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Original Citation:

Availability:

This version is available http://hdl.handle.net/2318/1566833 since 2016-06-16T11:04:21Z

Published version:

DOI:10.1038/npp.2015.339

A trade-off between traits that contribute to male and female function in hermaphrodites

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Running title. Genetic covariance in sexual functions

Abstract

Sex allocation theory assumes that male and female reproductive functions share a common limited resource pool and are negatively correlated in hermaphrodites. Here we report on the first artificial selection experiment designed to test the existence of genetically-based correlations between sex functions in hermaphroditic animals. The polychaete worm *Ophryotrocha diadema* has a long juvenile male phase, and then shifts to the simultaneously hermaphroditic phase. We selected two sets of lines of worms for a short male phase and, after 4 generations, worms had a significantly shorter male-phase than their generation-0 ancestors. As negatively correlated responses, generation-4 worms spent more time maturing eggs and produced a higher number of eggs at 1st laying than worms of generation 0. Both traits contributed to the female function and were not the target of the selection experiment. In contrast, selection was ineffective in the lines descending from phenotypically-hermaphroditic worms that reproduced only via their male function. Our results provide the first empirical support of a genetic basis for a trade-off between traits related to the male and female function in hermaphroditic animals and highlight that these trade-offs are complex. Our results also suggest that the trade-off between male and female functions breaks up as hermaphrodites evolve some sexual specialization where resources are channeled towards a single sexual function.

KEY WORDS: artificial selection, genetic covariance, correlated traits, phenotypic plasticity, sex allocation, protandry

INTRODUCTION

Natural selection cannot cause an unlimited increase in some components of fitness without simultaneously causing a decrease in others, as long as these components share the same, limited, resource pool. Therefore, selection favors those individuals that maximize their fitness by allocating resources differentially and appropriately to traits that share common, limited resources. This implies that competing traits are linked by a negative correlation (STEARNS & HOEKSTRA 2000; ROFF & FAIRBAIRN 2007; COX & CALSBEEK 2010).

An interesting case study of correlations among traits is offered by gender expression in hermaphrodites, in which the male and the female functions can behave as competing traits. Charnov was the first to address the consequences of the evolutionary association of sexual functions in a single organism and modeled how evolution shapes the differential allocation of resources to the two sexual functions (CHARNOV et al. 1976; CHARNOV 1982). Both the male and the female functions have nutritional and energetic demands associated with their expression. If they share the same limited resource budget when they are tied in the same organism, as it occurs in hermaphrodites, a larger resource allocation to, for example, the female function, will result in a smaller allocation to the male function and vice versa. In other words, sexual functions are linked by an intrinsic trade-off. Such a trade-off is expected in functional hermaphrodites but lessens in sexually-specialized phenotypes where resources are canalized towards only one sexual function (EHLERS & BATAILLON 2007).

Empirical evidence for the existence of trade-offs between sexual functions in hermaphrodites has often been looked for, but straightforward evidence is scarce and results often equivocal (CAMPBELL 2000; SCHÄRER 2009). Correlations among traits may be investigated by selecting on one trait and looking for correlated responses in other traits. If the other traits are altered even though the researcher imposed no direct selection on them, this indicates that they are genetically correlated with the trait under selection (LANDE 1979; STEARNS & HOEKSTRA 2000; ROFF & FAIRBAIRN 2007). Genetically based trade-offs between traits emerge when a change in a trait that increases fitness is linked to a change in another trait that decreases fitness (STEARNS 1992).

The experiment reported here is, to our knowledge, the first artificial selection experiment designed to test the existence of genetically-based trade-offs between sex functions in hermaphroditic animals. We chose the marine polychaete worm *Ophryotrocha diadema* as our study model. This hermaphrodite is strictly non-selfing, and, in pairs, worms regularly trade eggs with partners, producing large number of eggs (SELLA 1985). When the opportunities for mating increase, these worms drastically reduce their female allocation and desert their reciprocating partners for mating in the preferred male role (SELLA & LORENZI 2000; LORENZI et al. 2005; LORENZI & SELLA 2008). Since fitness is often more sensitive to changes in age at maturity than to changes in other life-history traits (STEARNS & HOEKSTRA 2000), we focused our analyses on the length of the juvenile, protandric, male phase that precedes the hermaphroditic phase, and we selected for shortening it. The length of the male phase is related to current fitness via the male function (SELLA & LORENZI 2003), and we tested whether it was negatively related to fitness via the female function, assuming a genetically-based trade-off between male and female functions. By selecting for shortening the male phase, we expected both a direct response to selection (a shorter male phase) and a correlated response in traits that contributed to the female function (e.g. more time devoted to mature/produce eggs). Our selection experiment included two sets of lineages, because *O. diadema* hermaphrodites have two different sexual phenotypes: functional hermaphrodites and functional males (i.e., worms that have a hermaphroditic phenotype but function only as males, DI BONA et al. 2010). It is likely that these different sexual phenotypes result from the different expression of the multiple sex loci underlying the two sexual functions, as it occurs in other species of the genus (PREMOLI et al 1996, LORENZI and SELLA 2013). In this hypothesis, functional males could represent a phenotypic class at one of the extremes of the range of sex-allocation variation and sex-loci combinations (MOORE & ROBERTS 2012). We predicted that selection outcomes in the two lineages would differ. Whereas we expected that a genetic correlation between traits related to sex functions emerged in the sets of lines of functional hermaphrodites, we expected no or little response to selection in the sets of lines of functional males, as the trade-off between sex functions may not work in these sexually-specialized worms.

MATERIALS AND METHODS

Study model

Ophryotrocha diadema is an iteroparous, non-selfing, simultaneously hermaphroditic, polychaete worm with external fertilization. A few worms were originally collected in Los Angeles harbor in August 1972 (ÅKESSON 1976). Since then, these worms have been cultured in the laboratory. *O. diadema* is small-sized (about 4 mm). Larvae hatch from eggs and develop inside their cocoon for about 8 days. When larvae are 4-segment-long, they leave their cocoon and soon enter their male phase (SELLA 1990). During the male phase they produce viable sperm (and no eggs) and successfully fertilize eggs that hermaphrodites lay (SELLA 1990; SELLA & LORENZI 2003). After 30 days, the young worms develop eggs, which can be seen in the coelom through their transparent body walls (under stereomicroscope, 40X magnification). At this point, worms stop their male-only phase and enter their simultaneously hermaphroditic phase, during which functional hermaphrodites reproduce via the male and female function alternately (SELLA 1985) (Fig. 1). Before their 1st spawning, young hermaphrodites court each other for 1-4 days. Then, one worm plays the female role and releases 20-25 eggs protected by a jelly egg-cocoon, and its partner plays the male role and releases its sperm inside the cocoon. Then, generally, worms take turns in either laying or fertilizing eggs in subsequent mating events (fig. 1) (SELLA 1985; SELLA & RAMELLA 1999; LORENZI & SELLA 2000). During the hermaphroditic phase, hermaphrodites adjust their sex allocation to male and female function rapidly and opportunistically (Lorenzi et al. 2005, 2008), basing on cues which inform them on mating opportunities (SCHLEICHEROVÀ et al. 2006, 2010). Hermaphrodites compete for egg fertilization and multiple paternity of single egg-cocoons is not rare (LORENZI et al. 2013).

In these worms, a dominant Y allele determines a yellow egg color, while the recessive y allele determines a white egg color (SELLA & MARZONA 1983). This marker is neutral (ÅKESSON 1976).

By means of this marker, we can identify focal worms in a group and assign their progeny.

Throughout this paper, we have classified worms as follows: 1) "worms in male phase" are individuals in the protandrous phase (i.e., between hatching and the first appearance of eggs in the coelom); 2) "young hermaphrodites" are worms that are maturing their first eggs (i.e., between the first appearance of eggs in the coelom and the 1st egg laying); 3) "hermaphrodites" are worms between the 1st egg laying and their death; and 4) "functional males" are phenotypically hermaphroditic worms that function only as males (they have eggs in their coelom but do not lay them, DI BONA et al. 2010).

Correlations between the traits used in the experiment

In a preliminary experiment we checked whether the length of the male phase is correlated to two traits that contribute to the female function (i.e. number eggs at 1st laying and length of the interval between the first appearance of eggs in the coelom and the 1st egg laying). To this aim, 256 functional hermaphrodites were randomly taken from our mass cultures and each of them was paired with a partner. We found that the longer the male phase, the fewer the number of eggs that worms laid at the 1st laying, suggesting that the trait "length of male phase" traded-off with the number of eggs at the 1st egg laying (Pearson's $r = -0.158$, $P = 0.011$). Vice versa, the longer the time worms spent as young hermaphrodites, the larger the number of eggs at the 1st laying ($r = 0.552$, $P < 0.0001$), suggesting that the trait "time as young hermaphrodite" contributed mostly to the female function. Additionally, in a subset of these paired worms, we measured lifetime fecundity and we found that, in paired worms, the number of eggs at the 1st laying was significantly and positively associated to lifetime fecundity ($r = 0.603$, $n = 51$, $P < 0.0001$). Therefore, the number of eggs at the 1st laying is a good proxy for lifetime fecundity. These traits are the main fitness components in *O. diadema* (PREVEDELLI et al. 2006)

Rearing conditions

We reared worms in sea water (density of 1024 $g{*}m^{-3}$), in 10-ml bowls, in darkened, thermostatic chambers at 20°C. We fed animals with spinach *ad libitum* and changed water in the bowls twice a week. In each sibship and generation, we kept sibling larvae and protandrous males together until they were young hermaphrodites.

Experimental design

We formed the two sets of lines from 269 homozygous yellow-egg hermaphroditic worms, randomly chosen from our lab population. The 269 selected worms composed the founding population and were the result of the first episode of selection. In order to use them as founders of the two sets of selected lines, we checked their functional gender as soon as they entered their hermaphroditic phase. We paired each of them to a white-egg hermaphrodite for 21 days (i.e. two thirds of their fertile life) and noted whether they laid eggs (functional hermaphrodites) or not (functional males). During 21 days, hermaphrodites are expected to lay 5-10 cocoons of eggs and functional males no cocoon at all (DI BONA et al. 2010). Of 269 worms, 256 were functional hermaphrodites and 13 functional males.

Generation 0 of the set of lines of functional hermaphrodites was composed of 20 pairs of functional hermaphrodites, randomly chosen among the 256 functional hermaphrodites of the founding population. Generation 0 of the set of lines of functional males was composed of 13 pairs, each formed by one of the 13 functional males and by a functional hermaphrodite. We based the selection for a short male phase on the phenotypic variation of this trait. At every new generation, we selected the worms with the shortest male phase from each sibships of homozygous yellow-egg hermaphrodites. The selected worms were backcrossed with one of their parents to produce the next generation. From every sibship, other 4 worms were used to measure the mean values of two traits (i.e. the number of eggs at 1st laying and the length of the young hermaphroditic phase). These two traits were correlated to the length of the male phase in the founding population (see above). To measure the direct and indirect responses to selection, the mean values of the traits of generation 4 were compared to those of generation 0 in the same set of lines. A significant direct response to selection was detected when the mean value of the length of the male phase was significantly smaller in generation 4 than in generation 0. Such a direct response would indicate that the trait "length of the male phase" is heritable. Significant indirectly correlated responses to selection were detected when the mean value of the traits that were not the target of selection (i.e. number of eggs at 1st laying and length of the young hermaphroditic phase) changed in concert with the length of the male phase, a result that would indicate that the two traits were genetically linked. At every generation, sibships were reared separately from both parents and other sibships. The selection procedure was performed on worms homozygous with yellow eggs. When it was necessary to recognize paired worms individually and to identify which partner in a pair laid cocoons, yellow-egg worms were paired to white-egg worms.

We backcrossed the selected offspring to their parents in order to strengthen the differences between lines in their genetic background. In this way we controlled the sex-related genetic contribution to the next generation to a larger extent than if we had performed crosses between randomly chosen worms within each line. Because the experimental procedure was the same in the two sets of lines, we imposed the same level of inbreeding and the same selection pressure on both lines. We did not include a set of control lines in our experimental design. This would have allowed us to control for environmental fluctuations, but it would have also reduced the available facilities by constraining us to reduce the number of replicates in the sets of selected lines. The reduction in the number of the selected lines would have increased their sampling variance and reduced the accuracy of the response estimate. Therefore we gave up the set of control lines, considering that while it is true that random changes in the environment reduce the precision with which the response to selection is estimated, nevertheless they do not bias the estimate of the response (FALCONER 1989).

Measures of phenotypic variation of the traits used in the experiment

It is easy to measure female allocation in these worms. After worms have entered the hermaphroditic phase, they repeatedly produce and lay eggs lifelong. Eggs are large and countable, and their contribution to the female function is obvious (SELLA & RAMELLA 1999). It is less easy to measure male allocation, which consists in almost invariant, low sperm counts and elusive mate competition (PREMOLI & SELLA 1995; LORENZI et al. 2006). These worms spend about one third of their life in the protandrous phase, before moving on to the simultaneous hermaphroditic phase. Therefore, in generation 0 and in generation 4 of the two selected sets of lines we measured the length of the male phase, the length of the young-hermaphrodite phase and the number of eggs at 1st laying. In a subset of worms of the founding population and of generation 4, we also checked lifetime fecundity (i.e., lifetime egg production). To measure lifetime fecundity, we paired each of these worms, which had a yellow-egg phenotype, to a white-egg hermaphrodite, and then we checked their egg production. We used these data also to analyze the lifetime temporal pattern of egg production. We did this in the set of lines of functional-hermaphrodites by comparing the proportion of eggs they laid in the first half of their layings with respect to lifetime egg production, in worms of generations 0 and 4. Additionally, we measured body size at the end of the male phase (number of chaetigerous body segments).

We also estimated the frequencies of functional hermaphrodites and functional males in the two sets of lines at generation 4, by pairing worms with novel partners until they laid eggs (or until they died). We classified worms as functional hermaphrodites when they laid their first egg-cocoon within 21 days since they had been paired and as functional males when they did not. We tested functional males for male-gender sterility by checking whether the eggs they fertilized developed into embryos.

Control for selection for ability to acquire resources

By selecting on phenotypic values of a trait it may happen that individuals are selected for their ability to acquire resources rather than for alleles directly connected to that trait. The two sets of selection lines originated from worms (founding population) that had different gender expression (functional hermaphrodites *vs* functional males). Therefore we checked whether there were differences between the two sets of lines in ability to acquire resources. To this aim, we compared the length of the male phase and body size at the end of the male phase between worms of generation 0 in the two sets of lines. We speculated that if we had selected for ability to acquire resources, the two sets of lines would have responded to selection in the same way (resulting in no significant differences between worms of generation 4). Additionally, in the functional hermaphrodite set of lines only, we checked whether lifetime fecundity increased from generation 0 to generation 4. If we had selected for better resource acquisition ability, we would have found an increase in lifetime fecundity between generations.

Statistical analyses

We performed the statistical tests to detect the responses to selection by comparing the trait values of the worms in generation 0 *vs* those in generation 4. In these comparisons, we avoided pseudo-replications by using the trait values of one worm per sibship. We used general linear models (GLMs) to test for the effects of line and generation on trait values. Data were not normally distributed and/or had non-homogenous variances. To account for assumptions of normality and homogeneity of variances, we ln-transformed the length of male phase, the interval of time spent as young hermaphrodite, the number of eggs at the 1st laying, and body size. We also transformed lifetime fecundity as 1/lifetime fecundity.

We also used generalized linear models (GZLMs) for binomial distributions with a logit link to test for differences in trait values when traits had a binomial distribution. We analysed in this way: 1) the proportion of worms that were either functional males or functional hermaphrodites within each sibship; 2) the proportions of time each worm spent either in the male phase or as a young hermaphrodite; and 3) the proportion of eggs each individual produced in the first half of its layings in relation to lifetime egg production (temporal pattern of lifetime egg production).

Because some worms died and/or we could not measure some traits, sample sizes vary among analyses. Descriptive statistics were reported as mean ± 1 SE. Tests were two-tailed and statistical analyses were performed using IBM SPSS statistics version 20.

RESULTS

Direct response to selection for a short male phase

The response to selection was significantly different between sets of lines (as indicated by a significant interaction term set of line * generation in the GLM, $F_{1,94} = 19.546, P < 0.0001$). In the set of lines of functional hermaphrodites, the male phase shortened significantly of about 10 days after selection ($F_{1,58} = 41.039, P < 0.0001$), whereas in the set of lines of functional males it did not change ($F_{1,36} = 0.320, P = 0.575$) (Fig. 2A). Adding body size as a covariate yielded substantially similar results.

Correlated responses to selection for a short male phase

Number of eggs at 1st laying

The correlated response in the number of eggs at 1st laying differed between sets of lines (GLM, interaction term set of lines*generation: $F_{1,85} = 9.855, P < 0.002$). The worms of generation 4 laid twice as many eggs as those in generation 0 in the set of lines of functional hermaphrodites ($F_{1,58} = 53.515, P < 0.0001$), whereas worms in the set of lines of functional males did not vary their the number of eggs at 1st laying significantly ($F_{1,27} = 0.222, P = 0.641$) (Fig. 2B). Adding body size as a covariate yielded substantially similar results.

Time spent as young hermaphrodite

The correlated response in the time that individuals spent as young hermaphrodites differed between sets of lines (GLM, interaction term set of lines*generation: $F_{1, 94} = 10.736, P = 0.001$) (Fig. 2C). Worms of generation 4 spent on average 3 days more than worms of generation 0 as young hermaphrodites in the set of lines of functional hermaphrodites ($F_{1, 58} = 9.921$, $P = 0.003$), whereas worms of the set of lines of functional males at generation 4 spent approximately as much time as their ancestors of generation 0 as young hermaphrodite ($F_{1, 36} = 2.895$, $P = 0.097$).

The trade-off between the proportions of time spent as males and as young hermaphrodites

The proportion of time that worms spent as males relatively to that spent as young hermaphrodites varied significantly between generations and lines (GZLM, interaction term set of lines*generation: Wald $\chi^2 = 66.314$, df = 1, $P < 0.0001$) (Fig. 3). It decreased significantly in the set of lines of functional hermaphrodites (Wald $\chi^2 = 61.238$, df = 1, $P < 0.0001$), whereas it increased significantly in the set of lines of functional males (Wald $\chi^2 = 17.297$, df = 1, $P < 0.0001$) (Fig. 3).

Control for selection on the ability to acquire resources and temporal pattern of lifetime egg production

The two sets of selected lines originated from worms that had similar abilities to acquire resources, as can be inferred by their similar length of male phase and body size at the end of male phase (GLM, generation 0 of the two sets of lines: length of the male phase, $F_{1,64} = 0.449$, $P = 0.505$, body size at the end of male phase, Wald $\chi^2 = 0.015$, $P = 0.904$). In contrast, at generation 4, the worms of the two sets of lines differed significantly in the length of their male phase (see above, $P < 0.0001$) but not in their body size (Wald $\chi^2 = 0.009$, $P = 0.926$). These results confute the hypothesis that worms were selected for ability to acquire resources.

Additionally, in the set of lines of functional hermaphrodites, worms of generation 4 had a lower lifetime fecundity than worms of generation 0 (generation 0: 665.92 ± 43.18; generation 4: 189.05 ± 37.626 eggs) (Welch statistic = 6.523, $df_1 = 1$, $df_2 = 19.238$, $P = 0.019$). If we had selected for a larger or faster ability to acquire resources, we would have found an increase (rather than a decrease) in lifetime fecundity across generations.

Finally, as a result of selection, the temporal pattern of lifetime egg production changed in the set of lines of functional hermaphrodites (Fig. 4). Worms of generation 4 produced a significantly smaller proportion of eggs in the first half of their layings (in relation to lifetime egg production) than worms of generation 0 (GZLM, Wald $\chi^2 = 348.996$, df = 1, $P < 0.0001$).

Proportion of functional males in generation 4

There was a significant difference between sets of lines in the proportions of functional males and hermaphrodites in generation 4 (Wald $\chi^2 = 6.943$, df = 1, $P < 0.008$). In the set of lines of functional hermaphrodites, the average proportion of males in generation 4 was 0.02 ± 0.02, whereas in that of functional males it was 0.22 ± 0.09 (Fig. 5).

Once paired to mature hermaphrodites, all these functional males fertilized their partners' eggs repeatedly and successfully. None of them laid any egg lifelong. Of their 13 ancestors in generation 0, only 7 behaved as males lifelong.

DISCUSSION

Our results represent the first test in hermaphroditic animals, to our knowledge, of the prediction of the sex allocation theory that assumes that, within the fixed budget of reproductive resources, resource-sharing between male and female reproductive traits generate a negative genetic correlation between reproductive traits. In effect, we selected functional hermaphrodites for a shorter male phase and after selection we found that worms allocated significantly less time to the male phase and more time to the young hermaphrodite phase (during which they mature the 1st batch of eggs) than their progenitors. The responses to selection were clear cut and consistent across replicates. Worms that have a long male phase will subtract a relatively larger amount of time and resources to the female function (i.e., egg production) than worms with a short male phase. Saving time devoted to the male function means saving time and resources that will be later devoted to the female function. The larger the savings during the male phase, the higher the number of eggs that will be produced in the hermaphroditic phase, and the longer the time available to produce them. In this sense, the length of the male phase and the length of the hermaphroditic phase are proxies for the partitioning of resources between male and female function.

During artificial selection, genetic correlations may be caused by genetic drift. This hypothesis is usually supported by large within-line variances in the values of the correlated traits (MAZER et al. 2007). Such large variances did not occur here. Instead, we found a consistency in the values of the two traits connected by the negative genetic correlation (the length of male phase and the number of eggs at 1st laying) among replicates of the set of lines of functional hermaphrodites. These observations led us to conclude that the responses to selection in the set of lines of functional hermaphrodites were the expression of a negative genetic correlation that was caused by pleiotropy or linkage disequilibrium and not caused by random genetic drift or selection for resource acquisition. The two traits connected by the negative genetic correlation seem to evolve interdependently and therefore they may be under the control of pleiotropic genes. These genes may affect one trait favorably and the other unfavorably. The link between traits constrains the action of natural selection within the limits of the trade-off and maintains the genetic variation underlying the trade-off (STEARNS 1992; ROFF 2002).

In fact, the differences in male and female investment between generation 0 and generation 4 might reflect the consequences of both inbreeding depression and artificial selection. Overall, we found more genetic variation than we expected under the inbreeding level caused by our backcross design, suggesting that *O. diadema* laboratory populations may have purged most deleterious alleles during the long period of laboratory rearing. However, inbreeding depression usually reduces lifetime fecundity (REED & FRANKHAM 2003) and this reduction occurred here. Yet, if inbreeding effects would have been the main cause of trait variations in our experiment, we could not explain why we observed a twofold increase in the number of eggs at the 1st laying in the set of lines of functional hermaphrodites.

It could be argued that the shortening of the male phase affected not only the length of the young hermaphroditic phase, but also the trade-off between current and future reproduction through the female function, with an increase in current egg production, disfavoring the future one. Our data do not support this hypothesis. Indeed, with respect to their ancestors in generation 0, worms in generation 4 decreased their current egg production in favor of the future one, notwithstanding a peak in the 1st laying and an overall decrease in lifetime fecundity.

Finally, we cannot rule out the hypothesis that a short juvenile male phase could be compensated by a larger investment in the male function during the functional hermaphroditic phase. In this hypothesis, the observed results would indicate an indirect effect of selection on the allocation of resources to the female function, but not a direct negative correlation between the two traits analyzed. We cannot rule out this hypothesis, but if this was the case, we could explain neither the peak in egg production at the 1st laying, nor that selected worms reciprocated eggs regularly.

The absence of a control line in the design of the experiment did not allow us to estimate the role of uncontrolled environmental effects on the response to selection. Therefore our estimates of the differences in trait values between generations may be flawed by random effects. Notwithstanding the limits of our experimental design, the differences in mean values of the selected traits were significant. The success of this selection experiment could be further validated by an experiment where two sets of lines are selected in opposite directions (i.e. for a short or long male phase). In this case, each set of selected lines would act as a control for the other and the response would be measured as the divergence between the upward and the downward set (FALCONER 1989).

Experiments on the genetic covariance between male and female functions have been performed on hermaphroditic plants (MAZER et al. 2007), but they have never been performed on hermaphroditic animals before, as far as we know, possibly because it is not easy to measure male and female traits. Generally, sexual functions may require different resource investments and we may not be able to compare the resource currencies of the two sexual functions (SCHÄRER 2009). In our study model we were able to compare the traits linked by a trade-off using the same currency – time, i.e., the proportion of time spent as male vs that spent as young hermaphrodite.

The trait targeted by artificial selection did not respond to selection in the set of lines of functional males. This indicates that the genetic architecture underlying sexual functions is different in this set of lines or that genetic variation for sex allocation was exhausted in this set of lines. We recall that these worms originated from founders that were identified through their male-biased gender expression: they had eggs but never laid them. They differed from the worms of the set of lines of functional hermaphrodites in the genetic background of the traits linked to gender expression and in the responses to selection on these traits. In functional males, the trade-off was almost fixed and most reproductive resources were channeled to the male function.

The results we obtained by selecting on functional hermaphrodites and functional males can help us to outline the first steps in the evolutionary transition from hermaphroditism to separate sexes. Theoretical evolutionary models indicate that the transition from hermaphroditism to separate sexes (or vice versa) requires changes in the allocation of reproductive resources in response to natural selection and a trade-off between the two sexual functions in hermaphrodites (CHARLESWORTH & CHARLESWORTH 1978; CHARNOV 1982; DELPH & WOLF 2004, PANNELL & VERDU 2006) Under these conditions, if mutant hermaphrodites appear that specialize in, for example, more male functions (at the expense of the female functions), natural selection will favor other hermaphrodites that specialize in the female function. Our results document that in hermaphroditic worms there was genetic variation for sex allocation patterns and there was a genetic covariance between the traits that contributed to the two sexual functions. There were also hermaphrodites specialized in more male function. The evolutionary pathway of the transition could be this: hermaphroditic progenitors lose their negative genetic correlation between the traits connected to the sexual functions, as it occurred to functional males, and this is the first step towards gonochorism. Then, natural selection will favor other hermaphrodites that specialize in the opposite function, as it was nicely shown in plants (DORKEN & PANNELL 2009). In this perspective, functional males and specialized hermaphrodites could be the ancestors of separate-sex descendants as suggested for another species of worms, in which multiple sexual phenotypes are present and trade-off between sexual functions are almost completely broken up (LORENZI et al. 2013).

Our results provide the first empirical support of a genetic basis for a trade-off between traits related to the male and female function in hermaphroditic animals and highlight that these trade-offs are complex. Our results also suggest that the trade-off between male and female functions breaks up as hermaphrodites evolve some sexual specialization where resources are channeled towards a single sexual function.

Acknowledgements

We wish to thank Sandro Cavicchi for his help in designing the experiment and discussing the results. We also thank Patrice David, Roberto Simonini and two anonymous referees who made precious comments on a previous version of the manuscript, and Vincent Marsicano for linguistic revision.

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Legends to Figures

Figure 1. Life cycle and sexual pattern in *O. diadema*. Soon after hatching, worms enter their male phase during which they fertilize eggs laid by mature hermaphrodites. When the male phase ends, worms mature eggs in their coelom (eggs are visible through their transparent body walls). During the hermaphroditic phase, mating events occur each 1 - 2 days between paired worms (fertilization is external and occurs via pseudocopulation). At each mating, worms either play the female role (i.e., they lay eggs) or the male role (i.e., they fertilize eggs). At the next mating event, the worm which played the female role will play the male role, and, vice versa, the one which played the male role will play the female role. Usually, 20 - 40 mating events occur during the hermaphroditic phase, before worms die when they are 80 - 100 days-old.

Figure 2. Direct and correlated responses to selection for a shorter male phase in generations 0 and 4 in the sets of lines of functional hermaphrodites and functional males. A: The shortening of the male phase occurred in the set of lines of functional hermaphrodites, whereas in that of functional males it did not. B: The variation in the number of eggs at 1st laying occurred in the set of lines of functional hermaphrodites, whereas in that of functional males it did not. C: The lengthening of the time as young hermaphrodite occurred in the set of lines of functional hermaphrodites, whereas in that of functional males it did not.

Figure 3. Proportion of time spent as male and as young hermaphrodite. The dashed lines between the bars show the variation across generations within line.

Figure 4. Variation in the temporal patterns of egg production in the set of lines of functional hermaphrodites. Worms of generation 4 produced a significantly smaller proportion of eggs in the first half of their layings (in relation to lifetime egg production) than their ancestors in generation 0

(notwithstanding the peak in the 1st egg laying).

Figure 5. Proportion of functional males and functional hermaphrodites in generation 4 in the two selected lines.