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Phenotypically plastic adjustment of sex allocation in a simultaneous hermaphrodite

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Sex allocation theory for simultaneous hermaphrodites predicts an influence of the mating group size on sex allocation. Mating group size may depend on the size of the group in which an individual lives, or on the density, but studies to date have not distinguished between the two factors. We performed an experiment in which we raised a transparent simultaneous hermaphrodite, the flatworm *Macrostomum* sp., in different group sizes (pairs, triplets, quartets and octets) and in different enclosure sizes (small and large). This design allows us to differentiate between the effects of group size and density. After worms reached maturity we determined their reproductive allocation patterns from microscopic images taken *in vivo*. The results suggest that the mating group size is a function of the group size, and not of the density. They support the shift to higher male allocation in larger mating groups predicted by sex allocation theory. To our knowledge, this is the first study that unambiguously shows phenotypically plastic sex allocation in response to mating group size in a simultaneous hermaphrodite.

Keywords: local mate competition; phenotypic plasticity; platyhelminthes; sex allocation; simultaneous hermaphrodite; sperm competition

1. INTRODUCTION

Sex allocation theory is an important branch of life-history theory (Stearns 1992). It attempts to predict the optimal investment of limited resources to male and female reproductive functions and has been formulated for all major types of sexuality, namely dioecy (Fisher 1930; Hamilton 1967; Charnov 1982), sequential hermaphroditism (Ghiselin 1969; Warner *et al.* 1975; Charnov 1982), simultaneous hermaphroditism (Charnov 1979; Charlesworth & Charlesworth 1981; Charnov 1982; Lloyd 1982) and cyclical parthenogenesis (Innes & Dunbrack 1993; Aparici *et al.* 1998). Sex allocation theory has stimulated a wide array of empirical studies, but recent reviews conclude that experimental tests are still required, particularly in hermaphrodites (Godfray & Werren 1996; Campbell 2000; Komdeur & Pen 2002; West *et al.* 2002). With the exception of plant studies, most work on sex allocation has been restricted to organisms with separate sexes.

We aimed to test a fundamental prediction of sex allocation theory for outcrossing simultaneous hermaphrodites: that the mating group size influences sex allocation (Charnov 1980, 1982). In Charnov's model mating group size is denoted as $K + 1$, where K is the number of sperm donors from which a recipient receives sperm at the time its eggs are fertilized. It is hence a measure of the strength of sperm competition, and higher allocation to male reproduction is predicted in larger mating groups. Charnov's argument is very similar to that of sex-ratio adjustment under local mate competition (Hamilton 1967). With increasing mating group size the related sperm from one hermaphrodite are increasingly in competition with unrelated sperm from other hermaphrodites.

Mating group size may influence sex allocation in two ways: first, in evolutionary terms, where the average mating group size encountered in subsequent generations selects for an optimal sex allocation; and second, in terms of phenotypic plasticity, where short-term adjustments in sex allocation are made in response to current mating group size (or to current environmental conditions that affect the mating group size). We focus on the latter mechanism.

First, we show that our model species, the free-living flatworm *Macrostomum* sp., is an outcrossing simultaneous hermaphrodite. We then demonstrate that growth, reproductive allocation and sperm transfer can be reliably determined by using non-invasive morphometry (*Macrostomum* sp. is almost completely transparent). In the main part of the study we then experimentally investigate the effect of mating group size on sex allocation. Direct manipulation of mating group size is difficult, as it requires experimental control over sperm donation. However, mating group size may be related to the size of the group in which an individual lives, and this factor can be manipulated experimentally (Trouvé *et al.* 1999; Schärer & Wedekind 2001). There is, however, a problem with this approach. By changing the group size one automatically changes the density at which individuals live, and density may itself affect reproductive allocation.

Higher density may, for example, lead to resource competition. We can control for this kind of density effect by keeping the worms in all group sizes under *ad libitum* food conditions. Higher density may also lead to higher concentrations of harmful metabolites. However, without a detailed understanding of how metabolites affect growth and reproduction, it is difficult to control for them. We therefore introduced a second factor that manipulated density independently of the group size. This was achieved

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by placing the worms in the different group sizes in both small and large enclosures. The variation in density caused by this factor was matched to that produced by the group size. If density has an effect on growth and reproduction, one would expect to see it in both factors.

We expected that larger amounts of sperm would be transferred in larger groups in response to more intense sperm competition. We further expected that individuals that mature in a larger group allocate more to male reproduction in order to replenish the sperm used up in sperm competition. Finally, under the assumption of a trade-off between male and female reproductive allocation, we expected a corresponding drop in female allocation. Such a trade-off may, however, be difficult to reveal under the *ad libitum* food conditions used.

2. MATERIAL AND METHODS

(a) *Study animal*

Macrostomum sp. (Rhabditophora: Macrostomida) is a member of the interstitial sand fauna of the Northern Adriatic Sea (Ladurner *et al.* 2000). It is an outcrossing simultaneous hermaphrodite (see § 2b) and reaches 1.5 mm in length when fully grown. It is transparent, allowing non-invasive observation of internal structures (figure 1). The paired testes are located anterior to the paired ovaries, and the female gonopore is anterior to the male gonopore. The female gonopore opens into the female atrium, into which sperm are transferred during copulation. The male gonopore is associated with a sclerotic stylet, which serves as a copulatory organ, and with a seminal vesicle, which contains the sperm to be used in future copulations. Copulations are frequent and reciprocal. Received sperm can often be observed in the female atrium, where sperm heads stick in a specialized tissue that connects to the oviduct, and sperm tails often beat vigorously. Eggs start to form posterior to the ovary, gradually increase in size during vitellogenesis and enter the female atrium, generally one at a time, where they remain for some time before being laid. Mass cultures of *Macrostomum* sp. have been maintained at the University of Innsbruck since 1995 according to culture conditions described elsewhere (Tyler 1981; Rieger *et al.* 1988). Briefly, worms are maintained at 20 °C in glass Petri dishes containing f/2 medium, a nutrient-enriched artificial sea water (Guillard & Ryther 1962), and fed with diatoms of the species *Nitzschia curvilineata*. During the experiments worms were under constant illumination. Generation time under these conditions is 18 days: 5 days from egg laying to hatching and 13 days from hatching to adult.

(b) *Check for self-fertilization*

To assess whether worms can reproduce via self-fertilization, we placed either one or two randomly chosen juvenile worms from mass cultures into wells of 24-hole tissue culture plates (24 replicates per treatment group), and scored the production of viable offspring 25 days later. Twenty-three of the replicates that we started with one hatchling contained an adult worm, but none of these produced eggs or offspring during the experiment, suggesting that self-fertilization is not possible. By contrast, 18 of the replicates we had started with two hatchlings contained two adult worms, and these produced between five and 41 hatchlings per pair, which clearly indicates that the worms we used in the experiments were fertile.

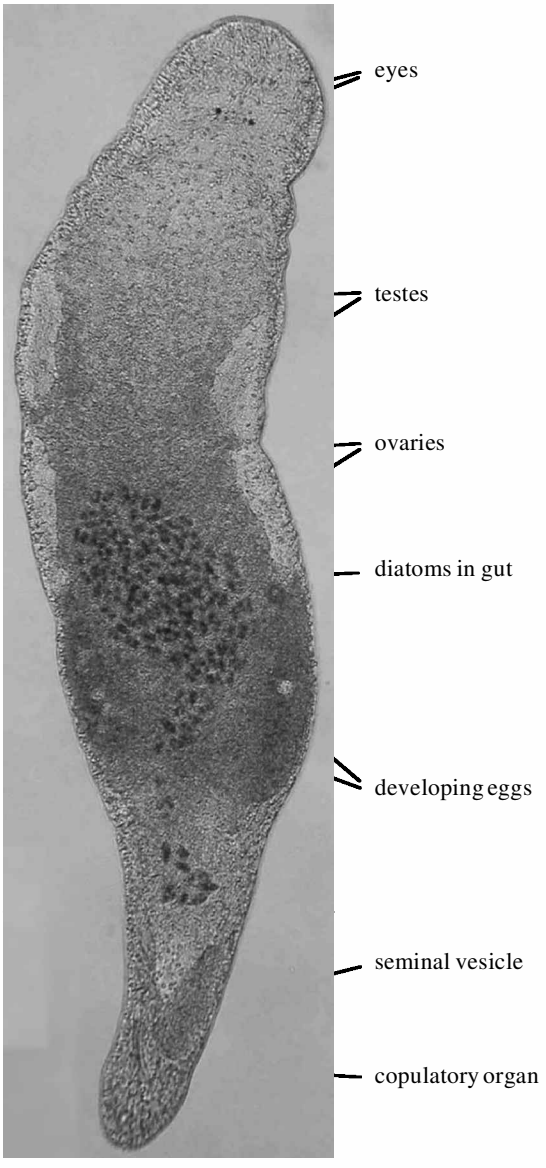

Figure 1. Adult *Macrostomum* sp. squeezed in a standardized way between two glass plates. The total length of the worm is *ca.* 1.5 mm.

(c) *Morphometry*

We anaesthetized worms in a 1 : 1 mixture of f/2 medium and 7.14% $MgCl_2$ solution, placed them on a glass slide and squeezed them dorsoventrally with a cover glass of a haemacytometer (using a plastic film of 35 μm thickness as a standardized spacer). We observed worms with a Leitz Diaplan compound microscope (figure 1), and estimated the received sperm score, i.e. the amount of received sperm in the female atrium, on a scale from 0 (no sperm visible) to 3 (many sperm visible). We then took digital pictures at magnifications of 40–400× with a c-mount video camera (Sony CCD Iris) connected to an Apple PowerMacintosh 8100/100 AV, running the public domain image-analysis software NIH-Image 1.62 (developed at the US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). From the pictures, we later determined the areas of the worm, of the testes (sum of both testes), of the ovaries (sum of both ovaries) and of the seminal vesicle (also called the vesicular sperm area). If present, we also determined the area of eggs in the female atrium (such eggs are fully formed and the area can therefore serve as a measure of

Table 1. The effects of the factors (group size and enclosure size) and the covariates (time of measurement and body area) on the dependent variables (reproductive parameters). The type of ANOVA used is as follows: body area (nested two-way ANCOVA), testis area (nested two-way ANCOVA), vesicular sperm area (nested two-way ANCOVA), received sperm score (two-way ANCOVA), ovary area (nested two-way ANCOVA), fecundity (two-way ANCOVA), and egg size (nested two-way ANCOVA). Note that JMP synthesizes non-integer denominator degrees of freedom because the nested factor is defined as random (SAS 1994, p. 257).

dependent variable	model fit	factor 1: group size			factor 2: enclosure size			interaction			nested factor: replicate			covariates: time			covariates: body area		
	r^2	F	d.f.	p	F	d.f.	p	F	d.f.	p	F	d.f.	p	F	d.f.	p	F	d.f.	p
body area	0.26	0.88	3,50.7	0.46	0.05	1,65.1	0.82	0.04	3,50.4	0.99	0.99	39,129	0.50	4.01	1,129	0.047	—	—	—
testis area	0.68	4.11	3,47.8	0.011	0.36	1,58.3	0.55	1.19	3,47.2	0.32	1.28	39,128	0.15	25.7	1,128	<0.001	121.4	1,128	<0.001
vesicular sperm area	0.59	7.50	3,46.2	<0.001	<0.001	1,54.6	0.99	0.68	3,45.7	0.57	1.57	39,128	0.031	93.2	1,128	<0.001	3.96	1,128	0.048
received sperm score	0.26	3.46	3,35	0.026	0.27	1,35	0.61	0.49	3,35	0.69	—	—	—	1.13	1,35	0.29	1.80	1,35	0.19
ovary area	0.58	1.46	3,52.1	0.24	3.20	1,67.7	0.078	0.63	3,51.2	0.60	0.88	39,128	0.67	13.2	1,128	<0.001	95.8	1,128	<0.001
fecundity	0.31	0.25	3,37	0.86	0.84	1,37	0.36	0.77	3,37	0.52	—	—	—	4.37	1,37	0.044	4.11	1,37	0.050
egg size	0.72	0.18	3,22.6	0.91	2.75	1,27.1	0.11	0.36	3,23.8	0.28	1.07	18,12	0.47	3.31	1,12	0.094	0.58	1,12	0.81

egg size). The pictures and the measurements were taken blind with respect to the treatment groups. After measurement, worms were placed into fresh medium and they quickly recovered from anaesthesia.

We determined the repeatability of the morphometric measurements by subjecting worms to two measurement sets. Each set consisted of the complete measurement sequence outlined above. The results suggested good repeatabilities for all morphometric measurements, especially given that they were performed non-invasively on live animals (single classification analysis of variance (ANOVA) and intraclass correlation coefficient r_I, as in Sokal & Rohlf (1995): body area, $F_{36,37} = 6.0$, $r_I = 0.71$; testis area, $F_{36,37} = 7.5$, $r_I = 0.76$; vesicular sperm area, $F_{36,37} = 9.4$, $r_I = 0.81$; received sperm score, $F_{36,37} = 4.0$, $r_I = 0.60$; ovary area, $F_{36,37} = 3.6$, $r_I = 0.57$; all $p < 0.001$). Owing to the distributional properties of the received sperm score, we also calculated a Spearman's rank correlation coefficient between the two replicate measurements as a measure of repeatability ($r_s = 0.63$, $p < 0.001$).

(d) *Experiment*

We randomly assigned juvenile worms from the mass cultures to a group size (pairs, triplets, quartets or octets) and an enclosure size (small or large, i.e. six-hole or 24-hole tissue culture plates containing 1.4 or 6.1 ml of medium, respectively). Each factor thus produced a fourfold variation in density. Each factor combination was replicated six times giving a total of 204 worms in 48 replicates. We achieved random assignment by pipetting individual worms to their assigned well according to a permutation of all the factor combinations, effectively avoiding sequence effects. On day 1 we added a standard amount of diatoms to the wells. Throughout the experiment worms were kept under *ad libitum* food conditions (i.e. a dense layer of diatoms on the bottom of the wells). We changed the medium on day 7, and transferred worms to fresh culture plates on days 13 and 14. On days 17 to 19 worms were chosen for analysis in a random order, removing one worm at a time from a well.

At the end of the experiment one pair replicate contained only one worm and was excluded from further analyses. Otherwise the final numbers of worms in the replicates closely matched the numbers we had intended to produce (pairs, mean = 2.0, $n = 11$; triplets, mean = 2.9, $n = 12$; quartets, mean = 4.0, $n = 12$; octets, mean = 7.5, $n = 12$). This suggested that pipetting errors and/or losses caused by mortality were insignificant. Eleven worms were lost during the measurement process, and seven worms appeared to be malformed (lack of or incomplete formation of the stylet, lack of seminal vesicle, no growth) and were excluded. Final sample sizes were 177 worms in 47 replicates. After removing the adult worms for measurement, we counted the number of hatchlings after all eggs had hatched. As a measure of fecundity we used the *per capita* number of hatchlings produced in a well during the experiment.

(e) *Statistical analysis*

The effects of the experimental factors on body area were analysed with a nested two-way analysis of covariance (ANCOVA), with group size and density as fixed factors, and with the replicate containing the measurements of each individual worm nested in both factors and treated as a random effect. The time of measurement was included as a covariate. Similarly, for the testis area, seminal vesicle area, ovary area and egg area the analyses were nested two-way ANCOVAs including time of measurement and body area as covariates. Egg area could be

measured only in 40 worms that had an egg in the female atrium. The amount of received sperm was measured on a discontinuous scale, and thus the nested two-way ANOVA approach was not used for this parameter. Instead, we calculated the average amount of received sperm per replicate. A received sperm score was obtained from 123 worms, yielding an average received sperm score for 45 replicates. As this parameter was approximately normally distributed, we analysed it with a two-way ANCOVA with the mean time and mean body size for the replicate as covariates. We further determined whether fecundity was affected by the treatments with a two-way ANCOVA with both mean time and mean body area as covariates. We graphically checked whether the data fulfilled the assumptions of parametric-test statistics, and transformed the data if necessary. If no suitable transformation could be found, we used non-parametric statistics. For all statistical tests we give two-tailed error probabilities. Averages are always given as mean ± 1 s.e. Data were analysed with JMP 3.2.2 (SAS 1994).

3. RESULTS

Adult size was not significantly affected by group size, enclosure size or their interaction (table 1; figure 2*a*), which suggests that the worms in the different treatments are comparable. As predicted by sex allocation theory, testis size was strongly affected by group size: worms that grew up in larger groups had significantly larger testes (table 1; figure 2*b*). Enclosure size, however, had no significant effect on testis size, nor was there a significant interaction between group size and enclosure size, suggesting that density was not the cause of the observed effect. The significantly lower vesicular sperm areas of worms in larger groups (table 1; figure 2*c*) suggested that, despite their larger testes, they were unable to replenish the sperm they used in mating. This view is supported by the positive effect of time on vesicular sperm area, which suggests that, owing to the continuous removal of worms for measurement, the number of mating partners gradually decreased, allowing sperm stores to recover. Further evidence for higher mating activity or larger ejaculate size per copulation comes from the significantly higher received sperm score observed in the larger groups (table 1; figure 2*d*). Like testes area, both vesicular sperm area and the received sperm score appeared to be unaffected by enclosure size. The combination of the results for the three male reproductive parameters clearly suggests that sperm competition is present, and that the manipulation of group size did lead to different mating group sizes.

Sex allocation theory predicts that female reproductive investment should trade off with male reproductive investment. Although ovary size decreased with increasing group size, the effect did not reach statistical significance (table 1; figure 3*a*). Further, there was no indication that worms in larger groups had a lower fecundity (table 1; figure 3*b*), or that they produced smaller eggs (table 1; figure 3*c*). The significant effects of time and body size on fecundity, however, suggest that we determined fecundity sufficiently accurately. Despite a trend for ovary size to increase with enclosure size, no corresponding pattern was detected in either fecundity or egg size. We conclude that female reproductive allocation was not strongly affected by the conditions of our experiment.

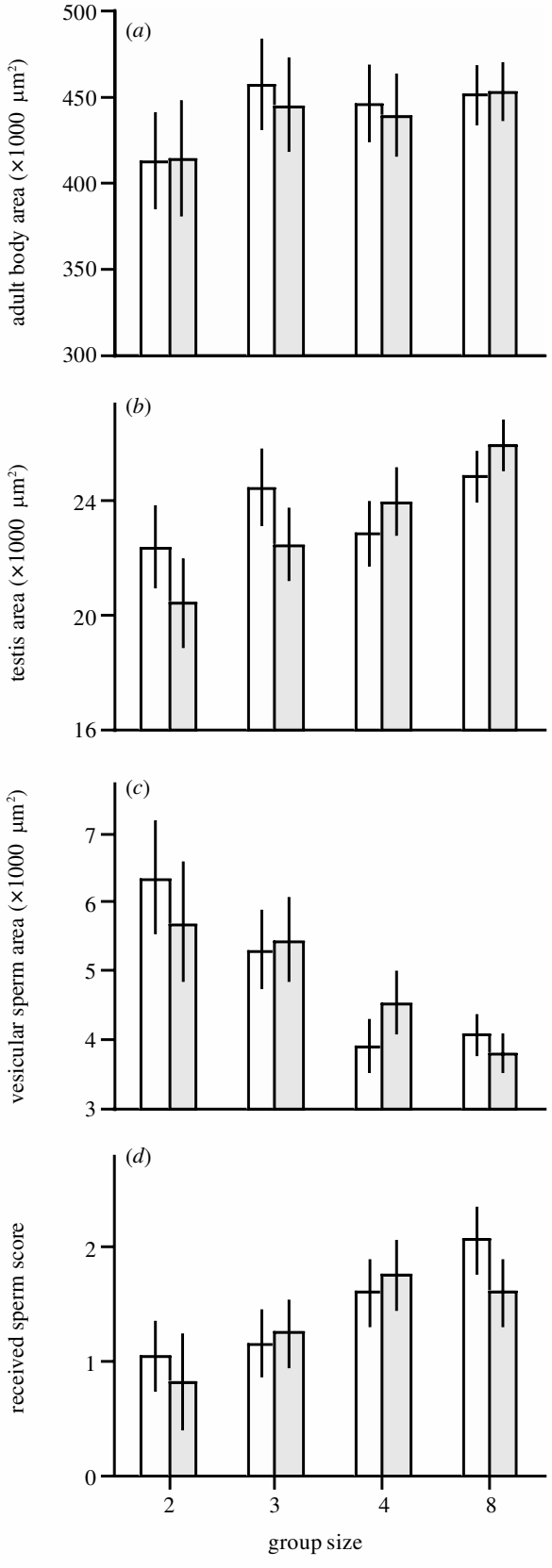

Figure 2. Effect of group size (*x*-axis) and enclosure size (white bars, small; grey bars, large) on adult body area and male reproductive parameters. We show the least-squares means (± 1 s.e.) of the analyses presented in table 1. Values were transformed back to the original scale when analyses were done on transformed parameters, which can lead to asymmetrical standard errors. (*a*) Adult body area, (*b*) testis area, (*c*) vesicular sperm area, and (*d*) received sperm score. See table 1 for statistics.

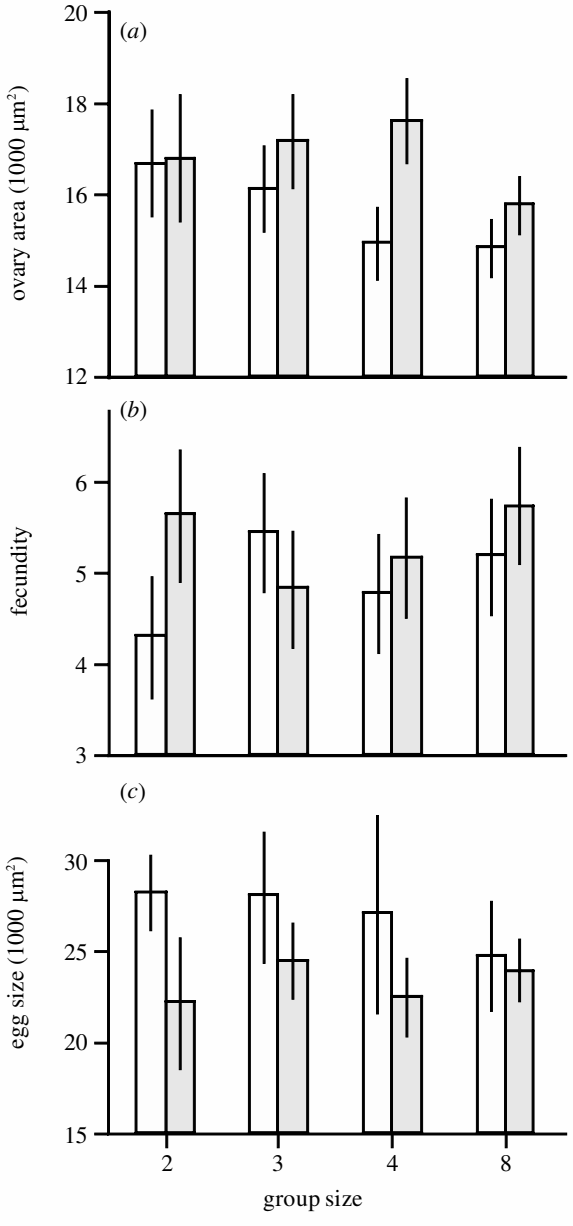

Figure 3. Effect of group size (*x*-axis) and enclosure size (white bars, small; grey bars, large) on female reproductive parameters. We show the least-squares means (± 1 s.e.) of the analyses presented in table 1. Values were transformed back to the original scale when analyses were done on transformed parameters, which can lead to asymmetrical standard errors. (*a*) Ovary area, (*b*) fecundity, and (*c*) egg size. See table 1 for statistics.

4. DISCUSSION

Our study provides the first unequivocal experimental evidence, to our knowledge, for a phenotypically plastic adjustment of sex allocation as a result of different mating group size in a simultaneous hermaphrodite. We show that, as evidenced by their larger testis size, individuals that were raised in larger groups did increase their male reproductive allocation in accordance with the predictions of sex allocation theory (Charnov 1982). Furthermore, the observed changes in the patterns of sperm transfer suggest that we did successfully manipulate mating group size in our experiment.

Other studies have suggested phenotypically plastic changes in sex allocation in response to changes in mating group size. However, these studies have either been purely descriptive (Raimondi & Martin 1991), or not experimentally controlled for the potentially confounding effects of density and body size (Trouvé *et al.* 1999). The former study provided correlational evidence for an influence of the mating group size on sex allocation in an outcrossing simultaneous hermaphrodite, the barnacle *Catomerus polymerus*. The study compared allocation patterns between individuals collected from patches with naturally occurring density differences (and hence presumably different mating group sizes) and found the predicted lower allocation to the male function at lower density.

The latter study experimentally infected mice with one, two or twenty individuals of the parasite *Echinostoma caproni*, and determined sex allocation after the parasites reached maturity (Trouvé *et al.* 1999). The study found a very strong increase in male reproductive allocation with increasing group size, and a corresponding decrease in female allocation, as predicted by theory. A former study had shown that this parasite can self- and cross-fertilize simultaneously, and that individuals in larger groups do receive sperm from several partners (Trouvé *et al.* 1996), suggesting that the mating group sizes in the different group sizes had indeed been different. However, there are at least two alternative explanations for the different sex allocation patterns in the different group sizes. First, density differed between the treatment groups, and the significantly smaller size of worms from larger groups indeed suggests that there was resource competition. This, in combination with recent evidence for size-dependent sex allocation in hermaphroditic animals, suggests an alternative explanation for these findings (Petersen & Fischer 1996; Schärer *et al.* 2001; Schärer & Wedekind 2001). Small individuals are generally more male-biased in their allocation, a finding that is well documented in plants (Lloyd & Bawa 1984; Klinkhamer *et al.* 1997). Hence, the smaller size attained by individuals at high density could explain the observed differences in sex allocation. Second, accumulation of harmful metabolites at higher densities may also affect growth and reproductive allocation. So-called crowding effects have been described for intestinal parasites (Zavras & Roberts 1984), which may cause a delay in the maturation of worms. Given the frequently mentioned pattern that male gonads mature earlier than female gonads in helminths and annelids (Ghiselin 1969), crowding could also explain the results.

Our study was designed to avoid the complications mentioned above. First, feeding was *ad libitum*, and the lack of effects of the factors on body size makes resource competition unlikely. Second, given that the fourfold variation in enclosure size had no significant effects on any of the parameters studied, accumulation of harmful metabolites is also unlikely to explain the patterns we observed. Rather, it appears that worms adjust their sex allocation to the number of potential mating partners, irrespective of the size of the enclosure in which they grow up. High density should lead to a higher encounter rate between worms, and hence to a higher perceived group size. Individuals can distinguish between a higher perceived group size and an actual increase in group size only if they have a mechanism to differentiate between previously and newly

encountered members of the group. The mechanism by which the worms achieve this warrants further investigation.

Contrary to the predictions from sex allocation theory the female function appeared to be unaffected by group size. A likely explanation for this is that individual differences in growth rates and in fecundity could have obscured a possible trade-off because the worms were fed *ad libitum* (Bell & Koufopanou 1986). Alternatively, male allocation could trade off with survival or regeneration ability.

Evidence for phenotypically plastic adjustment of male allocation in response to environmental variation is also scarce in organisms with separate sexes. We are aware of only four such studies (He & Tsubaki 1992; Gage 1995; Stockley & Seal 2001; Hellriegel & Blanckenhorn 2002), none of which distinguish between the effects of group size and those of density. The separation of these effects enables a better understanding of the factors that are responsible for phenotypic plasticity in reproductive allocation.

Sex allocation theory has the potential to unify conceptually the diverse patterns of sexuality, i.e. separate sexes, sequential hermaphroditism, simultaneous hermaphroditism and cyclical parthenogenesis, under a common theoretical framework. The recent shift from purely descriptive to experimental approaches is required to uncover the causal relationships that underlie observed sex allocation patterns (Godfray & Werren 1996; Campbell 2000; Komdeur & Pen 2002; West *et al.* 2002). Ultimately, knowledge of these will allow us to understand the similarities and the dissimilarities of different sexual modes. A unified view of sexuality is required to understand the evolution and maintenance of sexual reproduction, a question that was brought to our attention over a generation ago (Williams 1975; Maynard-Smith 1978; Bell 1982), and which remains an active area of research (Barton & Charlesworth 1998; West *et al.* 1999; Doncaster *et al.* 2000; Agrawal 2001; Siller 2001).

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