Size-dependent sex allocation in a simultaneous hermaphrodite parasite

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Abstract

Most models of sex allocation distinguish between sequential and simultaneous hermaphrodites, although an intermediate sexual pattern, size-dependent sex allocation, is widespread in plants. Here we investigated sex allocation in a simultaneous hermaphrodite animal, the tapeworm *Schistocephalus solidus*, in which adult size is highly variable. Sex allocation was determined using stereological techniques, which allow measuring somatic and reproductive tissues in a common currency, namely volume. We investigated the relationships between individual volume and allocation to different reproductive tissues using an allometric model. One measure of female allocation, yolk gland volume, increased more than proportionally with individual volume. This is in contrast to the measure of male allocation, testis volume, which showed a strong tendency to increase less than proportionally with individual volume. Together these patterns led to sex allocation being strongly related to individual volume, with large individuals being more biased towards female allocation. We discuss these findings in the light of current ideas about size-dependent sex allocation in, primarily, plants and try to extend them to simultaneous hermaphrodite animals.

Introduction

Models of sex allocation in hermaphrodites have classically made a clear distinction between sequential and simultaneous hermaphrodites. Sequential hermaphrodites are born into one sex and change to the other sex later in their lives. Models for this group of organisms look at allocation patterns of individuals in a population (i.e. at which time or size they change sex, Ghiselin, 1969; Warner, 1975; Charnov, 1982). They are based on the size-advantage argument, which states that an individual should change sex when it can attain a higher fitness in one sex when it is small and a higher fitness in the other sex once it attains a certain size. In other words, in a sequential hermaphrodite sex allocation changes dramatically (i.e. totally) in response to size, condition and/or population structure.

In contrast, models of sex allocation in simultaneous hermaphrodites, which express both sex functions at the same time, have generally assumed that all individuals in a population have the same sex allocation (Charnov, 1980, 1982, 1996; Charlesworth & Charlesworth, 1981; Petersen, 1991; Greeff & Michiels, 1999; for exceptions see: Charnov, 1987; Petersen & Fischer, 1996; St Mary, 1997). At the heart of those models are the so-called fitness gain curves, which relate investment into male or female function to the fitness gains derived from this investment. Gain curves are generally modelled as power functions where the exponent determines if the gain in fitness is an increasing or decreasing function of investment. The shapes of these gain curves can be used to assess the stability of hermaphroditism vs. separate sexes and to make predictions about patterns of allocation to

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male and female reproductive function (Charnov, 1982). In most models, a fixed amount of reproductive investment is partitioned into male and female investment in a way that maximizes the product of the fitness derived from the two investments. An individual that is larger may, however, have a higher absolute reproductive investment. This may place it at different positions on the male and female gain curves compared with other individuals in the population and hence may change its optimal sex allocation. More generally one can predict that unless the exponents of the male and female gain curves are identical, sex allocation in simultaneous hermaphrodites is expected to be size-dependent (Klinkhamer *et al*., 1997).

It has previously been noted that the distinction between the two types of hermaphroditism is somewhat artificial (Charnov & Bull, 1977; St Mary, 1997). There are several examples of intermediate sexual patterns in animals (e.g. polychaetes, Berglund, 1986; Premoli & Sella, 1995; and fish, Kuwamura *et al*., 1994; St Mary, 1994; review in Leonard, 1993) and intermediate patterns are the rule rather than the exception in plants (Lloyd & Bawa, 1984). We can view the classical distinction between the two forms of hermaphroditism as representing the two extremes, suggesting that size- and condition-dependent sex allocation should be widespread in simultaneous hermaphrodites. Continuous variation in sex allocation as a function of individual size is well documented in plants (Lloyd & Bawa, 1984; Klinkhamer & de Jong, 1997; Klinkhamer *et al*., 1997). An analytical framework based on allometric models has been proposed for its analysis (Klinkhamer *et al*., 1990). This approach has frequently been used to analyse data on sex allocation in plants (Klinkhamer & de Jong, 1993; Rademaker & Klinkhamer, 1999; Wright & Barrett, 1999; Koelewijn & Hunscheid, 2000). We are, however, aware of only three studies in animals that report size-dependent sex allocation (St Mary, 1994; Petersen & Fischer, 1996; Trouvé *et al*., 1999) and none of these studies used the above approach.

In this study, we test for size-dependent sex allocation patterns in a simultaneous hermaphrodite, the tapeworm *Schistocephalus solidus*, that are established during growth in its natural second intermediate host, the three-spined stickleback. We produced the large size range of worms needed to investigate size-dependent allocation patterns by exposing a large size range of fish to the parasite. In order to measure the allocation patterns we then developed a method based on stereology (e.g. Howard & Reed, 1998; Gundersen *et al*., 1988a,b), that allows for quantitative and unbiased estimation of individual volume and the volumes of male and female reproductive structures from histological sections. Further, we look for independent evidence for size-dependent sex allocation patterns by re-analysing data from a previous study (Wedekind *et al*., 1998), i.e. data on egg production of worms that were allowed to reproduce in an *in vitro* system replacing the final host. Finally, we discuss our findings with respect to current hypotheses regarding size-dependent sex allocation.

Materials and methods

Study species

The pseudophyllidean tapeworm *S. solidus* is a simultaneous hermaphrodite parasite that reproduces in the intestine of fish eating water birds and eggs pass into the water with the faeces. If after hatching the free swimming first larval stage, the coracidium, is ingested by the first intermediate host, a cyclopoid copepod (e.g. *Macrocyclops albidus*), the second larval stage develops in the haemocoel of this host. Infectivity to the second intermediate host, the three-spined stickleback, *Gasterosteus aculeatus*, is reached within 1–2 weeks (at the time of the formation of the cercomer). Infection of the fish occurs upon ingestion of an infected copepod. The third larval stage, the plerocercoid, grows in the peritoneum of the fish and reaches infectivity in the final host after 1–3 months (worms of >50 mg generally establish themselves in the final host, Tierney & Crompton, 1992). Smyth (1946) gives a detailed description of the anatomical organization of the plerocercoid. *Schistocephalus solidus* is unusual among tapeworms in that the larvae at this stage are completely segmented and all but the first 10 or so segments contain a full set of genitalia, which are in an advanced stage of development but immature. The reproductive system primarily consists of yolk glands, testes, and ovaries containing rather inactive cells (though mitosis occurs, Smyth, 1946). Spermatogenesis (i.e. meiosis) only takes place in the final host and is triggered by the high body temperature of the final host (Smyth, 1952), ruling out the possibility of sperm transfer in the fish. After ingestion by the final host, the larvae mature and start to produce eggs within 2 days (Smyth, 1946). *In vivo*, reproduction takes place within 1–2 weeks, after which the worms die (McCaig & Hopkins, 1963; Tierney & Crompton, 1992). Transmission rates are variable, but generally high (Tierney & Crompton, 1992), therefore worms in multiple infection have a chance of being transmitted together. A previous study suggested that self-fertilization is possible (Wedekind *et al*., 1998).

Exposure to parasites, maintenance of fish and recovery of worms

The parasites we used in this study came from 16 pairs of worms (each crosses of two parasite lines kept in our laboratory) that were allowed to reproduce in an *in vitro* system (Wedekind *et al*., 1998) and copepod cultures were kept according to Orr & Hopkins (1969). Copepods were each exposed to six coracidia following a standard

protocol (Wedekind, 1997) and their infection status was checked between day 11 and 20 after exposure. Copepods carrying a single procercoid with a developed cercomer were separated for exposure of the fish (33 days after exposure of the copepod).

We used 9–10-month-old sticklebacks of a large range of sizes that had been raised in our laboratory (values here and further are given as mean ± 1 SE, standard length, 41.2 ± 0.8 mm, range 30–50 mm, fish weight, 944 ± 55 mg, range 270–1520 mg, relative condition, after Le Cren, 1951; p. 204, 1.00 ± 0.02, range 0.68–1.22, $n = 40$). For exposure we individually placed fish into 3 L aquaria and treated them by adding two singly infected copepods. We then added several noninfected copepods and *Daphnia* to elicit feeding behaviour leading to the consumption of the infected copepods. Three days after exposure we distributed 10 fish each to four 60 L aquaria (16 : 8 h light : dark cycle, 12 ± 1 °C water temperature). We marked the fish by cutting dorsal spines, in order to distinguish them from other fish we held in the same aquaria (total of 23 fish per aquarium). Aquaria were balanced in respect to the mean of standard length, fish weight and relative condition. Fish were fed daily (except weekends) with live copepods, *Daphnia* and *Assellus*. Within the first 3 months, some mortality occurred as a result of unidentified pathogens (0, 5, 2 and 3 experimental fish died in aquaria 1, 2, 3 and 4, respectively). We, therefore, decided to distribute the fish to a total of 16 aquaria (18 L, 13 ± 1 °C water temperature, 3–6 fish per aquarium) after which only one fish died.

To recover the worms we killed the fish 4 months after exposure by a cerebrospinal cut and opened the body cavity with a pair of fine scissors. We determined standard length, fish weight and gut content weight and the number and weights of worms to the nearest 1 mg by subtractive weighing. Relative condition was calculated on the basis of net fish weight (fish weight – gut content weight – worm weight). We also determined the water displacement volume (Weibel, 1979; p. 240) of the worms as a reference for later histological volume determinations and in order to allow correction of tissue shrinkage during histological preparation. We then transferred each worm into a horizontally placed 50 mL polypropylene centrifuge tube containing 20 mL of 10% formalin in water (one part of 34% formaldehyde solution in nine parts of deionized water). The tubes were assigned a random number to ensure naive analysis later on.

Tissue preparation, sampling and stereology

The aim of the stereological analysis was to estimate the volume of the reproductive structures in each worm, denoted V(structure) (mm^3), where 'structure' stands for either yolk cells, testes or ovaries. We used the following approach. Volumes were estimated using the Cavalieri principle (Gundersen & Jensen, 1987). The bounded object (i.e. the worm) containing the structure of interest (i.e. any of the mentioned reproductive structures) is cut with a set of parallel plane sections a distance T apart and the cross-sectional area of the corresponding structure is measured on each section. An estimator V(structure), of the required volume is then

$$V(\text{structure}) = T \times \{\text{total cross-sectional area}\}.$$

For unbiased estimation, the position of the first section must be uniform random within an interval of length T. Cross-sectional area was estimated by point counting (e.g. Howard & Reed, 1998; p. 31), using a test system of regularly spaced points. The number of test points hitting a plane region, times the area associated with a test point, is an unbiased estimate of the area of that region. Again, unbiased estimation requires that the position of the test system is uniform random. The cross-sectional area of yolk cells and testes, which are more abundant and less localized than ovaries, were estimated by systematic subsampling on each section and using the fractionator principle (Gundersen, 1986). This principle, which is applicable to any additive, quantitative parameter, says that the sampling period times the sample total (of area, say), is an unbiased estimate of the population total.

Each worm was embedded in 2% agar and cut, with a random start, into a set of parallel slabs of thickness $T = 2.34$ mm using a tissue slicer (Fig. 2 in Michel & Cruz-Orive, 1988). The slabs were cut at an angle of about 45° to the longitudinal axis of the worm, so that each cut intersected several worm segments (this will reduce the variance of the estimates). Slabs were dehydrated through a graded series of the following solutions for 15 min each (ethanol: 70, 2 × 80, 2 × 95%; propanol: 3 × 100%; xylene: 2 × 100%), brought to paraffin and embedded. From each slab, one section of 3 μm nominal thickness was cut on a microtome (5–10 sections $worm^{-1}$). Sections were stained with haematoxylin-eosin and mounted on glass slides with a cover glass and Pertex mounting medium. Before point counting, the sections (155 in total) were numbered sequentially and then arranged and analysed in a sequence according to a random permutation of the numbers. This allowed a 'blind' analysis of the sections without knowing which worm a section represented.

Sections were analysed using a modified Olympus BH-2 microscope (20× objective), equipped with a projection arm and a stepping specimen stage (e.g. Fig. 3 in Gundersen *et al.*, 1988a). Microscopic images were projected onto the table at a final magnification of $M = 312\times$. As previously mentioned, each section was systematically subsampled to estimate the cross-sectional area of yolk cells and testes. Microscopic fields were therefore positioned according to a regular array pattern, by moving the specimen stage in predetermined

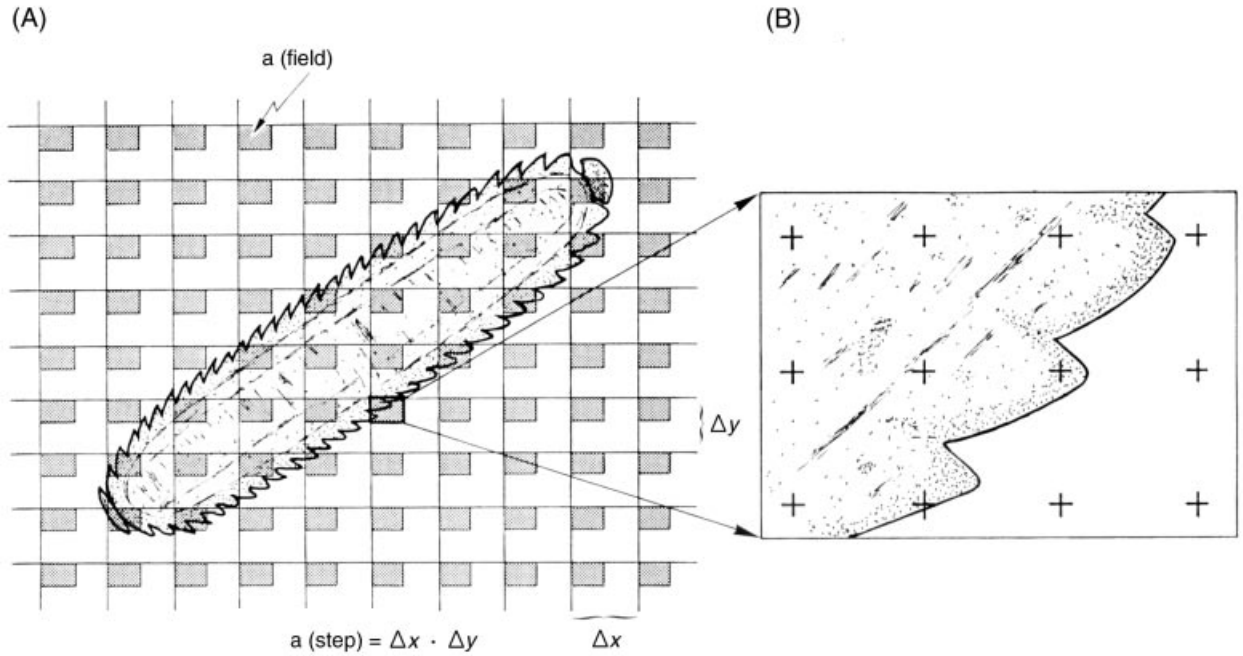

Fig. 1 Subsampling and point counting on the histological sections. (A) Starting at a random location the field of view is systematically moved over the stained section by means of a motorized stepping specimen stage covering the step area, *a*(step), with every step. (B) The number of points in the field of view, *a*(field), hitting the structures of interest is counted. The test system for the Cavalieri estimation of the worm volume is depicted.

steps (Fig. 1A). Thus, we sampled an average of about 17 fields section^{-1} (range 3–30). The sampling period k, was estimated as a(step)/a(field), where a(step) is the area associated with each step of the specimen stage and a(field) is the area of a microscopic field. Here, $a(\text{step}) = 0.9839$ mm^2, $a(\text{field}) = 0.4205$ mm^2, and hence $k = 2.34$ (i.e. we point counted on about 43% of the area of the section). The total cross-sectional areas of yolk cells and testes in the subsample were then estimated by point counting using a square grid of test points (Fig. 1B). The test system was printed on a transparent sheet, which was overlaid with uniform random position on the microscopic images, and the distance between test points was $d = 15$ mm. For all structures we only counted points that intersected with the stained portion of the structures (i.e. gaps between individual cells were not counted). The cross-sectional area of ovaries was estimated without subsampling of the sections (i.e. $k = 1$), by continuously moving the specimen stage and localizing all ovaries present on a section. Now the test system used for point counting had $d = 5$ mm. The final estimator of volume was

$$V(\text{structure}) = T \times k\left(\frac{d}{M}\right)^2 \times \sum_{i=1}^{n} P_i(\text{structure}),$$

where P_i(structure) is the total number of test points hitting the relevant structure on the ith section ($i = 1,2,\ldots,n$), and n is the number of sections used per worm. The distance d is divided with M to correct for magnification. Per worm we counted an average of about 237 points (range 39–477) in yolk cells, 92 points (range 34–137) in testes and 140 points (range 39–254) in ovaries.

The amount of shrinkage caused by the histological preparation procedure was estimated for each worm as shrinkage = (volume before–volume after)/volume before, where volume before and volume after denote the estimates of worm volume obtained using water displacement and the Cavalieri principle, respectively. The Cavalieri estimates of worm volume were obtained in a similar way as described above for the yolk cells and testes, but using a test system for point counting with $d = 45$ mm (Fig. 1B). Here we counted an average of 1684 points (599–2889) per worm. The volume estimates of the reproductive structures were then corrected for shrinkage (individually for each worm), to obtain their volume before tissue preparation. In correcting shrinkage we implicitly assumed that shrinkage of overall worm tissue and reproductive tissues was equal and independent of worm volume. Tissue shrinkage could probably be reduced if embedding were performed in a resin such as methyl methacrylate instead of paraffin (Howard & Reed, 1998), but resins are far more costly and more difficult to section. In all further analyses worm volume refers to water displacement volume.

Combined allocation measures

Using these stereological volume estimates, we calculated several combined measures. The reproductive volume (i.e. yolk gland volume + ovary volume + testis volume) represents a measure of total reproductive investment by a worm. Sex allocation (i.e. (yolk gland volume + ovary volume)/testis volume) is a ratio which is a measure of the relative allocation to female vs. male reproductive structures. Egg provisioning (i.e. yolk gland volume/ovary volume) is a ratio which compares the amount of yolk available per oocyte. Under the assumption of a positive correlation between ovary volume and the number of oocytes that can be produced in that volume, this ratio can be expected to be positively correlated to the volume of individual eggs produced by the worms. This is because each egg consists of many yolk cells surrounding one oocyte (Smyth, 1956). For similar reasons yolk gland volume can be expected to correlate positively with total egg volume (number of eggs × egg volume) produced by the worms because eggs primarily consist of yolk cells (Smyth, 1956).

Statistical analysis

We graphically checked if data fulfilled the assumptions of parametric test statistics and transformed if necessary. Data were analysed with JMPIN 3.2.1. (Sall & Lehman, 1996) and Systat 5.2.1. (Systat, 1992).

Results

Worm recovery

Nineteen of 29 fish were found to be infected, of which eight fish had one worm, 10 fish had two worms, and one fish had three worms (possibly as a result of an overlooked double infection in an administered copepod). We restricted all our further analysis to the infection level we had intended to produce, namely the worms in doubly infected fish. The initially large size range persisted through rearing ($n = 10$, standard length, 53.1 ± 1.7 mm, range 45–59 mm, fish weight 1892 ± 203 mg, range 1007–2661 mg, relative condition, 1.00 ± 0.03, range 0.86–1.19).

Histology and stereology

The overall histology of the worms was in good agreement with Smyth (1946), very anterior segments lacked reproductive structures and the different tissues were easily identifiable. Tissue shrinkage averaged 0.194 ± 0.008 (range 0.09–0.28, $n = 20$) and tended to be smaller in smaller worms (Pearson $r = 0.42$, $P = 0.07$), but in the range expected for paraffin embedding (Iwadare *et al.*, 1984; Howard & Reed, 1998). The ratio of worm water displacement volume to worm weight averaged 0.871 ± 0.003 $mm^3\ mg^{-1}$, and can be used to convert water displacement volume to worm weight, which has frequently been used in other studies. Detailed statistical analyses of the stereological results for the different reproductive structures are given in the next section.

Size-dependent allocation

The large size range of the fish that we exposed to the parasite yielded the large size range of worms needed to investigate size-dependent allocation patterns (worm volume, 242 ± 31 mm^3, range 86–382 mm^3, $n = 10$, means $fish^{-1}$) and there was a strong correlation between both measures of fish size and the mean volume of its worms (standard length, Pearson $r = 0.81$, $P = 0.005$; fish net weight, Pearson $r = 0.91$, $P < 0.001$). To assess the relationships between worm volume (x) and the volume of reproductive structures and the measure of egg provisioning (y), we used an allometric model ($y = a \times x^b$, Klinkhamer *et al.*, 1990, 1997). Linear regression on log-transformed data ($\log y = a + b \times \log x$) allows to estimate the slope b (i.e. the exponent of the nontransformed relationship) which is a measure for the rate of exponential increase ($b > 1$) or decrease ($b < 1$) of allocation with worm volume.

Because worms from the same fish are not statistically independent, it was not feasible to analyse the 20 worms together in one such analysis. In order to avoid pseudoreplication but nevertheless include information about all 20 worms, we fitted the allometric model to all possible data sets that can be generated by drawing one worm per fish at a time at random (10 fish with two worms each, $2^{10} = 1024$ possible combinations). For each of these analyses this yielded a r^2-value and an estimate of the slope (b) with its associated standard error (SE). In order to assess the overall relationship in the original data set we then calculated the mean r^2-value, the mean slope (b) and the mean of its associated standard error (SE), which we report in Table 1.

Further, in order to test if a correlation existed between the worm volume and a given reproductive measure, we calculated, for each data set, the t-ratio for the estimated slope (b) against a hypothesized slope of zero, i.e. $t = (b - 0/\mathrm{SE})$, yielding a total of 1024 t-ratios. In order to test the overall data set we then calculated the mean t-ratio of all tests (Table 1) and determined its associated P-value (Table 1) with the appropriate number of degrees of freedom (i.e. $n = 10$, d.f. = 8, two-sided). Similarly, to test if the relationship in each overall data set deviated from linearity we calculated the t-ratios for the estimated slopes (b) against a hypothesized slope of one, i.e. $t = (b - 1/\mathrm{SE})$ and determined the mean t-ratio and associated P-value (Table 1). This analysis was repeated for each of the six reproductive measures.

Table 1 Effect of worm volume on reproductive measures. For each of the six reproductive measures regression analyses (on log–log transformed data according to the approach outlined in the results) were performed on all possible data sets that can be generated by drawing one worm per fish at a time at random (10 fish with two worms each, $2^{10} = 1024$ possible combinations). Here we give, for each reproductive measure, the mean and the range of the 1024 r^2-values, the mean and the range of the 1024 estimated slopes (b) and the mean of the 1024 standard errors (SE) of these estimated slopes. We further give the mean t-values for all the 1024 tests for (a) an overall correlation between worm size and reproductive measure (i.e. H_0: $b = 0$), and the mean t-ratios for all the 1024 tests for (b) a deviation from linearity in the relationship between worm size and reproductive measure (i.e. H_0: $b = 1$). Finally we give the P-values associated with these mean t-ratios (d.f. = 8, two-sided).

	r^2		Slope (b)			Test (H_0: $b = 0$)		Test (H_0: $b = 1$)	
Reproductive measure	Mean	Range	Mean	Range	Mean SE	Mean t	P	Mean t	P
Yolk gland volume	0.95	0.91–0.98	1.36	1.14–1.65	0.11	13.0	<0.001	3.29	0.011
Ovary volume	0.94	0.88–0.98	1.12	0.97–1.34	0.10	12.1	<0.001	1.29	0.23
Testis volume	0.78	0.47–0.96	0.73	0.46–1.01	0.13	5.95	<0.001	−2.23	0.057
Reproductive volume	0.95	0.92–0.99	1.14	0.93–1.40	0.10	12.2	<0.001	1.44	0.19
Sex allocation	0.69	0.41–0.92	0.61	0.40–0.88	0.14	4.45	<0.002	−2.79	0.024
Egg provisioning	0.40	0.13–0.69	0.24	0.10–0.42	0.10	2.33	0.048	−7.63	<0.001

All regression analyses fitted well, as indicated by the high mean r^2-values (Table 1). Further, all reproductive structures increased with increasing worm volume, as shown by the significant P-values for the mean t-ratios (Table 1, Fig. 2). Interestingly we found that the worms allocated disproportionately to the female function with increasing volume, as yolk gland volume increased more than proportionally with worm volume (mean $b = 1.36$, Table 1, Fig. 2A). The other measure of female allocation also had a positive mean slope (mean $b = 1.12$, Table 1, Fig. 2B), however, this value was not significantly different from unity (Table 1). A different pattern was evident in the measure of male allocation. Larger worms also seemed to allocate disproportionately to male function, as testis volume showed a strong tendency to increase less than proportionally with worm volume (mean $b = 0.73$, Table 1, Fig. 2C). Finally, larger worms did not appear to invest disproportionately more in reproduction, as the overall reproductive volume did not increase more than proportionally with worm volume ($b = 1.14$, Table 1, Fig. 2D).

Sex allocation (which we defined here as the ratio of female to male reproductive tissue) increased significantly with increasing worm volume (mean $b = 0.61$, Table 1, Fig. 3A), as the mean slope was significantly different from a slope of zero. Small worms were only slightly female-biased in allocation, whereas larger worms were strongly female-biased. This increase was not linear, however, as evidenced by the test for a deviation from linearity (Table 1, Fig. 3A). Finally, egg provisioning (which we defined as the ratio of yolk volume to ovary volume) showed a positive relation to worm volume, but was increasing less than linearly (mean $b = 0.24$), and this deviation from linearity was also highly significant (Table 1, Fig. 3B).

We also tested if it was always the larger of the two worms within each fish that had the stronger female bias in sex allocation. There was no indication for such a pattern (Fisher exact test, $P = 1.0$), suggesting that sex allocation within a fish is not only determined by worm volume.

Size-dependence in egg volume and total egg volume

We re-analysed earlier data on egg production in *S. solidus* (from Wedekind *et al*., 1998) in the light of possible nonlinear allocation to female function and expected to find that egg volume (called egg size therein) increases less than proportionally and total egg volume (called egg mass therein) more than proportionally with worm weight (for rationale see section 'Combined allocation measures'). We used the analogous allometric approach as used above by fitting log(worm weight) to log(egg volume) and log(total egg volume), respectively, for individuals that were allowed to reproduce alone in the *in vitro* system (see Fig. 1C in Wedekind *et al*., 1998). We show only the analyses of these data because there were no significant correlations with worm weight in the data on pairs (Fig. 1D in Wedekind *et al*., 1998).

As reported earlier (Wedekind *et al*., 1998) egg volume increased with worm weight ($\log(\text{egg volume}) = 4.28 + 0.22 \times \log(\text{worm weight})$, $F_{1,30} = 16.6$, $P = 0.0003$, $r^2 = 0.36$, $n = 32$, Fig. 4A). Additionally, as expected from our stereological analyses, egg size increased less than proportionally with worm weight as $b = 0.22$, which is highly significantly smaller than 1 ($F_{1,30} = 203.6$, $P < 0.0001$, Fig. 4A). This observed deviation from linearity is in close agreement with the pattern observed in the present study for egg provisioning (for which mean b was 0.24), suggesting that egg provisioning actually reflects egg volume quite well. Total egg volume increased strongly with worm weight ($\log(\text{total egg volume}) = 4.76 + 1.91 \times \log(\text{worm weight})$, $F_{1,30} = 22.2$, $P < 0.0001$, $r^2 = 0.43$, Fig. 4B).

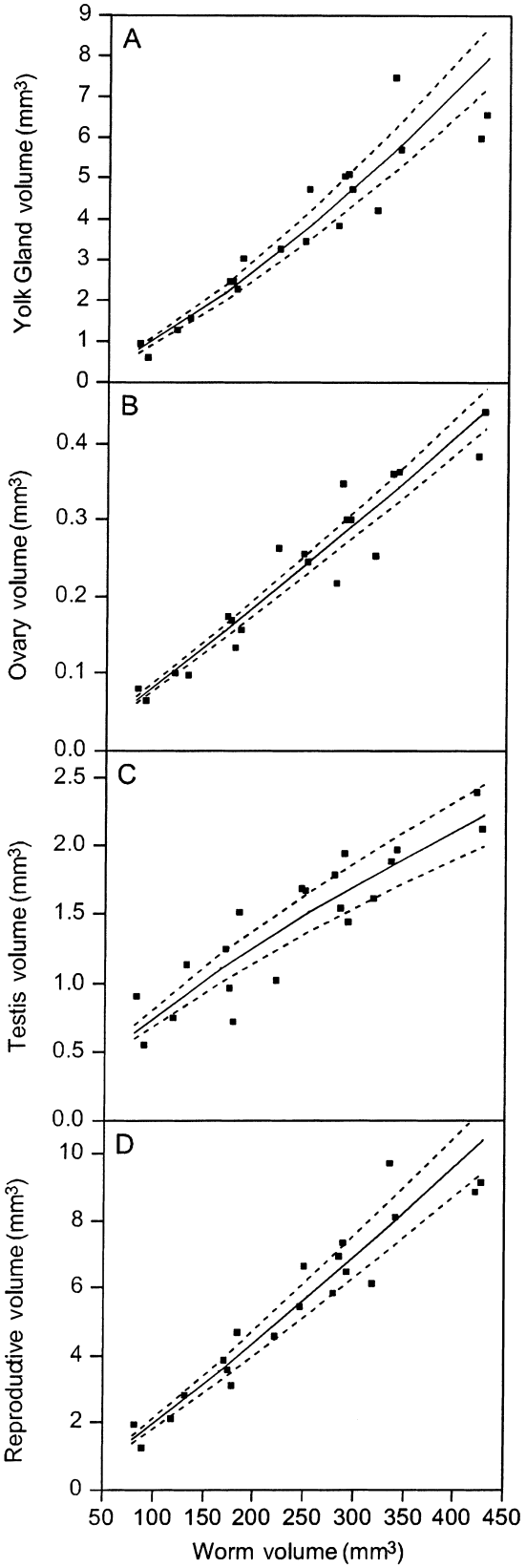

Fig. 2 Size-dependence in allocation to reproductive structures in *S. solidus*. The figure shows the data of all 20 worms that were analysed stereologically. Regression analyses (on log–log transformed data according to the approach outlined in the results) were carried out on all possible data sets that can be generated by drawing one worm per fish at random (10 fish with two worms each, $2^{10} = 1024$ possible combinations). The mean regression line derived from the 1024 regressions and its 95% confidence intervals are shown for yolk gland volume (A), ovary volume (B), testis volume (C), and reproductive volume (D). See Results and Table 1 for statistics.

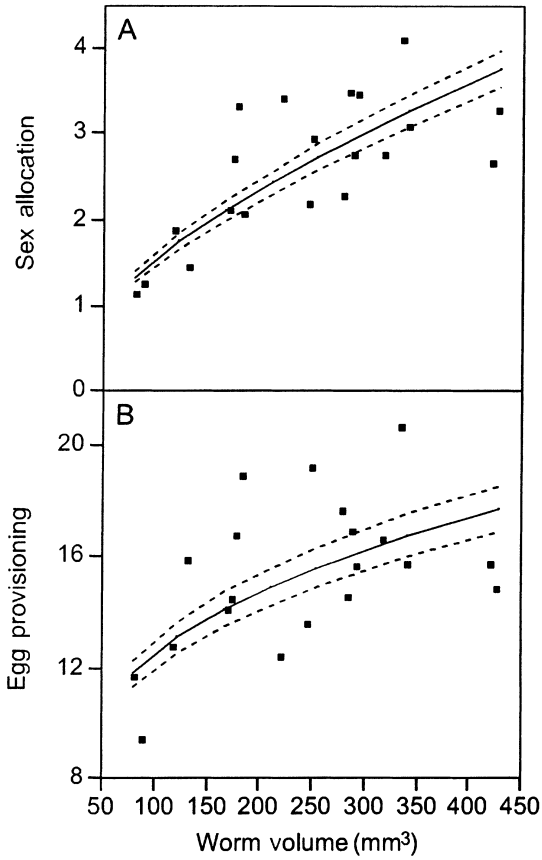

Fig. 3 Size-dependence in allocation ratios in *S. solidus*. The figure shows the data of all 20 worms that were analysed stereologically. The regression analyses were carried out as described in Fig. 2. Sex allocation (A), i.e. the ratio of the volumes of female divided by male reproductive structures (values <1 indicate male-biased allocation, and values >1 indicate female-biased allocation), and egg provisioning (B), i.e. the ratio of yolk gland volume divided by ovary volume, a measure that is expected to reflect egg volume produced by worms. See Results and Table 1 for statistics.

Again as expected from our stereological analyses, total egg volume increased more than proportionally with worm weight as $b = 1.91$, which is significantly larger than 1 ($F_{1,30} = 5.03$, $P = 0.032$, Fig. 4B), providing

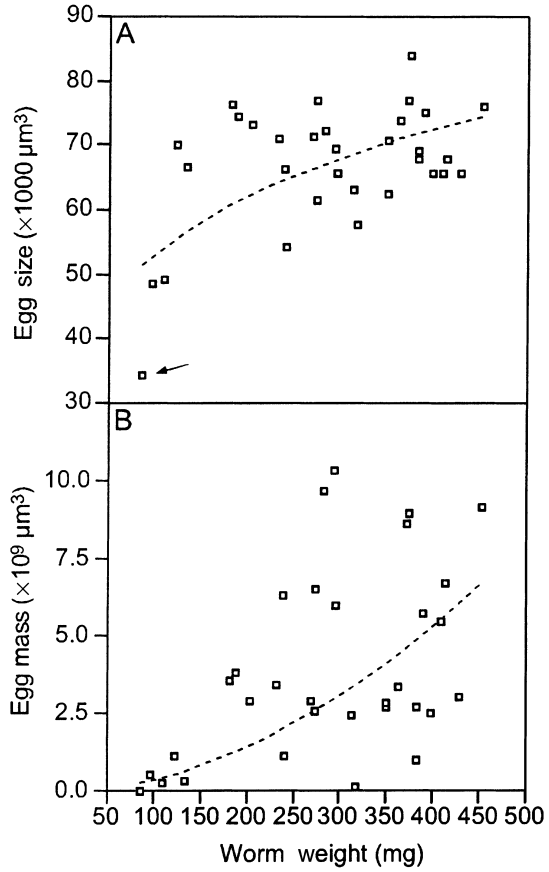

Fig. 4 Size-dependence in egg production in *S. solidus* kept isolated during the first 3 days of reproduction (re-analysis of data plotted in Fig. 1C in Wedekind *et al.*, 1998). Regression analyses were carried out according to the approach described in Results. Regression lines are shown for egg volume (A), and total egg volume (egg number × egg volume, B). See Results for statistics. Exclusion of the data point indicated by the arrow did not affect conclusions about deviation from linearity. Worm weight of 1 mg corresponds to about 0.871 mm^3 of worm volume as estimated from the data in the current study.

independent further evidence for nonlinear allocation to female function in *S. solidus*.

Discussion

Method

Stereology appears to be a suitable method for determining the reproductive allocation patterns of simultaneous hermaphrodites. It is especially useful in a modular organism, such as a tapeworm because it allows for unbiased and systematic subsampling of the repetitive and dispersed male and female reproductive organs. Stereological estimation of volumes is efficient if 100–200 points are counted per animal (Gundersen & Østerby, 1980, 1981), a range which was achieved for all reproductive structures in this study, except in the smallest worms. As a result of the unbiased nature of stereological methods, counting fewer than 100 points will, however, only increase the variance in the estimate and will not lead to a bias.

Overall sex allocation

All measured worms produced substantial amounts of both male and female reproductive organs, demonstrating that *S. solidus* clearly is a simultaneous hermaphrodite. Under the assumption that yolk glands, testes and ovaries require about equal energy investment per unit volume of structure, sex allocation in *S. solidus* is strongly female-biased (mean 2.6*x* more female structure than male structure, range 1.2–4.1*x*, Fig. 3A). This is in agreement with theoretical predictions for a partially selfing organism (Charlesworth & Charlesworth, 1981). We are not aware of published information about the relative costs of building male vs. female reproductive tissues in any organism (see also Baylis, 1981). Determination of caloric energy content may go some way to estimate energetic cost of building. For example, a recent study on reproductive allocation in a coral reef fish (Schärer & Robertson, 1999) found semen to contain 16% less energy than eggs per unit of dry weight. Most studies, however, use biomass alone as an estimate of reproductive allocation, which may be problematic if tissues differ greatly in their building costs. However, McKone (1987) found that estimates of sex allocation in several grass species were quite robust when different resource currencies were used (i.e. energy content and five different nutrients). For an overall sex allocation of unity in *S. solidus*, testes would have to be on average close to three times more costly to build than the female structures. For the reasons mentioned above this appears improbable. To gain hard data on this issue will probably require detailed knowledge about biochemical pathways involved in the construction of male and female structures.

Size-dependent sex allocation

We found size-dependent sex allocation in the simultaneous hermaphrodite parasite *S. solidus*, with larger individuals being more biased to female allocation, a pattern that has since been confirmed in another study of our group (L. Schärer & C. Wedekind, unpublished manuscript). *Schistocephalus solidus* is a very suitable species to investigate questions of size-dependent sex allocation for several reasons: (a) it is semelparous, reproducing within a short reproductive period after which the worms die, which avoids problems with assessing the relative importance of current vs. future reproductive success, (b) during reproduction it primarily uses energy accumulated during growth in the second intermediate host, which may allow to experimentally

manipulate the resources available for reproduction, (c) male and female reproductive structures are probably built at the same time which suggests that they may be built from a common resource pool and (d) it shows a large range of sizes in the adult phase (up to two orders of magnitude, Tierney & Crompton, 1992; L. Schärer & C. Wedekind, personal observation), which is generally rare in animals that are not colonial.

The observed size-dependence could be because of a difference in resource availability in small vs. large hosts. It would, however, be premature to conclude that resource levels are responsible for the observed patterns in reproductive allocation, as they have not been experimentally manipulated in this study. We cannot currently directly control the resource levels available to the parasite *in vivo*, because they depend (a) on the food uptake and resource allocation of the fish and (b) on the growth patterns of the worms and their influence on fish growth, food uptake and behaviour. Controlling the food levels of the fish would hence only partly control the resources available to the worm. An *in vitro* system replacing the second intermediate host, which would allow such control, is currently not available for *S. solidus*.

In two studies on a simultaneous hermaphrodite plant (Klinkhamer & de Jong, 1993; Rademaker & Klinkhamer, 1999) there was no evidence that equalizing the resources available to small and large plants removed the size-dependence in sex allocation. Similarly, Hughes (1989) performed a field study that manipulated resource levels available to bryozoans and found no effect on sex allocation, despite large effects on growth and an overall shift from male-biased to a more balanced allocation later in life. To conclude: evidence that size-dependent sex allocation is a response to varying resource levels seems currently not available.

The phenotypic allocation patterns measured in this study may allow us to speculate about the shape of the fitness gain curves for reproductive investment in *S. solidus*. Gain curves in simultaneous hermaphrodite animals have rarely been investigated (but see McCartney, 1997; Yund, 1998). In *S. solidus* the strong trend for a less than proportional increase in male allocation with volume, as seen in the data on allocation to testis, would be expected with a decelerating male gain curve. Given the proportional increase in overall reproductive investment with volume, this leads to a strongly accelerating allocation to female function with worm volume, as shown in the allocation patterns of yolk glands and the patterns in total egg volume. This would be expected with a linear, or at least less decelerating, gain curve for investment to the female function. However, the additional female allocation does not only increase the number of eggs produced, but is also distributed differently to eggs, as can be seen in the changes in egg provisioning and egg volume with worm volume.

A simple alternative explanation for the observed patterns of size-dependence could stem from the fact that the male and female organs are arranged in different ways in the worm and that they may therefore be constrained differently by worm volume. Yolk gland cells are distributed peripherally around the whole circumference of the worm, whereas testes are arranged in one layer in the centre of the worm (see plates in Smyth, 1946). It will be required to show that the observed patterns are actually adaptive to dismiss off this alternative explanation.

Hypotheses about size-dependent sex allocation

A number of hypotheses have been put forward to explain size-dependent sex allocation in simultaneous hermaphrodites. We will summarize them below and discuss their relevance to our study species. As some of these hypotheses have originally been formulated for plants only, we will attempt to extend them to animals.

(a) Local resource competition (Clark, 1978; Charnov, 1982) is the competition for limiting resources between related offspring and may lead a parent to invest less in the sexual function with lower gene dispersal. An example could be the higher spatial dispersal of pollen (the male function derived genes) compared with ovules (the female function derived genes) in plants, leading to competition between seedlings that have the same mother but different fathers (Lloyd & Bawa, 1984). Larger plants may produce more seeds and therefore competition between their seedlings maybe higher. This local resource competition may hence cause a shift in allocation to the sexual function with higher dispersal (the male function in this case) with increasing size. To explain the female-biased size-dependent sex allocation in *S. solidus*, the male function would need to achieve lower gene dispersal. Although it appears possible that related individuals would compete for resources in the same host and within host competition has been shown to reduce individual parasite size (Wedekind, 1997), local resource competition here is unlikely to be important in selecting size-dependent sex allocation because internal fertilization probably leads to similar dispersal of male and female function derived genes.

(b) Local mate competition (Hamilton, 1967) is the competition between genes derived from the male function of a single individual for the access to gametes derived from female function. It may lead to diminishing returns for the investment to male function. In a simultaneous hermaphrodite local mate competition is best viewed as the competition between sperm cells derived from the male function of an individual for the fertilization of female function derived oocytes. In the most extreme case of local mate competition those oocytes are from the same individual, which is the case of selfing. An obligate selfer should only produce the minimal amount of sperm to fertilize its own eggs (Charlesworth & Charlesworth, 1981). In outcrossing species small mating group size has been suggested to

lead to local mate competition and reduced male allocation (Charnov, 1982, 1987; Raimondi & Martin, 1991). Small mating group size can be a consequence of a sessile lifestyle (Charnov, 1987), parasitic lifestyle (Charnov, 1979) and reciprocal egg trading (Fischer, 1980; Sella, 1990; Petersen & Fischer, 1996). An increase in mating group size may lead to sperm competition (Parker, 1970), where the sperm derived from the male functions of several individuals compete for the access to female function derived gametes. Sperm competition in turn leads to increased male allocation, and may lead to the evolution of sperm digestion (i.e. the digestion of sperm received from a mating partner, Charnov, 1979; Greeff & Michiels, 1999), which may further equalize the investment in male and female function.

Local mate competition is likely in *S. solidus* for several reasons. It is self-fertile and has been shown to reproduce even when isolated during the reproductive period (Wedekind *et al*., 1998). Further, the mating group size depends on how the parasite population is distributed. Parasite distributions are generally overdispersed (see Pennycuick, 1971 for our study species), which may mean that there is a limited number of mating partners that a worm is likely to encounter, with little to be gained from investing more in male function than is needed to fertilize these. So larger individuals may gain more from investing in female function than to further invest in male function. Our finding of a more female biased sex allocation in larger individuals is in agreement with local mate competition.

(c) Differences in the temporal patterns of male and female reproduction in combination with size-differential mortality may also lead to size-dependent sex allocation (time commitment hypothesis, Day & Aarssen, 1997). For example, if small individuals have higher mortality and the male function requires less time commitment, small individuals may bias their investment to the male function and then try to fertilize other individuals before they die. Temporal patterns in male and female reproduction are likely to differ in our study species, as fertilization takes place before formation of the egg shell (Smyth, 1956). Thus the return for investment in the female function necessarily requires more time to materialize. Additionally, the storage of received sperm in the receptaculum seminis may further reduce the amount of time during which performing the male function is necessary. Size-differential mortality could be tested by infecting natural hosts (e.g. gulls, herons or kingfishers) with worms of a range of sizes and then looking for size-dependent excretion of worms. A previous study on establishment rates in *S. solidus* in an experimental final host (i.e. week-old chicken, Tierney & Crompton, 1992) found an effect in the expected direction but it was not statistically significant. However, the study assessed establishment rates 1 day after infection, which is probably to be too early as sperm only become available on the second day after host change (L. Schärer & C. Wedekind, unpublished manuscript). The time commitment hypothesis represents a viable hypothesis for size-dependent sex allocation in *S. solidus*.

(d) Geitonogamous selfing, i.e. selfing between flowers of an individual plant, has been suggested to select for female-biased allocation in larger individuals (de Jong *et al*., 1999), if the amount of selfing increases with the amount of male allocation. A similar pattern could be present in a simultaneous hermaphrodite animal that has little control over how much selfing it does (as in some self-compatible marine invertebrates, e.g. Hughes, 1989). The equivalent of geitonogamous selfing appears unlikely in *S. solidus*, as (1) the worms can be expected to have control over whether they self-fertilize and (2) there is no reason to believe that larger worms should have less control over this. As this mechanism would, however, predict the same pattern of size-dependent sex allocation as observed in our study, it would be careful to asses the levels of selfing in relation to the size of the worms before dismissing this hypothesis.

(e) The dispersal ability of pollen for outcrossing has been suggested to depend on plant size (Burd & Allen, 1988; Bickel & Freeman, 1993), with larger plants being more efficient at pollen dispersal (especially in wind pollinated plants). This would lead to large individuals being more male-biased in sex allocation. This mechanism is sometimes referred to as pollination syndrome (Wright & Barrett, 1999). A similar mechanism could be operating in animals if sperm donation ability depends on individual size. This could, for example, be the case in animals with hypodermic impregnation (injection of sperm through the body wall, Michiels, 1998; Michiels & Newman, 1998), if competitive ability or coercive ability depend on body size. This is essentially the local mate competition argument because larger individuals would fertilize more individuals and would hence experience a larger mating group size and possibly more sperm competition than small individuals. We find no support for the notion that larger individuals may be better sperm donors as the effect we observed goes in the opposite direction of the prediction.

Conclusions

Size-dependent sex allocation in simultaneous hermaphrodite plants is well documented (Lloyd & Bawa, 1984; Klinkhamer & de Jong, 1997; Klinkhamer *et al*., 1997; Wright & Barrett, 1999). We are aware of only three studies that show size-dependent sex allocation in simultaneous hermaphrodite animals and none of these studies provide a formal analysis of the patterns of size-dependence. The study by Petersen & Fischer (1996) found evidence for larger individuals being more female-biased in a small egg trading coral reef fish, *Serranus tortugarum*. This result is in agreement with the local mate competition hypothesis and is probably a result of low mating group size because of egg trading. Trouvé

et al. (1999) found a higher female allocation in larger individuals of a parasitic trematode for reasons that are probably similar to the ones in our study species. Another study in a small temperate fish, the goby *Lythrypnus dalli*, found that large nest-building males are strongly male-biased and size was implicated to play a role in the successful defence of nest sites (St Mary, 1994). This pattern is in close agreement to the patterns observed in sequential hermaphrodite fishes (Robertson & Warner, 1978; Warner & Robertson, 1978), but male-biased *L. dalli*, at least partly, retain the ability to change back to female reproduction when deprived of male mating opportunities (St Mary, 1994). These and our study clearly suggest that size-dependent sex allocation is present in simultaneous hermaphrodite animals, and that sex allocation theory will require to incorporate this phenomenon more formally.

Despite considerable work on size-dependent sex allocation in plants, the causal relationships between size and sex allocation remain poorly understood (Campbell, 2000). One problem in studying this issue in plants is the difference in timing between flower production (which entails both male and female investment) and seed set (which only represent female investment). Our system greatly reduces this problem, as male and female reproductive organs are built before the reproductive period, in the second intermediate host. Further, a technique that would allow for accurate control of resource levels available to the parasite during growth would be an extremely powerful tool to investigate the causal relationships in size-dependent sex allocation.

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