

Resource-dependent sex-allocation in a simultaneous hermaphrodite

D. B. VIZOSO^{1,2} & L. SCHÄRER^{1,2}

¹Division of Ultrastructural Research and Evolutionary Biology, Institute of Zoology, University of Innsbruck, Innsbruck, Austria

²Evolutionary Biology, Zoological Institute University of Basel, Basel, Switzerland

Keywords:

budget effects;
fitness-gain curves;
Macrostomum lignano;
resource dependence;
sex allocation;
simultaneous hermaphrodite;
size dependence.

Abstract

Most sex allocation theory is based on the relationship between the resource investment into male and female reproduction and the consequent fitness returns (often called fitness-gain curves). Here we investigate the effects of resource availability on the sex allocation of a simultaneously hermaphroditic animal, the free-living flatworm *Macrostomum lignano*. We kept the worms under different resource levels and determined the size of their testes and ovaries over a period of time. At higher resource levels, worms allocated relatively more into the female function, suggesting a saturating male fitness-gain curve for this species. A large part of the observed effect was due to a correlated increase in body size, showing size-dependent sex allocation in *M. lignano*. However, a significant part of the overall effect was independent of body size, and therefore likely due to the differences in resource availability. Moreover, in accordance with a saturating male fitness-gain curve, the worms developed the male gonads first. As the group size was kept constant, our results contrast with expectations from sex allocation models that deal with local mate competition alone, and with previous experiments that test these models.

Introduction

Sex allocation theory, 'one of the success stories of evolutionary biology' (Klinkhamer *et al.*, 1997), attempts to explain how organisms invest into male and female reproduction. It is often based on fitness-gain curves, which describe how much fitness is gained through the male and the female function for a given resource investment. The shape of these curves, and, importantly, the relation between them, can be used to determine the evolutionary stability of different mating systems (e.g. Charnov *et al.*, 1976; Seger & Eckhart, 1996), and to predict the optimal sex allocation of an organism under a variety of conditions (e.g. Charlesworth & Charlesworth, 1981; Charnov, 1982; Frank, 1987a).

In the presence of local mate competition (Hamilton, 1967), the fitness returns for male investment will increase at a diminishing rate as the amount of resources invested increases (Charnov, 1982; Frank, 1987b), since the competition among related sperm will increase (Greeff *et al.*, 2001; Schärer *et al.*, 2001). This scenario is depicted in Fig. 1a, which shows the fitness-gain curves for a hypothetical simultaneous hermaphrodite and its expected sex allocation. The male fitness gains increase with resources invested in a decelerating fashion (see Frank, 1987a; Charnov, 1982 for other examples), while the female fitness-gain curve increases linearly with resources invested. Up to a certain point, m (where the tangent of the male function equals that of the female's) the marginal gains per unit of investment are higher in the male function. Beyond that point, investing into the male function produces a lower fitness gain in comparison with the fitness that could be acquired through the female function. The lower graph shows the expected

Correspondence: Dita B. Vizoso, Evolutionary Biology, Zoological Institute, University of Basel, Basel, Switzerland. E-mail: dita.vizoso@unibas.ch

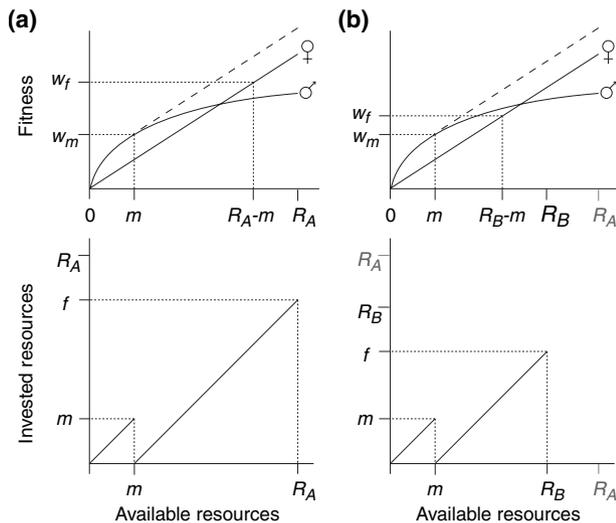


Fig. 1 Fitness gain curves and expected sex allocation of a hypothetical simultaneous hermaphrodite. The upper graphs show the fitness gained per resource invested into the male and the female functions. The male fitness gain curve shows diminishing returns, while the female returns increase linearly with resources invested. Maximal fitness returns are obtained by allocating m resources to the male function and the rest ($R-m$) to the female function. The lower graphs show the allocation to the male (m) and the female (f) functions expected from the gain curves. (a) and (b) differ only in the amount of resources available for reproduction, R , which is lower in (b).

allocation: an individual with resources R_A will invest m resources into the male function and the rest of its resources, $R_A - m$, into the female function. Simultaneous hermaphroditism is expected to be stable under the above conditions (Charnov, 1982; Lloyd & Bawa, 1984).

If, as shown in Fig. 1b, the amount of resources available for reproduction were reduced ($R_B < R_A$), it is expected that the absolute investment into the male function, m , will not change, and that only the amount of resources left to invest in the female function, $R_B - m$, will be reduced (Frank, 1987a,b). It is clear that under ever-diminishing resource availability, this model does not hold, as the predicted output would be a male-only allocation strategy that is unstable over evolutionary time (due to frequency-dependent selection). However, if the species is able to plastically adjust its sex allocation, we expect that individuals with relatively less resources will be more male-biased while individuals with more resources will be more female-biased (as suggested by e.g. Charnov, 1982, 1996).

The effect of resource availability on sex allocation in animals has been addressed as far back as 1913, when Chewyreu summarized and discussed studies on variation in sex allocation as a response to host size in parasitic Hymenoptera (for a summary see Charnov, 1979). Briefly, parasitoid wasps bias their sex ratio at oviposition depending on the size of the host they encounter, preferentially laying daughters in larger hosts.

Charnov (1979) showed that this pattern could be explained if the fitness returns through sons increase less than those through daughters with increasing host size (if the male fitness-gain curve decelerates faster). He concluded that it is this difference in fitness-gain curves what drives resource-dependent sex allocation, and not that 'small hosts are better for producing sons than daughters' (Charnov, 1979).

In hermaphroditic animals, differences between the male and female fitness-gain curves are thought to lead to size-dependent timing of sex change in sequential hermaphrodites (e.g. Charnov, 1982; Warner, 1984; see Schärer & Vizoso, 2003 for an indirect test), and to size-dependent sex allocation in simultaneous hermaphrodites (Petersen & Fischer, 1996; Klinkhamer *et al.*, 1997; St Mary, 1997; Schärer *et al.*, 2001), if at least one of the fitness-gain curves is non-linear. The amount of resources available for reproduction is likely to change with age, as organisms develop gonads, grow, and store energy. Following our example of Fig. 1, individuals with continuous growth should start their reproductive life as males, as the initial amount of resources available for reproduction will be relatively small. As they grow, allocation should then shift towards the female function. This argument has been formulated separately for sequential and simultaneous hermaphrodites. However, the distinction between sequential and simultaneous hermaphroditism is somewhat artificial, an idea recorded more than a century ago, when Montgomery (1895) stated that simultaneous and sequential hermaphroditism 'are closely connected with each other, and their differences are more of degree than of kind'. Instead of a dichotomy, a continuous shift in sex allocation between the two extremes can be obtained as a direct consequence of differences between male and female fitness-gain curves. Support for this idea has been recently provided by several authors (Petersen & Fischer, 1996; St Mary, 1997; Schärer *et al.*, 2001; Angeloni *et al.*, 2002; Cadet *et al.*, 2004), and indeed many species gradually shift their sex allocation as they grow. In plants, condition- and size-dependent sex allocation is the rule rather than the exception (e.g. reviews by Lloyd & Bawa, 1984; De Jong & Klinkhamer, 1989; Klinkhamer *et al.*, 1997; Klinkhamer & de Jong, 1997). In animals, simultaneous hermaphrodites with a brief protandrous phase are widespread (e.g. many groups, Montgomery, 1895, free-living flatworms, Bresslau, 1928–33, cestodes, Roberts & Davis, 1992, chaetognaths, Bone *et al.*, 1991; Alvarino, 1992), but we are aware of only a few studies that report size-dependent (Petersen & Fischer, 1996; St Mary, 1997; Trouvé *et al.*, 1999; Schärer *et al.*, 2001) or condition-dependent (Locher & Baur, 2002) sex allocation.

Using the simultaneous hermaphrodite *Macrostomum lignano* (Ladurner *et al.*, 2005), we specifically aim at testing the effect of resource availability on sex allocation. *Macrostomum lignano* can plastically adjust its sex allocation in response to its social environment (Schärer

& Ladurner, 2003; Schärer *et al.*, 2004b; Brauer *et al.*, in press), and, in restricted food conditions, a trade-off between male and female allocation has been found (Schärer *et al.*, 2005). These studies manipulated the mating group size and found that decreasing the number of mating competitors induces *M. lignano* to reduce its investment into male allocation. The idea that mating group size affects sex allocation in simultaneous hermaphrodites is a fundamental prediction of sex allocation theory (Charnov, 1982), which is analogous to the sex-ratio adjustment under local mate competition (which was formulated for gonochorists, Hamilton, 1967), as previously pointed out (e.g. Greeff *et al.*, 2001; Schärer *et al.*, 2001; Schärer & Ladurner, 2003). We thus expect that local mate competition produces a saturating male fitness-gain curve in *M. lignano*, which makes it a well-suited model organism to address the effect of resource availability on sex allocation. We experimentally manipulated resource availability, keeping worms under different diets, and morphometrically measured their allocation to the male and female gonads over time. This further allowed us to determine the effect of age on the relationship between resource and sex allocation.

Materials and methods

Study animal

Macrostomum lignano (Macrostomorpha, Platyhelminthes) is a member of the interstitial sand fauna of the Northern Adriatic Sea (Ladurner *et al.*, 2005). It is an outcrossing simultaneous hermaphrodite (Schärer & Ladurner, 2003), which reaches 1.5 mm in length when fully grown, and it is transparent, allowing non-invasive observation of internal structures (Fig. 2). 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 antrum, into which sperm are transferred during copulation. The male gonopore is associated with a 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 (Schärer *et al.*, 2004a). Mass cultures of *M. lignano* are maintained in conditions described elsewhere (Tyler, 1981; Rieger *et al.*, 1988). Briefly, worms are kept at 20 °C in glass Petri dishes containing f/2 medium, a nutrient-enriched artificial sea water (Guillard & Ryther, 1962; Andersen *et al.*, 2005), and fed with diatoms of the species *Nitzschia curvilineata*. Generation time under these conditions is 18 days: 5 days from egg laying to hatching and 13 days from hatching to adult (Schärer & Ladurner, 2003).

Morphometry

To quantify the allocation to female and male reproduction, we analysed the worms in the standardized way

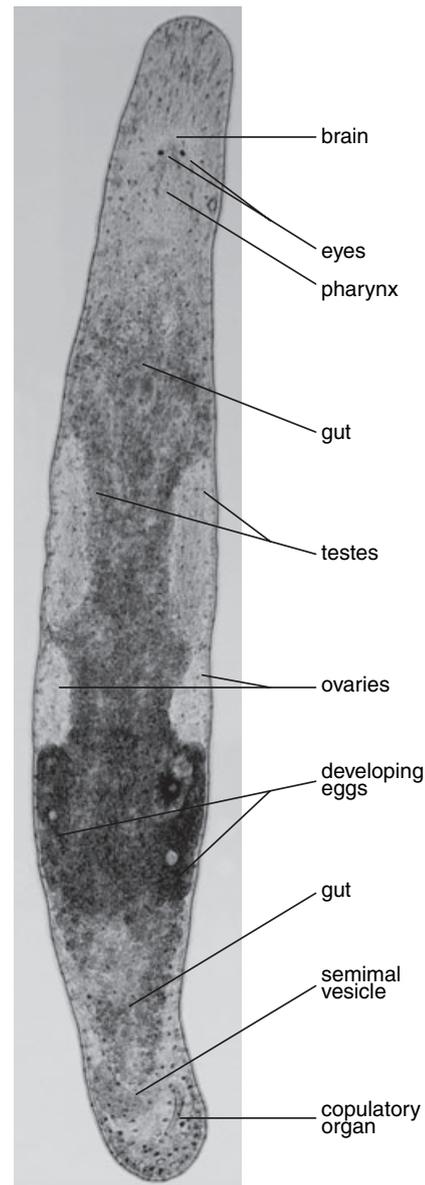


Fig. 2 Live adult specimen of *Macrostomum lignano* squeezed between two glass slides. Total length of this worm is approximately 1.7 mm.

described in Schärer & Ladurner (2003). Briefly, we squeezed anaesthetised worms dorsoventrally between a glass slide and haemocytometer cover glass kept apart by a plastic spacer of 35 µm thickness. We observed the squeezed worms with a compound microscope and took digital micrographs at 40–400x with a digital video camera, a Sony DFW-X700 (Sony Broadcast & Professional, Köln, Germany) connected to an Leitz Diaplan compound microscope (Leica Microsystems, Wetzlar, Germany) via a c-mount and connected to an Apple PowerMac G4 (running MacOS 9) via the built-in

FireWire connection. In order to acquire the images, we used the shareware image capture software BTV Pro (available on the Internet at <http://www.bensoftware.com>). The pictures were analysed with the public-domain image-analysis software Object-Image 2.09 (available at <http://simon.bio.uva.nl/object-image.html>) to determine the areas of the worm, of the testes (sum of both testes), and of the ovaries (sum of both ovaries). This method has good repeatability for all these morphometric measurements (Schärer & Ladurner, 2003).

Experiment

We collected 7-days old worms (2 days after hatching) from mass cultures and randomly produced groups of four worms in 12-well tissue culture plates with 4 mL of artificial sea water (ASW) per well. To test the effect of the amount of resources available for reproduction on sex allocation, we fed the groups of worms daily with eight different quantities of algae (hereafter called diets). The amount of algae per worm and day increased twofold between each diet, from 25 algae worm⁻¹ day⁻¹ in the lowest, to 3200 algae worm⁻¹ day⁻¹ in the highest (further called diets 1–8). This range was chosen according to the daily consumption of an adult *Macrostomum* in standard culture conditions. To prevent the algae from growing after being added to the wells, the plates were kept in the dark and ASW was used instead of f/2 medium. We sampled only one worm from each replicate well (5 worms per diet) at five different ages (i.e. 17, 21, 25, 29 and 33 days after being laid as an egg), and analysed them morphometrically as described above. Four replicates were lost during the last sampling date (from diets 4, 5, 6 and 8; see Table 1), which led to a total of 196 measured worms in as many replicates.

Statistical analyses

We are interested in the effect of resource level on the relative allocation to the male function. However, we expect that this allocation will change as worms grow.

Therefore, our predictor variables are the resource level, measured as the number of algae per worm added to a well each day (*diet*), and the age of the worms on the sampling date, measured as number of days after being laid as an egg (*age*). Sex allocation was calculated as the area of the testes of a worm divided by the total area of the gonads (ovaries + testes). As there is a trade-off between these two measures of investment in *Macrostomum* (Schärer *et al.*, 2005), this is a good measure of sex allocation. We also analysed the two separate components of sex allocation, testis area and ovary area, to determine in which way the variation in resources and age affect sex allocation.

Effects of age and diet on sex allocation

To test the presence of effects of age and diet on the sex allocation of the worms we performed a Multiple Regression Analysis, using Standard Least Squares (Kleinbaum *et al.*, 1998; Quinn & Keough, 2002). The model used is

$$\text{sex allocation} = \beta_0 + \beta_1 \text{ age} + \beta_2 \text{ diet} + \beta_3 \text{ age} \cdot \text{diet} + E, \quad (1)$$

where β_0, \dots, β_3 are the regression coefficients, and E the error component. The data either fulfilled the assumptions of the test (Kleinbaum *et al.*, 1998) or were transformed when necessary. To determine the significance of the difference in relative allocation to the testes between sampling dates, we performed a one-way ANOVA on the residuals of sex allocation regressed on resource level, and then compared the means with a Least Significant Difference (LSD) test (Kleinbaum *et al.*, 1998).

Effects of body size on sex allocation

Changes in sex allocation could potentially be mediated solely through effects of the age and diet on body size of the worms. We therefore also performed the Multiple Regression Analysis of eqn (1) with body size as the dependent variable instead of sex allocation. This analysis suggested strong effects of age and diet on body size (see Results). We therefore controlled for the effect of body size in our sex allocation measure by performing a

Table 1 Development of gonads in *Macrostomum lignano* at different ages and diets.

Diet	Algae	17	21	25	29	33
1	25	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0
2	50	0/0/0	0/0/0	0/0/0	60/40/0	40/20/40
3	100	0/0/0	20/20/20	100/100/100	80/80/60	100/100/100
4	200	40/0/0	100/80/80	100/100/100	80/80/80	75/100/100*
5	400	100/60/60	100/100/100	100/100/100	100/100/100	100/100/100*
6	800	80/80/40	100/100/100	100/100/100	100/100/100	100/100/100*
7	1600	80/80/80	100/100/100	100/100/100	100/100/100	100/100/100
8	3200	100/100/100	80/80/80	100/100/100	100/100/100	100/100/100*

For each cell (i.e. treatment combination of age and diet), we show the percentage (%) of worms in that cell (generally $n = 5$ per cell) that had developed testes, ovaries, and a copulatory stylet (e.g. for the cell with diet 2 and age 33, two of the five worms analysed had developed testes, one had developed ovaries, and two had developed a stylet: 40/20/40).

*Cells where one replicate was lost (i.e. $n = 4$ per cell).

Polynomial Regression Analysis using Standard Least Squares (Kleinbaum *et al.*, 1998; Quinn & Keough, 2002). The model used is

$$\text{sex allocation} = \beta_0 + \beta_1 \text{ body area} + \beta_2(\text{body area})^2 + E, \quad (2)$$

where β_0 , β_1 , β_2 are the regression coefficients, and E the error component. Again, the data fulfilled the assumptions of the test (Kleinbaum *et al.*, 1998).

We then performed a Multiple Regression as described in (1) on the residuals of the Polynomial Regression in eqn (2), and investigated if previously observed effects remained.

Effects of age and diet on testis and ovary size

The predicted changes in sex allocation can be due to changes in male allocation (testis size), female allocation (ovary size), or both. However, our proposed scenario suggests that as resources increase, more will be invested into the female function, while male investment will remain the same. Therefore, we also tested for the presence of effects of age and diet on the male and female allocation of the worms separately. To correct for body size, we performed a Polynomial Regression as described in eqn (2), for the testis and ovary area, to determine the relative male and female allocation, respectively. The models used are

$$\text{testis area} = \beta_0 + \beta_1 \text{ body area} + \beta_2(\text{body area})^2 + E, \quad (3.1)$$

$$\text{ovary area} = \beta_0 + \beta_1 \text{ body area} + \beta_2(\text{body area})^2 + E. \quad (3.2)$$

We then performed Multiple Regression Analyses as described in eqn (1), on the residuals of the Polynomial Regression in eqns (3.1) and (3.2).

$$\begin{aligned} \text{relative male allocation} &= \beta_0 + \beta_1 \text{ age} + \beta_2 \text{ diet} \\ &+ \beta_3 \text{ age} \cdot \text{diet} + E, \end{aligned} \quad (4.1)$$

$$\begin{aligned} \text{relative female allocation} &= \beta_0 + \beta_1 \text{ age} + \beta_2 \text{ diet} \\ &+ \beta_3 \text{ age} \cdot \text{diet} + E, \end{aligned} \quad (4.2)$$

where β_0, \dots, β_3 are the regression coefficients, and E the error component. The data fulfilled the assumptions of the test (Kleinbaum *et al.*, 1998) or were transformed when necessary.

Excluded individuals

Worms which did not develop gonads were not included in the analyses. In some diets, no worms had developed gonads at one or more sampling dates (diets 1, 2, and 3, see Results and Table 1). To preserve the orthogonality of the design, these diets were excluded from the analyses. In the remaining diets, a further eight worms had not

developed gonads (see Results and Table 1). The number of observations included in the analyses was thus 113.

Results

Overall effects of age and diet

The amount of resources provided to the worms had a strong effect on gonad development. Worms from the lowest resource level (diet 1) did not develop any gonads throughout the experiment. Worms from diet 2 only started developing gonads on the last two sampling dates, while worms from diet 3 started developing gonads on the second sampling date (see Table 1). Some worms from diets 4 and 5 showed developed testes before any sign of ovaries could be observed, suggesting that worms may allocate to the male function before allocating to the female function (Table 1).

Effects of age and diet on sex allocation

Sex allocation changed significantly with worm age (Table 2), primarily due to a higher relative allocation to the testes in the first sampling date (Fig. 3a). This was confirmed by the LSD test, where only worms 17 days old had a significantly higher allocation to the male function (MSE = 0.0077; $k = 5$; $l = 10$; $t = 2.87$; d.f. = 109, Fig. 3b). Overall, the increase in size as worms aged was stronger and steadier for the testes than for the ovaries (Table 4, Fig. 3b). The resource level had a significant overall effect on sex allocation (Table 2), with sex allocation decreasing as resource level increased (Fig. 4a). This effect was independent of the age of the worm, as shown by the lack of significance in the interaction term of the model (Table 2).

Body size and sex allocation

Body size was, as expected, strongly affected by age and resource level (multiple regression: $R^2 = 0.57$, $F_{3,109} = 27.73$, $P < 0.0001$; age, $F_{1,109} = 41.78$, $P < 0.0001$; diet, $F_{1,109} = 37.25$, $P < 0.0001$; interaction $F_{1,109} = 2.68$,

Table 2 ANOVA table for the multiple regression results on sex allocation of *Macrostomum lignano* regressed on age, diet and their interaction.

Source	d.f.	SS	F	P-value	R ²
Model	3	0.449	16.03	< 0.0001	0.31
Age	1	0.096	10.3	0.0017	
Diet	1	0.343	36.7	< 0.0001	
Age-diet	1	0.014	1.5	0.23	
Error	109	1.019			

Age was calculated as the number of days since the worm was laid as an egg.

Diet was included in the model as the $\log_{10}(\text{algae worm}^{-1} \text{ day}^{-1})$.

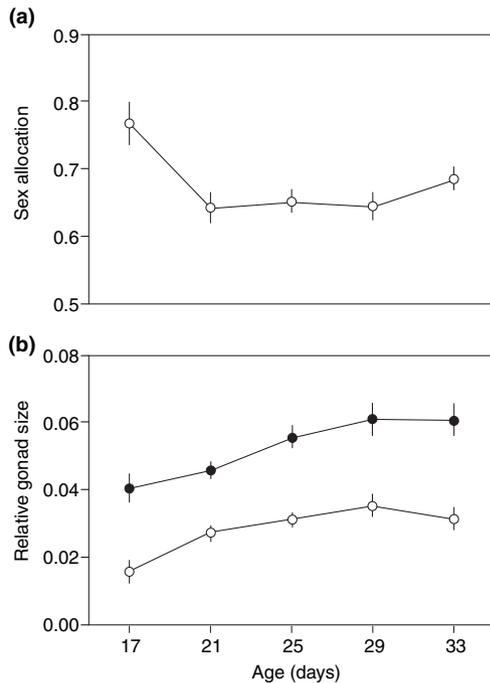


Fig. 3 Protandry in *Macrostomum lignano*. (a) Changes in sex allocation with age. Circles represent the mean sex allocation (testis area relative to total gonad area, averaged across diets) for each age at which the worms were sampled. (b) Changes in gonad size. Black circles represent the mean relative testis size (testis area divided by body area, averaged across diets) and white circles represent the mean relative ovary size (ovary area divided by body area, averaged across diets). Bars indicate standard errors.

$P = 0.11$). And body size affected sex allocation, with larger worms being more female-biased (polynomial regression: $R^2 = 0.34$, $F_{2,110} = 29.21$, $P < 0.0001$; size, $F_{1,110} = 56.91$, $P < 0.0001$; size², $F_{1,110} = 6.76$, $P = 0.01$). This effect was stronger in smaller worms, which is reflected in the significant quadratic term and which can be seen in the plot of size-dependent sex allocation (Fig. 5). Further note that in the lowest diets some individuals appeared to only have matured in the male function.

As it is possible that the observed shift in sex allocation is only due to body size effects, we re-analysed the effects of age and resource level correcting for body area, as described in the Materials and methods. As shown in Table 3, the previously observed effect of age (Table 2) disappears, whereas the effect of resource level on sex allocation diminishes but remains significant. This suggests that although body size explains an important part of the observed shift in sex allocation, there is an effect of the resource level on sex allocation which is independent of body size.

When analysed separately, the two components of sex allocation show a different response to the increase in resource availability (Table 4, Fig. 4b), with ovary size

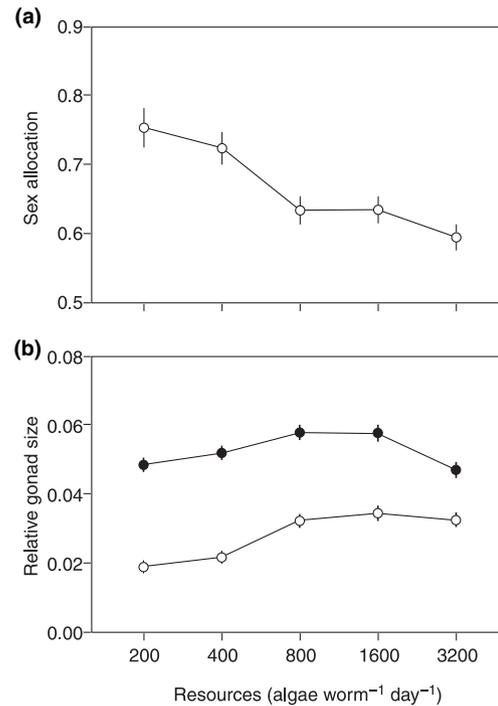


Fig. 4 Effect of the resource availability on sex allocation in *Macrostomum lignano*. (a) Changes in sex allocation with age. Circles represent the mean sex allocation (testes area relative to total gonad area, averaged across ages) for the different diets under which the worms were kept. (b) Changes in gonad size. Black circles represent the mean relative testis size (testis area divided by body area, averaged across ages) and white circles represent the mean relative ovary size (ovary area divided by body area, averaged across ages). Bars indicate standard errors.

increasing with the amount of resources, while the variation in testis was not significant.

Discussion

Our results show that the availability of resources can indeed affect sex allocation of *M. lignano* in a phenotypically plastic manner, with better-fed individuals having a more female-biased sex allocation, due to larger increase in investment into the female function. Higher female allocation with increasing resources is expected if the fitness-gain curve decelerates faster in the male than in the female function. Our results thus support the notion of a saturating male fitness-gain curve in *M. lignano* under restricted mating group size, as suggested by previous studies (Schärer & Ladurner, 2003; Schärer *et al.*, 2005; Brauer *et al.*, in press).

Size-dependent sex allocation

The observed effect of resources on sex allocation became weaker but remained significant when we corrected for

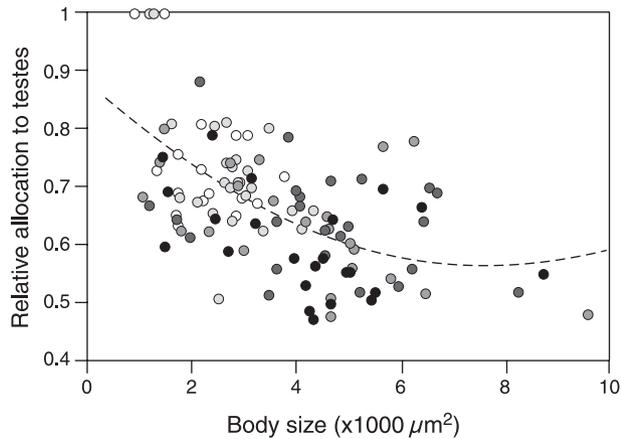


Fig. 5 Size-dependent sex allocation in *Macrostomum lignano*. Circles represent individual values of sex allocation and body size (measured as area, see Materials and methods). Grey values indicate the different diets, from diet 4 (white circles) to diet 8 (black circles). The dashed line shows the polynomial regression (for details see Statistical analyses).

Table 3 ANOVA table for the multiple regression results on sex allocation (corrected for body size, see Materials and methods) of *Macrostomum lignano* regressed on age, diet and their interaction.

Source	d.f.	SS	F	P-value	R ²
Model	3	0.120	5.2	0.002	0.13
Age	1	0.0002	0.03	0.87	
Diet	1	0.066	8.6	0.004	
Age-diet	1	0.054	7.0	0.009	
Error	109	0.839			

Age was calculated as the number of days since the worm was laid as an egg.

Diet was included in the model as the $\log_{10}(\text{algae worm}^{-1} \text{ day}^{-1})$.

body size. Therefore, the resource-induced shift in sex allocation occurred partly through a correlated change in body size, and partly through a size-independent component. Body size can affect sex allocation in two conceptually different ways, the so-called direct and budget effects (Klinkhamer *et al.*, 1997; Cadet *et al.*, 2004). Direct effects are those that come from advantages of size *per se*, for instance in territory defence or confrontations during copulation (for other examples see Schärer *et al.*, 2001). The likely outcome of this kind of size-dependent sex allocation, according to Klinkhamer *et al.* (1997), is an abrupt shift from one sex to the other at a particular size. For direct effects of size to explain our results, big individuals should have an advantage in being female solely due to their size. Although we cannot exclude this possibility with our present knowledge, the gradual shift in sex allocation with increasing resources observed in *M. lignano* makes the presence of direct size effects unlikely.

Budget effects of body size, on the other hand, are due to an increase of the resources available for reproduction with increasing size. Bigger individuals generally have a larger budget to invest into reproduction than smaller individuals, and can thus produce a greater amount of gametes. If this is combined with at least one of the fitness-gain curves being decelerating (or, in general, non-linear, see Klinkhamer *et al.*, 1997), size-dependent sex allocation because of budget effects is expected. Klinkhamer *et al.* (1997) predict that budget effects of body size will most likely lead to a gradual shift from one sex to the other, with the direction and magnitude of the shift depending on which of the fitness-gain curves decelerates faster. For example, in the presence of local mate competition, a gradual shift from male towards female allocation is expected. Our results are in accordance with such a scenario, as we observed a gradual shift from the male to the female function with size, and, moreover, a size-independent effect of resource availability on sex allocation.

Size-dependent sex allocation has been reported in many simultaneous hermaphrodites, not only in plants (e.g. Klinkhamer & de Jong, 1997 and references therein), but also in animals (e.g. Schärer *et al.*, 2001 and references therein). Previous experiments with *M. lignano* have not revealed size-dependent sex allocation, probably because these studies have usually tried to minimize rather than maximize variation in resource availability as in the present case.

Packaging costs

Another proposed explanation for size-dependent sex allocation via budget effects is the existence of differences in the 'cost of packaging' between female and male gametes (Frank, 1987a). Judging from the differences in size between eggs and sperm (see for instance Ladurner *et al.*, 2005), the amount of resources required to deliver one egg in *M. lignano* greatly exceeds the costs of producing a few sperm. This means that producing a single egg (i.e. the minimal required investment into female reproduction beyond fixed costs) needs more energy than producing potentially enough sperm to secure some returns via the male function (at least in theory, a single sperm is enough to fertilize an egg). In this scenario, worms with less resources would be expected to invest more into male reproduction. This will likely be an important issue when levels of resource availability are small and worms simply cannot produce an egg. The significance of this hypothesis for worms with a lot of resources is less clear, although the cost of each additional egg for mature, well-fed worms will still surpass the cost of a few sperm. This hypothesis only works if small males actually have access to fertilization and are not selected against in favour of larger individuals (as otherwise

Table 4 ANOVA table for the Multiple Regression results on relative male and female allocation (testis size and ovary size corrected for body size, see Materials and methods) of *Macrostomum lignano* regressed on age, diet and their interaction.

Source	d.f.	Relative male allocation				Relative female allocation			
		SS	F	P-value	R ²	SS	F	P-value	R ²
Model	3	6270.6	3.82	0.012	0.10	3904.1	2.97	0.035	0.076
Age	1	6143.8	11.25	0.0011		1685.5	3.84	0.053	
Diet	1	56.14	0.10	0.75		1953.2	4.45	0.037	
Age · diet	1	12.96	0.02	0.88		352.24	0.80	0.37	
Error	109	59 551				47 799			

Age was calculated as the number of days since the worm was laid as an egg.

Diet was included in the model as the $\log_{10}(\text{algae worm}^{-1} \text{ day}^{-1})$, and gonad size was square-root transformed.

they should invest into growth), an assumption which is testable in our study system.

Sequential vs. simultaneous hermaphroditism

In sex allocation theory, a clear distinction has usually been made between simultaneous and sequential hermaphroditism. While models for simultaneous hermaphrodites usually predict the optimal sex allocation for a population, models for sequential hermaphrodites usually predict the optimal moment when an individual should change sex. However, protandrous simultaneous hermaphroditism, where there is a brief phase in which individuals function as a male only before turning into a simultaneous hermaphrodite, has been described as a widespread phenomenon in animals as early as the late 19th century (Montgomery, 1895). One of the scenarios in which simultaneous hermaphroditism is predicted as an evolutionary stable strategy is the presence of local mate competition, which, as we have discussed above, is also predicted to lead to protandry. Therefore, local mate competition may be a common cause not only of hermaphroditism, but also of protandrous simultaneous hermaphroditism.

Recently, protandrous simultaneous hermaphroditism has been formally incorporated in models of sex allocation (e.g. St Mary, 1997; Angeloni & Bradbury, 1999; Cadet *et al.*, 2004), and their results suggest that it is the rule rather than the exception. Our data suggest that the testes develop before the ovaries, so that we could consider *M. lignano* to be a protandrous simultaneous hermaphrodite. However, in our study, the formation of the copulatory organ often lagged behind the formation of the testes, and the individuals with only testes could not yet mate in the male role. It is conceivable that as the testes started developing before the ovaries, the male gametes will be ready to be given away before the oocytes are ready to be fertilized, and thus *M. lignano* may still start its reproductive life as a functional male. A future study will have to clarify in more detail the timing of the maturation of different male and female reproductive morphologies and behaviours.

Resource-dependent sex-allocation: is this scenario possible?

An interesting result of the current study is that sex allocation varied despite the fact that all worms were grown in the same group size. This contrasts with the observed effects of group size on sex allocation in *M. lignano*, where worms raised in larger groups have been shown to develop a more male-biased sex allocation (Schärer & Ladurner, 2003; Schärer *et al.*, 2004b, 2005). Moreover, it also contrasts with theoretical results, which predict that the effect of the resource availability in an individual will be relative to the amount of resources available to the other individuals in the population (e.g. Charnov *et al.*, 1981; Angeloni *et al.*, 2002). If that were true, we should not have found any effect, because in our experiment all individuals within a treatment had access to the same amount of resources.

Several hypotheses could explain this discrepancy. First, the observed sex allocation could simply be maladaptive. This can easily happen in laboratory conditions, where the worms are being tested in different resource levels and population sizes than those under which they have evolved. Although phenotypic plasticity in sex allocation has been demonstrated in *M. lignano*, it could still be possible that the sex allocation we measured is a transient one, i.e. the worms were still in the process of reaching their optimal sex allocation. If this hypothesis were true, we would expect that the worms would eventually adjust their sex allocation to the one expected for a group size of four. Our data, however, do not support this, as the effect of resource level remained significant throughout the experiment (results not shown), and previous data showed that *M. lignano* can change their sex allocation within a shorter time (Brauer *et al.*, in press).

Alternatively, resource level may serve as a cue for mating group size under natural conditions, as worm density is likely to depend on resource availability. Intuitively, one would expect that resource limitation would lead to smaller mating group sizes, therefore producing the reverse pattern as the one we observed

(i.e. that individuals in poorer environments would be more female-biased rather than more male-biased as we observed here). However, high densities of worms will likely lead to resource limitation under natural conditions, in which case low resources may signal larger group sizes. A previous study has revealed that, under constrained resource availability density-related factors that can affect sex allocation in *M. lignano* (Schärer *et al.*, 2005), but the same is not true for *ad libitum* conditions (Schärer & Ladurner, 2003), suggesting that resource availability may affect the way the worms perceive and/or react to other factors. A very interesting experiment would be to determine mating group size under different levels of resource availability in large populations.

Finally, an interesting possibility would be if the optimal sex allocation for a given mating group size depended on resource availability. Due to frequency dependence, the effect of the availability of resources tends to be relative rather than absolute (i.e. that individuals make allocation decisions in response to the amount of resources they have in relation with the resources of other individuals in the population, e.g. in parasitoid wasps, Charnov, 1979; Charnov *et al.*, 1981). However, the total amount of resources available in a population may still affect the male and female fitness-gain curves (a prediction suggested by Frank, 1987a and Klinkhamer *et al.*, 1997). For example, it is likely that limiting resources may impose a stronger physiological constraint on female reproduction due to the 'cost of packaging' hypothesis outlined above. In a resource-limited population the ratio of costs of producing eggs vs. sperm will thus be relatively larger than in a resource-rich population, consequently changing the shape of the investment-return functions. Interestingly, internal fertilizers with sperm storage (as *M. lignano*) may be particularly sensitive to changes in resource availability (Charnov, 1996), if the size of the sperm storage organ (and thus the optimal amount of sperm required to displace sperm of rivals) is relatively invariant to body size. Variable resources may thus change the relative cost of producing this optimal amount of sperm, and could thus lead to variation in sex allocation.

We are unaware of sex allocation models that explicitly include variation in the total amount of resources at the population level and its interaction with mating group size (i.e. local mate competition), and hope that our study will promote their development. Experimental data (both on phenotypic plasticity and evolutionary responses) on sex allocation under varying group sizes and resource levels are required to elucidate this problem.

Conclusions

We have shown that the simultaneous hermaphrodite *M. lignano* phenotypically adjusts its sex allocation in response to the amount of resources available. The direction of change (a gradual shift towards female

allocation with increasing resources) is expected for a hermaphrodite with a saturating male fitness-gain curve (or at least one that is more saturating than that of the female function), supporting that sex allocation is affected by local mate competition. Body size greatly affected sex allocation, likely due to larger worms having more resources to invest in reproduction. We also show that *M. lignano* develop their male organs first, as expected for a decelerating male fitness-gain curve. Our results further suggest a discrepancy with the sex allocation expectations derived from local mate competition arguments, which may imply that sex allocation in *M. lignano* may be under different selection pressures.

Acknowledgments

We are deeply grateful to the late Reinhard Rieger for his invaluable contribution and support throughout our work in Innsbruck. We will dearly miss him. We would also like to thank Peter Ladurner for help and support in the laboratory, and John Pannell and one anonymous reviewer for helpful discussion and comments on the manuscript. During this study, L.S. was supported by a Lise-Meitner-fellowship (FWF, Austria), a start-up fellowship of the Tyrolean Science Foundation (TWF, Austria), and an advanced researcher fellowship (SNF, Switzerland).

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Received 3 November 2006; accepted 17 November 2006