The consequences of self-fertilization and outcrossing of the cestode *Schistocephalus solidus* in its second intermediate host

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SUMMARY

Many hermaphroditic parasites reproduce by both cross-fertilization and self-fertilization. To understand the maintenance of such mixed mating systems it is necessary to compare the fitness consequences of the two reproductive modes. This has, however, almost never been done in the context of host–parasite coevolution. Here we show the consequences of outcrossing and selfing in an advanced life-stage of the cestode *Schistocephalus solidus*, i.e. in its second intermediate host, the three-spined stickleback (*Gasterosteus aculeatus*). Each juvenile stickleback was simultaneously exposed to 2 experimentally infected copepods, one harbouring outcrossed the other selfed parasites. At 60 days p.i. parasites were removed from the fish's body cavity and, with microsatellite markers, assigned to either outcrossed or selfed origin. Prevalence was not significantly higher in outcrossed parasites. However, those fish that were infected contained significantly more outcrossed than selfed parasites. Thus the probability of a selfed parasite to progress in the life-cycle is reduced in the second intermediate host. Furthermore, we found that even the multiply infected fish increased in weight during the experiment. Nevertheless, total worm weight in multiply infected fish was significantly lower than in singly infected ones, which thus might be a parasite life-history strategy.

Key words: outcrossing, selfing, inbreeding depression, host–parasite coevolution, cestode.

INTRODUCTION

Studying the benefits of outcrossing (cross-fertilization) as compared to selfing (self-fertilization) is of major importance for understanding the evolution of mixed mating systems (Thornhill, 1993). The consequences of the 2 reproductive modes for individual fitness in terms of life-time reproductive success are often difficult to obtain. Many hermaphrodite tapeworms can reproduce by outcrossing as well as by selfing (Ghiselin, 1969; Storch & Welsch, 1991), and self-fertilize to some extent even if a mating partner is available (Lüscher & Milinski, manuscript submitted) thus adopting a mixed mating strategy. Mixed mating systems are found also in other organisms, for example, in ascidians and snails (Bishop & Ryland, 1993; Jarne & Charlesworth, 1993). Selfing is the most extreme case of inbreeding, as it comprises the fusion of gametes which have been produced by the same individual, i.e. a hermaphrodite plant or animal. The relatedness between selfed offspring and their parents is greater than that between outcrossed ones and their parents, which implies a transmission cost of outcrossing (Charlesworth, 1980; Charlesworth & Charlesworth, 1998; Lively & Lloyd, 1990). This cost of outcrossing as compared to selfing is similar to the 2-fold cost of obligately sexual females as compared to obligately parthenogenetic ones (Fisher, 1941; Maynard Smith, 1971, 1978). However, outcrossing can be more favorable due to the detrimental consequences of selfing, i.e. due to inbreeding depression, which can be severe especially in individuals that are outbred (Charlesworth & Charlesworth, 1987; Lande & Schemske, 1985; Wright, 1977). Inbreeding depression can be caused by (1) rare deleterious alleles being more frequently expressed in the homozygous condition (dominance hypothesis), (2) the relative worse performance of homozygotes relative to heterozygotes (overdominance hypothesis) or, as has been discussed by Thornhill (1993), (3) a combination of both causes (Wright, 1977). Furthermore, outcrossing produces genetically more heterogenous offspring than does selfing, which may be especially important in temporally fluctuating biotic environments such as in host–parasite interactions (Hamilton, 1980; Jaenike, 1978; Williams, 1975). The fitness consequences of the two reproductive modes, i.e. outcrossing and selfing, have only rarely been investigated in the context of host–parasite coevolution, especially from the parasite's point of view (Christen, Kurtz & Milinski, 2002; Gemmill, Viney & Read, 1997; Green, Kraaijeveld & Godfray, 2000; Kaltz & Shykoff, 1999; Wedekind & Ruetschi, 2000). Increased genetic variation can be beneficial for the

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host as a defence against its parasites but also to the parasite to circumvent host defence strategies (Bell & Maynard Smith, 1987). It has been shown that selfed *Plasmodium* have lower infection rates in the mosquito vector than outcrossed ones in laboratory crosses (Walker-Jonah *et al*. 1992; Walliker *et al*. 1987).

Our study species, the tapeworm parasite *Schistocephalus solidus*, is a simultaneous hermaphrodite which can reproduce either by outcrossing or by selfing in the final host's, i.e. any fish eating bird's, gut. The parasite has a complex life-cycle involving a cyclopoid copepod (first intermediate host) and the three-spined stickleback (second intermediate host). In an earlier study (Christen *et al*. 2002) we have shown that *S. solidus* can benefit from outcrossing as compared to selfing in terms of higher hatching rate (Christen *et al*. 2002; Wedekind, Strahm & Schärer, 1998) higher infection success and larger size in its first intermediate host (Christen *et al*. 2002). If those differences between outcrossed and selfed parasites persisted throughout the life-cycle, i.e. in the second intermediate host and up to the final host, selfed parasites would have a lower probability to reach the final host than outcrossed ones. However, it has been pointed out that inbreeding depression can be concentrated at late life-stages (Carr & Dudash, 1995; Charlesworth, Lyons & Litchfield, 1994; Meagher, Penn & Potts, 2000; Thornhill, 1993) and in the case of a parasite with the complex life-cycle might be underestimated if not all, and especially, the late life-stages are taken into account. We therefore tested in the present study whether additional differences between outcrossed and selfed parasites would arise especially in the second intermediate host. The transmission of the parasite from the copepod to the fish might be a critical stage to overcome (Smyth & McManus, 1989) and outcrossed parasites may be better in becoming established in the fish host. Furthermore, larval development and growth in the second intermediate host is important because over 98% of the parasite's final weight is attained in the fish (calculated from Orr & Hopkins (1969) and from own observations) and as larger parasites have a higher reproductive success in the final host (Lüscher & Milinski, manuscript submitted; Lüscher & Wedekind, 2002; Schärer *et al*. 2001; Wedekind *et al*. 1998). In addition, final parasite size, i.e. reproductive size, is reached already in the fish. We exposed laboratory reared fish singly to 2 experimentally infected copepods simultaneously, one harbouring outcrossed parasites and the other the same number of selfed parasites. Selfed and outcrossed parasites were thus in a competitive situation in the fish. At 60 days post-infection we dissected the fish and defined prevalence, intensity of infection and weight of outcrossed and selfed parasites. Microsatellite markers were used to distinguish between outcrossed and selfed parasites.

MATERIALS AND METHODS

Sampling and culturing of tapeworm parents

We caught three-spined sticklebacks, *Gasterosteus aculeatus*, the second intermediate host of the hermaphrodite tapeworm *Schistocephalus solidus*, from a large population in the brackish bay 'Binnenwasser' in Neustadt, Northern Germany, in September 2001. The fish were housed at 16 °C, under a 16:8 light:dark cycle in big tanks (200 l) and were fed with a mixture of frozen food (chironomid and artemia larvae, daphnia and copepods). After 2 months, sticklebacks were dissected and checked for plerocercoids (3rd larval stage of *Schistocephalus solidus*) in their body cavity. Plerocercoids were removed aseptically from the fish (Orr & Hopkins, 1969; Smyth, 1946) and weighed to the nearest mg before placing them into an *in vitro* system for reproduction for a period of 5 days. The *in vitro* system was designed to replace the final host's (fish-eating bird's) gut and consisted of nylon mesh bags (200 μm mesh size) which contained the worms and which were suspended in 0·5 l culture bottles filled with a sterile medium. The tapeworm eggs pass through the mesh and assemble at the bottom of the bottle (for details on the *in vitro* system see Schärer & Wedekind (1999) and Wedekind *et al*. (1998)). The culture bottles must be kept at 40 °C (equal to a bird's body temperature) as temperature is an important trigger for parasite reproduction (Smyth, 1946). We used worms only from singly infected sticklebacks for the experiment and placed them into the culture bottles either alone ($n=9$) or in weight-matched pairs ($n=9$). Single worms could thus reproduce only alone whereas pairs of worms were allowed to outcross. Lüscher & Milinski (manuscript submitted) found that outcrossing does indeed occur when pairs of worms are given the opportunity to outcross and outcrossing rate was especially high in weight-matched worm pairs with the following weights per worm: 222 mg (100%), 294 mg (98%) and 262 mg (96%). We used a similar range of worm weight in this study (see Table 1). However, even if the worms did not fully outcross, this would have a conservative effect on our results as we would not find any difference between outcrossing and selfing if outcrossing rate were zero.

Outcrossed and selfed tapeworm offspring

We collected the tapeworm eggs from the culture bottles, rinsed them and placed them into a refrigerator at 4 °C in the dark. The worms (parents) that produced the clutches were stored in 70% alcohol for microsatellite analysis (see below). At 20 days before hatching a subsample of each clutch was transferred to 20 °C for the start of development. The day before hatching, the eggs were exposed to light to induce hatching (Dubinina, 1966).

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Table 1. Experimental protocol with weight-matched worm clutch combinations, i.e. outcrossed and selfed worm clutches that were produced by weight-matched selfing and outcrossing parent worms

(The last column gives the number of fish which were exposed to worm larvae originating from each worm clutch combination. Fish which were excluded from the statistical analysis (see Materials and Methods section) are not included in this table.)

Worm clutch combinations	Selfed clutches	Outcrossed clutches	Selfing parent worm weight (g)	Outcrossing parent worm 1 weight (g)	Outcrossing parent worm 2 weight (g)	Exposed fish (*n*)
1	1a	10b	0·340	0·344	0·346	3
2	2a	11b	0·223	0·224	0·227	6
3	3a	12b	0·310	0·328	0·331	11
4	4a	13b	0·207	0·208	0·209	2
5	5a	14b	0·257	0·212	0·214	10
6	6a	15b	0·325	0·295	0·302	10
7	7a	16b	0·245	0·230	0·234	11
8	8a	17b	0·285	0·286	0·288	9
9	9a	18b	0·311	0·271	0·273	8

To control for differences in egg size between outcrossed and selfed eggs we measured egg size from the remaining subsample of each clutch of eggs using a CASY® Cell Counter (Schärfe) (for details see Christen *et al*. (2002) or Lüscher & Milinski (manuscript submitted)).

Infection of the first intermediate host: copepods

Before sticklebacks (second intermediate host) could be exposed to infected copepods (first intermediate host) we had to expose copepods to the 1st larval stage (coracidia) of the parasite. Male copepods of equal age were filtered from the *Macrocyclops albidus* cultures (see Christen *et al*. (2003)) 1 day before exposure, and were transferred singly into individual wells (about 2 ml) of 24-well ELISA plates. We exposed copepods randomly to either 6 selfed or 6 outcrossed parasite larvae originating from worm parents which had been matched for weight not only between the two worms within a pair but also between the single worm and the pair of worms (see Table 1). Copepods were kept at 20 °C and 16:8 light:dark cycle and fed every second day with 3 *Artemia salina* starting 1 day after exposure and continuing until the day before parasite screening. At 12 days p.i. we placed each copepod carefully with a pipette in a drop of water on a microscope slide and screened it from both sides for the number of parasites (procercoids) at 100× magnification with a brightfield microscope. The person screening the copepods was blind with respect to the treatment, i.e. selfing or outcrossing respectively, and counted the number of procercoids per copepod. At the end of each screening day pairs of copepods harbouring the same number (1, 2, 3 or 4) of either outcrossed or selfed parasites originating from the weight-matched worm parents (as described above) were established. They were match-labelled on their home wells for simultaneous exposure to sticklebacks on the following day. We did not measure parasite size in this study for the following reasons: in an earlier experiment (Christen *et al*. 2002) we had shown that neither infection rate nor mean worm size or intensity of infection differed between outcrossed and selfed parasites in a non-competitive situation i.e. where copepods had been infected with either only selfed or only outcrossed parasites, as in the present study. Furthermore, copepod condition might have been affected by the relatively long *in vivo* measurement procedure.

Infection of the second intermediate host: three-spined sticklebacks

Three-spined sticklebacks, *G. aculeatus* were bred in the laboratory from adults that we had caught from the Vierersee, Northern Germany. Offspring from 6 pairs were raised under standardized conditions (Bakker, 1986). At the age of 2 months (27–32 days before infection), 84 fish were placed singly in individual home tanks (21 × 35 × 25 cm) as follows: 13–15 fish were taken randomly from each sibship and were housed each in an individual tank. The tanks were distributed evenly across the shelfs in the aquaria room (18 °C and 16:8 light:dark cycle). Each fish was fed every second day on a fixed ration of dissolved frozen food, i.e. 1 pipette (vol. 5 ml) from a well stirred solution of 100 g frozen cyclops or Daphnia per litre.

One day before exposure to infected copepods fish were transferred into small tanks (2 l). On the day of exposure, each fish ($n=84$) was given 2 copepods each harbouring the same number (1, 2, 3 or 4) of outcrossed and selfed procercoids (see above). All the fish that were exposed to copepods harbouring parasites from worm parents which had been matched for weight will be referred to as belonging to the same *worm clutch combination* (see Table 1). Worm clutch combinations were composed of fish from different sibships such that parasite origin was randomized across fish sibships. To increase the probability of copepod consumption and thus eventually parasite transmission we did not feed the fish during

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2 days before and 1 day after exposure to infected copepods. Two days after exposure fish were placed back into their home tanks and their small exposure tanks were filtered for detecting copepods that might not have been consumed. In each of only 2 tanks 1 dead copepod was found. None of the respective 2 fish was infected at the end of the experiment and therefore automatically excluded from the comparison between outcrossed and selfed parasites (otherwise, i.e. in all analyses containing exposed non-infected fish, those 2 fish were excluded). At 60 days p.i. fish were dissected and the body cavity was searched for plerocercoids under a binocular microscope. Each larva was weighed to the nearest 0·1 mg as soon as it was detected and removed from the fish. The plerocercoids (offspring) were stored in 70% alcohol for microsatellite analysis (see below). We measured fish wet weight and fish length (fork length) at the start of the experiment, i.e. at 13 days p.i. and at the end of the experiment, i.e. at 60 days p.i. before (in case of fish length) and after (in case of fish weight) the worms had been removed from the fish's body cavity. During the whole experiment 30 non-exposed control fish were treated in exactly the same way as all the exposed ($n=84$) ones.

Microsatellite analysis

To assign each plerocercoid (offspring) found in the fish to its origin, i.e. to single parent worms (selfing) or to pairs of parent worms (allowed to outcross), we used microsatellite markers (Binz *et al*. 2000) and followed a protocol which had been developed by Lüscher & Milinski (manuscript submitted). DNA extraction followed the Chelex© extraction method (Walsh, Metzger & Higuchi, 1991). A sample of about 1 mm^2 from each parent worm as well as of each offspring worm was mixed in 70 μl of 5% Chelex© suspension. Proteinase K solution was added and incubation carried out for 15 min at 55 °C and 100 °C. PCR reactions were set up with 1 μl of extract and 1 of 2 pairs of locus-specific fluorescent-labelled forward primer and non-labelled reverse primer (see Binz *et al*. (2000)). Fluorescent PCR fragments were separated and visualized on a ABI PRISM® 3100 Genetic Analyzer using the POP4™ Polymer (Applera Corporation). We used the following 2 marker loci: B6 (Genbank accession number AF 247829) and A22 (Genbank accession number AF 247830). We first tried to assign offspring to their origin using only 1 marker locus. This worked out in the case of offspring which shared alleles with only either the single parent (selfing) or the pair of parents (allowed to outcross). However, there were cases where offspring shared alleles with both types of parents and therefore could not be unambiguously assigned. In such cases we used the second primer. In each of 7 fish we could not assign all worm offspring with neither the first nor the second primer and therefore excluded those fish from our analysis (see below). Thus, worm offspring had to share at least 1 allele at 1 locus with 1 of the parent types and it had to be excluded that the same allele could originate from the other parent type as well.

Data analysis

All data were analysed using JMP Version 4.0.1. for Macintosh (© SAS, 1989–2000). We had to exclude 14 out of 84 fish from our analysis (a) in 7 fish it had not been possible to assign unambiguously all worms within the fish to their origin (i.e. outcrossed or selfed) with the two marker loci that we had used, (b) in each of 5 fish we had found more parasites than the fish had been exposed to, which was most probably due to mistakes when screening the copepods and (c) 2 fish, as already mentioned earlier, did not consume both copepods they had been exposed to.

RESULTS

Egg size

Outcrossed and selfed eggs did not significantly differ in size (paired t-test, $n=9$, $t=0{\cdot}001$, $P=0{\cdot}9996$, when taking worm clutch combinations matched for weight as the statistical unit, see Table 1; one-way Anova, $n_{\text{selfed}}=9$, $n_{\text{outcrossed}}=9$, $F=1*10^{-6}$, $P=0{\cdot}999$, when taking individual worm clutches as the statistical unit) as we have already shown in an earlier study (Christen *et al*. 2002).

Prevalence

We exposed each fish simultaneously to 2 copepods, one of which was infected with outcrossed parasite larvae while the other was infected with the same number of selfed larvae. In total, fish were exposed to either 2, 4, 6 or 8 larvae. We found that 40 out of 70 fish (57·14%) were infected with at least 1 worm. More than half of the infected fish (57·5%) harboured both outcrossed and selfed worms, whereas 15% had only selfed and 27·5% had only outcrossed worms.

Table 1 shows the experimental set up with the weight-matched worm clutch combinations. It can be seen, that always several fish (e.g. 3 fish in worm clutch combination 1, i.e. in the first row of the table) were exposed to parasites that had been derived from the same 2 clutches (e.g. from selfed clutch number 1a and from outcrossed clutch number 10b). The selfing parent worms (e.g. 0·340 g) had always been closely matched for weight to the outcrossing parent worms (e.g. worm 1 = 0·344 g, worm 2 = 0·346 g). The 9 weight-matched worm clutch combinations were considered as the statistical units but in addition we also give the statistics where individual fish were taken as the statistical unit to allow

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for comparison with a previous study (Christen *et al*. 2002). For each worm clutch combination, the prevalence (the proportion of fish infected) was calculated for outcrossed and selfed worms separately and the difference between those values was compared: outcrossed worms had a higher prevalence in 5 out of 9 and selfed worms in only 1 worm clutch combination (in 3 worm clutch combinations the difference was zero), however, this was not significant (Wilcoxon signed-ranks test, $T=5{\cdot}5$, $n=9$, $P=0{\cdot}313$; Fig. 1A; or when taking individual fish as the statistical unit, out of 40 infected fish 34 were infected with outcrossed and 29 with selfed worms: $\chi^2=0{\cdot}529$, D.F. $=1$, $n=40$, $P=0{\cdot}467$).

Intensity of infection

In 1 fish we found up to 6 plerocercoids and 13 fish had only 1 plerocercoid. The frequency of multiple infections was as follows: 2 worms ($n=8$), 3 worms ($n=9$), 4 worms ($n=6$), 5 worms ($n=3$). The number of outcrossed and selfed plerocercoids found per fish increased with increasing number of procercoids (larvae within copepods) that the fish had been exposed to (outcrossed worms: Spearman's $r_s=0{\cdot}469$, $n=40$, $P<0{\cdot}002$; selfed worms: $r_s=0{\cdot}435$, $n=40$, $P=0{\cdot}005$; all worms: $r_s=0{\cdot}580$, $n=40$, $P<0{\cdot}0001$). To compare the infection intensity of outcrossed and selfed worms the differences in the number of outcrossed and selfed worms per fish were averaged over the fish belonging to the same worm clutch combination. It was found that outcrossed worms had a significantly higher infection intensity than selfed ones (Wilcoxon signed-ranks test, $T=18$, $n=9$, $P=0{\cdot}008$; Fig. 1B) as 8 out of 9 worm clutch combinations had more outcrossed than selfed worms (in 1 worm clutch combination the difference was zero; when taking individual fish as the statistical unit: Wilcoxon signed-ranks test, $T=93$, $n=40$, $P=0{\cdot}039$).

Parasite growth

The smallest plerocercoids weighed only 0·2 mg and came from a multiply infected fish with 4 plerocercoids whereas the largest worm (113 mg) came from a singly infected fish. We compared the average and total weight of outcrossed and selfed worms by first calculating the difference between the two types of worms within each infected fish and then averaging the differences over the fish belonging to the same worm clutch combination. Although data indicate somewhat higher values in outcrossed than in selfed worms (the average weight as well as the total weight of outcrossed worms was larger in 7 out of 9 worm clutch combinations) there was no significant difference in either of the two parameters (average worm weight: Wilcoxon signed-ranks test, $T=10{\cdot}5$, $n=9$, $P=0{\cdot}250$; total worm weight: Wilcoxon signed-ranks test, $T=12{\cdot}5$, $n=9$, $P=0{\cdot}164$; Fig. 1C; when taking individual fish as the statistical unit: average worm weight: Wilcoxon signed-ranks test, $T=76$, $n=40$, $P=0{\cdot}313$; total worm weight: $T=57$, $n=40$, $P=0{\cdot}451$).

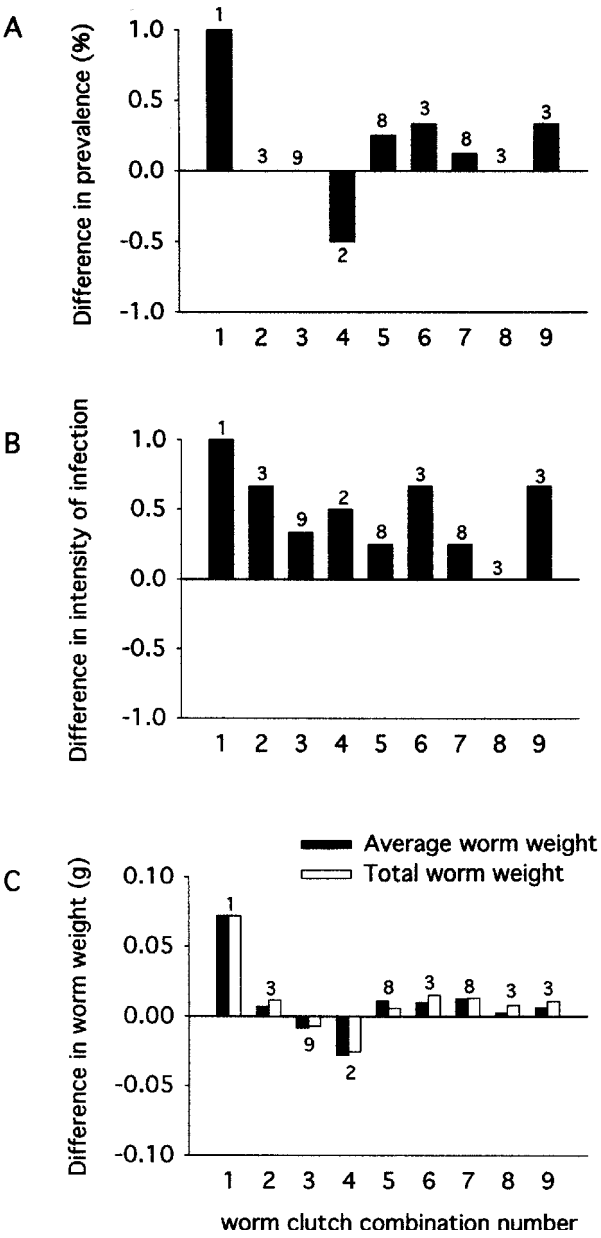

Fig. 1. The mean difference between outcrossed and selfed worms in (A) prevalence, (B) intensity of infection and (C) worm weight in the second intermediate host, the three-spined stickleback. The mean differences are shown for each of the 9 weight-matched worm clutch combinations (see Table 1). Values are positive when outcrossed worms were superior to selfed ones. The numbers above and below the bars show the number of replicates, i.e. the number of fish the calculations within the worm clutch combinations are based on.

Singly and multiply infected fish

Irrespective of whether fish harboured outcrossed or selfed parasites, the total worm weight of fish

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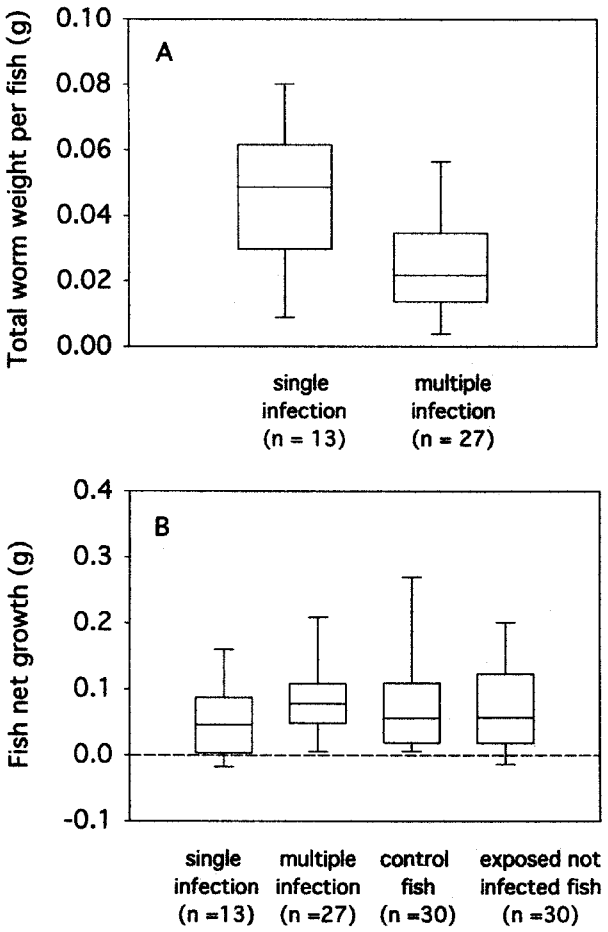

Fig. 2. Parasite weight and stickleback net growth. (A) Total worm weight in singly and multiply infected fish. Median and quantiles (10, 25, 75, 90%) are shown. (B) Fish net growth in singly infected, multiply infected, non-exposed control and exposed but not infected fish. Fish net growth was calculated as the difference between fish net weight at the end of the experiment after removal of the worms, i.e. 60 days p.i., and the fish weight at the start of the experiment, i.e. 13 days p.i. Median and quantiles (10, 25, 75, 90%) are shown.

harbouring only 1 worm (singly infected fish) was significantly higher than that in fish harbouring multiple worms (multiply infected fish) (Wilcoxon two-sample test, $Z=2{\cdot}123$, $n_{\text{single}}=13$, $n_{\text{multiple}}=27$, $P=0{\cdot}034$; Fig. 2A). In singly infected fish ($n=13$) 8 fish (61·54%) harboured outcrossed worms and in multiply infected fish ($n=27$) 26 fish (96·30%) were infected with outcrossed and 24 (88·90%) with selfed worms, 23 fish (85·19%) harboured both outcrossed as well as selfed worms.

Fish growth

Fish net growth (difference between fish net weight at the end of the experiment after removal of the worms, i.e. 60 days p.i., and the fish weight at the start of the experiment, i.e. 13 days p.i.) was calculated to see whether singly and multiply infected fish had grown differently. Interestingly, neither an overall difference in fish net growth between singly infected, multiply infected, non-exposed control and exposed but not infected fish was found (Kruskal-Wallis test, $\chi^2=2{\cdot}777$, $n_{\text{single}}=13$, $n_{\text{multiple}}=27$, $n_{\text{control}}=30$, $n_{\text{non-infected}}=30$, $P=0{\cdot}427$; Fig. 2B) nor a difference between singly and multiply infected fish (Wilcoxon two-sample test, $Z=-1{\cdot}502$, $n_{\text{single}}=13$, $n_{\text{multiple}}=27$, $P=0{\cdot}133$; Fig. 2B; Bonferoni correction was applied to correct for multiple testing, only $P<0{\cdot}05/2=0{\cdot}025$ would have been considered significant). However, all 4 types of fish increased significantly in weight from the start to the end of the experiment (Wilcoxon signed-ranks tests: control fish: $T=222{\cdot}5$, $n=30$, $P<0{\cdot}001$; exposed non-infected fish: $T=198{\cdot}5$, $n=30$, $P<0{\cdot}001$; single infected fish: $T=34{\cdot}5$, $n=13$, $P=0{\cdot}013$; multiple infected fish: $T=176$, $n=27$, $P<0{\cdot}001$; Bonferoni correction was applied to correct for multiple testing, only $P<0{\cdot}05/4=0{\cdot}0125$ is considered significant; see Fig. 2B).

Fish condition factor

To see whether fish condition might have influenced the outcome of infection and whether infection might have changed fish condition, we calculated the fish condition factor using the formula described in (Frischknecht, 1993) (100*fish weight in g/fish length$^{3\cdot102}$ in cm). The condition factor of singly and multiply infected fish was compared at the start (i.e. 13 days p.i.) and at the end (60 days p.i. using fish net weight after removal of the worms) of the experiment: we found a trend for the condition factor to be higher in singly than in multiply infected fish at the start of the experiment (one-way Anova, $F=3{\cdot}104$, D.F.$=1$, $n_{\text{single}}=13$, $n_{\text{multiple}}=27$, $P=0{\cdot}086$). At the end of the experiment the situation has reversed, i.e. singly infected fish had a significantly lower condition factor than multiply infected ones (one-way Anova, $F=4{\cdot}923$, D.F.$=1$, $n_{\text{single}}=13$, $n_{\text{multiple}}=26$, $P=0{\cdot}0327$; the length of 1 multiply infected fish had not been measured, therefore the sample size was 26 instead of 27). The condition factor in singly infected fish decreased significantly from the start to the end of the experiment (Wilcoxon signed-ranks test, $T=-45{\cdot}5$, $n=13$, $P<0{\cdot}001$) whereas there was only a trend for the condition factor to decrease in multiply infected fish (Wilcoxon signed-ranks test, $T=-59{\cdot}5$, $n=26$, $P=0{\cdot}133$).

DISCUSSION

In this study we show that the cestode *Schistocephalus solidus* benefits from outcrossing in an advanced stage of the life-cycle, i.e. in the second intermediate host, the three-spined stickleback. It was found that infection intensity of outcrossed parasites was significantly higher than that of selfed ones

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in the stickleback. As a consequence, the probability of outcrossed larvae reaching the final host and reproducing will be higher than that of selfed ones. Outcrossed worms tended to have a higher average and total weight per fish than selfed worms. Similarly, parasite prevalence was slightly but not significantly higher in outcrossed worms. The two types of parasite were in a competitive situation in the fish as each fish was exposed simultaneously to 2 copepods (first intermediate host), one of which had been infected with outcrossed procercoids (parasite larvae in the copepod) while the other had been infected with the same number of selfed procercoids. A competitive situation, i.e. a stressful one, reflects better the conditions met in the field, and it has been pointed out that, for example, inbreeding depression might remain undetected under too benign conditions (Bijlsma, Bundgaard & Van Putten, 1999; Meagher *et al*. 2000; Szafraniec, Borts & Korona, 2001). In contrast to a competitive situation, we did not find benefits of outcrossed parasites in a non-competitive situation in copepods, i.e. in the first intermediate host of *S. solidus*, in an earlier study (Christen *et al*. 2002).

In the advanced life-stage that we investigated in this study, inbreeding depression might have decreased the parasite's ability to evade the host's defences or to get established in the host. A procercoid has to overcome mechanical and immunological stages of host defence before reaching the fish's body cavity, similar to a coracidium (first larval stage of *S. solidus*) before reaching the copepod's haemocoel (Van der Veen & Kurtz, 2002) and selfed procercoids might more often have failed to do so. After the copepod has been ingested by the fish a critical stage for the procercoid might be to leave the copepod within the fish's gut and to pass through the fish's gut wall (Clarke, 1954). If, for example, the typically well-developed procercoid glands which produce secretions that serve to aid passage through the host tissues (Kuperman & Davydov, 1982; Smyth & McManus, 1989) have a reduced function as a consequence of inbreeding, this might have prevented more selfed than outcrossed parasites to reach the fish's body cavity.

Another reason for higher infection intensity of outcrossed parasites in our study might be the difference in the degree of genetic heterogeneity between outcrossed and selfed offspring. Outcrossed offspring are genetically more variable than selfed ones and the probability that allelic combinations exist which could evade the host's immune defence is higher in outcrossed than in selfed offspring. With this reasoning we would expect also a higher prevalence of outcrossed parasites. We found, however, only a slightly higher prevalence of outcrossed parasites. Thus, inbreeding depression might be the more likely cause of the difference in infection intensity between the two types of parasites. When controlling for inbreeding depression, Wedekind & Ruetschi (2000) showed that prevalence in copepods was higher when they were exposed to a mixture of outcrossed parasites (*S. solidus*) from 2 sibships (high heterogeneity) than when they were exposed to only 1 outcrossed sibship (low heterogeneity). However, in their study high and low heterogeneity exposures occurred in different host individuals (copepods) whereas in our study each fish host was exposed to both selfed (low heterogeneity) and outcrossed (high heterogeneity) parasites simultaneously.

Although we found that the differences in average and total worm weight between outcrossed and selfed parasites pointed to the expected direction (outcrossed worms are expected to be heavier than selfed ones) they fell short of statistical significance. In the first intermediate host of *S. solidus*, the copepod, the same 2 parameters were found to be significantly higher in outcrossed than in selfed parasites in a competitive situation, i.e. where individual copepods had been exposed to both outcrossed and selfed parasites simultaneously (Christen *et al*. 2002). For this case we suggested that unequal partitioning of limited resources between the two types of worms within the copepod had caused the difference in size. In contrast to the copepod intermediate host, the space and resources available in the fish might not have been limiting yet at the time of dissection as the worms were still relatively small at 60 days p.i. However, parasite total weight decreased with increasing intensity of infection and such a density-dependent effect rather suggests that constraints in available host resources already existed (see discussion on fish growth below). It thus seems that outcrossed and selfed parasites once they are established in the second intermediate host are almost equal competitors. This may not be the case if differences in size between outcrossed and selfed parasites had already existed in the copepod as a result of competition (Christen *et al*. 2002). In the present study we avoided such a situation in the infected copepods in order to detect additional differences between outcrossed and selfed parasites in the fish. Under natural conditions, however, benefits from earlier life-stages of the parasite would probably be transmitted from one stage to the next. When new benefits arise in a later life-stage (such as the increased infection intensity found in the fish in this study) they would add up to those from the earlier life-stages rendering the total benefits of outcrossing greater the further the parasites advance in their life-cycle. Whether the performance of outcrossed and selfed progeny in later life-history stages is affected by the performance in earlier ones, however, may depend on which trait is measured, as shown in plants in Carr & Dudash (1995).

When comparing singly and multiply infected fish we found a surprising result suggesting that reduced parasite growth in multiple infections as

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compared to single infections might have been a life-history strategy of the parasite instead of a density-dependent competition effect (see Parker *et al*. (2003) for a model). All groups of fish in our study, i.e. not exposed control fish, exposed but not infected fish, fish with only 1 worm (singly infected) as well as those harbouring multiple worms (multiply infected) had significantly increased in weight from the start to the end of the experiment. It is surprising that infected fish grew at all, especially the ones in multiple infections. It has been shown that *S. solidus* converts energy more efficiently than its stickleback host (Walkey & Meakins, 1970). Therefore, we would have expected that fish with multiple infections grow less than singly infected fish as a consequence of the greater energy drain. However, although the fish grew, the total worm weight per fish was significantly lower in multiply than in singly infected fish, which might indicate a parasite life-history strategy of restrained growth. Such a strategy can be expected because *S. solidus* within its small copepod intermediate host will most often be transmitted to very small sticklebacks as a consequence of stickleback optimal prey choice (Christen & Milinski, manuscript submitted). Therefore, multiply infected fish need to grow to a larger size than singly infected ones in order to allow any of the plerocercoids to become infective to the final host. Thus, in the case where all parasites started growing immediately as fast as possible they might overexploit their host and risk damaging or killing it before being transmitted to the final host, especially in relatively small hosts. Supporting this hypothesis we found that the condition factor of singly infected fish decreased significantly from 13 to 60 days p.i. whereas it did not in multiply infected fish. Pennycuick (1971) found that mean number and weight of plerocercoids in dead fish from Priddy Pool in Somerset was higher than in living fish and that the parasite indices were also greater.

In conclusion, our study has shown that in addition to the benefits of outcrossing found in earlier life-stages of *S. solidus* a new benefit in terms of increased infection intensity arises in the second intermediate host. Thus, a decrease in fitness due to selfing occurs also at an advanced stage of the parasite's life-cycle, i.e. before transmission to the final host where reproduction takes place. The most probable explanation why selfing, despite its detrimental fitness consequences, is maintained in this species is the unpredictability of finding a partner and thus assurance of reproduction (Lloyd, 1979) in case the parasite ends up in the final host alone or does not find a mating partner. There are only few reports on the frequency of infection in the final host (fish eating bird) of *Schistocephalus solidus* in the field (Nybelin, 1919; Vik, 1954) and they show that multiple infections do occur. The frequency of infection will, however, depend on the frequency of stickleback infection which fluctuates to a large extent among years (Arme & Owen, 1967; Jakobsen, Johansen & Larsson, 1988; McPhail & Peacock, 1983; Meakins & Walkey, 1975). Therefore, the probability of a parasite to end up alone in the final host and be forced to self will vary from year to year. However, even if a mating partner was available a significant proportion of the eggs were found to be selfed as a consequence of a mixed mating strategy and of conflict in the Hermaphrodite's Dilemma (Lüscher & Milinski, submitted).

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