

Gender expression and group size: a test in a hermaphroditic and a gonochoric congeneric species of *Ophryotrocha* (Polychaeta)

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Accepted 20 January 2010

SUMMARY

Hermaphroditism and gonochorism are two contrasting forms of sexuality. Hermaphroditic species are generally seen as species adapted to conditions of low density, stabilized by poor mate search efficiency and high costs of searching. They can adjust allocation of reproductive resources to each sex function in response to current social conditions, making reproduction more efficient, at least in principle. By contrast, gonochorism (separate sexes) is advantageous when mates are frequent, making it ineffective to maintain two sex functions in a single individual. This, however, also rules out the need for a flexible response to mating opportunities as known for hermaphrodites. In the hermaphroditic marine polychaete worm *Ophryotrocha diadema* we showed earlier that group size is assessed through a chemical cue. In this study we verified the accuracy of the response to gradients of the chemical cue used to assess group size by *O. diadema* by checking reduction in egg production as the group of partners increases, as expected according to sex allocation theory. Furthermore we compared the effect of such a gradient with a similar gradient in a closely related gonochoric species (*O. labronica*). Here sex allocation adjustment is not predicted, thus an adaptive change in egg production in response to group-size cues is not expected. In fact, our results show that the group-size effect only occurs in *O. diadema* and not in *O. labronica*. Moreover, our study provides evidence of high perceptual abilities of chemical cues in *O. diadema*, suggesting that perceiving social cues and adjusting sex allocation appropriately are special properties of hermaphrodites.

Key words: hermaphroditism, gonochorism, female reproductive investment, social cues, perceptual abilities.

INTRODUCTION

Simultaneous hermaphrodites have functional male and female reproductive systems simultaneously active in the same individual for the greatest part of their life. Hermaphroditism can be adaptive only when individual reproductive success is greater than that of a pure male or a pure female (Charnov et al., 1976; Charnov, 1982). The conditions that lead to higher reproductive success in hermaphrodites must be associated with diminishing returns in at least one of the two sex functions (Charnov, 1982).

Sex allocation theory (Charnov, 1982) assumes that the optimal hermaphrodite divides a fixed amount of resources between sperm and eggs in response to social conditions. In hermaphrodites living in monogamous pairs both partners are expected to allocate the smallest amount of resources to sperm that is still sufficient to fertilize the partner's eggs. All remaining resources are then diverted to egg production. As the number of reproductive competitors or potential partners increases, allocation to the male function should increase and allocation to the female function decrease. Hence, sex allocation should reflect the current social conditions, with a trade-off between the male and female function. Unfortunately, there is no straightforward evidence for such a trade-off from empirical studies in animals (Raimondi and Martin, 1991; Yund et al., 1997; Trouvé et al., 1999; Locher and Baur, 2000; Schärer and Wedekind, 2001; Locher and Baur, 2002; Newlon et al., 2003; Schärer and Ladurner, 2003; Tan et al., 2004; Lorenzi et al., 2005; Brauer et al., 2007). One explanation for this is that a single reproductive resource budget that is perfectly divided into male and female allocation does not exist. In some papers, absolute investment in male and female

function is used – but this is prone to confounding effects caused by budget differences. In other papers, the same measure of male and female investment (e.g. gonad surface) is used to calculate relative male or female investment (Schärer et al., 2005; Brauer et al., 2007). This procedure controls for different budgets, but needs to be used with care because it also hides effects that may be of importance (e.g. individual differences). Male and female allocation may not compete for a common resource pool if there are temporal differences in the activity of both sex functions or when the nutrients required to build male and female tissues differ. Under such conditions, male or female investment may not trade-off with each other, but with other life-history traits (Schärer et al., 2005; Lorenzi et al., 2006). According to Locher and Baur (Locher and Baur, 2002) trade-offs in resource allocation may not occur or be less pronounced under favourable conditions, whereas under stressful conditions, such as limited food supply, high temperature or drought, the energy intake might be carefully shared among different functions, including reproduction (Reznick, 1992).

In contrast to hermaphrodites, sex allocation adjustments are not predicted for gonochorists. Thus, egg production in gonochoric species is expected to be independent of group size variation.

The ability of organisms to assess relevant cues about their social environment may be a major factor constraining the extent to which individuals adaptively adjust their sex allocation (West and Sheldon, 2002). *Eisenia andrei*, an epigeic hermaphrodite earthworm, detects the mating status of their partners and responds by adjusting their ejaculate tripling the donated sperm when mating with a non-virgin mate (Velando et al., 2008). In aquatic

environments, chemical signals are crucial in inducing behavioural reactions connected with reproduction. This includes finding a mate [as in the gonochoric crayfish *Pacifastacus leniusculus* (Stebbing et al., 2003) and the scale worm *Harmothoe imbricata* (Watson et al., 2000)], synchronizing release of gametes [e.g. in polychaetes (Hardege et al., 1998; Hardege and Bentley, 1997; Ram et al., 1999) and in corals (Twan et al., 2003)], and sperm activation and sperm attraction by eggs (Müller, 1976; Foltz, 1995). Hermaphroditic sea slugs of the genus *Aplysia* release a pheromone from their egg masses, which attracts potential mates and stimulates them to lay eggs on the same site (Painter et al., 2004). In hermaphroditic animals pheromones are also used to influence the fate of the donated sperm [e.g. in the pond snail *Lymnaea stagnalis* (Koene and Ter Maat, 2005)]. Among polychaetes, species-specific sex pheromones are known from few species (Andries, 2001). In the genus *Ophryotrocha*, females of the gonochoric *O. labronica* and of the sequentially hermaphroditic *O. puerilis* produce water-borne pheromones to attract males (Berglund, 1990), and the 'pair culture effect' [resulting from the fact that placing two mature *O. puerilis* females together causes one of the two to change sex (Hartmann and Huth, 1936)] is induced by a lipidic pheromone (Marchionni and Rolando, 1981). Little is known about the chemical cues that promote perception of social environment. In *O. diadema*, simultaneous hermaphrodites adaptively adjust their sex allocation to the mating opportunities independently of population density or metabolite accumulation (Lorenzi et al., 2005) and produce a species-specific chemical signal which allows an assessment of group size and hence presumably the number of potential mates (Schleicherova et al., 2006).

Therefore, in the present study we wanted to check perceptual abilities of chemical cues in *O. diadema* verifying the accuracy of the response to a 'chemical signal' known to affect allocation to the female function in this species (Schleicherova et al., 2006). Moreover, since hermaphrodites allocate their reproductive resources according to the mating group size, whereas gonochorists do not, we expected high perceptual abilities and sensitivity to the 'chemical signal' in the hermaphroditic species *O. diadema* and not in the gonochoric species *O. labronica*. Therefore, if the response is accurate, sex allocation to the female function (i.e. egg production) should decrease with increasing levels of the group-size cue in the hermaphroditic species, consistent with sex allocation theory. By contrast, in the gonochoric species, egg production is expected to be independent of group size variation.

As a test, we compared the effect on egg production of water from a large group of individuals in concentrations increasing from 0% to 100% in the hermaphroditic *O. diadema* and the gonochoric *O. labronica*.

MATERIALS AND METHODS

Study organisms

Ophryotrocha diadema

Ophryotrocha diadema, a 2–2.5 mm long marine polychaete, is a simultaneous hermaphrodite. All the *O. diadema* life cycle data (Åkesson, 1976; Åkesson, 1982) and information on the main features of its mating system (Sella, 1985; Sella, 1988; Sella, 1990; Sella, 1991; Premoli and Sella, 1995; Sella and Lorenzi, 2000) have been obtained through laboratory observations. *O. diadema* was originally collected from organic sediments of Californian harbours. According to D. J. Reish (personal communication to G.S.) they typically live in low-density populations. However, since adults produce a network of mucous trails that can be followed by

conspecifics, their spatial distribution is probably clustered (G.S., unpublished data).

The life cycle starts with a 21-day protandrous phase, followed by a 30- to 40-day simultaneously hermaphroditic phase (Åkesson, 1982). Mating is preceded by courtship during which both partners mutually rub against each other, and is achieved by pseudocopulation, a process of external fertilization in which partners maintain close physical contact before releasing their gametes [as described for *O. gracilis* by Westheide (Westheide, 1984)]. Partners regularly alternate sexual roles by reciprocally exchanging a transparent mucous cocoon with 20–25 eggs every second day. The costs of the investment in male or female gametes are unknown in this species. The only available information is that eggs are huge in comparison to sperm (Sella and Ramella, 1999) but high levels of sperm production can slow down juvenile body growth (Sella and Lorenzi, 2003) and sex adjustments load no costs in adults (Lorenzi et al., 2008). Since cocoons are transparent, egg development can be followed under a stereomicroscope. Nine days after egg-laying, offspring are released from the cocoon as small four-segment individuals, soon ready to produce their first sperm. When worms reach a body length of 14–17 segments they become simultaneous hermaphrodites with sperm in the fourth and fifth segment and eggs in all following segments (Åkesson, 1976).

We utilized individuals of an *O. diadema* strain derived from individuals collected by B. Åkesson in 1976 and 1980 in Long Beach harbour (Los Angeles). In order to increase genetic variability and to refresh our laboratory populations, new individuals were added to the strain in 1995 and again in 2001. These animals came from other laboratory cultures, and were kindly sent to G. Sella by B. Åkesson.

Ophryotrocha labronica

The life cycle of *O. labronica* La Greca and Bacci, as well as its rearing conditions, have been described by Åkesson (Åkesson, 1967; Åkesson, 1970a; Åkesson, 1970b; Åkesson, 1973) and Berglund (Berglund, 1991). *O. labronica* is geographically widespread and lives in the fouling community of harbours. Natural populations are patchily distributed and show different, but seasonally constant female–male sex ratios (Sella and Zambaldi, 1985; Prevedelli and Simonini, 2003). The development is completed in less than 1 month and life span is 6–7 months.

Berglund (Berglund, 1991) observed that *O. labronica* reproductive success increases significantly with female body size but not with male body size. During the courtship the male follows the female around, then they stay close together and pseudocopulate (Åkesson, 1970a; Åkesson, 1970b; Rolando, 1981). Egg production rate is comparable to that of *O. diadema* (Premoli and Sella, 1995), but temporally clustered. A female produces a tubular egg mass (an average of 120 eggs per cocoon, egg diameter: 120 µm) every second week for 3 months or more and provides maternal care to the developing embryos for about 6–7 days (Åkesson, 1973; Simonini, 2002). Since the sexes show only little morphological dimorphism, sex is best determined at sexual maturity on the basis of (1) the presence or absence of eggs in the coelomic cavity and (2) the number of the star-shaped glands on the dorsal side of the last segments (4–7 in males, 2–3 in females) (Pfannenstiel, 1976). We used individuals from an *O. labronica* strain collected in Rimini harbour (Ferrara, Italy) by R. Simonini in 2005.

Both *O. diadema* and *O. labronica* can be cultured under the same standard conditions (20°C, artificial sea water with 35‰ salinity and parboiled spinach as food).

Table 1. Group size simulation using different concentrations of water from high population density cultures of the same species

Treatment	% High pop density water	Volume of high pop density water (ml)	% Pure water	Volume of pure water (ml)
1	0	0	100	10.0
2	1	0.1	99	9.9
3	5	0.5	95	9.5
4	10	1.0	90	9.0
5	25	2.5	75	7.5
6	40	4.0	60	6.0
7	75	7.5	25	2.5
8	100	10.0	0	0

Experimental set-up

All experiments were carried out in 6×10 ml well plates. Up to 12 plates were placed together in loosely capped plastic boxes to reduce evaporation and kept in a thermostatic cabinet at 20°C. Animals were kept in groups of their own species. They were fed *ad libitum* with a constant amount (130 µl) of liquid spinach suspension once per week.

O. diadema group set-up

To generate a sufficient number of virgin hermaphrodites of the same age, 40 pairs of *O. diadema* were taken from the mass culture, isolated and allowed to reproduce. Their progeny were reared with their siblings until sexual maturity (i.e. until they had matured both male and female function). At the start of the experiment, 320 adults were randomly assigned to 160 *O. diadema* pairs. We assured that only non-siblings were paired.

O. labronica group set-up

We used 160 virgin adults (80 males, 80 females), selected from the offspring produced by 50 isolated pairs. Since the sex of *O. labronica* can be determined only at sexual maturity, individuals were reared with their siblings until they became adults. Afterwards a male and a female were randomly assigned to form a group of 80 *O. labronica* experimental, non-sibling pairs.

Treatment

The experiment started on the same day for both species and lasted 4 weeks. Plates and position within plates were randomized with respect to treatment and species. The position of the plates was also rotated within the thermostatic cabinet. Observations were blind for treatment. In order to obtain 'high population density' water, high-density source populations had been created for each species with a similar high density (approximately 400 adult individuals per 60 ml). From these populations, we obtained the '*O. diadema* high population density water' and the '*O. labronica* high population density water'. This 100% density water was diluted as indicated in the Table 1 and was then transferred to the respective treatment pair.

Twice a week each pair was checked and water replaced with a fresh mixture of the same concentration. By keeping all individuals in pairs and only varying exposure to the concentration of the chemical cue (see below), we excluded confounding effects caused by different group sizes, such as egg cannibalism or competition for food or fertilization. Instead, following Schleicherova et al. (Schleicherova et al., 2006), we simulated the group-size effect by using different concentrations of 'chemical signal' in water from high population density cultures of their own species (100% density water). We set up eight treatments per species with increasing concentrations of the 100% density water (0%, 1%, 5%, 10%, 25%, 40%, 75% and 100%; Table 1). Pairs treated with clean water (0% of chemical signal) were considered as control pairs. We started

with 20 pairs of *O. diadema* and 10 pairs of *O. labronica* for each treatment. Processing more than 30×8 pairs in parallel was not feasible and we decided *a priori* that a higher resolution would be more important for *O. diadema*. An *a posteriori* control on the data obtained confirmed that an increase in the number of *O. labronica* replicates to 20 would not have affected our conclusions.

As both partners produce eggs in *O. diadema*, we evaluated female reproductive investment of *O. diadema* pairs by counting the number of cocoons per pair and the number of eggs per cocoon twice a week. In subsequent calculations, we used the average number of eggs per individual. In *O. labronica*, we evaluated female reproductive investment of pairs by the number of cocoons per individual and the number of eggs per individual twice a week. At each census cocoons were removed from plates in order to avoid variation of the social conditions due to the presence of progeny developing from cocoons.

Statistical analyses

Pairs in which one individual died ($N=3$, *O. labronica* only) were ignored for analysis. All other pairs produced eggs during the 4-week observation period and could be used for analysis. Distributions of egg and cocoon numbers were tested for normality using the Kolmogorov–Smirnov one-sample test and for homogeneity of variance using Levene's test. All data were normally distributed ($P>0.05$).

Since Pearson's test revealed a significant correlation between the number of eggs and the number of cocoons per individual, only the number of eggs per individual was considered for the analyses. Statistical analyses were performed using the general linear model (GLM). As first analysis we checked whether the two species responded differentially to the treatment by univariate ANOVA with number of eggs per individual (i.e. female reproductive output) as the dependent variable, species as a fixed factor and treatment as covariate. Moreover, we compared female reproductive output between treatments using univariate ANOVA with number of eggs per individual as the dependent variable, and treatment as a fixed factor. Statistics are reported as means ± standard deviation (s.d.). Moreover, we checked differences in female reproductive output between treatments by means of contrasts in one-way ANOVA using the first treatment (0% of chemical cue) as a reference. Finally, we used linear regression to verify the relationship between female reproductive output and treatment. Probabilities are two-tailed. Statistical analyses were performed using SPSS 17.0.0 (SPSS Inc, Chicago, IL, USA).

RESULTS

O. diadema and *O. labronica* responded differently to the treatment (univariate ANOVA interactions: factor species: $F_{1,1011}=108.522$, d.f.=1, $P<0.001$; factor treatment: $F_{1,1011}=1.117$, d.f.=1, $P=0.291$; interaction species×treatment: $F_{1,1011}=6.903$, d.f.=1, $P<0.009$).

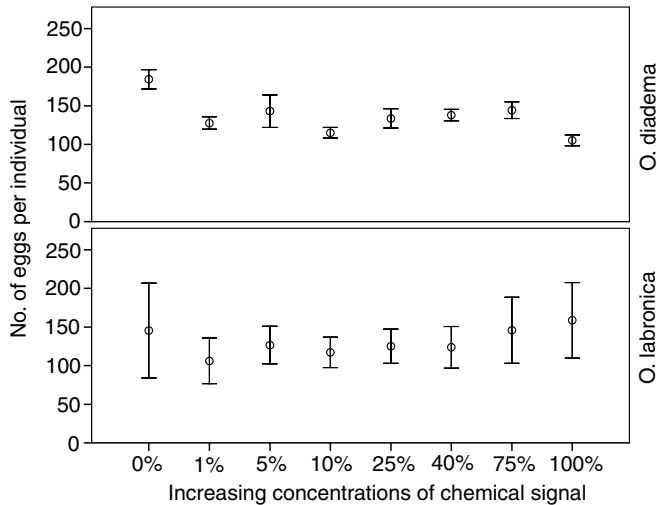


Fig. 1. Linear regression of the mean number of eggs per individual in relation to the treatment in *O. diadema* and in *O. labronica*.

Female reproductive output of *O. diadema* significantly decreased with increase of the chemical signal concentration (linear regression: $r = -0.232$, $F_{1,157} = 8.902$, $P = 0.003$), whereas such a relationship did not exist in *O. labronica* (eggs: linear regression: $r = 0.092$, $F_{1,57} = 0.477$, $P = 0.493$; Fig. 1).

Moreover, highly significant differences between treatments in female reproductive output in *O. diadema* pairs were observed (number of eggs per individual: ANOVA, $F_{7,150} = 4.17$, $P < 0.001$). However, not all pairwise comparisons between treatments showed a significant difference in egg production. Using treatment 1 (0% of chemical signal) as a reference, *O. diadema* control pairs, laid significantly more eggs per individual than all the treated pairs (ANOVA contrasts, $t = 4.42$, d.f. = 150, $P < 0.001$). In particular, the direct comparison between the 0% and 1% treatment showed that *O. diadema* control pairs (0% of chemical signal) laid significantly more eggs per individual than *O. diadema* pairs treated with 1% of chemical signal (ANOVA contrasts, $t = 3.46$, d.f. = 150, $P = 0.001$).

DISCUSSION

Our results confirm findings of a previous study by Schleicherova et al. (Schleicherova et al., 2006), i.e. that reduced female allocation in the hermaphroditic *O. diadema* is induced by a chemical signal that conveys information about group size (Schleicherova et al., 2006). More precisely we can now state that the response to the chemical cue occurs even at 1% concentration. Such a high perceptual ability is a new finding for the *Ophryotrocha* genus. Our results confirm that the reduced egg production in high mating group size (Lorenzi et al., 2005) is not a consequence of the stress caused by accumulation of toxic metabolic by-products or by direct behavioural interactions with rivals (Lorenzi et al., 2006). Such interactions are known to have negative effects on fecundity in other polychaetes (Peschet et al., 1983) but were absent in our experimental design. Reduced egg production is an adaptive response to perceived social conditions (i.e. large group size) and consistent with predictions of sex allocation theory (Charnov, 1982; Fischer, 1984).

Although in *O. diadema* the general trend of egg production is negatively correlated with increasing concentration of the group size cue, not all pairwise comparisons between treatments showed a significant difference in egg production. This finding indicates

that the ability to discriminate between different concentrations of the chemical cue is limited, as if what matters to the receiver is not the knowledge of the exact number of potential partners but only whether they are one or more than one. Consequently, such findings support the results of Schleicherova et al. (Schleicherova et al., 2006), who showed that the ability of *O. diadema* to discriminate the number of partners is limited. These polychaetes are not able to discriminate between 6 and 12 partners, but only between one partner and more than one partner. This can explain why a significant response in sex allocation variation occurs even at a 1% concentration of 'high density water'. Nevertheless, since the response in sex allocation occurs at such a low concentration of the chemical cue, our results also show high perceptual abilities in this hermaphroditic species. Similar high perceptual abilities of social environment by means of sex pheromones have been shown in other invertebrates. In the nematode *Diplosteron potohikus* 1 p.p.m. of tryptamine, a pheromone-like substance produced by females, is effective in inducing maleness through the inhibition of the development of the female reproductive system when potential mates are available (Clark, 1977). In the marine copepod *Temora longicornis* males are able to perceive a chemical gradient of female pheromones and adjust their behavioural responses accordingly (Yen et al., 1998). In the parasitoid wasp *Trissolcus suffidus* the low traces of pheromones produced by females are sufficient to regulate the sex ratio of offspring (Viktorov and Kochetova, 1973). In the case of the polychaete *Platynereis dumerilii* (Zeeck, 1996) a threshold concentration of $0.6 \mu\text{mol l}^{-1}$ of uric acid is effective in inducing spermatozoid release. However, high perceptual abilities are known only in a few polychaete species (Bartels-Hardege et al., 1996; Müller et al., 1999; Ram et al., 1999; Rohl et al., 1999).

In contrast to *O. diadema*, in the gonochoric *O. labronica*, where sex allocation adjustments are not predicted by theory, egg production was unaffected by gradients of seawater from the 'high population density water'. This result is in accordance with the expectation that in a gonochoric species egg production is independent of group size and not influenced by mating opportunities. Moreover, it is a further demonstration that foul seawater was not harmful to egg production for either *O. diadema* or *O. labronica*.

To date, only in one of the hermaphroditic species of the genus *Ophryotrocha* has accuracy of social cue perception been studied. Thus, further studies are necessary to better understand the mechanisms that allow hermaphrodites to assess their social environment.

ACKNOWLEDGEMENTS

We thank Gregor Schulte for his great technical support in the laboratory, Marianne Kaiser and Beatriz Sanchez Navarro for their help with the experiment. We are particularly grateful to Daniella Prevedelli and Roberto Simonini (University of Modena) who kindly provided *O. labronica* individuals. We also thank Sergio Castellano for advice on the use of GLM. This project was funded by the Deutscher Akademischer Austausch Dienst (DAAD, Germany).

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