Social situation, sperm competition and sex allocation in a simultaneous hermaphrodite parasite, the cestode *Schistocephalus solidus*

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Abstract

Evolutionary theory predicts an influence of mating group size on sex allocation in simultaneous hermaphrodites. We experimentally manipulated the social situation during reproduction in a simultaneous hermaphrodite parasite, the tapeworm *Schistocephalus solidus*, by placing worms as singles, pairs or triplets into an *in vitro* system that replaces the final host. We then determined the reproductive allocation patterns after 24 h (i.e. before the start of egg release) and after 72 h (i.e. around the peak of egg release rate) using stereology. After 24 h, sex allocation strongly depended on worm volume (which is determined in the second intermediate host), but was not significantly affected by the social situation experienced during reproduction. After 72 h, worms in groups had less vesicular sperm (i.e. sperm to be used in future inseminations) than singles. They also stored significantly more received sperm in their seminal receptacles than singles, suggesting that more sperm had been transferred in groups. Moreover, worms in triplets stored significantly more received sperm than worms in pairs, suggesting that they either mated more often and/or transferred more sperm per mating. This suggests a behavioural response to the increased risk of sperm competition in triplets. We further discuss the relative importance of sex allocation decisions at different life-history stages.

Introduction

Sex allocation theory predicts the optimal investment into male and female reproduction in sexually reproducing organisms (Charnov, 1982). In simultaneous hermaphrodites, in which individuals invest in sperm and eggs at the same time, sex allocation is best viewed as the ratio of investment into male vs. female gametes, and several studies have shown that this ratio may vary in ways that are consistent with predictions from sex allocation theory (e.g. Raimondi & Martin, 1991; Klinkhamer & de Jong, 1997; Klinkhamer *et al.*, 1997; Johnston *et al.*, 1998; Trouvé *et al.*, 1999). Mating group size, i.e. the number of sperm donors from which recipients receive sperm, is suggested to influence sex allocation in simultaneous hermaphrodites (Charnov, 1982). As mating group size increases, sex allocation is predicted to shift from female-biased investment to more equal investment (Charnov, 1982, 1996). This theoretical prediction has rarely been experimentally investigated in animals (but see, Trouvé *et al.*, 1999).

The factors that are responsible for the change in optimal sex allocation with changing mating group size are local mate competition (Hamilton, 1967) and sperm competition (Parker, 1970). Local mate competition is the competition among related individuals (usually brothers) for mates, whereas sperm competition is the competition between sperm of (unrelated) males for the fertilization of eggs of a female. In simultaneous

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hermaphrodites, local mate competition can be viewed as the competition between sperm of the *same* or *related* individuals for the fertilization of oocytes. It is here therefore in a sense the contrary of sperm competition, which represents the competition between sperm of *unrelated* individuals.

In an isolated, self-fertilizing individual self-sperm compete for the fertilization of self-oocytes, and the individual will experience a mating group size of one. This is a situation with maximal local mate competition and no sperm competition. It is easy to see that such an individual should only produce the minimal amount of sperm required to fertilize the self-oocytes, because any additional sperm would be wasted in local mate competition. Sex allocation theory predicts that such an individual should have a strongly female-biased sex allocation, because it is assumed that resources not invested to the male function can be channelled to the female function.

With two individuals the situation becomes more complex, because in a facultatively selfing organism the mating group size depends on how mating takes place. If worms just self-fertilize the situation remains as outlined above, i.e. mating group size is one and sex allocation is not expected to change. If the worms only cross-fertilize, mating group size is also one, and the optimal investment in sperm depends on the number of eggs of the partner. Such a restriction to outcrossing can be viewed as a form of co-operation between the mating partners (Fischer, 1980; Leonard, 1990). If both self- and cross-fertilization take place, self-sperm of individual A will be in competition with sperm of individual B for the fertilization of the oocytes of A (and vice versa for individual B). In this case mating group size will be two, local mate competition will be reduced, sperm competition will be present and sex allocation is expected to be less female-biased, because of an increase in male allocation to counteract sperm competition.

In a group of three individuals, we can expect a further increase of mating group size (unless individuals exclusively self-fertilize irrespective of group size). If mating is promiscuous and only cross-fertilization takes place, mating group size will be two, i.e. each recipient will be inseminated by two partners. If both self- and cross-fertilization take place, mating group size will be three. Thus reduced local mate competition and sperm competition are expected to be present even if cross-fertilization were the only type of mating. We can therefore expect male allocation to be higher in groups of three individuals than in isolated or paired individuals.

Here we experimentally investigate the influence of the social situation encountered during reproduction on sex allocation and look for evidence for sperm competition in a facultatively self-fertilizing simultaneous hermaphrodite parasite, the tapeworm *Schistocephalus solidus*. Worms were allowed to reproduce in an *in vitro* system replacing the final host, while either being isolated (singles), in groups of two (pairs) or in groups of three (triplets). If sex allocation can be adjusted by a phenotypically plastic response to the social situation encountered during reproduction in the final host, we expect a less female-biased sex allocation with increasing group size. The size of the effect on sex allocation of social situation can be predicted by a simple model (Charnov, 1982, p. 245). Although this model assumes outcrossing, it can be used to get at least a qualitative estimate of the expected effect size (a more realistic model that incorporates the selfing rate and inbreeding depression, e.g. Charlesworth & Charlesworth, 1981, cannot be applied to our situation, because there are currently no estimates for either parameter).

In Table 1 we summarize the predicted sex allocation patterns under the expected mating group sizes for two different mating scenarios: (a) self-fertilization in single worms and cross-fertilization in larger groups only, and (b) self-fertilization in single worms and both self- and cross-fertilization in larger groups. These may not be the only possible scenarios, but we think they are the most likely ones, given earlier results on behaviour and egg production of *S. solidus* under different group sizes (Wedekind *et al.*, 1998; Schärer & Wedekind, 1999; Lüscher & Wedekind, 2001).

If self-fertilization leads to inbreeding depression (e.g. Jarne & Delay, 1990), individuals in groups may tend to avoid self-fertilization and prefer to cross-fertilize. However, some factors may favour self-fertilization over

Table 1 Expected mating group sizes and the predicted optimal male allocation (as a proportion of total allocation) under two different mating scenarios (following Charnov, 1982, p. 245). Large effect sizes between the different social situations are predicted under both scenarios. An optimal allocation of zero stands for minimal male allocation required to fertilize all eggs present, and an allocation of one would represent that all resources are channelled into the male function.

	Scenario			
	Singles self-fertilize, others cross-fertlilize only		Singles self-fertilize, others both self- and cross-fertlilize	
Group size	Mating group size	Optimal male allocation	Mating group size	Optimal male allocation
Single	1	0	1	0
Pair	1	0	2	0.333
Triplet	2	0.333	3	0.4

cross-fertilization, such as the retention of locally adapted genotypes, or the higher relatedness with offspring derived from self-fertilization (two copies of own genes per offspring instead of only one copy in the case of cross-fertilization). Therefore, it is possible that worms in groups show a combination of both types of mating. Such simultaneous occurrence of self- and cross-fertilization has been documented in other cestodes (Smyth & Smyth, 1969; Nollen, 1975) and trematodes (Trouvé *et al*., 1996; Nollen, 1997).

Further clues regarding mating group size may come from investigating sperm transfer patterns under the different social situations, and may allow to exclude some alternative mating scenarios. For example, no differences in the levels of vesicular sperm (i.e. sperm to be used in future inseminations) or received sperm would be expected if self-fertilization were the only mating mode employed under all social situations. Moreover, differences in these parameters could indicate that worms react to different perceived levels of sperm competition risk in the different social situations. For example, if larger group size leads to more sperm competition, we expect worms in larger groups to store larger amounts of received sperm either because of a higher mating rate or a higher amount of sperm transferred per mating in response to sperm competition.

Materials and methods

Study species

The pseudophyllidean tapeworm *S. solidus* is a simultaneous hermaphrodite parasite that reproduces in the intestine of fish eating birds. Eggs pass into the water with the birds' faeces. After hatching, the free-swimming first larval stage is ingested by the first intermediate host, a cyclopoid copepod. 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. 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 to the final host after 1–3 months (plerocercoids of >50 mg generally establish themselves in the final host, Tierney & Crompton, 1992). After ingestion of an infected fish by the final host, the plerocercoids mature to become adult worms and start to produce eggs within 2 days (Smyth, 1946).

Schistocephalus solidus is unusual among tapeworms in that the plerocercoids in the fish are completely segmented and all but the first 10 or so segments contain a full set of genitalia. These are in an advanced stage of development but immature. The reproductive system at this stage primarily consists of yolk glands, testes and ovaries containing rather inactive cells (Smyth, 1946).

Spermatogenesis (i.e. meiosis) is triggered by the high body temperature of the final host (Smyth, 1952), which rules out the possibility of sperm transfer in the fish. Mature sperm are collected in the vasa deferentia and the muscular seminal vesicles. The anatomical organization of the reproductive organs suggests that insemination takes place via copulation. The cirrus, a penis like structure, can be everted (L. Schärer, personal observation) and sperm is probably ejaculated into the vagina by contraction of the musculature of the seminal vesicles. The amount of sperm ejaculated could hence be under muscular control. Sperm then migrates to the receptaculum seminis. Smyth (1956) gives a detailed account of the fertilization and egg formation process.

Reproduction takes place within 1–2 weeks, after which the worms die (*in vivo*: McCaig & Hopkins, 1963; Tierney & Crompton, 1992; *in vitro*: Schärer & Wedekind, 1999). Transmission rates to the final host are variable, but generally high (i.e. between 60 and 100% of the worms successfully establish themselves in the final host, Tierney & Crompton, 1992), therefore worms in multiple infection have a chance of being transmitted together. Growth does not appear to take place in the final host, as worms both lose weight with increased time in the *in vitro* system (see below), and *in vivo* (e.g. Tierney & Crompton, 1992). A previous study suggested that self-fertilization is possible, and that it may lead to inbreeding depression (Wedekind *et al*., 1998). We further previously established that allocation to reproductive tissues and sex allocation is strongly size-dependent in plerocercoids, i.e. the larval stage in the fish, with larger individuals being more biased to female allocation than small individuals (Schärer *et al*., 2001). Here, we concentrate on the adult stage of *S. solidus*.

General methods

To obtain the worms used in the experiments, we killed infected fish by a cerebrospinal cut and opened their body cavity with a pair of fine scissors. We determined fish standard length, net fish weight (fish weight minus the sum of the weights of its worms), and the number and weights of worms to the nearest 1 mg by subtractive weighing. This allowed to calculate the infection level (i.e. the number of worms coinfecting a fish), and the parasite index (the total parasite weight divided by the net fish weight). We further calculated relative condition of the fish based on net fish weight (after Le Cren, 1951). All worms came from fish that had been infected with one of three different parasite lines that had been inbred in our laboratory for two generations (lines 'A', 'D' and 'N'). We only used worms coming from fish that were multiply infected (range 2–9 worms, average 4). After removal from the fish, we stored the worms in culture medium at room temperature for a maximum of 10 h before starting the experiments.

We used an *in vitro* system to replace the final host (a detailed description is given in Wedekind *et al*., 1998; Schärer & Wedekind, 1999). It consisted of nylon nets

(20 × 80 mm) that we suspended into polypropylene medium bottles containing tissue culture medium, which had been preheated to 40 °C in a climate chamber. In an earlier study we found that the worms started to produce eggs after around 46 h and that egg production rate peaked on the third day; around 70–80% of the eggs were produced within the first 5 days (Schärer & Wedekind, 1999).

After 24 h (i.e. before release of the first eggs) or after 72 h (i.e. at about the peak of egg release rate, Schärer & Wedekind, 1999), we removed the worms from the *in vitro* system and determined their weights (to the nearest 1 mg), and their water displacement volume (Weibel, 1979, p. 240) as a reference for later histological volume determinations and in order to allow for correction of tissue shrinkage during histological preparation. We then fixated each worm by placing it into a horizontal 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.

Stereology

The aim of the stereological analysis was to estimate the volumes of different reproductive structures in each worm and the volume of the worm itself. Stereology is based on quantitative measurements of histological structures from serial sections. Volumes of the reproductive structures were estimated using the Cavalieri principle (e.g. Gundersen & Jensen, 1987) and point-counting (e.g. Howard & Reed, 1998, p. 31). Details and rationale of the method are published elsewhere (Schärer *et al*., 2001). To reduce the stereological sampling effort we picked one worm per replicate at random (i.e. all singles, one worm from each pair and one worm from each triplet).

We analysed an average of seven sections per worm (range 5–9). For estimation of reproductive structures, we generally point-counted in the area occupied by a structure, rather than only the heavily stained portion of the structure as we had performed in a earlier study (Schärer *et al*., 2001). For yolk glands and ovaries it was clearly visible where the boundaries of the structures were. For testes (i.e. site of sperm production), we point-counted within the boundary of the testis vesicles. We then calculated a measure of sex allocation as (yolk gland volume + ovary volume)/testis volume, which represents the relative allocation to female vs. male reproductive structures. During sperm production the testis vesicles swell up and contain loose developing sperm surrounded by empty lumen. Therefore the measure of sex allocation we report here (a) cannot be directly compared with that of the earlier study (Schärer *et al*., 2001), and is (b) likely to be too male-biased, because of the lumen being included in the measure of testis volume. The following reproductive structures were only point-counted in worms collected in the second experiment (see below), as they only appear after about 2 days in the *in vitro* system. For the volume of vesicular sperm (i.e. produced sperm in the vasa deferentia and seminal vesicles that is ready to be used in future inseminations) we used the cell layer lining the ducts as the boundary. Finally, for the volume of received sperm (i.e. stored sperm in the receptaculum seminis that was received by copulation) we point-counted only the portion of the compartment occupied by the sperm, because many receptacles were not completely filled. The volume estimates of the reproductive structures were then corrected for shrinkage (individually for each worm), to obtain their volume before tissue preparation (see Schärer *et al*., 2001). In all further analyses worm volume refers to the measured water displacement volume.

Study design

Worms were randomly assigned to a social situation (single, pair, or triplet). This was performed in a way which assured that (a) worm lines were balanced over treatment groups, (b) worms placed together in pairs or triplets came from different lines (to possibly enhance the likelihood of cross-fertilization), (c) worms within groups were of similar weight (again to possibly enhance the likelihood of cross-fertilization, see Lüscher & Wedekind, 2001), and (d) the mean worm weight was balanced over replicates. We then placed these worms in the *in vitro* system in medium bottles containing either 260 mL (small) or 520 mL (large) of culture medium. We added this second experimental factor to the design for the following reason. The fact that we balanced for mean worm weight over replicates necessarily lead to higher total worm weight per unit of culture medium in the larger groups. The additional factor made it possible to distinguish between effects of the social situation and effects of the total worm weight. A weight effect, if present, would be expected to lead to a significant effect of bottle size and/or a significant interaction term between bottle size and social situation. We made two experiments that were set up as shown in Fig. 1. To check if experiments were balanced for the variables describing the donor fishes we calculated their average values per replicate (e.g. mean standard length of the three donor fishes for worms in triplets).

Experiment 1

In this experiment we investigated if the size-dependent sex allocation patterns that we have previously found in plerocercoids of *S. solidus* (Schärer *et al*., 2001) were affected by the social situation into which the worms were placed. The worms were removed from the *in vitro* system after 24 h, and then analysed stereologically.

Social situation	Bottle size: Small	Bottle size: Large
Singles	**A** D N	A D N
Pairs	AD DN AN	AD DN AN
Triplets	ADN ADN ADN	ADN ADN **ADN**

Fig. 1 Experimental design of Experiments 1 and 2: two-way ANOVA design with three social situations (single, pairs and triplets) and two bottle sizes (small and large). Families were initially balanced within and among all experimental factors. In Experiment 2 we lost the replicates in bold typeface (for details see Methods).

From a previous study we know that no eggs are released until that point (Schärer & Wedekind, 1999). We were particularly interested to see whether the predicted shift towards male allocation occurred in worms that we placed in groups. Because of a randomization error, the mean worm weight per replicate was higher in the large bottles than in small bottles (two-way ANOVA, social situation, $F_{2,12} = 2.33$, $P = 0.14$; bottle size, $F_{1,12} = 31.4$, $P < 0.001$; interaction, $F_{2,12} = 0.13$, $P = 0.88$, $n = 18$). However, the fact that the social situation was completely balanced regarding bottle size allowed us to drop the factor bottle size from the analysis (further Experiment 2 suggests no bottle size effects). All variables describing the donor fishes were balanced over the different social situations, i.e. standard length, net fish weight, relative condition, infection level and parasite index (one-way ANOVAS, all $P > 0.59$). The experimental design was also balanced for the mean worm weight in the replicates (singles, 258.6 ± 46.3 mg; pairs, 326.5 ± 40.9 mg; and triplets, 317.6 ± 36.2 mg, one-way ANOVA, $F_{2,15} = 0.8$, $P = 0.47$).

We have previously used the worm volume to describe worm size (Schärer *et al.*, 2001). Here, however, the worm volume could have been affected by the treatment. If so, it would not be suitable as a covariate in an analysis looking for effects of the treatment. We checked this possibility by comparing the mean worm weights before the experiment to the mean worm weights after the experiment, by calculating a repeated measures ANOVA with social situation as a factor. Despite a significant drop in the mean worm weight over the time in the *in vitro* system ($F_{1,15} = 8.6$, $P = 0.01$), there was no effect of social situation ($F_{2,15} = 0.83$, $P = 0.46$) and no interaction with time ($F_{2,15} = 0.50$, $P = 0.62$). Because, further, the mean worm weight after the experiment and the worm volume were highly correlated ($n = 18$, Pearson $r = 0.997$, $P < 0.001$), we decided that worm volume was suitable to describe worm size.

Experiment 2

Here we extended the duration until sampling to 72 h in order to also investigate the effect of the social situation on the amount of vesicular and received sperm. At removal from the *in vitro* system, one single worm had a transparent area in the centre of the body (10–15% of the body), and it appeared as if the internal structures were damaged. Further, one triplet was lost because of the worms escaping from the nylon net into the medium bottle. These replicates were excluded from the analysis (see Fig. 1 for the final design).

Nevertheless, the two-way ANOVA design (factor 1: social situation, factor 2: bottle size) remained balanced for most of the parameters describing the donor fishes, i.e. standard length ($P_1 = 0.56$, $P_2 = 0.85$, $P_{1*2} = 0.28$), net fish weight ($P_1 = 0.75$, $P_2 = 0.85$, $P_{1*2} = 0.33$), relative condition ($P_1 = 0.47$, $P_2 = 0.87$, $P_{1*2} = 0.79$), infection level ($P_1 = 0.15$, $P_2 = 0.14$, $P_{1*2} = 0.05$) and parasite index ($P_1 = 0.36$, $P_2 = 0.92$, $P_{1*2} = 0.44$). The design also remained balanced for mean worm weight in the different social situations (singles, 301 ± 64 mg, $n = 5$; pairs, 297 ± 37 mg, $n = 6$; and triplets, 306 ± 54 mg, $n = 5$, two-way ANOVA, social situation, $F_{2,10} = 0.005$, $P = 0.99$; bottle size, $F_{1,10} = 0.38$, $P = 0.55$; interaction, $F_{2,10} = 0.006$, $P = 0.99$).

As in Experiment 1 we checked for the suitability of the worm volume as a covariate. Despite a significant drop in the mean worm weight over the time in the *in vitro* system ($F_{1,10} = 85.6$, $P < 0.001$), there was no effect of the factors (social situation, $F_{2,10} = 0.01$, $P = 0.99$; bottle size, $F_{1,10} = 0.58$, $P = 0.46$; interaction, $F_{2,10} = 0.02$, $P = 0.98$). Also there were no significant interactions between the factors and time ($P = 0.24$, 0.27 and 0.67, respectively). Because the mean worm weight after the experiment and the worm volume were highly correlated ($n = 16$, Pearson $r = 0.999$, $P < 0.001$), we again used the worm volume to describe worm size.

Correlations between worm volume and donor fish parameters

In order to better understand the importance of worm size for sex allocation decisions, we checked how well the different parameters describing a donor fish (i.e. standard length, net fish weight, relative condition, infection level and parasite index) were correlated to the mean weight of its worms. For this analysis we used all the fish we sacrificed for this and a related study (i.e. including the singly infected fish, $n = 79$).

Statistical analysis

To assess the relationships between worm volume (x) and the different reproductive measures (y) we used an allometric model ($y = ax^b$, Klinkhamer *et al.*, 1990). An

ANCOVA on log-log-transformed data ($\log y = a + b \log x$) allows to estimate the regression coefficient b (i.e. the exponent of the untransfomed relationship). It here represents a measure for the rate of exponential increase ($b > 1$) or decrease ($b < 1$) in allocation to a given structure with increasing worm size. We graphically checked if data fulfilled the assumptions of parametric test statistics. If no suitable transformation could be found we used nonparametric test statistics. Data were analysed with JMPIN 3.2.1 (Sall & Lehman, 1996) and SYSTAT 5.2.1 (Systat, 1992).

Results

Experiment 1

None of the reproductive volumes were significantly affected by the social situation we placed the worms into (Table 2), suggesting that there was no strong phenotypically plastic alteration in allocation patterns in response to the experimental factor. All reproductive volumes increased with increasing worm volume, as shown by the significant positive effects of the covariate (Table 2). In addition, worms allocated disproportionately more to the female function with increasing size, as yolk gland volume was increasing significantly more than proportionally with worm volume ($b = 1.25$, Table 2). A similar pattern was found for ovary volume, but it was not statistically significant ($b = 1.22$, Table 2). Larger worms also allocated disproportionately less to male function, in that testis volume increased significantly less that proportionally with worm volume ($b = 0.80$, Table 2).

There was also no significant effect of the social situation on the combined measure of sex allocation (Table 2, Fig. 2a, see below for details on the statistical power of this test). However, the measure of sex allocation varied about four-fold with worm volume. Larger worms were more strongly biased towards the female function, as the measure of sex allocation

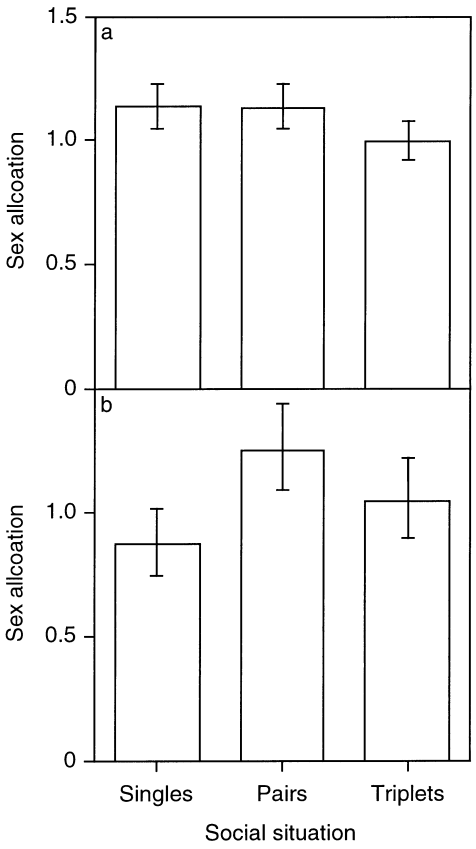

Fig. 2 Sex allocation of *S. solidus* as a function of the social situation. Sex allocation is expressed as the ratio of female volume (yolk gland volume + ovary volume) by male volume (testis volume). Least squares means (±1 SE) of the ANCOVA on log-transformed data. For greater clarity, data were transformed back to the original scale for graphing, leading to asymmetrical standard errors. (a) Worms removed from the *in vitro* system after 24 h. See Table 2 for statistics. (b) Worms removed from the *in vitro* system after 72 h. See Table 3 for statistics.

Table 2 One-way ANCOVAS for reproductive measures in *S. solidus* after 24 h in the *in vitro* system (Experiment 1). Sex allocation is expressed as the ratio of female volume (yolk gland volume + ovary volume) by male volume (testis volume). Data were log-log transformed for analysis (see Materials and methods). For each reproductive measure (dependent variable) we give the proportion of explained variance by the full model (r^2), and the F-test and probability for the effect of the factor. We further give the slope of the regression b and the F-tests and probabilities for (a) the null hypothesis that there is no relationship between the covariate and the dependent variable (H_0: $b = 0$), and (A) for the null hypothesis that the relationship between covariate and the reproductive parameter is linearly increasing function (H_0: $b = 1$).

				Covariate: worm volume				
		Factor: Social situation			Test (H_0: $b = 0$)		Test (H_0: $b = 1$)	
Dependent variable Reproductive measure	Full model r^2	$F_{2,14}$	P	Slope b^1	$F_{1,14}$	P	$F_{1,14}$	P
Yolk gland volume	0.95	0.87	0.44	1.25	233.6	<0.001	9.5	0.008
Ovary volume	0.89	1.06	0.37	1.22	111.2	<0.001	3.6	0.080
Testis volume	0.96	0.41	0.67	0.80	283.3	<0.001	17.7	0.001
Sex allocation	0.64	0.92	0.42	0.45	22.3	<0.001	33.5	<0.001

significantly increased with increasing worm volume (Table 2). Finally, this increase was not linear, but got weaker with increasing worm size, as the slope was significantly smaller than 1 ($b = 0.45$, Table 2). All findings regarding size-dependence are in close agreement with an earlier study of our group (Schärer *et al.*, 2001), and confirm that sex allocation is strongly size-dependent in our study species.

Experiment 2

In this experiment we found an overall effect of social situation on the amount of vesicular sperm stored by worms (Table 3, Fig. 3), with singles storing most, and worms in triplets storing least vesicular sperm, suggesting that more sperm had been used in larger mating groups. In a *post hoc* test, vesicular sperm volume was significantly higher in singles than in pairs or triplets, but the difference between pairs and triplets was not significant (Scheffé's test: singles vs. pairs, $P = 0.035$; singles vs. triplets, $P = 0.008$; pairs vs. triplets, $P = 0.55$). Vesicular sperm volume was not significantly affected by bottle size or its interaction with social situation, indicating that the amount of culture medium provided to the worms does not explain this result.

The pattern observed for vesicular sperm is in good agreement with our findings regarding received sperm. Here worms in the larger mating groups stored more received sperm than the singles. Because there were three null values in the received sperm volume of single worms, we could not use the above parametric approach for an overall analysis. We therefore performed two analyses. We (a) excluded the singles, and examined the difference in this parameter between pairs and triplets alone (statistics reported in Table 3). This analysis revealed a significantly higher received sperm volume in triplets than in pairs (Fig. 4a). To include information about singles, we (b) calculated a nonparametric two-way ANOVA (Sokal & Rohlf, 1995, p. 446) on the data corrected for worm volume (i.e. received sperm volume/worm volume). This analysis revealed a significant overall effect of social situation in the same direction as observed in the former analysis ($\chi^2_2 = 13.3$, $P = 0.0013$, Fig. 4b), with singles storing least and worm in triplets

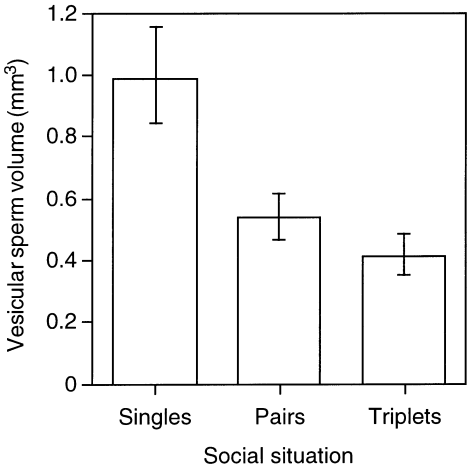

Fig. 3 Amount of vesicular sperm stored by *S. solidus* after 72 h in the *in vitro* system as a function of the social situation. Least squares means (±1 SE) of the two-way ANCOVA on log-transformed data. For greater clarity data were transformed back to the original scale for graphing, leading to asymmetrical standard errors. See Table 3 for statistics.

Table 3 Two-way ANCOVAS for reproductive measures in *S. solidus* after 72 h in the *in vitro* system (Experiment 2). Sex allocation is expressed as the ratio of female volume (yolk gland volume + ovary volume) by male volume (testis volume). Data were log-log transformed for analysis (see Materials and methods). For each reproductive measure (dependent variable) we give the proportion of explained variance by the full model (r^2), and the F-tests and probabilities for the effects of the factors and their interaction. We further give the slope of the regression b and the F-tests and probabilities for (a) the null hypothesis that there is no relationship between the covariate and the dependent variable (H_0: $b = 0$), and (b) for the null hypothesis that the relationship between covariate and the reproductive parameter is linearly increasing function (H_0: $b = 1$). The analysis for received sperm volume only includes pairs and triplets because of singles having null values that cannot be analysed parametrically (see Results for a nonparametric approach). This reduces the degrees of freedom for this analysis (i.e. $\text{d.f.}_{\text{num}} = 1$ and $\text{d.f.}_{\text{den}} = 6$ for all tests).

		Factor 1: Social situation		Factor 2: Bottle size		Interaction		Covariate: worm volume				
									Test (H_0: $b = 0$)		Test (H_0: $b = 1$)	
Dependent variable / Reproductive measure	Full model r^2	$F_{2,9}$	P	$F_{1,9}$	P	$F_{2,9}$	P	Slope b	$F_{1,9}$	P	$F_{1,9}$	P
Yolk gland volume	0.85	0.29	0.76	0.25	0.63	0.57	0.58	1.04	36.9	<0.001	0.04	0.84
Ovary volume	0.88	0.38	0.69	1.90	0.20	0.40	0.68	1.18	47.7	<0.001	1.14	0.31
Testis volume	0.88	2.21	0.17	0.24	0.63	3.4	0.08	0.82	55.1	<0.001	2.66	0.14
Uterus volume	0.71	0.25	0.78	1.34	0.28	0.55	0.59	1.00	19.4	0.002	<0.001	0.99
Vesicular sperm volume	0.81	8.09	0.01	0.87	0.38	0.73	0.51	0.90	19.9	0.002	0.23	0.64
Received sperm volume	0.86	9.00	0.024	0.16	0.70	2.35	0.18	1.49	10.5	0.018	1.13	0.33
Sex allocation	0.55	1.62	0.25	0.57	0.47	2.14	0.17	0.22	1.32	0.28	–	–

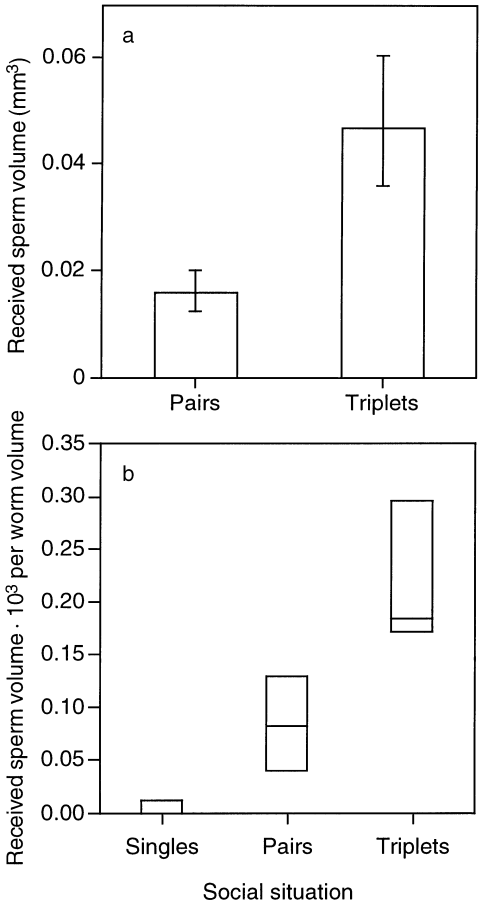

Fig. 4 Amount of received sperm stored by *S. solidus* after 72 h in the *in vitro* system as a function of the social situation. (a) Parametric approach (singles excluded from analysis): least squares means (±1 SE) of the two-way ANCOVA on log-transformed data. For greater clarity, data were transformed back to the original scale for graphing, leading to asymmetrical standard errors. See Table 3 for statistics. (b) Nonparametric two-way ANOVA approach: Medians and lower and upper quartiles of received sperm volume per worm volume (the median in singles is zero).

storing most received sperm. No effect of bottle size ($\chi^2_2 = 0.008$, $P = 0.93$) nor a significant interaction ($\chi^2_2 = 0.48$, $P = 0.79$) could be found, again suggesting that the amount of culture medium did not have a strong influence on the observed patterns. The same approach revealed significant differences between all pairs of possible comparisons of the social situation at a Bonferroni adjusted error probability (three comparisons, $\alpha/3 = 0.0166$, singles vs. pairs, $\chi^2_1 = 7.33$; singles vs. triplets, $\chi^2_1 = 84.2$; pairs vs. triplets, $\chi^2_1 = 7.93$; all $P < 0.01$).

None of the other reproductive volumes were significantly affected by the experimental factors (Table 3), suggesting no strong influence of the social situation or the amount of culture medium on these parameters. There was also no significant effect of the social situation on the combined measure of sex allocation (Table 3, Fig. 2b). However, all reproductive volumes still strongly increased with increasing worm volume (Table 3, see effect of the covariate), but none of the patterns of nonlinear allocation to reproductive structures we had observed in Experiment 1 remained significant after 72 h in the *in vitro* system. Similarly, the combined measure of sex allocation was not significantly increasing with size anymore (Table 3). The loss of the nonlinear relationships is probably because of the fact that the reproductive structures are being used up by reproduction and that this weakens the relationships. An earlier study showed that around half of all eggs had been released by that time (Schärer & Wedekind, 1999). Because of the large variance in the different measures in Experiment 2 we restrict the following analyses to the allocation patterns observed in Experiment 1.

Power analysis and directional hypothesis testing

Under both scenarios outlined in Table 1 a large effect on sex allocation is predicted, either between singles and pairs vs. triplets in the absence of selfing in larger groups, or between singles vs. pairs and triplets in its presence (note that the data appear more consistent with the first scenario, Fig. 2a). Given the expectation of a large effect (i.e. of a standardized effect size of $f = 0.4$ following the convention of Cohen, 1992 for ANOVA) we can compare the observed and predicted raw effect sizes given the observed error variance. The observed raw effect size in the comparison of sex allocation after 24 h was 0.027, which, given the observed error variance, corresponds to a standardized effect size of 0.32 and a power of 0.18. This is fairly close to the expected raw effect size of 0.034 which would have corresponded to a large standardized effect size *sensu* Cohen (1992), and which would have led to a power of 0.26. This analysis suggests that an effect of social situation on sex allocation may be present in our data, but that we failed to detect it with our 18 replicates. Given the observed error variance and effect size, if there were an effect, the number of replicates required for a statistically significant difference would have been 62.

An alternative to increasing the sample size is to make an analysis that takes advantage of the fact that we have a clear prediction about the direction of the expected effect (namely that the group means should be ordered as $\text{mean}_{\text{singles}} \geq \text{mean}_{\text{pairs}} \geq \text{mean}_{\text{triplets}}$ with one inequality strict). This can be achieved by combining an ordered heterogeneity test (Rice & Gaines, 1994a), which includes information about how well the data meet the expected ordering, and a directed test (Rice & Gaines, 1994b), which includes information about whether the observed effect goes in the expected direction. Because of the fact that the ordering of the means is as expected and that the effect goes in the expected direction (Table 1,

Fig. 2a), such an analysis suggests a nonsignificant trend for sex allocation to be affected by social situation ($r_sP_c = 0.58$, $P_{dir} = 0.088$).

Correlations between worm volume and donor fish parameters

All the correlations between the parameters describing the donor fishes and the mean weight of the worms of those fishes were significant (Spearman r_s, $n = 79$, standard length, $r_s = 0.57$, $P < 0.001$, net fish weight, $r_s = 0.64$, $P < 0.001$, relative condition, $r_s = 0.25$, $P < 0.03$, infection level, $r_s = -0.75$, $P < 0.001$, parasite index, $r_s = -0.50$, $P < 0.001$).

In Fig. 5a we illustrate the correlation between infection level and worm size of worms measured in Experiment 1. In Fig. 5b we further show how the correlation between worm size and sex allocation leads to sex allocation being correlated to infection level. Note that for worms that had been kept in groups in the *in vitro* system it was not possible to assign the worm we analysed stereologically to its original donor fish. We therefore report the average infection level of the donor fishes contributing to a group, which at least partly explains why the effects appear weaker in the larger groups.

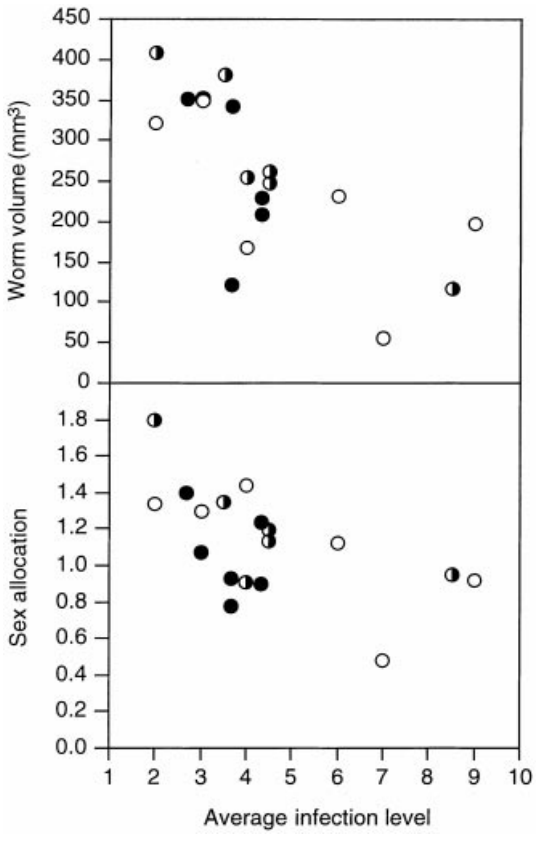

Fig. 5 Relationships between the mean infection level of donor fishes and (a) worm volume, or (b) sex allocation of the worms measured in Experiment 1. Sex allocation is expressed as the ratio of female volume (yolk gland volume + ovary volume) by male volume (testis volume). Singles (open circles), pairs (half circles) and triplets (closed circles).

Discussion

Sperm storage

When placed alone during reproduction, individual *S. solidus* store more vesicular sperm (i.e. produced sperm that can be used in future copulation) than when placed in groups of two or three individuals. Further worms that were allowed to reproduce in groups of three individuals store more received sperm (i.e. sperm that was received through copulation) than worms that were kept alone or in groups of two individuals.

The observed patterns in both vesicular and received sperm strongly suggest that worms in pairs and triplets do not exclusively self-fertilize. Further, we observed very low levels of received sperm volume in singles, which may either suggest that little sperm is required when mating alone, or that self-fertilization is constrained in the *in vitro* system. As a consequence, we will not attempt to interpret the observed differences between singles and worms in pairs and triplets. The rest of the discussion regarding sperm storage focuses on the larger mating groups only, and assumes that worms do not self- and cross-fertilize simultaneously in these groups. As we are interested in the occurrence of sperm competition, this assumption, although it may be wrong, is conservative because even low levels of self-fertilization in groups will automatically lead to sperm competition between self-sperm and foreign received sperm.

If worms in pairs and triplets mated at a fixed overall rate and transferred a fixed amount of sperm per mating, we would expect that all worms would store equal amounts of received sperm (i.e. worms in pairs would receive all their partner's mating effort, whereas each worm in triplets would each receive half of the mating effort of their two partners). The difference in received sperm we observed between worms in pairs and triplets therefore requires that worms in triplets increase their mating effort (either by mating more often, and/or by transferring more sperm per copulation). It further appears unlikely that the observed difference is an effect of increased mating rate caused by a higher encounter probability between worms in larger groups, because the nylon nets into which we placed the worms are small in comparison with the size of the worms, and worms spend much time in direct physical contact. Direct observation of reproductive behaviour was unfortunately not possible in the *in vitro* system we used. It is difficult to measure mating rates because the site of copulation is concealed by the worms being in close contact.

To conclude, our observation suggests a behavioural response to the different social situations, probably as a response to an increased risk of sperm competition in the larger mating groups. To our knowledge, this is the first study that provides evidence for a behavioural response to an increased risk of sperm competition in a tapeworm.

Sex allocation in the final host

We found no strong evidence that the social situation caused a phenotypically plastic response that altered the allocation patterns in the direction predicted by sex allocation theory (Table 1). This is despite the fact that sperm competition appears to be taking place in larger mating groups, indicating that our experiment successfully manipulated mating group size. However, both the power analysis and the directional hypothesis test suggest that we could have failed to find an effect because of our relatively low sample size.

In contrast, our results after 24 h in the *in vitro* system are in close agreement with the results of an earlier study on size-dependent sex allocation in plerocercoids where worms exhibited a roughly four-fold variation of sex allocation with worm size (Schärer *et al*., 2001). In the present study, we found the same patterns of size-dependence in allocation to yolk glands, ovaries and testes, leading to a strongly positive relationship between worm size and sex allocation. After 72 h in the *in vitro* system, the influence of worm volume on the volume of reproductive structures remained strong, but was clearly weaker than after 24 h. Further, the relationships were no longer significantly nonlinear.

Given the observed size-dependent sex allocation, it appears interesting to examine the relative contribution of social situation and individual size to variation in sex allocation. If we assume that the observed variation in sex allocation as a result of social situation is in the right order of magnitude, it is relatively small in comparison with large variation with individual size. As worm size is determined during growth in the second intermediate host, this suggests that sex allocation is largely determined at that stage, and that worms may only partly be able to modify this predetermined allocation pattern according to the social situation encountered in the final host.

Sex allocation in the second intermediate host

One reason why sex allocation patterns appear to be strongly determined in the second intermediate host, i.e. the fish, may be that the very short reproductive period in the final host constrains the magnitude of a plastic response that is possible there. As already mentioned, 70–80% of the eggs are laid within the first 5 days (Schärer & Wedekind, 1999), and worms die shortly after this. It hence seems that worms are under a strong time-constraint, and that they, during reproduction, just 'use' the reproductive structures they have built before.

The conditions encountered during growth in the fish could provide reliable information on the optimal sex allocation and the long growth-period gives ample opportunity to fine-tune allocation. We have previously discussed the factors that could select for size-dependent sex allocation in *S. solidus* (Schärer *et al*., 2001). We here briefly discuss an issue that is relevant to the new evidence of sperm competition.

The analysis of the correlations between the various parameters describing the donor fishes and the mean worm weight of these fishes suggests that the infection level is a very important correlate to worm weight, and worm size has been shown to be strongly correlated to sex allocation (both this study and Schärer *et al*., 2001), which in turn appears to lead to a correlation between infection level and sex allocation. Could the size that a worm attains in the intermediate host be a predictor of the social situation, and hence the mating group size and the level of local mate competition that a worm will experience in the final host? This appears plausible for two reasons. First, the infection level in the fish may, because of the generally high transmission rates from the fish to the final host (72% of the worms larger than 50 mg successfully establish themselves in the host, Tierney & Crompton, 1992), approximately translate to the mating group size in the final host. If this were the case then one would expect that, because of some losses during transmission, sex allocation would be close to optimal for mating group sizes which are somewhat smaller than the group sizes experienced in the fish. Secondly, the infection level within a fish may to some extent indicate the parasite prevalence in the local fish population, and hence the likelihood that the final host ingests several infected fish (which should lead to a higher mating group size than experienced in the fish). This is because infection levels and prevalence can vary dramatically from year to year (e.g. Arme & Owen, 1967; Pennycuick, 1971), and because they are expected to covary (e.g. Guyatt *et al*., 1990; Anderson & May, 1992), with fish being more heavily infected when many fish are infected. The predicted relationship between infection level and prevalence (a negative binomial probability distribution) is approximately met in the field data on infection levels and prevalence in *S. solidus* of Arme & Owen (1967), and the correlation between the parameters is suggestive ($n = 7$, Spearman $r_s = 0.68$, $P = 0.094$). If this relationship is sufficiently strong in nature, worms in fish with high infection level may expect that prevalence is high and that they are likely to be joined by worms from other fish ingested by the final host. This would lead to a higher mating group size than the group size experienced in the fish, and worms would be expected to be more male-biased than what would be optimal for the given group size in the fish.

An interesting prediction we can derive from this kind of reasoning is that because of the higher mating group sizes, worms would experience in the following scenarios the average sex allocation in a local population should be more male-biased if (a) the parasite prevalence in the fish is high, or has been high over several years, and/or (b) if the primary final host species is a large bird (e.g. heron or cormorant) which eats many sticklebacks per unit time, rather than a small bird (such as a kingfisher) which eats few.

Other evidence for plastic sex allocation

We are aware of only one published study that experimentally manipulated the social situation during reproduction in a simultaneous hermaphrodite with the aim of testing the effect of mating group size on sex allocation (Trouvé *et al*., 1999). They found a strong shift in sex allocation in the predicted direction in the parasitic trematode *Echinostoma caproni*. They experimentally infected mice, a possible final host of *E. caproni*, with a defined number of metacercariae. These metacercariae then grew to become adult worms, which were sampled after 24 days and analysed for sex allocation. They found that worms in larger groups allocated relatively more to the male function. In addition to this, they also found a strong effect of individual size, with smaller individuals being more biased towards male function than larger individuals. The main difference between their system and ours is that *E. caproni* grows in the final host, whereas *S. solidus* does not. *Echinostoma caproni* is hence constantly exposed to a reliable stimulus regarding the expected mating group size during the time of formation of their reproductive structures. Further, *E. caproni* can reproduce over an extended period, and may hence be able to further adjust the sex allocation to changing conditions.

Conclusions

We provide evidence for a plastic response in mating behaviour under different risks of sperm competition in a tapeworm. Our evidence suggests that tapeworms can assess their environment, and modify their reproductive behaviour as a function of the social situation they experience in the final host. We did not, however, find support for the notion that worms strongly adjust their sex allocation at that stage. This finding is in contrast to another published study in a simultaneous hermaphrodite parasite (Trouvé *et al*., 1999), but the discrepancy between their and our results could potentially be explained by the differences in the life-histories of the two study species, with most allocation decisions in *S. solidus* happening during growth in the second intermediate host.

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