Trade-off between male and female allocation in the simultaneously hermaphroditic flatworm *Macrostomum* sp.

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**Introduction**

Simultaneous hermaphrodites possess both functional male and female reproductive organs at the same time. A hermaphrodite may thus be able to adjust the allocation of reproductive resources in response to environmental conditions in an opportunistic way (Charnov, 1979, 1982; Michiels, 1998). It is thereby expected to allocate the reproductive resources in a way that maximizes the fitness gained through both functions, and predicting this optimal allocation is the goal of sex allocation theory. A general feature of most sex allocation models is that individuals have a fixed reproductive resource budget, of which a proportion $r$ is invested in the male function, leaving a proportion $f = 1 - r$ for the female function (Charnov, 1982). These models therefore imply a direct trade-off in allocation to the male and female functions. Such physiological trade-offs generally can result from life-history decisions that compete directly with one another for limited resources within a single individual (Stearns, 1992). Following Bell & Koufopanou (1986) a negative phenotypic correlation between two such processes indicates a trade-off.

Although the trade-off between male and female allocation in simultaneous hermaphrodites is a fundamental assumption of sex allocation theory, there is only conflicting evidence for it from empirical studies that deal with animals (e.g. Raimondi & Martin, 1991; Yund *et al.*, 1997; Trouvé *et al.*, 1999; Locher & Baur, 2000; Schärer & Wedekind, 2001; Locher & Baur, 2002; Newlon *et al.*, 2003; Schärer & Ladurner, 2003; Tan *et al.*, 2004). One possible explanation for this is that a single reproductive resource budget that is perfectly divided into male and female allocation does not exist. Indeed, theoreticians have often been careful about the trade-off assumption and mention other possibilities (e.g. Charnov *et al.*, 1976). For example, male and female allocation may not compete for a common resource pool if there are temporal differences in the investment to male and female function or when the limiting nutrients required to build the male and female tissues are different. Under such conditions male and female allocation may not trade-off with each other, but with other life-history traits.

**Keywords:**

local mate competition; mating group size; platyhelminthes; resources; sex allocation; simultaneous hermaphrodite; sperm competition.

**Abstract**

Sex allocation theory for simultaneous hermaphrodites assumes a direct trade-off between the allocation of resources to the male and female reproductive functions. Empirical support for this basic assumption is scarce, possibly because studies rarely control for variation in individual reproductive resource budgets. Such variation, which can have environmental or genetic sources, can generate a positive relationship between male and female investment and can thus obscure the trade-off. In this study on the hermaphroditic flatworm *Macrostomum* sp. we tried to control for budget effects by restricting food availability in a standardized way and by using an inbred line. We then manipulated mating group size in a two-way design (two group sizes × two enclosure sizes) in order to induce phenotypic variation in male allocation, and expected to find an opposing correlated response in female allocation. The results suggest that we only managed to control the budget effects under some conditions. Under these the sex allocation trade-off emerged. Under the other conditions we found a strongly positive correlation between male and female allocation. We discuss possible causes for the observed differences.
A very different explanation for the ambiguous support for the sex allocation trade-off may be that, despite the fact that the trade-off exists, experiments to date have not been performed under the right conditions. Whether a phenotypic trade-off between the allocation to the two sex functions is found in empirical studies may depend on two main factors: the amount of variation in the reproductive resource budget and the amount of variation in sex allocation (cf. van Noordwijk & De Jong, 1986). Variation in budgets has been found in several empirical studies (e.g. Petersen & Fischer, 1996; Trouvé et al., 1999; Schärer et al., 2001) and is starting to be included in recent sex allocation models (Klinkhamer et al., 1997; Angeloni et al., 2002). When variation in the reproductive resource budget is large, but variation in sex allocation small, positive rather than negative correlations will result (Fig. 1). Hence, in order to demonstrate a trade-off, it may be imperative to minimize variation in the reproductive resource budget, while increasing variation in sex allocation (Campbell, 2000, cf. van Noordwijk & De Jong, 1986), and it is this approach that we take here. Failure to confirm the trade-off assumption using such an approach would make it necessary to question its validity.

Variation in reproductive resource budget can have genetic and environmental sources, and we will here try to limit the impact of both. To reduce genetic variation, for example, the ability to acquire, store, and convert food (Campbell, 2000; Mangel & Stamps, 2001) one can use an inbred line. To reduce environmental variation feeding needs to be standardized. A commonly used way to achieve this is to provide food ad libitum. However, life-history trade-offs are rarely found when organisms are cultured under ad libitum laboratory conditions (Bell & Koufopanou, 1986), and the same holds for several sex allocation studies in simultaneously hermaphroditic animals (e.g. Locher & Baur, 2000; Schärer & Ladurner, 2003; Tan et al., 2004). Apparently, individuals with access to a lot of resources can reach high values for many life-history functions. Negative correlations were found more often in natural populations or under resource-restricted or stressful conditions (Bell & Koufopanou, 1986). In fact those studies that support a sex allocation trade-off in hermaphroditic animals were carried out either in the field or under fairly natural conditions (e.g. Raimondi & Martin, 1991; Trouvé et al., 1999). This suggests that an experiment to test for the presence of the sex allocation trade-off should be done under appropriate, resource-limited conditions. Too strong a resource limitation may prevent an organism from developing normally, which may again obscure the trade-off. When used carefully, a direct manipulation of the resource availability remains preferable over other

**Fig. 1** Graphical representation of the effect of reproductive resource budget variation on the phenotypic trade-off between male and female reproductive allocation in a simultaneous hermaphrodite. The grey lines depict the sex allocation trade-offs given the minimum and maximum resource budgets in the example. Variation in the resource budget shifts these lines diagonally (as indicated by the grey arrows). The black lines depict the minimum and maximum sex allocation for different resource budgets in the example. Variation in sex allocation opens and closes these lines (as indicated by black arrows). The shaded areas depict the sex allocation values we expect to observe given different combinations of variation in budget and sex allocation. Note that the trade-off between male and female allocation can only be observed if the variation in the sex allocation is large and the variation in the budget is low (lower left panel). Failure to control for resource budget therefore leads either to none or a positive correlation (adapted from van Noordwijk & De Jong, 1986).
kinds of stress that have been used in similar contexts, such as temperature (e.g. Doums et al., 1998) or pathogens (e.g. Schrag & Rollinson, 1994).

While keeping resources constant, we induced variation in sex allocation by varying mating group size. The local mate competition model (Hamilton, 1967; Charnov, 1982), predicts that the optimal investment in male function is low in the absence of sperm competition as only few sperm are required to fertilize all eggs produced. As mating group size increases, sperm competition drives the optimal male allocation up. This prediction is supported by descriptive (Petersen, 1991; Raimondi & Martin, 1991), and experimental studies (Trouvé et al., 1999; Karlsson, 2001; Hughes et al., 2003; Schärer & Ladurner, 2003; Tan et al., 2004; but see Locher & Baur, 2000; Schärer & Wedekind, 2001).

In the present study we test for a phenotypic trade-off between male and female allocation in the free-living flatworm *Macrostomum* sp. We constrain the environmental and genetic variation in resource budgets by limiting food availability and by using an inbred isofemale line, respectively. To induce phenotypic variation in male allocation we manipulate group size (Schärer & Ladurner, 2003). We expect that a larger group size leads to a higher male allocation, and, assuming a trade-off, to a reduced allocation to the female function. As manipulating group size also changes the density at which the worms live, we further check for effects of density by using two enclosure sizes. Although density is not important for sex allocation under *ad libitum* conditions (Schärer & Ladurner, 2003), it may be under a restricted diet. If density affects sex allocation, the same effect is expected by decreasing enclosure size as by increasing group size.

**Materials and methods**

**Study organism**

*Macrostomum* sp. is a free-living marine flatworm (Platyhelminthes: Rhabditiforma: Macrostomida) that occurs in fine intertidal sands of the northern Adriatic Sea. It is 1.5 mm in size and has a generation time of 18 days at 20 °C and with *ad libitum* food supply. It is an outcrossing simultaneous hermaphrodite with copulation and internal fertilization (Schärer & Ladurner, 2003; Schärer et al., 2004). Because it is transparent, the size of the gonads and other structures can be estimated morphometrically in living animals. The prominent paired testes are located anterior to the smaller paired ovaries (Fig. 2). Animals can be reared in glass petri dishes filled with f/2 medium, which is nutrient enriched artificial sea water (Guillard & Ryther, 1962), and fed with the unicellular diatom *Nitzschia curvilineata* (Heterokontophyta: Bacillariophyceae). Under *ad libitum* food supply mature individuals lay approximately one egg per day.

The laboratory culture of *Macrostomum* sp. we use here appeared as a contamination of a culture of another species, *Macrostomum pusillum*, that was originally established in 1995. It was probably founded by few individuals and is expected to have reduced genetic diversity. However, we estimate that between 50 and 100 generations have passed since the establishment and population sizes were usually large (i.e. thousands of individuals), so the input of mutations may have led to some recovery of genetic diversity.

**Experimental set-up**

To limit genetic diversity the worms used in the experiment came from an isofemale line that was generated as follows: two virgin worms were mated, and one of them was placed into a fresh enclosure with algae and allowed to lay eggs. Three of the resulting offspring were then isolated from their parent and allowed to mate (we used three offspring to reduce the chance of the line dying out). This procedure was repeated for three consecutive generations. A population was then allowed to grow
from three founders for approximately five generations. We expect this population to have a fairly reduced genetic diversity.

At the start of the experiment, a sample of 240 hatchlings that had hatched within 32 h was randomly assigned to pairs or groups of 10 worms (further called groups), in either large or small enclosures in a fully-factorial design. For large enclosures, we used 6-well plates containing 5 mL 1/2 medium per well, and for small enclosures we used 24-well plates containing 1 mL of 1/2 medium per well. Ten per cent of all individuals died before day 35 and 25 worms remained sexually immature throughout the experiment and were excluded from the analysis (the proportion of dead and immature worms was not affected by treatment). This resulted in a final sample size of 185 worms in 37 replicates (pairs in large enclosures, \( n = 7 \); pairs in small enclosures, \( n = 10 \); groups in large enclosures, \( n = 9 \); groups in small enclosures, \( n = 11 \)). Mean actual group size was 2.0 for pairs and 8.4 for groups.

All worms were reared on a restricted diet. To avoid competition for food among group members we restricted feeding time rather than the amount of food. Once a day, diatoms from a single, freshly made diatom suspension were added ad libitum to each enclosure. Two and a half hours later the worms were transferred to enclosures without algae until the next day.

**Morphometric measurements**

As a proxy for the growth and the overall resource budget available to a worm we estimated body size at the age of 35 days. Reproductive investment was estimated using testis size for the male function and ovary size for the female function. In *Macrostomum* sp. testis and ovary size are both linearly related to the number of active stem cells in the gonads and, hence, gonad size indicates gonad productivity (Schärer et al., 2004b; L. Schärer, unpublished data). The sum of testis plus ovary size served as a proxy for the reproductive resource budget. However, summing these two parameters implicitly makes the untested assumption that they are measured in a comparable resource currency, and this measure should thus be used with some caution. We measured body and gonad size as described by Schärer & Ladurner (2003). Briefly, anaesthetized worms were dorsoventrally compressed to a fixed thickness of 35 \( \mu m \) between a microscope slide and a cover slip of a hemacytometer. Pictures of the body and of the gonads were taken in vivo with a Sony DFW-X700 digital colour camera (Sony Broadcast & Professional, Köln, Germany) mounted on an Olympus BH-2 microscope (Olympus Europa GmbH, Hamburg, Germany) at 36X and 400X. Body area, total testis and ovary area were measured using Object-Image 2.09 (available at: http://simon.bio.uva.nl/object-image.html). Because the thickness of the compressed worms was fixed, these areas are probably good measures of the actual volumes of the structures. Measurements were taken blind with respect to treatment group.

**Fecundity measurements**

We determined cumulative egg volume as an additional measure for female investment. After the above measurements were taken we placed worms individually in 100 \( \mu l \) of 1/2 medium in an observation chamber consisting of two microscopy slides separated by 215 \( \mu m \) spacers and sealed with pure white Vaseline. Worms remained there until the age of 53 days without food, and were checked every other day for eggs. Eggs were digitally photographed and their volume was estimated from the cross sectional area (eggs are approx. spherical). Two additional measures of female reproduction were taken at the level of the replicate. First, as all individuals were removed from their enclosure after daily feeding, the eggs that had been deposited remained in the enclosure until hatching. We counted the number of hatchlings and calculated the per capita fecundity per fertile worm present in that replicate on that day. Secondly, the date of the first egg laying was used as an estimate of the time of female maturation of the worms in the replicate.

**Statistical analyses**

As worms within a replicate are not statistically independent, all analyses were carried out using averages per replicate. In groups, these were calculated from two randomly chosen members per replicate in order to obtain comparable variances for both groups and pairs.

We first checked if our experimental design led to the intended comparable overall resource budgets in all the factor combinations. For this we used a fully factorial two-way ANOVA with group and enclosure size as factors, and body area as the dependent variable. This analysis (see below) suggested that our approach had not led to comparable growth of worms in large and small enclosures. However, worms raised within the same enclosure size were comparable in size. We therefore analysed the data separately for the two enclosure sizes.

Next we determined the effects of the group size on (a) the time of female maturation (i.e. day of first egg laying) using a Wilcoxon rank sum test and (b) the reproductive resource budget (i.e. the sum of testis and ovary size) using a \( t \)-test. Further, we compared the morphologically determined sex allocation measures, and the additional female fecundity measurements using \( t \)-tests. Finally, we checked for a correlation between the morphologically determined sex allocation estimates, i.e. between testis and ovary area.

Data were visually checked for normality and homoscedasticity and transformed if necessary. Nonparametric
tests were used if no suitable transformations could be found. All statistical analyses were carried out using JMP 3.2.2 (SAS Institute Inc., Cary, NC, USA).

**Results**

Body area was not significantly affected by group size, but worms raised in large enclosures grew considerably larger than those raised in small enclosures (fully factorial two-way anova: group size, $F_{1,33} = 0.58$, ns; enclosure size, $F_{1,33} = 13.0$, $P < 0.001$; interaction, $F_{1,33} = 0.15$, ns; Fig. 3). This suggested that, while worms raised in different group sizes grew equally well, our controlled feeding failed to produce similar overall resource budgets in large and small enclosures. We thus analysed large and small enclosures separately.

**Large enclosures**

In large enclosures worms raised in pairs and groups did not differ significantly in time of first egg laying (Wilcoxon rank sum test with $\chi^2$-approximation, $\chi^2 = 1.62$, ns, Fig. 4a). However, as it on average took 26 instead of about 13 days for the first egg to be laid, maturation was clearly delayed under the restricted diet compared with the previously studied ad libitum condition (Scharer & Ladurner, 2003). Further, in large enclosures worms raised in pairs and groups did not differ significantly in the reproductive resource budget (sum of testis and ovary size; $t$-test, $t = -0.95$, d.f. = 14, ns, Fig. 4b). This suggests that the worms from pairs and groups in the large enclosures were comparable in these life-history parameters, and that we can expect to see the sex allocation trade-off if present. Indeed, testis and ovary size showed the expected responses to the group size manipulation (testis area: $t = -2.71$, d.f. = 14, $P < 0.05$, Fig. 5a; ovary area: $t = 3.39$, d.f. = 14, $P < 0.01$, Fig. 5b), suggesting that the trade-off occurred. The other estimates of female fecundity further confirmed this pattern (cumulative egg volume: $t = 2.60$, d.f. = 14, $P < 0.05$, Fig. 5c; hatchlings per capita and day: $t = 4.30$, d.f. = 14, $P < 0.001$, Fig. 5d). And although there was a negative correlation between testis size and ovary size in...
the large enclosures, the relationship was not statistically significant (Pearson $r = -0.29$, ns, Fig. 7a, $n = 16$), despite the fact that the responses for testis and ovary size were both significant, and went in the expected opposing directions.

Small enclosures

In contrast, in small enclosures worms raised in pairs and groups differed strongly and significantly in time of first egg laying (Wilcoxon rank sum test with $\chi^2$-approximation, $\chi^2 = 9.96$, $P < 0.01$, Fig. 4c), and the reproductive resource budget ($t$-test, $t = 2.65$, d.f. = 19, $P < 0.05$). This suggests that in the small enclosures we failed to achieve balanced budgets across group size treatments. Although all measures of female fecundity showed the expected patterns (ovary area: $t = 3.93$, d.f. = 19, $P < 0.001$, Fig. 6b; cumulative egg volume: $t = 2.08$, d.f. = 19, $P = 0.051$, Fig. 6c; hatchlings per capita and day: $t = 7.07$, d.f. = 19, $P < 0.001$, Fig. 6d) male allocation showed a pattern opposite to our expectations (testis area: $t = 1.93$, d.f. = 19, $P = 0.068$, Fig. 6a). As a result, in small enclosures there was a highly significant positive correlation between testis and ovary size (Pearson $r = 0.81$, $P < 0.001$, Fig. 7b, $n = 21$), suggesting strong budget effects.
Discussion

Sex allocation trade-off

The scarce support for a sex allocation trade-off in simultaneous hermaphrodites may stem from the fact that most studies to date have not adequately controlled for budget effects, which can obscure the visibility of life-history trade-offs (van Noordwijk & De Jong, 1986; Campbell, 2000). Indeed, an earlier study in our study species that was performed under ad libitum conditions did not reveal the trade-off (Schärer & Ladurner, 2003). The results we present here suggest that under restricted food conditions a trade-off between the allocation to male and female allocation can sometimes be shown (as in the large enclosures), but that uncontrolled density effects can lead to budget effects and destroy the relationship (as in the small enclosures).

Trade-off in large enclosures

In the large enclosures worms grown in pairs and groups reached a comparable size, and were also similar in other life-history traits, such as time to female maturation and investment to reproduction. This suggests that under these conditions we managed to produce worms with comparable reproductive resource budgets, and that we thus could expect to find the trade-off. Indeed, male and female allocation did vary as expected, with worms allocating significantly more to the male function in groups than in pairs, and with female allocation showing the opposite pattern. A significant negative correlation was not found, but that may be due to the limited sample size.

Budget effects in small enclosures

In the small enclosures worms grown in pairs and groups also reached a comparable size (although a smaller one than in the large enclosures), but they differed significantly in time to female maturation and investment in reproduction. Worms in groups matured later and invested less in reproduction, suggesting that the budget for growth and reproduction was lower than that of worms in pairs. Whereas worms in groups had smaller testes, and thus a lower allocation to the male function than worms in pairs, female allocation was also lower in groups. In both pairs and groups there appeared to be a positive relationship between male and female allocation instead of a trade-off, and overall this relationship was strongly positive.

Density effects

The results suggested two effects of density on resource allocation. First, under restricted food conditions body size in Macrostomum sp. is affected by the size of the enclosure in which the worms are held. Second, in small enclosures male allocation was lower in groups than in pairs. Both observations are in contrast to findings of an earlier study, in which worms were fed ad libitum (Schärer & Ladurner, 2003). These effects were mainly caused by worms held at the highest density (i.e. groups in small enclosures), which diverged most from our a priori expectations.

Because of the time-limited ad libitum feeding regime, we consider it unlikely that the observed differences are because of different levels of competition for food. Algae were added in such large numbers that no apparent reduction was perceivable after the 2.5 h feeding time. Moreover, algae were added in suspension, and we made sure that the fill level in small and large enclosures was equal, and thus that the time until the algae settled on the floor – from where worms could pick them up – was the same in both treatments. However, standardizing the fill-level led to a ratio of wall to floor surface that was 2.1 times larger in small enclosures. As the worms often move along the walls of the enclosures, the ones in small enclosures may have spent somewhat less time on the floor, and may thus have had a reduced feeding time and a more restricted diet. Furthermore, behavioural interactions may have been more frequent in smaller wells, particularly in groups, potentially directing attention away from feeding.

The group size manipulation aimed at producing different mating group sizes, and a higher mating rate, and/or a higher sperm transfer per copulation in larger groups, as suggested by earlier results (Schärer & Ladurner, 2003). This could have led to disproportionately high spending on behaviour aimed at acquiring, courting, assessing and inseminating partners in groups in small enclosures. This part of male investment, however, is hard to quantify.

In the pond snail, Lymnaea stagnalis Bohlken et al. (1987) observed a three- to tenfold decrease in egg production in snails that were maintained in groups relative to the egg production of snails in isolation (L. stagnalis can self-fertilize). Reduced egg-laying appeared to be caused by increased investment in mating itself rather than sperm (see also Van Duijvenboden et al., 1985; de Visser et al., 1994). Hence, testis size may well be an incomplete measure of male allocation in Macrostomum sp., and this may explain the seemingly low male allocation of worms held at the highest density.

Conclusions

Our experiment lends support to a phenotypic trade-off between male and female allocation in Macrostomum sp. held in large enclosures. In worms kept in small enclosures spatially-, socially- and/or sexually-induced behavioural changes seem to result in uncontrolled budget effects, possibly by reducing resource acquisition. Moreover, behaviours involved in sexual interactions
may act as a drain on reproductive resources. Future studies should attempt to include such parameters. To gain a more complete understanding of the energy flows we further suggest that future studies should try to quantify other important life-history traits, such as the time of male maturation, size at male and female maturation, and longevity.

The study of phenotypic correlations to test for life-history trade-offs has previously been criticized by, for example, Reznick (1992), who suggested that the study of genetic correlations or selection experiments are preferable. However, there is increasing experimental evidence that simultaneously hermaphroditic animals can adjust sex allocation in a phenotypically plastic way in response to mating group size (Trouvé et al., 1999; Karlsson, 2001; Hughes et al., 2003; Schärer & Ladurner, 2003; Tan et al., 2004), but several of these studies did not observe the trade-off. The selective advantage of phenotypic plasticity in sex allocation, however, requires that resources that are economized in one function can be plastically allocated to the other. Thus the study of phenotypic correlations in sex allocation is important to understand the adaptive value of phenotypic plasticity. The current study shows that the expected phenotypic correlations can be observed under at least some conditions.

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