Rolando 1984; Lorenzi and Sella 2013) show that in several, so-called gonochoric species of the genus *Ophryotrocha* intermediate sexual phenotypes are present and that there is a certain degree of plasticity in sexual expression.

Taking into account all the experimental evidence, it is possible to state that in this genus there is a strong environmental contribution to sex determination, but, at the same time, sex determination is also affected by genetic factors.

### **The genetic basis of sex determination in *Ophryotrocha***

In order to understand the selective mechanisms underlying the transition between hermaphroditism and gonochorism, it is important to know the genetic contribution to sex determination.

When the transition occurs from hermaphroditism to gonochorism, generally a polygenic sex determination is expected, since at least two mutations (e.g., the first silencing the female function and the second silencing the male function) are necessary to produce females and males from a hermaphroditic ancestor (Delph and Wolf 2005; Bachtrog et al. 2014). Under a polygenic sex determination system, sex is not determined by a single genetic locus acting as a “master switch” and channelling for either the female or the male - or the hermaphroditic - developmental pathways, but it is instead determined by a quantitative threshold trait controlled by multiple regions in the genome (Bulmer and Bull 1982; Moore and Roberts 2013; Bachtrog et al. 2014). Polygenic sex

determination is also known to be sensitive to environmental effects, where abiotic (e.g. temperature) and biotic factors (including social factors, e.g. exposure to the opposite sex) influence gene expression (Falconer 1981; Bull 1983).

We know very little about the genetic basis of sex determination in *Ophryotrocha* worms. A few studies, whose results are based on selection experiments or appropriate crossings, were performed in sequential hermaphrodites (*O. puerilis* Bacci and Bortesi 1961; Bacci 1965; Sella 1969) and in gonochorists (*O. labronica*; Premoli et al. 1996). These experiments suggested that sex determination is probably polygenic in the genus and that environmental factors (i.e., social effects, such as exposure to individuals of the same or the opposite sex, and mating opportunities) have an effect on sex expression.

For example, in the simultaneous hermaphrodite *O. diadema*, Di Bona et al. (2015) showed with backcrossing experiments that the functional-male phenotype is a heritable trait, i.e., the expression of a strongly male-biased allocation, where individuals have a hermaphroditic phenotype but reproduce only as males, is under genetic control. Indeed, in only four generations, the frequency of the male phenotype increased from 5% to 25%. The authors investigated also the variation of life history traits related to sex expression across generations. The hermaphrodites of the lines originating from crosses between functional males and hermaphrodites had a longer protandrous phase (i.e. developed oocytes later) and produced fewer eggs at the first laying compared to hermaphrodites originating from the control lines resulting from hermaphrodite x hermaphrodite crosses. These results suggest that sex allocation is a heritable trait, that there is a genetic association between the functional-male phenotype and a male-biased hermaphrodite sex allocation, and that a genetic trade-off may exist between traits associated with male and female functions (Di Bona et al. 2015).

Similarly, Bacci and Bortesi (1961) selected for either a longer or a shorter duration of the initial male phase in the sequential (male-first) hermaphrodite *O. puerilis*. Again, after only 4-5 selected generations, the worms spent almost their entire life either as males or as females. Crosses between individuals with a long male phase and those with a long female phase produced individuals that changed sex at a body length similar to that of the worms in the original population (Bacci and Bortesi 1961). In another study, Sella (1969) confirmed that body size at the moment when the first sex change occurs in *O. puerilis* has a certain degree of heritability (estimated around 0.3-0.4). These studies suggest again a genetic contribution to sex determination in *O. puerilis* where several genes are involved.

Slightly different results were obtained by Premoli et al. (1996) for *O. labronica*. The authors investigated the heritability of sex ratio in a laboratory population in order to propose a model that could explain the sex determining mechanism; they found that the mean sex ratio of the offspring whose fathers came from families with a male-biased sex ratio significantly differed from the mean sex ratio of the offspring whose fathers came from female-biased sex ratio families, whereas this difference was not significant for the mother lineages. On the basis of their experiment the authors hypothesised that in this species sex is determined by a multilocus genetic system that combines the effects of a female major sex gene (which could give rise to a form of female heterogamety) and masculinising modifiers (Premoli et al. 1996).

If *O. labronica* represents a taxon towards the end point of the transition, we might speculate that polygenic sex determination switched towards a mechanism based on major sex genes at some point along the evolutionary pathways, thus reducing the level of plasticity in favour of a more deterministic (and rigid) system. A genetic sex determination mostly linked to major sex genes (genotypic sex determination) is expected to facilitate the evolution of sex-specific traits (Karlin and Lessard 1986) and, since sex is determined at conception, the individual can begin ‘developing into’ its gender early probably allowing that individual to become “a better male or a better female”; this could represent the major advantage of a genotypic sex determination (Charnov and Bull 1977; Leonard 2013).

## Conclusions

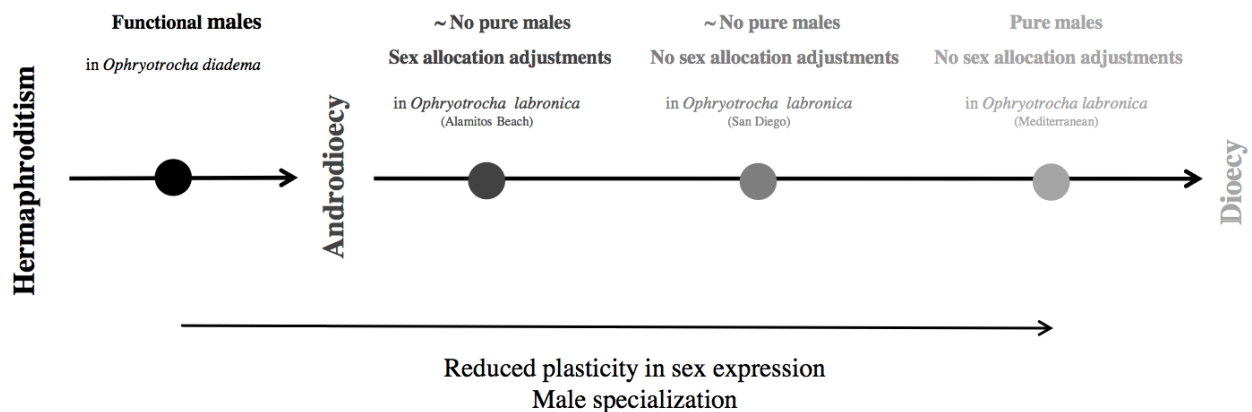
The several experiments conducted in recent years on the genus *Ophryotrocha* allow us to speculate on the evolutionary transition between hermaphroditism and gonochorism in this taxon. As in this genus hermaphroditism is thought to be the basal condition (Heggøy et al. 2007; Thornhill et al. 2009), we discuss the likely pathway that may have led from the hypothetical hermaphroditic ancestors to gonochoric species. On the basis of the experimental evidence available on *Ophryotrocha* species, we may hypothesize that the transition occurred via a pathway similar to that proposed by Delph and Wolf (2005) and via androdioecy even if some significant differences may exist. Indeed, in simultaneously hermaphroditic plants, mutations of mtDNA that cause inability to produce viable pollen grains (cytoplasmic male sterility) have been described in several species (Lewis 1941; Touzet and Meyer 2014) and frequently represent the first step of the aforementioned pathway. Such a mutation has never been reported in animals (Budar et al. 2003)

and the first step of the transition is probably longer than in plants, involving gradual changes in sex allocation.

The hermaphroditic species *Ophryotrocha diadema* is able to adjust its sex allocation plastically as a function of mating opportunities, reducing the proportion of resources invested in the female role when mating opportunities increase (Lorenzi et al. 2005) – and this pattern is common to other hermaphroditic species in the genus (Schleicherová et al. 2014). We also know that *Ophryotrocha* hermaphrodites allocate relatively more resources into aggressive behaviours that are likely to increase male reproductive success, when mating opportunities increase (mate competition, Lorenzi et al. 2006). Furthermore, the occasional presence of functional-male-phenotype worms, whose frequency depended on mating opportunities (Di Bona et al. 2010) but was based on a heritable trait (Di Bona et al. 2015), has been reported in hermaphroditic populations. Overall, this evidence suggests that if population density rises in a hermaphroditic population, we could expect that some hermaphrodites may allocate more and more resources to face mate competition i.e., to male function and aggressive behaviour at the expenses of egg production (and indeed, overall egg production is lower in high than in low density populations, Lorenzi et al. 2014). This set the stage for an increase in the frequency of functional males – hermaphrodites “specialized” on the male function. Behaving more often as male is probably advantageous when mating opportunities are large: since finding partners is easier and deserting them is less costly, worms behaving as males have a higher fitness than less male-biased hermaphrodites; they save resources for gamete production (and may have a longer lifespan, Di Bona et al. 2010). If the high population-density condition (and the concurrent strong sexual selection) is stable across a sufficiently long time period, the male-biased phenotype will spread in the population and will be selected favourably to the point that some male-biased hermaphrodites will switch off the female function, leading to some sort of androdioecious population (i.e., a population composed of hermaphrodites + pure males). Such a population has never been described in *Ophryotrocha* but something similar as been described in the Mediterranean population of *O. labronica*, where females able to fertilize eggs and pure males coexist (see below). In turn, the presence of pure males or strongly male-biased hermaphrodites in the population is expected to impose a selective pressure on the other hermaphrodites in favour of a female-biased allocation. In the presence of pure males or strongly male-biased hermaphrodites, the female-biased hermaphrodites will get a higher reproductive success than hermaphrodites with relatively even sex allocation and this selective pressure will act to the point that the population will be entirely composed of pure males and pure females – as gonochorist populations are. The three *O. labronica* populations studied by Lorenzi and Sella (2013) seems to represent well three

intermediate stages of this second step: the worms are indeed functionally gonochoric and exhibit sexual dimorphism, supporting the hypothesis that they “specialized” for either the male or the female function. Yet, these sexually specialised worms maintain some vestigial hermaphroditic traits. One population – the Alamitos Beach population - retained the most hermaphroditic traits, as pure males were absent and functional males were still able to plastically adjust their sex allocation to face mate competition. Another population (San Diego) included individuals that had lost the ability to make sex allocation adjustments, but pure males were still absent. Eventually, in the most truly gonochoric population – the Mediterranean one - pure males were quite frequent and functional males were no longer plastic in their sex allocation.

Such an evolutionary scenario is depicted in Figure 3 where the experimental evidence for intermediate steps is highlighted.



**Figure 3:** Scheme of the hypothetical transition from hermaphroditism to gonochorism in *Ophryotrocha* worms.

The selective pressure behind this whole evolutionary process is the one emerging from competition for mating, suggesting that sexual selection may impose disruptive selection on hermaphroditic populations favouring the emergence of specialized sexual phenotypes and ultimately males and females (Lorenzi and Sella 2008; Anthes et al. 2010).

This scenario assumes a simultaneous hermaphroditic ancestor, from which gonochoric species have evolved. According to the phylogenetic tree proposed by Dahlgren and co-authors (2001; Fig. 2), the most parsimonious hypothesis predicts that also sequential hermaphroditism has evolved from simultaneous hermaphroditism, if the ancestor is a simultaneous hermaphrodite.

Following the theory of “quantitative gender” (Klinkhamer et al. 1997; Klinkhamer and Jong 2002; Cadet et al. 2004), sequential hermaphroditism is favoured over simultaneous hermaphroditism when “direct” effects of size (i.e., the benefits of size *per se*) exceed “budget”

effects (i.e., the benefits derived from greater energetic resources). This could be the case of the genus *Ophryotrocha*. The preference of *O. puerilis* females for smaller males may represent a strong “direct” effect of size: this mating preference makes disadvantageous for larger individuals to continue to produce sperm while they are producing also eggs, since the mating success of large males is probably low (Berglund 1990). Therefore, if in populations of simultaneous hermaphrodites, mutants that prefer to have their eggs fertilized by smaller individuals appear and spread this may set the stage for the evolution of sequential hermaphroditism from simultaneous hermaphroditism.

How and why a preference for small males could emerge is still not clear, and further experiments will be necessary to throw light on the mechanisms underlying this evolutionary pathway.

However, this is not the only scenario that has been proposed. Thanks to the experimental work on *O. puerilis* it was also hypothesized (Berglund 1991; Premoli and Sella 1995) that both simultaneous hermaphrodites and gonochorists evolved from protandrous sequential hermaphrodites. This hypothesis was based on the observation that sequential hermaphrodites (such as *O. puerilis*) synchronized their sex change to the point that they behaved as simultaneous hermaphrodites when they were reared in pairs for long time periods (Berglund, 1986). In Berglund’s view, this suggests that there is the potential for simultaneous hermaphrodites to evolve from sequential hermaphrodites, if sequential hermaphrodites have the opportunity to form relatively long-term pair bonds in natural populations, as it may happen if they live at a low population density and partners meet at mucus nesting sites.

In the view of Berglund (1991) and Premoli and Sella (1995), separate sexes could originate as well from sequential hermaphrodites. In sequential hermaphrodites (e.g., *O. puerilis*) small males have a higher reproductive success than large ones, since females prefer them as mates (Berglund 1986, 1990). In such populations, if mutant females appear, which exhibit a preference for large males, sex changers might be selected against and populations with pure males will appear. This is especially true if population density increases, which results in increased mate competition where large males may be better competitors.

Although reasonable, this second hypothesis gets weak support from the phylogenetic analyses of the genus *Ophryotrocha*, where sequential hermaphroditism originated recently, relative to the point where simultaneous hermaphrodites and gonochorists separated (Dahlgren et al. 2001).

Although the androdioecious pathways seems more likely to explain the transition from hermaphroditism to separate sexes among *Ophryotrocha* polychaetes, more work is needed to

confirm whether the transition really occurred along this way. For example, to list some points which we see as crucial for our understanding of the mechanisms of this biological transition, we need to know in more detail which safeguards exist against non-reciprocating worms (i.e., against functional-male-phenotype worms) in hermaphroditic populations and how such safeguard mechanisms, which prevent cheaters from spreading, may be disrupted in populations evolving towards separate sexes. We need to know how sexual dimorphism emerged in hermaphroditic populations and how dimorphic traits are linked to fitness - where recent work has failed to find any association (Meconcelli et al. 2015b). We also need to know whether higher mate competition abilities confer higher fitness returns to “sexually specialised” hermaphrodites in high density populations.

Merging data on phylogeny, sexual systems and mating systems has been useful for our understanding of the transition between sexual systems in several families of teleost fishes (reviewed in Erisman et al. 2013). In particular, this kind of study made it possible to appreciate how life history traits, such as mating systems (e.g. group spawning, spawning of pairs) and sperm competition, contribute to shape sexual systems and their study provide experimental support to theoretical models, like the Size Advantage Model proposed by Ghiselin (1969) (Erisman et al. 2013)

Similarly, working on a genus with such a wide variety of sexual systems has proved fruitful. The presence of several species that can be defined neither as fully simultaneous hermaphroditic nor as clearly gonochoristic offers the opportunity to increase our understanding of a fine-scale evolutionary transition, highlighting the likely intermediate steps of this pathway and throwing light on how sexual selection works in driving sexual systems.

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## Supplementary materials

**Table S1:** A non-exhaustive list of polychaete genera where both hermaphroditic and gonochoric species are described.

Family	Species	Sexual system	Sexual system described through	Selfing in hermaphrodite	Habitat	Population density	Sexual dimorphism in gonochorists	Notes on reproductive biology	References
Capitellidae	<i>Capitella</i> sp. S	Gonochorist	Presence of sperm or eggs		Intertidal sediments	NA	Genital spines in males	Larval development in parental brood tube	Linke-Gamenick et al. 2000
	<i>C. iatapiuna</i>	Trioecious	Presence of sperm or eggs or both	NA	Deep sea whale-fall inside bones	High	Genital spines in males; females with enlarged Lateral genital pores		Silva et al. 2016
	<i>C. sp. I</i>	Trioecious	Presence of sperm or eggs or both	No	Disturbed habitats	Rapid population explosions	NA		Linke-Gamenick et al. 2000; Peitraitis 1985
	<i>C. sp. M</i>	Trioecious	Presence of sperm or eggs or both	NA	Shallow hydrothermal vent areas	NA	NA		Linke-Gamenick et al. 2000
	<i>C. sp. Y</i>	Trioecious	Presence of sperm or eggs or both	Yes (?)	Estuaries	NA	Genital spines in males	Brooding behaviour in females and hermaphrodites	Méndez 2006

\*\* Four sexual phenotypes are described (pure males, males with oocytes, females with sperm, pure females) but individuals behave only as males or females (see text)

NA No data available

(?) Doubtful evidence

**Table S1:** A non-exhaustive list of polychaete genera where both hermaphroditic and gonochoric species are described.

<b>Family</b>	<b>Species</b>	<b>Sexual system</b>	<b>Sexual system described through</b>	<b>Selfing in hermaphrodite</b>	<b>Habitat</b>	<b>Population density</b>	<b>Sexual dimorphism in gonochorists</b>	<b>Notes on reproductive biology</b>	<b>References</b>
Cirratulidae	<i>Aphelochaeta</i> spp.	Simultaneous hermaphrodite	Presence of sperm and eggs	NA	NA	NA			Petersen 1999
	A spp.	Gonochorist	Presence of sperm or eggs		NA	NA	Sexual dimorphism in <i>A. marioni</i> sensu Gibbs (1971) : cellular differences		Petersen 1999
Cirratulidae	<i>Caulleriella parva</i>	Simultaneous hermaphrodite	Presence of sperm and eggs	NA	NA	NA			Petersen 1999
	C. spp.	Gonochorist	Presence of sperm or eggs		NA	NA	No	Viviparity	Petersen 1999
	C. spp.	Gonochorist	Presence of sperm or eggs		NA	NA	No		Petersen 1999
Cirratulidae	<i>Chaetozone vivipara</i> (as <i>T. vivipara</i> )	Simultaneous hermaphrodite	Presence of sperm and eggs	NA	NA	NA		Viviparity	Petersen 1999

Family	Species	Sexual system	Sexual system described through	Selfing in hermaphrodite	Habitat	Population density	Sexual dimorphism in gonochorists	Notes on reproductive biology	References
	<i>C. spp.</i>	Gonochorist	Presence of sperm or eggs		NA	NA	NA	Viviparity	Petersen 1999
Cirratulidae	<i>Dysponetus pygmaeus</i>	Simultaneous hermaphrodite	Presence of sperm and eggs	NA	NA	NA		Protandrous and presence of penial papilla	Tzelin 2002
	<i>D. spp.</i>	Gonochorist	Presence of sperm or eggs		NA	NA	NA		Tzelin 2002
Dorvillidae	<i>Ophryotrocha labronica</i>	Functional gonochorist **	Presence of sperm and eggs, fertility experiment		Interstitial habitat in nutrient-rich water	Clumped distribution	Male with wider prostomium, thicker jaws and more rosette glands	Pseudocopulation	Sella and Ramella 1999; Lorenzi and Sella 2013
	<i>O. macrovifera</i>	Functional gonochorist **	Presence of sperm and eggs, fertility experiment		Interstitial habitat in nutrient rich water	NA	Male with wider prostomium, thicker jaws and more rosette glands	Pseudocopulation	Sella and Ramella 1999; Meconcelli et al. 2015

\*\* Four sexual phenotypes are described (pure males, males with oocytes, females with sperm, pure females) but individuals behave only as males or females (see text)

NA No data available

(?) Doubtful evidence

**Table S1:** A non-exhaustive list of polychaete genera where both hermaphroditic and gonochoric species are described.

Family	Species	Sexual system				Sexual dimorphism in gonochorists	Notes on reproductive biology	References
		Sexual system	Sexual system described through hermaphrodite	Selfing in hermaphrodite	Population density			
	<i>O. robusta</i>	Functional gonochorist **	Presence of sperm and eggs, fertility experiment	No	Interstitial habitat in nutrient rich water	Male with wider prostomium, thicker jaws and more rosette glands	Pseudocopulation	Sella and Ramella 1999; Meconcelli et al. 2015
	<i>O. diadema</i>	Simultaneous hermaphrodite	Presence of sperm and eggs, fertility experiment	No	Interstitial habitat in nutrient rich water		Pseudocopulation	Sella and Ramella 1999
	<i>O. gracilis</i>	Simultaneous hermaphrodite	Presence of sperm and eggs	No	Interstitial habitat in nutrient rich water		Pseudocopulation	Sella and Ramella 1999
	<i>O. hartmanni</i>	Simultaneous hermaphrodite	Presence of sperm and eggs	No	Interstitial habitat in nutrient rich water		Pseudocopulation	Sella and Ramella 1999
	<i>O. socialis</i>	Simultaneous hermaphrodite	Presence of sperm and eggs	No	Interstitial habitat in nutrient rich water		Pseudocopulation	Sella and Ramella 1999
	<i>O. puerilis</i>	Protandrous, sequential hermaphrodite	Presence of sperm or eggs, fertility experiments	No	Interstitial habitat in nutrient rich water		Pseudocopulation	Sella and Ramella 1999

Family	Species	Sexual system	Sexual system described through	Selfing in hermaphrodite	Habitat	Population density	Sexual dimorphism in gonochorists	Notes on reproductive biology	References
Maldanidae	<i>Micromaldane nutricula</i>	Gonochorist	Presence of sperm or eggs		Mats of red algae in the littoral zone	NA	NA	Presence of spermathecae	Rouse 1990
	<i>M. pamela</i>	Gonochorist	Presence of sperm or eggs		Mats of red algae in the littoral zone	NA	NA	Presence of spermathecae	Rouse 1990
	<i>M. rubrospermatheca</i>	Gonochorist	Presence of sperm or eggs		Mats of red algae	NA	NA	Presence of spermathecae	Rouse 1990
	<i>M. androgyne</i>	Simultaneous hermaphrodite	Presence of sperm and eggs	Expected	Mats of red algae	NA	NA	Absence of spermathecae	Rouse 1990
Nereididae	<i>Hediste japonica</i>	Gonochorist	Presence of sperm or eggs		Shallow brackish waters	NA	NA		Sato 1999; Sato and Nakashima 2003
	<i>H. atoka</i>	Gonochorist	Presence of sperm or eggs		Shallow brackish waters	NA	NA	Female spawning within burrows	Sato and Nakashima 2003
	<i>H. diadroma</i>	Gonochorist	Presence of sperm or eggs		Shallow brackish waters	NA	NA		Sato and Nakashima 2003

\*\* Four sexual phenotypes are described (pure males, males with oocytes, females with sperm, pure females) but individuals behave only as males or females (see text)

NA No data available

(?) Doubtful evidence



**Table S1:** A non-exhaustive list of polychaete genera where both hermaphroditic and gonochoric species are described.

<b>Family</b>	<b>Species</b>	<b>Sexual system</b>	<b>Sexual system described through</b>	<b>Selfing in hermaphrodite</b>	<b>Habitat</b>	<b>Population density</b>	<b>Sexual dimorphism in gonochorists</b>	<b>Notes on reproductive biology</b>	<b>References</b>
	<i>H. diversicolor</i>	Gonochorist	Presence of sperm or eggs		Shallow brackish waters	NA	NA	Female spawning within burrows	Sato 1999
	<i>H. limnicola</i>	Simultaneous hermaphrodite	Presence of sperm and eggs	Yes	Fresh waters	NA		Viviparity	Sato 1999
Nereididae	<i>Platynereis dumerilii</i>	Gonochorist	Presence of sperm or eggs		NA	NA	NA	Broadcast spawning	Helm et al. 2014
	<i>P. massiliensis</i>	Protandrous sequential hermaphrodite	Presence of sperm or eggs	NA	NA	NA			Helm et al. 2014; Schneider et al. 1992
Sabellidae	<i>Oriopsis</i> spp	Gonochorist	Presence of sperm or eggs		NA	NA	NA		Rouse 1994

Family	Species	Sexual system	Sexual system described through	Selfing in hermaphrodite	Habitat	Population density	Sexual dimorphism in gonochorists	Notes on reproductive biology	References
	<i>O. androgyne</i>	Simultaneous hermaphrodite	Presence of sperm and eggs	NA	NA	NA			Rouse 1994
	<i>O. androgyne</i>	Simultaneous hermaphrodite	Presence of sperm and eggs	NA	NA	NA			Rouse 1994
Sabellidae	<i>Parasabella microphthalma</i> ( <i>Demonax microphthalmus</i> of previous authors)	Gynodioecious (simultaneous hermaphrodites and females)	No sperm in some specimens	NA	NA	NA	NA	Spermatheca (=sperm storage organ)	Gregory 1905; Weeks 2012
	<i>P. pallida</i>	Simultaneous hermaphrodite	Presence of sperm and eggs	NA	NA	NA		External fertilization (?)	Tovar-Hernández et al. 2009

\*\* Four sexual phenotypes are described (pure males, males with oocytes, females with sperm, pure females) but individuals behave only as males or females (see text)  
NA No data available  
(?) Doubtful evidence

Table S1: A non-exhaustive list of polychaete genera where both hermaphroditic and gonochoric species are described.

<b>Family</b>	<b>Species</b>	<b>Sexual system</b>	<b>Sexual system described through</b>	<b>Selfing in hermaphrodite</b>	<b>Habitat</b>	<b>Population density</b>	<b>Sexual dimorphism in gonochorists</b>	<b>Notes on reproductive biology</b>	<b>References</b>
	<i>Parasabella</i> sp. cf. <i>P. aulacnota</i>	Gonochorist (?)	Presence of eggs in some specimens		Harbour and port environment	NA	NA		Capa and Murray 2015
	<i>Parasabella</i> sp. cf. <i>P. japonica</i>	Gonochorist (?)	Presence of eggs in some specimens		In dead coral rubble, associated with sponges, bryozoa, and algae, or in coarse sand	NA	NA		Capa and Murray 2015
	<i>P. crassichaetae</i>	Gonochorist (?)	Presence of eggs in some specimens		Tropical and temperate waters	NA	NA		Capa and Murray 2015
	<i>P. aberrans</i>	Gonochorist (?)	Presence of eggs in some specimens		Fouling communities in wharf piles	Low	NA		Capa and Murray 2015

<b>Family</b>	<b>Species</b>	<b>Sexual system</b>	<b>Sexual system described through</b>	<b>Selfing in hermaphrodite</b>	<b>Habitat</b>	<b>Population density</b>	<b>Sexual dimorphism in gonochorists</b>	<b>Notes on reproductive biology</b>	<b>References</b>
	<i>P. bioculata</i>	Gonochorist (?)	Presence of eggs in some specimens		Tropical waters	NA	NA		Capa and Murray 2015
Sabellidae	<i>Perkinsiana litoralis</i>	Gonochorist	Presence of sperm or eggs		Antarctic waters	NA	NA		Gambi et al. 2000
	<i>P. borsibrunoi</i>	Gonochorist	Presence of sperm or eggs		Antarctic waters	NA	NA		Gambi et al. 2000

\*\* Four sexual phenotypes are described (pure males, males with oocytes, females with sperm, pure females) but individuals behave only as males or females (see text)  
 NA No data available  
 (?) Doubtful evidence

**Table 1:** A non-exhaustive list of polychaete genera where both hermaphroditic and gonochoric species are described.

<b>Family</b>	<b>Species</b>	<b>Sexual system</b>	<b>Sexual system described through</b>	<b>Selfing in hermaphrodite</b>	<b>Habitat</b>	<b>Population density</b>	<b>Sexual dimorphism in gonochorists</b>	<b>Notes on reproductive biology</b>	<b>References</b>
	<i>P. antarctica</i>	Simultaneous hermaphrodite	Presence of sperm and eggs	NA	Antarctic waters	High			Gambi and Patti 1999; Gambi et al. 2000
	<i>P. riwo</i>	Simultaneous hermaphrodite	Presence of sperm and eggs	NA	NA	High			Gambi and Patti 1999; Gambi et al. 2000
	<i>P. milae</i>	Asexual reproduction	No sperm nor eggs		NA	NA			Gambi et al. 2000

Family	Species	Sexual system	Sexual system described through	Selfing in hermaphrodite	Habitat	Population density	Sexual dimorphism in gonochorists	Notes on reproductive biology	References
Sabellidae	<i>Sabellastarte spectabilis</i>	Trioecious	Sperm or eggs in most the specimens (85%), both in few others (15%)	NA	Intertidal and subtidal reefs	High	NA	Spawning	Bybee et al. 2007
Serpulidae	<i>Hydroides diramphus</i>	Gonochorist	Presence of sperm or eggs		Benthic marine assemblages	Highly variable	NA	External fertilization	Allen and Marshall 2014
	<i>H. elegans</i>	Protandrous sequential hermaphrodite	Presence of sperm or eggs	NA	NA	NA		External Fertilization	Miles and Wayne 2009

\*\* Four sexual phenotypes are described (pure males, males with oocytes, females with sperm, pure females) but individuals behave only as males or females (see text)  
NA No data available  
(?) Doubtful evidence

**Table S1:** A non-exhaustive list of polychaete genera where both hermaphroditic and gonochoric species are described.

Family	Species	Sexual system	Sexual system described through	Selfing in hermaphrodite	Habitat	Population density	Sexual dimorphism in gonochorists	Notes on reproductive biology	References
Serpulidae	<i>Spirobranchus polycerus</i>	2 morphs (possibly two species) morph 1: simultaneous hermaphrodite; morph 2: gonochorist	Presence of sperm/eggs; experimental breeding in lab	Yes	<b>Gonochorist:</b> cliffs and boulders exposed to wave action; <b>Hermaphrodite:</b> obligate association with fire corals <i>milleporacomplanata</i> in patch reefs	Usually found singly or in small groups	NA	Broadcast spawners (both forms)	Marsden 1992
	<i>S. giganteus</i>	Gonochorist	Presence of sperm and eggs		Tropical and subtropical seas associate with live hermatypic coral	Clustered distribution	NA	External fertilization	Hunte et al. 1990
	<i>S. lamarckii</i> (as <i>Pomatoceros lamarckii</i> )	Protandrous sequential hermaphrodite with a small percentage of simultaneous hermaphrodites	Presence of sperm or eggs in some specimens, both sperm and eggs in few others	NA	NA	NA	NA		Cotter et al. 2013; Castric-Fey 1984

Family	Species	Sexual system	Sexual system described through	Selfing in hermaphrodite	Habitat	Population density	Sexual dimorphism in gonochorists	Notes on reproductive biology	References
	<i>S. triqueter</i> (as <i>Pomatoceros triqueter</i> )	Protandrous sequential hermaphrodite with a small percentage of simultaneous hermaphrodites	Presence of sperm or eggs in some specimens, both sperm and eggs in few others	NA	NA	NA			Cotter et al. 2013; Castric-Fey 1984
Syllidae	<i>Syllis prolifera</i>	Gonochorist	Presence of sperm only		NA	NA	No	Stolonization	Musco et al. 2010
	<i>S. gertlachi</i>	Gonochorist	Presence of sperm only		NA	NA	No	Stolonization	Musco et al. 2010
	<i>S. vittata</i>	Simultaneous hermaphrodite	Presence of sperm and eggs	NA	NA	Low		Stolonization	Musco et al. 2010

\*\* Four sexual phenotypes are described (pure males, males with oocytes, females with sperm, pure females) but individuals behave only as males or females (see text)

NA No data available

(?) Doubtful evidence





**3.**

**Quantitative matching of clutch size  
in reciprocating hermaphroditic  
worms**

Laura Picchi, Guénaél Cabanes, Claire Ricci-Bonot, and Maria Cristina Lorenzi

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## Abstract

Reciprocity (Trivers 1971) is one of the most controversial evolutionary explanations of cooperation among non-kin (Clutton-Brock 2009; Taborsky 2013). For some authors, cognitive capacity of non-human organisms is limiting, and more parsimonious mechanisms should apply (Hammerstein 2003; Clutton-Brock 2009; West et al. 2011); for others, the debate is mainly semantic (Taborsky 2013; Carter 2014) and empirical evidence can be found in a wide range of taxa (Taborsky et al. 2016). However, while the ability to alternate cooperative behaviors does not settle the reciprocity controversy, the capacity to adjust cooperative behavior to the value of received help could prove decisive. Marine polychaete worms *Ophryotrocha diadema*, as several simultaneous hermaphrodites, do not self-fertilize and have unilateral mating (i.e., they behave either as females or as males during each mating event). They are also external fertilizers and thus cannot store allosperm, which contribute to make them ideal model organisms to investigate reciprocity, since partners usually alternate sexual roles with each other, repeatedly exchanging egg clutch of variable size (Fischer 1980; Petersen 2006; Sella 1985; Sella et al. 1997; Hart et al. 2016). However, whether the alternation of sexual roles is the result of conditional reciprocity rather than by-product reciprocity has never been tested (Schärer et al. 2014). Here we show that *O. diadema* worms reciprocate eggs conditionally to the partner's behavior and adjust the quality of cooperation according to that of their partners. Moreover, only egg reciprocation offers similar fitness returns via both the female and the male function with respect to non-reciprocating laying strategies. These results document that fine-tuned forms of conditional reciprocity can emerge in cognitively unsophisticated animals, broadening the criteria to recognize conditional reciprocity among animals.

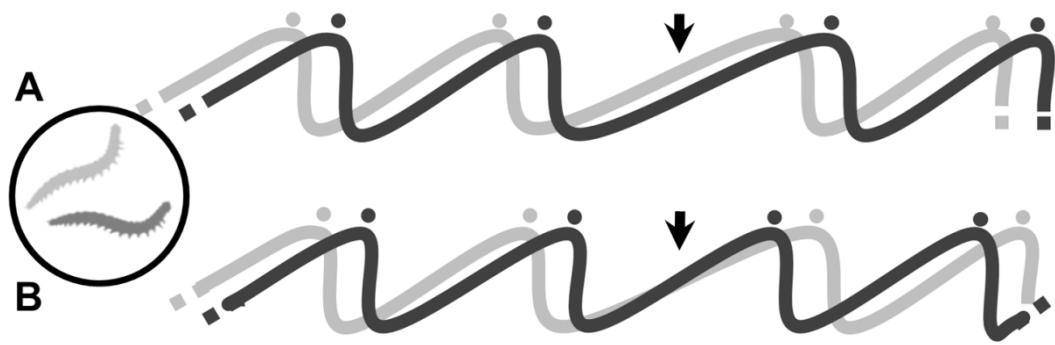
**Keywords:** Egg-trading, Cooperation, *Ophryotrocha diadema*, Conditional reciprocity, Polychaete, Sexual conflict.

## Results

We daily observed the egg laying activity of 39 isolated dyads of sexually mature hermaphroditic *Ophryotrocha diadema* worms for up to 49 days. For each dyad, we identified a “focal” individual taking advantage of a natural polymorphism for the egg color: wild strain worms produce yellow eggs, whereas albino worms produce white eggs. Since eggs are visible through the transparent body walls before they are laid, and through the transparent jelly cocoons after that, we could identify the individuals in the dyads and assign clutch maternity (see STAR Methods for details).

Overall, worms produced 507 egg clutches (each containing several eggs) (focal worms: 257; their partners: 250 clutches), out of which 79.5% (N = 403) were reciprocated (i.e., laid after the partner laid, Figure S1). We tested whether the number of reciprocated egg clutches was larger than that expected in random sequences with runs tests, and we found that, in 30 out of 39 dyads, worms significantly alternated sexual roles ( $p \leq 0.05$ ), and overall did so significantly more often than expected by chance when all p-values were combined (Fisher’s combined probability test,  $p < 0.0001$ ).

Regular alternation between layings and fertilizations of egg clutches of variable size has been documented in several simultaneous hermaphrodites with unilateral mating (see below), such as fish (e.g., genera *Hyploplectrus*, and *Serranus* [Fischer 1980; Petersen 2006; Hart et al. 2016]), and marine worms (genus *Ophryotrocha* [Sella 1985; Sella et al. 1997]). Indeed, even if simultaneous hermaphrodites produce both sperm and eggs, and reproduce via both the female and the male sexual functions, some of them must have a partner to reproduce and behave either as females or as males during a single mating event, i.e., they are outcrossers and have unilateral mating. Unilateral mating sets the stage for a conflict between partners, since partners may share the preference for the same sexual role (e.g., the male role may be preferred because sperm are cheaper than eggs to produce [Bateman 1948; Charnov 1979; Michiels 1998; Anthes et al. 2010; Schärer et al. 2014]). In the aforementioned species, the alternation of sexual roles has been interpreted as conditional egg reciprocation (in which giving eggs is conditional upon receiving them), evolved as a solution to the conflict over the sexual role (Axelrod and Hamilton 1981; Leonard 2005). However, regular alternations of sexual roles may arise irrespective of the partner behavior as random alignments of two separate, relatively regular, rhythms of egg maturation and release, since the egg laying by one individual and the next by its partner necessarily occur at a certain delay (“by-product reciprocity” [Schärer et al. 2014]; Figure 1).

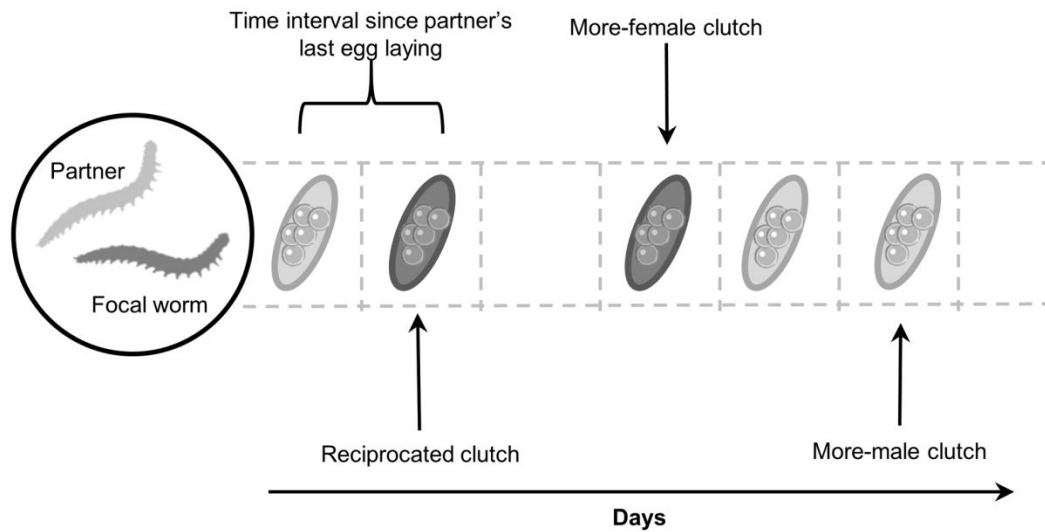


**Figure 1:** Schematic representation of conditional reciprocity vs. by-product reciprocity in egg-trading. The lines represent the rhythms of egg-maturation and egg laying of two partners, and each dot represents a single egg laying. Under the conditional reciprocity paradigm (A), a change in the egg laying rhythm by one individual leads to a change in the egg laying rhythm by its partner (black arrow). Under the alternative hypothesis of by-product reciprocity (B), a change in the egg laying rhythm by one individual does not alter the partner's rhythm (black arrow). In our study, conditional reciprocity (A) has been observed in real worms, whereas by-product reciprocity (B) emerged from the agent-based simulation.

We therefore investigated whether the alternation of sexual roles is the result of actual conditional reciprocity, testing whether egg laying activity was conditional upon the partner behavior, and, if so, whether hermaphrodites assessed the quality of received cooperation and responded accordingly. Previous studies suggest that *O. diadema* worms prefer to mate as males (Sella and Lorenzi 2000; Lorenzi et al. 2006; Di Bona et al. 2010) making it likely that a conflict over the sexual role arises and that conditional reciprocity emerges as a solution to the conflict.

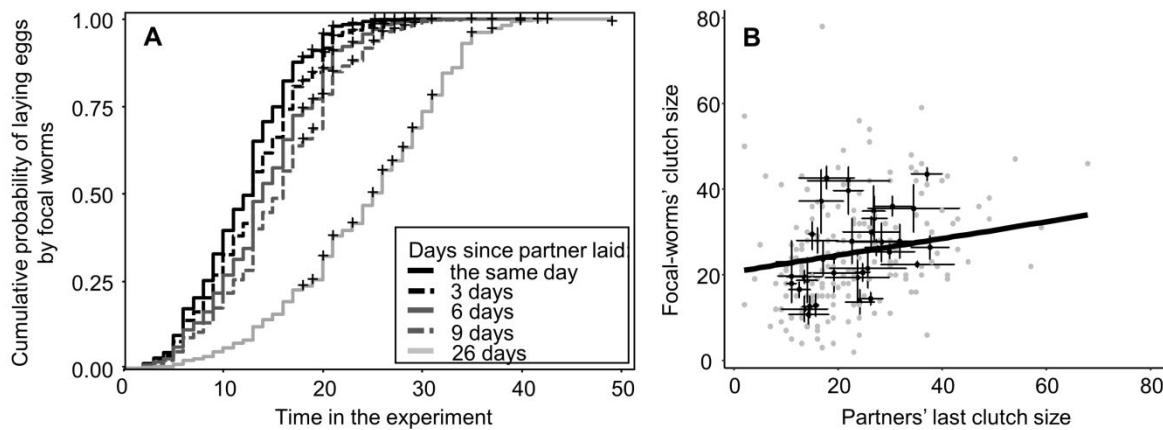
### **Conditionality in egg reciprocation**

To confirm that the alternation of egg clutches between partners was the result of conditional egg reciprocation, we tested whether the probability of egg laying by the focal worms was affected by the time elapsed since the partner's last egg laying (Figure 2). Under the hypothesis that worms reciprocated eggs conditionally, we expected that the likelihood of egg laying by the focal worms was the highest soon after their partners laid and gradually decreased later on. *Vice versa*, under the hypothesis that the alternation of sexual roles emerged by chance from the alignment of the two independent physiological rhythms of egg maturation and release of two partners (by-product reciprocity), we expected that focal worms laid eggs regardless of their partner's behavior.



**Figure 2:** Schematic representation of observations (on isolated dyads) and data processing criteria. Each box delimited by dashed, light-grey lines is a different day. Oblong-shapes represent egg clutches (each containing several eggs) by the focal worms (dark grey) and by their partners (light grey). See the main text for the description of clutch type. See also Figure S1.

As expected under the hypothesis of conditional reciprocation, focal worms were significantly more likely to lay soon after their partners laid (Andersen-Gill proportional hazard model:  $\beta = -0.081 \pm 0.028$ ,  $\chi^2 = 8.290$ ,  $n = 39$ ,  $p = 0.004$ ; Figure 3A). (Worms laying depended also significantly on age, with younger worms being more likely to lay than older ones:  $\beta = -0.895 \pm 0.103$ ,  $\chi^2 = 39.272$ ,  $n = 39$ ,  $p < 0.0001$ ; and on body size, with longer worms laying less often than shorter ones:  $\beta = -0.112 \pm 0.038$ ,  $\chi^2 = 8.471$ ,  $n = 39$ ,  $p = 0.004$ , likely because longer worms laid larger cocoons (see below) and thus needed more time to mature them.)



**Figure 3:** Conditional reciprocation in egg-laying activity. (A) Cumulative probability of laying eggs by focal worms as a function of the time since the partner's last laying at different time intervals, as predicted by the Andersen-Gill proportional hazard model. Black crosses represent censoring points. See also Figure S2. (B) Relationship between the size of the clutches (number of eggs) laid by focal worms and those laid by their partners within a maximum time interval of 7 days. The line shows the predicted value from the LMM and was computed allowing the partners' clutch size vary and keeping the other variables constant (average values). Gray dots represent raw data, while black dots represent the average values by pair ( $\pm$  s.e.). See also Figure S3 and S4.

We further tested whether conditionality in egg reciprocation (i.e., the effect of the partner's behavior on focal worm's laying probability) might have emerged by chance as a result of two mathematical series that keep repeating. To this aim, we performed an agent-based simulation where 'virtual worms' ( $N = 78,000$ ) were programmed to lay eggs following the simple rule "lay when your eggs are mature" (i.e., the probability of laying by 'virtual worms' depended only on when they laid the last time; such probability was computed from the data collected in real worms; see STAR Methods for further details). We randomly paired such 'virtual worms' and created 1000 groups, each composed of 39 different pairs of worms. For each group, we tested whether the time since the partner's last egg laying affected the probability that the virtual focal worms laid, and compared the results with those obtained from real worms. Only 37 out of 1000 models showed results comparable to those from real worms (i.e.,  $\beta < 0$  and  $p \leq 0.05$ ), which indicated that the probability to get data which suggested conditional reciprocity when instead worms laid eggs irrespective of their partners' behavior was small (probability to get a false positive result:  $p = 0.037$ , Figure S2). Conversely, the simulated data highlighted that the simple alternation of sexual roles can easily emerge when worms lay regardless of the partner behavior:  $67\% \pm 12.4$  (mean  $\pm$  s.d.) of layings were alternated. Moreover, running a runs test on a group of 39 virtual dyads, in which the probability of laying by focal worms was not significantly affected by the time

elapsed since the partners' last egg laying (Andersen-Gill proportional hazard model:  $\beta = -0.010 \pm 0.017$ ,  $p = 0.576$ ), indicated that worms alternated sexual roles more often than expected by chance (70% of laying where alternated; run tests were significant ( $p \leq 0.05$ ) in 11 out of 39 dyads; overall  $p < 0.0001$  with Fisher's combined probability test). These results strongly suggest that the alternation of sexual roles arises also from the random alignment of two separate rhythms, while the modulatory effect of the partner behavior does not (Figure 1).

### **Quantitative matching between offered and received cooperation**

Since the focal worms' clutch size was significantly different among- (ANOVA:  $F = 3.454$ ,  $n = 39$ ,  $p < 0.0001$ ), but weakly correlated within-individuals (Intraclass Correlation Coefficient, ICC1:  $r_1 = 0.327$ ; Figure S3), we investigated whether focal worms reciprocated conditionally not only in terms of quantity (i.e., number of clutches) but also in term of quality (i.e., clutch size, measured as number of eggs/clutch). Worms significantly adjusted the quality of their cooperation to that received by partners, as shown by a significant positive effect of the partners' clutch size on focal worms' (LMM:  $\beta = 0.210 \pm 0.065$ ,  $F = 10.274$ ,  $n = 39$ ,  $p = 0.002$ ): focal worms laid large clutches after their partners laid large clutches (Figure 3B; Figure S4). (In addition, younger and longer worms laid significantly larger clutches, LMM: Time in the experiment:  $\beta = -0.511 \pm 0.072$ ,  $F = 50.445$ ,  $n = 39$ ,  $p < 0.0001$ ; Body size:  $\beta = 3.644 \pm 0.559$ ,  $F = 42.492$ ,  $n = 39$ ,  $p < 0.0001$ ).

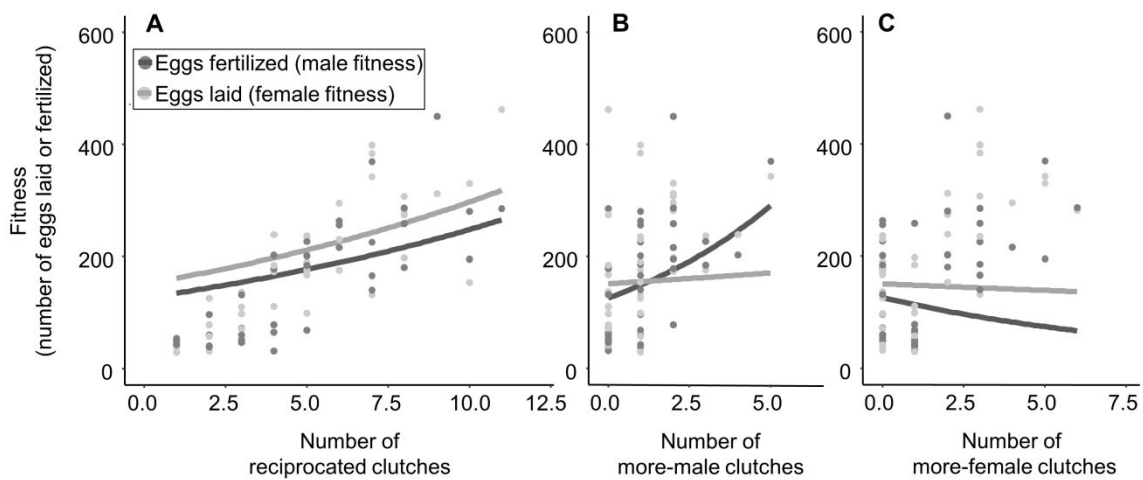
### **Fitness returns for reciprocating vs non-reciprocating laying strategies**

We showed in the previous paragraph that, when the clutches were reciprocated, focal worms adjusted their size to that of the received clutches, which suggested that worms that fertilized more eggs also laid more eggs. However, it was unclear whether worms adjusted their clutch size also when they received, or laid, more than one clutch in a row. For example, worms might have adjusted the size of their clutches to the total number of eggs received since their last laying (in case they had laid more than one clutch in a row) or might have reduced their clutch size in case their partners did not reciprocated eggs. Thus, we classified clutches as either reciprocated (when the worms laid after their partners), more-male (when the partners laid twice in a row, and thus the focal worms *fertilized* twice in a row), or more-female (when the focal worms laid twice in a row; Figure 2 and STAR Methods). Then, we tested whether the fitness of focal worms was affected by how often they adopted the different



laying strategies (fitness was measured as the number of eggs laid and fertilized, since hermaphrodite fitness has both a male and a female component).

Worms that reciprocated more often, gained similar total fitness returns in the male and in the female function (GLMM:  $\beta = 0.07 \pm 0.025$ ,  $\chi^2 = 7.64$ ,  $n = 38$ ,  $p = 0.006$ ; Figure 4A), while the other laying strategies promoted fitness in different ways depending on the sexual function [as shown by the significant interactions between sexual function and the number of more-male (GLMM:  $\beta = 0.14 \pm 0.054$ ,  $\chi^2 = 6.96$ ,  $n = 38$ ,  $p = 0.008$ ) and more-female clutches (GLMM:  $\beta = -0.09 \pm 0.039$ ,  $\chi^2 = 5.23$ ,  $n = 38$ ,  $p = 0.022$ ), Figure 4B-C].



**Figure 4 :** Fitness returns for reciprocating vs non-reciprocating laying strategies. Relationship between the fitness of the focal worms via the male (dark grey) and the female (light grey) function, and the number of reciprocated (A), more-male (B) and more-female (C) clutches. Fitness via the male and the female function was calculated as the number of eggs fertilized and laid by the focal worms. Solid, dark- and light-grey lines show the predicted values from the GLMM for the fitness via the male and the female function. Lines were computed allowing the variable of interest to vary, while keeping the others constant and equal to 0 (e.g., the lines of figure A depict the increase of fitness with the increase in reciprocated clutches, when more-male and more-female clutches were 0; analogously, the lines of figure B depict the increase of fitness with the number of more-male clutches when reciprocated and more-female clutches were 0). The time worms spent in the experiment was kept constant to the median value. Dots represent raw data.

Analyzing the data by sexual function, fitness via the male function significantly increased with the number of more-male clutches (GLMM:  $\beta = 0.17 \pm 0.049$ ,  $\chi^2 = 12.55$ ,  $n = 38$ ,  $p < 0.001$ ), while that via the female function did not. In contrast, there was a significant, negative effect of the number of more-female clutches on fitness via the male function (GLMM:  $\beta = -0.12 \pm 0.04$ ,  $\chi^2 = 8.29$ ,  $n = 38$ ,  $p = 0.04$ ), and no effect on that via the female function.

## Discussion

Since Trivers' seminal paper publication in 1971, reciprocity has been considered as a key explanation for cooperation among non-kin in a large variety of animals (from fish [Milinski 1987]; to rats [Rutte and Taborsky 2008]; and bats [Wilkinson 1984]; up to primates [Parker 1977], and humans [Gintis 2000]). However, the power of reciprocity as a mechanism favoring cooperation has been, and still is, hotly debated, because reciprocation is considered too cognitively demanding (Stevens and Hauser 2004; West et al. 2011) and therefore rare (if not absent) outside humans (West et al. 2011).

Our results add to those of recent papers (Carter and Wilkinson 2013; Schweinfurth and Taborsky 2018) in providing strong evidence of reciprocity in non-human organisms: worms were more likely to lay soon after their partners. A short-term temporal relation between cooperative action and reciprocation is considered the hallmark of hard-wired conditional reciprocity (Schino and Aureli 2010; Tiddi et al. 2011), which is expected to work in cognitively unsophisticated animals, as it does not require partner recognition or stable social relationships, and recipients are expected to respond to cooperation by returning the “favor” quickly (Schino and Aureli 2017).

An increase in the stability of cooperation with a diminishing time delay between giving and receiving is in accordance also with theoretical predictions (van Doorn et al. 2014), which show that cooperation is particularly stable when two individuals act simultaneously; under the authors' mathematical assumptions, introducing a delay between giving and receiving help, adds a cost to cooperation, making its evolution less likely (van Doorn et al. 2014). The fact that worms pay a cost by reciprocating at intervals of days suggests a strong selective pressure for the evolution of reciprocity in these animals, which may be represented by the benefit of resolving the conflict over the sexual role (Michiels 1998; Schärer et al. 2014). Under this view, providing evidence for conditional reciprocity provides support for the existence of a sexual conflict in *O. diadema* worms and, thus, that there is a consistent preference for mating in the same sexual role.

Our data show how reciprocating eggs represents a solution to such a conflict, whereby partners share reproductive costs and achieve equal fitness pay-offs (Leonard 2005; Schärer et al. 2014): it is indeed only by reciprocating eggs that worms gained similar fitness returns via both the male and female function.

Conditional egg reciprocation is a key behavior for several hermaphrodites with unilateral mating, and its crucial importance is not limited to solving the conflict over the sexual role,

but it extends to promoting the evolutionary stability of hermaphroditism. In fact, simultaneous hermaphroditism is thought to be favored under low population density, where mating may often occur in isolated dyads (Ghiselin 1969). Egg reciprocation might play a crucial role in the maintenance of hermaphroditism when population density increases, as shown theoretically by Henshaw and colleagues (2015). As egg-trading increases the value of eggs (which are used as “bargaining chips”), hermaphrodites which invest large amounts of their reproductive resources in the female function are favored. The female-biased sex allocation prevents pure females from invading egg-trader populations, because the fitness of pure females should be twice as high as the fitness of trading hermaphrodites via the female role (Charnov 1982; Henshaw et al. 2015). Experimental evidence of the central role of egg-trading in the maintenance of hermaphroditism comes from the chalk bass *Serranus tortugarum*, which live in relatively dense social groups, where partners of monogamous pairs regularly alternate sexual roles throughout spawning bouts (Hart et al. 2016).

Finally, this study is, to our knowledge, the only one providing evidence of reciprocity in invertebrates, which are often not reported in papers about reciprocity [e.g., Clutton-Brock 2009; Taborsky et al. 2016]. The rarity of clear evidence of reciprocating invertebrates makes even more interesting that here we document, for the first time, a quantitative matching between received and reciprocated cooperative acts; a quantitative assessment may prove to be decisive in settling the reciprocity controversy along with the qualitative effect on reciprocation propensity recently documented in rats (Dolivo and Taborsky 2015).

It remains unclear whether worms recognize their partners and are more likely to offer their eggs to the worms that offered them before (“direct reciprocity”). Worms may indeed follow the simple rule “lay eggs after receiving them” regardless of their partner identity, thus performing “generalized reciprocity”, which is evolutionary stable when individuals interact in small groups (Pfeiffer et al. 2005) (as expected in this species [Sella and Ramella 1999] and, in general, in hermaphrodites [Ghiselin 1969]; see STAR Methods).

In conclusion, this study documents that cognitively unsophisticated animals not only reciprocate conditionally to the behavior of another individual, but also assess the value of the received help and finely tune their behavioral response.

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## STAR Methods

### Experimental model and subject details

*Ophryotrocha diadema* worms are 4-mm-long, interstitial, marine polychaetes, which live among the detritus and fouling fauna of polluted harbours (Åkesson 1976). They are expected to live at low population density, as is often the case with interstitial organisms (Svedmark 1964; Clark 1978; Westheide 1984).

They are outcrossing simultaneous hermaphrodites with unilateral mating, meaning that individuals cannot self-fertilize, they need a partner to mate and cannot release sperm and eggs at the same time: they mate either as females or as males (Sella 1985). Before reaching full sexual maturity, worms go through a 5-6-week-long protandric, adolescent phase that begins soon after larvae hatch from the egg cocoon at a body length of 6 chaetigerous segments (i.e., the segments bearing the setae which are characteristic of polychaete worms); during this phase they produce functional sperm (Åkesson 1976). When worms reach a body length of 14-15 chaetigerous segments, they become fully sexually mature simultaneous hermaphrodites and produce both sperm and eggs (egg are laid each 3-4 days, in a jelly cocoon containing on average 25 eggs) (Sella 1985; Åkesson 1976).

Fertilization is external and achieved by pseudo-copulation, which follows a relative complex behavioural sequence where the two partners stay in physical contact for a long time (8+ hs [Sella 1985]), prior to the rapid synchronous release of gametes inside the jelly cocoon (as described in *O. gracilis* by Westheide [1984]).

In this study, we used worms derived from individuals collected by B. Åkesson in 1976 and 1980 in the Los Angeles and Long Beach harbours (Åkesson 1976; Sella and Lorenzi 2000). New individuals were added to the laboratory strain in 1995 and 2001 (kindly sent to G. Sella by B. Åkesson), and again in 2008, when worms of *O. diadema* were collected at Porto Empedocle (Sicily, Mediterranean Sea) by R. Simonini (Simonini et al. 2009).

Individual identification of worms is made possible via a genetically determined polymorphism in the colour of the eggs, which are either yellow (wild strain) or white (“albino” strain). The yellow coloration is determined by a dominant allele that controls for the storing of lutein in the egg vitellum, while the white coloration is due to a recessive allele that prevents lutein uptake (Sella and Marzona 1983).

For the experiment, worms were kept in 10-ml bowls, and, following Åkesson (1976), kept in artificial marine water, in a thermostatic cabinet at 20 °C, in the dark, and fed spinach *ad libitum*.

## Method details

Individuals used in the experiment were sexually mature, virgin worms (i.e., they had ripe oocytes in their coeloms), selected from the offspring of 78 isolated pairs (39 pairs per egg-color phenotype). Worms were reared with their siblings until they began maturing oocytes (9-12 chaetigerous segments) and then kept in isolation until they had ripe oocytes, when they were introduced into the experiment (at an age of 6-8 weeks). Worms used in the experiment were not siblings (they came from different parental pairs).

We set up 39 pairs of worms, each composed of a yellow-phenotype worm (focal individual) and a white-phenotype worm, which allowed us to identify worms and assign maternity and paternity. Worms were randomly paired with regard to body size; however, due to their similar age and development conditions, they had approximately the same size ( $18 \pm 1$  segments (mean  $\pm$  s.d.)).

Each day, for up to 49 days, we checked which worm laid eggs, we counted the number of eggs per cocoon, if any, and measured the body size of the focal worm (i.e., the number of chaetigerous segments). The development of *O. diadema* eggs can be followed at low magnification (Sella and Ramella 1999), therefore, we used the developmental stage of the eggs to assess which worm laid first, when two cocoons were laid the same day.

Egg cocoons were removed once per week (before larvae left the egg cocoon), when water and food were renewed.

The experiment lasted 49 days and was replicated 4 times (reaching a total of 39 pairs); in case worms died or got sick (i.e., they were tangled in their mucus or lost body parts and became shorter than 15 segments), data collection stopped (data collection lasted on average  $34.1$  days  $\pm$  11.7 s.d.).

## Quantification and statistical analysis

### Data processing

We measured the time interval between two consecutive egg layings by the focal worms and that between the last egg laying by the partners and the next egg laying by the focal worms. We classified each clutch as reciprocated when the focal worm laid after its partner, as a more-female clutch when the focal worm laid twice in a row, and as a more-male clutch when the focal worm's partner laid twice in a row (i.e., the focal worm fertilized the partners' eggs twice in a row and did not lay in between). As a measure of aging, we counted the number of days worms spent in the experiment, where the worms were paired at day 0 and day 1 is the first day of observation. When possible (see below), we used this variable to account for the effect of aging since it accounted for the time worms spent reproducing.

### Statistical analyses

Since worms belonged to two strains (yellow- or white-phenotype), we first checked whether there was a difference between strains in the clutch size and in the time interval necessary to produce two consecutive layings by the same individual. We did that using a generalized linear mixed model (GLMM) for Poisson distributed data, including the time spent in the experiment (as a fixed effect) to account for the potential effect of aging, and pair ID and experimental replicate (as random effects). There was no significant difference between strains in the time interval between consecutive layings (focal worms laid each  $4.1 \text{ days} \pm 0.1$  (mean  $\pm$  s.e.) and their partners each  $3.9 \text{ days} \pm 0.1$ ; GLMM:  $\beta = 0.057 \pm 0.044$ ,  $\chi^2 = 1.668$ ,  $n = 39$  pairs,  $p = 0.196$ ). In contrast, there was a significant, difference in the size of the clutches produced by white-phenotype worms (partners) (on average  $22.2 \pm 0.7$  eggs/clutch), and those produced by focal worms ( $24.6 \pm 0.8$  eggs/clutch) (GLMM:  $\beta = -0.107 \pm 0.043$ ,  $\chi^2 = 6.367$ ,  $n = 39$ ,  $p = 0.012$ ) as described by Åkesson (1976).

(G)LMMs (including the ones below) were performed with the R package *lme4* (Bates et al. 2015).

Following Hart and colleagues (2016), we tested whether reciprocation of egg clutches within dyad occurred more often than expected by chance with runs tests performed with the R package *randtests* (Caeiro and Mateus 2014) and we used the Fisher's method for combining independent p values to assess whether the number of alternation of sexual roles

was greater than expected in random sequences when all data were combined (R package *metap*; [Dewey 2018]).

To investigate whether the alternation of sexual roles was the result of conditional reciprocity or the outcome of by-product reciprocity, we tested whether the probability of laying by the focal worms was affected by the time elapsed since the partner's last egg laying with an Andersen-Gill proportional hazard model (Andersen and Gill 1982), which accounted for left truncated and right censored data (R package *survival*; [Therneau and Grambsch 2000]). We included in the model the body size of focal worms and their age as fixed effects, and we clustered the observation by pair ID and experimental replicate. In this analysis we included the age of the worms rather than the time they spent in the experiment to account for aging, since, in this kind of models, the observation time is included as the response variable. The time since the partner's last egg laying was higher than 9 days in only 3 cases; however, removing these cases did not affect the model results (Andersen-Gill proportional hazard model: Time since the partner's last egg laying:  $\beta = -0.070 \pm 0.033$ ,  $\chi^2 = 4.422$ ,  $n = 39$ ,  $p = 0.036$ ; Age:  $\beta = -0.898 \pm 0.105$ ,  $\chi^2 = 37.594$ ,  $n = 39$ ,  $p < 0.0001$ ; Body size:  $\beta = -0.113 \pm 0.039$ ,  $\chi^2 = 8.699$ ,  $n = 39$ ,  $p = 0.003$ ).

We obtained similar results when running an analogous model on the probability of laying by partners (Andersen-Gill proportional hazard model: Time since the yellow-phenotype partner's last egg laying:  $\beta = -0.083 \pm 0.031$ ,  $\chi^2 = 22.726$ ,  $n = 39$ ,  $p < 0.0001$ ; Age:  $\beta = -0.887 \pm 0.098$ ,  $\chi^2 = 40.560$ ,  $n = 39$ ,  $p < 0.0001$ ), which documents that the results are not strain-dependent.

We tested at what extent clutch size was correlated within (focal) worms using the Intraclass Correlation Coefficient (ICC1) (R package *multilevel* [Bliese 2016]), which was calculated from the ANOVA model that included the number of eggs/clutch as response variable and the worm ID as predictor (we report the F and p value for the ANOVA).

As the clutch size was highly variable within individuals, we investigated whether worms adjusted the cooperation level to that received by the partner, testing with a LMM whether the size of the clutches reciprocated by the focal worms was associated with that of the last clutch laid by the partner no longer than 7 days before (such a time interval includes 95% of the clutches and represents the maximum interval eggs were left in the bowl before removal). We also included in the model the body size of focal worms, the time spent in the experiment, the time since the last egg laying by the focal worms, the time since the partner's last egg laying and the interaction between the latter and the number of eggs in the partners' clutch as fixed

effects (pair ID and experimental replicate were entered as random effects). The time since the last egg laying by the focal worms was included as we could expect larger clutches after longer time interval, while the time since the last partner's laying and its interaction with the size of the last partner's clutch were included to test whether the quantitative matching was stronger when the two layings (one by the partner and the other by the focal worm) were closer in time. The two variables and the interaction were then dropped from the model since they were non-significant and the simplified model had a lower AIC (LMM: Time since the focal worm's last laying:  $\beta = -0.094 \pm 0.461$ ,  $F = 0.042$ ,  $n = 39$ ,  $p = 0.838$ ; Time since the partner's last laying:  $\beta = -0.384 \pm 0.540$ ,  $F = 0.505$ ,  $n = 39$ ,  $p = 0.478$ ; Interaction time since the partner's last laying\* size of the last partner's clutch:  $\beta = -0.048 \pm 0.045$ ,  $F = 1.102$ ,  $n = 39$ ,  $p = 0.295$ ).

To investigate how reciprocated and non-reciprocated clutches affected the fitness via the male and female function we used a GLMM for Poisson distribution (log link function). Here, the response variable was the cumulative number of eggs laid or fertilized by the focal individuals (since 90% of the eggs typically develop in free-swimming larvae [Sella 1991]) and we included the number of reciprocated, more-male and more-female clutches and the sexual function (male or female) through which fitness was achieved as fixed effects, as well as the interactions between sexual function and the number of reciprocated, more-male and more-female clutches. The time worms spent in the experiment was included as an exposure variable. For each worm we measured fitness via both the male *and* the female function; therefore, each worm was represented twice and we included the pair ID and experimental replicate as random effects; we also added an observation-level random effect to account for overdispersion.

The interaction between the number of reciprocated layings and the sexual function was not significant (GLMM:  $\beta = 0.038 \pm 0.028$ ,  $\chi^2 = 1.736$ ,  $n = 38$ ,  $p = 0.188$ ) and thus dropped.

Before running the model, we checked for outliers using Tukey's method (Tukey 1977), which uses an interquartile (IQR) approach. A pair of worms had a number of less-cooperative layings which ranged above  $1.5 \times \text{IQR}$  and was excluded from the analysis.

We performed two separate GLMMs (Poisson distribution) to test for the association between laying strategy and fitness via the female or male function. We included in the analysis the same variables as above, except for the sexual function and pair ID (each focal worm was represented once).

In all the analyses, we formulated models that addressed specific hypotheses and evaluated the model fit as well as the significance levels of the variables; non-significant



terms and interactions were dropped one by one, and we retained the models with the lowest AIC (Akaike Information Criterion). Results are reported for the reduced models.

Analyses were performed in R 3.4.4 (R Core Team 2018).

The graphs resulting from GLMMs were drawn with the R package *ggplot2* (Wickham 2009), using the estimate values of the intercept and the predictor variables of interest, while keeping the values of the other covariates constant (either as mean values or as zero) and averaging the effect of the random factors.

### **Agent-based simulation**

In the agent-based simulation, we programmed virtual worms to use the simple rule “lay when your eggs are mature”. The physiological rhythm of egg-maturation and release (that is, the time interval between two consecutive egg layings by the same individual) was computed using real data obtained from the 78 experimental worms (regardless of their strain). We built a logistic regression model (GLMM for binomial distribution) where the probability of laying eggs by worms depended on the time since their own last laying (we included pair ID as a random effect) and we obtained the logistic function linking the probability of egg laying to the time since the last laying, which we used to program virtual worms (GLMM: Intercept = -1.87; Slope = 0.15; n = 78). In this way, each virtual worm had a given probability to lay eggs which varied as a function of the time since its last laying. For example, at time  $t$ , worms which laid at time  $t-1$  had a probability of laying of 0.15  $[1/(1+\exp(-0.15*1-1.87))]$ ; the worms which did not lay at time  $t$ , had a probability of laying at time  $t + 1$  of 0.17  $[1/(1+\exp(-0.15*2-1.87))]$  since two days had passed since their last laying, while worms which laid at time  $t$  had again a probability of 0.15.

We established when the virtual worms laid their first clutch and how much time they spent in the virtual experiment using the empirical distribution of the two variables (i.e., the values were randomly chosen from the list of the real data, so that more common values were chosen more often).

We simulated 39,000 virtual dyads of worms and generated a dataset analogous to the real one, with 1,000 groups of 39 individuals each. On each group, we performed an Andersen-Gill proportional hazard model, where we tested whether the probability of laying eggs by focal virtual worms was affected by the time since the last partner’s egg laying, and we clustered by pair ID. We then compared the results of these models with those of the model

on real data, which included the same variables, and we computed the probability to get the same results from simulated data (i.e., a negative coefficient and a significant p value).

Finally, on one group of 39 individuals, randomly chosen among groups with non-significant results, we performed standard runs tests to test whether the virtual worms alternated sexual functions more often than expected by chance, and we combined the p values with the Fisher's methods (as we did on real dyads).

The agent-based simulation was performed with Python 3.6 and, as above, the data analyses with R 3.4.4 (R Core Team 2018).

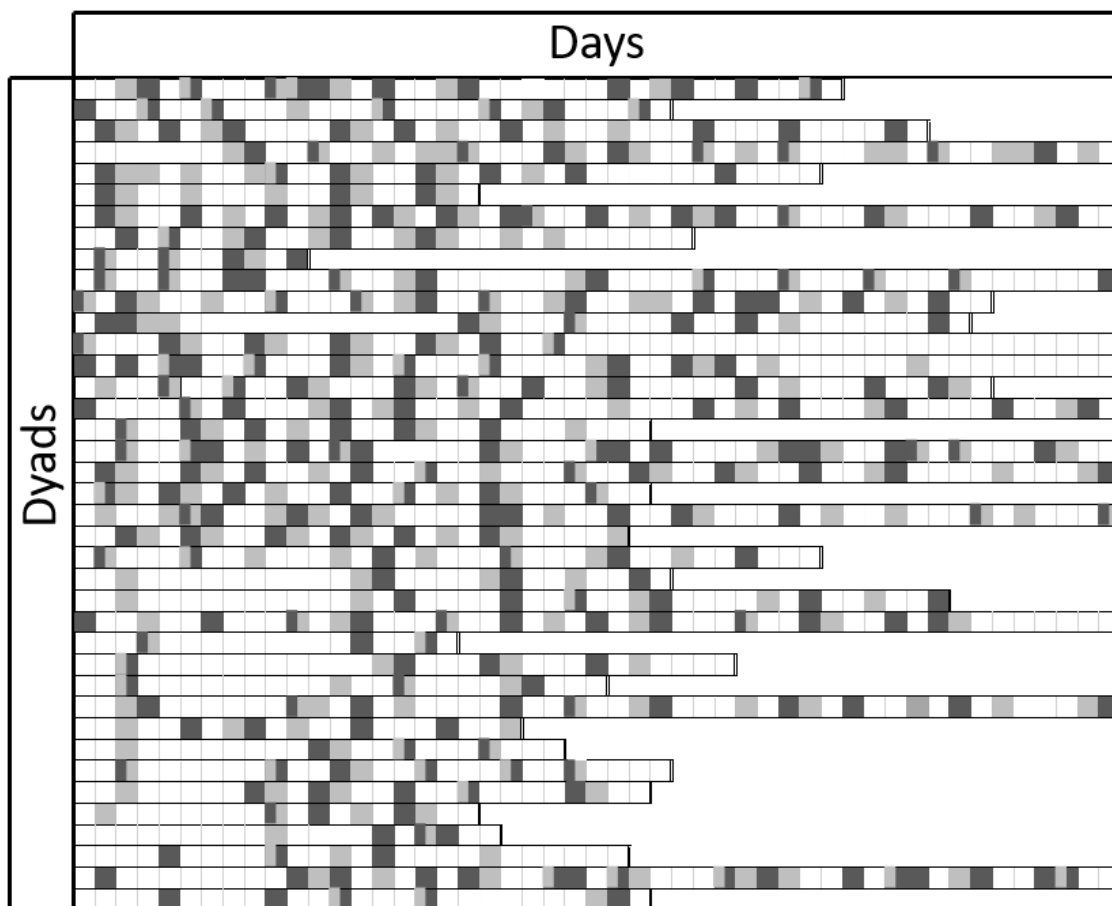
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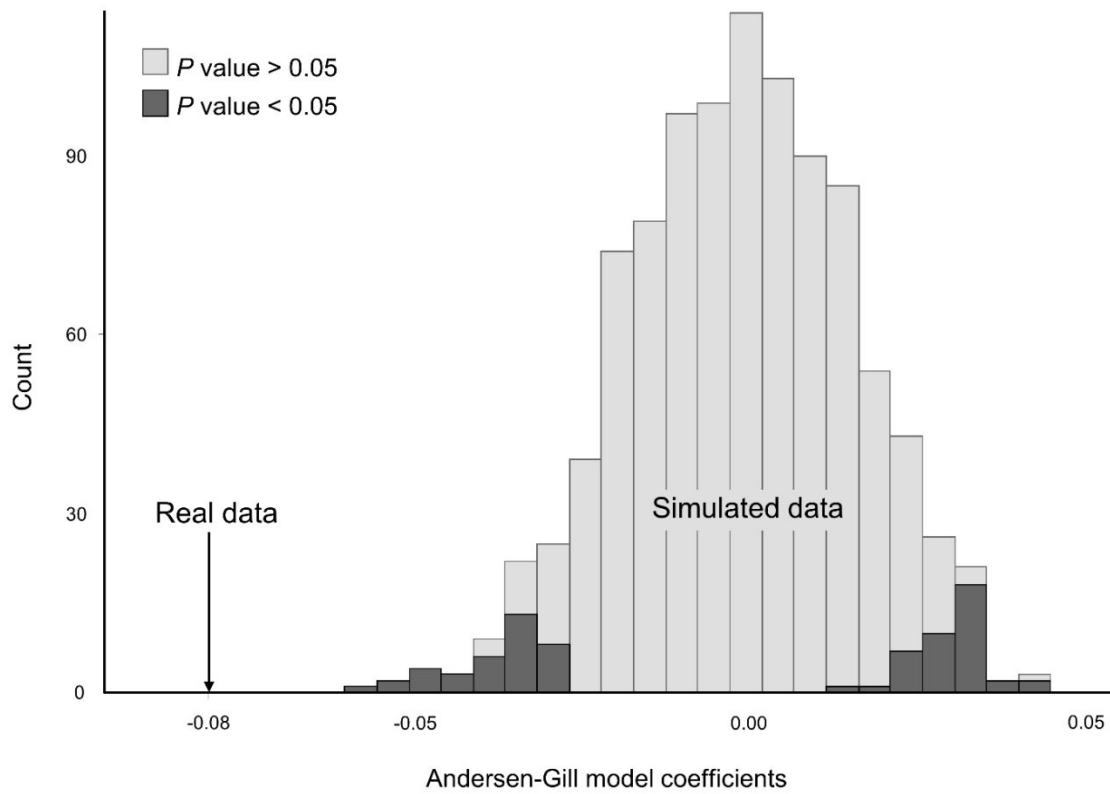
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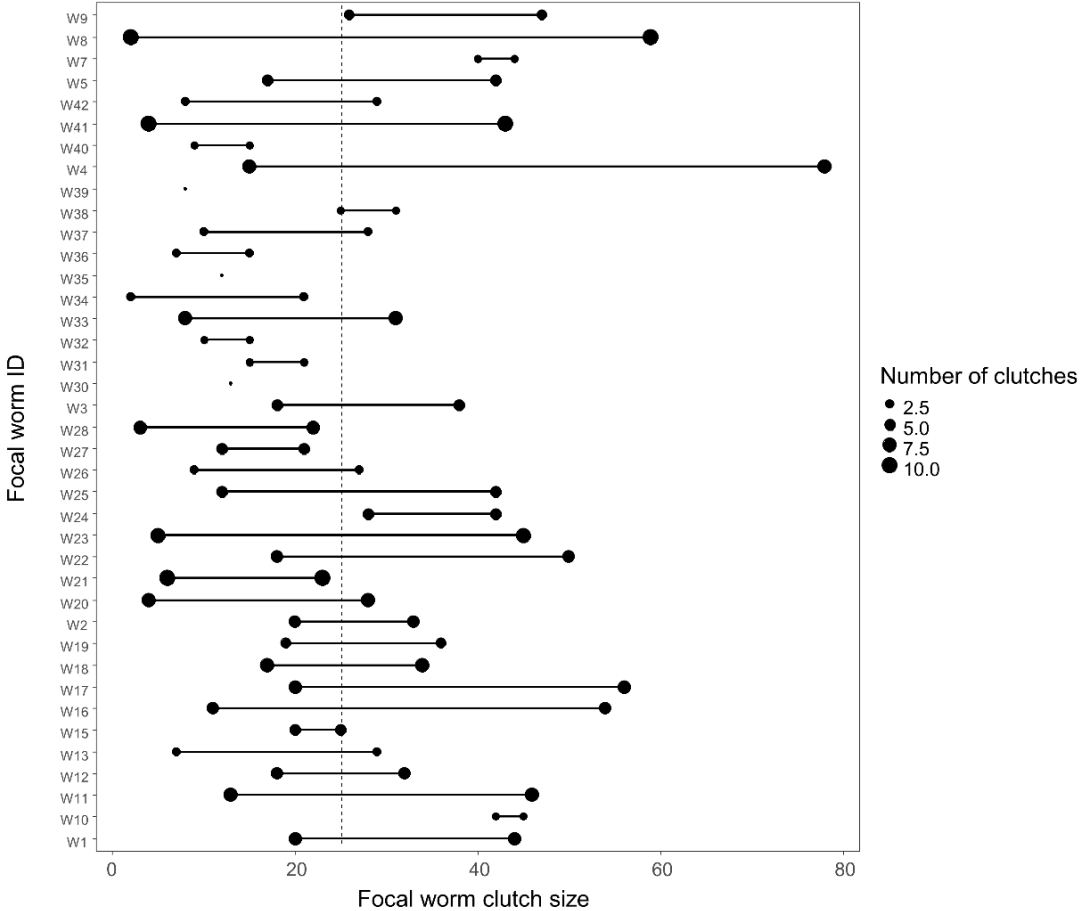
**Supplementary materials**

**Figure S5:** Graphic representation of the whole data set. Related to Figure 2. Each cell corresponds to a different day and each line to a different pair. Dark-grey cells represent egg layings by the focal worms, while light-grey cells represent egg layings by their partners. White cells without any frame represent missing data.

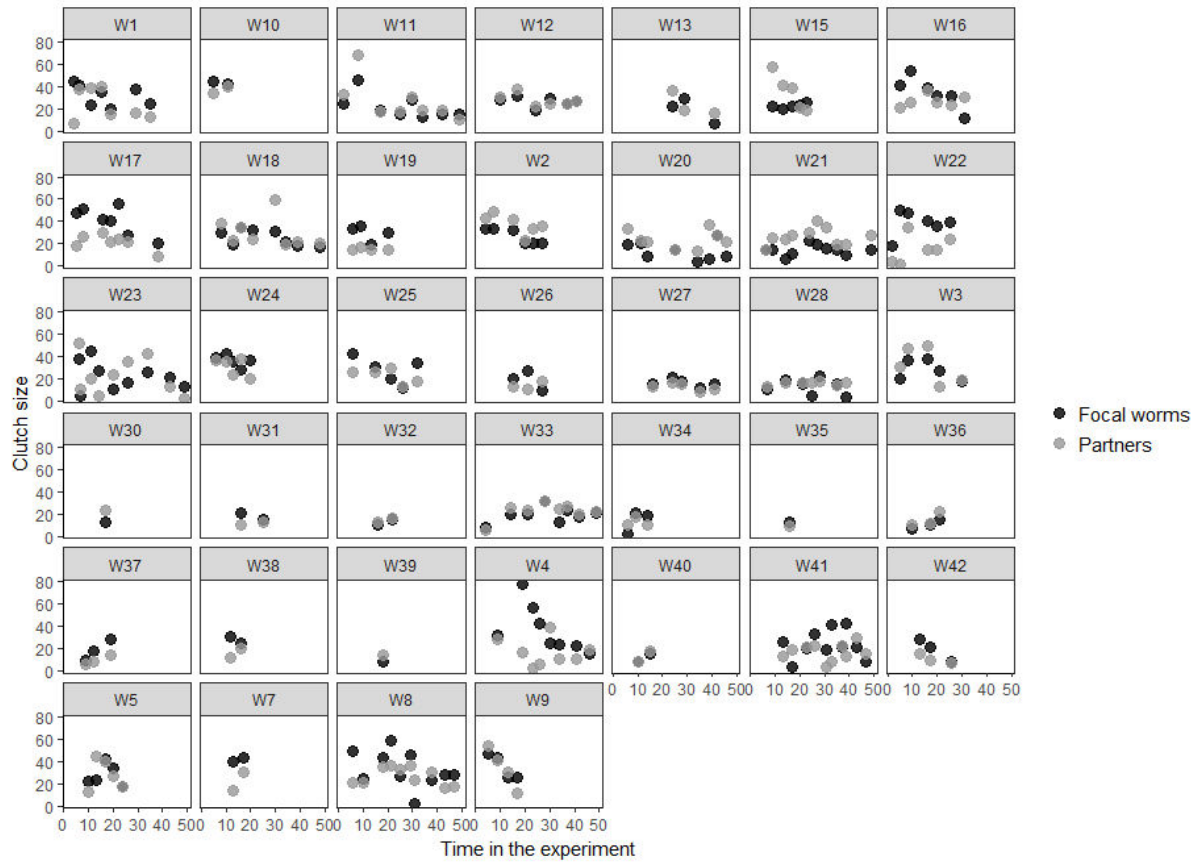


**Figure S6:** Results of the Andersen-Gill model on simulated and real data. Related to Figure 3A. Comparison between the results (coefficients) of the Andersen-Gill models on simulated and real data. Negative coefficients indicate that partners' egg layings increase focal worms' laying probability, while positive coefficients indicate that partners' egg layings decrease focal worms' laying probability.





**Figure S7:** Within subject variation in focal-worm clutch size. Related to Figure 3B. Dot size corresponds to the number of clutches laid by each focal worm. The vertical, dotted line corresponds to the average clutch size in the population.



**Figure S8:** Size of reciprocated clutches as a function of time by pair ID. Related to Figure 3B. Dark-grey dots represent the size of the reciprocated clutches by focal worms and the light-grey dots represent the size of the last clutch laid by the partner within a maximum time interval of 7 days.



# 4.

## **Dynamic modulation of reproductive strategies in a simultaneous hermaphrodite and preference for the male role**

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## Abstract

Reproductive traits are often tightly linked to variations in the social environment. In animals with separate sexes, producing male or female offspring results in different fitness gains depending on population structure. In simultaneous hermaphrodites, allocating a given proportion of resources to the two sexes results in fitness gains that depend on mating opportunities, and extremely plastic hermaphrodites may have a selective advantage. To test to what extent hermaphrodites have flexible and reversible sex allocation at the individual level, we exposed hermaphroditic *Ophryotrocha diadema* worms (an obligatorily nonselfing polychaete, with low numbers of aflagellate, immotile sperm and ‘pseudocopulation’) to weekly changes in actual or simulated mating opportunities and mate competition (controlling for density effects). We found that worms rapidly switched from high investment in the female function (eggs and courting) to high investment in the male function (aggressive behaviour) with increasing mating opportunities. Simulated mate competition and competition over the male role triggered similar variations in egg production (i.e. worms laid fewer eggs), indicating that these worms preferred to mate in the male role. These results highlight the key role of behaviour in the study of reproductive investment in hermaphrodites and the importance of plasticity in sex allocation (including precopulatory behaviours) in hermaphrodites as a way to compete for the male role and to keep hermaphroditism stable in fluctuating environments.

**Keywords:** mate competition, mating opportunity, *Ophryotrocha diadema*, sex allocation, trade-off

## Introduction

Reproduction is the biological process most straightforwardly linked to Darwinian fitness, and an organism's fitness therefore depends on it reproducing efficiently. To maximize its reproductive success, however, an organism needs to match its phenotype to the environment which changes constantly over space and time. For example, in several species of animals, the time of reproduction is strongly affected by fluctuations in temperature and photoperiod (e.g. birds, Dawson 2008; Visser et al. 2009; lizards, Carretero 2006). The ability to adjust phenotypic trait values (e.g. time of/age at reproduction) to environmental conditions is generally referred to as phenotypic plasticity (Pigliucci 2001) and is especially intriguing when animals respond to social cues (i.e. to the social environment, West-Eberhard 2003), which are crucial for reproduction. Indeed, in many species of animals, the social environment triggers changes in the phenotypic traits involved in mating, such as sperm production (Pizzari et al. 2003; Ramm and Stockle, 2009), morphology (Immler et al. 2010), body coloration (McGraw et al. 2003; Karubian et al. 2011) and mate choice (Bailey and Zuk 2009; Wong et al. 2011).

Animals are also able to adjust the amount of resources that are allocated to male and female offspring in response to the social environment, that is, they can adjust their sex allocation (Charnov 1982). According to Fisher's theory, organisms should invest equally in the two sexes (Fisher 1930); in animals with two sexes that invest equally in producing sons and daughters, this leads to an overall population sex ratio of 50:50. If, for some reason, the sex ratio is biased towards one sex, the reproductive value of the less represented sex will increase, and a frequency-dependent selection will favour individuals that overproduce the rarer sex, until the sex ratio stabilizes back to 50:50. However, Fisher's theory does not take into account the effect that cooperative or competitive interactions with relatives may have on the optimal sex ratio (West 2009). If, indeed, such interactions occur mainly with individuals of one sex, this may favour an increased or decreased investment in this sex, selecting for biased sex allocation (Hamilton 1967). Seychelles warblers, *Acrocephalus sechellensis*, for example, in which males disperse while females stay at the nest as helpers, adjust their offspring sex ratio according to the number of helpers at the nest (Komdeur et al. 1997). In *Nasonia vitripennis* parasitoid wasps, mothers adjust their offspring sex ratio in response to the presence of eggs laid by other females in the patch (i.e. the host; Shuker and West 2004). Males are wingless and short lived and mating generally takes place in the patch; if only one female lays in the patch, males will compete only with related individuals for access to the

female (local mate competition; Hamilton 1967) and it is advantageous from the mother's perspective to produce female-biased offspring. If, instead, several females lay in the patch, local mate competition will decrease (i.e. males will compete mostly with unrelated individuals) and less female-biased sex allocation will offer higher fitness returns (Hamilton 1967).

However, sex allocation decisions occur also in simultaneous hermaphrodites in how they allocate resources to the female and male functions. In these organisms, sex allocation is extremely plastic and has been proposed as one of the main advantages of hermaphroditism over separate sexes (Charnov 1982; Michiels 1998).

In simultaneous hermaphrodites (hereafter called hermaphrodites), a process similar to Hamilton's local mate competition, and recently named local sperm competition by Schärer (2009), may apply. When hermaphrodites mate in monogamous pairs or self-fertilize their eggs, the sperm released by one individual (i.e. the sperm donor) will compete only with the sperm produced by the same individual (competition among related sperm); thus, from the sperm donor's perspective it does not pay to invest in sperm production, since an increase in sperm number will not lead to an increase in reproductive success. Under such conditions, sex allocation is expected to be strongly biased towards the female function. If the mating group increases, so does competition among unrelated sperm (or, more generally, competition over the male role), and hermaphrodites will be advantaged if they shift resources from the female to the male function (Charnov 1982).

Several studies have tested how sex allocation is adjusted to the social environment (i.e. the social group size, or, following Charnov (1982), to the mating group size) and the majority found a decrease in female allocation and/or an increase in male allocation with increasing group size (reviewed in Schärer 2009). However, in some organisms, sex allocation was relatively fixed, as, for example, in the tapeworm *Schistocephalus solidus* (Schärer and Wedekind, 2001). Schärer and Wedekind (2001) suggested, as a possible explanation, that these worms lack the physiological time to shift resources between sexual functions during their short reproductive period (about 5 days), whereas they may adjust their sex allocation to the social conditions they experienced during development (i.e. population density in the intermediate host; Schärer and Wedekind, 2001). Moreover, in the flatworm *Macrostomum lignano*, which adjusts sex allocation in response to changes in group size (Schärer et al. 2005; Janicke et al. 2013), sex allocation seems to be weakly subject to sexual selection, since no differences were found between sex allocation traits in worms evolved under monogamy versus polygamy (Janicke et al. 2016); nevertheless, sex allocation was still

extremely plastic in both selection regimes after 20 generations (Janicke et al. 2016).

These studies highlight, from different perspectives, the importance of investigating costs and limits (or their absence) of sex allocation plasticity in simultaneous hermaphrodites. On the one hand, in *S. solidus* worms, the short reproductive period limits the advantages of adopting a flexible sex allocation during adulthood; on the other hand, however, the extremely flexible (and perhaps not too costly) sex allocation of *M. lignano* may limit the strength of sexual selection on this trait, greatly reducing the rate of evolution (Janicke et al. 2016).

To comprehend the distribution of sex allocation plasticity among hermaphrodites, and its link to evolutionary processes, we need to assess to what extent plasticity allows individuals to approach their fitness optimum. To this aim, investigating intraindividual variation in sex allocation in response to changes in the social environment may prove to be decisive for understanding whether sex allocation is a flexible trait (i.e. adjustments are reversible), and whether the adjustments allow a good match between phenotype and social environment.

To investigate this, we performed three longitudinal experiments on the hermaphroditic worm *Ophryotrocha diadema*. These worms have a relatively long reproductive period as fully mature hermaphrodites (up to 3 months, Di Bona et al. 2010), and can adjust their female allocation plastically to social group size (Lorenzi et al. 2005, 2006; Schleicherová et al. 2006, 2010). We repeatedly measured sex allocation in the same individuals as they were exposed to changing conditions, which reflected different (actual or simulated) levels of mating opportunities and/or mating competition. We aimed to test (1) to what extent (and speed) sex allocation can be adjusted in one direction (e.g. in favour of the female function), and then reversed (e.g. at the expense of the female function); and (2) what cues trigger the adjustments, that is, whether sex allocation adjustments are promoted by competition over the fertilization of the eggs or rather by competition over laying eggs (i.e. by competition over the male or the female role, respectively). We therefore measured how individual (focal) worms adjusted their sexual allocation when they were exposed to (1) actual changes in mating opportunities, (2) simulated changes in mating opportunities or (3) changes in the level of competition over the male role. An additional aim was to infer from these experiments which sexual role these simultaneous hermaphrodites preferred.



## Methods

### Study species

*Ophryotrocha diadema* (Dorvilleidae) is a marine, polychaete worm, a few millimetres long, living among fouling fauna. It is an obligate outcrossing, protandrous, simultaneous hermaphrodite with unilateral mating; worms need a partner to fertilize their eggs, and, during mating, each individual plays only one sexual role (either lays or fertilizes eggs), while the partner plays the opposite role (unilateral mating).

In this species, mating occurs after a long courtship (8+ h, Sella, 1985), during which partners are likely to take decisions on their reciprocal sexual roles (Lorenzi et al. in press). At the moment of laying, one partner releases its eggs and the other its sperm inside the jelly cocoon produced by the egg donor. Therefore, fertilization is external, but occurs through the release of immotile sperm (aflagellate sperm; Morrow 2004) inside the egg cocoon while partners are in physical contact (a mating behaviour that was called pseudocopulation in the congeneric species *Ophryotrocha gracilis*, Westheide 1984; Lorenzi et al. in press). A single egg cocoon can be fertilized by more than one worm, which suggests that hermaphrodites compete to fertilize the eggs (Lorenzi et al. 2014).

Before reaching full sexual maturity, worms have a 40-day protandrous phase which begins soon after they leave the egg cocoon as larvae (four to five segments); during the protandrous phase, worms produce sperm, but not eggs, and successfully compete with mature worms at fertilizing eggs (Sella 1990; Sella and Lorenzi 2003). At a body size of 13-14 segments, worms reach full sexual maturation and begin to produce both eggs and sperm. Mature hermaphrodites produce on average 25 eggs per bout as often as every 3 days, so that they are ready to play the female role intermittently every 3+ days. In contrast, sperm are continuously produced and available (Sella 1990), although released in low numbers (approximately 50 sperm per egg; Sella 1990; Premoli and Sella 1995). Because sperm are smaller than eggs (3 versus 180  $\mu\text{m}$  diameter, respectively, Sella 1990), they presumably consume fewer resources, at least in terms of volume of resources allocated (Sella and Ramella 1999).

The mating system of these worms is special in many respects. In isolated pairs, partners reciprocate egg clutches (Sella 1985; Picchi et al. in press) and alternate sexual roles over several weeks, taking turns at laying and fertilizing their partner's cocoons. When mating opportunities increase, as occurs when they are kept in groups of more than two individuals,

the worms no longer alternate their sexual roles; they diminish their egg production, in favour of a putative larger allocation to the male function (Lorenzi et al. 2005, 2006).

We used an albino and a wild strain to identify the worms: albino worms lay white eggs, which are also visible through the transparent body wall before they are released, whereas wild-type worms produce yellow eggs. Hereafter, we refer to the worms as ‘white-phenotype’ (albino strain) and ‘yellow-phenotype’ (wild-type strain). Yellow-phenotype worms were used as ‘focal’ worms in the experiments, and white-phenotype worms were used as ‘partners’.

### **Rearing worms**

For each experiment, we produced a new generation by breeding pairs of worms and using their newly sexually mature, same-age virgin offspring, at 13-15 segments long. At the beginning of an experiment, worms had visible mature eggs in their coelom and were ready to mate in both sexual functions (unless otherwise stated). In all experiments, nonsibling worms were paired or grouped and kept in separate bowls, each including a ‘focal’ worm. Focal worms were exposed to weekly changes in mating conditions for 3 or 4 weeks and data collection began after 1 acclimation week. Previous experiments have shown no significant effects of population density (Lorenzi et al. 2005, 2006) except in extremely restricted water volumes (Cannarsa and Meconcelli 2017). However, where reasonable, we also took population density into account by using bowls of different sizes.

Worms were kept according to Åkesson (1976) in artificial sea water, in thermostatic cabinets at 21 °C, and fed with spinach ad libitum. Sea water in the bowls was changed weekly.

### **Experimental design**

#### *Experiment 1: alternating mating opportunities*

In this experiment, we aimed to test whether focal worms were able to switch their sex allocation by rapidly tracking mating opportunities, which were alternated, every week, for 4 weeks. We predicted that worms would change repeatedly from a female-biased to a relatively more male-biased sex allocation (and vice versa), depending on the presence of single or multiple partners/rivals, that is, depending on mating opportunities.

We set up 36 pairs, each composed of a yellow-phenotype (focal) worm and a white-phenotype worm. Each pair was kept either in a large (80 ml, low population density,  $N = 18$  pairs) or in a small (20 ml, high population density,  $N = 18$  pairs) bowl, to control for putative population density effects. The first week, each bowl contained the focal worm and the partner; worms built their mucous trails, met their partners and started to reproduce (week 0, acclimation period). Then, we introduced three more white-phenotype worms in each bowl (i.e. high mating opportunities: the focal worm and four partners/rivals) and began to collect data (week 1). After another week, we removed three worms from each bowl (randomly chosen among the four white-phenotype worms), so that focal worms again had only one partner (i.e. low mating opportunity, week 2). In the next 2 weeks, we switched again to four (week 3) and one (week 4) mating opportunities as above.

*Experiment 2: simulated changes in mating opportunities*

‘Conditioned water’ from mass cultures of these worms contains chemical cues conveying information about the number of worms (i.e. the quantity of potential sexual partners and/or rivals), as inferred by the fact that the worms adjust their female allocation accordingly (Schleicherová et al. 2006, 2010). These chemical cues are likely to be species specific, because conditioned water from a different hermaphroditic species (same genus) does not trigger such adjustments (Schleicherová et al. 2006). Therefore, we exposed focal worms to conditioned water, where either conspecific worms or worms of a different species (*Ophryotrocha adherens*) had been kept. In this way, we checked for sex allocation adjustments in response to simulated variations in mating opportunities, while accounting for the effects associated with population density (such as oxygen consumption, pH changes, metabolite concentration and/or any stress effect due to overcrowding). We predicted that conditioned water from both conspecific and heterospecific worms would cause density-related effects, but only that from conspecific worms would elicit changes in allocation to the female role.

We set up 60 pairs, each composed of a focal worm and a partner housed in small (20 ml) bowls. Each week, for 4 weeks (1 acclimation week + 3 weeks of data collection), each pair was exposed alternately to conditioned water collected from mass cultures of either the same (*O. diadema*) or another (*O. adherens*) hermaphroditic species.

*Experiment 3: competition over the male role*

Ideally, we would have tested whether sex allocation adjustments resulted from competition over the male or the female role by exposing hermaphroditic focal worms to ‘rivals’ ready to play only one sexual function. However, competition over the female role is difficult to test empirically (these worms are rarely sperm depleted). We were, however, able to test whether female allocation adjustments resulted from competition over the male role (i.e. competition for fertilizing eggs), because adolescent worms produce sperm but not eggs.

We predicted that, if hermaphrodites competed for the male role, they would diminish their allocation to the female function (and produce fewer eggs) to face increased competition over the fertilization of the partner's eggs. In contrast, if hermaphrodites competed for the female role, they would increase their allocation to the female function (and produce more eggs) when multiple potential sperm donors were present.

Therefore, we tested the focal worms’ responses by exposing them to weekly changes in competition over the male role, by adding/removing adolescent males, 9-11 segments long, to/from a pair of mature hermaphrodites.

We set up 60 pairs of sexually mature hermaphrodites, each composed of the focal worm and a partner, housed in either large (80 ml,  $N = 30$ ) or small (20 ml,  $N = 30$ ) bowls to control for population density effects. We then added four adolescent males to 30 of the 60 pairs (15 pairs in small bowls and 15 in large bowls, experimental group 1) immediately and to the other 30 bowls a week later (experimental group 2), to get two experimental groups temporally out of phase and control for a putative synchronisation in egg output. Therefore, during week 0, half of the bowls contained the focal worm and the sexually mature partner (i.e. there was no competition over the male role), and another 30 bowls contained the focal worm, its partner and four adolescent males (i.e. the focal worms were expected to compete with adolescent males for egg fertilization if there was a preference for the male role). In the subsequent 4 weeks, we repeatedly changed the level of competition by adding/removing four adolescent males to/from the bowls once per week, so that the presence/absence of adolescent males was constantly reversed between experimental groups (e.g. during week 1, we removed the adolescent males from the pairs belonging to experimental group 1, and we added them to the pairs of experimental group 2). Adolescent males were white-phenotype worms obtained by rearing egg cocoons laid about 12 days before they entered the experiment (they were used only once).

## **Experimental measures of sex allocation**

### *Female allocation*

Twice a week for 3 or 4 weeks (six-eight censuses, depending on the experiment), we counted the cocoons laid by the focal worms and by their partners as well as the eggs inside each cocoon; focal worms were yellow-phenotype and therefore laid yellow eggs, which allowed us to distinguish between their cocoons and those laid by their partners (white eggs). After counting, cocoons and eggs were removed from the bowls to avoid changes in the density level.

In data analysis, we used the number of (yellow) eggs laid by (yellow-phenotype) focal worms as a measure of individual female allocation. We did not measure egg size. Indeed, although variable, egg size has a limited range of variation (especially with respect to the number of eggs per cocoon, which varies between five and more than 80, Picchi et al. in press) and we do not foresee any biological reason for a trade-off between egg size and egg number in relation to mating opportunities. Moreover, egg survival rates do not differ as a function of mating opportunities, further suggesting that the investment in quantity and quality of nutrients in each egg does not depend on mating opportunities (in contrast, the amount of parental care on the cocoons varies with mating opportunities and affects egg survival, Picchi and Lorenzi unpublished data).

### *Male allocation*

We measured male allocation by focusing on behavioural interactions between worms. Previous research on the same model species (Lorenzi et al. 2005, 2006) has suggested that mate acquisition through behavioural competition may be relatively more important than postmating competition between sperm (particularly because sperm are immotile, Morrow 2004). Additionally, counting sperm requires the worm to be squeezed, which is an undesirable manipulation in long-term experiments.

In experiment 1, we performed 15 min behavioural observations on 36 focal worms, once at one and once at high mating opportunities (both population density treatments). For logistic reasons, 18 worms were observed in weeks 1 and 2 and 18 in weeks 3 and 4. We recorded whether the focal worms initiated (or received) the following behaviours: Following, Being Followed, Contact, Received Contact, Attacking, Received Attack, Rubbing and Withdrawing (see Table 1 for the descriptions of the behaviours). All behaviours were recorded as frequency.

**Table 1:** Descriptive statistics on the observed behaviours by mating opportunities

Behaviours	Mean frequency $\pm$ s.e. [% of total behavioural interactions]	
	High mating opportunities	Low mating opportunities
Following	7.389 $\pm$ 1.477 [12.3%]	7.694 $\pm$ 1.155 [17.3%]
Being followed	15.861 $\pm$ 2.638 [26.3%]	10.472 $\pm$ 1.863 [23.5%]
Contact	10.694 $\pm$ 1.608 [17.7%]	9.639 $\pm$ 1.147 [21.6%]
Received Contact	18.500 $\pm$ 2.780 [30.7%]	12.639 $\pm$ 1.854 [28.4%]
<b>Attacking</b>	<b>1.528 <math>\pm</math> 0.435</b> <b>[2.5%]</b>	<b>0.00 <math>\pm</math> 0.00</b> <b>[0.0%]</b>
<b>Received Attack</b>	<b>1.028 <math>\pm</math> 0.348</b> <b>[1.7%]</b>	<b>0.00 <math>\pm</math> 0.00</b> <b>[0.0%]</b>
<b>Rubbing</b>	<b>1.417 <math>\pm</math> 0.280</b> <b>[2.4%]</b>	<b>3.083 <math>\pm</math> 0.334</b> <b>[6.9%]</b>
<b>Withdrawing</b>	<b>3.833 <math>\pm</math> 0.643</b> <b>[6.4%]</b>	<b>1.028 <math>\pm</math> 0.220</b> <b>[2.3%]</b>

Following: while walking rapidly on the substrate, one worm follows the other at a close distance (less than a worm body length);

Contact: usually brief (< 2 s), the focal worm touches the other worm's body with its head, tail or body side);

Attacking: the focal worm bites another;

Rubbing: first described by Sella (1985) as the typical behavior during courtship, it occurs when two worms slide along each other's bodies, often in an antiparallel direction, then often one makes a U-turn and they repeat the sliding. This behaviour is reciprocal, and was scored as an active behaviour;

Withdrawing: a rapid backward movement, where the focal worm subtracts itself from physical interactions with the opponent; it was classified as passive.

In bold the behaviours summarised by the PCs that varied with mating opportunities.

### **Statistical analyses**

In the three experiments, we tested whether female allocation (number of eggs produced by focal worms at each census) was associated with mating opportunities (two levels: high versus low). As data on female allocation were count data and included zeroes, we used generalized linear mixed models (GLMM) for Poisson-distributed data (log link function) and included an observation level random factor rendering the residuals log-normal Poisson distributed, after checking for overdispersion. Time (continuous variable: census 1-8) was entered as a covariate, to account for the effect of time in the experiment. To control for non-independence of data (female allocation was measured six or eight times during 3 or 4 weeks on every focal worm), we entered focal worm identity as a random factor. Our model selection procedure involved formulating models that addressed specific hypotheses and evaluating the model fit as well as significance levels of the variables in the model (non-significant terms were removed, and the simplified model was rerun).

In experiments 1 and 3 we also tested for density effects and thus we included enclosure size (two levels: large versus small) in the model. In experiments 2 and 3, fully sexually mature worms were always in pairs, and we know that paired worms alternate sexual roles and engage in egg exchange, conditional upon receiving eggs from the partner, which makes worms more likely to lay soon after the partner (Sella 1985; Picchi et al. in press). For this reason, we included in the model whether the focal worm's partner had laid eggs in the 2 days before (yes/no) to account for the 'facilitating' effect of reciprocal laying.

Finally, in experiment 1, we tested for an association between mating opportunities and behaviour. We performed two principal component analyses (PCA) on active or passive behaviours. Both PCAs (correlation matrix, varimax rotation) produced two principal components (PCs) with eigenvalue >1. We then tested whether there was a significant association between the PCs and mating opportunities (two levels: high or low), population density (two levels: large or small), sexual role (see below), presence/absence of partners ready to mate in both sexual roles and time (continuous variable, four observation sessions) using linear mixed models (LMM) with focal worm identity as a random factor. The values of the PCs were  $\log_{10}$  transformed to fulfil normality assumptions (after adding a constant value to avoid negative values ( $\log(\text{PC} + 2.5)$ )), except for the PC on attacking and courting, whose values were normally distributed.

We classified the focal worm as (1) ‘ready to mate in the male role’ when it had laid eggs during the 48 h before the behavioural observations, which would imply the worm had sperm and was ready to play the male role, but was relatively egg depleted (see above) or (2) ‘ready to mate in both sexual roles’ when it had not laid eggs during the 48 h before, an indication the worm had mature eggs (besides sperm).

In all our models, we ran preliminary models with all main effects and biologically meaningful interactions and reran models after removing non-significant interactions. Statistical analyses were performed in R version 3.4.1 (R Core Team 2016), except for PCA, which was run in IBM SPSS Statistics 23.0 (IBM Corp., Armonk, NY, U.S.A.). We applied GLMMs based on restricted maximum likelihood estimates using the package lme4 (Bates et al. 2015).

### **Ethical note**

The species used in this study are not protected. Worms originated from laboratory cultures and were kept under optimal rearing conditions (temperature, food availability, population density, Åkesson 1976). The worms were manipulated only to move them in and out of the experimental bowls (no more than once per week), and the measurements of sex allocation traits did not involve any adverse impacts on their welfare. After the study, the worms were returned to the laboratory populations.

## **Results**

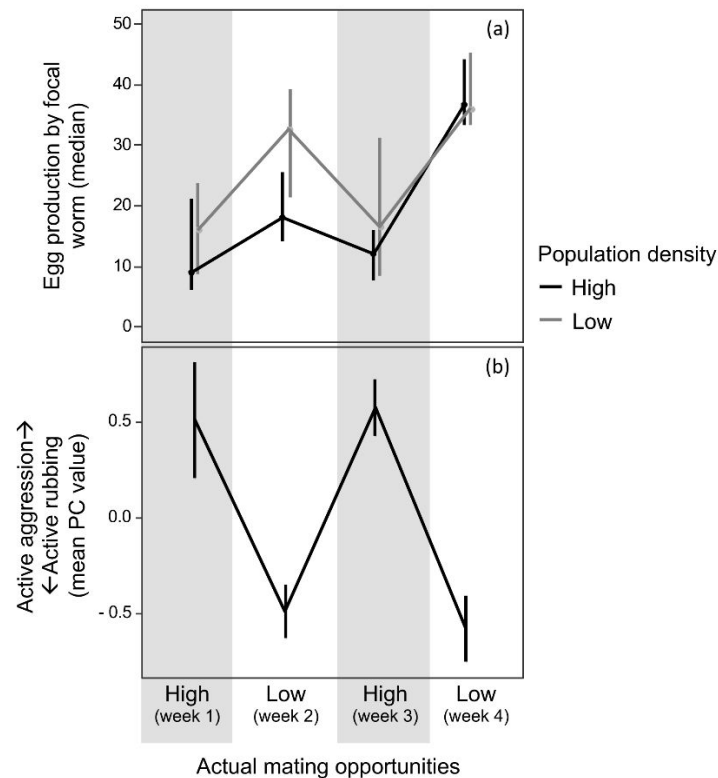
### **Experiment 1: alternating mating opportunities**

#### *Female allocation*

In the 4 weeks, the 36 focal worms laid a total of 223 cocoons and 3401 eggs (that is, on average, 1.6 cocoons per worm per week). However, focal worms significantly varied their egg production with changes in mating opportunities (GLMM, mating opportunities [low]:  $\beta = 1.038 \pm 0.215$ ,  $\chi^2 = 23.317$ ,  $N = 36$ ,  $P < 0.001$ ), whereas population density had a marginal effect, if any (population density [low]:  $\beta = 0.342 \pm 0.190$ ,  $\chi^2 = 3.251$ ,  $N = 36$ ,  $P = 0.071$ ); focal worms produced significantly more eggs when exposed to low mating opportunity, and significantly decreased their egg production when mating opportunities were large (Figure



1a). Focal worms also increased their egg production over time (time in the experiment:  $\beta = 0.156 \pm 0.047$ ,  $\chi^2 = 11.253$ ,  $N = 36$ ,  $P < 0.001$ ).



**Figure 1:** Phenotypic plasticity in female and male allocation in focal worms exposed to weekly changes in mating opportunities. (a) Variation in egg production (median and interquartile ranges); (b) variation in the values of the principal component summarizing aggression and rubbing (i.e. courting) as actively performed by focal worms (mean  $\pm$  SE). Grey areas: high mating opportunities (i.e. focal worm + four partners/rivals); white areas: low mating opportunity (i.e. focal worm + one partner).

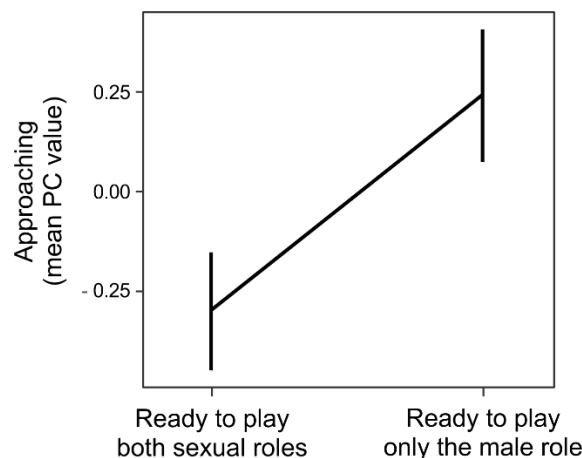
### Male allocation

Focal worms also changed their behaviour depending on mating opportunities; while paired worms often followed each other and exhibited Contacts and Rubbing, in larger groups they also performed Attacks and subsequent Withdrawal.

Indeed, the active behaviours of the focal worms were effectively summarized by a PCA which produced two PCs (PC1 capturing 48.6% of the variance; PC2: 27.5%). Contacts and Following both loaded (factors loadings  $> 0.980$ ) on PC1, suggesting that the underlying nature of PC1 could be summarized as ‘Approaching’ where increasing PC1 values meant that focal worms approached other hermaphrodites more often. Interestingly, PC2 correlated positively with Attacking (biting, factors loadings = 0.749) and negatively with Rubbing (i.e. courting, loading = - 0.735), highlighting a negative correlation between these behaviours and suggesting that PC2 could be labelled as ‘Attacking or Courting’ (larger, positive values

meaning that focal worms exhibited more aggression, while smaller and negative values indicate more courtship).

The variable ‘Attacking or Courting’ varied with mating opportunities (LMM on ‘Attacking and courting’ PC, mating opportunities [one]:  $\beta = -1.075 \pm 0.197$ ,  $F_{1, 44.013} = 29.904$ ,  $N = 27$ ,  $P < 0.001$ ), irrespective of the focal worm’s sexual role: all worms attacked other worms more often (and courted them less often) under high mating opportunities and courted other worms more often (and attacked them less often) under low mating opportunities (Figure 1b). Instead, focal worms that were ready to mate in the male role only were more likely to Follow and Contact (i.e. Approach) other hermaphrodites (LMM on ‘Approaching’ PC, sexual role [male only]:  $\beta = 0.086 \pm 0.035$ ,  $F_{1,66.691} = 5.995$ ,  $N = 27$ ,  $P = 0.017$ ), irrespective of mating opportunities, suggesting that these risk-taking behaviours (potentially linked to mate searching) may be associated with the male function (Figure 2).



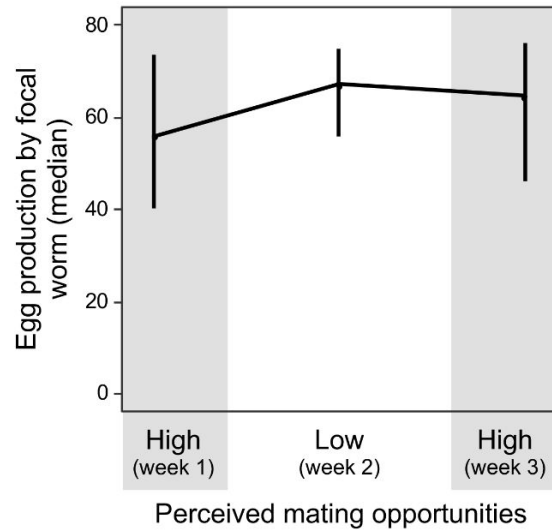
**Figure 2:** Behavioural plasticity in ‘Approaching’ behaviours as a function of the sexual role, and represented as the variation in the values of the principal component summarizing contacts and following actively performed by focal worms (mean  $\pm$  SE). Worms ready to play both sexual roles had laid no eggs in the 48 h preceding the observations, whereas worms ready to play only the male role had, and thus had no available eggs.

Focal worms were also the target of behaviours of other worms. A second PCA on the passive behaviours resulted in another two PCs, which together accounted for 88.7% of the total variance (PC1: 52.3%; PC2: 36.4%). PC1 mainly summarized ‘Received Contacts’ and ‘Being followed’ (both factors loadings  $> 0.976$ ), which suggested that PC1 could be labelled as ‘Being approached’. PC2 correlated with receiving Attacks and Withdrawing (factor loadings = 0.922 and = 0.759, respectively), and was thus labelled ‘Receiving aggression and withdrawing’. Focal worms were the target of attacks by other worms and withdrew

significantly more often when mating opportunities were high than when they were in pairs and mainly involved in courting their partners (LMM on ‘Receiving aggression and withdrawing’, mating opportunities [low]:  $\beta = 0.116 \pm 0.027$ ,  $F_{1,69} = 18.654$ ,  $N = 27$ ,  $P < 0.001$ ). The switch between aggression and courtship occurred irrespective of the focal worm’s sexual role, but with a significant effect of population density (LMM on ‘Receiving aggression and withdrawing’, population density [low]:  $\beta = -0.064 \pm 0.031$ ,  $F_{1,69} = 4.259$ ,  $N = 27$ ,  $P = 0.043$ ): all worms received aggression and withdrew more often under high than low mating opportunities as well as under high population density. In contrast, focal worms were the targets of approaches by other worms irrespective of their sexual role, current mating opportunities, population density or presence of partners with mature eggs.

### **Experiment 2: simulated changes in mating opportunities**

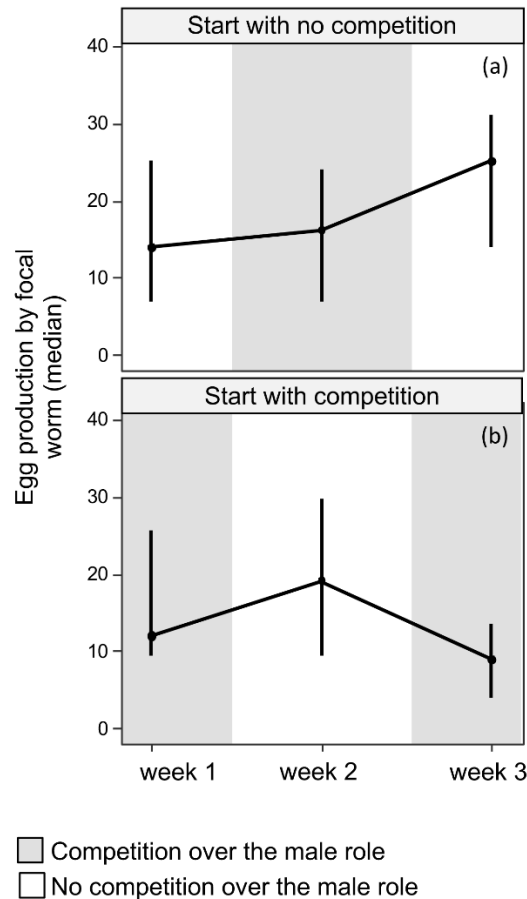
Focal worms exposed to water (and waterborne chemicals) from groups of worms of their own or another species (and thus perceived mating opportunities as high or low, respectively) adjusted their sex allocation as expected depending on perceived mating opportunities (GLMM, mating opportunities [low]:  $\beta = 0.244 \pm 0.123$ ,  $\chi^2 = 3.918$ ,  $N = 50$ ,  $P = 0.048$ ): they decreased their female allocation when exposed to conditioned water from their own species and increased it when exposed to conditioned water from another species (Figure 3). However, they also increased egg production over time (GLMM, time in the experiment:  $\beta = 0.070 \pm 0.034$ ,  $\chi^2 = 4.193$ ,  $N = 50$ ,  $P = 0.041$ ), which partially masked the decrease in egg production during week 3, when worms were exposed to conspecific-conditioned water and therefore perceived mating opportunities as high (Figure 3). Furthermore, the focal worm’s egg production was positively affected by the partner’s laying activity (GLMM, partner lays [yes]:  $\beta = 0.826 \pm 0.213$ ,  $\chi^2 = 15.102$ ,  $N = 50$ ,  $P < 0.001$ ), as expected under a reciprocation paradigm.



**Figure 3:** Plasticity in egg production (median and interquartile ranges) in focal worms with weekly changes in perceived mating opportunities. Grey areas: simulated high mating opportunities, i.e. conditioned water from groups of conspecific worms; white areas: low mating opportunity, i.e. conditioned water from groups of heterospecific worms.

### **Experiment 3: competition over the male role**

Focal worms significantly varied their female allocation depending on the level of competition over the male role (GLMM, mating opportunities [low]:  $\beta = 0.513 \pm 0.194$ ,  $\chi^2 = 6.974$ ,  $N = 56$ ,  $P = 0.008$ ), irrespective of population density and experimental group, but with a significant, positive effect of whether the partner laid eggs (GLMM, partner lays [yes]:  $\beta = 0.987 \pm 0.216$ ,  $\chi^2 = 20.916$ ,  $N = 56$ ,  $P < 0.001$ ), as happens when worms reciprocate egg cocoons (Figure 4). Thus, focal worms produced more eggs when there was no competition over the male role (i.e. when adolescent males were not present) than when adolescent males were present.



**Figure 4:** Plasticity in egg production (median and interquartile ranges) in focal worms with weekly changes in competition over the male role. (a) Experimental group starting with no competition; (b) experimental group starting with competition. Competition over the male role: focal worm + one partner + three adolescent males. No competition over the male role: focal worm + one partner.

## Discussion

### Plasticity in female allocation

This longitudinal study shows that the obligatorily outcrossing, simultaneously hermaphroditic *O. diadema* worm shows extreme plasticity in its allocation of reproductive resources. Individual worms were able to change their female allocation as often as once per week, tracking changes in mating opportunities and reversing their investment in sexual resources as often as four times a month. These changes were consistent across experiments, even if the overall egg output varied. Worms in pairs, with low mating opportunities, produced almost three times as many eggs as when four potential sexual partners and/or rivals

where there (experiment 1), a condition that we defined as high mating opportunities (following Dorken and Pannell 2009).

These findings partly confirmed the theoretical prediction by Charnov (1979, 1982) that hermaphrodites have a plastic sex allocation, and raised two questions. First, where did the worms allocate (i.e. trade off, Stearns 1989) the resources spared from the female function when there were high mating opportunities (roughly equivalent to two-thirds of the weekly egg production of a worm)? Second, does mate competition in hermaphrodites imply they compete to lay or fertilize eggs, that is, do hermaphrodites have a preferred sexual role (Schärer et al. 2015)? The two questions may be intrinsically linked by ‘tracking’ which life history traits increase at the same time as egg production diminishes (and vice versa). Measuring behaviours in experiment 1 allowed us to make such a link.

### **Plasticity in male allocation**

Resources spared from the female function when multiple, sexually mature hermaphrodites were present were redirected to behavioural traits such as attacks (which included both initiating attacks and responding with aggression to attacks initiated by other worms). Indeed, aggressive behaviour was not particularly associated with any given physiological status; it was performed by any worm, irrespective of the sexual role it was ready to play, whenever it had high mating opportunities. These worms are rarely sperm depleted, which means that they are virtually always ready to fertilize eggs (Sella 1990), whereas eggs are not always available, as they mature a clutch of eggs, then release it, then begin to mature another, and so on, approximately every 4 days (Sella 1985; Picchi et al. in press). Attacks and defence were common to all individuals, irrespective of whether they had or had not ready-to-lay eggs, but all were ready to fertilize eggs; this strongly suggests that these behaviours were mainly associated with the male function.

A rise in aggressiveness (which may include mate competition and/or mate guarding) with an increase in mating group size has been reported both in hermaphrodites (Wong and Michiels 2011) and in males of several species with separate sexes (Bateman et al. 2001; Candolin and Reynolds 2002). In particular, in the grass goby, *Zostera sp.* reproductive success of territorial males is affected by body size (large body size is likely to increase the efficiency of mate guarding) rather than by testis size (Scaggiante et al. 2005) (which does not correlate with body size, Pujolar et al. 2012). Indeed, it has been shown that precopulatory

aggression towards rivals can pay more in term of fitness than increasing sperm production, provided mate guarding is efficient (Alonzo and Warner 2000).

By documenting a trade-off between egg production and aggressive behaviour, the results of experiment 1 provide a possible explanation for why no clear-cut change in sperm production in response to increased mate competition has been documented in this species. In previous experiments, worms were isolated for 3 days before sperm were counted (Lorenzi et al. 2005, 2006). However, here we found that 7 days were enough for worms to change the number of eggs produced and laid, so that we may hypothesize that a period of 3 days of isolation (no mating opportunities) might mask changes in sperm production, promoting a reinvestment of the resources in the female function.

The relative higher efficiency of aggression compared to gamete production in acquiring mates may be particularly straightforward in *O. diadema* worms, owing to their peculiar mating behaviour: fertilization is external, it occurs inside the mucous egg cocoon by means of immotile sperm, and takes place immediately after egg release when the two partners are in tight physical contact (Westheide 1984; Lorenzi et al. in press). This mating behaviour, called pseudocopulation, is likely to make postcopulatory sperm competition weak compared to precopulatory competition, thus favouring selection on traits (such as aggressive behaviour) that allow the worms to compete with rivals, and to try to monopolize the appropriate physical position to mate, and relaxing selection on traits that increase the number of sperm. As argued by Schärer and Pen (2013, p. 3) ‘potential male-driven, precopulatory components aimed at either obtaining more mates or gaining exclusive access to mates’ need to be taken into account as the beneficiary of resources moves away from the female function.

More generally, aggressive behaviour is associated with the sex whose reproductive success is limited by access to partners; indeed, in species where the male sex is the limiting sex, females are often larger and more aggressive than males, as occurs for example in wattled jacanas, *Jacana jacana* (Emlen and Wrege 2004).

### **Competition for the male role**

Our results provide further evidence that, in this species, hermaphrodites compete for the male, rather than the female, role. Indeed, hermaphrodites without ready-to-lay eggs consistently exhibited a special behavioural trait that consisted of contacting and following other worms, often approaching them. This suggests that worms ready to play only the male role, possibly more mobile than those whose body was swollen with mature eggs, were more

risk prone (contacting other worms often resulted in being attacked): they might be more active in mate searching and/or in keeping rivals at a distance.

Furthermore, experiment 3 confirmed the interpretation that worms reduced their allocation to the female role when they competed for mating in the male role: when in the presence of adolescent worms, which were ready to mate as males but unable to produce eggs (Sella 1990), adult hermaphrodites decreased their egg production, possibly to increase resources available for competing with the adolescent males, that is, by monopolizing access to partners in the female role and/or keeping rivals at a distance. Indeed, even though adolescent males are usually ignored as partners and sexually mature hermaphrodites are preferred (Sella 1988), it has been shown that adolescent males compete successfully with mature hermaphrodites for egg fertilization (Sella and Lorenzi 2003).

Instead, if the female role had been preferred, we would have expected these worms to invest in egg production, and got the eggs fertilized either by the other adult hermaphrodite and/or by one of the four adolescent males. This conclusion adds to previous indirect evidence that the male role is preferred in these hermaphrodites: they lay less frequently when paired with adolescent males than with mature hermaphrodites (Sella 1988), abandon their reciprocating partners for worms with riper eggs (Sella and Lorenzi 2000), mate repeatedly in the male role only (Di Bona et al. 2010) and reduce investment in the female function and compete for mating in the male role (Lorenzi et al. 2005, 2006; this study).

### **Cues to level of mating opportunities**

Finally, experiment 2 ruled out the hypothesis that egg production was affected by population density: the conditioned water from both conspecific and heterospecific worms was likely to contain metabolites (and/or other stressing agents), but only that from conspecific worms caused the decrease in egg production. This result suggests that conspecific-conditioned water contains waterborne chemical cues that inform *O. diadema* hermaphrodites about the presence of other conspecific hermaphrodites close by and sets the stage for a change in sex allocation towards a less female-biased one. This change usually occurs in response to increased mating opportunities (see also Schleicherová et al. 2006, 2010). Further studies are needed to identify the chemical compounds responsible for sex allocation adjustments.



### **Stability of hermaphroditism**

Overall, the results of this study show that *O. diadema* worms can rapidly adjust their sex allocation in both directions (from strongly to weakly female biased and vice versa), and that they can do it as a response to chemical cues in the water. This capacity of performing rapid, reversible and precise phenotypic changes strongly reduces the cost associated with phenotypic plasticity (Piersma and Drent 2003) and limits the penalty associated with a mismatch between phenotype and environment, which is considered one of the main costs of phenotypic plasticity (De Witt et al. 1998). Moreover, the ability to adjust sex allocation does not entail short-term fitness costs, further supporting the hypothesis that plasticity in sex allocation requires few resources (Lorenzi et al. 2008).

The low risk of mismatch between phenotype and environment, and the lack of costs, may result in a weak selective pressure against the plasticity. Indeed, these worms still exhibit extremely plastic sex allocation after over 200 generations in the laboratory under constantly high mating opportunities (they are only slightly less plastic than wild worms; Schleicherová et al. 2013).

The low cost of phenotypic plasticity in sex allocation, which allows individuals to express the appropriate sexual phenotype in various social environments, may strongly affect the rate of evolution of sex allocation in this species. A phenotypic trait that varies with the environment can indeed weaken the effect of natural selection on the genes responsible for that trait and prevent its evolution (Price et al. 2003; Chevin et al. 2013). In view of this, the highly plastic sex allocation of *O. diadema* worms may prevent sex allocation evolution and we may expect no significant variation in sex allocation adjustments in individuals exposed to selection for several generations (i.e. strong and weak selection for male-biased sex allocation), similarly to that recently documented in *M. lignano*: worms reared for several generations in pairs or groups did not differ in sex allocation traits (Janicke et al. 2016). However, it seems that *M. lignano* worms cannot allocate their sexual resources optimally, at least under monogamy (Schärer and Ladurner 2003; Schärer and Vizoso 2007). In monogamy, hermaphrodites are expected to invest a minimum amount of resources in the male function, because (pre- and postcopulatory) mate competition is minimal (Charnov 1982). In contrast, *M. lignano* worms kept in pairs produced more sperm than those needed to fertilize their partner's eggs (Schärer and Ladurner 2003; Schärer and Vizoso 2007). This is not the case in *O. diadema* worms, which have consistently small sperm counts of 50 sperm per egg (Sella 1990); this is one of the lowest levels of sperm redundancy in the animal

kingdom (Cohen 1975). This suggests that these worms almost perfectly match the optimal sex allocation when mating opportunities are scarce, that is, investment in the male function is minimal. As the current results show, this low investment also includes virtually no allocation to ‘male’ behaviours; in experiment 1, no aggression was reported when worms were kept in pairs.

To conclude, investigating individual adjustments in sex allocation offered an insight into the costs of plasticity in *O. diadema* worms, which suggest that sex allocation might be shielded from the action of sexual selection. The fact that sex allocation may evolve slowly compared to other traits may be crucial to the maintenance of hermaphroditism in this species. While phylogenetic analyses suggest that, in animals, the transition between sexual systems probably occurred more often from gonochorism (i.e. separate sexes) to hermaphroditism (Sasson and Ryan 2017), it seems that it occurred in the opposite direction in the genus *Ophryotrocha* (Dahlgren et al. 2001). Organisms with separate sexes may evolve from hermaphroditic ancestors due to the pressure exerted by sexual selection on hermaphrodites for optimal sex allocation (Charnov 1982). According to theory, hermaphroditic individuals should invest more in the male function with increasing mate competition; if mate competition is constantly high, sexual selection should thus favour those individuals that are more male biased, which will lead to a further increase in the level of sperm competition. Under these circumstances, individuals that invest all their resources in the male role (pure males) should gain a higher siring success. Once pure-male individuals have invaded the population, they will impose a selective pressure on the remaining hermaphrodites for allocating progressively more resources to the female function (as observed in plants; Dorken and Pannell 2009). However, if sex allocation does not evolve due to its plasticity, because hermaphrodites can bias their sex allocation in one direction, but easily revert to the original condition, this may also prevent the evolution of separate sexes. This is the case in plants, where phenotypic plasticity prevents the evolution of fully separate-sex species (Delph and Wolf 2005) and may be the case also in at least some of the hermaphroditic *Ophryotrocha* worms.

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**5.**

**Gender-related behaviors: evidence  
for a trade-off between sexual  
functions in a hermaphrodite**

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## Abstract

According to sex allocation theory, hermaphrodites are expected to allocate most reproductive resources to the female function under low level of mate competition and to shift them to the male function when mate competition increases. Therefore, sex allocation theory assumes a trade-off between sexual functions. Although several studies highlight some adjustments of sex allocation with mating opportunities, empirical support for the trade-off between sexual functions is surprisingly scarce. Here we argue that this lack of support for a trade-off might depend on the exaggerated focus on gamete production in sex allocation studies at the expense of other gender-related traits. We investigated whether parental care (a putative female behavior) and motility (a proxy for mate searching, and a putative male behavior) varied plastically with mating opportunities in the hermaphroditic polychaete worms *Ophryotrocha diadema*. We found that parental care was higher under low mating opportunities while motility increased under high mating opportunities, and that the two behaviors were negatively correlated with each other, that is, there was a trade-off between them. Moreover, observing the behavior of the closely-related, separate-sex species *Ophryotrocha labronica*, we found that males moved more than females in the separate-sex species and that mothers performed more parental care than fathers in both *Ophryotrocha* species, although the difference was larger in the separate-sex species. Our results provide convincing evidence in support of a trade-off between sexual function and highlights the importance to investigate sex-allocation adjustments in reproductive traits others than gametes.

**Key words:** mate competition, mate searching, motility, parental care, sex allocation.

## Introduction

Simultaneous hermaphrodites (hereafter called hermaphrodites) are organisms which produce and use both male and female gametes and thus may be viewed as both males and females at the same time. However, compared to separate-sex organisms, the relative amount of resources that hermaphrodites invest in the two sexual functions during their life is not fixed, but can be adjusted to environmental conditions in order to maximize the fitness (Charnov 1979, 1982; Michiels 1998). Predicting the optimal investment of reproductive resources in the female *versus* the male function is the aim of sex allocation theory (Charnov 1982; Hardy 2002; West 2009). According to this theory, if the proportion of resources allocated to the male function increases, the investment in the female function should necessarily decrease: there is a trade-off between the allocation to the female and male function (Charnov 1982). Thus, to maximize their fitness, hermaphrodites should invest a minimum amount of resources into the male function (e.g., sperm production) when they are isolated or in monogamous pairs, that is, when sperm competition is virtually absent and related sperm (i.e., sperm produced by the same individual) compete with each other for egg fertilization [a process similar to the Local Mate Competition proposed by Hamilton (1967) and recently called Local Sperm Competition (Schärer 2009)]. Indeed, since eggs are costlier to produce than sperm (Bateman 1948), hermaphrodites which invest a minimum amount of resources in sperm production when exposed to low sperm competition, not only produce enough sperm to fertilize the available eggs while decreasing competition among related sperm, but save also resources to invest in the female function (Charnov 1979; Taylor 1981). However, when the mating group increases, so does sperm competition, and, from the individual's perspective, it pays to reallocate a certain amount of resources from the female to the male function.

Sex allocation adjustments via phenotypic plasticity have received considerable attention in the literature and a significant amount of evidence in support of sex allocation theory has been collected both in natural and laboratory conditions (reviewed in Schärer 2009). Nevertheless, the empirical support for a trade-off between the male and female function is surprisingly scarce and often contradicting.

Several hermaphrodites, indeed, respond to variation in the social group size (often used as a proxy of mating group size) adjusting the investment in one sexual function, while

leaving the other unchanged. For example, the chalk basses *Serranus tortugarum* adjust only the female allocation, so that fish which live at (or are experimentally transplanted to) low density populations have smaller ovaries, but not larger testis (Hart et al. 2010, 2011); likewise, the polychaete worms *Ophryotrocha diadema* significantly decrease their egg production when kept in groups rather than in pairs, while producing approximately the same amount of sperm (Lorenzi et al. 2005, 2006). The leeches *Helobdella papillornata*, instead, increase their testisac, but not egg, volume with social group size, thus adjusting only their male allocation (Tan et al. 2004).

More surprisingly, in some species of hermaphrodites, the two sexual functions seem to be positively correlated, since individuals that invest more in one sexual function do so also in the other, as happens in the land snails *Arianta arbustorum*, where individuals that produce higher amount of sperm also produce more eggs (Locher and Baur 2000).

Finally, in the few cases where a decrease in one sexual function leads to an increase in the other, it remains unclear whether the trade-off between female and male investment is the actual cause of this negative correlation. In the freshwater snail *Lymnaea stagnalis*, the experimental cutting of a nerve, which determines whether or not individuals may copulate as males, leads to a significant increase in the egg production with respect to both control and sham-operated snails (De Visser et al. 1994; Koene 2006). The increase in egg production after the suppression of the male reproductive activity has been considered one of the clearest evidence of a trade-off between sexual functions. However, recent studies provide alternative explanations: in this freshwater snail, egg-laying activity is negatively affected by the receipt of chemicals of the seminal fluid (Koene et al. 2009; Koene et al. 2010); in the study from De Visser and colleagues (1994), snails prevented from mating in the male role were kept with each other and therefore were not exposed to those components of seminal fluid that reduce egg-laying activity, which would cause the increase in egg production.

To explain the striking lack of evidence for a trade-off between sex functions, it has been argued that the trade-off may not exist (Schärer 2009). At the origin of the trade-off, there is indeed the strong assumption that each individual has a fixed reproductive resource budget, separated from the resource budget of other traits (Charnov 1982). If, however, there is not such a strict partition between reproductive and non-reproductive resources, male and female allocation may not trade off with each other but with other life-history traits, such as asexual body growth (e.g., in *Botryllus schlosseri* ascidians, Yund et al. 1997; or developing *Lymnaea stagnalis* snails, Koene and Ter Maat 2004) or lifespan and body growth (e.g. *Ophryotrocha diadema*, Lorenzi et al 2006; Di Bona et al. 2010).

Alternatively, the experimental conditions at which the trade-off has been tested did not allow highlighting it: variations in the reproductive resource budget could mask the trade-off between male and female function. Individuals might vary, for example, in their assimilation efficiency or food-finding ability and therefore, even when organisms are reared in the same environmental conditions, the amount of resources they invest in reproduction (i.e., the resource budget) may differ among individuals. If the variation in the resource budget exceeds the variation in sex allocation, the tests on the relationship between female and male investment will split individuals on the basis of their amount of resources, which would result in a positive correlation between female and male function (that is, individuals with larger resource budget have higher investment in the female and male function than individuals with smaller budget, Schärer et al. 2005). In the flatworm *Macrostomum* sp., an increase in male investment *and* a decrease in female one with increasing social group size have been described only under specific environmental conditions, which minimize the variation in reproductive resource budget (i.e., food restriction; Schärer et al. 2005).

A last explanation for the paucity of support for the trade-off between sexual functions in hermaphrodites relies on the excessive focus on post-copulatory traits, that is, those traits which increase fertilization success, but not mating rate (Parker 1970). Sexual selection on pre-copulatory traits (i.e., involved in mate acquisition) is expected to be considerably weaker in hermaphrodites than in separate-sex animals (Greeff and Michiels 1999) and the hermaphroditic literature has mainly focused on sperm competition (Charnov 1979; Michiels 1998). The reproductive investment has therefore been measured almost exclusively as gamete production (either as gamete number or gonadal size), leaving largely unexplored other reproductive traits, such as gender-related behaviors (Schärer 2009; Schärer and Pen 2013).

Here we aimed at investigating whether changes in mating opportunities (i.e., social group size) triggered adjustments in the amount of male- and female-related behaviors in the hermaphroditic polychaete worms *Ophryotrocha diadema*, and whether these behaviors traded off between each other.

Due to their peculiar reproductive mode (see Methods), post-copulatory competition is expected to be weak in *O. diadema* worms (although extant, Sella and Lorenzi 2003; Lorenzi et al. 2014), and it has been shown that male-reproductive resources may be invested in aggressive behaviors rather than in sperm production (Lorenzi et al 2006; Santi et al. unpublished data).

We hypothesized that, since sperm competition is likely low, gamete production may represent only a small fraction of the reproductive resource investment and worms should allocate a considerable amount of resources towards behaviors enhancing sex-specific reproductive success. Under this assumption, we expected that worms exposed to low mating opportunities will bias their investment to the female function, increasing not only their egg production (Lorenzi et al. 2005, 2006) but also their investment in behaviors enhancing the female fitness (e.g., parental care). In contrast, worms exposed to high mating opportunities (high mate competition), should decrease their investment in the female function (e.g., the amount of parental care provided) to reallocate those resources to behaviors increasing the male reproductive success (e.g., mate searching).

Therefore, we tested whether parental care and motility (a proxy for mate searching) decreased and increased respectively with increasing mating opportunities.

Eventually, with the aim to confirm that these behaviors were actually gender-specific, we investigated whether and how parental care and motility differed between males and females in pairs of the closely-related, separate-sex species, *Ophryotrocha labronica*.

## **Methods**

### **Study Species**

*Ophryotrocha* worms are few-mm-long marine polychaetes, usually found among the fouling fauna of eutrophic water (e.g., harbor; Åkesson 1976; Thornhill et al. 2009).

They are external fertilizers, but mating is achieved through “pseudocopulation”, a complex mating behavior in which the two partners are in close physical contact during gamete release (Westheide 1984; Sella and Ramella 1999; Lorenzi et al. unpublished data). Pseudocopulation generally occurs after a long and time-consuming courtship [from several hours in *O. diadema* (Sella 1985) to several days in *O. labronica* (Åkesson 1974)] when the female (or the “egg-donor” in hermaphrodites) releases its eggs within a jelly cocoon; soon after the egg release, the male (or the “sperm-donor”) enters the jelly cocoon and releases its sperm (Westheide 1984; Lorenzi et al. unpublished data), which are aflagellate and immotile (Morrow 2004). The short time-interval between egg and sperm release, the close physical contact between partners, and the almost null motility of sperm suggest a key role of pre-copulatory competition in determining the reproductive success of males (or “sperm-donors”):

it may be more important to be in the right position at the moment of egg release, rather than produce high amount of sperm. This hypothesis is further supported by the low number of sperm produced by the hermaphroditic species (50 sperm/egg, one of the lowest sperm redundancies among animals, Cohen 1975; Premoli and Sella 1995).

Fertilized eggs develop into free-swimming larvae within about 8 days, during which cocoons are cared by one or both parents (depending on the species), which either rest or move back and forth on the cocoon, likely in order to keep it free from fungi and bacteria (Sella 1991; Sella and Ramella 1999).

Simultaneously hermaphroditic *O. diadema* worms are obligate outcrossers (they cannot self-fertilize) and have unilateral mating, that is, during a single mating, each worm either plays the male role (and releases sperm) or plays the female role (and releases eggs). Sexual maturity is reached after a 40-days-long protandrous, adolescent phase in which individuals produce and use only sperm and which begin soon after larvae hatch from the cocoon (Sella 1990; Sella and Lorenzi 2003); once they reach a body length of 13-14 chaetigerous segments, worms start to produce also eggs which they lay each 3-4 days, in cocoons containing on average 25 eggs (Sella 1985; Picchi et al. in press).

*O. diadema* worms have a natural genetic marker for the color of the eggs which allows distinguishing two strains: the wild and the albino strain (Sella and Marzona 1983). We used this polymorphism to assess maternity (and, when possible, paternity) and to identify the “focal” individuals in each experimental group: since eggs are visible within the body cavity through the transparent body walls, wild-strain worms have a yellow phenotype and lay yellow eggs, while albino-strain worms have a white phenotype and lay white eggs.

*O. labronica* is instead a sexually-dimorphic, separate-sex species. Sexual maturity is reached in less than 1 month, even if with slight differences between males and females: female become sexually mature at a larger body size, but grow faster (Paxton and Åkesson 2007; Lorenzi and Sella 2013). Once they reach sexual maturity, males can be distinguished by their relatively wider prostomium and thicker jaws, while females have eggs in the coelomic cavity and a larger body size.

Egg production is similar to *O. diadema* worms, but temporally clustered differently: *O. labronica* worms lay about 120 eggs in a tubular jelly cocoons each one or two weeks (Premoli and Sella 1995).

Experiments were performed with worms coming from laboratory cultures, established several years ago: *O. diadema* worms were collected in California (Long Beach) in 1976 and 1980, and in Italy (Porto Empedocle) in 2008; *O. labronica* worms were collected in

California (Long Beach, LA) in 2005. Experimental individuals were selected from the offspring of isolated pairs of worms taken from the laboratory cultures, so that worms were all virgin and we knew their age (in weeks). We reared the newly generated worms with their siblings until they started to have visible oocytes in the coelomic cavity and then we kept them in isolation (in 9mL Petri dishes) until they entered the experiment.

During the experiment, worms were kept in 20mL bowls with artificial sea water (salinity 32‰), fed *ad libitum* with spinach and kept in thermostatic cabinets, at a constant temperature of 21°C (according to Åkesson 1976). Individuals used in the experiment were sexually mature; “focal” worms for *O. diadema*, and males and females for *O. labronica*, were not siblings.

### **Experimental set up**

#### *Ophryotrocha diadema*

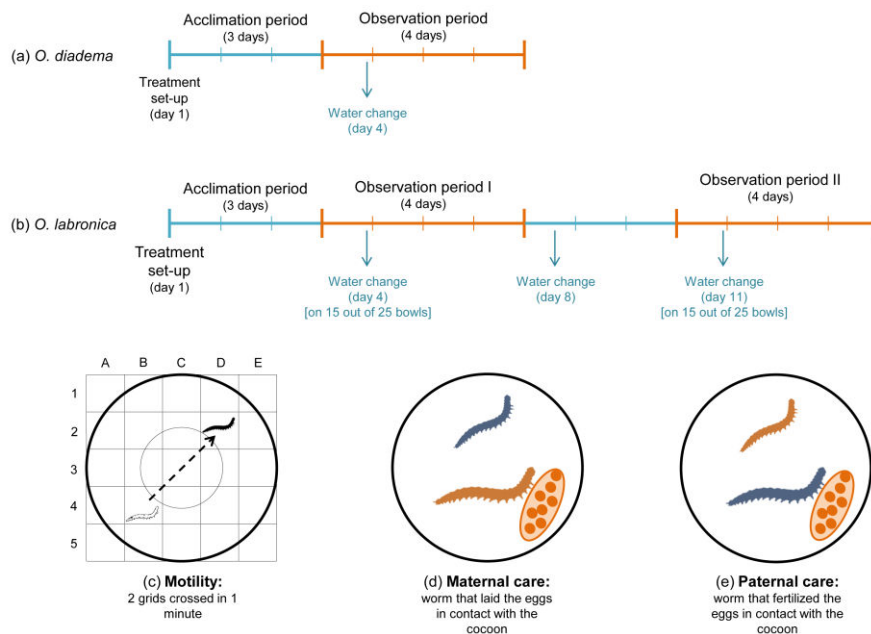
*O. diadema* worms perceive mating opportunities by means of species-specific chemical compounds, as shown by the fact that they adjust their female allocation when exposed to water where conspecifics have been kept (called homospecific conditioned water), but they do not when exposed to water from congeneric species (i.e., heterospecific conditioned water) (Schleicherová et al. 2006; Santi et al. unpublished data).

In order to test whether parental care and motility changed with mating opportunities in hermaphrodites, we randomly assigned *O. diadema* worms to 4 different treatments: 1) “Pair” (i.e., actual low mating opportunities), with 2 mature hermaphrodites per bowl (1 wild-phenotype + 1 albino-phenotype, N = 24); 2) “Group” (i.e., actual high mating opportunities), with 5 mature hermaphrodites per bowl (1 wild-phenotype + 4 albino-phenotype, N = 20; 1 albino-phenotype + 4 wild-phenotype, N = 15); 3) “Heterospecific Conditioned Water” (i.e., low mating opportunities), with 2 mature hermaphrodites per bowl (1 wild-phenotype + 1 albino-phenotype, N = 25) exposed to water where congeneric worms (*O. labronica*) had been kept; 4) “Homospecific Conditioned Water” (i.e., simulated high mating opportunities), with 2 mature hermaphrodites per bowl (1 wild-phenotype + 1 albino-phenotype, N = 25) exposed to water where conspecific worms (*O. diadema*) had been kept. We prepared conditioned water keeping 20 worms (either *O. diadema* or *O. labronica*) in 60 mL bowls for 3 days.

This experimental set up allowed disentangling between the effects of mating opportunities and those of density stress (due to metabolites, oxygen depletion, etc.) and/or

encounter rate: worms in Group were indeed exposed to higher mating opportunities than those in Pair, but also to higher density stress and encounter rate; worms in Homospecific Conditioned Water were instead exposed to higher (perceived) mating opportunities and density stress, but not to higher encounter rate compared to pairs; finally, worms in Heterospecific Conditioned Water were only exposed to higher density stress. Therefore, if worms in Pairs and Heterospecific Conditioned Water did not differ with each other, but differed from Groups and Homospecific Conditioned Water, we could rule out density and encounter rate as causes of behavioral changes.

The experiment lasted 2 weeks, and for logistic reasons was replicated once. Each week, we exposed four new set of worms, each to one of the four treatments, and we observed simultaneously their behavior for four days. We let worms a 3-days acclimation period before starting the behavioral observations on the fourth day. Since the first observation, we checked daily for the presence and color of new cocoon in the bowls and we counted the number of eggs in both newly-and previously laid cocoons; we also measured the body size (i.e., number of chaetigerous segments) of focal worms and the water salinity (Figure 1a). Water and food were renewed once (the fourth day, after the first observation).



**Figure 1:** Experimental set up and behavioral observation schedule for (a) *O. diadema* and (b) *O. labronica*, and schematic representation of the observed behaviors: (c) Motility, that is, the estimation of the number of grids crossed per minute on the basis of the position of the worms at each observation; (d) Maternal care, when the phenotype of the hermaphroditic worm in contact with the cocoon matched the phenotype of the eggs (or when the female was in contact with the cocoon in the separate-sex worms); (e) Paternal care, when the phenotype of the hermaphroditic worm in contact with the cocoon did not match the phenotype of the eggs (or when the male was in contact with the cocoon in the separate-sex worms).



*Ophryotrocha labronica*

To investigate whether and how parental care and motility differed between males and females in *O. labronica*, we set up 25 pairs of worms (1 female + 1 male) in which we observed maternal and paternal care; we observed instead male and female motility in 15 of those pairs. We observed the behavior only in pairs since a previous study showed that the separate-sex *O. labronica* worms do not adjust their sex allocation to the group size (Schleicherová et al. 2010).

To account for the longer time-interval between egg-layings in *O. labronica*, we kept the worms paired for two weeks. As for the other species, the experiment was replicated once. Analogously to what we did for *O. diadema* worms, we paired new worms, let them a 3-days acclimation period, and the fourth day we started the behavioral observations, which lasted 8 non-consecutive days. In the 15 pairs in which we observed both parental care and motility, water and food were renewed three times (once each 3 days), while in the remaining 10 they were renewed once (after the first week). Each day in which we performed the behavioral observations, we checked the bowls for newly laid cocoons, we counted the eggs per cocoon (when present) and we measured the body size (i.e., number of chaetigerous segments) of both males and females and water salinity. We scored the number of eggs/cocoon as discrete classes (each with a range of 15 eggs), noted as the upper-limit of the classes (i.e., 15, 30, 45, etc.), since the tubular shape of the cocoons makes it difficult to count the exact number of eggs (Figure 1b).

In all the experiments, we performed measurements under a stereomicroscope Leica EZ4, while we performed behavioral observations with a magnifying glass, in order to avoid disturbing the worms by repeatedly moving the bowls to and from the microscope.

*Behavioral measurements*

We performed 10 behavioral observation for motility and 10 for parental care per day and per bowl for four days with the scan sampling technique, which consists in noting the current activity of an individual at regular time intervals (Altmann 1974). We observed parental care each 15 minutes, while we measured motility each minute, in order to reduce underestimation of the distance travelled.

To measure motility, we put the transparent-glass bowls over a 6 X 6-cm grid consisting of 25 smaller squares (each with a side-length of 1.2 cm). Each minute, we noted which square the focal worm was in, and then computed motility as the minimum number of grids that worms crossed to move from their position at time  $t$  to position at time  $t+1$ . Worms could

move in any direction, including diagonal, and we considered 1 grid crossed when worms moved from the bowl bottom to the top or *vice versa*. For the hermaphroditic *O. diadema* worms, we measured motility of the wild-phenotype worms (i.e., the yellow-phenotype worms), while, for the separate-sex *O. labronica* worms, we measured motility of both males and females (Figure 1c).

Observations on parental care were temporally separated from those on motility and were carried out the same days. We considered that the worms were performing parental care if they were in physical contact with a cocoon. Since we aimed to distinguish between maternal and paternal care (where possible), we focused on a single cocoon per bowl: *O. labronica* laid only one egg cocoon per bowl (which was therefore the focus of our observation), while in *O. diadema* we focused on the first laid yellow-egg cocoon (laid by the wild-yellow-phenotype worms) for half of the bowls (N = 50) and on the first laid white-egg cocoon for the remaining half (N = 59). In *O. labronica* we considered that there was maternal and/ or paternal care when the female and/or the male respectively were in contact with the focal cocoon; in *O. diadema* we considered care as maternal when the focal cocoon was cared by the worm which laid it (i.e., yellow cocoons cared by yellow worms or white cocoons cared by white worms) and as paternal when the worm and the cocoon had different colors (paternal care was not measured in the treatment Group, since we could not assign paternity) (Figure 1d-e).

## **Statistical analyses**

### *Motility*

Since motility was measured as the number of grids crossed by the worms, we tested whether it differed between Pair and the other three treatments in *O. diadema* worms with a Generalized Linear Mixed Model (GLMM) for Poisson distributed data. We further controlled for the body size of the focal worms, their age (in weeks), the time they had spent in the experiment, the presence/absence of cocoons in the bowl, and for water salinity; we included the bowl ID and the observation number (10 observations per day) as random factors.

We analyzed motility with GLMM for Poisson distributed data also in *O. labronica*. Here we tested whether the number of grids crossed in a minute differed between males and females, controlling for the effect of body size, age, and water salinity; since half of the observations was done when there was no cocoon in the bowl, we further checked whether the

presence of the cocoon affected motility and we included the bowl ID and the observation number as random factors. Time in the experiment was excluded as it correlated with age.

### *Parental care*

Parental care was instead a binomial variable (worms either did or did not parental care) and therefore it was analyzed with a GLMM for binomial distributed data. Here we tested whether performing maternal care in *O. diadema* differed in Pairs compared to the other three treatments and whether it depended on the phenotype of the focal worms and on the number of eggs in the cocoon. As above, we controlled for body size, age, the time spent in the experiment, and water salinity, and we included the bowl ID and the observation number as random factors.

We further investigated whether parental care offered a selective advantage, testing whether the egg developmental success was affected by the amount of maternal care received. We therefore tested whether the proportion of eggs still present in the bowl the last day of the behavioral observation (quasibinomial distributed data to account for overdispersion) depended on the proportion of times mothers were observed caring for the cocoon over the entire observation period, and whether it depended on the treatment, the age of the worms and the egg-color phenotype of the mother. Since each individual was represented once, we did not include any random factors (Generalized Linear Model - GLM).

Eventually, we compared maternal and paternal care in *O. diadema* and *O. labronica*. We tested whether parental care depended on the species, parenthood (i.e., whether it was maternal or paternal care), and on the interaction between these two variables. We controlled for the number of eggs in the cocoon, age, and the time spent in the experiment, and we included, as random factors, the bowl ID, the observation number, and the experimental replicate, since *O. labronica* parental care were observed with two, slightly different, experimental set up (see Experimental set-up section and Figure 1b). Here, the number of eggs was considered as 15-egg-range classes also for *O. diadema* to allow comparison between species. We excluded both body size and salinity from this model since we lacked this information for 10 pairs of *O. labronica*; however, both variables were non-significant when tested on a subset (N = 15) of data from *O. labronica* worms (GLMM, Body size:  $\beta = 0.042 \pm 0.911$ ,  $z = 0.046$ ,  $p = 0.964$ ; Salinity:  $\beta = 1.079 \pm 0.762$ ,  $z = 1.416$ ,  $p = 0.157$ ). Finally, in order to test whether mothers provided a different amount of care than fathers in both species, we checked for a difference between maternal and paternal care separately for

the two species (including only the significant variables and the random factors of the previous model).

*Trade-off between parental care and motility in O. diadema*

We eventually tested whether there was a trade-off between parental care (female-behavior) and motility (male-behavior). Here the response variable was the proportion of times wild-phenotype worms (motility was measured only on these worms) were observed performing (either maternal or paternal) parental care during the entire observation period. We thus performed a GLM for quasibinomially distributed data (to account for overdispersion) where the predictors were the total number of grids crossed over the observation period, the treatment, the total amount of eggs worms had cared for, whether the care was paternal or maternal, and the age of the worm.

In all the models, we checked for multicollinearity and (where needed) for overdispersion. In case the data were collinear, we excluded one of the correlated variables on the basis of the Likelihood Ratio Test.

In all the analyses, we selected the variables on the basis of their biological relevance, we evaluated the model fit and the significance levels of the predictors, and then we dropped non-significant interactions and variables one by one and selected the model variables on the basis of the Likelihood Ratio Test (when models differed significantly we selected the model with the smallest AIC, otherwise, for parsimony, we selected the model with less degrees of freedom, that is, the simplest model). Results are reported for the reduced models. All the variables included in the model were scaled and centered.

Statistical analyses were conducted in R v.3.5.0 (R Core Team 2018) using the *lme4* package (Bates et al. 2015). Figures represent Least-Squares Means (LSM) computed from the models with the R package *emmeans* (Lenth 2018).

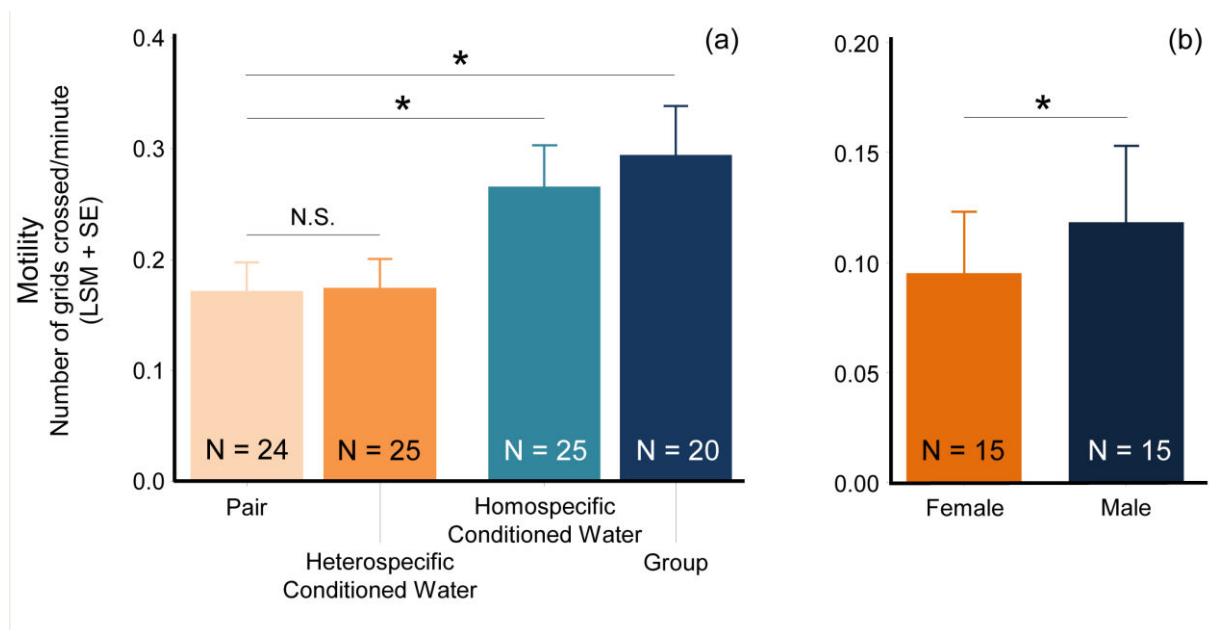
Descriptive statistics are reported as means  $\pm$  Standard Errors (SE).

## Results

### Motility

*Ophryotrocha* worms usually move relatively slowly and indeed, they crossed on average less than one grid per minute, although they were able to cross a 1.2 x 1.2 cm square in less than 10 s. Despite the overall low mobility, the number of grids crossed by the hermaphroditic *Ophryotrocha diadema* worms significantly varied according to mating opportunities (Figure 2a; Table 1a): worms moved less when exposed to low mating opportunities (Pair and Heterospecific Conditioned Water:  $0.197 \pm 0.017$  and  $0.222 \pm 0.018$  grids crossed per minute respectively) compared to worms exposed to high mating opportunities (Group and Homospecific Conditioned Water:  $0.340 \pm 0.023$  and  $0.321 \pm 0.020$  grids crossed).

Moreover, males of *O. labronica* moved significantly more than females ( $0.245 \pm 0.016$  vs  $0.197 \pm 0.019$  grids crossed respectively; Figure 2b; Table 1b), although both males and females moved significantly less when there was a cocoon in the bowl (Table 1b).



**Figure 2:** Motility of *Ophryotrocha* worms as a function of (a) mating opportunities in the hermaphroditic worms, and (b) the sex in the separate-sex species. The values in the figures represent the Least-Squares Means ( $\pm$  SE) computed from the models. N represents the number of focal individuals observed per group. Hermaphroditic worms in Pair and Heterospecific Conditioned Water were exposed to low mating opportunities, while those in Group or Homospecific Conditioned Water were exposed to (actual or perceived) high mating opportunities.

**Table 1:** Results of the GLMMs testing whether (a) hermaphroditic worms adjusted their motility to mating opportunities; and (b) separate-sex male and female worms differed in their motility level.

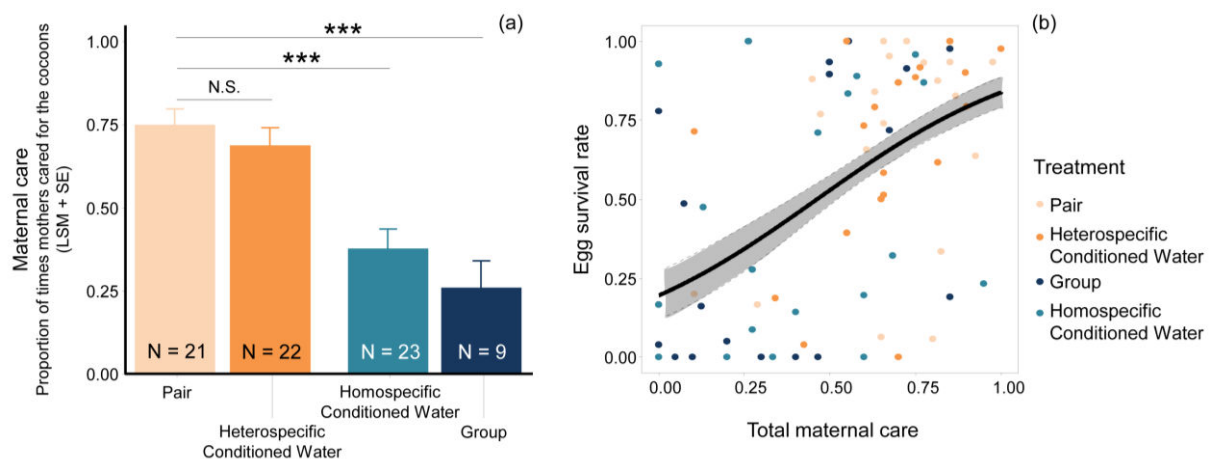
		<b>Motility</b>				
	Predictors	N	Estimate	SE	z-value	p-value
(a) <i>O. diadema</i>	Intercept		- 1.764	0.153	- 11.492	< 0.001
	Treatment [Heterospecific Conditioned Water] <sup>a</sup>		0.010	0.214	0.048	0.962
	<b>Treatment [Homospecific Conditioned Water]<sup>a</sup></b>		<b>0.436</b>	<b>0.206</b>	<b>2.114</b>	<b>0.035</b>
	<b>Treatment [Groups]<sup>a</sup></b>		<b>0.537</b>	<b>0.214</b>	<b>2.505</b>	<b>0.012</b>
	Presence of cocoons [yes]		N.S.	N.S.	N.S.	N.S.
	Body size focal worm		N.S.	N.S.	N.S.	N.S.
	Age (in weeks)		N.S.	N.S.	N.S.	N.S.
	Time in the experiment		N.S.	N.S.	N.S.	N.S.
	Salinity		N.S.	N.S.	N.S.	N.S.
	<b>Random factors</b>					
	Bowl ID	94				
	Observation number	9				
<b>Observations</b>		3104				
	Predictors	N	Estimate	SE	z-value	p-value
(b) <i>O. labronica</i>	Intercept		- 1.773	0.309	- 5.738	< 0.001
	<b>Sex [male]</b>		<b>0.219</b>	<b>0.093</b>	<b>2.356</b>	<b>0.018</b>
	<b>Presence of cocoons [yes]</b>		<b>- 1.170</b>	<b>0.246</b>	<b>- 4.754</b>	<b>&lt; 0.001</b>
	Body size focal worm		N.S.	N.S.	N.S.	N.S.
	<b>Age (in weeks)</b>		<b>- 0.769</b>	<b>0.209</b>	<b>- 3.673</b>	<b>&lt; 0.001</b>
	Salinity		N.S.	N.S.	N.S.	N.S.
	<b>Random factors</b>					
	Bowl ID	15				
	Observation number	9				
<b>Observations</b>		2070				

The significant results are in bold. N.S. represents the non-significant variables which were dropped from the preliminary models. <sup>a</sup> Values are relative to the comparison with the treatment Pair.

## Parental care

When a cocoon was present, *O. diadema* worms often cared for it. However, as it happened for motility, hermaphroditic worms adjusted the amount of maternal care to mating opportunities (Figure 3a; Table 2): mothers exhibited parental care  $70.2\% \pm 1.8$  and  $65.6\% \pm 2.0$  of times in Pair and Heterospecific Conditioned Water, respectively (i.e., low mating opportunities); in Group and Homospecific Conditioned Water, instead, mothers cared for their cocoons only  $35.3\% \pm 3.3$  and  $39.6\% \pm 2.0$  of times. Worms performed maternal care regardless of their phenotype, but adjusted the amount of care to the number of eggs contained in the cocoon (Table 2).

Moreover, the amount of parental care provided to the cocoons was the only variable (among those tested) which affected the egg survival rate: more eggs survived in the cocoons which received more care (GLM, parental care:  $\beta = 0.858 \pm 0.209$ ,  $N = 78$ ,  $t$ -value = 4.100,  $p < 0.001$ , Figure 3b).



**Figure 3:** (a) The variation in maternal care provided by the hermaphroditic worms in response to mating opportunities, represented as the Least-Squares Means ( $\pm$  SE) computed from the model. N represents the number of different focal individuals analyzed per group. (b) Egg survival rate in response to the amount of maternal care received by the cocoon. Dots represent raw data. The line represents the value predicted by the model ( $\pm$  SE). Worms in Pair and Heterospecific Conditioned Water were exposed to low mating opportunities, while those in Group and Homospecific Conditioned Water were exposed to (actual or perceived) high mating opportunities.

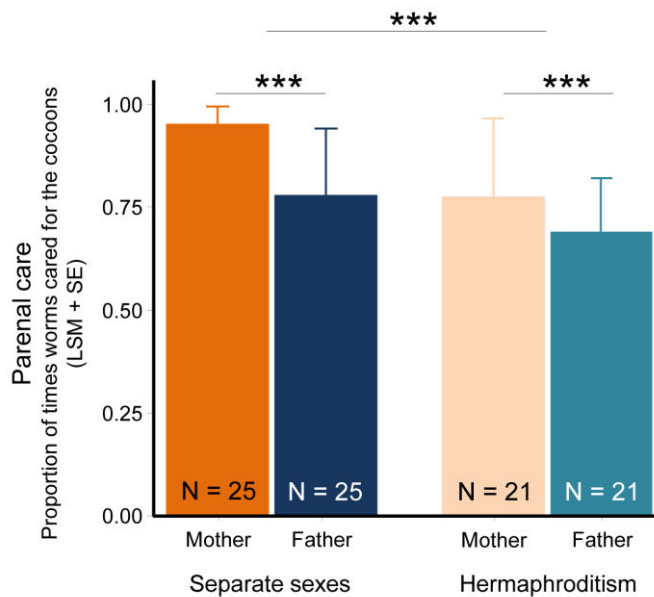
**Table 2:** Results of the GLMM testing whether hermaphroditic worms adjusted maternal care to mating opportunities

<b>Maternal care</b>					
Predictors	N	Estimate	SE	z-value	p-value
Intercept		- 1.095	0.259	4.230	< 0.001
Treatment [Heterospecific Conditioned Water] <sup>a</sup>		- 0.310	0.364	- 0.851	0.395
<b>Treatment [Homospecific Conditioned Water]<sup>a</sup></b>		<b>- 1.599</b>	<b>0.365</b>	<b>- 4.384</b>	<b>&lt; 0.001</b>
<b>Treatment [Groups]<sup>a</sup></b>		<b>- 2.150</b>	<b>0.503</b>	<b>- 4.276</b>	<b>&lt; 0.001</b>
Phenotype focal worm		N.S.	N.S.	N.S.	N.S.
<b>Number of eggs in the focal cocoon</b>		<b>1.009</b>	<b>0.119</b>	<b>8.472</b>	<b>&lt; 0.001</b>
Body size focal worm		N.S.	N.S.	N.S.	N.S.
Age (in weeks)		N.S.	N.S.	N.S.	N.S.
<b>Time in the experiment</b>		<b>0.312</b>	<b>0.061</b>	<b>5.075</b>	<b>&lt; 0.001</b>
Salinity		N.S.	N.S.	N.S.	N.S.
<b>Random factors</b>					
Bowl ID	75				
Observation number	10				
<b>Observations</b>		1993			

The significant results are in bold. N.S. represents the non-significant variables which were dropped from the preliminary models. <sup>a</sup> Values are relative to the comparison with the treatment Pair.



Comparing the amount of parental care provided by mothers and fathers in *O. diadema* and *O. labronica* we found species-specific differences between maternal and paternal care: in both species, mothers provided more care than fathers (Table 3b-c; results remain significant even after Bonferroni correction), but the difference was wider in the separate-sex species, as shown by the significant interaction between parenthood and species (Figure 4; Table 3a).



**Figure 4:** Differences between maternal and paternal care as a function of species. In the separate-sex species, maternal care was performed by the female while paternal care by the male; in the hermaphroditic species, maternal care was performed by the worm who laid the eggs while paternal care by the worm who fertilized them. The values in the figures represent the Least-Squares Means ( $\pm$  SE) computed from the model and N represents the number of different individuals analyzed per group.

**Table 3:** Results of the GLMMs testing for species-specific sex-differences in the amount of parental care provided [(a) general model; (b) difference in maternal vs paternal care in *O. didactyla*; and (c) in *O. labronica*].

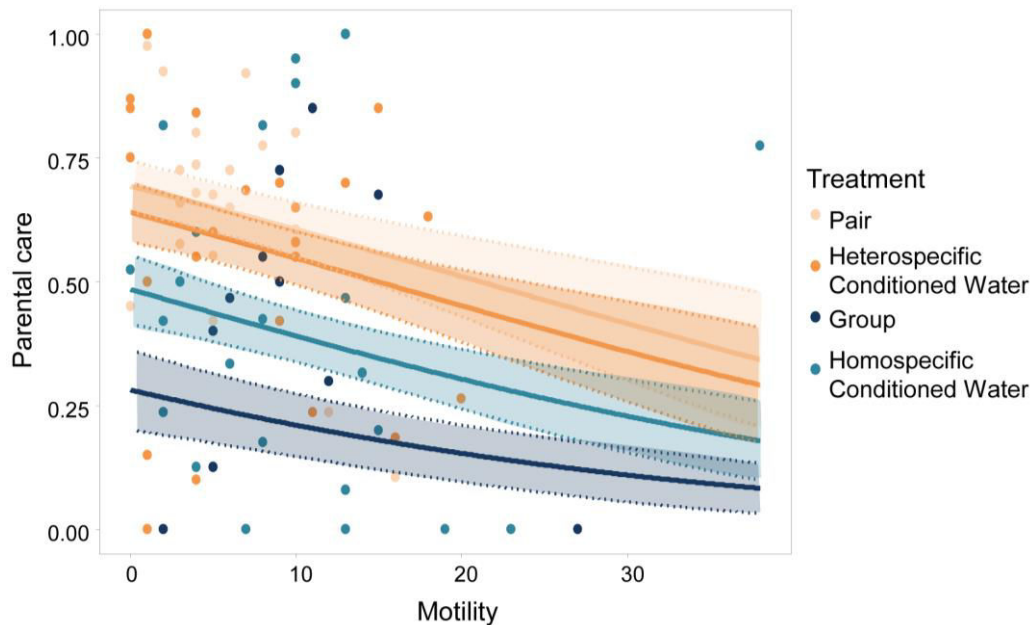
Predictors	(a) Maternal vs paternal care					(b) <i>O. didactyla</i>					(c) <i>O. labronica</i>				
	N	Estimate	SE	z-value	p-value	N	Estimate	SE	z-value	p-value	N	Estimate	SE	z-value	p-value
Intercept		1.232	1.365	0.903	0.367		0.997	0.215	4.636	< 0.001		5.042	0.1645	7.820	< 0.001
Parenthood [father]		<b>-0.442</b>	<b>0.131</b>	<b>-3.367</b>	<b>&lt; 0.001</b>		<b>-0.435</b>	<b>0.130</b>	<b>-3.353</b>	<b>&lt; 0.001</b>		<b>-1.758</b>	<b>0.216</b>	<b>-8.133</b>	<b>&lt; 0.001</b>
Species [ <i>O. labronica</i> ]		1.725	1.259	1.370	0.171		-	-	-	-		-	-	-	-
Kinship * Species		<b>-1.266</b>	<b>0.249</b>	<b>-5.083</b>	<b>&lt; 0.001</b>		-	-	-	-		-	-	-	-
Number of eggs in the focal cocoon		<b>1.227</b>	<b>0.512</b>	<b>2.394</b>	<b>0.017</b>		<b>0.263</b>	<b>0.128</b>	<b>2.055</b>	<b>0.040</b>		<b>3.772</b>	<b>0.611</b>	<b>6.175</b>	<b>&lt; 0.001</b>
Age (in weeks)		N.S.	N.S.	N.S.	N.S.		-	-	-	-		-	-	-	-
Time in the experiment		<b>0.761</b>	<b>0.234</b>	<b>3.255</b>	<b>0.001</b>		<b>0.239</b>	<b>0.067</b>	<b>3.568</b>	<b>&lt; 0.001</b>		N.S.	N.S.	N.S.	N.S.
<b>Random factors</b>															
Bowl ID	46					21					25				
Observation number	10					10					10				
Experimental replicate	2					-					2				
<b>Observations</b>	<b>2920</b>					<b>1300</b>					<b>1620</b>				

The significant results are in bold. N.S. represents the non-significant variables which were dropped from the preliminary models.

□

### Parental care vs motility in *O. diadema*

Lastly, we found that worms which cared for their cocoons more often (either maternal or paternal care), were the worms which moved less: there was a negative correlation between caring for the eggs and moving (Figure 5; Table 4).



**Figure 5:** The amount of parental care (total proportion of time hermaphroditic worms were observed performing parental care) as a function of motility (the total number of grids crossed) and mating opportunities. Worms in Pair and Heterospecific Conditioned Water were exposed to low mating opportunities, while those in Homospecific Conditioned Water and Group were exposed to (actual or perceived) high mating opportunities. Dots represent raw data. Lines represent the values predicted by the model ( $\pm$  SE).

**Table 4:** Results of the GLM testing whether parental care traded-off with motility.

Total parental care					
Predictors	N	Estimate	SE	t-value	p-value
Intercept		0.473	0.223	2.122	0.037
Treatment [Heterospecific Conditioned Water] <sup>a</sup>		-0.264	0.308	-0.772	0.442
<b>Treatment [Homospecific Conditioned Water]<sup>a</sup></b>		<b>-0.874</b>	<b>0.317</b>	<b>-2.761</b>	<b>0.007</b>
<b>Treatment [Groups]<sup>a</sup></b>		<b>-1.750</b>	<b>0.443</b>	<b>-3.954</b>	<b>&lt; 0.001</b>
<b>Total number of grids crossed</b>		<b>-0.264</b>	<b>0.121</b>	<b>-2.188</b>	<b>0.032</b>
<b>Total number of eggs cared for</b>		<b>0.721</b>	<b>0.150</b>	<b>4.826</b>	<b>&lt; 0.001</b>
Parenthood [father]		N.S.	N.S.	N.S.	N.S.
Age (in weeks)		N.S.	N.S.	N.S.	N.S.
<b>Observations</b>	<b>78</b>				

The significant results are in bold. N.S. represents the non-significant variables which were dropped from the preliminary models. <sup>a</sup> Values are relative to the comparison with the treatment Pair.

## Discussion

These results show that hermaphrodites are able to plastically adjust gender-related behaviors to mating opportunities; hermaphroditic worms moved more when exposed to high mating opportunities, that is, when they were expected to exhibit a relatively more male-biased sex allocation, and provided more parental care when exposed to low mating opportunities, a condition in which sex allocation is predicted to be female-biased (Charnov 1982). The first conclusion we can draw is that hermaphrodites express gender-related behaviors and that, in *O. diadema* worms, parental care is a female-related behavior while motility (likely linked to mate searching) seems to be associated to the male function. It may be argued that both motility and parental care may vary in response to density, either assessed by direct contact with other individuals or by means of cues. Dispersal can be density-dependent, where individuals move to avoid resource competition (Denno and Roderick 1992; Poethke and Hovestadt 2002); analogously, the amount of parental care may depend on food availability, with parents caring less for their offspring under food-limiting conditions (Carlisle 1982; Arcese and Smith 1988). In our experiment, density-related stressors are unlikely to drive the observed behavioral plasticity: if this was the case, we would have expected similar values of parental care and motility in the three treatments were density was (or was perceived as) high (i.e., Group, Heterospecific Conditioned Water and Homospecific Conditioned Waters). Instead worms in Pair and Heterospecific Conditioned Water (where mating opportunities were low) exhibited similar values of parental care and motility, which were significantly lower and higher respectively in worms kept in Group and Homospecific Conditioned Water, where mating opportunities were (or were perceived as) high.

To further support the hypothesis that parental care and motility are mainly female- and male-related behaviors, we found that the closely-related, separate-sex species *O. labronica* showed sex-specific behavioral patterns: males moved more, while females provided more parental care. These findings are consistent with conventional sexual roles, as defined by the Darwin-Bateman paradigm (Darwin 1871; Bateman 1948; Dewsbury 2005): in general, males, which produce cheap gametes and whose reproductive success increases linearly with mate number, are eager to mate and invest resources in finding partners and mating more often; females, which produce fewer large gametes and whose reproductive success is loosely linked to mating success, are more selective and invest relatively more in the offspring (including providing more care, Trivers 1972). Although the Darwin-Bateman paradigm has been severely criticized and is thought to reflect more the culture of the period than a general

rule (Knight 2002; Dewsbury 2005), there is burgeoning empirical evidence supporting the theoretical framework behind this paradigm. In a recent comparative study, Janicke and colleagues (2016) have shown that Bateman gradients are steeper in males than in females, which support the hypothesis that sexual selection acts strongly on males; they also provided evidence that a stronger sexual selection on males was linked to female-biased parental care and to a male-biased dimorphism (that is, males exhibited more elaborated traits, including behaviors). Observations on separate-sex species have indeed highlighted that females usually provide more care, even in species with biparental care (reviewed in Kokko and Jennions 2012). Likewise, enhanced motility has been defined by Parker (1978) “the first disparity in reproductive strategy between the sexes to follow anisogamy”. Although female mate searching can emerge under certain selective pressure (McCartney et al. 2012), in the majority of species, males search for females and are subjected to a stronger selection to evolve higher motility than females (Hammerstein and Parker 1987; Fromhage et al. 2016). Moreover, in *Caenorhabditis elegans*, in which sexually mature individual can be either hermaphrodites or pure males, mate-searching is a behavior stimulated by male-specific neurons, and, therefore, only the males move in search for hermaphrodites (Barrios et al. 2008).

The results of the present study, providing evidence for hermaphrodites investing resources in reproductive behaviors, highlights the importance of expanding the traits observed in sex-allocation studies. Sex-specific behaviors have indeed received little attention in the hermaphroditic literature and are surprisingly a missing topic in sex-allocation studies (Schärer 2009; Schärer and Pen 2013), despite a few papers have focused on differences in courtship driven by the sexual role individuals are willing to play (e.g., Fisher 1980; Leonard and Lukowiak 1984; Lorenzi et al. unpublished data). In separate-sex species, instead, resource allocation to pre-copulatory behaviors in response to mate competition has been shown to offer higher fitness returns than increased sperm production (Alonzo and Warner 2000). Males of both gobies and bitterlings, for example, increase aggression towards rivals when exposed to higher level of mate competition, while they do not modify sperm production (Scaggiante et al. 2005; Candolin and Reynolds 2002); likewise, crickets plastically adjust their investment in mate-guarding, but not that in ejaculates (Bateman et al. 2001). Higher levels of aggression in larger social groups have been found also in the few hermaphroditic species in which the investment in pre-copulatory behaviors in response to mate competition has been investigated (i.e., the shrimps *Lysmata amboinensis*, Wong and Michiels 2011; and *O. diadema*, Lorenzi et al. 2006; Santi et al. unpublished data).

The focus on gender-related behaviors allowed highlighting the negative correlation between female and male investment predicted by sex allocation theory, which has so rarely been observed in hermaphrodites. Hermaphroditic worms which were exposed to high mating opportunities and which moved more during the observation period were those which performed the least parental care. We could therefore recognize worms that invested more in the male function (i.e., moved more) and thus invested less in the female function (i.e., provided less care) and *vice versa*, providing one of the first experimental evidence for a behavioral trade-off (i.e., a negative correlation) between male and female allocation.

Behavioral trade-offs have been the focus of many evolutionary and ecological studies and have concerned several behaviors. For example, animals trade off foraging with behaviors aimed at avoiding predators (e.g., vigilance, sheltering, etc.) (e.g., Sih 1980; Verdolin 2006; Scharf et al. 2011); likewise, reproductive traits (including courtship) trade off with predation avoidance behaviors, foraging activity, shelter use and/or territorial defense (reviewed in Scharf et al. 2013). Interestingly, in separate-sex species, males trade off the investment in parental care with mate effort (i.e., behaviors aimed at gaining additional matings) (Magrath and Komdeur 2003). Males are expected to invest more in mate effort when population density (or the proportion of females) increases: for example, males of the cichlid fish *Herotilapia multispinosa* are more likely to desert their nests when the population sex-ratio is more female-biased (Keenleyside 1983). In contrast, males are expected to increase their investment in parental care with clutch size, and, indeed, in the European starling (*Sturnus vulgaris*), experimentally enlarged clutches triggered an increase in paternal investment and a decrease in courting behaviors (Komdeur et al. 2002). These findings are consistent with our results. Hermaphroditic worms performed more maternal and paternal care when the clutches they were caring for were larger, and decreased parental investment in favor of a higher motility, when population density was (or was perceived as) high.

Overall, this study provides evidence in support of sex-allocation theory. It is worth noting, however, that sex allocation theory, although studied mainly in relation to phenotypic plasticity, aims to predict evolutionary outcomes: one of the main goals of sex-allocation models is indeed to understand the environmental conditions which favor a sexual system over another (e.g., separate sexes over simultaneous hermaphroditism or *vice versa*) (Charnov 1982; West 2009). We may therefore speculate on how our results would translate in evolutionary terms. According to sex allocation theory, at low population density, female-biased hermaphrodites should be favored over separate sexes, since increasing the investment in the male role would not translate into higher fitness (saturating male fitness gain curve;

Charnov 1979, 1982). *O. diadema* worms are indeed expected to live in low density populations (Sella and Ramella 1999). Under these conditions, worms should be selected for investing more in the female function: producing more eggs (Lorenzi et al. 2005, 2006; Schleicherová et al. 2006, 2010) and providing more parental care, which further increases the reproductive success (this study). If population density raises so does mate competition and the opportunities to mate in the male role; selection should thus favor those individual that allocate fewer resources to egg production and parental care in favor of those behaviors that increase male reproductive success [i.e., aggression towards rivals (Lorenzi et al. 2006; Santi et al. unpublished data) and mate searching (this study)]. Despite empirical evidence for the adaptive value of aggression and motility in this species are still lacking, the greater motility of adolescent males compared to hermaphroditic adults has been proposed as the main selective advantage of the protandrous phase in *O. diadema* worms (Sella 1988; Sella and Ramella 1999).

The genus *Ophryotrocha* is a species-rich taxon, with more than 40 species; within the same genus, eight species are simultaneous hermaphrodites, while the others have separate-sex (except one sequentially hermaphroditic species). Phylogenetic analyses showed that simultaneous hermaphrodites represent a monophyletic clade, which seems to be the ancestral state (Dahlgren et al. 2001): the transition between sexual systems occurred only once, from hermaphroditism to separate sexes. On the basis of recent studies on *Ophryotrocha* worms, it has been hypothesized that hermaphrodites evolved into separate-sex species via androdioecy (i.e., hermaphrodites + pure males) under the pressure exerted by sexual selection on sex allocation (Picchi and Lorenzi in press). According to this hypothesis, increased mating group size (and thus mate competition) selected for hermaphrodites allocating more and more resources to the male function until the emergence in hermaphroditic populations of individual specialized in the male function (i.e., pure males); pure males allocate reproductive resources only in the male function, and thus should exhibit higher motility and lower parental care. Once pure males spread in hermaphroditic populations, the overall sex ratio would be biased towards males; which should increase the egg reproductive value, and favor hermaphrodites which invest more resources into the female function, until the population sex-ratio stabilize to 50:50, when remaining hermaphrodites specialize in pure females (Düsing 1884; Fisher 1930); pure females should therefore exhibit higher level of parental care and lower level of motility compared to pure males. In support of this hypothesis, hermaphroditic species in the genus *Ophryotrocha* have usually biparental care, while care tends to be mainly maternal in the separate-sex species (at least in the species where this trait

has been investigated, Sella and Ramella 1999). In contrast, less is known about motility in *Ophryotrocha* worms, if we exclude the higher motility described for adolescent males in hermaphroditic species. However, from a broader perspective, motility has been associated to the transition between hermaphroditism and separate sexes, and species having separate sex have been shown to have also the potential to move fast (Eppley and Jesson 2008).

In conclusion, our study, providing convincing evidence in favor of a trade-off between sexual functions, sheds new light on the importance of gender-specific behaviors in simultaneous hermaphrodites and highlights the need to consider these traits as part of the reproductive investment in sex allocation studies.

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# 6.

## **Egg availability and flexible mate preference in the hermaphroditic worm *Ophryotrocha diadema***

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In preparation



## Abstract

In recent years, an increasing number of studies have highlighted that, in hermaphrodites, mate choice often benefits only one of the two sexual functions and that which sexual function benefits depends on the preferred trait. Therefore, hermaphrodites that are willing to mate as males are expected to prefer different partners with respect to hermaphrodites willing to mate as females. Here, we advanced the hypothesis that, in the hermaphroditic worms, *Ophryotrocha diadema*, the egg maturation level should influence the mate preference, as it affects the mating role worms are able to play. We, therefore, tested whether individuals preferred partners with or without ready-to-lay eggs and whether the preference was consistently expressed by worms with and without ready-to-lay eggs. As an additional aim, we investigated the mechanisms of mate choice in these hermaphroditic worms, performing the tests in a Y-maze apparatus which prevented physical contact between worms. Our results show that hermaphrodites without eggs, and therefore ready to mate in the male role only, significantly preferred partners ready to mate as females (i.e., with ripe eggs), though this preference was less strong after the worms were allowed to mature new eggs. The fact that the worms exhibited a preference even when prevented from physically contacting other worms documents that chemical cues released by conspecifics in the water convey information on the presence and egg maturation level of potential partners, and play a key role in mate choice. Overall, this study shows that *O. diadema* hermaphroditic worms express a flexible mate preference, which depend on the egg maturation level, suggesting that the mating role hermaphrodites are willing to play may affect the traits they prefer in their partners.

**Key words:** chemical communication, choice test, mate choice, Polychaetes

## Introduction

Mate choice is a major component of sexual selection and plays a central role in shaping evolution (Darwin 1871; Andersson 1994). For example, female (and sometimes male) preference for certain traits drives the evolution of exaggerated ornaments and behaviors (Fisher 1930; Zahavi 1975; Hamilton and Zuk 1982; Kirkpatrick 1982; Kokko et al. 2003; Andersson and Simmons 2006; Ryan and Cummings 2013), such as the long tails of the widowbirds (Andersson 1982) or the dancing display performed by the blue-crowned manakins (Durães et al. 2009). Non-random mating can also be responsible for reproductive isolation, representing one of the driving forces of speciation (Lande 1981; Boughman 2002; Kirkpatrick and Ravigné 2002; Verzijden et al. 2005); for instance, it has been shown that divergent evolution in the visual system of cichlid fish (*Pundamilia* sp.) is associated with polymorphisms for male coloration and female preference, which have favored the sympatric speciation of this genus (Seehausen et al. 2008). Thus, it is not surprising that mate choice has received considerable attention (about 500 papers/year since 2008, Edwards 2014), increasing enormously our understanding of this evolutionary process in separate-sex animals.

Unfortunately, mate choice did not receive the same attention in research on simultaneous hermaphrodites (hereafter hermaphrodites), since hermaphroditic organisms, which produce and use both sperm and eggs, have struggled to be considered as targets of sexual selection. Darwin himself (1871), while laying the foundations of sexual selection, claimed that the “lowest classes” of animals (which include the majority of hermaphroditic species) “have too imperfect senses and too low mental powers to feel mutual rivalry, or to appreciate each other’s beauty or other attractions” (Darwin 1871, p. 321). Later on, despite the idea that hermaphrodites are the targets of sexual selection made its way in the scientific community (Charnov 1979; Arnold 1994; Leonard 2006; Lorenzi and Sella 2008; Anthes et al. 2010), the lack of sex-specific traits (such as ornaments) suggested that pre-copulatory sexual selection was weak in hermaphroditic organisms (Greeff and Michiels 1999) and biased the research focus on post-copulatory traits (Schärer and Pen 2013).

Only in recent years, scientists have begun to study mate preference in hermaphrodites, finding non-random mating in several species, though the evidence remains scarce (Anthes 2010). So far, empirical studies have shown that mate choice in hermaphroditic animals is based mainly on: 1) relatedness and immune function, 2) body size, and 3) mating status (Anthes 2010). Inbreeding is indeed often associated with fitness reduction (usually through

reduced offspring viability) and animals are therefore expected to avoid mating with related individuals. One of the mechanisms to avoid inbreeding depression is kin-discriminatory mate choice (Pusey and Wolf 1996). For example, the freshwater snails *Physa gyrina* and *P. acuta* recognize their relatives, which are rejected as partners more often than non-siblings (McCarthy 2004; Facon et al. 2006). Interestingly, the most “picky” snails (i.e. those that are more likely to reject related snails) are those mating in the female role (i.e., the individual providing the eggs), which are expected to pay for inbreeding depression higher costs than snails providing sperm (Jarne et al. 2000). Likewise, in freshwater snails *Biomphalaria glabrata* selected to resist to parasitic infection, individuals actively refused to mate as females, but not as males, with infected partners (Webster et al. 2003).

Another relatively common form of non-random mating in hermaphrodites is size-assortative mating. In hermaphrodites, indeed, the link between body size and fecundity (i.e., egg production) is often particularly strong, as larger individuals, not only should possess more resources to invest in reproduction, but may allocate proportionally more resources to the female function (as predicted by Klinkhamer et al. 1997 and Angeloni et al. 2002, and documented by Vizoso and Schärer 2007). Size-assortative mating is therefore expected to emerge as the preference for larger partners is shared by all individuals. A spectacular example of size-assortative mating comes from the flatworm *Dugesia gonocephala*, in which the two potential partners assess each other size by stretching out their body when in tight physical contact, assuming a “sandwich posture”, which is followed by copulation when the worms’ body sizes match (Vreys and Michiels 1997). Size-assortative mating has been found in several hermaphroditic species also in the meta-analysis performed by Graham and colleagues (2015), though they found random mating with respect to body size in their experimental test on *Physa acuta*.

As larger hermaphrodites are more fecund and are expected to invest more in the female function, the preference for larger partners should benefit sperm donors (individuals mating in the male function), rather than the sperm receivers. Consistently with this expectation, size-assortative mating is more common in hermaphrodites with reciprocal mating, where both partners mate contemporary as males (and as females), than in hermaphrodites with unilateral mating, in which only the individual mating in the male role is expected to prefer larger partners (Graham et al. 2015).

Finally, hermaphrodites have been shown to select their partner on the basis of their mating history. Internally fertilizing hermaphrodites can store allosperm for long periods (e.g., Nakadera and Koene 2013 for gastropods) resulting in high level of sperm competition. If the

cost of producing sperm is not trivial, hermaphrodites might be selected to avoid sperm competition. For example, in the flatworms *Dugesia polychroa*, previously isolated individuals (i.e., which did not mate for several weeks) are more attractive as partners and non-isolated worms are more likely to mate with them (Michiels and Bakovski 2000). Likewise, the opisthobranchs *Aeolidiella glauca* are more likely to reject previously mated individuals, which they recognize by the presence of external spermatophores deposited on their skin by previous partners (Haase and Karlsson 2004).

A particularly interesting aspect of the aforementioned examples is that, although hermaphrodites can mate both as females and as males, the preference for certain traits seems to benefit mainly one of the two functions: the relatedness-based mate choice can be defined as a female choice, while both size-assortative mating and preference for unmated individual favor primarily the male component of hermaphroditic fitness. The preference for a given partner should therefore depend on the mating role individuals are willing to play, so that hermaphrodites willing to mate as males and those willing to mate as females, may exhibit different mate choice. Whether and when hermaphrodites should prefer to mate in either one or the other role is debated; however, it has been argued that mating history, which affects the relative availability of gametes (eggs, autosperm and allosperm), may modify the mating role hermaphrodites are willing to play, resulting in a flexible mating role preference (Anthes et al. 2006). Assuming, for instance, no preference for mating in either sexual role, both allosperm depletion (following long period with no mating) and autosperm depletion (following recent mating in the male role) can drive a temporary preference for the female role (i.e. mating as females).

The theoretical framework for a flexible mating role preference, named “gender-ratio hypothesis”, was originally formulated for copulating hermaphrodites with internal fertilization and the ability to store sperm (Anthes et al. 2006); however, we might expect that the same framework applies also to external fertilizers, though with some significant differences. For instance, sperm storage is limited to internal fertilizers and cannot be accounted for in determining the mating role preference of external fertilizers. In contrast, egg availability is more likely to affect the preference for a given mating role in external fertilizers, since in hermaphrodites able to store sperm, copulation and egg fertilization (and sometimes egg production) are temporally separated. Therefore, we predict that in external-fertilizing hermaphrodites, recent mating as a female (that causes egg depletion) should result in a marked preference for mating in the male role, while long periods with no matings in the

female role, might favor a temporary preference for mating as a female, or, at least, should reduce the preference for mating as a male.

Since hermaphrodite mate choice benefit mainly one of the two sexual function, we predicted that the preference for a certain partner should reflect the preference for mating in a certain role and tested whether egg depletion affected the mate preference in the simultaneous hermaphrodite *Ophryotrocha diadema*.

The marine polychaete worms, *O. diadema*, conditionally reciprocate egg clutches with their partners (Sella 1985; Picchi et al. in press) and are more likely to lay eggs when paired with an ovigerous worm (i.e., which is able to produce eggs) (Sella 1988; Premoli and Sella 1995). They are external fertilizers and at each mating event they play either the male or the female role (that is, they have unilateral mating). Mating in the male role does not cause sperm depletion and worms still have available sperm after a fertilization event (Sella 1990); in contrast, during egg laying, hermaphrodites usually release all their mature eggs and need about 4 days to mature a new clutch. *O. diadema* worms are therefore virtually always ready to play the male role, while only intermittently ready to play the female role (hereafter we will refer at the availability or not of ready-to-lay eggs as egg maturation level).

We investigated whether *O. diadema* hermaphroditic worms preferred individuals with ready-to-lay eggs as partners and whether their preference was affected by their egg maturation level (i.e., whether the worms had or not ready-to-lay eggs). Moreover, since we lack information about the mechanisms involved in mate assessment and choice (Anthes 2010), we tested whether the preference for a certain partner could be mediated by chemical cues.

With this aim, we developed a 3D-printed Y-maze which prevented physical contact between worms and allowed the focal worms to move freely in the maze. We then investigated whether worms were attracted by chemical cues released by conspecific worms and did that in both the hermaphroditic species (*O. diadema*) and a closely-related, separate-sex species (*O. labronica*); we could thus investigate whether the attraction to conspecifics was linked to mate choice, testing whether males and females in the separate-sex species were significantly more attracted to individuals of the opposite sex.

Finally, we tested whether the hermaphroditic and separate-sex species differed in their responsiveness and ability to discriminate between potential partners.

## Materials and methods

### Study species

The genus *Ophryotrocha* is a species-rich taxon of polychaete worms (> 40 species), which includes both hermaphroditic and separate-sex species. According to phylogenetic analyses, hermaphrodites (8 species) belong to a monophyletic clade and likely represent the ancestral condition, from which separate sexes evolved once (Dahlgren et al. 2001).

The two *Ophryotrocha* species used in this experiment are a few millimetres long, transparent, marine worms, which are part of the infauna and inhabit eutrophic water (e.g., polluted harbor, Åkesson 1976; Thornhill et al. 2009).

In both *O. diadema* and *O. labronica* fertilization is external (there is not sperm storage) and achieved via pseudocopulation, a particular mating behavior in which gamete release occurs when the two partners are in tight physical contact (Westheide 1984; Sella and Ramella 1999; Lorenzi et al. in press). Pseudocopulation follows a long courtship (from several hours in *O. diadema* (Sella 1985) to several days in *O. labronica* (Åkesson 1974)) during which worms usually engage in tactile contacts and rubbing, possibly assessing the partner condition. During pseudocopulation, the worm mating in the female role (or the female, in separate-sex species) releases all its ripe eggs in a jelly cocoon, in which the worm in the male role (or the male) releases its sperm soon after.

In the separate-sex species, *O. labronica*, females release about 120 eggs per cocoon every one-two weeks (Premoli and Sella 1995), while hermaphroditic *O. diadema* worms lay about 25 eggs every 3-4 days (Sella 1985; Picchi et al. in press). Eggs take about 8 days to develop into free-swimming larvae, period during which one or both parents take care of the cocoon (Sella 1991; Sella and Ramella 1999; Picchi and Lorenzi unpublished data).

In the separate-sex species, sexual maturation is achieved in less than one month, when males start to produce sperm and females eggs (Paxton and Åkesson 2007; Lorenzi and Sella 2013). From this moment, males and females can be easily distinguished through secondary sexual characters: males are relatively smaller than females and have larger prostomium and thicker jaws, while females have visible eggs in their coelomic cavity.

*O. diadema* are protandrous simultaneous hermaphrodites in which sperm production start few days after free-swimming larvae hatch from the cocoon. Full sexual maturity is reached about 40 days later, when worms start to produce also eggs, and are able to mate both as males and as females; however, since they have unilateral mating, during pseudocopulation

they either release eggs (and mate in the female role) or release sperm (and mate in the male role).

Unilateral mating sets the stage for a conflict over the sexual role each individual should play during mating: since sperm are cheaper to produce than eggs, the fitness pay-offs are higher for the partner mating as a male (which gains the same fitness but invests less resources in gamete production), which leads to a shared preference for mating in the male role (Michiels 1998; Schärer et al. 2015). To solve this conflict, *O. diadema* worms conditionally reciprocate egg clutches, that is, they donate eggs in order to receive eggs by their partner (Sella 1985; Picchi et al. in press). It has been shown that hermaphroditic *O. diadema* worms prefer to mate with partners able to reciprocate: they lay eggs sooner when paired with a sexually mature worm than when paired with an adolescent male (which produces only sperm) (Sella 1988) and they desert their partners when offered worms with riper eggs (Sella and Lorenzi 2000). Conditional egg reciprocation makes *O. diadema* worms an ideal model to investigate the mechanisms of mate choice, since a preference for a given partner is predictable.

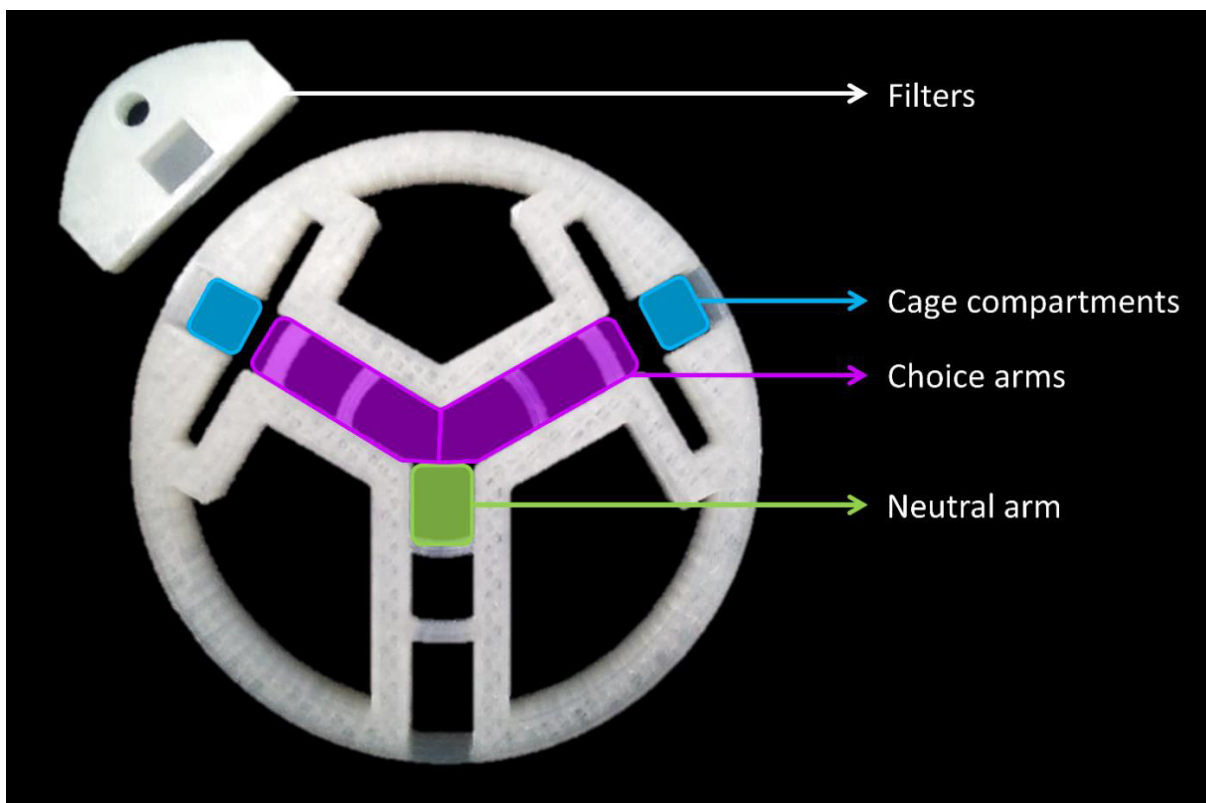
In the hermaphroditic species it is possible to identify individuals and assign egg maternity thanks to a natural polymorphism for the color of the eggs (Sella and Marzona 1983): wild-type worms produce yellow eggs, while albino worms produce white eggs and eggs are visible while they mature in the coelom through the transparent body walls. In the experiments, focal worms (i.e., the worms that made the choice) were always wild-type (i.e., yellow phenotype) individuals, while the “caged worms” (i.e., the worms offered to focal worms as a choice) were either wild-type or albino worms (depending on the experiment, see below).

The individuals used in the experiment were selected from the offspring of isolated pairs of worms, randomly taken from our laboratory cultures. This allowed us to know the age and mating history of the worms, which were sexually mature and virgin when they entered the experiment (unless otherwise stated). Worms were reared with their siblings in 20mL bowls until they started to mature eggs, then they were isolated in 9mL Petri dishes until they entered the experiment. Focal worms were not siblings.

Worms were kept according to Åkesson (1976) in artificial sea water (salinity 32‰), in the dark, in a thermostatic cabinet at a constant temperature of 21° C, and fed *ad libitum* with spinach.

### Y-maze apparatus

To test whether *Ophryotrocha* worms exhibited a preference for certain partners on the basis of the chemical cues they release, we molded semi-transparent plastic into the walls of Y-maze with the 3D-printer Ultimaker 2 Extended, which were then glued to Petri dishes with the silicon for aquaria JBL Aquasil. The Y-maze (Figure 1) consisted of two 2.7-cm-long arms (the choice arms) radiating from a third 1.4-cm-long arm (the neutral arm), each with a square cross section with sides of 1 cm. At the end of each choice arm there was a compartment (the “cage compartment”), separated from the arms by a 120  $\mu\text{m}$  mesh supported by a plastic frame which served as filter. In this way, water and chemical cues could flow freely in the maze, while the caged worms could not. The focal worm was instead released in the neutral arm and was free to move in the apparatus and visit either choice arms.



**Figure 1:** The Y-maze apparatus used for the experiment. Focal worms were free to move in the green and purple areas (the neutral and choice arms). All worms were prevented from entering the black areas.



### **Experimental set up**

Each day, before performing the behavioral observations, we measured the body size (i.e., the number of chaetigerous segments) of the experimental worms under a stereomicroscope Leica EZ4. Once measured, the caged worms were moved with a Pasteur pipette to the cage compartments of the Y-mazes (randomizing for side, to control for potential lateral biases). The Y-mazes were then filled with 6 mL of marine water, which we poured at the center of the maze, to fill the arms symmetrically and centripetally. Eventually, we released the focal worms in the neutral arm. The Y-mazes were then covered with a lid, to limit water evaporation (and the consequent increase in salinity), and kept under a cardboard box, to reduce light exposure.

After the start of the experiment, each 10 minutes for 70 minutes, we noted whether the focal worm was 1) in the neutral arm, 2) in the choice arm corresponding to the “correct” choice (i.e., the choice we expected) or 3) in the choice arm corresponding to the “wrong” choice (i.e., the non-expected choice). The observation time period was selected after testing for the preference of hermaphroditic worms which were offered the choice between conspecifics and food-enriched water (see below, Experiment 1) and documenting that 70 minutes were sufficient for worms to exhibit a preference.

After the observations, the mazes were thoroughly washed with tap and sea water before next use.

### **Experiment 1: Are *Ophryotrocha* worms attracted to conspecific chemical cues? (*O. diadema* and *O. labronica*)**

To test whether worms perceive and are attracted by the chemical cues produced by conspecifics, we offered focal worms the choice between a conspecific and food-enriched water (as traces of food might have been present in the cage with the conspecific worm). We tested in such way hermaphroditic *O. diadema* worms (N = 40) and both male (N = 40) and female (N = 40) *O. labronica* worms. Separate-sex individuals were given the choice between a worm of the opposite sex (male for females and female for males) and food-enriched water, while hermaphrodites (with ripe eggs) were given the choice between wild-type worms with

ready-to-lay eggs and food-enriched water. We predicted that focal worms would be more attracted to conspecifics than to water with food.

In this experiment, hermaphrodites were observed each 30 minutes for 150 minutes, while the separate-sex worms were observed each 10 minutes for 70 minutes.

### *Statistical analyses (experiment 1)*

To test whether worms preferred conspecific worms to food-enriched water, we performed Generalized Linear Mixed Models (GLMMs) for binomially distributed data where we included the focal worm ID as a random factor to account for repeated measures on the same individuals. We excluded all the observations in which the focal worms were in the neutral arm (i.e. they expressed no preference) and noted as 1 the observation when the worms were in the correct arm (i.e., attraction to conspecific) and 0 when they were in the wrong arm (i.e. attraction to food). According to the method proposed by Krackow and colleagues (2002) to test for biased sex-ratio, we performed intercept-only GLMMs to test whether the intercept differed from 0, so that non-significant values suggest that worms expressed no preference, positive significant values suggest that worms were in the correct arm more often than expected by chance and negative, significant values that worms made the wrong choice. We further tested (with GLMMs for binomially distributed data) whether the choice was affected by species, age (measured as the number of days since worms had been separated from their siblings), and body size of the focal worms. In *O. labronica*, we also tested for sex.

We also investigated whether responsiveness differed between hermaphroditic and separate-sex worms. Here we tested whether how often worms were in the neutral arm vs either choice arms (binary response variable, 0/1) depended on species, age and body size of the focal worms. As above, we tested for the effect of sex in *O. labronica*.

In each analysis, we selected the model using the Likelihood Ratio Test (LRT). The results of the model that explained the most variance (or of the intercept-only model, if models did not differ between each other), and the results of the LRT comparing the selected model with the models including all predictors are reported.

Statistical analyses (including the ones reported below) were performed in R v.3.5.0 (R Core Team 2018) using the *lme4* (Bates et al. 2015) and the *survival* (Therneau and Grambsch 2000) packages.

All variables were scaled and centered.

### **Experiment 2: Is attraction driven by mate searching? (*O. labronica*)**

We investigated whether chemical cues conveyed information about sex and whether males and females were attracted to opposite sex individuals. With this aim, we offered *O. labronica* males (N = 40) and females (N = 40) the choice between a male and a female worm, and we predicted that, if the choice was associated with mate searching, they would exhibit a preference for worms of the opposite sex.

#### *Statistical analyses (experiment 2)*

As above, we tested whether *O. labronica* worms expressed a preference for partners of the opposite sex with an intercept-only GLMM for binomially distributed data where we included the focal worm ID as a random factor. We further tested (with GLMMs for binomially distributed data) whether the choice was affected by the sex, age and body size of the focal worms.

### **Experiment 3: Does the egg maturation level affect the choice of hermaphroditic worms? (*O. diadema*)**

Finally, we tested whether the egg maturation level (i.e., whether worms had ready-to-lay eggs) of both the caged and the focal individuals (N = 40) affected the mate preference in hermaphrodites. To obtain sexually mature hermaphrodites without eggs, we paired the experimental worms with a partner and let each worm mate once in the female role. Worms without ready-to-lay eggs entered the experiment less than 24 hr after mating as female, while worms with ready-to-lay eggs were isolated at least 5 days after mating, to allow them to mature eggs again. Furthermore, to assess whether mate preference was flexible, we tested the same individuals as focal worms twice; first soon after they had mated as females (i.e., they had no eggs) and again after an isolation period (i.e., when they had ripe eggs). In this experiment both caged worms were albino (they produced white eggs) and each focal worm was offered the choice between a worm with ripe eggs (ready to mate in both sexual roles) and one without eggs (ready to mate in the male role only). We expected that focal worms had a preference for worms ready to play both roles (i.e., with ripe eggs), as these would be more likely to reciprocate eggs soon.

We eventually investigated whether the preference expressed by the focal worms with ready-to-lay eggs mirrored their mating behavior. With this aim, after the choice test, we randomly removed one of the two filters of the Y-maze (the filters separate caged worms from

focal worms) and allowed the focal and one of the caged worms to come into contact. We then observed the worms twice a day, for 5 days, and measured how long it took for the focal worms to lay eggs.

### *Statistical analyses (experiment 3)*

As in the previous experiment, we tested whether *O. diadema* worms expressed a preference for partners with ready-to-lay eggs with an intercept-only GLMM for binomially distributed data (focal worm ID as a random factor). We further tested whether the choice was affected by egg maturation level, age and body size of the focal worms, and, since body size is key factor in hermaphrodite mate choice, the relative body size of the two caged worms measured as a 3-levels factor (whether the caged worm corresponding to the correct choice was larger, smaller or the same size of the other caged worm).

We also investigated whether responsiveness differed between the hermaphroditic and separate-sex worms when they had to choose between two conspecifics, comparing the data of experiment 2 and 3. As in the first experiment, we tested (with a GLMM for binomial data) whether the likelihood to find the focal worms in the neutral arm depended on its species, age and body size. In *O. labronica* we further tested for the effect of sex, while in *O. diadema* we tested for the effect of egg maturation level of the focal worm.

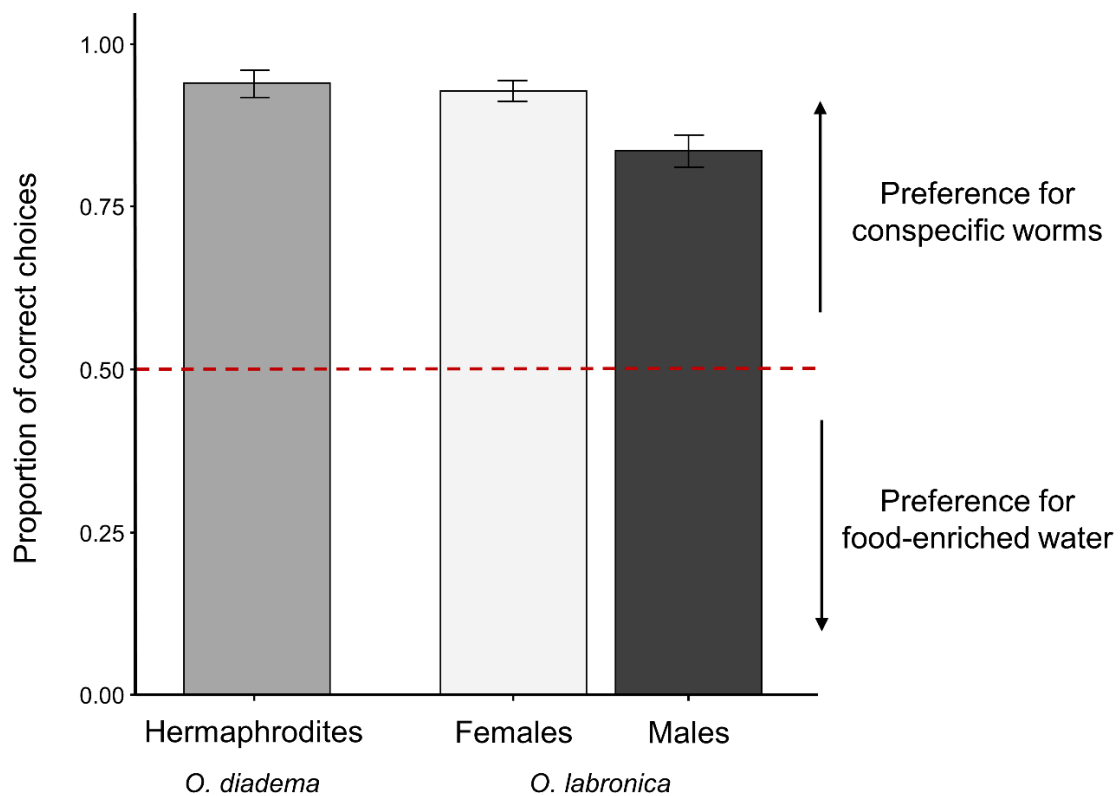
Eventually, in the hermaphroditic species, we tested whether the time interval to the first egg clutch laid by focal worms depended on the egg maturation level of the partner with a Cox's proportional hazard model, controlling for age and body size of the focal worm, body size of the partner and the time period spent in isolation after they had mated as females.

## **Results**

### **Experiment 1: Are *Ophryotrocha* worms attracted to conspecific chemical cues? (*O. diadema* and *O. labronica*)**

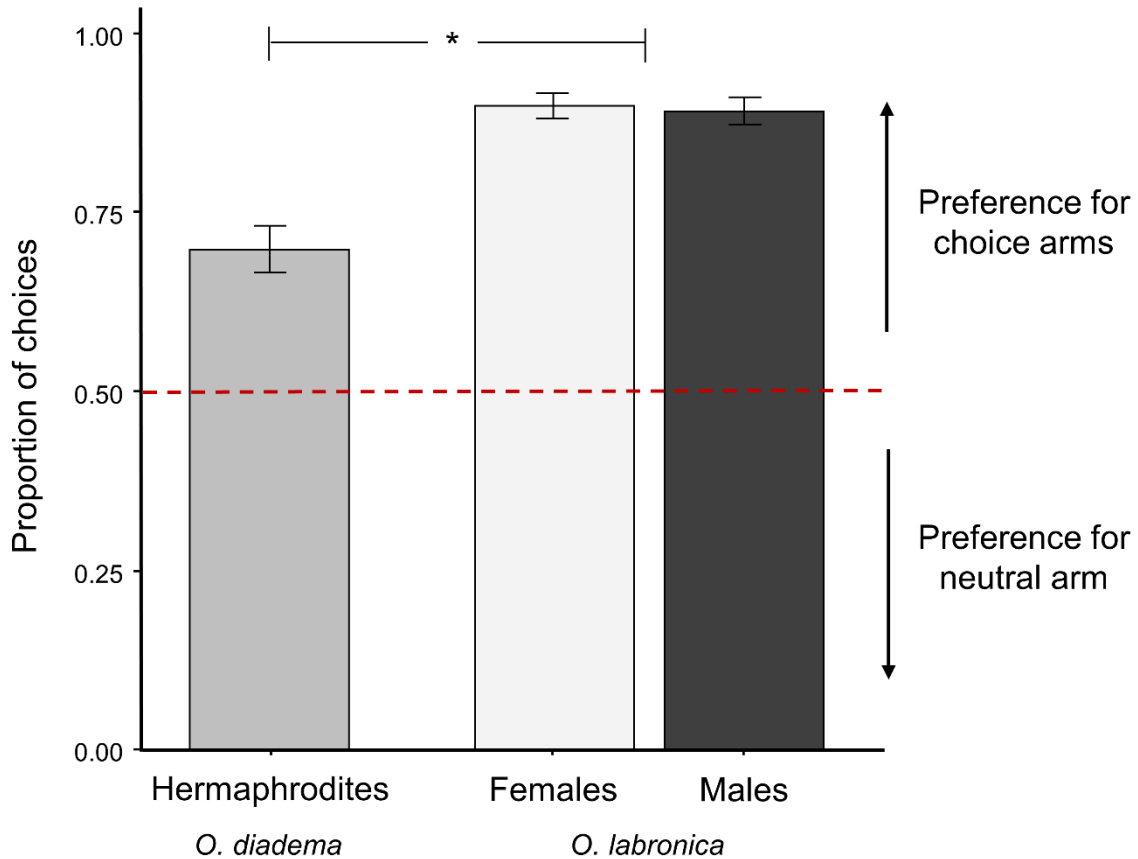
Overall, the focal worms visited the choice arm where a conspecific was caged (i.e., they made the correct choice) 76% of times while they visited the arm with food-enriched water 9% of the times; the remaining 15% of times the worms stayed (or moved back) in the neutral arm.

When we focused only on the observations in which the worms entered either choice arms, the proportion of correct choices was significantly higher than expected by chance (GLMM Intercept: Estimate  $\pm$  SE =  $7.316 \pm 1.106$ ,  $Z = 6.614$ ,  $n = 106$ ,  $p < 0.001$ ; Figure 2). Furthermore, focal worms made the correct choice regardless of species, age and body size, as shown by the lack of significant difference between the intercept-only model and the model including the predictors (LRT:  $\chi^2 = 1.277$ ,  $\Delta df = 3$ ,  $p = 0.734$ ). Likewise, choice was not affected by the sex of the focal worms in *O. labronica* (LRT:  $\chi^2 = 3.242$ ,  $\Delta df = 3$ ,  $p = 0.356$ ).



**Figure 2:** Proportion of choices for conspecifics vs food-enriched water in *O. diadema* hermaphrodites and *O. labronica* males and females. Bars represent mean values  $\pm$  S.E.

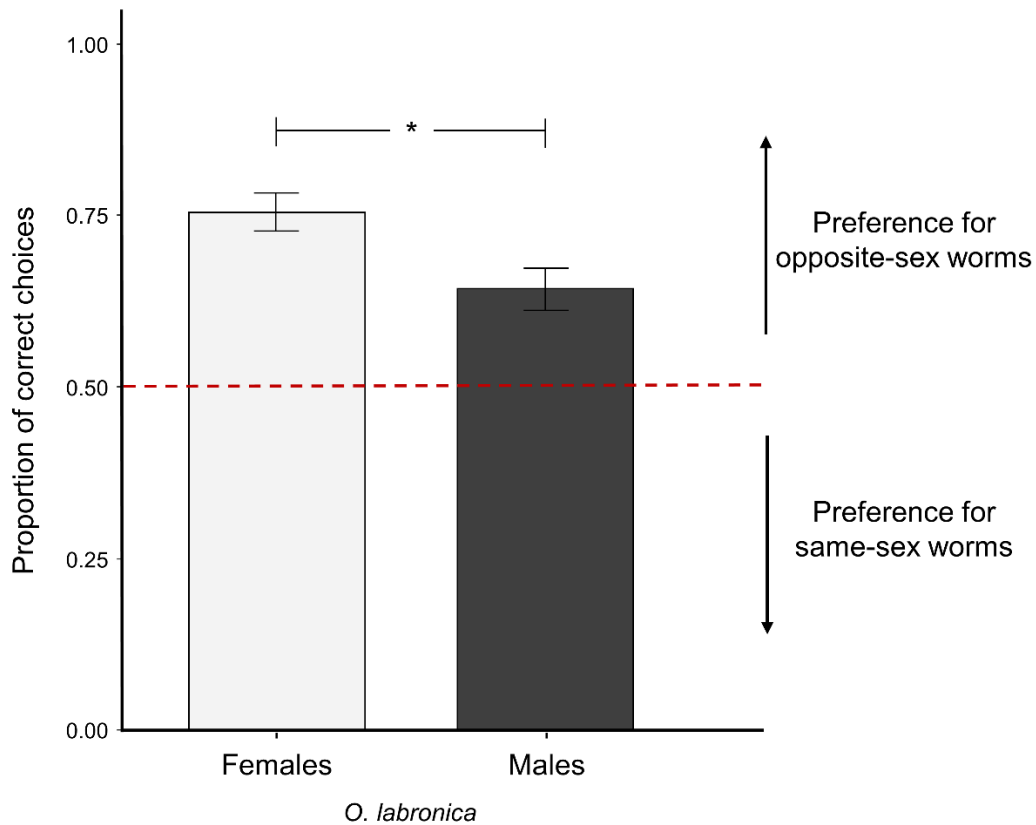
In contrast, as for responsiveness, the proportion of observations in which worms were in the neutral arm differed significantly between the two species: the separate-sex, *O. labronica*, focal worms entered more often the choice arms than the hermaphroditic *O. diadema* (GLMM Species [*O. labronica*]: Estimate  $\pm$  SE =  $2.576 \pm 1.114$ ,  $Z = 2.312$ ,  $n = 116$ ,  $p = 0.021$ ; Figure 3), though, on average, worms were more often in the choice arms than in the neutral arm (GLMM Intercept: Estimate  $\pm$  SE =  $3.551 \pm 1.540$ ,  $Z = 2.306$ ,  $n = 116$ ,  $p = 0.021$ ; Figure 3). There was no significant effect of age and body size (LRT:  $\chi^2 = 0.375$ ,  $\Delta df = 2$ ,  $p = 0.829$ ), as well as sex in *O. labronica* (LRT:  $\chi^2 = 0.900$ ,  $\Delta df = 3$ ,  $p = 0.825$ ).



**Figure 3:** Proportion of choices for the two choice arms vs the neutral arm when worms were offered the choice between conspecifics and food-enriched water in *O. diadema* hermaphrodites and *O. labronica* males and females. Bars represent mean values  $\pm$  S.E.

### **Experiment 2: Is attraction driven by mate searching? (*O. labronica*)**

When offered the choice between two conspecifics, *O. labronica* males and females visited the correct arm 64% of times, the wrong one 28% and stayed in the neutral arm the remaining 8% of times. Overall, *O. labronica* males and females were significantly more likely to visit the arm with the worm of the opposite sex than that with the worm of the same sex (GLMM Intercept: Estimate  $\pm$  SE =  $4.424 \pm 1.346$ ,  $Z = 3.287$ ,  $n = 72$ ,  $p = 0.001$ ; Figure 4), even though the proportion of correct choices was lower in males (GLMM Sex [male]: Estimate  $\pm$  SE =  $-1.416 \pm 0.688$ ,  $Z = -2.058$ ,  $n = 72$ ,  $p = 0.040$ ; Figure 4) and decreased with the age of the focal worms (GLMM Age: Estimate  $\pm$  SE =  $-1.764 \pm 0.805$ ,  $Z = -2.191$ ,  $n = 72$ ,  $p = 0.028$ ), while was not affected by body size (LRT:  $\chi^2 = 0.999$ ,  $\Delta df = 1$ ,  $p = 0.317$ ).



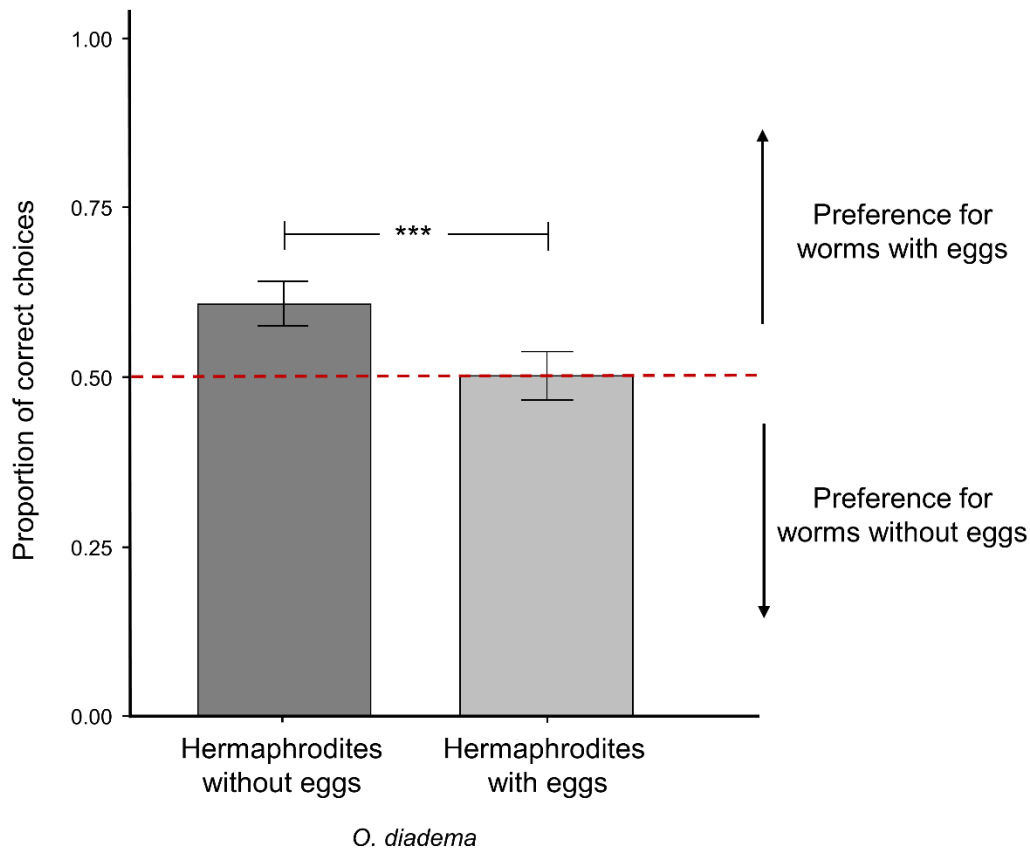
**Figure 4:** Proportion of choices for opposite-sex conspecifics vs same-sex conspecifics in *O. labronica* males and females. Bars represent mean values  $\pm$  S.E.

### **Experiment 3: Does the egg maturation level affect the choice of hermaphroditic worms? (*O. diadema*)**

In the hermaphroditic species *O. diadema*, focal worms which were offered the choice between two conspecifics that differed in their egg maturation levels, visited the arm with the partner with ripe eggs 42% of times, the arm with the worm without ripe eggs 34% of times (24% of times they stayed in the neutral arm).

When focusing only on the observations in which the worms made a choice, the proportion of correct choices was significantly higher than expected by chance (GLMM Intercept: Estimate  $\pm$  SE = 1.422  $\pm$  0.474, Z = 2.999, n = 41, p = 0.003; Figure 5). However, focal worms visited the correct arm more often when they had no eggs (GLMM Egg maturation level [ripe]: Estimate  $\pm$  SE = - 2.119  $\pm$  0.474, Z = - 4.474, n = 41, p < 0.001; Figure 5) and when they were larger (GLMM Body size: Estimate  $\pm$  SE = 1.300  $\pm$  0.367, Z = 3.546, n = 41, p < 0.001); instead, nor the relative size of the potential partners or the age of the focal worms had an effect (LRT:  $\chi^2 = 5.982$ ,  $\Delta$ df = 3, p = 0.112). Similarly, there was no

effect of the isolation period needed for egg maturation (when testing only worms with ripe eggs; LRT:  $\chi^2 = 4.236$ ,  $\Delta df = 3$ ,  $p = 0.237$ ).

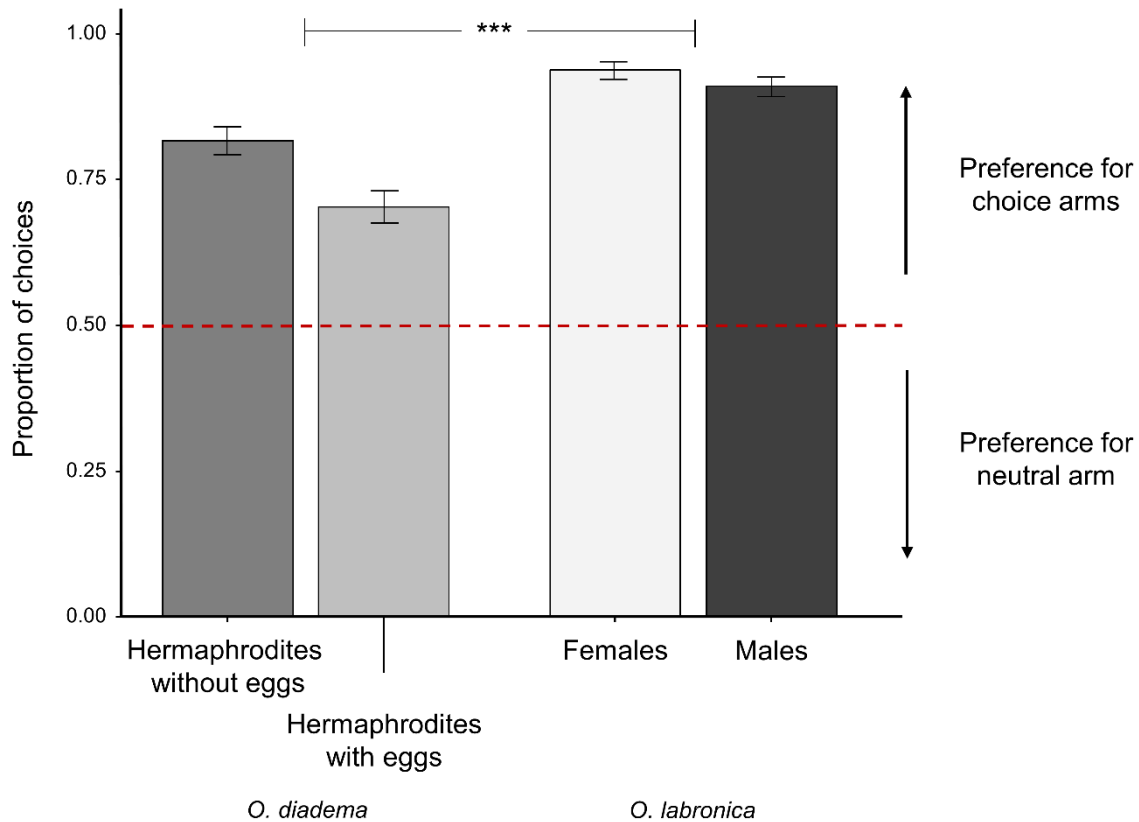


**Figure 5:** Proportion of choices for the worms with ripe eggs vs the worm without eggs in *O. diadema* hermaphrodites with and without ready-to-lay-eggs. Bars represent mean values  $\pm$  S.E.

The proportion of times in which the focal worms stayed in the neutral arm differed significantly between species also when worms were offered a choice between two conspecifics, with *O. labronica* worms visiting more often the choice arms than *O. diadema* (GLMM Species [*O. labronica*]: Estimate  $\pm$  SE =  $1.879 \pm 0.470$ ,  $Z = 3.996$ ,  $n = 115$ ,  $p < 0.001$ ; Figure 6), while none of the other factors affected focal worms' responsiveness (LRT:  $\chi^2 = 0.455$ ,  $\Delta df = 2$ ,  $p = 0.797$ ).

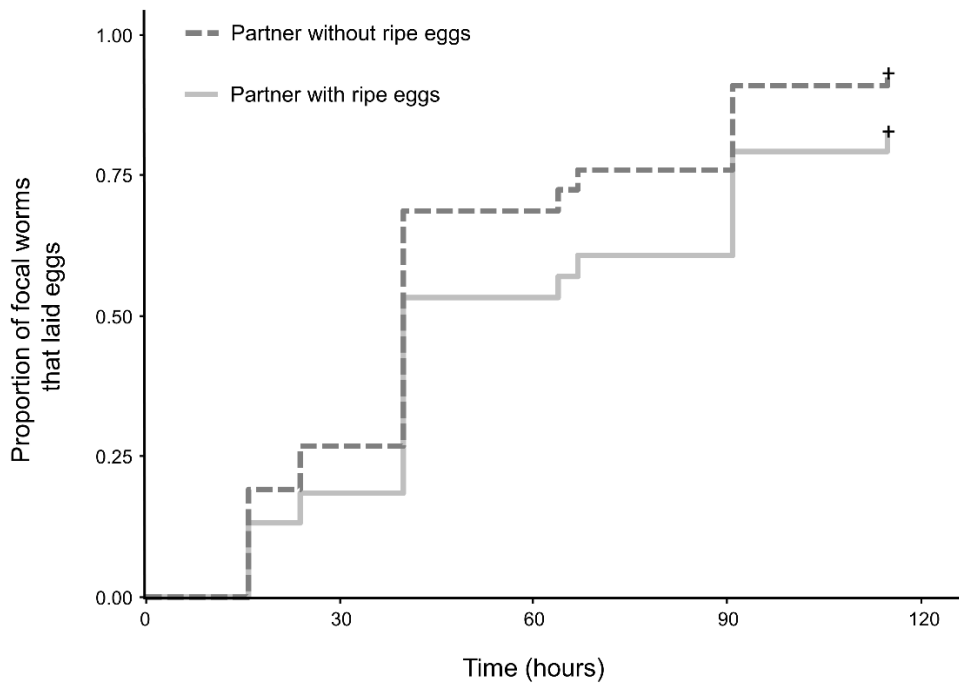
The proportion of times in which the worms stayed in the neutral arm was not significantly different between hermaphroditic worms with or without eggs (LRT:  $\chi^2 = 2.818$ ,  $\Delta df = 3$ ,  $p = 0.421$ ) nor between males and females in *O. labronica* worms (LRT:  $\chi^2 = 5.174$ ,  $\Delta df = 3$ ,  $p = 0.159$ ).





**Figure 6:** Proportion of choices for the two choice arms vs the neutral arm when worms were offered the choice between two conspecifics in *O. diadema* hermaphrodites with and without ripe eggs and *O. labronica* males and females. Bars represent mean values  $\pm$  S.E.

Finally, among the worms which were allowed to mate in the maze, 82.5% (33 out of 40) successfully laid eggs within 5 days. Focal worms were equally likely to lay eggs irrespective of the egg maturation level of the partner (Figure 7). Likewise, neither their body size, nor that of their partners nor the duration of the isolation period had an effect; in contrast, older worms took significantly longer to lay eggs (Cox-regression model Age: Estimate  $\pm$  SE = -1.070  $\pm$  0.367, Z = -2.917, n = 40, p = 0.003).



**Figure 7:** Probability of laying eggs by *O. diadema* focal worms as a function of time and depending on the egg maturation level of the partners. Lines represent the probability when the age is kept constant at the mean value. Crosses represent the censoring point (5<sup>th</sup> day).

## Discussion

Overall, these results show that *Ophryotrocha* worms are attracted by the chemical cues produced by conspecifics, which they use to get information about their potential partners. Both separate-sex and hermaphroditic focal worms preferred conspecifics with whom they could mate soon; in the hermaphroditic species, however, this choice depended on the egg maturation level, so that worms ready-to-mate-in-the-male-role-only preferred potential partners ready to mate also in the female role, while this preference was less clear-cut in worms ready to mate in both sexual roles.

The first conclusion we can draw from these results concerns the mechanisms of the mate choice. Using the Y-maze apparatus, we showed that the chemical cues produced by conspecific worm, not only are attractive for *Ophryotrocha* worms, but also convey information on the sex of individuals in *O. labronica* worms and on the egg maturation level in *O. diadema*. Focal worms, regardless of the species and the sex, were attracted to conspecifics and discriminated between males and females in the separate-sex species and between worms with or without ripe eggs in the hermaphroditic species.

The role of chemical communications in reproduction was firstly highlighted in hermaphroditic *Ophryotrocha* worm when they were shown being able to adjust their egg production in response to water-borne chemical compounds produced by their conspecifics, which they likely use to assess group size (Schleicherova 2006, 2010; Santi et al. in press). Moreover, males of the separate-sex *O. labronica* and of the sequential hermaphrodite *O. puerilis* are attracted by water where females were previously kept (Berglund 1990, 1991). However, this is the first time (to our knowledge) that chemical cues were proven to be attractive in hermaphroditic *O. diadema* worms, highlighting their potential role in mate searching and assessment.

Chemical communication has been deeply investigated in separate-sex animals, in which the central role of pheromones in searching for and choosing mates is well established (see for example Wyatt 2014); while less attention has been given to chemical communication in hermaphrodites, in particular with regard to mate choice, although some evidence has been collected over the years. For example, in the model organism *Caenorhabditis elegans*, in which adult individuals can be either simultaneous hermaphrodites or pure males (i.e. androdioecy), several chemical cues involved in mating behaviors have been identified and shown to mediate male and hermaphrodite attraction (recently reviewed in Chute and Srinivasan 2014). Likewise, mate searching is chemically mediated in the simultaneous hermaphrodite *Echinostoma* sp., a parasitic flatworm (Trouvé and Coustau 1999) and in the shrimp *Lysmata wurdemanni* (Zhang and Lin 2006). In the cestode *Schistocephalus solidus*, instead, the chemical cues produced by the individuals convey information about their inbreeding status, so that outcrossed cestodes prefer outcrossed partners over selfed ones, even if prevented from physical contact with them (Schjørring 2009).

It may be argued that attraction to conspecifics may involve social interactions not necessarily linked to mate searching. Animals often seek conspecifics to obtain benefits other than reproduction, such as protection from extreme weather condition (e.g., grouping for warmth), improved motility (e.g., the aerodynamic V formations of several migratory birds), increased foraging success, and diluted predation risk (Davis et al. 2012). It was, indeed, Hamilton (1971) who suggested that protection against predators should favor attraction to conspecifics and animal aggregation even in non-gregarious species. For example, guppies are able to count the number of conspecifics in a group and prefer to join larger shoals, where predation risk is diluted (Agrillo et al. 2008). Although offering worms the choice between a conspecific and food-enriched water do not allow disentangling between reproduction and other benefits provided by groups, results from the experiment in which we offered separate-

sex individuals the choice between a male and a female strongly suggest that *O. labronica* worms were seeking for potential partners: females were indeed attracted by males, and males by females. It is, however, less clear why females exhibited a stronger preference; we speculate that it may depend on the higher motility of males (Picchi and Lorenzi unpublished data), which might make them more likely to explore the entire maze in search for the partner. However, we have no data to support this hypothesis and further tests are needed to understand the cause of this difference between sexes.

In the hermaphroditic species, the hypothesis that worms were choosing mates is more difficult to document as all individuals are potential sexual partners. Nevertheless, worms ready to play the male role only (worms without ripe eggs) expressed a significant preference for worms ready to play the female role (i.e., with ready-to-lay eggs), that is, they preferred the worm with whom they could mate soon.

Interestingly, when the same individuals were allowed maturing eggs and were offered the choice between a partner with ripe eggs and another without, the clear preference they exhibited in the previous experiment disappeared: focal worms visited the choice arm with the partner ready to mate in female role significantly less often when they have matured new eggs. Consistently, focal worms allowed to mate with one of the caged partners did not lay their eggs sooner when paired with the worms with ripe eggs, which we interpreted as a further suggestion that focal worms had no preference when they had ready-to-lay eggs. Overall, these results suggest that hermaphroditic *O. diadema* worms were plastic in their mate preference for partner with ripe eggs.

In many animals, mate preference is not fixed, and both the preference function (that determines which trait values are preferred) and the “choosiness” (which determines the threshold to select a partner) are adjusted to extrinsic and intrinsic factors (Jennions and Petrie 1997; Ah-King and Gowaty 2016). Searching for and choosing a partner, indeed, implies several costs (Reynolds and Gross 1990), whose relative importance, compared to benefits, depends on the social environment and on endogenous factors, favoring a plastic strategy over a fixed one (Real, 1990). For example, in the threespine sticklebacks, females exposed to female-biased Operational Sex Ratio - OSR (where mate availability was low) both increased responsiveness to and decreased choosiness for partners as they aged, since with time passing and scarce mating opportunities, it paid-off to mate with a lower quality male rather than not mating at all. In contrast, when mate availability was high (male-biased OSR) and the cost of choosing a partner was lower, and choosiness was not affected by the age of the individuals (Tinghitella et al. 2013).

In hermaphrodites, however, a flexible mate preference (like the one found in this study) may reflect a flexible preference for the mating role. Hermaphrodites might indeed prefer to mate as males or as females depending on their mating history (Anthes et al. 2006). *Physa acuta* freshwater snails usually prefer to mate as males; however, individuals isolated for long period (which are allosperm depleted) prefer to mate as females, thus reversing their preference (Facon et al. 2007). Similarly, in the sea hare *Aplysia californica*, individuals that have mated in the female role prefer to mate in the male role, while those that have mated as males exhibit no preference (Ludwig and Walsh 2008).

Experimental evidence is accumulating which suggests that *O. diadema* worms prefer to mate in the male role (Sella 1988; Sella and Lorenzi 2000; Lorenzi et al. 2006; Di Bona et al. 2010; Santi et al. in press). Nevertheless, the availability of ready-to-lay eggs and the relatively long period of isolation (necessary to allow worms to mature new eggs) could have biased the preference for the female role. In this perspective, worms that have just mated sought partners ready to mate as females, since they preferred to mate in the male role, while worms which did not mate for several days, had no preferred role, which reflected in the absence of a mate preference, resulting in a flexible mate preference.

The results of this study highlight interesting differences between hermaphroditic and separate-sex worms in the responsiveness to the chemical cues produced by conspecifics. Hermaphrodites were less responsive than separate-sex worms, which exhibited more often a preference. The reasons for such a difference may be identified in the fact that, for hermaphrodites, any conspecific is a potential partner, while in the separate-sex species, finding the right partner (i.e., the one belonging to the opposite sex) is essential for mating. Indeed, since hermaphroditism double the likelihood of finding a suitable partner, hermaphroditic organisms should be favored when individuals face a low mate-encounter rate (Tomlinson 1966; Ghiselin 1969; Charnov 1976). If mate-encounter rate increases, hermaphroditism may become less stable and separate sexes are expected to be favored. Under these conditions, suitable partners are no longer limiting and having separate sexes reduces the effect of inbreeding depression and increases reproduction efficiency through division of labor (Charnov 1976; 1982; Leonard 2010). Mate-encounter rate can increase simply because population density rises and individuals are more likely to meet each other. However, even at equal population density, improved mate searching efficiency can increase the likelihood of finding a suitable partner and favor the evolution of separate sexes (Puurtilinen and Kaitala 2002, but see Eppley and Jesson 2008 for a comparative study). Our results are consistent with these prediction (and findings) since they show that separate-sex

worms were “better” than hermaphrodites at mate searching, as they spent more time in the choice arm corresponding to the correct choice, even when worms were offered the simple choice between a potential partner and food-enriched water.

In conclusion, this study offers interesting insight on mate searching and assessment in the hermaphroditic *O. diadema* worms, highlighting that egg availability, affecting the mating role worms are able to play, influences the preference for the mating partner. Furthermore, the higher responsiveness separate-sex, *O. labronica*, worms suggests that mate searching efficiency may have played a crucial role in the transition between sexual systems in the genus *Ophryotrocha*.

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**7.**

## **Discussion**

In both plants and animals, separate sexes have repeatedly originated from hermaphroditic ancestors (Renner 2014; Iyer and Roughgarden 2008; Eppley and Jesson 2008; Sasson and Ryan 2017), yet, in animals, the evolutionary pathway is still unclear (Weeks 2012, Leonard in press). Charnov (1979, 1982; but see also Janzen, 1977; Thomson and Barrett, 1981) advanced the hypothesis that sexual selection (particularly sperm competition) may act on the male and female functions of hermaphrodites and promote sexual specialization and the evolution of separate sexes. In other words, the social environment can shape hermaphrodite sex allocation and, thus, drive the evolution of sexual systems. The aim of this thesis has been to investigate whether and how cooperative and competitive interactions between individuals affect sex allocation pattern in *Ophryotrocha* polychaete worms. This aim has been addressed from different perspectives, as we tested 1) whether worms exhibited forms of cooperation that trigger biased sex allocation (**chapter 3**), 2) how competitive interactions shape sex allocation (**chapter 4** and **5**), and 3) whether the availability of (female) gametes affect the mate choice (**chapter 6**).

## Cooperation and the stability of simultaneous hermaphroditism

Egg-trading (Fischer 1980), in which “individuals give up eggs to be fertilized in exchange for the opportunity to fertilize the eggs of a partner” (Fischer 1988, p. 119), is a peculiar form of conditional reciprocity (Trivers 1971) exhibited by certain simultaneous hermaphrodites. The results reported in **chapter 3** strongly support the hypothesis that *Ophryotrocha diadema* hermaphroditic worms trade eggs with each other: worms are more likely to lay eggs after receiving them from their partner and they adjust the number of eggs per clutch to the amount of eggs donated by their partners.

A clear sex-role alternation during mating has been described in several hermaphroditic species and many hypotheses have been advanced to explain the evolution of this behavior. Like in *O. diadema*, the regular alternation of layings and fertilizations in the fish *Hypoplectrus* sp. and *Serranus* sp. may be explained by egg-trading (Fischer 1980, 1984, 1988; Petersen 2006; Hart et al. 2016). In the sea slugs *Navanax inermis* and *Chelidonura hirundinina*, it has been proposed that individuals trade sperm rather than eggs (Leonard and Lukowiak 1984; Anthes et al. 2005). The main difference between putative egg- and sperm-traders lies in the form of fertilization: egg-traders typically have external fertilization, while sperm-traders are internal fertilizers. If fertilization is internal, sperm recipients (but not

sperm donors) have control over fertilization, as the sperm might be digested rather than used for egg fertilization. Therefore, sperm donors experience higher variance in their reproductive success and, as Leonard (1990, 2005) suggested, these hermaphrodites might prefer to mate in the female role since the risk to waste female gametes is lower. In support to this hypothesis, *Chelidonura hirundinina* sea slugs experimentally prevented from transferring sperm (but allowed mating) received less penis intromissions and were abandoned as partners more often than control individuals (Anthes et al. 2005).

Non-random alternation of sexual roles has been described also in the snails *Physa acuta* and *Lymnaea stagnalis*, though, in these two species, alternation was not the result of conditional reciprocity, but rather the by-product of changes in the preference for the mating role (Facon et al. 2008; Koene and Ter Maat 2005).

To unravel the effects of conditional reciprocity on the evolution of sexual systems is crucial to investigate the causes underlying the regular alternation of sexual roles. We may identify two ways through which conditional reciprocity contribute to the stability of simultaneous hermaphroditism: maintaining a small mating group size, and biasing sex allocation towards the female function. If partners exchange gametes (either eggs or sperm) within a pair, the mating group size can be roughly considered as 1, even if this occurs at high population density, since reproductive interactions occur mainly within the pair (Charnov 1982). Furthermore, egg-trading (i.e., conditional egg reciprocation) increases the value of the eggs, favoring hermaphrodites that bias their sex allocation towards the female function, and preventing invasion from pure sex individuals (Henshaw et al. 2015). In an egg-trader population, pure males cannot invade because they have nothing to trade and, thus, have a low mating success; pure females, instead, might get a higher mating success than pure males, but need to get the double of the female fitness of a hermaphrodite to be able to successfully invade the population, which is hindered by the female-biased sex allocation of the hermaphrodites (see Charnov 1979 and Henshaw et al. 2015 for “the twice-the-fitness rule”).

### **Mate competition and sexual specialization**

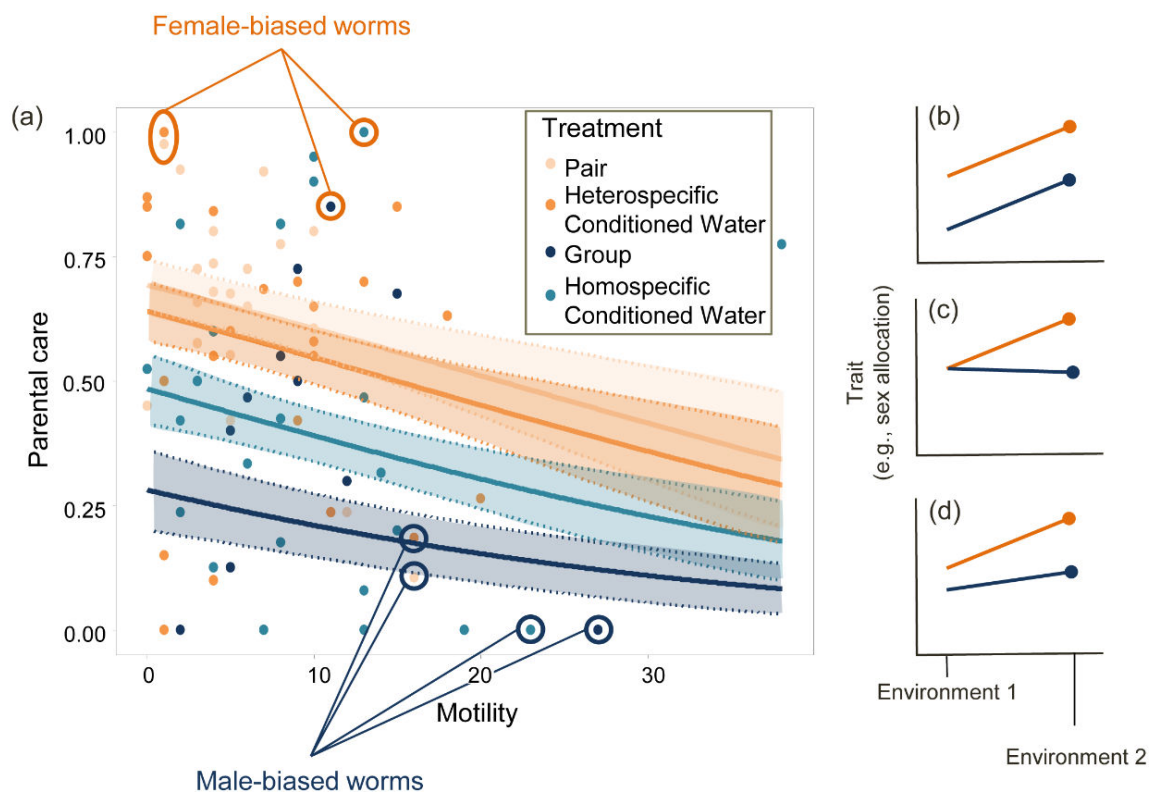
If cooperation may bias sex allocation towards the female function and stabilize hermaphroditism, (mate) competition may favor a male-biased sex allocation and promote the evolution of separate sexes. Evidence in support for more male-biased sex allocation when hermaphrodites are exposed to high mating opportunities has been found in several organisms



(both in laboratory condition, e.g., Trouvé et al. 1999; Tan et al. 2004; Janicke et al. 2013; Giannakara et al. 2016; and in the wild, e.g., Raimondi and Martin 1991; Hart et al. 2010; see Schärer 2009 for a review), including *O. diadema* worms (Lorenzi et al. 2005, 2006; Schleicherová et al. 2006, 2010, 2014). Yet, evidence for a trade-off between sexual functions (one of the main assumption of sex allocation theory) is surprisingly scarce. With this respect, the results of **chapter 4** and **5** offer a relatively unexplored perspective in the study of sex-allocation trade-offs in simultaneous hermaphrodites (Schärer and Pen 2013): the investment of reproductive resources may involve gender-specific behaviors. Until now, the focus on gamete production in most hermaphroditic animals, including *O. diadema* worms, has provided only partial confirmation of sex allocation theory prediction: hermaphrodites usually adjust either the male or the female investment to the group size. For instance, *O. diadema* worms adjust their investment into the female function to current mating opportunities, but do not modify their male investment accordingly, when male investment is measured as sperm production (Lorenzi et al. 2005, 2006). Including potential gender-related behaviors in sex-allocation measurement, however, highlighted that hermaphroditic worms adjust the allocation to the female function, measured both as egg production and parental care, *and* to the male function, which we measured as aggression and motility. Pre-copulatory traits have often been considered less effective than post-copulatory traits in contributing hermaphrodite reproductive success (Greeff and Michiels 1999) and therefore are usually ignored in sex allocation studies (Schärer and Pen 2013). However, in a recent study on the hermaphroditic snail *Physa acuta*, Pélissié and colleagues (2014) found that 40% of the variance in male reproductive success arises at the pre-copulatory stage, with a strong first-male sperm precedence (the first individual that donates sperm sires most of the offspring). These results suggest that selection acts on hermaphrodite pre-copulatory traits, favoring those snails that find and fertilize their partners earlier (Pélissié et al. 2014).

From an evolutionary perspective, our findings suggest that gender-related behaviors may be subject to selection promoting sexual specialization; this hypothesis is further supported by the behavioral sexual dimorphism exhibited by the closely-related gonochoric species, *O. labronica*: males are usually more motile, while females perform more parental care (**chapter 5**). Although appealing and partially supported by evidence, this hypothesis remains speculative to some extent. Two points need further investigations before we confirm that selection acting on gender-related behavior, has favored the emergence of separate sexes: 1) the heritability of these behaviors, and 2) their effect on male and female fitness. Despite these two aspects were not the focus of this thesis, results of **chapter 5** offer some interesting

insights. For logistic reasons, we could not associate motility with the reproductive success in the male role, but we were able to document that parental care affected egg survival (confirming a previous study by Sella 1991). On the other hand, though we did not investigate whether motility and parental care were genetically inherited, our results showed that worms kept in the same experimental conditions exhibited a certain degree of variation in their relative investment in motility and parental care; within the same treatment, we could identify individuals that moved more and performed less parental care (male-biased worms) and individuals that exhibited the reverse pattern (female-biased worms) (Figure 1a). The inter-individual variation in sex allocation within the same treatment suggests that there might be a certain degree of variation either in the genes coding for the relative investment in motility versus parental care (Figure 1b) or in those coding for the response to environmental cues (Figure 1c) (or an interaction between the two, Figure 1d): that is, the worms might express different sex allocation patterns or might have different reaction norms.



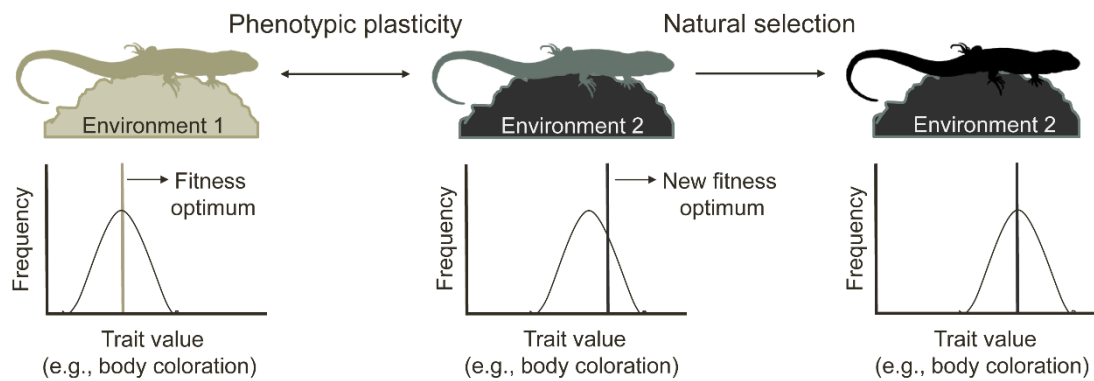
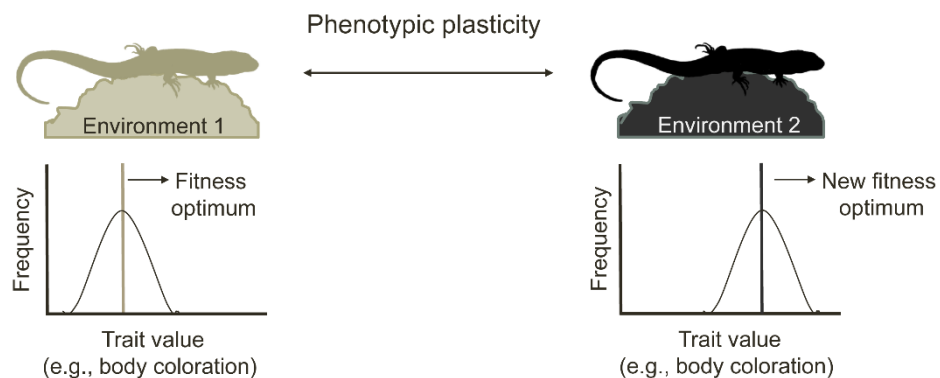
**Figure 1:** (a) Trade-off between female (i.e., parental care) and male (i.e., motility) investment; a behaviorally female-biased (dark orange) and a male-biased (dark blue) worm per treatment are highlighted. (b, c, d) Potential explanations for the within treatment, inter-individual variation are: (b) Worms differed in their average sex allocation, regardless of the environment; (c) worms differed in their reaction norms; (d) worms differed in their average sex allocation and in their reaction norms.

Another interesting point highlighted in this research, and in particular in **chapter 4**, is the extreme phenotypic flexibility of these worms relative to sex allocation adjustments. In one week, worms are able to modify both their egg production and the amount of aggressive behaviors to face the (actual or perceived) changes in the level of mate competition. The link between phenotypic plasticity and genetic adaptation is a hotly debated topic in evolutionary biology, since, theoretically, phenotypic plasticity might either enable or hinder genetic adaptation (see, for instance, Pigliucci 2007; Perry et al. 2018).

### **Phenotypic plasticity and evolution**

On one hand, phenotypic plasticity allows individual to survive in novel environments, enabling natural selection to act on the extant genetic variation or on *de novo* mutations (e.g., Baldwin 1896; West-Eberhard 2003; Crispo 2007; Schlichting et al. 2014; Levis and Pfennig 2016; see also Schwander and Leimar 2011 and Levis and Pfennig 2018 for empirical evidence; Figure 2a).

A recent study by Corl and colleagues (2018) provides empirical evidence of the genetic basis underlying this synergy between plasticity and evolution in the side-blotched lizards, *Uta stansburiana*. These lizards usually have light coloration, which facilitates crypsis in the light soil that characterizes their typical natural (lava-off) environment; one population, however, inhabits the Pisgah Lava Flow (southern California) and is characterized by a melanic coloration, allowing the matching with the dark color of the lava flow. Keeping (dark) lava and (light) off-lava individuals in dark and light substrates, the authors showed that both type of lizards were phenotypically plastic with respect of body coloration; however, the lizards inhabiting the lava flow were able to achieve a darker coloration when exposed to a dark substrate, which suggested a genetic adaptation to the novel dark environment. Exome sequencing and the analyses of genetic differentiation of the lava and the nearby off-lava populations showed that relatively few genes were highly differentiated between populations, two of which are involved in melanin production. The variants of these genes were not found in the off-lava populations or in lava populations other than the Pisgah Lava Flow population, which suggests that they have a recent origin. In view of these results, the authors advanced the hypothesis that, in side-blotched lizards, the extant phenotypic plasticity allowed lizards to survive in the novel environment (the lava flow) while natural selection acted on *de novo* mutations emerged over time in the Pisgah Lava Flow population, favoring those individuals with a darker coloration.

**(a) Phenotypic plasticity enabling evolution****(b) Phenotypic plasticity hindering evolution**

**Figure 2:** Schematic representation of the role of phenotypic plasticity in genetic evolution. (a) Phenotypic plasticity for body coloration allows colonization of and survival in the dark environment but does not allow reaching the fitness optimum; natural selection then acts on the population favoring the darker individuals (which match the dark environment better) and shifts the body coloration towards a darker color. (b) Higher phenotypic plasticity allows “perfect” matching in both the original and the novel environment and permits reaching the fitness optimum.

Under the hypothesis that phenotypic plasticity enables genetic adaptation, the ability of hermaphrodites to plastically adjust sex allocation can be viewed as the first step in the transition from hermaphroditism to separate sexes (Lorenzi and Sella 2013).

**Phenotypic plasticity versus evolution**

On the other hand, it has been hypothesized that, if plasticity is adaptive and allows approaching the fitness optimum in the novel environment, it can prevent genetic evolution, as individuals attain high fitness via the plastic phenotypic response (Price et al. 2003; Ghalambor et al. 2007; Figure 2b). This hypothesis has received poor empirical support, since

organisms typically have moderate plasticity which does not allow reaching the fitness optimum (Price et al. 2003); however, some hermaphroditic organisms may tell a different story.

In an experimental evolution study on the hermaphroditic flatworm *Macrostomum lignano*, Janicke et al. (2016) showed that worms which evolved under enforced monogamy differed from worms evolved under higher mate competition levels in the shape of the male copulatory organ and in sperm morphology; other traits, however, did not change, such as, for instance, sex allocation (i.e., testis area divided by total gonad area). Interestingly, most of the traits that did not evolve were also the most plastic, which may suggest that in this species phenotypic plasticity prevents the genetic evolution of certain traits (Janicke et al. 2016). Similarly, it has been proposed that phenotypic plasticity (in particular in the form of labile gender expression) may prevent the evolution of separate sexes from gynodioecious ancestors, since males may maintain the ability to produce fruits and seeds (Delph and Wolf 2005).

Unfortunately, the relation between phenotypic plasticity and evolution has never been investigated in *Ophryotrocha* worms and, as mentioned before, phenotypic plasticity has been considered as the first step of the evolutionary transition between sexual systems in this genus (e.g., Lorenzi and Sella 2013); however, the high flexibility of sex allocation highlights that it would be worth investigating whether plasticity can hinder genetic evolution in this genus. Interestingly, there is between-species variation in the level of female allocation plasticity of the hermaphroditic *Ophryotrocha* worms (Schleicherová et al. 2014): *O. diadema* and *O. adherens* worms are highly plastic (consistently with the results presented here for *O. diadema*) and adjust their female allocation to current mating opportunities, while *O. gracilis* worms are not. Male allocation (measured as sperm production), instead, is not plastic in the three species. If plasticity can limit genetic evolution in *Ophryotrocha* species, these results suggest two non-mutually exclusive scenarios for separate sexes to evolve in this genus: gonochorism may originate more frequently from hermaphroditic species with low level of plasticity, so that the hypothetical hermaphroditic ancestors of the current *Ophryotrocha* gonochoric species were likely more similar to *O. gracilis* than to *O. diadema* and *O. adherens*; on the other hand, selection may have acted more strongly on those traits that are less plastic, such as sperm number.

## Egg availability and reproductive behavior

In the light of the results discussed until now, sex allocation appears as a key feature in the evolution of sexual systems; **chapter 6** offers yet another perspective on this subject. **Chapter 6** highlights that egg availability may play a central role in mate preference in hermaphrodites. Worms without ripe eggs have a clear preference for partners with ready-to-lay eggs; however, this preference is no longer expressed when worms mature eggs. We discussed these results in the perspective of the “gender ratio hypothesis” proposed by Anthes and colleagues (2006); according to the Authors the preference for mating in the female or male role is not fixed, but can be affected by several factors and notably, by the mating history of the individual. For instance, in internal fertilizers, long periods of isolation, and the consequent depletion in allosperm, can trigger a preference for mating in the female role (or a less strong preference for mating in the male role) (e.g., Facon et al. 2007; Ludwig and Walsh 2008). According to the gender ratio hypothesis, we interpreted the flexible mate preference exhibited by *O. diadema* worms as a switch in the preference for the mating role triggered by egg availability; worms without ready-to-lay eggs expressed a marked preference to mate as males, while worms with ready-to-lay eggs preferred to mate in the female role or had no preference. Under this view, we may speculate that sex allocation pattern might have a similar effect on mate preference of hermaphrodites, so that more male-biased individuals should prefer more female-biased partners, as they should strongly prefer to mate as males. If this was the case, the preference expressed by the male-biased individuals would result in a higher frequency of matings between individuals with opposite phenotype (i.e., disassortative mating: male-biased individuals mating with female-biased ones). Interestingly, this outcome recalls some key aspects in the evolution of anisogamy where selection favored the small gametes (sperm) that fused disassortatively with large gametes (eggs) (Parker 1978).

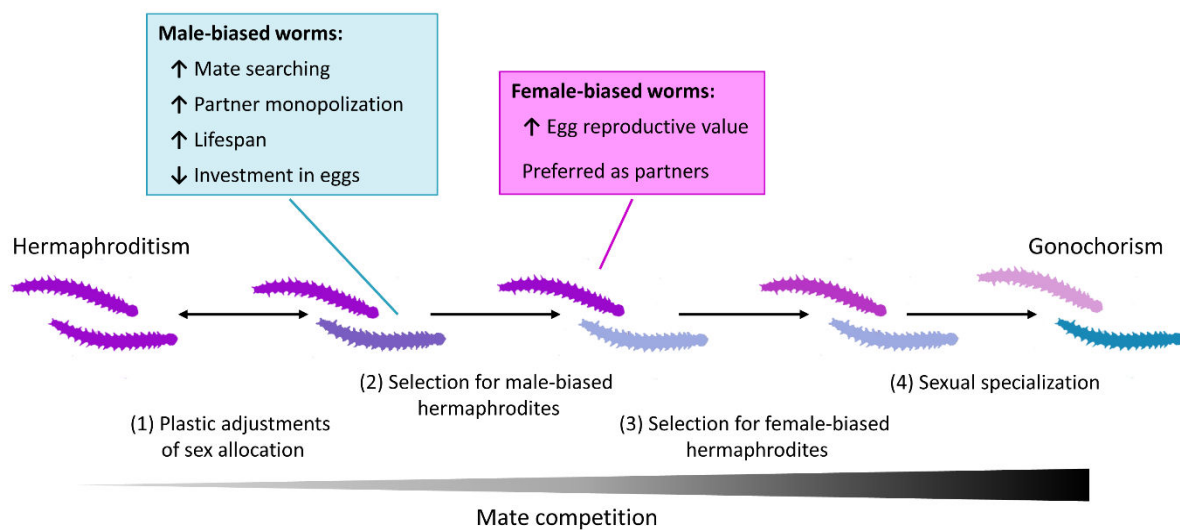
In animals, to our knowledge, there are no studies on the role of disassortative mating in promoting sexual specialization; however, in plants, negative frequency-dependent selection is expected to maintain sexual polymorphism in presence of disassortative mating (Baker et al. 2000; Barrett 2002; Gleiser et al. 2008; Shang et al. 2012).

## Conclusions

### **The (hypothetical) transition from simultaneous hermaphroditism to separate sexes in *Ophryotrocha* worms**

We would like to conclude this manuscript re-proposing a hypothetical pathway from hermaphroditism to separate sexes in light of the new findings of this thesis. In **chapter 2**, we advanced the hypothesis that gonochorism might have evolved from hermaphroditism via androdioecy, an intermediate step in which pure males and hermaphrodites coexist in the same population. Androdioecy is expected to bias the average reproductive investment of the population towards the male function and, according to Fisher's theory of equal investment, should increase the reproductive value of hermaphrodites with female-biased sex allocation, favoring the evolution of separate sexes. As a consequence, androdioecy is expected to be evolutionary unstable and short living and, consistently with this expectation, it has been shown that it typically has recent origin (Zierold et al. 2007). In *Ophryotrocha* worms, androdioecy has never been reported, which might depend upon its evolutionary instability; however, there may be another explanation. Compared to plants, where gonochorism is expected to evolve via gynodioecy that originates from a mutation of the mtDNA (Delph and Wolf 2005; Charlesworth 2006), animals may have undergone a more gradual transition (**chapter 2**; Leonard in press). The gradual transition in *Ophryotrocha* worms, instead of resulting in the evolution of pure males first and pure females later on, might favor the spread of male-biased hermaphrodites under certain conditions. Male-biased hermaphrodites, before evolving in pure males, would exert a pressure on the other hermaphrodites for a larger allocation to the female function; disruptive selection would then favor the contemporary emergences of pure males and pure females. This second hypothesis seems to be more consistent with the presence of both "males with oocytes" and "female with sperm" in functionally gonochoric species, such as *O. labronica*, *O. macrovifera*, and *O. robusta* (Lorenzi and Sella 2013; Meconcelli et al. 2015). Notwithstanding that actual androdioecy may never evolve, the key steps and selective pressures favoring the evolution of separate sexes proposed in **chapter 2** remain basically unchanged. Indeed, as we explained in **chapter 2**, we expect that the first step in the transition to separate sexes involves an increased allocation of reproductive resources from the female to the male function in a subset of the population, triggered by a rise in population density (and, thus, increased mate competition). In dense population, therefore, hermaphrodites are expected to reduce their allocation to egg

production and parental care while increasing motility and aggression. These male-biased hermaphrodites should then spread in the population, as they spare resources from egg production and parental care (Lorenzi et al. 2005; 2006; Schleicherová et al. 2010; 2014; **chapter 4** and **5**), find and monopolize partners more easily (Lorenzi et al. 2006; **chapter 4** and **5**), seek actively for partners with ready-to-lay eggs (**chapter 6**), and are likely to have longer lifespan (Di Bona et al. 2010). Once male-biased hermaphrodites spread in a population, negative frequency-dependent selection should favor those individuals which invest relatively more of their reproductive resources in the female function (egg production and parental care), and whose reproductive success (as females) would be further increased by the fact that they are preferred as partners by male-biased hermaphrodites. Eventually, disruptive selection should favor individuals that specialize in one sexual function only (Figure 3). Consistently with this pathway, some gonochoric species of the genus *Ophryotrocha* are thought to maintain vestigial traits of their hermaphroditic past (i.e., the ability to produce, but not use, gametes of the opposite sex) and a labile sex determination mechanism (Lorenzi and Sella 2013; Meconcelli et al. 2015).



**Figure 3:** Schematic representation of the hypothetical transition from hermaphroditism to separate sexes in *Ophryotrocha* worms.

In further support of this hypothesis, recent phylogenetic analyses have shown that the level of mate competition is associated with the evolution of sexual systems (Yusa et al. 2012; Erisman et al. 2013) and that the presence of pure males can bias hermaphrodite sex allocation towards the female function (Dorken and Pannell 2009; Dreyer et al. 2018).



Overall, this thesis shows that cooperation and competition between individuals are crucial factors in hermaphrodite sex allocation (as interactions between relatives are crucial to determine sex ratios in gonochoric species, West 2009) and, as a consequence, in the evolutionary transition between sexual systems.

## Future directions

Although this thesis has the merit, in our opinion, to draw the attention on hermaphrodite reproductive behaviors (traits which are often ignored in the hermaphroditic literature), it also highlights that our knowledge of the genetics and reproductive physiology of *Ophryotrocha* worms is too scarce to unravel the complexity of the mechanisms underlying the evolution of sexual systems. Indeed, to understand the evolutionary processes, it is essential to know the genetic mechanisms involved in these processes. For instance, we hypothesized that disassortative mating might favor the evolution and maintenance of the sexual polymorphism (i.e., separate sexes). This hypothesis assumes that disassortative mating favors individuals with biased sex allocation, since female-biased hermaphrodites are preferred as partners (**chapter 6**) and male biased hermaphrodites are more motile and aggressive, and, likely, better at searching for and monopolize mates (**chapter 4 and 5**). For disassortative mating to favor the evolution of separate sexes, however, not only sex allocation patterns must have a genetic basis (Di Bona et al. 2015), but the phenotype of the offspring must also reflect the phenotype of only one of the two parents; that is, pairs of one male-biased and one female-biased hermaphrodite should originate worms that are either male-biased or female-biased. However, assuming a polygenic determination of sex allocation (Di Bona et al. 2015) we may also hypothesize another scenario. A study on the timing of sex change in the sequential hermaphrodite, *O. puerilis*, has shown that disassortative mating between individuals selected for late and early sex change reversed the effect of selection: the offspring changed sex at a time intermediate between that of the parents (Bacci and Bortesi 1961). Similarly, disassortative mating between male- and female-biased simultaneous hermaphrodites may produce offspring with a more even sex allocation. Studies on the genetics of sex allocation are therefore needed to understand whether or not disassortative mating might be involved in the transition between sexual systems.

Another key aspect of *Ophryotrocha* reproductive biology that deserves further investigation is chemical communication. Here we show that chemical compounds are used to

estimate group size and adjust sex allocation accordingly (see also Schleicherová et al. 2006; 2010). However, despite their key role, these chemical compounds are still unidentified and it remains unclear whether they are simple cues or have evolved to become signals. We also found that the chemical compounds produced by conspecifics are attractive for *Ophryotrocha* worms and are used for mate searching and assessing; although not surprising, these findings describe a new function of chemical communication in *O. diadema* hermaphroditic worms and suggest that chemical compounds might mediate other kind of inter-individual interactions. For instance, we speculate that chemical compounds may be involved in the reciprocal exchange of egg clutches: *O. diadema* worms might trigger egg maturation and release in their partners to reduce the risk of cheating (i.e., non-reciprocating partners). Compounds with this kind of effect have been described in several nereid polychaetes, which are broadcast spawners and avoid wasting gamete through synchronous gamete release (Hardege 1999; Watson et al. 2003). Unfortunately, we do not have empirical data on the role of chemical signals in reciprocating *O. diadema* worms, but further investigation on this subject may provide interesting information about the nature of sexual conflict in hermaphrodites. Sperm donors should indeed benefit from inducing partners to produce more eggs, and hermaphrodites are expected to evolve physiological mechanisms enabling partner “feminization” (Charnov 1979; Michiels 1998). In the freshwater snail, *Lymnaea stagnalis*, it has been shown that proteins transferred with the ejaculate delay egg laying and promote the production of larger eggs and the reduction of the male investment in the partners (Koene et al. 2010; Hoffer et al. 2012; Nakadera et al. 2014; reviewed in Koene 2017). However, it remains unclear whether this manipulation implies some costs for the partner and whether it may be interpreted in the light of sexual conflict (Koene 2017). From this perspective, it will be particularly interesting to be able to identify the chemical compounds produced by *O. diadema* worms and test their effects.

Eventually, a point that is particularly fascinating and deserves further investigation is the link between phenotypic plasticity and evolution. We believe that experimental evolution studies aimed at comparing the evolvability of sex allocation between species or between traits (within species) that express different levels of plasticity may prove fruitful in this regard. Such studies will indeed allow to test whether plasticity can hinder genetic evolution in those species (or those traits) that are more plastic, while enabling it in less plastic species (or traits) (e.g., the faster evolution of less plastic traits in Janicke et al. 2016). Investigating whether phenotypic plasticity hampers evolution may also offer new insights on the current distribution of sexual systems across the Metazoa (i.e., the William’s paradox), providing an

explanation for higher stability of sexual systems (in particular of hermaphroditism) compared to other reproductive traits, such as the mode of spawning (Kerr et al. 2011): if plasticity hinders evolution, hermaphrodites may be less likely to evolve separate sexes.

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# **Annex A.**

## **Courtship behavior as a war of attrition in a simultaneous hermaphrodite**

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## **Abstract**

In outcrossing hermaphrodites with unilateral mating, where at each mating interaction one individual assumes the female role and the other the male role, each individual must take a sexual role opposite to that of its partner. In the polychaete worm *Ophryotrocha diadema*, the decision on sexual role is likely at stake during the day-long courtship. Here we describe for the first time courtship and pseudocopulation in this species, quantify their pre-copulatory behavior, and search for behavioral traits predicting the prospective sexual role (i.e., behavioral sexual dimorphism), by analyzing the courtship behavior of pairs of worms during the day preceding a mating event. We did not find any behavioral cue predicting the sexual role worms were to play; partners' pre-copulatory behaviors were qualitatively and quantitatively symmetrical. We interpret this as the outcome of a war of attrition where partners share the preference for the same sexual role, and both hide their 'willingness' to play the less preferred one, until one individual reaches its cost threshold and accepts the less preferred sexual role.

**Keywords:** Behavioral sexual dimorphism; communication; mating; sexual conflict; sexual roles

## Introduction

In animals with separate sexes, males — whose reproductive success is often limited by access to females — typically spend resources and time in male-male scramble competition, which involves searching for and attracting females, and/or in contest competition; in contrast, females make larger investments in gametes and their reproductive success is more limited by the quality of mate (Andersson 1994; Bradbury and Vehrencamp 2011). Courtship behaviors between males and females have traditionally been interpreted as cooperative behaviors between partners, serving mutual species identification, synchronization of mating activity, and establishment of a pair bond. They are now often interpreted as components of a process during which individuals mutually assess their partner's traits, physiological condition [e.g., nutritional status, mating history, readiness to mate (Jennions and Petrie 1997)] and quality [good genes, health status, etc. (e.g., Milinski and Bakker 1990; Kotiaho 2002)] come at the expense of the other individual. While mating may involve the common goal of partners to pass on genes via offspring production, it also involves potential conflict between partners as to how resources are to be invested (Parker 2006).

If courtship is the expression of the conflict of interests between sexes in separate-sex animals, what about hermaphrodites? In simultaneous hermaphrodites (hermaphrodites hereafter) the two sexual functions coexist in the same individual, and each individual is fully functional via both sexual roles. When the two partners meet, each individual may have some degree of choice about whether it will donate or receive sperm, i.e., play the male or the female role, or both, during mating (Schärer et al. 2014). The conflict over which partner invests more resources in the offspring can, therefore, be extremely strong in hermaphrodites (Michiels 1998) and may be expressed in their pre-copulatory behavior. Unfortunately, we often lack descriptions of these behaviors, since there has been more focus on the post-copulatory component of sexual selection in hermaphrodites (Parker 1970; Schärer and Pen 2013).

Simultaneous hermaphroditism is widespread among animal taxa, and there are substantial differences in the reproductive biology and reproductive modes of hermaphroditic organisms (Jarne and Auld 2006, Leonard 2006). Hermaphroditic partners may exchange sperm reciprocally in a single mating (bilateral mating) or do it in separate mating events (unilateral mating) and may have internal or external fertilization. The amount and kind of conflict between the interests

of the two individuals, and their pre-copulatory behavior may thus vary. For example, courtship is virtually absent in the planarian *Dugesia polychroa*, where sex roles are fixed: two partners reciprocally exchange sperm within seconds after they meet, as soon as they adjust their physical positions so that their ventrally-located gonopores come into contact (Peters et al. 1996). In contrast, in hermaphrodites with unilateral mating, courtship may be decisive and may play a key role in the decision about the sexual role the two partners will play. The case of the opisthobranch *Navanax* may represent an example of this situation. Leonard and Lukowiak (1984) described how these mollusks with internal fertilization and sperm storage alternate playing the male role during ‘bouts’ of repeated copulations, which occur over a period of 3-7 h; in each mating, one individual donates and the other receives sperm. Courtship (which implies exploring the partner’s parapodia with the head) is initiated by the ‘male’ partner, which advertises its ‘willingness to risk currency gametes’ — in this case, sperm, since the sperm receiver can digest part of them (Leonard and Lukowiak 1984, 1985). Therefore, there is a behavioral dimorphism between partners, as the courtship behavior of each individual depends on its prospective role (Leonard 2006).

In the fish *Hypoplectrus nigricans*, partners alternate sexual roles up to eight times during a 2-h-long time period of daily mating interactions (Fischer 1980). Their displays predict the sexual role: head snapping and quivering, as well as who begins the courtship behavior, denote the ‘female’, and partners switch between behaviors as they switch role (Fischer 1981). Likewise, the serranine fish, *Serranus tortugarum*, and *S. tigrinus*, which form long-lasting monogamous pairs, display their prospective sexual role during courtship (Pressley 1981; Hart et al. 2016;).

Hermaphroditic *Ophryotrocha* polychaete worms have repeated mating interactions where partners alternate sexual roles, but alternations occur at intervals of days: each worm lays a clutch of eggs approximately every four days (Sella and Ramella 1999). The relatively long time interval between clutches means that pairs are more likely to break up between two successive matings (e.g., Sella and Lorenzi 2000). The conflict of interest over who is going to play which role may therefore be stronger: the cost of playing the less preferred role is higher when the likelihood of successfully switching to the preferred role is lower.

Pre-copulatory behaviors are expected to provide exchanges of information about the partner’s quality (e.g., egg abundance and ripeness, body size, etc.), and to promote synchronization between egg and sperm release, but also involve decisions about the role each

partner is 'willing' to play during mating, particularly if the two partners share a preference for the same role. Mate assessment can occur through chemical and/or tactile signals, but, when behavioral interactions occur, we expect that they contribute to the exchange of information.

In this study we describe for the first time the courtship behavior and pseudocopulation of simultaneously hermaphroditic *Ophryotrocha diadema* Åkesson 1976 polychaetes, quantify their behavioral interactions, and examine the relationship between these behaviors and subsequent sexual roles.

We reasoned that, if individuals have no preference for a particular sex role, and courtship has the main function of attracting partners and synchronizing gamete release, mates should make clear to each other the sexual role they are going to play during copulation soon after courtship starts; the prospective sexual role should be unambiguously advertised during courtship so that partners take complementary sexual roles. Likewise, if individuals change their sex role preferences at each mating interaction depending on the potential fitness gains in copulating as a male vs. as a female (as predicted by the gender ratio hypothesis, Anthes et al. 2006), we expect that individuals should readily advertise their preference. In both cases, courtship is expected to be an honest advertisement of individual quality and decisions.

Alternatively, in case there is a shared preference for a sexual role and a consequent conflict of interests between partners over who is going to play what role, we expect that courtship will be symmetrical, with partners performing the same behaviors, each concealing its motivation to make concessions in terms of sexual roles, and 'resisting' the partner's solicitation to accept the less preferred sexual role.

## **Materials and Methods**

### **Study species**

*Ophryotrocha diadema* polychaete worms (Dorvilleidae) are 3-4-mm-long simultaneous hermaphrodites living in nutrient-rich marine areas, such as harbors.

In this species, mating between mature hermaphrodites occurs after a long courtship involving repeated body contacts and rubbing (8 h or more, Sella 1985). Fertilization is external

and is thought to occur in a way similar to that described for the congeneric species *O. gracilis*: one worm releases eggs and the other its sperm inside a jelly egg-cocoon, a process called pseudocopulation, as it implies a physical contact between partners (Westheide 1984). Pseudocopulation usually occurs only between undisturbed worms, in the dark, making it very difficult to observe.

When they are sexually mature (at a body length of about 13-14 segments), paired worms can lay eggs as often as every four days (Picchi, unpublished) and often alternate egg laying and fertilization between partners (Sella 1985). Body walls are transparent, which makes it easy to assess the egg maturation level of each individual. Eggs are relatively large (diameter: 180  $\mu\text{m}$ ) and each clutch contains 25 eggs on average (Sella 1990). Sperm (diameter: 3  $\mu\text{m}$ ) are immotile (aflagellate sperm, Berruti et al. 1978), and produced in low numbers (Sella 1990; Premoli and Sella 1995), two characteristics which strongly suggest limited post-copulatory sperm competition (Morrow 2004).

During the experiment, worms were kept in artificial sea water, in thermostatic chambers, at 21 °C. Sea water was changed once a week and worms were fed spinach ad libitum. Egg-cocoons were regularly removed from the bowls.

## **Experimental procedure**

### *Description of pseudocopulation*

We recorded a pseudocopulation sequence under restricted space (the lid of a 1.5 mL Eppendorf tube), and here, for the first time, describe the process in *O. diadema* worms.

### *Pre-copulatory behavior*

We set up 50 pairs of mature hermaphrodites in 20 mL glass bowls, half full of artificial sea water, and each pair was composed of an ‘albino’ (white phenotype) and a wild (yellow phenotype) worm (the focal worm, see below); color phenotype allowed unambiguous individual recognition of the worms in the pair and made it possible to assign egg maternity, and therefore, sexual roles during mating (i.e., which worm laid eggs, which implies the other fertilized them). Paired worms had similar body size (number of segments at the beginning of the experiment in  $n = 26$  pairs: median = 18 segments for both focal worms and their partners, range 14-20; sign test,  $z = 15$ ,  $p = 0.134$ ).

At variable time intervals after setting up pairs (range: 1-39 days, mean  $\pm$  s.d.:  $27 \pm 14$  days), we recorded the worms' behavior for 30 consecutive mins. Before video-recording, we noted the body size of the focal (yellow) worm and the relative level of egg maturation of the two partners (higher in the focal worm, higher in its partner, or equal between worms). We analyzed the videos by measuring any interaction between the focal worms and their partners. We scored Rubbing, Following and Staying Side by Side, as time spent (duration); we scored discrete behaviors involving contacts between partners as frequency (number of occurrences) (see below for the description of the 18 behaviors observed). We considered any behavior as active when the focal individual initiated it and as passive when the partner initiated it.

The day after recording the video, we checked whether one of the two worms had laid eggs, which implied that it had played the female role and that the partner had played the male role. Thirty-nine pairs laid eggs within 24 h. Excluding five pairs where both worms laid eggs, we obtained 34 videos where the focal worm was to play either the male or the female role within 24 h, i.e., 17 h of video that we used in the present data analysis.

All observations were done using a video camera (Leica IC80 HD) integrated to a Leica M80 stereomicroscope (magnification 7.5).

### **Statistical analyses**

We performed a PCA (correlation matrix, Varimax rotation) on all 18 behaviors that the focal worms performed or received, in order to identify correlated behaviors. The PCA extracted six principal components (PCs), which cumulatively explained 69.9% of the variance.

To determine the relationship between behaviors and sexual role, we ran Generalized Linear Models (GLMs, binomial distribution, logit link) whose binary response variable was whether the focal worm would play the male or the female role within the next 24 h (i.e., whether it laid or fertilized eggs) and whose predictors were the relative egg-maturation level (as a factor, three levels), the focal worm body size, the six PCs summarizing the behavioral interactions, and the duration of the acclimatization period (days) (to account for synchronization within the pair) (as continuous covariates).

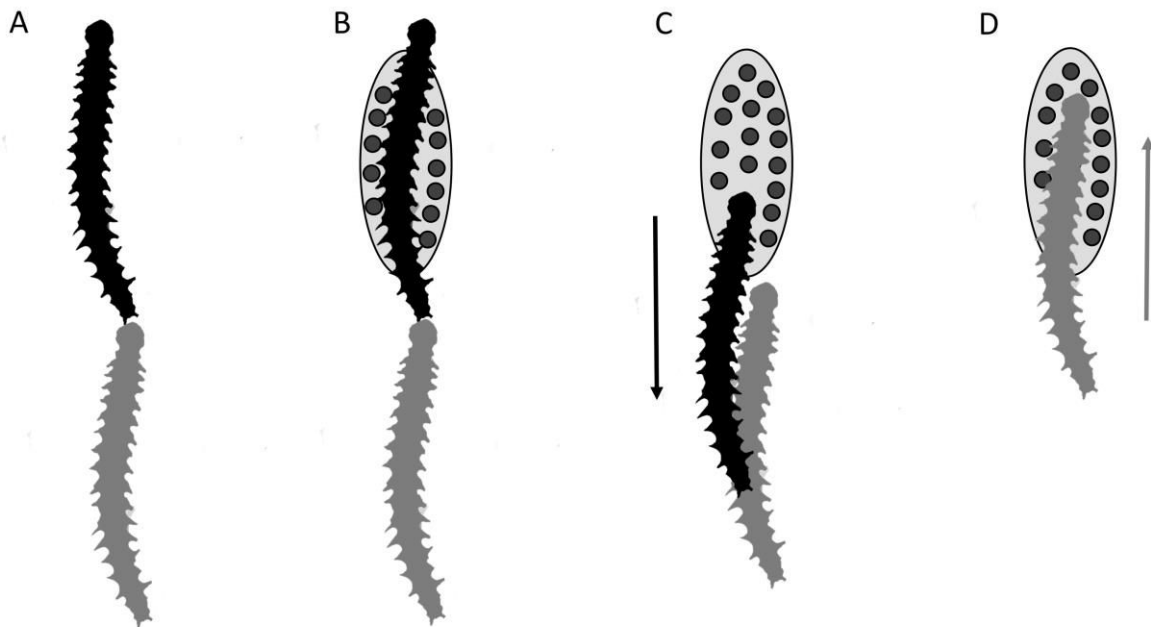
Statistical analyses were performed in R, version 3.4.1 (R Development Core Team 2016), except for PCA, which was run in IBM SPSS Statistics 23.0.



## Results

### Description of pseudocopulation in *O. diadema*

Immediately before pseudocopulation, the two worms engaged in a 2-min-long Head-Tail contact, during which they were almost immobile, and the female-role worm (i.e., the one who was to lay eggs) was in front of the male-role partner. Then, the ‘female’ began to lay eggs, until she left the cocoon, sliding over the ‘male’. Soon the ‘male’ entered the egg-cocoon, passed over the eggs (presumably laying its immotile sperm) and left the cocoon, emerging from the opposite side (See the video in the Supplementary Material and Figure 1).



**Figure 1.** Schematic representation of pseudocopulation in *Ophryotrocha diadema*: (A) Worms stay immobile in Head-to-Tail contact; (B) the female-role worm (in black) lays eggs inside the jelly cocoon and then (C) leaves the cocoon by sliding over the male-role worm (in gray); (D) the male-role worm enters the cocoon and (likely) releases its sperm. The arrows indicate the direction of movement of the female-role worm (black arrow) and of the male-role worm (gray arrow).

The pseudocopulation sequence (i.e., egg laying + egg fertilization) lasted one minute, and was characterized by egg spawning preceding sperm release. Worms stayed in physical contact up to the moment when the male entered the egg cocoon.

*Pre-copulatory behavior*

The pre-copulatory behavior of the worms involved the following behavioral interactions (Figure 2):

- Rubbing: first described by Sella (1985). Worms slide along each other's bodies, often in an antiparallel direction, then one makes a U-turn and they repeat the sliding. Rubbing occurred up to once per minute, and each rubbing sequence had an average duration of  $13.3 \text{ s} \pm 2.8$  (mean  $\pm$  s.d.).
- Staying Side by Side: the two worms stay still, their bodies in contact all along their sides, usually in an antiparallel orientation.
- Following: while walking rapidly on the substrate, one worm crawls after the other at a close distance (less than a worm's body length).

We also scored a large variety of brief contacts between worms ( $< 2 \text{ s}$ ), where one worm 'tapped' the other lightly. Depending on the body parts involved, the contacts observed were scored as follows: Head to Head Contacts, Head to Body-Side Contacts, Head to Pygidium (hereafter, Head to Tail) Contacts; Tail to Tail Contacts, Tail to Head Contacts, Tail to Body-Side Contacts (also 'Kicks'); Body-Side to Head Contacts, and Body-Side to Tail (Figure 2 and Table 1).

Long-lasting behaviors		
Behavior type	Duration (s) (mean $\pm$ s.e.) [% of total time]	Schematic representation
Rubbing	143.2 $\pm$ 22.8 s [8%]	
Staying Side by Side	107.8 $\pm$ 33.2 s [6%]	
Following active & Following passive	41.2 $\pm$ 14.1 s [2.3%]	
Contacts		
Behavior type	Frequency (mean $\pm$ s.e.) [% of contacts]	Schematic representation
Head to Head	2.0 $\pm$ 0.5 [19.3%]	
Tail to Tail	0.4 $\pm$ 0.1 [3.7%]	
Head to Tail & Tail to Head	6.2 $\pm$ 1.0 [58.5%]	
Head to Body-Side & Body-Side to Head	1.8 $\pm$ 0.3 [17.3%]	
Tail to Body-Side & Body-Side to Tail	0.1 $\pm$ 0.1 [1.2%]	

**Figure 2.** Schematic representation of the behaviors described in this study and their mean frequency in 30 min (A: active worm; P: passive worm). For the long-lasting behaviors (i.e., Rubbing, Staying Side by Side, and Following), the mean duration and the mean percentage of the time dedicated to these behaviors during the observations are reported. For the contacts, the mean number of occurrences and their percentages of the total number of contacts are reported. The arrows indicate the direction of movement of the worms.

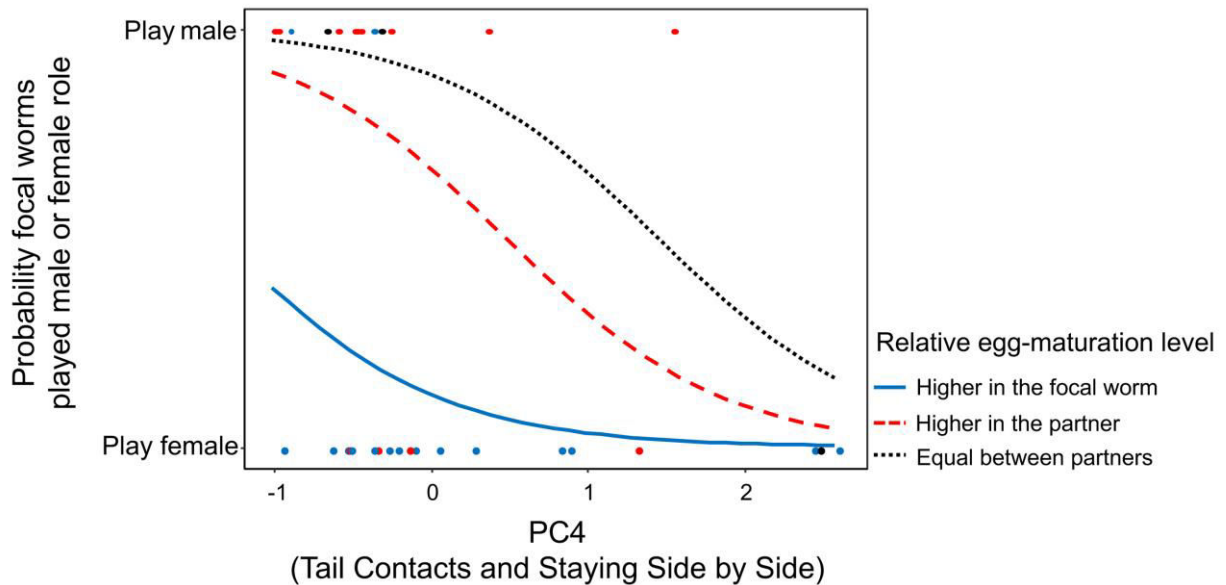
**Table 1.** Factor loadings on the 6 PCs (Rotated Component Matrix). The factor score loadings (> 0.650) of the behavioral variables on the PCs, where high loadings indicate that the variable was highly correlated with the PC (variables with lower loadings have been removed from the table). The PCs are sorted by the relative variance they explain. In bold the variables the load on the same PC and represent the same behavior, in its active and passive form. PC4 was the only significant predictor of sexual role.

Behaviors	Component					
	1	2	3	4	5	6
Active Head-Tail: Focal taps partner's tail with head	0.827					
Passive Head-Tail: Partner taps focal's head with tail	0.653					
Rubbing	0.654					
<b>Active Head-Head: Focal taps partner's head with head</b>		<b>0.786</b>				
Passive Tail-Head: Partner taps focal's tail with head		0.749				
<b>Passive Head-Head: Partner taps focal's head with head</b>		<b>0.653</b>				
Active Tail-Body: Focal taps partner's flank with tail			0.882			
Passive Head-Body: Partner taps focal's head with flank			0.686			
Active Tail-Tail: Focal taps partner's tail with tail				0.800		
Active Tail-Head: Focal taps partner's head with tail (kick)				0.659		
Staying side by side				0.651		
<b>Passive Following: Partner follows focal</b>					<b>0.755</b>	
<b>Active Following: Focal follows its partner</b>					<b>0.658</b>	
Active Body-Head: Focal taps partner's head with flank						0.845
Passive Body-Tail: Partner taps focal's flank with tail						0.789
Variance explained (%)	13.84	12.95	12.38	11.45	9.7	9.61

**Pre-copulatory behavior and the prospective sexual role**

Of the 34 focal worms that mated by 24 h after the video recording, 19 played the female role and 15 the male role. Head-Tail Contacts and Rubbing determined the largest variation in behavior as they loaded on PC 1 (Table 1), which suggested that PC1 mainly summarized behaviors closely associated with the very last steps before pseudocopulation (see above). Both Head-Tail Contacts performed by the focal worms and the Head-Tail Contacts that the focal worms received from their partners loaded on PC1, with the same sign. This indicated a strong correlation between performed and received contacts between the head of the focal worm and the tail of the partner, and highlighted that worms behaved symmetrically in the quality and in the amount of the behaviors they exhibited — the more one worm tapped its partner's tail with its head, the more it was touched on its head by its partner's tail.

Other behaviors were also matched between partners, as both their active and passive expressions loaded on the same principal component, with the same sign and with similar loading factors, indicating not only qualitative, but also quantitative matching. Indeed, passive and active Head-Head Contacts both positively loaded on PC2, and passive and active Following both loaded on PC5: the focal worms followed their partners as much as partners followed focal worms (Table 1). These results anticipated and clarified the next analyses (GLM): overall, the interactions initiated by the focal worms corresponded qualitatively and quantitatively to those initiated by their partners. The only significant predictors of the sexual role the focal worms were to play were, as expected, whether 1) they had riper eggs than their partners (GLM,  $\chi^2 = 11.7203$ ,  $df = 2$ ,  $P = 0.003$ ) and 2) they were tapping the partner's head and tail with their own tail more often (PC4:  $\chi^2 = 6.9285$   $df = 1$ ,  $P = 0.009$ ). Therefore, the focal worms with riper eggs and which did more Tail-Head or Tail-Tail Contacts were more likely to adopt the female role (Figure 3). Prolonged Tail-Head Contacts immediately preceded pseudocopulation.



**Figure 3.** The probability of the focal worm playing either the male or the female role at the next mating interaction as a function of the relative egg maturation level and the amount of Tail Contacts and time spent Staying Side by Side (as summarized by PC4). Focal worms that performed more Tail Contacts and spent more time Side by Side with their partners (i.e., higher PC4 values) were more likely to play the female role, especially if they had more mature eggs than their partners. When partners were equally matched for egg maturation, behavioral predictors (PC4) were relatively less effective.

## Discussion

We recorded, for the first time, pseudocopulation between two *O. diadema* worms and we show that their symmetrical courtship fails to predict what sexual role each partner will adopt.

During the pseudocopulation, two worms stay almost immobile in a Tail-Head position — the head of the male-role worm in contact with the tail of the ‘female’; likely, this is the moment when the ‘female’ produces the transparent jelly cocoon that would envelop the eggs. After the ‘female’ has laid its eggs, it exits the cocoon, and the ‘male’ penetrates it and releases its sperm (not visible but in accordance with what was described in *O. gracilis* — Westheide 1984).

The behavioral interactions occurring before pseudocopulation consist of long, repeated series of reciprocal Rubbing, Following, rapid Contacts between the partners’ bodies, and time intervals spent Staying Side by Side in close contact, that continue all during the 24 h (but may be performed even before, Picchi, pers. comm.). Behavioral interactions were ineffective predictors

of what sexual role (releasing eggs vs. releasing sperm) the worms were to play. Most of the behaviors were symmetrical, meaning that worms engaged in reciprocal behaviors, where the behaviors one worm exhibited towards its partner corresponded to the behaviors it received, both in quality and in quantity. The only factors that foreshadowed what role the focal worms were to play were the relative maturity of their eggs, and whether they tapped their partners with their tails — the Tail-Head position being the one that begins the pseudocopulation sequence: the worms with more mature eggs and which tapped their partners' tail or head with their tails more often were more likely to act as females.

Two key aspects distinguish the pre-copulatory behavior of *O. diadema* worms from that of other hermaphrodites: we observed no aggression, and behavior was symmetrical within partners. We will therefore discuss these points.

First, although mating does not involve aggressive interactions in the majority of hermaphroditic species, aggression has been reported in the pre-copulatory behavior of certain hermaphrodites (Michiels 1998). For example, *Pseudoceros bifurcus* flatworms stab each other with their penis (Michiels and Newman 1998); earthworms pierce their partner's body with their setae during copulation (Koene et al. 2005) and land snails dart their partners with their calcareous stylets, known as 'love darts' (Lodi and Koene 2016). With the exception of Tail Kicks (when the focal worm taps the partner's tail, or head, with its tail) which might be interpreted as mild aggression (perhaps to avoid mating in the female role?), we did not observe any aggression between paired worms (whereas worms attack each other when they are in groups larger than two individuals and compete for mates, Lorenzi et al. 2006). *Ophryotrocha* hermaphrodites are external fertilizers where the release of eggs by one worm and the release of sperm by its partners follow each other after a lapse of a few seconds. In contrast, stabbing flatworms, piercing earthworms and darting land snails are internal fertilizers, and egg fertilization occurs days, weeks or even years after sperm receipt (e.g., see Nakadera and Koene 2013, for time intervals between receiving sperm and releasing eggs in gastropods). Fertilization type makes a difference: in internally fertilizing hermaphrodites, when there is a time gap between the uptake of allosperm and the fertilization of the eggs, one individual can manipulate the partner's physiology and increase its own reproductive success (for example by enhancing the partner's female function, or limiting the digestion of donated sperm; e.g., Koene and Chase 1998; Landolfi et al. 2001; Koene et al. 2005). This cannot occur in externally fertilizing

hermaphrodites, where egg and sperm release is virtually simultaneous and the manipulation of the partner's reproductive decisions (e.g., sperm digestion) cannot occur via 'aggression'.

The lack of aggression does not mean that mating conflict does not occur among externally fertilizing hermaphrodites; indeed, we interpret the symmetry in courtship behaviors as an expression of the conflict over the sexual role.

In the externally fertilizing hermaphroditic serranine fish *H. nigricans* and *S. tigrinus* courtship is relatively short, and the fish which is to play the female role first, advertises its readiness to spawn by beginning courtship and exhibiting particular behaviors (Fischer 1980; Hart et al. 2016). In contrast, in *O. diadema* worms, courtship is prolonged for one day or more and there is no behavioral display of the prospective sexual role. Although the mating systems of serranine fish and *O. diadema* worms have important similarities, they also differ in some relevant details. For example, *H. nigricans* fish spawn repeatedly during a 2-h period (egg parceling, Fischer 1980) and alternate sexual role between partners every few minutes, whereas paired *O. diadema* worms typically spawn every two days (so that each worm alternates between sexes every four days). Additionally, while *H. nigricans* fish cannot store their eggs (which will become largely infertile overnight, Fischer 1981), *O. diadema* worms can keep ripe eggs for days (Lorenzi, unpublished). These differences make the conflict between partners much stronger in *O. diadema* worms if partners share a preference for the same sexual role, since the relatively long time interval between matings and the possibility to restrain from laying mature eggs make desertion likely (Sella and Lorenzi 2000).

We interpret the long courtship between *O. diadema* partners as the outcome of a difficult-to-reach agreement between partners, where the preferred sexual role is at stake. According to previous studies (Sella 1988; Sella and Lorenzi 2000), we expect that worms are more likely to court hermaphrodites with riper oocytes; however, once the worms are involved in courtship, it is unclear which partner will give up playing the preferred sexual role sooner. For example, worms with riper eggs (e.g., stronger motivation to lay eggs) might accept playing the female role sooner, which would suggest that worms with relatively less ripe eggs would have higher resource-holding potential, which seems unlikely. However, the scarcity of information on the reproductive physiology of these worms makes these predictions speculative.

It may be argued that worms spend a long time courting each other because their perceptual ability is limited and they need a long time interval to acquire the relevant information for



assessing their mate. This was indeed Darwin's claim that "hermaphrodites have too imperfect senses and much too low mental powers to appreciate each other's beauty or other attractions, or to feel rivalry" (Darwin 1871). While this may well be the case and suggests a need for focused investigations on hermaphrodite neurophysiology (in particular, we have only scarce and patchy information about polychaete sensory abilities and their responsiveness to external stimuli — Bartels-Hardege et al. 1996; Müller et al. 1999; Ram et al. 1999), it is worth noting that hermaphrodites are highly responsive to chemical cues, particularly to those coming from conspecifics (Schleicherová et al. 2006; Velando et al. 2008; Alvarez 2017). Among polychaetes, the separate-sex worm *Platynereis dumerilii* has an extremely low response threshold to the female sexual pheromone, as they respond to concentrations of 0.4 ml of a  $10^{-5}$  M solution (Zeeck et al. 1996, 1998). Likewise, *O. diadema* worms respond to concentrations as small as 1% of the chemical cues produced by conspecifics (Schleicherová et al. 2010), which indicates that they have sophisticated perceptual abilities.

Some pre-copulatory behaviors described in hermaphrodites have been associated with assessing the partner's mating status or whether it is a new partner (e.g. Anthes et al. 2006; Koene and Ter Maat 2007; Velando et al. 2008). This led some authors (e.g., Schärer and Pen 2013) to consider that these cues can be used as proxies for the likelihood of encountering sperm competition, and that they are therefore strongly linked to post-copulatory sexual selection. While this may well be true for some hermaphrodites, it is not the case in *Ophryotrocha* worms, where fertilization is external (i.e., there is no sperm storage). Therefore, we can rule out the hypothesis that courtship functions to assess sperm competition.

In conclusion, we interpret symmetries in the pre-copulatory behavior as the consequence of partners sharing a preference for one sexual role and competing for playing it. Such a preference would be kept stable irrespective of individual condition; for example, size-dependent sex allocation (Ghiselin 1969; Angeloni et al. 2002) does not affect the relative investment in male and female functions in paired worms (Lorenzi et al. 2005). We hypothesize that, during their enigmatic pre-copulatory behavior, *O. diadema* worms display symmetrically, just because their preference for the sexual role is symmetrical (as also supported by the equal number of focal worms which eventually mated as females or males). This is not a typical outcome among hermaphrodites. Behavioral sexual dimorphism has been reported in many different species of hermaphrodites, that is, individuals behave differently when they are to assume the male or

female role (i.e., they advertise their prospective sexual role with sex-specific displays; reviewed in Leonard 2006), and only one exception is known, to our knowledge; banana slugs *Ariolimax californicus*, have symmetrical courtship (Leonard et al. 2002; in Leonard 2006).

Courtship is costly, both in terms of resources invested in displaying and in terms of time (i.e., opportunities lost for foraging, searching for other mates, etc.). In this perspective, the long *O. diadema* courtship resembles a war of attrition (Maynard Smith and Price 1973; Parker and Rubenstein 1981; Payne 1998), where the two contestants display symmetrically (matching each other's behaviors in both quality and quantity) until the cumulative costs for one of them reach a threshold; at this point the loser stops displaying and ends the competition (i.e., it accepts playing the less preferred role, while the partner will play the preferred role). This explains why pre-copulatory interactions were prolonged and symmetrical.

This research highlights that in a species where post-copulatory competition is limited, pre-copulatory investment may be especially important, addressing a trade-off between these two components of sexual selection (Parker et al. 2012).

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## Supplementary materials

**Video S1:** The clip shows the pseudocopulation between two *Ophryotrocha diadema* worms. Both partners have mature eggs visible in the coelom, which have a yellowish color in the female-role worm, and a whitish color in the male-role worm. A third worm is visible on the right of the screen; it does not take part in the mating interaction. The red circle at the beginning of the sequence highlights the Head-Tail contact between the partners. See text for details.

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# Coopération et compétition comme éléments moteurs de la transition évolutive de l'hermaphroditisme vers des sexes séparés chez les vers du genre *Ophryotrocha*

## Résumé

Chez les animaux, l'hermaphroditisme et le gonochorisme sont tous deux apparus plusieurs fois de manière indépendante. Cependant, les voies évolutives impliquées dans la transition entre les systèmes sexuels sont en grande partie inconnues. L'objectif de cette thèse était donc d'étudier la transition évolutive de l'hermaphroditisme vers des sexes séparés chez les vers du genre *Ophryotrocha*, en s'intéressant en particulier à la manière dont la coopération et la compétition entre individus peuvent déterminer l'allocation de ressources reproductives chez les hermaphrodites, soit en favorisant l'évolution de sexes séparés ou en stabilisant l'hermaphroditisme. Nos résultats ont révélé que les vers hermaphrodites échangent réciproquement des œufs, une forme de coopération qui favorise une allocation des ressources sexuelles biaisée en faveur de la fonction femelle et qui stabilise ainsi l'hermaphroditisme. Toutefois, lorsque les hermaphrodites sont exposés à une forte compétition pour l'accouplement, ils transfèrent les ressources de la fonction femelle vers la fonction mâle, ce qui diminue la production d'œufs et les soins parentaux, mais augmente la motilité et l'agressivité. À des niveaux élevés de compétition pour l'accouplement, motilité et agression peuvent améliorer la recherche et la monopolisation du partenaire, favorisant ainsi la propagation des hermaphrodites biaisés en faveur de la fonction mâle, promouvant ainsi l'évolution de mâles purs et, ensuite, de femelles pures. Enfin, nous avons montré que les hermaphrodites prêts à s'accoupler en tant que mâles uniquement (sans œufs matures) préfèrent des partenaires hermaphrodites prêts à s'accoupler en tant que femelles, qui, en revanche, n'expriment aucune préférence. Globalement, ce travail montre que la coopération et la compétition sont tous deux des facteurs importants dans la répartition des sexes chez les hermaphrodites et suggère que des changements dans l'environnement social ont pu jouer un rôle central dans l'évolution des systèmes sexuels chez les vers du genre *Ophryotrocha*.

**Mots clés :** systèmes sexuels, compétition pour la reproduction, allocation sexuelle, polychètes

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## Cooperation and competition as drivers of the evolutionary transition from simultaneous hermaphroditism to separate sexes in *Ophryotrocha* worms

### Abstract

In animals, both simultaneous hermaphroditism and gonochorism originated independently several times. Yet the evolutionary pathways involved in the transition between sexual systems are largely unknown. The aim of this thesis is therefore to investigate the evolutionary transition from hermaphroditism to separate sexes in *Ophryotrocha* worms, focusing on how cooperation and competition between individuals shape hermaphrodite sex allocation, either promoting the evolution of separate sexes or stabilizing hermaphroditism. Our results revealed that hermaphroditic worms reciprocally exchange egg-clutches, a form of cooperation that favor a female-biased sex allocation and stabilizes hermaphroditism. However, when hermaphrodites are exposed to high level of mate competition, they reallocate resources from the female to the male function, decreasing egg production and parental care and increasing motility and aggression. Under high mate competition level, motility and aggression may improve mate searching and partner monopolization and may favor the spread of male-biased hermaphrodites, promoting the evolution of pure males and, later, pure females. Finally, we showed that hermaphrodites ready to mate as males only (without ripe eggs) prefer hermaphrodites ready to mate as females as partners, which, in contrast, do not express any preference. Overall, this work shows that both cooperation and competition are important factors in hermaphrodite sex allocation, and suggests that changes in the social environment may have played a central role in the evolution of sexual systems in *Ophryotrocha* worms.

**Key words:** sexual systems, mate competition, sex allocation, polychaetes

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